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GENOMIC INSTABILITY AND AGING

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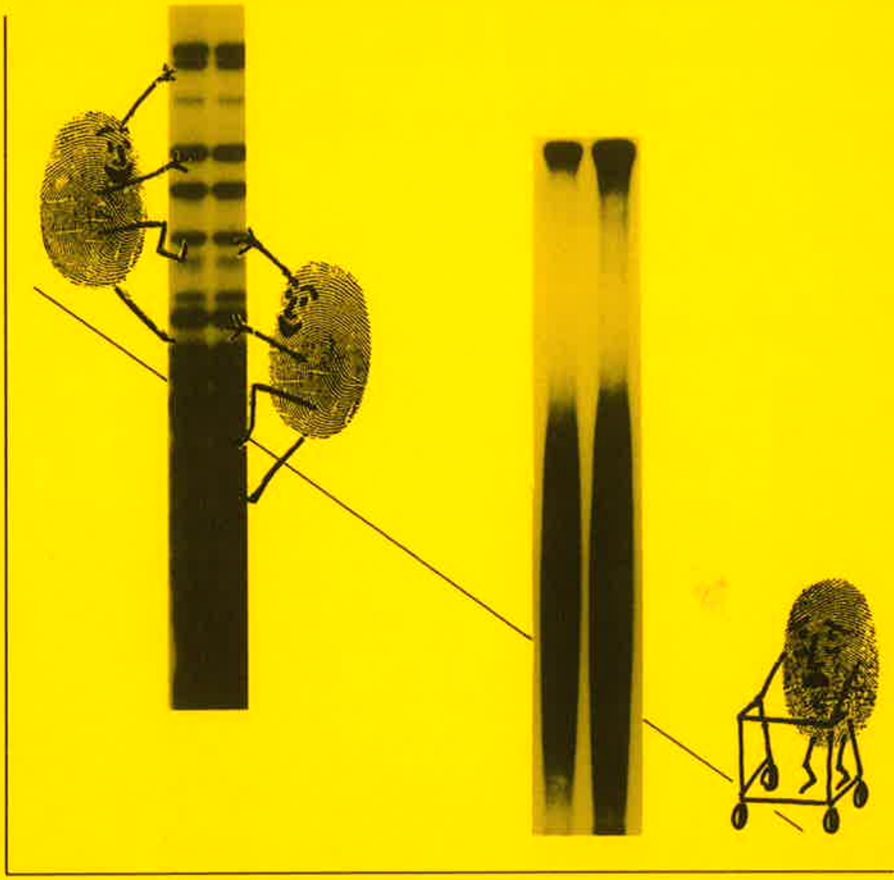

age

P. Eline Slagboom

Stellingen

behorende bij het proefschrift "Genomic instability and aging"
van P.E. Slagboom
Leiden, 30 november 1993.

STELLINGEN

1. Voor het meten van veranderingen in genexpressie in relatie tot de leeftijd is een kwantitatieve bepaling van de mRNA's van GAPDH en ß-actine als controle niet geschikt.
Dit proefschrift

2. Functionele achteruitgang van mitotische cellen na het bereiken van de "Hayflick-limiet" (beëindiging van delingsactiviteit) is slechts relevant voor veroudering indien bewezen kan worden dat deze limiet *in vivo* wordt bereikt.
Dit proefschrift

3. Ondanks het feit dat genregulatie en chromosomale integriteit door telomeerverkorting worden aangetast, zou dit mechanisme zowel een negatief als een positief effect kunnen hebben op de levensduur van de mens.
Dit proefschrift

4. Aan het optreden van mitochondriale deleties als functie van de leeftijd wordt voorbarig veel betekenis gehecht als mechanisme van veroudering, gezien de frequentie waarmee deze deleties optreden.
Dit proefschrift

5. Micro- en minisatelliet loci met de hoogste frequentie van heterozygositeit zijn voor koppelingsonderzoek beperkt bruikbaar vanwege hun verhoogde kans op somatisch en geslachtslijn mosaïcisme.
Jones M.H. en Nakamura Y. Hum Mut 1992, 1: 224-228

6. Dat in PCR-reacties met een enkele "random" gekozen "arbitrary primer" met humaan DNA een produkt wordt verkregen, impliceert dat het genoom voor een groot deel bestaat uit inversies.
Williams et al., Nucl Acids Res 1990, 18: 6531-6535.

7. Collageengenen verkeren door hun mutatiegevoelige structuur in een extreem evolutionair spanningsveld tussen behoud en variatie.

8. Het verdient aanbeveling het *ulcus duodeni* met onder andere antibiotica te behandelen tenzij bewezen is dat er geen *Heliobacter pylori* in het spel is.

9. Aangezien heterozygositeit de kans op ziekten, veroorzaakt door recessieve mutaties, verkleint zal vermenging van rassen de fitness van onze soort verhogen.

10. Hopelijk zullen daadwerkelijke keuzen in de zorg in de spreekkamer gemaakt blijven worden.
Kiezen en delen. Advies in hoofdzaken van de commissie Keuzen in de zorg. 1991.

11. Gezien het feit dat het voor fruitvliegen loont om "laat aan kinderen te beginnen" zouden overeenkomstige uit het feminisme voortvloeiende tendensen kunnen bijdragen tot een verlengde levensduur van de mens.

12. Aan het woord "onmenselijk" wordt een pijnlijk misleidende betekenis gehecht.

13. Het meest wonderlijke in de muziek is dat majeur en mineur slechts een halve toon verschillen.

14. Bij het bestrijden van criminaliteit worden de messen net zolang geslepen tot men zelfs het heft niet meer in handen heeft.

15. Een westerse wijsheid is een oosters cliché.

16. Gebrek aan diepgang is geen bezwaar als je voor de wind vaart.

NOGMAALS DANK

Het leggen van een ei is een eenzaam proces. Daarom is het goed dat je dat niet alleen doet. Neem iemand als Jan, die de eerste periode zo inspirerend heeft meegepuft. Of Gerjan en André die door het bulderen van "de diligence" een sfeer creëren die je nooit meer vergeet. Het is ook heerlijk om door het gekakel van Karin, Ingrid en Simone in het lab te worden afgeleid. En dat terwijl ze zoveel klusjes voor je opknappen. Daarnaast zijn er mensen nodig als Dick en Kees die je terug op het ei jagen als je het broeden zat bent. Ik denk aan Paul die je steeds weer een goede chinees voorzet want het moet ook leuk blijven. Chris van wiens pijp en ideeën je helemaal high wordt. Of neem Adriaan die met eindeloos geduld je lief en leed aanhoort in café Cliché. Martin, waarmee het goed emmers leeggooien is. Anderen, zoals Joey, Taeke, Henk en Johan die je aansporen om nog veel meer eieren te leggen en Jacques die je dan overhaalt om het met computers te doen. En Dorret dan, het rekenwonder dat alles in het onderzoek dubbel ziet. Op een dag komt dat ei uit en dan heb je Gert-Jan nodig om de overtollige veren van het kuiken te plukken en dat waren er heel wat. Marlène, Ted, Ita en Corrie die orde in de chaos scheppen en het menselijke behouden waar zo vaak geen tijd voor is. Boeken Bert is daar trouwens ook sterk in. Veel hoogbejaarde actieve tweelingen hebben me meer energie gegeven dan ze ooit zullen vermoeden. Dan heb ik het nog niet eens gehad over mijn ouders die steeds weer enthousiast zijn als je zegt dat het dit jaar echt gaat gebeuren. Eelco, de chauffeur die de Lange Kleiweg wel kan dromen en de luisterende oren van zes Ton en Bernadette. Dan is daar nog mijn Toon, die er steeds opnieuw muziek in blaast. Nee, men zal mij niet horen zeggen dat ik dat ei alleen gelegd heb. Sterker nog, ik bedank iedereen die hier staat genoemd en alle anderen, die overigens niet vergeten zijn, voor alle steun en belangstelling voor mijn onderzoek die ik de afgelopen jaren heb ondervonden.

GENOMIC INSTABILITY AND AGING

Proefschrift

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op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op dinsdag 30 november 1993
te klokke 16.15 uur

door

Pieternella Eline Slagboom

geboren te Dordrecht in 1960

Promotiecommissie:

Promotoren:	Prof. Dr. D.L. Knook
	Prof. Dr. G.J.B. van Ommen
Referent:	Prof. Dr. T.B.L. Kirkwood (University of Manchester, U.K.)
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aan mijn ouders
voor Toon

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Chapter 6	Two-dimensional DNA fingerprinting of human individuals. Uitterlinden, A.G., P.E. Slagboom, D.L. Knook and J. Vijg. Proc. Natl. Acad. Sci. USA 1989; 86: 2742-2746.	85
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CHAPTER 1

GENERAL INTRODUCTION

1.1 A description of aging

The aging process of an organism may be defined as the, mainly intrinsic, progressive accumulation of changes with time, associated with or responsible for the increasing risk of dying. Only some of these changes are clearly associated with disease. Aging studies of many different species have revealed that at time points at which age-related changes become detectable, they can be found at all levels of organismic organization. These involve changes in hormone status, response mechanisms to stress, structural components, such as the intra- and extracellular matrix and chromatin, the primary DNA sequence, the protein synthesis machinery, etc.. In principle, aging starts at any time point between conception and death. Consequently, any change occurring during the life of an organism which contributes to decline of function is relevant for its process of aging.

Due to a decrease of infectious diseases, the human life expectancy in industrialized countries has increased 40 to 50 years over the last 400 years. The human aging rate, however, remained essentially unchanged. Although aging is considered to be a general phenomenon among most species (except for species with 'negligible senescence', Finch, 1990), there is a large interspecies variability in the aging rate (Comfort, 1979). Still, some unifying patterns of aging can be recognized. Placental animals, for example, spend roughly 1/3 of their life span to reach sexual maturity and to reproduce. In this period, there is a maximum of physiological response. In the next 1/3, there is a decline of functional responses such as reproduction, immunity, reaction time, and synchrony of biorhythms. The last 1/3 of the life span is associated with an exponential increase of spontaneous disease and mortality rate (Finch, 1992). The concept of 'aging' in these species, which reproduce repeatedly (iteroparous species), is clearly different from the post-reproductive death of species that reproduce only once in their lifetime (semelparous species) (Kirkwood and Cremer, 1982). Aging in iteroparous species is associated with a gradual accumulation of a diverse spectrum of pathological conditions (Zurcher and Hollander, 1982).

Aging research in the past decades has been dedicated to the search for common factors and mechanisms determining the rate of aging and the nature of aging phenomena. There are four characteristics by which aging in different species is usually described and compared: the maximum life span (which depends on a single last survivor), the 50% survival age (chosen because the occurrence of multiple pathology is generally significantly less in those dying before that age), the initial mortality rate (IMR, determined by the main cause of death

at a relatively young age), and the mortality rate doubling time (MRDT, expressing the acceleration of mortality).

1.2 A theoretical framework for the evolution of aging

Apart from Eve's unfortunate appetite there must be an explanation for the fact that life apparently must come to an end. Strehler (1986) stated that any theory of aging should explain the universality, intrinsicality, progressiveness and deleteriousness of the aging phenomenon. The maximum life span and the MRDT appear to be specific for the species, even if diverged populations are compared, which indicates that these characteristics are determined by a significant genetic component. Life span is correlated with species characteristics such as body size and temperature, metabolic rate, blood glucose levels, etc.. The IMR, in contrast, can vary extensively among different populations of the same species, which may be caused both by environmental influences (if, for example, a population has many predators) and genetic ones (if a strong genetic predisposition for a disease causing an early death prevails in a population). The fact that the largest differences in life span and MRDT are those between species (40-fold between mammalian species) suggests a genetic basis for the interspecies variations in aging rate. A useful basis for the discussion of how aging may be caused, is provided by the theories hypothesizing why aging exists. These theories, explaining the relationship between aging and evolution, are controversial essentially in respect of which of two phenomena evolved: aging or longevity. This is reflected in three contrasting concepts that were put forward to provide a genetic basis for the evolution of aging (as summarised by Kirkwood, 1985, 1991):

Programmatic theory of aging

Aging could be a programmed process designed to actively bring life to an end. Such a programme may have evolved as a necessary adaptation to guarantee the turn-over and renewal of populations in terms of competition for food, preservation of genetic variation, etc.. This programmatic, adaptive theory predicts an active control of aging through a limited number of gene products. It has been shown that such a programme is unlikely to have evolved universally, since this would require selection for advantages to the species to be more effective than selection among individuals for the reproductive advantages of a longer life (Maynard Smith, 1976; Kirkwood, 1981).

A second argument against the programmatic theory of aging is that in most natural populations aging is not the main contributor to mortality (Medawar, 1952), because most organisms in the wild die from accidents and predators. Since the probability of dying enhances with time for each individual, it is unlikely that there has been any need for extra life-terminating mechanisms.

Reproductive selection has little interaction with late aspects of most species' life histories. Survival selection of a group, however, may act to the advantage of longevity. Populations may require balanced proportions of older individuals to survive (Mergler and

Goldstein, 1983). This can be observed in some vertebrate species which exhibit slow maturation rates, long individual life span and population age structures which extend over several generations and harbour a limited number of highly aged individuals (Finch, 1992). If the period of maximal reproductivity of long-lived individuals is not extended or improved, they arise continuously in a population but do not increase in number.

Pleiotropic gene theory of aging

In the second concept, aging is regarded as a by-product of selection, evolved by a gradual accumulation of germ-line mutations, the deleterious effects of which are not expressed or becoming phenotypically evident until 'late' in life, after the period of maximal reproductivity (Medawar, 1952). Such late-acting deleterious mutations will not have selective disadvantage if they do not interfere with reproductive capacity, and may therefore be tolerated in the germ-line. By this mechanism so-called pleiotropic genes may have evolved, simultaneously exerting advantageous effects on reproduction and deleterious effects late in life (Williams, 1957). As a result of late-acting deleterious or pleiotropic germ-line mutations, new gene variants releasing modified gene products, or differently regulated expression patterns of existing genes, may continuously be generated. These may underlie a broad spectrum of aging phenomena.

Disposable soma theory of aging

Aging may have evolved as a consequence of a trade-off between early fecundity and late-survival (Kirkwood, 1977). This idea is expressed in the 'disposable soma' theory, which explains the evolution of somatic aging as a result of selection to optimise reproductive success, by adjusting the balance in expenditure of finite resources between somatic maintenance and reproductive effort. The disposable soma theory implies that for the sake of maximum fitness, the optimum level of investment in somatic maintenance is less than would be required for indefinite somatic longevity. The level of mortality from environmental causes imposes the selection for a longer or shorter life. As a result, populations propagating at a high risk of dying will have an early fecundity, a high reproductive effort, a low somatic maintenance and a short life. This theory predicts that i) aging is under direct control of genes reassuring somatic maintenance (e.g. the network of genes involved in cellular defense, growth, food-seeking and processing), ii) these genes do not function entirely without error leading to the gradual accumulation of somatic defects in biomacromolecules. Both intrinsic and extrinsic factors modulate the risk of damage, the quality of the 'longevity assurance genes' (Sacher, 1978; Cutler, 1979) and the integrity of their network form the basis of the protection against such damage. The somatic defects accumulating as a resultant of these activities form the basis of aging in the disposable soma theory.

Many segmental theories of aging fit into the evolutionary framework of the disposable soma theory. An example is the free radical theory which predicts that aging is caused by superoxide radicals, the products of cellular oxygen metabolism (Harman, 1981; 1984). These reactive oxygen species have direct damaging effects on membranes, lipids, proteins and nucleic acids, and influence gene expression. Free superoxide radicals are eliminated by

a chain of scavenging reactions and the damaged molecules are degraded or repaired. The genes involved in these somatic maintenance functions are regarded as longevity assurance genes.

1.3 Manifestations of three evolutionary concepts of aging

The aim of this thesis is not to falsify or favour any of the three evolutionary concepts, but to use their main predictions as a tool in distinguishing molecular mechanisms of aging. The main topic of this thesis is a discussion of these mechanisms and their contribution to the progressive functional decline of aging organisms (section 1.6 of this Chapter and Chapters 3-8). The three evolutionary concepts of aging, as outlined in the previous paragraph, will be used as a frame of reference throughout this thesis. First, however, a brief overview will be given on factors and genes involved in the regulation of maximum life span.

Early life span studies were focused on the comparison of different species and strains within species. In addition, mutant individuals were investigated with a life span significantly aberrant from the life span of the population from which they arise. Most of these cases had a decreased life span. Hence, it is doubtful if accelerated 'normal' aging is studied, or plain disease. Indeed, most of the short-lived mouse strains, for example, die from disease (cancer, in many cases) rather than from accelerated aging. These strains do not prove a genetic basis for longevity, but suggest that specific genes may set limits on life span through their influence on age-related diseases. The most informative experiments on life span regulation were performed by manipulating nematodes and fruit-flies by selective breeding, selection of chemically induced mutants and implantation of candidate longevity genes in the germ line and by food restriction experiments on laboratory rodents.

Programmatic theory of aging

The most obvious examples of programmed 'aging' can be found in semelparous species. This process is triggered by hormones in most instances (Finch, 1990). It has been suggested that aging of iteroparous species is likewise governed by a neuroendocrine clock. The length of the maturational phase of life correlates with the length of the period of aging across many mammalian species (despite vast differences in total life span). Hence, similarly programmed genetic processes may control maturational development and aging (Russel, 1978). Many hormones governing circadian rhythms, development and seasonal sexual cycling (such as pituitary, thyroid, adrenocorticoid, ovarian, and testicular hormones) have major effects on the length of the reproductive period and on median and maximal survival in nematodes, fruit-flies, rodents and primates (Everitt and Meites, 1989). Hypophysectomy, ovariectomy and castration delay age-related physiological changes and aging in mice and rats (Regelson, 1983). In old rats the activity of the hypothalamo-pituitary-adreno cortical axis is increased. This is associated with an age-related derangement of thyroid function. The beneficial effect of hypophysectomy is supposed to involve enhancement of thyroid hormonal action. Other hormones such as growth hormone (Gresik et al., 1986) and melatonin, the pineal hormone,

have many rejuvenating and longevity-prolonging effects when administered to old mice (Reiter, 1987; Pierpaoli et al., 1991). A reduction in pineal melatonin levels, especially during darkness, is associated with aging of rats and humans (Pierpaoli et al., 1991).

These observations could indicate the widespread existence of aging programmes. Hormone-driven programmed processes indeed affect tissues of iteroparous species (menopause, thymic involution), and the age of onset of age-related diseases. There is no evidence, however, that these programmed processes also determine the functional decline of the whole organism. Several lines of evidence indicate a general inability of higher organisms to maintain the homeostatic balance of the endocrine and nervous system at later ages. This may result from late-life alterations in the level of key hormones, neurotransmitters and peptides. Instead of being programmed, such alterations may well be due to random loss of transcriptional control of the gene systems involved in neuroendocrine functions.

Certain aspects of aging are strongly influenced by environment and lifestyle. From one batch of insect eggs, for example, both worker and queen bees arise, living up to 10 months or five years, respectively. Such longevity variations must be due to differential gene expression under environmental control, since both types have a highly homologous genetic background. The genetic constitution of individuals is determined not only by the primary DNA sequence but also by the programmed responses to epigenetic factors regulating the expression of genes. Thus, organisms may harbour an intrinsic life span potential much greater than the maximum life span observed even under relatively favourable conditions.

Pleiotropic gene theory of aging

The genome of species with a post-reproductive period are expected to carry mutations which are advantageous and/or neutral to reproduction with late-acting deleterious effects. These pleiotropic genes may determine life span through late-life alterations in expression. Alternatively, late-acting deleterious effects may arise from genes with 'mild' mutations, the declined quality of their products becoming a hazard gradually. Such mild mutations may accumulate in gene regions coding for protein domains on which selection is not too stringent. Late-acting deleterious variants of key genes in multi gene-networks may affect large functional units composed of sets of genes.

Some indications in favour of the pleiotropic gene theory came from the study of rapidly aging species, such as the nematode *Caenorhabditis elegans* and *Drosophila melanogaster*. For both species, an acquired longer life span was inversely related to early fitness characteristics such as fecundity and (for the flies) egg-laying rate. Although no direct proof, these reciprocal effects are in accordance with the hypothesis of antagonistic pleiotropy and, as a matter of fact, also with the disposable soma theory.

Selection for late reproduction in *Drosophila* strains has lead to the isolation of strains with a 35-100% increase in average and maximum life span (Lunckinbill et al., 1988; Rose et al., 1993). The quantitative genetic traits responsible for these extensions in life span, which are believed to involve hundreds of loci, have not yet been identified; some recessive genes have now been localized to the 3rd chromosome (Arking et al., 1993). Apart from

genetic variation, epigenetic (neuroendocrine) mechanisms may also be involved in *Drosophila* life span variations, since developmental conditions of the larvae have a major effect on the life span of the selected strains.

From *Caenorhabditis* recombinant inbred long-lived strains were isolated as well as chemically induced mutants with a two-fold increased maximum life span and a 50% increased average life span. A mutant gene was characterised in one of the long-lived strains, designated *age-1* (Friedman and Johnson, 1988a, 1988b). The resistance to oxidative stress is increased in the age-1 mutants (Lithgow et al., 1993; Larsen, 1993). The reduced fecundity and late survival of the mutant strain could be due to a combined effect of the age-1 gene and the closely linked fertility locus *fer-15*. Recently, two more mutations at another locus (*gro-1* and *gro-2*) were identified which confer improved heat shock resistance, impaired reproductive capacity, extended larval period and increased life span (Larsen, 1993). It is expected that at least 4 more loci are involved in longevity regulation of the nematode (Ebert et al., 1993). Age-1 was the first long-lived strain for which the age-related acceleration of mortality is slowed down (MRDT). The pathology of the nematode, however, is as yet largely unknown which is a serious drawback in comparing aging of the nematode to higher species. This also holds true for the fruit-flies.

Disposable soma theory of aging

By comparative studies, relations were found between life span and species-specific characteristics such as metabolic rate, growth rate, etc.. These relations are compatible with the disposable soma theory. Two major lines of research have dominated the experimental investigations into the disposable soma concept. One line was focused on identification of candidate longevity genes; another line on monitoring the accumulation of somatic defects.

Especially the free radical theory of aging and the prediction of free radical scavenging genes to be longevity assurance genes, have received much attention. A few representative data are discussed. For about 12 species, a weak correlation was found between the species maximum life span potential and the activity of some of the antioxidants (Cutler, 1991). In the long-lived *Drosophila* and *Caenorhabditis* strains discussed above, the antioxidant protection was enhanced. Suppletion of vitamin E or other antioxidants increased both the mean and maximum life span of *Drosophila* (Miquel and Lindseth, 1983) and *Caenorhabditis* in culture (Kahn and Enesco, 1981), while it had no effect on survival of house-flies (Sohal et al., 1985). On the basis of the evidence reported thus far, the free radical hypothesis of aging is neither proven nor disproved (Sohal 1993).

Although the gene coding for superoxide dismutase (SOD), a radical scavenging protein, SOD-gene would logically be a longevity assurance gene, manipulation towards higher expression did not result in life span extension of *Drosophila* (Seto et al., 1990). In transgenic mice carrying a human SOD-gene, pathological changes were observed in neuromuscular junctions similar to those occurring in old mice (Yarom et al., 1988); the life span of the mice was not extended. The phenotypic characteristics also resembled those found in human Down's patients which carry a trisomy of chromosome 21 containing the SOD-gene. Expression of human SOD in bovine adrenocortical cells by transfection is cytotoxic

(Norris and Hornsby, 1990). These negative effects may be explained by stating that human SOD has negative effects in cells from other species, or that over-expression of SOD leads to accumulation of hydrogen peroxide and hydroxyl radicals, intermediates of O^{3-} detoxification, which are even more reactive than oxygen. Given this possibility, the free radical hypothesis and, thus, the potential role of oxygen radical scavenging genes as longevity genes, can only seriously be tested in a balanced system in which the whole network of free radical scavenging is improved. A complicating factor is that the balance between different antioxidant enzymes may vary among species.

Transgenic *Drosophila* with an extra copy of the elongation factor EF1α-gene, under control of a temperature sensitive heatshock promotor (Shepherd et al., 1989) had an extended life span of 41% when the body temperature was increased. These experiments would suggest EF1α to be a longevity gene. However, in *Drosophila*, the production of this protein drops 95% with aging. Therefore, the experimentally generated gene dosage effect probably compensates an age-related change which may be specific for this species. What may be concluded is that the altered expression level of the native EF1α gene with age is a pleiotropic aging effect.

The only way in which the life span of higher species could thus far be extended is by caloric restriction (CR) of laboratory rodents. A diet of low but sufficient caloric intake, delays most age-related physiological changes, the onset of age-related chronic diseases (especially cancer), and extends the average and maximum life span (Masoro et al., 1991). Although the mechanism underlying these effects of CR has not been revealed, two aspects are likely to be involved: induction of a beneficial change in hormone status and a reduction of damage-inducing metabolic by-products. The first explanation refers to the role of the neuroendocrine and nervous systems in aging (programmed or pleiotropic effects), the second to the role in aging of stochastic oxidative damage in biomacromolecules (disposable soma). Combinations of both explanations are most likely. The decrease in the rate of aging and cancer by CR is often accompanied by a decrease in the ability to reproduce. This has been interpreted as part of both the disposable soma and pleiotropic gene theory. It has been speculated that a hormonal switch in starving animals decreases reproductive capacity and increases the maintenance functions that prolong life. Interestingly, a recent study in the People's Republic of China suggested that age at menarche significantly increases by low protein intake (Chen et al., 1990), which acts in much the same way as restricted calorie intake. A longer life could be associated with a reduced reproduction effort and an extended reproductive period.

So far, suggestive and mainly circumstantial evidence is found supporting each of the three evolutionary concepts. The main predictions emerging from these concepts are the existence of:

1. programmed events and processes contributing to functional decline of individuals;
2. pleiotropic effects mediated by alterations in gene expression and late-deleterious effects of mild mutations;
3. a gradual loss of the integrity of networks of longevity genes and, as a result, accumulation of somatic defects.

These predictions should ultimately lead to the identification of dominant factors determining the life span of strains and species, and of defined physiological age-related changes within individuals. The most critical life span-limiting function may vary from species to species. When a dominant factor shortens life span, a small life-extension can be obtained by improving that factor until it is offset by the next dominant factor. The search for life span determining genes therefore may not lead to the finding of a unifying principle of aging in different species, even if it were present.

1.4 Cellular life span variation

A logical question arising from the search for causal factors in aging is whether functional decline at the cellular level forms the basis for organismal aging. In species with a programmed death (e.g. semelparous species), aging occurs under genetic control at a higher (e.g. neuroendocrine) level rather than by cellular aging. In humans, however, aging is not associated with reproducible failure of one organ system before the other. The onset and progression of aging in different organs is expected to be more or less harmonised (Kirkwood, 1991). Cellular aging may be the principle cause of organismal aging. In somatic cells of higher species, there are numerous changes representing a time-dependent functional decline, which may be referred to as cellular aging. Cell types differ, however, in replicative capacity, differentiation status and death. The germ line is not subject to aging although germ cells within aging organisms do accumulate somatic defects (Edwards, 1989). Little is known about age-related deterioration of stem cell pools.

Cells may become transformed into 'immortal' cancer cells. This mainly occurs when most cells of an organism are expected to have undergone deleterious age-related changes. Either these age-changes are reversible or a sub-set of cells within an aged tissue (maybe former progenitors) remain viable enough to replicate indefinitely. Tumours arising from highly differentiated cell types such as neurons and muscle cells, are rarely found, which may indicate that for some cell types, release from the post-mitotic state is rare, if not impossible.

The differences in life span of different cell-types evoke three questions: 1) Which functions are unique to germ cells and lacking in somatic cells that lead to immortality of the germ line; 2) Is cellular aging a common deleterious process, leading to a progressive functional decline in all cell types; 3) Is cellular aging the basis for organismal aging, and if so, do all or just a sub-set of cells determine the aging rate? In the following section, we will focus on questions 2 and 3. Section 1.6.2.5. shows an example of the first issue: a repair function which is present in germ cells but absent in somatic cells.

1.5 The relevance of cellular aging for organismal aging

Aspects of aging at the cellular level can be studied by isolating and comparing cells from

young and old individuals. Most of the studies on cellular aging, however, have been focused on the behaviour of cells *in vitro* (Hayflick, 1985). If transferred into culture conditions, mitotic cells exert a finite capacity to replicate (the 'Hayflick limit') after which a process of terminal differentiation sets in (Hayflick and Morehead, 1961). Human fibroblasts in culture reach the Hayflick-limit (M1 cell-cycle arrest) after $\pm$ 50 population doublings. The population doubling potential of these cells is, in many cases, correlated to the life span of the species from which they originate (Röhme, 1981), and negatively correlated to the age of the donor (Martin et al., 1970). *In vitro*, the cells show a diverse spectrum of physiological alterations. The limited replicative life span of cells *in vitro* was frequently presented as a model of cellular aging *in vivo*. More likely, this system reflects what it is actually selected for, the regulation of cell replication. The limiting number of population doublings, is probably not reached in the living organism (Hayflick, 1991). Genes that were thus far identified to be involved in the Hayflick-limit are the c-fos, the retinoblastoma and the interleukin 1α gene (Seshadri and Campisi, 1990; Stein et al., 1990). These are indeed generally involved in the regulation of cell replication within mature organisms. Cultured cells may become immortalized through bypassing two steps of cells-cycle arrest: M1 and M2 (Wright and Shay, 1992). Bypass of M1 occurs in the presence of T-antigen, which was suggested to bind the retinoblastoma and p53 proteins. The M2 arrest may be passed if telomere shortening is is prevented by, for example, de-repression of the telomerase function (1.6.2.5; Chapter 8).

Some cell types harbour a self-destruction programme (apoptosis). Human erythrocytes, for example, are eliminated by phagocytosis following the programmed expression of a 'senescent cell' antigen (SCA) on the cell surface (Kay, 1991). Another trigger of cell death is phospholipase A_2 (PLA_2), a key mediator in the cell membrane damaging system (Regelson and Franson, 1991). PLA_2 and SCA seem vital factors governing the homeostatic balance between normal, damaged and 'senescent' cells. From this point of view, the SCA and PLA_2 genes may be regarded as longevity genes.

A third manifestation of functional decline at the cellular level, is atrophy and death of post-mitotic cells. In humans between 85 and 94 years of age, about 21% of the nerve cells is atrophic. This process in the human cortex has been associated with a decrease in the turnover of the glycolytic pathway (Meier-Rugge et al., 1991). The rate of neuronal loss does not seem to be under strong genetic (programmed) influence. It may be expected that in these post-mitotic cells, functional decline is mainly due to pleiotropic effects and accumulation of somatic defects.

The question whether cellular aging is the basis for organismal aging cannot be answered yet. The life span of whole organisms may well depend on the functional decline and death of sub-sets of damage-sensitive cells with key functions in the organism. Most likely, these are post-mitotic cells. Damage in mitotic cells, however, will be transferred to daughter cells. Replication, therefore, can not be regarded as cell renewal.

1.6 Aging monitored at the molecular level

A discussion of molecular mechanisms of aging can be focused on two questions: which molecular and biochemical mechanisms lead to an age-dependent decrease of bodily functions and how does this process relate to the multiple pathological conditions of the aged (Zurcher and Hollander, 1982).

1.6.1 Gene expression

Observations reported at the protein and RNA-level indicate both qualitative and quantitative alterations (Chapter 3). The relevance of age-related changes in gene expression for deterioration and aging is often unclear. Many conflicting data have been obtained due to the lack of appropriate controls (parameters proven to remain unaltered during aging), the lack of appropriate techniques and the choice of the age groups of the organism examined. It has thus far not been possible to identify age-related alterations in gene expression as causal factors in aging. Still, the data provide insight into major mechanisms of functional loss, associated with aging.

1.6.1.1 Translation

So far, there is no evidence in higher organisms that ageing is associated with a distinct universal pattern of degenerative switches in gene expression, as could have been expected if aging was programmed analogous to developmental processes. Although the available data are somewhat contradictory, a progressive decline of **overall** protein synthesis has been observed in almost all aging systems (for reviews, see Makrides 1983; Richardson and Semsei, 1987). This has been attributed to the decreased activity of initiation and elongation factors in translation (Castaneda et al., 1986) and to decreased ribosome aggregation to mRNA (Egilmez and Rothstein, 1985). In addition, a decreased rate of **overall** protein degradation has been described (Makrides, 1983; Dice and Goff, 1987). In the levels of individual proteins, however, both up and down variations were observed with aging (Horbach et al., 1987). These quantitative alterations may reflect intrinsic loss of transcriptional control, adaptive alterations in gene regulation and programmed changes in gene expression.

Quantitative changes occur, for example, in the proteins of the oxidative phosphorylation (OXPHOS) system with age, which changes are thought to play a major role in aging. In humans, the number of skeletal and heart muscle fibres deficient in cytochrome c oxidase, increases progressively with age (Müller-Hocker, 1989, 1990) and both the respiration rate and OXPHOS enzyme activities of Complex I and IV decline progressively with age in human skeletal muscle (Trounce et al., 1989) and liver (Yen et al., 1989). Since the OXPHOS system is the major source of adenosine triphosphate (ATP) for many tissue types,

an accumulation of bio-energetically deficient cells may be the consequence of the declined OXPHOS activity. The relation of this decline to the increased instability of the mitochondrial genome with age is discussed in section 1.6.3.

There is no evidence indicating an accumulation of proteins with altered primary structure during aging (Reff, 1985; Rothstein, 1987). This could be interpreted as evidence against the protein error catastrophe theory (Orgel, 1963, 1973), and the somatic mutation theory of aging (Curtis, 1966). These theories predict that by stochastic accumulation of erroneous proteins and DNA mutations, eventually key functions of somatic maintenance (synthesis, degradation, repair) will become affected leading to a cascade of detrimental consequences. Any conclusion with respect to the original theories, however, seems premature (Rosenberger and Kirkwood, 1986) in view of the limitations of the detection level of the techniques used, and the lack of insight as to what minimum level of erroneous proteins is required to cause an error catastrophe.

Individual proteins with aberrant heat stability, specific activity and immune responsiveness, have frequently been observed in tissues of aged animals (Ohrloff et al., 1980; Sharma and Rothstein, 1980). These are likely to result from post-translational modifications (Rothstein, 1987) such as glycosylation, glycation and oxidative damage. Non-enzymatic glycation of proteins and nucleic acids by glucose leads to loss of enzymatic activity, altered gene expression, altered binding of regulatory molecules (Lee and Cerami, 1990) and inappropriate cross-linking (Molnar et al., 1986). The latter process affects especially long-lived extracellular matrix proteins such as lens crystallines, basal membrane proteins, collagens and elastin. Cross-links in these proteins may contribute to functional loss in the eye (cataract, loss of accommodation), blood vessels, kidneys, skin and cartilage.

Observations of Oliver et al. (1987) indicate an increase in the level of oxidized key metabolic enzymes in fibroblast cultures from old as compared to young human donors. The accumulation of oxidized enzymes may have severe consequences for protein metabolism. Stadtman and Oliver (1991) showed that aging is associated with inactivation of glyceraldehyde-3-phosphate dehydrogenase, aspartate aminotransferase, phosphoglycerate kinase, and glucose-6-phosphate dehydrogenase. Interestingly, all of these toxic processes and the accumulation of glycated and oxidized proteins is retarded in calory or protein restricted rats (Youngman et al., 1992).

Lipid peroxidation of cell membranes and mitochondria, appear to be very important in aging and especially in disease. Intracellular aging pigments (lipofuscin) accumulate in cells of various tissues and organs as a result of lipid peroxidation (Tappel et al., 1973). These and other cellular and molecular age-associated changes occur in different species, though at a different speed.

1.6.1.2 Transcription

The quantitative variations in protein levels, discussed in the previous section, may indicate that the control of gene expression is affected during aging. The overall RNA synthesis

decreases with age in human fibroblasts and in various organs and tissues from experimental animals (for a review, see Richardson et al., 1983, 1985). In addition, the overall rate of mRNA synthesis decreases with age (for a review, see Richardson and Semsei, 1987). This, however, is not reflected in the level of all proteins since both up and down regulations were observed (previous section). Likewise, no general trend can be derived as to the effect of age on the level of individual mRNAs, since some mRNA levels go up, others goes down or remain unaltered with advancing age (Chapter 3). A few of these changes, which illustrate different mechanisms underlying loss of transcriptional control, will be discussed.

In the liver of 24- and 36-month-old WAG/Rij rats, a 65% decrease in the steady state level of the tyrosine amino transferase (TAT) mRNA was observed as compared to 6-month-old animals (Chapter 4). The altered mRNA level of this liver-specific gene, which was measured by Northern hybridization analysis of total RNA, paralleled quantitative changes at the protein level observed in Sprague-Dawley rats (Richardson and Semsei, 1987). Also for other inducible gene systems, the constitutive mRNA levels become altered with age. The mRNA level of albumin, for example, is decreased in the liver of old mice (Post et al., 1991) and humans (Pacifi et al., 1986). Albumin is a protein that becomes repressed in the acute phase response.

The constitutive mRNA level of 'positive' acute phase response proteins, such as T-kininogen, α1-acid glycoprotein (Rutherford et al., 1986) and α1-antitrypsin is increased in the liver of aged rodents. For some aged animals, the altered mRNA levels thus far discussed may be associated with individual pathologies leading to the induction of acute phase responses. In many cases however, the observed change was not associated with detectable pathological conditions. A loss of transcriptional control may underlay these alterations, for example by chromatin structure changes (Matocha et al., 1987). The role of chromatin in the regulation of acute phase gene transcription was clearly observed for the $\alpha_{2\mu}$-globulin gene family, coding for negative acute phase reactants. Transcriptional activation of this gene family at puberty and cessation of transcription at old age correlated with the association and dissociation of the gene domain with the nuclear matrix (Murty et al., 1988). A combination of chromatin changes and a decreased availability of hormone receptors leads to the age-related extinction of this gene (Roy et al., 1983).

In addition to effects on the constitutive expression, also the inducibility of the acute phase gene systems is affected with age, resulting in a slower induction, a lower peak value and sometimes also a decreased or delayed extinction (repression of gene activity; Dilella et al. 1982; Wellinger and Guigoz 1986; Post et al., 1991). This may be caused by altered levels of hormones, and factors stimulating growth and transcription in the organism, or a decreased cellular response. The latter may result from a reduced availability of receptors, or by deficient interaction between DNA-binding sites and trans-acting transcription factors, which may be caused by chromatin changes at the binding sites, or by changes in the structure and/or activity of the transcription factors. Evidence is growing that the latency of the heat shock and acute phase responses in aged animals result from decrements in DNA-protein binding (Post et al., 1991).

DNA methylation changes (section 1.6.2.2) have been suggested to cause another type

of transcriptional alterations with age: the de-repression of genes in tissues where they are normally not expressed (dysdifferentiation: Cutler, 1985; Ono and Cutler, 1978; Ono et al., 1985; see also Chapter 3) and the de-repression of X-linked genes. DNA methylation is postulated to be involved in the maintenance of X-chromosome inactivation (Lock et al., 1987). The de-repression of the X-linked ornithine carbamoyl transferase (OCT) gene in hepatocytes from old mice (Wareham et al. 1987) may indeed illustrate a decrease in the stability of X-chromosome inactivation with age.

Apart from inducible and tissue-specific genes, also the transcriptional control of constitutively expressed housekeeping genes becomes affected with age (Chapter 5). The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin mRNA were increased 200% and 100%, respectively, in the spleen of 36-month-old animals as compared to 6- and 24-month-old rats. In liver and brain from the same rats, however, no age-associated alterations in the level of these mRNAs were observed. These results demonstrate that β-actin and GAPDH are not suitable as internal standard in comparative studies. A considerable inter-tissue and inter-individual variation was observed in the mRNA levels of these genes in all age-groups as compared to the level of 28S rRNA, which was used as an internal control (de Leeuw et al., 1989). The yield of total RNA isolated per mg tissue remained unaltered with age. The inter-individual variation in the level of GAPDH mRNA paralleled the variation observed in the β-actin mRNA level in the same tissue. The possible involvement of DNA-methylation for the altered β-actin mRNA level in the spleen of aged WAG/Rij rats is discussed in the section dedicated to the topic of DNA methylation (section 1.6.2.2).

We may conclude that intrinsic age-associated alterations in gene expression occur widespread. The alterations are generated by changes in membrane parameters, chromatin structure, function and levels of transcription factors, hormones and hormone receptors. The data obtained thus far in this field, also include alterations in RNA processing and stability. These molecular changes may form the basis for the functional decline of homeostasis, structure and reproduction during aging.

Following the predictions arising from the evolutionary aging-concepts (section 1.3) the observations on the translation and transcription level can be divided in two categories:

1. Age-related stochastic accumulation of damaged and posttranslationally modified proteins. This observation is in support with the disposable soma concept. The generation of oxidized and glycated proteins is predicted by the free radical theory of aging. If chromatin and regulatory molecules are among these proteins, the regulation of gene expression may be affected stochastically. Genes coding for some types of proteases may be regarded as longevity genes (Davies, 1988). Modified proteases may loose their specificity, a source of decline in itself, which may represent a non-random (seemingly programmed) accumulation of aberrant proteins. The decline of protein degradation, so generally observed, might illustrate either a programmed or pleiotropic change of protease gene expression.

2. Age-related alterations in the patterns of gene expression. This may reflect a loss of transcriptional control, which may be of both genetic (mutations) and epigenetic origin. Such deregulation may lead to the quantitative changes that have been observed in the level of many proteins. This occurs probably in a growing number of cells with increasing age. The basis for such events may be stochastic. Repression or de-repression in a more consistent mode may represent or be due to pleiotropic effects. Some protein levels are observed to become altered relatively shortly after maturation, in a regulated fashion. Such changes may be remnants or distinct parts of a developmental programme, they might be early steps of a (deleterious) aging programme or they may be an early (non-programmed) pleiotropic gene effect. Some of these changes might be part of a phenomenon described as dysdifferentiation (Ono and Cutler, 1978).

Having discussed various ways by which alterations in gene expression become manifest, we will now focus on the role of the genome. Instabilities of the aging somatic nuclear and mitochondrial genome are discussed separately in the following sections. An overview of other phenomena, such as the appearance of extra-chromosomal circular DNA with age, is given in Chapter 3. Instabilities of the nuclear genome involve the macroscopic and microscopic level (DNA damage, DNA modification and DNA sequence alterations in coding and repetitive structures).

1.6.2 Instability of the nuclear genome

Somatic mutational theories of aging were formulated more than 30 years ago (Curtis, 1966; Failla, 1958). Somatic mutations are associated mechanistically with age-related abnormalities in "proliferative homeostasis" and with progeroid aging syndromes (Martin 1991). An age-related accumulation of somatic mutations is likely to be due to a species-specific level of error proneness of DNA-processing enzymes (Burnet, 1974). Endogenous DNA-damaging factors are expected to be far more important for aging than environmental ones (Ames, 1983). Large quantities of DNA-oxidation products are excreted by mammals, corresponding to 10^5 oxidative DNA hits per cell per day in rats and 10^4 in humans (Shigenaga et al., 1989; Fraga et al., 1990). The rate of free radical generation, of their detoxification and of the removal/repair of damaged target molecules is specific for the species. In spite of the inherent logic of ascribing a great number of age-changes to the continuous induction of irreversible mutational alterations in DNA, the role of somatic instabilities of the genome in the etiology of aging has not been proven yet.

In order to properly investigate this issue, six aspects should be studied which may vary for each species, tissue and cell type separately: (1) the frequency at which somatic DNA-defects occur spontaneously; (2) the type of such defects; (3) their location in the nuclear or mitochondrial genome; (4) the stage at which they accumulate, since mutations occurring during development may result in somatic mosaicism; (5) the effect of the defects on the regulation of gene expression and (6) the relation between the rate at which DNA instabilities

arise and the aging rate.

1.6.2.1 Macroscopic chromosomal rearrangements

In a number of studies an age-related increase of gross chromosomal aberrations was detected by cytogenetic analysis of metaphase chromosomes. Among these abnormalities were chromosomal translocations, inversions, deletions, breaks, dicentric- and ring-chromosomes, and chromosome losses. A seven- and five-fold increase in chromosomal aberrations was found with age in mouse liver (Curtis, 1966) and kidney (Martin et al., 1985) cells, respectively. The age-associated increase in chromosomal aberrations in peripheral blood lymphocytes of human donors varied from two to nine-fold (Hedner et al., 1982; Prieur et al., 1988; Chapter 3 and 6). Anderson et al., (1988) found no increase in chromosomal aberrations in relation to increasing donor age. The incidence of chromosomal aberrations was found to be greater in females than in males (Hedner et al. 1982). The level of sister chromatid exchange (SCE) was found to increase with age and was also higher in females than in males (Soper et al., 1984). This finding was, however, not confirmed by the study of Dewdney et al. (1986).

In spite of the variation among these data, the majority of the studies concluded that spontaneous chromosomal abnormalities occur at a considerable rate in young individuals and that this level increases with age. In addition, it was demonstrated that chromosomes in lymphocytes from old donors are more susceptible to induction of DNA damage than those from young ones (Esposito et al., 1989). Aging is further associated with aneuploidy (Schneider, 1985) and a preferential loss of the inactivated X chromosome (Abruzzo et al., 1985).

These chromosomal abnormalities accumulate stochastically; no site-specific aberrations could be associated with aging, except for the loss of DNA sequences from the chromosome ends, the telomeres. Instability of the telomeres may result in telomere fusion, subsequent formation of ring chromosomes and breakage of the chromosomes involved. The subject of telomere alterations will be discussed later (section 1.6.2.5), since these involve changes in repetitive DNA structures which can not be detected macroscopically.

1.6.2.2 DNA damage and methylation

The data on spontaneous occurrence of DNA damage with aging, are controversial (as discussed thoroughly by Mullaart, 1990). An exponential accumulation of DNA damage, expected on the basis of the error-catastrophe theories, (see also section 1.6.1.1) was not found. On the whole DNA repair activities are comparable in young and old individuals. The inadequacy of repair systems, however, to remove 100% of the damage explains the gradual age-related accumulation of DNA damage that was frequently observed.

The rate at which such accumulation occurs and the type of damage depend on the tissue-

and cell-type. This is illustrated by the study of Mullaart et al. (1988) who found an age-related increase in the number of single strand DNA breaks in the post-mitotic parenchymal cells of the rat liver, while this could not be observed in the mitotic Kupffer cells of the same animals. DNA damage occurring in post-mitotic cells can only be restored by repair systems, not by replication. Especially in post-mitotic cells, a gradual accumulation of DNA damage is expected and may be most effectively involved in aging. An age-related increase in the number of modified bases was found in neurons obtained from human donors of different ages (Finch, 1990). In view of the key role of these cells in the organism, the consequences of such accumulation may be widespread. An exponential accumulation of somatic defects in all cell types therefore, seems not a prerequisite for an organismal catastrophe.

Apart from DNA damage, alterations in DNA methylation patterns have also frequently been associated with aging (see Chapter 3). By using high performance liquid chromatography (HPLC) analysis, an age-related overall loss of 5-methylcytosine (5-mC) from genomic DNA of various cell types of the mouse and human donors (Wilson et al., 1987; Drinkwater et al., 1989).

Age-related loss of 5-mC can have multiple causes, such as imperfect transmission during replication (Holliday, 1987), incomplete remethylation after excision repair (Kastan et al., 1982) and deamination of 5-mC to thymine. This last mechanism is particularly interesting, since it does not only result in a methylation change, but can also lead to a C-T transition mutation (Youssoufian et al., 1986).

It is conceivable that age-related changes in gene expression (section 1.6.1) stem from age-related changes in genomic methylation patterns. In mammalian cells, the methylation of cytosines is thought to occur largely at CpG dinucleotides. DNA methylation of specific CpG sites is associated with the control of tissue- and stage-specific gene transcription (Adams and Burdon, 1985), cell differentiation (Razin et al., 1984) and X-chromosome inactivation (Lock et al., 1987). In general, hypermethylation is associated with transcriptional silence (Cedar, 1988). Methylation controlled tissue-specific expression usually involves demethylation of particular sites at the 5' end of the gene in the tissue where the gene is expressed. Housekeeping genes, in contrast, generally contain unmethylated CpG rich "islands" at their 5' or 3' ends (Bird, 1986). These are only methylated on inactivated X chromosomes (Yang and Caskey 1987). Housekeeping genes are transcriptionally active in all cell types, although at different levels.

It was investigated whether alterations in methylation patterns could be involved in the age-related decrease of the TAT mRNA level in the rat liver, and the increased β-actin and GAPDH mRNA levels in the rat spleen (section 1.6.1.2; see also Chapter 5). Methylation patterns of these genes were investigated by Southern hybridization analysis using the isoschizomeric restriction enzymes HpaII and MspI. Although both enzymes recognize the same sequence (5'-CCGG-3'), HpaII does not cut the site when the internal cytosine is methylated (Waalwijk et al., 1978). The TAT gene was found to be hypomethylated in the liver as compared to spleen and brain at six CpG sites within the gene. Methylation at these sites, however, remained unchanged during aging. The β-actin gene, on the other hand, became slightly demethylated with age in spleen DNA at the single CpG site for which

tissue-specificity was observed; it was methylated in spleen but not in liver and brain. The demethylation was observed in DNA from the spleen of 24- and 36-month-old rats, while an increase in β-actin mRNA level was found only in the spleen from 36-month-old rats. These methylation changes at least precede the age-related increase in β-actin mRNA level, which could indicate that these events are mechanistically related. Such influence of methylation on the expression of a housekeeping gene would be quite exceptional, but so is the methylation difference of the site involved, between spleen and other tissues. In spite of tissue-specific and age-related variation in the GAPDH mRNA levels, no significant age-related or tissue-specific alterations were observed in the methylation pattern of this gene.

A large number of individual genes has now been examined for the occurrence of methylation changes (see Chapter 3). The data indicate that age-associated alterations in DNA methylation occur in some genes, but not in all. The overall demethylation as determined by HPLC is not generally reflected in the methylation patterns of individual genes. Thus, a role for DNA methylation changes in the age-related alterations of transcriptional control has not directly been confirmed. More CpG sites of regulatory relevance must be investigated to test the role of methylation changes in aging.

Methylation changes relate to the predictions from the evolutionary concepts as follows:

1. The overall loss of 5-mC from human and animal genomes with age may represent a stochastic process of demethylation. This process is determined by the rate of loss through mechanisms discussed above, and the rate of remethylation by methylases. The genes coding for methylases may be regarded as longevity genes. Pleiotropic or programmed alterations in the expression of these genes will have major consequences for the maintenance of developmentally imprinted methylation patterns.

2. Random demethylation may account for a stochastic scatter of quantitative changes in gene expression. Consistent methylation changes (in a larger proportion of cells) may form the basis for both (unprogrammed) pleiotropic effects and programmed events.

1.6.2.3 Instability of gene sequences

Investigations into the frequency of somatic mutations indicated large variabilities among healthy human individuals (see, for a review, Mohrweiser and Jones, 1990). This has been subscribed to environmental factors and to donor factors such as age, sex and life-style habits. Donor age had the only significant effect on somatic mutation variability (Davies et al., 1992). Somatic mutation analysis of endogenous DNA sequences of the human genome were performed mainly at four different loci (see also Chapter 3 and 7):

1. The human hypoxanthine phosphoribosyltransferase (HPRT) gene and the rodent adenine phosphoribosyltransferase (APRT) gene, both selectable in T-lymphocytes, using 6-

thioguanine or other purine analogues as a selective agent.

2. The human lymphocyte antigen (HLA) locus on chromosome 6, which gene codes for multi-allelic cell surface antigens the presence of which can be detected by allele specific antibodies.

3. The glycophorin A (GPA) locus on chromosome 4. Red blood cells are used as the reporter cells in assays to measure the frequency of variants of this locus.

By far, the most information about *in vivo* somatic mutations comes from studies of the HPRT locus in lymphocytes. The general consensus emerging from these studies is that the spontaneous somatic mutation frequency increases with age. It raises from 0.6×10^{-6} in newborns via 6×10^{-6} in young adults to 16×10^{-6} in aged twins (Carrano, 1989). The frequency of HPRT mutants in mouse splenic T cells increased from 0.9×10^{-5} to 1.5×10^{-5} (Inamizu et al., 1986) with age, whereas in mouse kidney and muscle cells, the frequency (5.0×10^{-5}) remained unaltered (Horn et al., 1984). These data illustrate that somatic mutation frequencies vary among species, tissues and cell types. From the human HPRT studies, it could be concluded that minor spontaneous mutations, base substitutions and somatic deletions, predominate in adult humans whereas major mutations such as gross deletions or other alterations, predominate in lymphocytes of the cord blood from newborns (McGiniss and Albertini, 1989). The difference may be due to the action of recombinases associated with T cell-antigen receptor gene maturation in the thymus (Marrack and Kappler, 1987). Such early occurring mutations might, by introducing somatic mosaicism, be of major relevance for the functional capacities of the aging organism.

Since the HPRT mutation assay is based on selection, only mutations that abolish gene activity are detected. This suggests that the somatic mutation frequency in the HPRT gene is likely to be higher than suggested by these data. In fact, the frequency of mutations involving the HLA-A locus is 10-fold higher than the frequency of HPRT-deficient lymphocytes (Janatipour et al., 1988). This may reflect differences in selection (with HLA in favour of variability and with HPRT probably against). This different selection may well have produced different mutation mechanisms at each locus.

Spontaneous deletions at the selected HLA-A locus were frequently coupled with loss of heterozygosity (LOH) for the unselected HLA-B locus (Turner et al., 1988), which is located 0.8 cM apart from the HLA-A locus. LOH can be the result of a) the loss of one allele (hemizygosity) or b) the loss of one allele and duplication of the retained allele (homozygosity). The LOH events at the HLA locus frequently involved duplication of the unselected HLA-A allele by mitotic recombination, gene conversion, or chromosomal loss and duplication. The occurrence of LOH at genomic regions of considerable size, by which mechanism genes carrying recessive mutations become uncovered, is one of the basic steps in the pathogenesis of many types of cancer (Lasko, 1991).

The mutation frequency at the GPA locus was 1 to 33 in 10^6 cells (Jensen et al., 1987). A difficulty about the GPA somatic mutation detection is the fact that mutants are estimated

at the level of expression so that epigenetic alterations can not be excluded. The increase in frequency of mutant cells in older individuals is greater for GPA than for HPRT. This observation may point at an age-related loss of epigenetic control as discussed in the previous section. Although only a few genes are being studied at present, it is evident that loci differ in their roles as reporters of somatic mutation.

The spectrum of spontaneous mutations indicated specific events at the nucleotide level. For example, in the APRT gene of Chinese hamster cells *in vitro* (de Jong et al., 1988) predominance for GC to AT transition was detected at a prominent hot spot in the gene (25% of the mutations), which resembles the germ cell hot spot (Cooper and Clayton, 1988; Cooper and Krawezak, 1990).

The mutation frequency as measured in mitotically active blood lymphocytes probably differs from that in post-mitotic cells. Mutations in the latter, however, are difficult to measure. A study of Van Leeuwen et al. (1989) revealed thus far unknown manifestations of genomic alterations in the hypothalamic neurons of Brattleboro rats. These rats are homozygous for a single-base deletion in the vasopressin gene, which is the cause of diabetes insipidus in these animals. In the neurons of these rats, an age-related increase of reverse mutations was observed. The reversion was suggested to result from gene conversion of the mutated vasopressin and the highly homologous oxytocin gene, which is positioned close to the vasopressin gene. The number of neurons which reversed to normal vasopressin production increased with age from 0.1% to 3%. This would be the most dramatic evidence for a spontaneous mutational hot spot in post-mitotic somatic cells. And, if the mechanism is substantiated, this would be the first example revealing the presence of a gene conversion mechanism in post-mitotic cells.

We may conclude that the frequency of somatic gene mutations varies from locus to locus at least ten-fold. Locus structure and function, constrains on mechanisms of mutation *in vivo*, selection against certain mutant cells and ease of molecular analysis of recovered mutations are all relevant. Although the somatic mutation frequency at each locus increased with age, it remained rather low. The data discussed thus far do not support widespread instability of gene sequences to be generated with age.

The next section deals with the instability of repetitive DNA-structures, randomly dispersed putative mutation hot spots. Since most of these loci are not coding for proteins, the instability can be monitored largely in the absence of selective pressure.

1.6.2.4 Instability of repetitive DNA structures

Mutation frequencies in both germ and somatic cells can vary at the basepair level, among different genes and gene regions (e.g. exons vs introns, transcribed vs untranscribed strands) and from one chromosomal region to another. Mutation-prone sites, are a common phenomenon among the characterised human disease loci (Cooper and Clayton, 1988). Such mutation hot spots have been found at the human insulin gene (Chakravarti et al., 1986), around the fragile X (Oberlé et al., 1985) and at the Duchenne muscular dystrophy (DMD)

locus (Wapenaar et al., 1988). Many of these loci contain repetitive DNA which is a mediator of genetic instability. The rate of spontaneous germ line mutations at minisatellite loci, for example, varies from $4x10^{-3}$ to $5x10^{-2}$ per DNA fragment per gamete (Jeffreys et al., 1988). The variation depends on the locus, with the same loci expressing high mutation frequencies in both germ and somatic cells (Jeffreys et al., 1990). Minisatellites consist of blocks of tandemly arranged repetitive DNA dispersed over the genome of almost all mammalian species. Mutations at these loci include single base changes and changes in the number of repeats per block. Such length variations mainly result from polymerase slippage during replication.

Some repetitive DNA elements are highly unstable because they can undergo transposition from one location to another. Such elements are also assumed to reside in the human genome. Transposition, however, thus far appears to occur at a very low rate in the germ line. For *Drosophila melanogaster* it has been demonstrated that the so-called P element is mobile in both germ and somatic cells. Only recently it has been reported that transposition of this element in somatic cells reduces the life span of *Drosophila* (Woodruff 1992).

To scan for hot spots of DNA sequence variation, a novel technique was developed, based on the separation in two dimensions of restriction enzyme digested genomic DNA followed by hybridization analysis, using minisatellite and simple sequence motifs as probes (Chapter 6). DNA-sequence alterations within or surrounding the repeat loci result in variant migration patterns of the DNA fragments during electrophoresis, or in the disappearance of spots (e.g. for LOH-events). When this technique was applied to the study of human tumours, variations in the 'spot'patterns between tumour and normal DNA from the same patient were frequently detected. The two-dimensional (2-D) DNA-typing system appeared to lend itself well to the random analysis of the aging genome for hot spots of somatic instability (Vijg et al., 1987).

The 2-D technique was used for the analysis of clones from primary fibroblast cultures established from skin biopsies of rats of different ages (Chapter 7). Spot variants were detected between the clones at frequencies varying from $0.7x10^{-3}$ to $4.5x10^{-3}$ per analyzed DNA fragment. Assuming that these spot variations represent mutations, the somatic mutation frequency at these loci is high, as was expected from the data on germ line mutations of minisatellite loci (see above). Age-related variations in the somatic mutation frequency of these genomic regions, however, were not observed in the fibroblast clones derived from young and old rats. The mutation frequency measured might either represent genetic heterogeneity of cells *in vivo* within the tissues biopted, or mutations generated during cell passage in tissue culture.

1.6.2.5 Instability of the human telomeres

The ends of human chromosomes consist of tandem arrays of the (TTAGGG)n repetitive motif (Moyzis et al., 1988). Comparable motifs are found at the telomeres of many other species. In human somatic cells, the telomeric repeat arrays undergo a length reduction as

a function of age. *In vitro* telomere reduction occurs progressively with serial passage of human fibroblasts. Moreover, the initial telomere length of these cells predicts their replicative capacity (Harley et al. 1990; Allsopp et al. 1992). The average length of telomeric restriction fragments (TRFs) in white blood cells and colorectal mucosa was found to be shorter in old than in young human individuals, corresponding with a rate of telomere loss of 33 bp per year *in vivo* (Hastie et al., 1990). It was postulated that telomere shortening ultimately leads to cell-cycle exit and cellular senescence (Harley, 1991).

A loss of telomeric repeats occurs at the discontinuously replicating DNA strand during each cell cycle. Removal of the RNA primer at this strand results in a 3' terminal overhang; thus, the DNA molecule will loose sequences from the telomeric end with each round of replication. In germ cells, this mechanism is compensated by the activity of the telomerase enzyme, which repairs telomeric repeat gaps (Blackburn, 1991). Telomerase activity may be regarded as a vital function contributing to the indefinite lifespan of the germline, which is lacking in somatic cells. Telomerase may become reactivated in somatic cells during tumorigenesis and immortalization in culture. The absence of telomerase activity in normal somatic cells and hence, the loss of telomeric DNA, is considered a recording system of the number of replication rounds. Beyond a critical length, telomere shorting may result in instability of flanking genomic regions, in chromosomal aberrations and it may effect expression of adjacent genes (see also section 1.6.2.1, Chapter 8) (for reviews, see Broccoli and Cooke, 1993; Biessmann and Mason, 1992). The fact that the highest concentration of genes and transcriptional activity in the human genome is present at the telomeres (Saccone et al., 1992) indicates the relevance of investigating the origin and effects of somatic alterations at these regions.

In addition to differences in TRF length between age groups, a considerable variation was observed among subjects of the same age at all ages (15-80 years) studied by Hastie et al.(1990). If telomere length reduction is relevant to aging, TRF length might be a marker of the biological age of the individual. From this perspective, two important questions arise: how the individual rate of telomere loss is determined as a function of age and whether a constant amount of telomeric DNA is lost with each cell replication that occurs during life. In order to investigate the possibility that the individual rate of telomere loss is genetically determined, the mean TRF length in white blood cells from mono- and dizygotic human twins of different ages was measured (Chapter 8). The TRF size can be determined by Southern hybridization analysis of digested genomic DNA, using a radioactively labelled (TTAGGG)n sequence as a probe. Since the TRF length of different chromosomes varies from cell to cell, the hybridization pattern is a smear. The average TRF length of blood DNA was determined by densitometric scanning of these smeared patterns. The mean TRF length in twins of ages between 2 and 95 years decreased in a range from 10 to 5 Kb. The variation in average telomere length among unrelated twin pairs of the same age group was maximally 3 Kb. TRF length variation among pairs (unrelated individuals), was found to be higher than the variation within twin pairs. The variation within monozygotic twin pairs was found to be the smallest. Statistical analysis and model-fitting of our data indicated a heritability of 60% in 4-year old twins and 85% in 17- and 44-year old individuals. The data

suggest that individual differences in TRF length are almost entirely genetic in origin (Chapter 8).

Telomere length variation between individuals of the same age may be explained in four ways: 1) variation in initial telomere length in the germ line; 2) variation in incidental decrease of telomeric repeats in somatic cells at some time point after conception; 3) variation in the number of telomeric repeats lost at each cell division, or 4) variation in turn over rate of cells at a constant loss of telomeric repeats per cell division. Most likely, a combination of these mechanisms accounts for the interindividual variation.

Telomeric repeat loss could contribute to a decline of cellular functions if this mechanism increases the risk for chromosome fusion, mutations in flanking genes or alterations in the stability of expression of these genes by chromatin changes. Indeed, an increase in terminal chromosomal rearrangements was observed with age (Bender et al., 1989). Data from the study of telomere length in transformed human embryonic kidney cells (Counter et al., 1992) indicated that chromosomal instabilities indeed occur as well as increased cell death, when a critical minimum telomere length of 1.5 Kb is passed. Even in the oldest individuals included in our study, however, such critical values are not reached (Chapter 8).

1.6.3 Instability of the mitochondrial genome

It has been proposed, that accumulation of mutations in the mitochondrial genome is a major factor contributing to human aging and to degenerative diseases (Linnane et al., 1989). Human mtDNA is a 16 kb closed circular molecule which encodes 13 sub-units of the mitochondrial energy transducing system, oxidative phosphorylation (OXPHOS), plus the mitochondrial ribosomal and transfer RNA genes. The OXPHOS system in mitochondria is the main source of adenosine triphosphate (ATP) for a variety of organs and tissues. The biogenesis of OXPHOS requires, apart from the mitochondrial genes (Wallace, 1992a), hundreds of nuclear genes (Anderson et al., 1981).

By biochemical and molecular studies it was established that the OXPHOS system declines with age in rats and humans (section 1.6.1.1). This decline is associated with an increase in mtDNA damage. A variety of low-frequency insertion-deletion mtDNA mutants accumulate in old rats (Piko et al., 1988). In heart, liver and blood of human donors of ages above 35, an accumulation of distinct mtDNA deletions, the most common of which has a length of 5 kb, was observed (Cortopassi and Arnheim, 1990). The proportion of deleted mtDNA to undamaged DNA increases with age. The level of 8OH-dG and mtDNA deletions were increased in diaphragm muscle from aged human individuals (Hayakawa et. al., 1991). Interestingly, a regional variability of the age-related increase in the frequency of mtDNA deletions was observed in the human brain (Corral-Debrinsky et al., 1992). In the cortex, the deleted to total mtDNA ratio ranged from 0.00023 to 0.034 in 67 to 80-year-old subjects, whereas the cerebellum remained relatively devoid of mtDNA deletions. This can be compared to a ratio of 0.00007 in ischaemic hearts.

An exponential increase in mtDNA deletions might contribute to muscle weakness, declining mental capacity or the development of presbycardia, a non-atherosclerotic heart dysfunction, associated with old age. In fact, heritable mitochondrial mutations have already been associated with a number of degenerative diseases, the clinical manifestations of which include blindness, deafness, dementia, movement disorder, cardiac failure, diabetes, renal and liver dysfunction. Most of the heritable diseases associated with mtDNA deletions progress with age. The fact that the manifestations mentioned above are also found in a variety of common degenerative diseases, among which late-onset diabetes mellitus, Alzheimer's, Parkinson's and Huntington's disease (Wallace, 1992a,b) might suggest that mitochondrial diseases may be more common than thus far recognized. Although there is no direct evidence that somatic mtDNA mutations cause these diseases, it is interesting that they affect those brain regions where the highest levels of mtDNA deletions were observed.

The most likely cause for an age-related increase in mtDNA damage is oxidation. MtDNA is 16 times more prone to oxidative damage than nuclear DNA (Richter et al., 1988). This may be due to the lack of protective histones and the limited DNA repair systems (Ames, 1989; Bandy and Davison, 1990). The high information content of the mitochondrial genome is expected to result in a high frequency of gene mutations. Thus far however, the occurrence of point mutations in the mitochondrial genome has not been studied in relation to aging.

Even though a significant proportion of mtDNA's may be defective in elderly people, each cell contains hundreds of mitochondria and thousands of copies of the mitochondrial genome. tDNA has a considerable turnover both in mitotic and post-mitotic cells. This raises the question how defective mtDNA's could accumulate to such levels that might explain the age-related decline in mitochondrial function. This can be explained since deleted mtDNA molecules have a replicative advantage because mtDNA replication is directly proportional to the length of the molecule. Since all mtDNA replication enzymes are encoded by the nucleus (Wallace, 1992), deletions in the mitochondrial genome (except for those at the origin of replication) will not inhibit the replication of such genomes. Deleted mtDNA molecules may be generated during local stagnation of replication as a result of oxidative damage. At this point slip-replication or recombination may occur at repetitive sequences which would lead to deletions.

1.7 Summary of General Introduction

There are two major lines of research from which insight into aging phenomena may be expected: one line poses the *ultimate* question of why organisms age, the second the *proximate* question of how organisms age. From the first question three concepts have emerged which explain aging essentially as:

1. (a) programmed process(es);
2. a by-product of selection, executed by gene variants with late-acting deleterious and pleiotropic effects and

3. the result of insufficient energy resources spent on the achievement of indefinite somatic maintenance in favour of optimal reproduction.

Examples of all three concepts can be found among different species. From life span studies it can be concluded that the most critical and life span-limiting function may vary among species. If such a function is a single factor, a small increase of the maximum life span can be obtained by improving that factor until the next dominant factor becomes life-limiting. More likely, a network of functional steps determines the life span, in which case a significant increase in life span can only be obtained if all the steps of the network are altered concordantly. Within the limits of the genetic constitution of a species, epigenetic variations may exert a considerable effect on individual and strain-specific life span. The studies directed to discovery of life span determining genes, although informative, do not necessarily lead to conclusions regarding a unifying basis for aging in different species, even if there is any. Simple organisms are very suitable for the investigation of evolutionary principles, even if their cause of death is not known. Extrapolation to species exerting a diverse spectrum of pathological conditions with age, however, may be a bit far-fetched.

Possibly such studies can be better performed along the proximate line of aging research. Three main predictions emerging from the evolutionary concepts which may be helpful tools in this field are the following.

1. Species-specific programmed events, processes and accompanying gene expression patterns and products are expected to be identified, which play a major role in the age of onset of diseases that contribute to an increased chance of dying and in other causes of death (by behavioral mechanisms, for example).

2. Species-specific gene variants may be identified in the germ line, the structure and/or the expression patterns of which have late detrimental effects. Early effects of such gene variants may be beneficial for reproductive fitness, or neutral.

3. Somatic defects are expected to accumulate in somatic tissues at a frequency determined by the rate at which such defects are spontaneously generated, and the (species-specific) quality of a network of somatic maintenance activities executed by longevity genes. The defects are known to arise from exogenous, and especially form endogenous sources.

These predictions were used to discriminate patterns of physiological alterations and mechanisms which may underlie a decreased functionality of homeostasis, structure and reproduction with age in species for which aging is associated with multiple pathological conditions. Intrinsic age-associated alterations at the protein expression level occur with age in most of the tissues and cells of the species studied thus far. The constitutive expression levels of housekeeping, tissue-specific and inducible genes becomes affected with age. In addition, the inducibility and extinction of the latter type of genes are delayed and/or decreased with age. Some of these effects involve changes at the level of gene transcription which may, among others, be caused by alterations in DNA methylation, DNA-protein

interactions and availability of hormones, transcription factors and/or their receptors. Other effects at the protein level, involve post-transcriptional changes and post-translational modifications.

These intrinsic age-changes in protein expression may be epigenetically controlled (via hormone levels, for example), in which case a programmed sequence of deleterious events may be expected to occur. This has not been established as a universally operating mechanism. Alternatively, the effects described may reflect a loss of epigenetic, developmentally imprinted control, which may be due to or associated with damage to biomacromolecules. These possibilities reflect the predictions described above. These changes may form the basis for functions which clearly become affected with time, such as a loss of homeostatic capacity. Molecular alterations appear to start occurring relatively early in life, in the absence of plain disease and may finally contribute to disease.

Diverse somatic instabilities (both programmed and stochastic) of nuclear and mitochondrial DNA increase with age. However, in order to properly investigate their relevance for aging, 6 aspects should be studied, which may vary for each species, tissue and cell type separately:

1. the frequency at which somatic DNA defects occur spontaneously;
2. the type of defects;
3. the location within the nuclear and mitochondrial genome at which they occur;
4. the effect of the defects on the regulation of gene expression;
5. the time frame in which most of the somatic defects are generated; and
6. the relation between spontaneous DNA instabilities, the aging rate and development of age-related pathology.

In general, the frequency of mutations is low. Both in the nuclear and mitochondrial genome of mitotic and post-mitotic cells, a diverse spectrum of DNA defects accumulates as a function of age. Amongst a stochastic accumulation some site-specificity is observed at hot spots of instability corresponding to sites of instability in the germ line. Mutations are generated at a relatively high frequency at birth. Their effect with respect to gene expression patterns, to aging and disease, however, still has to be assessed.

Especially in cell types involved in systemic functions, gradual accumulation of DNA damage and mutations may be involved in aging. An exponential accumulation of somatic defects in all cell types seems not a prerequisite for an organismal catastrophe. The shortening of human telomeres, whether important to aging or not, shows that replication is not equal to cell renewal. If telomere shortening is a programmed process in humans, purposefully prevented in the germ-line, and if cell-cycle exit and functional repression and accumulation of somatic DNA defects in subtelomeric regions occurs as a consequence, telomere shortening may be (part of) a cell-based organismal aging programme. This would illustrate the overlap between different concepts of aging.

References

Abruzzo MA, Mayer M, Jacobs PA. Aging and aneuploidy: evidence for the preferential involvement of the inactive X chromosome. Cytogenet Cell Genet 1985; 39: 275-278.

Adams RLP, Burdon RH. Molecular Biology of DNA methylation. Springer Verlag, New York, 1985, pp 115-161.

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci 1992; 89: 10114-10118.

Ames BN. Dietary carcinogens and anti-carcinogens. Oxygen radicals and degenerative diseases. Science 1983; 221: 1256-1264.

Ames BN. Endogenous DNA damage as related to cancer and aging. Mutat Res 1989; 214: 41-46.

Anderson D, Jenkinson PC, Dewdney RS, Francis AJ, Godbert P, Butterworth KR. Chromosomal aberrations, mitogen-induced blastogenesis, and proliferative rate index in peripheral lymphocytes from 106 control individuals of the UK population. Mutat Res 1988; 204: 407-420.

Anderson S, Bankier AT, Barrel BG, De Bruijn MHL, Coulson AR, Drouib J, Eperois IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. Nature 1981; 290: 457-465.

Arking R, Dudas S, Buck s, Force A, Nicholson M and Baker G. Genetic and molecular analysis of longevity assurance genes in Drosophila. Abstractbook Molecular Biology of Aging. 1993

Bandy B, Davison AJ. Mitochondrial mutations may increase oxidative stress implications for carcinogenesis and aging? Mutat Res 1990; 8: 523-539.

Biessmann H, Mason JM. Genetics and molecular biology of telomeres. Adv Genet 1992; 30: 185-233.

Bird AP. CpG-rich islands and the function of DNA methylation. Nature 1986; 321: 209-213.

Blackburn EH. Structure and function of telomeres. Nature 1991; 350: 569-572.

Broccoli D, Cooke H. Aging, healing and the metabolism of telomeres. Am J Hum Genet 1993; 52: 657-660.

Burnet FM. Intrinsic mutagenesis: a genetic approach to aging. J. Wiley and Sons, New York, 1974.

Carrano AV. Summary of the workshop on mammalian in vivo somatic mutation. Genome 1989; 31: 458-459.

Castaneda M, Vargas R, Galvan S. Stagewise decline in the activity of brain protein synthesis factors and relationship between this decline and longevity in two rodent species. Mech Ageing Dev 1986; 36: 197-210.

Cedar H. DNA methylation and gene activity. Cell 1988; 53: 3-4.

Chakravarti A, Elbein SC, Permutt MA. Evidence for increased recombination near the human insulin gene: implication for disease association studies. Proc Natl Acad Sci USA 1986; 83: 1045-1049.

Chen J, Campbell TC, Li J, Peto R. Diet, life style and mortality in China: A study of the characteristics of 65 Chinese Counties. Oxford Univ Press, Oxford, UK, 1990.

Comfort A. The biology of senescence. 3rd ed. Edinburgh and London: Churchill Livingstone, 1979.

Cooper DN, Clayton JF. DNA polymorphism and the study of disease association. Hum Genet 1988; 78: 299-312.

Cooper DN, Krawezak M. The mutational spectrum of single base-pair substitutions causing human genetic disease: patterns and predictions. Hum Genet 1990; 85: 55-74.

Corral-Debrinsky M, Horton T, Lott MT, Shoffner JM, Flint Beal M, Wallace DC. Mitochondrial DNA deletions in human brain: regional variability and increase with advancing age. Nature Genet 1992; 2: 324-329.

Cortopassi GA, Arnheim N. Detection of a specific mitochondrial DNA deletion in tissues of older humans. Nucleic Acid Res 1990; 18: 6927-6933.

Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bachetti S. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 1992; 11: 1921-1929.

Curtis HJ. Biological mechanisms of aging. C.C. Thomas Springfield, Illinois, 1966.

Cutler RG. Evolution of human longevity: a critical overview. Mech Ageing Dev 1979; 9: 337-354.

Cutler RG. Dysdifferentiative hypothesis of aging: a review. In: Molecular biology of aging: gene stability and expression. Sohal RS et al, eds. Raven Press, New York, 1985, pp 307-340.

Cutler RG. Human longevity and aging: possible role of reactive oxygen species. Ann N Y Acad Sci 1991; 621: 1-28.

Davies KJA. Proteolytic systems as secondary antioxidant defenses. In: Cellular antioxidant defense mechanisms Vol 2. Chow CK et al, eds. CRC Press, Boca Raton, FL, 1988, pp 25-67.

Davies MJ, Lovell JP, Anderson D. Thioguanine-resistant mutant frequency in T-lymphocytes from a healthy human population. Mutat Res 1992; 265: 165-171.

De Jong PJ, Grosovski AJ, Glickman BW. Spectrum of spontaneous mutations at the APRT locus of Chinese hamster ovary cells: an analysis at the DNA sequence level. Proc Natl Acad Sci USA 1988; 85: 3499-3503.

De Leeuw WJF, Slagboom P, Vijg J. Quantitative comparison of mRNA levels in mammalian tissues: 28S ribosomal RNA level as an accurate internal control. Nucl Acids Res 1989; 17: 10137-10138.

Dewdney RS, Lovell DP, Jenkinson PC, Anderson D. Variation in sister chromatid exchanges among 106 members of the general UK population. Mutat Res 1986; 171: 43-51.

Dice JF, Goff A. Error catastrophy and aging: future directions of research. In: Modern biological theories of aging. Warner HR et al, eds. Raven Press, New York, 1987, pp 155-168.

Dilella AG, Chiang JY, Steggles AW. The quantitation of liver cytochrome P450-LM_2 mRNA in rabbits of different ages and after phenobarbital treatment. Mech Ageing Dev 1982; 19: 113-125.

Drinkwater RD, Blake TJ, Morley AA, Turner DR. Human Lymphocytes in vivo have reduced levels of methylation in transcriptionally active and inactive DNA. Mutat Res 1989; 219: 29-37.

Ebert R, Cherkasova V, Dennis R, Wu J, Ruggles S, Perrin TE and Shmookler Reis R. Life span-determining genes map to at least 7 chromosomal loci in C. Elegans. Abstractbook Molecular Biology of Aging. 1993.

Edwards JH. Familiarity, recessivity and germ line mosaicism. Ann Hum Genet 1989; 53: 33-47.

Egilmez NK, Rothstein M. The effect of aging on cell-free protein synthesis in the free-living nematode Tubatrix aceti. Biochim Biophys Acta 1985; 840: 355-363.

Esposito D, Fassina G, Szabo P, De Angelis P, Rodgers L, Weksler M, Siniscalco M. Chromosomes of older humans are more prone to aminopterin-induced breakage. Proc Natl Acad Sci USA 1989; 86: 1302-1306.

Everitt A, Meites J. Aging and anti-aging effects of hormones. J Gerontol 1989; 44: B139-B141.

Failla G. The aging process and carcinogenesis. Ann N Y Acad Sci 1958; 71: 1124-1135.

Finch CE. Longevity, senescence and the genome. University of Chicago Press, Chicago, IL, 1990.

Finch CE. Mechanisms in senescence: some thoughts in April 1990. Exp Gerontol 1992; 27: 7-16.

Fraga CG, Shigenaga MR, Park JW, Degan P, Ames BN. Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc natl Acad Sci 1990; 87: 4533-4537.

Friedman DB, Johnson TE. A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. Genetics 1988a; 118: 75-86.

Friedman DB, Johnson TE. Three mutants that extend both mean and maximum life span of the nematode, Caenorhabditis elegans, define the age-1 gene. J Gerontol 1988b; 43: B102-B109.

Gresik EW, Wenk-Salamone K, Onetti-Muda A, Gubits RM, Shaw PA. Effect of advanced age on the induction by androgen or thyroid hormone of epidermal growth factor and epidermal growth factor mRNA in the submandibular glands of C57Bl/6 male mice. Mech Ageing Dev 1986; 34: 175-189.

Harley CB. Telomereloss: mitotic clock or genetic time bomb? Mutat Res 1991; 256: 271-282.

Harley CB, Futcher AB, Greider CW. Telomeres shorten during aging of human fibroblasts. Nature 1990; 345: 458-460.

Harman D. The aging process. Proc Natl Acad Sci 1981; 78: 7124-7128.

Harman D. Free radical theory of aging. The "free radical" diseases. Age 1984; 7: 111-131.

Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colorectal carcinoma and with ageing. Nature 1990; 346: 866-868.

Hayakawa M, Torii K, Sugiyama S, Tanaka M, Ozawa T. Age-associated accumulation of 8-hydroxydeoxyguanosine in mitochondrial DNA of human diaphragm. Biochem Biophys Res Commun 1991; 179: 1023-1029.

Hayflick L. Theories on biological aging. Exp Gerontol 1985; 20: 145-149.

Hayflick L. Aging under glass. Mutat Res 1991; 256: 69-80.

Hayflick L, Moorhead PS. Recent advances in the cell biology of aging. Exp Cell Res 1961; 25: 585-621.

Hedner K, Högstedt B, Kolnig AM, Mark-Vendel E, Strömbeck B, Mitelman F. Sister chromatid exchanges and structural chromosome aberrations in relation to age and sex. Hum Genet 1982; 62: 305-309.

Holliday R. The inheritance of epigenetic defects. Science 1987; 238: 163-170.

Horbach GJMJ, Van Bezooijen CFA, Knook DL. Age-related changes in the sythesis of individual liver-specific proteins. Rev Biol Res in Aging 1987; 3: 485-494.

Horn PL, Turker MS, Ogburn, CE, Disteche CM, Martin GM. A cloning essay for 6-thioguanine resistance provides evidence against certain somatic mutational theories of aging. J Cell Physiol 1984; 121: 309-315.

Inamizu T, Nobusha N, Chang M, Makinodan T. Frequency of 6-thioguanine-resistant T cells is inversely related to the declining T-cell activities in aging mice. Proc Natl Acad Sci USA 1986; 83: 2488-2491.

Janatipour M, Trainor KJ, Kutlaca R, Bennet G, Hay J, Turner DR, Morley AA. Mutations in human lymphocytes studied by an HLA selection system. Mutat Res 1988; 198: 221-226

Jeffreys AJ, Royle NJ, Wong Z. Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature 1988; 332: 378-281.

Jeffreys AJ, Neumann R, Wilson V. Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. Cell 1990; 60: 473-485.

Jensen RH, Bigbee WL, Langlois RG. *In vivo somatic mutations in the glycophorin* A locus of erythroid cells. In: Banbury Report 28: Mammalian Cell Mutagenesis. Moore MM, DeMarini DM, De Serres FJ, Tindall KR, eds. Cold Spring Harbour Laboratory, Cold Spring Harbour, 1987, pp 149-159.

Kahn M, Enesco HA. Effect of a-tocopherol on the life span of Turbatrisc aceti. Age 1981; 4: 109-115.

Kastan MB, Gowans BJ, Lieberman MW. Methylation of deoxycytidine incorporated by excision-repair synthesis of DNA. Cell 1982; 30: 509-516.

Kay MMB. Bands in aging and neurological disease. Ann N Y Acad Sci 1991; 621: 179-204.

Kirkwood TBL. Evolution of aging. Nature 1977; 270: 301-304.

Kirkwood TBL. Repair and its evolution: survival versus reproduction; in Townsend, Calow, Physiological ecology. 1981: 165-189.

Kirkwood TBL. Comparative evolutionary aspects of longevity. In: Handbook of the biology of aging, 2nd ed. Finch CE, Schneider EL, eds. Van Nostrand, New York, NY, 1985, pp 27-45.

Kirkwood TBL. Genetic basis of limited cell proliferation. Mutat Res 1991; 256: 323-328.

Kirkwood TBL, Cremer, T. Cytogerontology since 1881: a reappraisal of August Weismann and a review of modern progress. Hum Genet 1982; 60: 101-121.

Larsen PL. Increased life span exhibited by two new mutations in C. Elegans. Abstractbook Molecular Biology of Aging. 1993

Lasko D, Cavenee W. Loss of constitutional heterozygosity in human cancer. Annu Rev Genet 1991; 25: 281-314.

Lee AT, Cerami A. Modifications of proteins and nucleic acids by reducing sugars: possible role in aging. In: Handbook of the biology of aging, 3rd ed. Schneider EL, Rowe JW, eds. Academic Press, San Diego, 1990, pp 116-130.

Linnane AW, Ozawa T, Marzuki S, Tanaka M. Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases. Lancet 1989; March 25: 642-645.

Lithgow GJ, Tedesco P, Hutchonson EW, Melov SL, Jensen AJ, Duhon S and Johson TE. Molecular genetics of C. Elegans life span. Abstractbook Molecular Biology of Aging. 1993

Lock LF, Takagi N, Martin GR. Methylation of the HPRT gene on the inactive X occurs after chromosome inactivation. Cell 1987; 48: 49-46.

Luckinbill LS, Graves JL, Reed AH, Koetsawang S. Localizing genes that defer senescence in Drosophila melanogaster. Heredity 1988; 60: 367-374.

Makrides SC. Protein synthesis and degradation during aging and senescence. Biol Rev 1983; 58: 343-422.

Marrack P, Kappler J. The T-cell receptor. Science 1987; 238: 1073-1078.

Martin GM. Genetic and environmental modulations of chromosomal stability: their roles in aging and oncogenesis. Ann N Y Acad Sci 1991; 621: 401-417.

Martin GM, Curtis A, Sprague BS, Epstein CJ. Replicative life span of cultivated human cells. Lab Invest 1970; 23: 86-92.

Martin GM, Smith AC, Ketterer DJ, Ogburn CE, Disteche CM. Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice. Isr J Med Sci 1985; 21: 296-301.

Masoro IJ, Shimokawa I, Yu BP. Retardation of the aging process in rats by food restriction. Ann N Y Acad Sci 1991; 621: 337-352.

Matocha MF, Cosgrove JW, Atack JR, Rapoport SI. Selective elevation of c-myc transcript levels in the liver of the aging Fischer-344 rat. Biochem Biophys Res Commun 1987; 147: 1-7.

Maynard Smith J. Group selection. Q Rev Biol 1976; 51: 277-283.
McGiniss MJ, Albertini RJ. Deletions predominate in "spontaneous" hprt mutant T-cells of the human newborn, Environ Mol Mutagen 1989; 14: 229-237.
Medawar PB. An unsolved problem of biology. H.K. Lewis, London, 1952.
Meier-Rugge W, Hunziker O, Iwangoff P. Senile dementia: a threshold phenomenon of normal aging? A contribution to the functional reserve hypothesis of the brain. Ann N Y Acad Sci 1991; 621: 104-118.
Mergler NL, Goldstein MD. Why are there old people. Hum Dev 1983; 26: 72-90.
Miquel J, Lindseth K. Determination of biological age in anti-oxidant treated drosophila and mice. In: Intervention in the Aging Process. Pt. B. Basic Research and Preclinical Screening. Regelson W, Sinex M, eds. Alan R. Liss, New York, 1983, pp 317-358.
Mohrweiser HM, Jones IM. Review of the molecular characteristics of gene mutations of the germ line and somatic cells of the human. Mutat Res 1990; 231: 87-108.
Molnar JA, Alpert N, Burke JF, Young UR. Synthesis and degradation rates of collagens in vivo in whole skin of rats, studied with $^{18}O_2$ labelling. Biochem J 1986; 240: 431-435.
Moyzis RK, Buckingham JM, Scott Cram L, Dani M, Deaven LL, Jones MD, Meyne J, Ratcliff RL, Wu J. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci USA 1988; 85: 6622-6626.
Mullaart E. DNA damage and repair in relation to aging. Thesis, Krips Repro, Meppel, 1990.
Mullaart E, Boerrigter METI, Brouwer A, Berends F, Vijg J. Age-dependent accumulation of alkali-labiled sites in DNA of post-mitotic but not in that of mitotic rat liver cells. Mech Ageing Dev 1988; 45: 41-49.
Müller-Hocker J. Cytochrome-c-oxidase deficient cardiomyocytes in the human heart- an age-related phenomenon. A histochemical ultracytochemical study. Am J Pathol 1989; 134: 1167-1173.
Müller-Hocker J. Cytochrome c oxidase deficient fibres in the limb muscle and diaphragm of man without muscular disease: an age-related alteration. J Neurol Sci 1990; 100: 14-21.
Murty CVR, Mancini MA, Chatterjee B, Roy A. Changes in transcriptional activity and matrix association of α_{2u}-globulin gene family in the rat liver during maturation and aging. Biochim Biophys Acta 1988; 949: 27-34.
Norris K, Hornsby PJ. Cytotoxic effects of expression of human superoxide dismutase in bovine adrenocortical cells. Mutat Res 1990; 237: 95-106.
Oberlé I, Drayna D, Camerino G, White R, Mandel JL. The telomeric region of the human X-chromosome long arm: presence of a highly polymorphic DNA marker and analysis of recombination frequency. Proc Natl Acad Sci USA 1985; 82: 2824-2828.
Ohrloff C, Lange G, Hockwin O. Post-sythetic changes of glutathione peroxidase (EC 1.11.1.9) and glutathione reductase (EC 1.6.4.2) in the ageing bovine lens. Mech Ageing Dev 1980; 14: 453-458.
Oliver CN, Ahn B, Moerman EJ, Goldstein S, Stadtman ER. Age-related changes in oxidized proteins. J Biol Chem 1987; 262: 5488-5491.
Ono T, Cutler RG. Age-dependent relaxation of gene expression: increase of endogenous murine leukemia virus-related and globin-related RNA in brain and liver of mice. Proc Natl Acad Sci USA 1978; 75: 4431-4435.
Ono T, Dean RG, Chattopadhyay SK, Cutler RG. Dysdifferentiative nature of aging: Age-dependent expression of MuLV and globin genes in thymus, liver and brain in the AKR mouse strain. Gerotology 1985; 32: 362-372.
Orgel L. The maintenance of the accuracy of protein synthesis and its relevance to aging. Proc Natl Acad Sci USA 1963; 49: 517-521.
Orgel LE. Ageing of clones of mammalian cells. Nature 1973; 243: 441-445.
Pacifici GM, Viani A, Taddeucci-Brunelli G, Rizzo G, Carrai M, Schulz HU. Effects of development, aging and renal and hepatic insufficiency as well as hemodialysis on the plasma concentrations of albumin and alpha 1-acid glycoprotein: implications for binding of drugs. Ther, Drug Monit 1986; 8: 259-263.
Pierpaoli W, Dall'Ara A, Pedrinis E, Regelson W. The pineal control of aging. The effects of melatonin and pineal grafting on the survival of older mice. Ann N Y Acad Sci 1991; 621: 291-313.
Piko L, Hougham AJ, Bulpitt KJ. Studies of sequence heterogeneity of mitochondrial DNA from rat and mouse tissue: evidence for an increased frequency of deletions/additions with aging. Mech Ageing Dev 1988; 43: 279-293.

Post DJ, Carter KC, Papaconstantinou J. 1991. The effect of aging on constitutive mRNA levels and Lipopolysacharide inducibility of acute phase genes. Ann N Y Acad Sci 1991; 621: 66-77.

Prieur M, Achkar WAI, Aurias A, Couturier J, Dutrillaux AM, Flury-Herard A, Gerbault-Seureau S, Hoffschir F, Lamoliatte E, Lefrancois D, Lombard M, Muleris M, Ricoul M, Sabatier L, Viegas Pequignot E. Acquired chromosome rearrangements in human lymphocytes: effects of aging. Hum Genet 1988; 79: 147-150.

Reff ME. RNA and protein metabolism. In: Handbook of the biology of aging. Finch CE, Schneider EL, eds. VNR, New York, 1985, pp 225-254.

Regelson W. The evidence for pituitary and thyroid control of aging: is aging reversal, a myth or a reality? The search for a "death hormone". In: Interventions in the Aging Process. Regelson W, Sinex FM, eds. Alan R. Liss, New York, 1983, pp 3-52.

Regelson W, Franson R. Phospholipase A_2 as a death trigger in the aging process. The use of PLA_2 inhibitors as antiaging substances. Ann N Y Acad Sci 1991; 621: 262-276.

Reiter RJ. The melatonin message: duration versus coincidence hypothesis. Life Sci 1987; 40: 2119-2131.

Richardson A, Birchenall-Sparks MC, Staecker JL. Aging and transcription. In: Review of biological research in aging, Vol 1. Rothstein M, ed. A.R. Liss, New York, 1983, pp 275-294.

Richardson A, Rutherford MS, Birchenall-Sparks MC, Roberts MS, Wu WT, Cheung HT. Levels of specific messenger RNA species as a function of age. Aging 1985; 29: 229-236.

Richardson A, Semsei I. 1987. Effect of aging on translation and transcription. In: Review of biological research in aging, Vol 3. Rothstein M, ed. A.R. Liss, New York, 1987, pp 467-483.

Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci 1988: 85: 6465-6467.

Rõhme D. Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo. Proc Natl Acad Sci USA 1981; 78: 5009-5013.

Rose MR, Tyler RH, Spicer GS, Ayala FJ, Nusbaum TJ, Mueller LD, Graves JL and Fleming J. Genetic and physiological mechanisms of postponed aging in Drosophila. Abstractbook Molecular Biology of Aging. 1993

Rosenberger RF, Kirkwood TBL. Errors and the integrity of genetic information transfer. In: Accuracy in molecular processes. Kirkwood TBL, Rosenberger RF, Galas DJ, eds. Chapman and Hall, London, 1986, pp 17-35.

Rothstein M. Evidence for and against the error catastrophy theory. In: Modern biological theories of aging. Warner HR et al, eds. Raven Press, New York, 1987, pp 139-154.

Roy AK, Nath TS, Motwani NM, Chatterjee B. *Age-dependent regulation of the polymorphic forms of* α_{2u}-globulin. J Biol Chem 1983; 258: 10123-10127.

Russel ES. Genetic origins and some research uses of C57BL/6, DBA/2 and B6D2F1 mice. In: Development of the rodent as a model system of aging. Gibson DC, Adelman RC, Finch CE, eds. NIH Publication DNEW, Bethesda, MD, 1978, pp 37-44.

Rutherford MS, Baehler CS, Richardson A. Genetic expression of complement factors and α1-acid glycoprotein by liver tissue during senescence. Mech Ageing Dev 1986; 35: 245-254.

Saccone S, De Sario A, Della Valle G, Bernardi G. The highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes. Proc Natl Acad Sci 1992; 89: 4913-4917.

Sacher GA. Longevity, aging and death: an evolutionary perspective. Gerontologist 1978; 18: 112-119.

Seshadri T, Campisi J. Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts. Science 1990; 247: 205-209.

Schneider EL. Cytogenetics of aging. In: Handbook of the biology of aging. Finch CE, Schneider EL, eds. VNR, New York, 1985, pp 357-373.

Seto NOL, Hyashi S, Tener GM. Overexpression of Cu-Zn superoxide dismutase in Drosphila does not affect life span. Proc Natl Acad Sci USA 1990; 87: 4270-4274.

Sharma HK, Rothstein M. Altered phosphoglycerate kinase in aging rats. J Biol Chem 1980; 255: 5043-5050.

Shepherd JCW, Walldorf U, Hug P, Gehring WJ. *Fruit* flies with additional expression of the elongation factor EF-1α live longer. Proc Natl Acad Sci USA 1989; 86: 7520-7521.

Shigenaga MK, Gimeno CJ, Ames BN. Urinary 8-hydroxy-2'-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proc Natl Acad Sci 1989; 86: 9697-9701.

Sohal RS, Allen RG, Farmer KJ. Effects of exogenous antioxidants on the level of endogenous antioxidants, lipid-soluble fluorescent material and life span in the housefly, Muca domestica. Mech Ageing Dev 1985; 31: 329-336.

Sohal RS. The free radical hypothesis of aging: an appraisal of the current status. Aging Clin Exp Res 1993; 5; 1; 3-17.

Soper KA, Stolley PD, Galloway SM, Smith JG, Nichols WW, Wolman SR. Sister chromatid exchange (SCE) report on control subjects of occupationally exposed workers. Mutat Res 1984; 129: 77-88.

Stadtman ER, Oliver CN. Metal-catalysed oxidation of proteins: physiological consequences. J Biol Chem 1991; 266: 2005-2008.

Stein G. Failure to phosphorylate the retimoblastoma gene product in senescent human fibroblasts. Science 1990; 249: 666-670.

Strehler B. Genetic instability as the primary cause of human aging. Exp Geront 1986; 21: 283-319.

Tappel A, Fletcher B, Deamer D. Effects of antioxidants and nutrients on lipid peroxidation fluorescent products and aging parameters in the mouse. J Gerontol 1973; 28: 415-424.

Trounce I, Byrne E, Marzuki S. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in aging. Lancet 1989; 1: 637-639.

Turner DR, Grist SA, Janatipour M, Morley AA. Mutations in human lymphocytes commonly involve gene duplication and resemble those in cancer cells. Proc Natl Acad Sci USA 1988; 85: 3189-3192.

Van Leeuwen F, Van der Beek E, Seger M, Burbach P, Ivell R. Age-related development of a heterozygous phenotype in solitary neurons of the homozygous Brattleboro rat. Proc Natl Acad Sci USA. 1989; 86: 6417-6420.

Vijg J, Uitterlinden AG. A search for DNA alterations in the aging mammalian genome: an experimental strategy. Mech Ageing Dev 1987; 41: 47-63.

Waalwijk C, Flavell RA. Msp I, an isoschizomer of Hpa II which cleaves both unmethylated and methylated Hpa II sites. Nucl Acids Res 1978; 5: 3232-3236.

Wallace DC. Diseases of the mitochondrial DNA. Annu Rev Biochem 1992a; 61: 1175-1212.

Wallace DC. Mitochondrial genetics: a paradigm for aging and degenerative diseases? Science 1992b; 256: 628-632.

Wapenaar MC, Kievits T, Hart KA, Abbs S, Blonden LA, Den Dunnen JT, Grootscholten PM, Bakker E, Verellen-Dumoulin Ch, Bobrow M, Van Ommen GJB, Pearson PL. A deletion hot spot in the Duchenne muscular dystrophy gene. Genomics 1988; 2: 101-108.

Wareham KA, Lyon MF, Glenister PH, Williams ED. Age-related reactivation of an X-linked gene. Nature 1987; 327: 725-727.

Wellinger R, Guigoz Y. The effect of age on the induction of tyrosine aminotransferase and tryptophan oxygenase genes by physiological stress. Mech Ageing Dev 1986; 34: 203-217.

Williams GC. Pleiotropy, natural selection, and the evolution of aging. Evolution 1957; 11: 398-411.

Wilson VL, Smith RA, Ma S, Cutler RG. Genomic 5-methyldeoxycitidine decreases with age. J Biol Chem 1987; 262: 9984-9951.

Woodruff RC. Transposable DNA elements and life history traits. Transposition of P DNA elements in somatic cells reduces the life span of Drosophila melanogaster. Genetica 1992; 86: 143-154.

Wright WE, Shay JW. The two-stage mechanism controlling cellular senescence and immortalization. Exp Gerontol 1992; 27: 383-389.

Yang TP, Caskey C. Nuclease sensitivity of the mouse HPRT gene promotor region: differential sensitivity on the active and inactive X chromosomes. Mol Cell Biol 1987; 7: 2994-2998.

Yarom R, Sapoznikov D, Havivi Y, Avraham KB, Schickler M, Groner Y. Premature aging changes in neuromuscular junctions of transgenic mice with an extra human Cu/Zn SOD gene: a model for tongue pathology in Down's syndrome. J Neurol Sci 1988; 88: 41-53.

Yen TC, Yen YS, King KL, Yen SH, Wei YH. Liver mitochondrial function declines with age. Biochem Biophys Res Commun 1989; 165: 994-1003.

Youngman D, Kim Park JY, Ames BN. Protein oxidation associated with aging is reduced by dietary restriction of protein or calories. Proc Natl Acad Sci 1992; 89: 9112-9116.

Youssoufian H, Kazazian Jr HH, Phillips DG, Aronis S, Tsiftis G, Brown VA, Antonarakis SE. Recurrent mutations in haemophilia A give evidence for CpG mutation hotspots. Nature 1986; 324: 380-382.

Zurcher C, Hollander CF. Multiple pathological changes in aging rat and man. In: Research animals and concepts of applicability to clinical medicine. Gärtner K, Hackbarth H, Stolte H, eds. Exp Biol Med Vol 7, Karger, Basel, 1982, pp 56-62.

CHAPTER 2

SUMMARY AND GENERAL DISCUSSION

2.1 Summary and general discussion

In view of the multifactorial character of aging in higher species, the discussion of experimental work described in this thesis (Chapter 3-8) is integrated in a interpretation of aging as a multicausal dynamic process. This interpretation is based on three different angles from which studies on aging are usually performed: the control of longevity at the level of species and populations; the characteristics of the aging process of individuals and the molecular changes that may form a basis for such a process. Finally, the concepts and mechanisms discussed in this thesis will be evaluated in the context of the aging process of humans.

2.1.1 Evaluation of evolutionary concepts of aging

Unlike developmental processes, aging has not yet been recognized to persist and having developed from lower to higher species. Because of the absence of evolutionary selection on traits that influence late-life events without negatively affecting reproduction, a large diversity of mechanisms may underlie aging in different species. The mortality rate doubling time (MRDT) and the maximum life span, however, appear to be stable characteristics among different populations of the same species (Finch, 1990). This points to a strong genetic influence on aging. Genetic control of longevity in lower species has indeed been demonstrated in selection experiments and by construction of transgenic animals carrying candidate longevity genes (section 1.3). It is thus far not clear whether findings in these species can be extrapolated to gradually aging species such as humans, with MRDT's determined by multi-organ pathology. Aging in semelparous species is programmed and executed by a limited number of hormones. Generally it is believed that such programmed processes do not dominate mortality in gradually aging species. Except for some life-shortening genotypes, no genes or gene clusters have yet been shown to be directly involved in the control of the maximum life span of higher species.

The only manipulation that has thus far resulted in successful retardation of aging phenomena and in alteration of the MRDT of a mammalian species is the application of caloric restriction (CR), starting before maturity, in rodents (Masoro, 1992). There are three criteria that support this view: a) CR leads to expansion of both the MRDT and the

maximum life span; b) CR leads to retardation of numerous physiological age-changes and c) CR leads to retardation of age-associated diseases such as cancer. These effects are believed to result from a change in metabolic characteristics during CR, that enable carbohydrates and oxygen to be used in a less harmful fashion (diminished production of oxygen radicals and glycation products; section 1.3). The effects of CR illustrate that longevity is not only determined by existence of specific genes but also by factors that influence the control of gene expression. These factors are both of exogenous (diet, larval space, etc.) and endogenous (presence of oxygen radicals; damaged DNA and proteins, etc) origin. They may form the basis of the life span variation observed in inbred laboratory animals (section 1.3). Other tools than CR may become available that will reveal a longevity potential exceeding the maximum life span presently observed for laboratory animals.

In an aging population, different subgroups may be discriminated for which the nature of the factors determining longevity varies. The survival curve of gradually aging species can hypothetically be divided into three parts (Figure 1). The 'A' part would be dominated by the initial mortality rate (IMR), as a result of trauma, early onset diseases caused by severe genetic, physiological or developmental defects, etc.. Short-lived genotypes of mice (and inbred offspring) are frequently associated with early onset diseases, which may resemble late onset diseases, but do not otherwise generally accelerate senescence. These shorter-lived genotypes may ultimately reveal candidate genes for age-related diseases, if late and early onset versions of disorders have a common basis. Thus, interventions may be found more easily than in longer-lived genotypes where the disease occurs among many other age-changes.

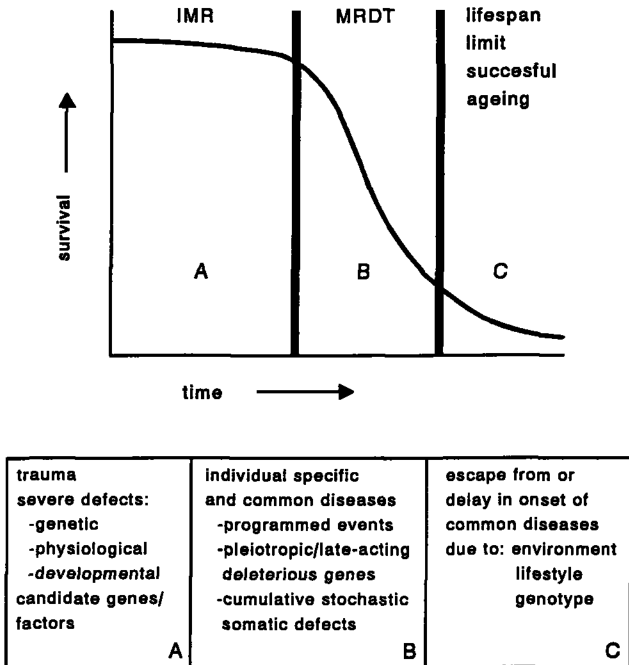

Figure 1. Hypothetical separation of subpopulations in a schematic survival curve of a gradually aging species.

The 'B' part of the curve is determined by the MRDT; mortality in this age-group is brought about by common and individual-specific susceptibilities and disease patterns. The type of disease and the rate and age of onset at which a disease becomes clinically expressed, are influenced by the genetic background and the aging process of the individual (see section 2.1.2). A better understanding of aging and diseases which dominate the mortality kinetics of part B may be obtained by applying the main predictions emerging from the evolutionary concepts of aging:

1. programmes with deleterious effects on cells and tissues executed by specific genes;
2. gene variants which, through their structure or levels of expression, have unprogrammed late-acting deleterious effects (diseases), and
3. networks of genes involved in somatic maintenance exerting their effect on longevity from the moment of conception.

Which of these is the most dominant genetic or epigenetic factor determining the MRDT of an aging population has not been established for any mammalian species.

The 'C' part of the curve is determined by relatively long-lived individuals. The process of aging in these individuals may occur more slowly than in other subjects, or more uniformly, not dominated by diseases arising in a single tissue or organ. Many physiological age-changes and dysfunctions are indeed retarded in these individuals and a general decline of organ reserve and impairment of homeostasis is present, which probably results in the diverse pathology at death (Eulderinck et al., 1993).

The variability of overall health status, disease patterns, pathological and molecular age-changes in part 'B' and 'C' increases. This even occurs in inbred laboratory animals (Zurcher and Slagboom, 1993). Studies aimed at the identification of the factors involved must be performed with subsets of individuals with comparable biological age (overall health status) and not only on the basis of chronological age. The study of specific subpopulations in the age-groups of section 'B' may lead to identification of genes determining the MRDT, which may represent functions involved in developmental programmes that set the homeostatic capacity, and risk alleles for late-onset diseases (late-acting deleterious alleles). The study of exceptionally healthy subpopulations in section 'B' and 'C' may reveal genes involved in protection from common disease patterns, and successful alleles of somatic maintenance (longevity) genes involved in functions such as DNA repair/proofreading, accuracy of protein synthesis, protein turnover, oxygen radical scavenging and acute phase response. These genes are expected to be organized as networks, the quality and synchronization of which may vary greatly from lower to higher species.

2.1.2 The aging process of individuals

Three determinants are of major influence on the physiological alterations occurring throughout the lifetime of an individual organism: genotype, lifestyle and environment (see Figure 2). From the moment of conception till the moment of death, patterns of gene expression and functional capacity of biomacromolecules are a resultant of interactions

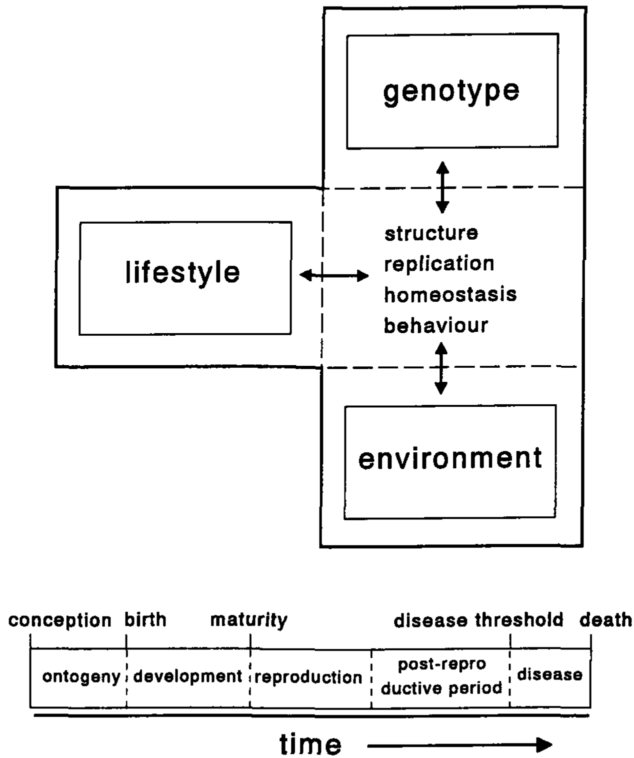

Figure 2. Factors involved in the generation of time-related alterations in physiological components of an aging organism.

between these determinants. Alterations in the physiology of the individual that are thus generated, can be monitored in structural components, in replicative capacity of germ and somatic cells, in homeostatic capacity of adaptive, immune and reproductive systems and in behavioral aspects. Among structural components are long-lived molecules of the nuclear envelope, cytoskeleton, membranes, extracellular matrix, chromatin (DNA, histones and methylation), etc.. Through their influence on the physiology of the individual, the three determinants influence each other as well, within a certain range. An altered environment, for example, may lead to altered patterns of food intake and behaviour and subsequent changes in metabolic parameters, health status and life span.

In Figure 3, the connection and mutual influence of deleterious age-changes, homeostatic decline and pathophysiological processes is schematically represented. This pathway will determine the individual pattern and age of onset of disease and mortality. Logically, the etiology of aging comprises individual and common physiological and pathological elements. This is reflected in the age-related alterations of the tyrosine aminotransferase (TAT), the glyceraldehyde dehydrogenase (GAPDH) and ß-actin mRNA levels in inbred rats (Chapter 4 and 5). The decreased TAT mRNA level in the liver of all 24- and 36-month-old inbred rats as compared to 6-month-old animals (Chapter 5), may reflect a common mechanism of aging (section 1.6.1.2). Alterations in the expression of inducible genes such as those involved in the acute phase response, for example, may underlie a general decline of

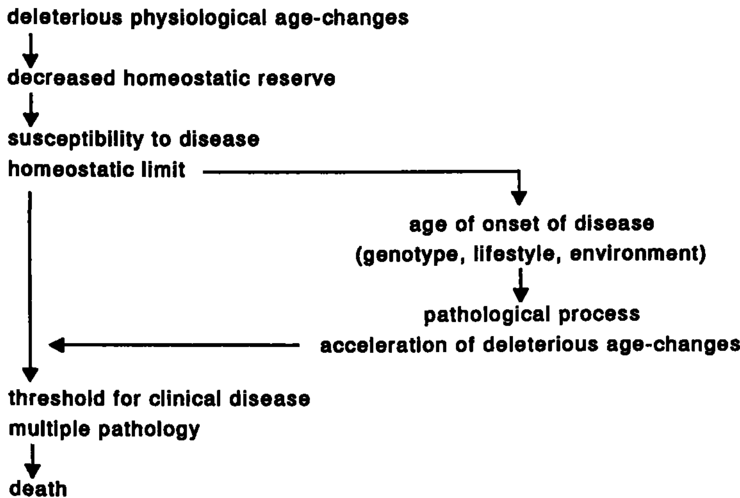

Figure 3. Schematic representation of the possible contribution of random deleterious changes in cells to organismal aging.

homeostasis. The individual variation in life span and pathophysiological processes even observed in inbred rats, however, can not be explained by common molecular alterations. The study in depth of mechanisms involved in the variability of GAPDH and β-actin mRNA levels, for which considerable individual variation was observed in inbred rats (Chapter 4), may reveal factors involved in individual aspects of aging, determining the age of onset and progress of age-related diseases.

One of the most widespread problems in aging research is to uncover direct causal relationships between molecular age-changes and decreased organismal functions. Apart from the individual variation of gradually aging populations, this is also due to the fact that among many age-related changes that occur, some are deleterious and irreversible, whereas others are without any effect or even compensatory. Molecular age-changes can be observed at ages when diseases do not become clinically manifest. The lack of markers indicative for biological age as opposed to chronological age has also hampered the causal interpretation of molecular age-changes. Still, the molecular alterations observed with age, provide a suggestive background for the declining health status of animal and human populations and the increasing interindividual variation associated with this.

2.1.3 Relevance of molecular age-changes in somatic cells

A diverse spectrum of alterations is observed at the cellular level as a function of age which may be the consequence of programmed, pleiotropic and stochastic events. Programmed events, for example, are involved in red blood cell atrophy and in cessation of cell proliferation *in vitro*. Pleiotropic and/or programmed events are thought to be involved in apoptosis (inducible by glucocorticoids, ionizing radiation and deprivation of growth factors). Stochastic somatic defects (inducible by oxygen radicals and glucose) are expected to

accumulate especially in post-mitotic cells and may be involved, for example, in neuronal atrophy.

The molecular mechanisms which are hypothesized to underlie changes observed at different levels of cellular organization, frequently involve a combination of programmed, pleiotropic and stochastic effects. This can be illustrated by discussing the putative role of *telomere shortening in aging (see also section 1.6.2.5).*

Telomere shortening

The telomerase gene product repairs the loss of sequences at the chromosome ends, generated during each cell division at the discontinuously replicating DNA strand. The gene may be regarded as a cellular maintenance gene, but it is expressed only in germ cells, and possibly in stem cells. The repression of this gene in somatic cells may be part of a differentiation programme and may start a clock monitoring the number of cell divisions each somatic cell has completed. The necessity of such registration, and the consequence of telomere shortening for cellular functions, is not fully understood. Recently, it has been demonstrated that some immortalized SV40 transformed human cells in culture express telomerase activity (Counter et al., 1992). In these immortalized cells, which have bypassed both the M1 and M2 cell-cycle arrests, telomere shortening and accumulation of dicentric chromosomes is stabilized. In the absence of telomerase expression, instabilities of the telomeric regions and cell death occurred in other immortalized cellines, when a critical minimum telomere length of 1.5 Kb was passed (Counter et al., 1992). Apart from chromosomal instabilities and DNA sequence changes, telomere shortening might lead to repression of adjacent genes in the gene-rich subtelomeric region (Biesmann and Mason, 1992). If telomeres of replicating cells *in vivo* would indeed become reduced beyond the critical length, such a mechanism could contribute to various pathological processes. If, on the other hand, such reduction forms a signal for cessation of cell division *in vivo*, the mechanism could be a protective function involved in the replicative arrest of 'old' cells. In this case, the repression of the telomerase gene in somatic cells may be regarded a longevity-assuring function. If telomere shortening is involved in organismal aging, the mechanism appears to have a programmed (repression of telomerase gene) as well as a stochastic aspect (accumulation of chromosomal instabilities).

Although telomere shortening may well be involved in cell-cycle control and cellular senescence, a causal relation between telomere shortening and organismal decline has not been established. In mice, no shortening of the telomeres (> 80 kb) could be measured as a function of age (Kipling et al., 1990). In peripheral blood lymphocytes of humans, however, such reduction of telomere length with age occurs. In addition, a significant individual variation in the length of telomeric restriction fragments (TRFs) was observed among twin pairs within the same age-groups at all ages (Chapter 8). This variation in adolescents and adults could be attributed for 85% to genetic influences, and in 4-year old individuals for 60%. These telomere size differences may reflect TRF length differences in the germ line even in the presence of telomerase; differences in the amount of telomeric DNA lost per replication round or differences in turnover rate of cells in vivo. The genetic

basis for this variation, as indicated by our data, may be found in the presence of telomerase gene variants with variable activity in germ line and/or stem cell pools or in a genetically determined variation of immunological response to antigenic exposure.

Telomere loss occurred at an average rate of 31 bp/year, making it unlikely that the critical length of 1.5 Kb (Counter et al., 1992) is passed *in vivo* during an individual's lifespan. Indeed, the mean TRF length of the oldest twin pairs in our study was not found to become less than 5 Kb. It can not be ruled out, however, that in subsets of cells a few chromosome ends have reached the critical TRF length in blood from these individuals. In addition, the large variation in telomere length among young twins of the same age-groups, indicates that at a constant rate of cell division and/or telomere loss per replication, the critical telomere length will be reached by some individuals in vivo. Any group of old individuals may therefore represent a selection and hence reveal telomeres above the critical length observed by Counter et al. (1992). In order to test the possibility that telomere size represents a biomarker of human aging, age-structures and disease patterns may be compared to telomere length in families with high and low rates of telomere loss. It is not clear yet whether telomere loss per cell division occurs at a constant rate in vivo during life. The cell division rate in pools of cell populations present in blood does not occur at a constant rate with age. Therefore, the individual rate of telomere loss, which is genetically determined, should be measured longitudinally.

Gene expression

Somatic age-changes occur abundantly at each level of cellular organization (section 1.6, and Chapter 3-8). A schematic overview of the nature of these changes is presented in Figure 4. Naturally, most of these changes occur through inter-related mechanisms. Stochastic damage at the DNA level may induce cellular defense mechanisms (DNA repair, heat shock response etc.) and qualitative and quantitative changes in specific gene products. Stochastic damage to proteins may affect translation, transcription and may also induce cellular defense mechanisms.

Controlled degenerative switches in gene expression could thus far not be recognized as being causal to aging in higher species. It must be noted, however, that subtle changes of gene activity at later ages would hardly be recognized as part of a programme. Many specific age-changes with respect to gene expression were observed in the absence of disease (section 1.6.1; Chapter 3-5). Partly these may underlie a decline of homeostasis. Among changes in mRNA levels of aging rats were both inducible (TAT; Chapter 5) and housekeeping genes (GAPDH, β-actin; Chapter 4). Changes in the expression of the latter were not observed in all tissues of the animals investigated. Demethylation of a specific CpG site in the gene may be involved in the increased β-actin mRNA level in the spleen of 36-month-old animals, although the methylation changes were also observed in 24-month-old animals (Chapter 4). In general, the numerous changes in gene activity may be due to a loss of epigenetic control (section 1.6.1) by altered DNA/protein interactions (histones, regulatory DNA elements, transacting factors and receptors), alterations in methylation patterns and altered heterogeneous nuclear RNA (hnRNA) processing. These can be regarded as endogenous to

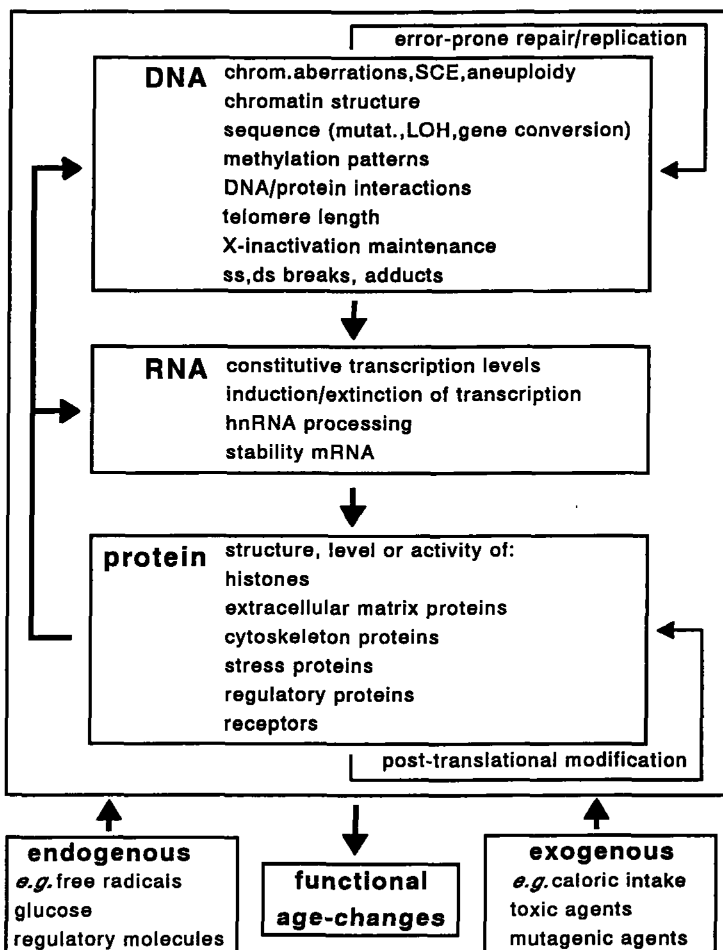

Figure 4. Schematic overview of the nature of molecular age-changes observed in somatic cells of gradually aging species.

the cell and its direct surroundings. Such consistent changes, most clearly observed in older subjects, when the number of affected cells increased to a detectable level, may be regarded as pleiotropic effects. The inter-individual variation of the effect is usually considerable.

Two major pathways may lead to the alterations in gene activity that occur with age. Both pathways have a circular character which may lead to an exponential accumulation of alterations in the expression patterns of most gene systems (snowball effect). In the first place an accumulation of stochastic defects may occur, generated by endogenous mechanisms such as oxidation of lipids, glycation and oxidation of long-lived slowly replaced molecules (DNA, collagen) and of other Advanced Glycation End (AGE) products (section 1.6.1.2 and 1.6.2). Whereas intermediates of these mechanisms are reversible, end products such as lipid peroxides, crosslinks and DNA mutations are not. The rate at which the defective molecules accumulate depends on the rate at which they are generated (depending on glucose levels,

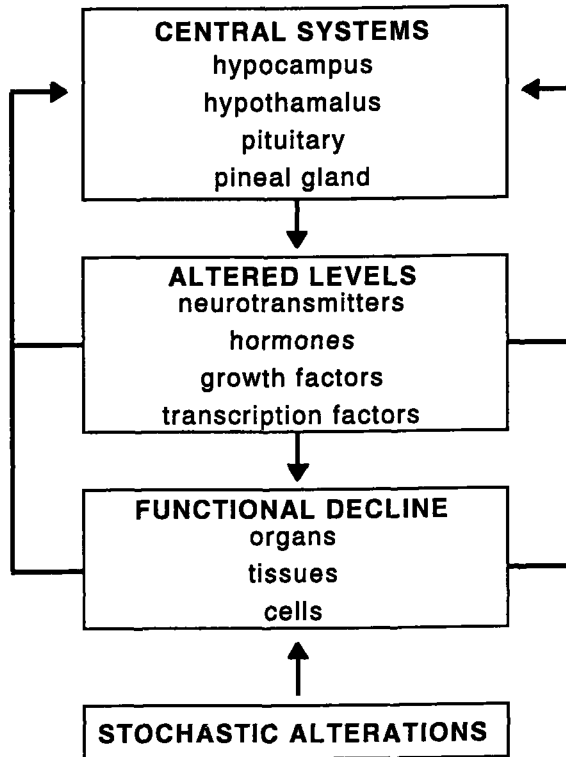

Figure 5. Schematic overview of the pathway along which systemic and cellular age-changes may accumulate and contribute to organismal aging.

free radical concentrations, etc.) and at which they are removed (depending on levels and quality of proteases, antioxidants, DNA repair enzymes, etc.). For AGE products, this rate is subject, for example, to local levels of sugar substrates, metals and metabolites that influence degradation of glycated intermediates. It is therefore expected that tissue areas that accumulate metals, such as zinc in the hippocampus, may accumulate glycated proteins and DNA (Massie et al., 1979; Finch, 1990). This illustrates that somatic defects may not accumulate completely stochastic and may be causally related to functional consequences only if the sites become known at which they occur at the highest frequencies. Another example of such area specificity is the accumulation of mitochondrial DNA (mtDNA) deletions in the cortex of the human brain (section 1.6.3). In general, the functional importance of somatic defects is now expected to be found in accumulation at specific areas of tissues, specific cell types, specific sites in the cell and, as we will discuss below, in the genome.

A second pathway through which age-related alterations in gene expression are induced, is by changes in the homeostatic network, involving systemic levels of hormones and growth- and transcription factors etc. (Figure 5). These levels are influenced by lifestyle and environmental factors. Many of the small changes in hormone levels and temporal patterns of secretion during non-pathological aging are expected to alter gene activity in target cells. An example of systemic age-related alterations in humans is given below (Finch, 1990):

- sex hormones: estrogen (ovarium), decrease

- melatonin night peak levels (pineal gland), decrease
- parathyroid hormone (parathyroid gland), increase
- growth hormone, nocturnal secretion, decrease
- luteinizing hormone (hypothalamus), testosterone, pulse frequency, decrease
- corticosteroids (adrenal gland), increase
- dopaminergic-2 receptor (neurostriatum), decrease
- blood glucose level, decrease
- growth factors: IGF-1 decrease

Altered levels of systemic factors are partly due to pleiotropic effects and partly to programmed events during mid-life, such as the menopause (decrease of estrogen) and thymic involution (decrease of thymic hormone levels). The importance of programmed systemic changes to longevity varies widely among species. In many domestic animals, male castration has increased longevity (Hamilton et al., 1967; Comfort, 1979). The effects of postnatal castration, associated with decreased levels of testicular hormones, result in the increase of male life span towards that of females. These effects are partly associated with decreased androgen-dependent dangerous behaviour. More than in humans, the menstrual cycle in rats (regulated via hypothalamus and pituitary) affects aging of the organism as a whole. Apart from programmed switches in the level of specific hormones, more diverse systemic alterations may result from endogenous accumulation of somatic defects in cells from the neuroendocrine and endocrine system (Figure 5). These may affect patterns of gene expression in target cells distributed over the whole organism.

DNA sequence organization

Besides age-changes in gene activity, also general genomic changes are relevant to aging. The overall loss of DNA methylation from the genome with age in mice, rats and humans, was reflected in the methylation patterns of only a few genes (section 1.6.2.2; Chapter 3-5). To seriously test the loss of this type of epigenetic control of gene expression as a basic mechanism of aging, many more specific methylation sites, directly involved in such control, must be investigated.

The role of the genome in aging may further be mediated by the occurrence of mutations. Except for transposons in *Drosophila* (Woodruff et al., 1992) and mtDNA deletions in *Podospora anserina* (Osiewatch et al., 1988), no direct causal relationship has thus far been established between accumulation of spontaneous genomic instability and longevity. The spontaneous somatic mutation frequency as thus far established, is low and ranges from 10^{-3}-10^{-5} per cell, depending on the locus. In general, mutation frequencies at coding regions occur at a much lower frequency than at non-coding loci (section 1.6.2). Partly this may be due to the fact that excision repair of DNA damage occurs preferentially in transcribed sequences (P. Hanawalt and I. Melton, 1993. Curr. Biol. 3: 67-69). Secondly, there is a close connection between the proteins involved in transcription and those involved in excision repair of DNA damage (Bootsma and Hoeijmakers, 1993). The somatic mutation theory of aging predicts that somatic mutations will eventually accumulate exponentially. If somatic mutations occur completely stochastic during aging, their accumulation can not be detected

Table 1. The total number of bands and band variants detected by one-dimensional DNA typing of human monozygotic twins using multi-locus core probes. Probe sequence given in Uitterlinden et al. (1992).

	monozygotic			
probe	twin pairs	total bands	total unique	% unique
33.15	63	4671	1	.0002
33.6	35	2602	0	0
INS	63	1946	0	0
T_2AG_3	37	2890	0	0
CAG_2	5	212	0	0
CAC	23	1858	0	0
total:	226	14179	1	-

in DNA isolated from whole tissues but must be measured in single cells. If, on the other hand, mutations are generated more consistently, at hot spots of genetic instability, their accumulation may be detected in DNA isolated from whole tissues. In the latter case, functional consequences of somatic DNA sequence changes in such genomic regions may be more easily investigated than would be the case for stochastically accumulating mutations.

The occurrence of consistent somatic DNA sequence changes was studied at a large number of selectively neutral repeat loci dispersed over the human and rat genome. Two-dimensional DNA typing of single cell clones at such loci (section 1.6.2.3; Chapter 7) indicated a high number of DNA sequence alterations. Their frequency did not become increased with age. It cannot be excluded that the DNA sequence variations observed, were generated during mitotic cell divisions *in vitro*. A comparable study *in vivo* was performed for human blood lymphocytes. Minisatellite and simple sequence loci were investigated in genomic DNA from white blood cells of human monozygotic twins of different ages. One- and two-dimensional DNA typing patterns were compared between the twins of a pair. Band- and spotdifferences that could be reproduced were scored (Table 1 and 2). One band- and two spotvariants were observed among 14.000 bands and 8000 spots investigated. DNA sequence variations between monozygotic twins, although expected, have not frequently been observed. It can be concluded that an (exponential) accumulation of consistent changes in aged individuals was not detected at the 'hypervariable' loci investigated in this thesis. At the same loci, DNA sequence variations occurred abundantly in human tumours (data not shown).

In spite of the data discussed in section 1.6 and Chapters 3-8, there is no direct evidence that genomic instability causes aging. The hypothesis has neither been falsified because there are no methods available for measuring the somatic mutation frequency at random, native sites on a single cell basis. By the frequently applied HPRT-assay, events occurring at a low

Table 2. The total number of spots and spot variants detected by two-dimensional DNA typing of human monozygotic twins using multi-locus core probes. +1 represents the only two variants observed.

		no. spots detected by								
twin pair	age	33.15	33.6	INS	CAC	CAG_2	GAA	CAGA	T_2AG_3	total
1	40	495	558		712				69	1834
2	48	613	238	82	512				51	1496
3	73	673	406	450	309	247+1			39	2124
4	94	649	536	178	594+1	354	270	196	68	2845
total spots:		2430	1738	710	2127	601	270	196	227	8299

frequency can be detected. The technique is based, though, on a single locus, the cells are subjected to short-term culture and only mutations that inactivate the gene are detected. By applying the 2-D typing system, many loci are analyzed simultaneously, but low-frequency alterations can not be detected. If Taq polymerases can be used under conditions at which the erroneous incorporation of nucleotides is low, the polymerase chain reaction (PCR) and subsequent mutation analysis of PCR-products would provide the tools to monitor low-frequency mutations at functionally relevant loci. The role of somatic mutations in aging, whether based on particular genomic sites, or on random instability, may be tested in transgenic animal models with defective somatic maintenance genes.

On the basis of the germ-line and somatic instabilities thus far observed, it can be concluded that mutations do not occur completely stochastic. Chromosomal instabilities and genetic rearrangements may occur at a different rate than nucleotide substitutions in coding regions. Genetic instabilities involving large regions of the genome may support recessive mutations to become homo- or hemizygous. In the pathogenesis of various types of human cancer such Loss Of Heterozygosity (LOH) is an important, well documented event occurring at a large number of loci in the human genome and revealing formerly silent mutations. The underlying mechanisms such as homologous recombination or non-disjunction may lead to the induction of randomized LOH in somatic cells during aging. Both germ-line and somatic defects may then become expressed as a mosaic and contribute to aging and disease. The studies discussed in section 1.6.2.3 indicate that LOH indeed occurs spontaneously in the absence of cancer.

Variations in the mutation frequency of different human disease loci are thought to be a consequence of both the DNA sequence and the genomic architecture of that region. High somatic mutation frequencies can be expected at germ-line mutation hot spots (section 1.6.2.3 and 4). Detailed analysis of mutation hot spots in functional sequences as a function of age, would be highly relevant. The germ-line mutation frequency appears to be highest at CpG dinucleotides (Cooper and Youssoufian, 1988) and in repetitive DNA structures. The

frequency of substitutions involving the CpG dinucleotide sequence is 42-fold higher than expected from random mutations and is consistent with a mutational mechanism which involves methylation-induced deamination of 5-methyldeoxycitidine. Among mutations in repetitive DNA structures are coding and non-coding areas of the genome. Instability of repetitive DNA structures, due to gene conversion, deletions, duplications, recombination and slippage replication (expansion and contraction), occur at multicopy gene families, such as tRNA and rRNA genes; between functional and pseudogenes, such as for the steroid 21-hydroxylase gene system (Armor et al., 1988); between partly homologous genes, such as the vasopressin and the oxytocin genes (van Leeuwen et al., 1989); between internal repetitions within the coding region of a gene, such as for the procollagen type I, II and III genes and at repetitive sequences within genes, such as Alu repeats in the LDL receptor gene (Lehrman et al., 1987) and trinucleotide repeats at the fragile X and myotonic dystrophy loci (Riggins et al., 1992). Heritable and somatic deletions in the mitochondrial genome occur at sites containing direct repeats (Holt et al., 1988). Finally, instability may occur at functional non-coding repetitive structures, such as telomeres and protein receptor sites.

What may be the consequence of stochastic somatic mutations ? Most of these will be recessive and are not likely to affect cellular functions to the extend that is expected to be necessary for a causal role in the etiology of aging. Mild defects, leading to sub-optimal expression of genes however, may have deleterious consequences. Especially if they occur in genes with a central role in a functional network. Thus far, somatic mutation studies were mainly performed on replicative tissues and cells. Genomic instability of post-mitotic cells was observed at the vasopressin gene in rats and in the mitochondrial genome (section 1.6.2.3). Instead of through random exponential accumulation in all cells, somatic mutations may be relevant in aging through accumulation at specific loci in cell types executing key functions in the organism (such as those of the neuroendocrine system, Figure 5). The information on the mutation-sensitivity of specific genomic regions may be relevant in two ways. The spontaneous somatic mutation frequency at these loci may contribute to aging and disease and second, such loci may harbour late-acting deleterious germ-line mutations. Mutations with a mild phenotypic effects may predispose to common diseases of old age, whereas more severe mutations at the same loci may be involved in early-onset diseases.

2.1.4 Human aging and disease

A genetic influence on human longevity is indicated by familial trends showing, among a huge variation of environmental influences, that long-lived parents have long-lived children (Abbott et al., 1978). This trend is confirmed by twin studies: the life span difference of monozygotic twins was smaller than for dizygotic twins (about 3 and 6 years, respectively) (Jarvik et al 1980). This may reflect the genetic influence both on the maximum life span and MRDT.

The genetic influence on human disease patterns can be discussed following the hypothetical division of phases in the survival curve depicted in Figure 1. Examples of

human premature disease genotypes (part A in Figure 1) are given in Table 3. The study of these syndromes may reveal genes involved in segmental aging phenomena. Telomere reductions as a function of age in peripheral blood lymphocytes of Down's syndrome patients is increased as compared to age-matched controls (Vaziri et al., 1993). In Down's syndrome patients with partial trisomy of chromosome 21 gene clusters and aspects of the total progeroid phenotype may eventually be dissociated and causally related. In addition to the progeroid syndromes, there are a number of early onset heritable disorders which shorten life span, such as atherosclerosis, dementia of the Alzheimer's type, diabetes type I, cancer and osteoarthrosis. These resemble (multifactorial) disease patterns dominating the MRDT and represent aspects of premature senescence, although none of them shows a uniform acceleration or intensification of the usual spectrum of age-related degenerative changes.

Table 3. Human progeroid syndromes.

Disease	Life span (years)	Genotype
Hutchinson-Guilford	10-20	autosomal recessive
Werner's	50-60	autosomal recessive
Huntington's	40-70	autosomal dominant
Down's	50-70	trisomy 21
Cockayne	50	autosomal recessive
Ataxia telangiectasia	40	autosomal recessive

One of the most stable components of the MRDT (part B, Figure 1) in different human populations is atherosclerosis. Heritable risk factors for heart disease are associated with defects in lipid transport proteins, of which low density lipoprotein (LDL) and high density lipoprotein (HDL) are important, as well as their receptors. These defects may lead to increases in the LDL/HDL ratio (Ross et al., 1986; Sing et al., 1985). LDL is the major carrier of cholesterol; HDL protects against atherosclerosis by the reverse cholesterol transport from peripheral tissues to the liver, where it may be recycled. The common basis for early and late onset atherosclerosis may be found in the damaging effect of oxidized lipids/LDL particles. Any dysfunction leading to high levels of blood LDL, which is under polygenic control, may result in accumulation of oxidized and glycated LDL. The importance of lipid peroxidation for atherosclerosis is confirmed by the fact that the disease in humans (Langlois et al., 1988) and Watanabe rabbits which have a defect in the LDL-receptor gene, is retarded by treatment with the antioxidant probucol (Kita et al., 1987). It can be understood that the age of onset of atherosclerosis may be severely influenced by age-related dysfunctions in the metabolic homeostatic systems regulating the accumulation of free oxygen radicals and glycation products. The pattern of human age-related diseases that dominate the MRDT, varies among different populations. Programmed and pleiotropic events, late-acting deleterious germ-line mutations and accumulation of somatic defects all seem to influence the

age of onset of human diseases occurring in subpopulations determining the MRDT and longevity.

Programmed events, such as the menopause, and switches in hormones connected to this process, have diverse effects on the onset of human diseases. Switches in levels of sex steroids during menopause are protective for atherosclerosis, and cancer of the mammary and female reproductive tract; decreased estrogen secretion is associated with increased osteoporosis and loss of bone, which can be partly prevented by steroid supplements.

Pleiotropic changes in the level of growth factors and melatonin are expected to be involved in a disturbed proliferative homeostasis leading to abnormal cell growth and metaplastic syndromes. Also late-acting deleterious germ line mutations in oncogene and tumour suppressor genes play an important role in the pathogenesis of cancer (predisposition). Pleiotropic changes in transcription factors and hormone levels, among which parathyroid hormone, are expected to be involved in altered bone formation during ongoing remodelling by osteoblast and osteoclasts in osteoporosis. In general, we may consider any risk allele predisposing for a late-onset disease as belonging to the late-acting deleterious mutations expected to be present at high numbers in our germ-line. The late-onset (mild) phenotypic expression of such mutations will hamper their identification; especially for mutated alleles from quantitative trait loci.

Accumulation of stochastic somatic defects are likely to be involved in carcinogenesis: somatic mutations, genomic instabilities leading to the revelation of recessive germ-line mutations (by LOH mechanisms) and methylation changes. Glycation of osteocalcin may be involved in osteoporosis; collagen cross-links in osteoarthrosis; in diabetes the glycation of hemoglobins and collagens is expected to be important (Bunn 1981; Garlich et al., 1988). Accumulation of lipid peroxides is involved in atherosclerosis, whereas cross-link formation may increase trapping of plasma proteins that could lead to aggregation of blood platelets, important in ischaemic heart disease and stroke. A role for nuclear and mtDNA damage and mutations may be expected especially in diseases which involve functional changes in post-mitotic cells (neurons, heart muscle cells) such as neuro-degenerative diseases and ischaemic heart disease.

In centenarians (part C, Figure 1) death, in the absence of substantive organ pathology, appears to occur mainly as a result of impairment of homeostatic functions. This is indicated by the low organ reserve and the "cascade breakdown" (Eulderinck et al., 1993) frequently occurring once one disease is treated. In these individuals, stochastic somatic defects might be the main source of deleterious age-effects, possibly accumulating also at a rate slower than that of the population average. Non-enzymatic crosslinks in collagen of the skin, for example, are generated at such a low rate in a number of 80-year-old individuals that their levels resembled those in 40-year-old individuals (Sell and Monnier, 1990). The presence of collagen crosslinks is a common characteristic of the aged. In addition, some specific genotypes increase life span above the average for the species by lowering incidence of specific diseases. Examples of this are the rare heritable hypo-β or hyper-α lipoproteinemias in humans, which are associated with life spans 5 to 10 years above the general population (Saito, 1984; Glueck et al., 1976, 1977). These genotypes have two to three-fold less LDL

levels than the general population, which may reduce the risk for myocardial infarction and ischaemic heart disease. These alleles enhance longevity by protecting against atherosclerosis and may resemble mouse Ath-1r and Ath-2r alleles which confer resistance to atherogenic diets (Paigen et al., 1989, 1990). By studying the healthy old, potential factors in environment, lifestyle and genotype may be identified that minimize the generation of deleterious changes over lifetime.

Improvements in prevention or intervention of human age-dependent diseases may be expected if a decline of homeostasis can be retarded. This may become feasible when more information becomes available about age-related changes in physiological control factors and their effects on endogenous cellular alterations at relevant sites in tissues, cells and molecules. For threshold diseases, successful intervention may be expected if retardation of age-changes that contribute to the disease can be accomplished before irreversible stages are reached. Early diagnosis of age-related diseases and/or identification of environmental, lifestyle or genotypic risk factors may provide an improved chance for successful intervention. Treatment could be directed to compensation of hormone, growth factor, etc. levels, and retardation of oxidation and glycation (antioxidants) in relevant tissues. It is not unlikely that also in humans a general decline of homeostasis and the occurrence of diseases can be retarded by CR. It was suggested that reduction in some age-related diseases such as cancer, Alzheimer's, Parkinson's and cardiovascular disease, is associated with dietary antioxidant suppletion (vitamin E and β-carotene) and reduced food intake (Slater and Block, 1991). The occurrence of age-related diseases seems inevitable in the light of all molecular changes discussed in this thesis. The age of onset and progression, however, may be delayed and modifiable, at least for some groups of patients. Since the average maximum life span in industrialized countries approaches the maximum life span of the human species, elimination of one common cause of death will establish a new equilibrium between the remaining causes, thereby hardly leading to extension of the average life expectancy (Lohman et al., 1992). Subpopulations in part 'B' (Fig. 1) may however be identified with an increased environmental and/or genetic risk for common diseases to occur at earlier ages. Insights into genetic factors involved in multifactorial age-related diseases (late-acting deleterious alleles), may be obtained by association studies in the population, the study of siblings and twins, and from the study of centenarians.

References

Abbott MH, Abbey H, Bolling DR, Murphy EA. The familial component in longevity. A study of the offspring of nonagenarians. 3. Intrafamilial studies. Am J Med Genet 1978; 2: 105-120.
Amor MKL, Parker H, Globerman MI, New, White PC. Mutation in the CYB21B gene (Ile172-Asn) causes steroid 21-hydroxylase deficiency. Proc Natl Acad Sci USA 1988; 85: 1600-1604.
Biessmann H, Mason JM. Genetics and molecular biology of telomeres. Adv Genet 1992; 30: 185-233.
Bootsma D, Hoeijmakers JHJ. Engagement with transcription. Nature 1993; 363: 114-115.
Bunn HF. Nonenzymatic glycosylation of protein: Relevance to diabetes. Am J Med 1981; 70: 325-330.
Comfort A. The Biology of Senescence. 3rd edition. Edinburgh and London: Churchill Livingstone, 1979.
Cooper DN, Youssoufian H. The CpG dinucleotide and human genetic disease. Hum Genet 1988; 78: 151-155.

Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bachetti S. Telomere shortening associated with chromosome instability is arrested in immortal cells which express teolerase activity. Embo J 1992; 11: 1921-1929.

Eulderink F, Heeren TJ, Knook DL, Ligthart GJ. Inleiding gerontologie en geriatrie. Bohn, Stafleu, Van loghum, Houten/Zaventem 1993.

Finch CE. Longevity, senescence and the genome. University of Chicago Press, Chicago, IL, 1990.

Garlick RL, Bunn HF, Spiro RG. Nonenzymatic glycation of basement membranes from human glomeruli and bovine sources: Effect of diabetes and age. Diabetes 1988; 37: 1144-1155.

Glueck CJ, Garside PS, Fallat RW, Sielski J, Steiner PM. Longevity syndromes: Familial hypobeta and familial hyperalpha lipoproteinemia. J Lab Clin Med 1976; 88: 941-957.

Glueck CJ, Garside PS, Mellies MJ, Steiner PM. Familial hypobetalipoproteinemia. Studies in 13 kindreds. Clin Res 1977; 25: 517.

Hamilton JB, Terada H, Mestler GE, Tirman W. 1. Coarse sternal hairs, a male secondary sex character that can be measured quantitatively: The influence of sex, age and genetic factors. 2. Other sex-differing characters: Relationship to age, to one another, and to values for coarse sternal hairs. In: Advances in Biology of Skin Hair Growth. Proceedings of a syposium held at the University of Oregon Medical School, 1967. Oxford and New York: Pergamon Press 1967; 9: pp 129-151.

Holt LJ, Harding AE, Morgan-Hughes JA. Deletions of muscle mitochondria DNA in patients with mitochondrial myopathioes. Nature 1988; 331: 717-719.

Jarvik LF, Ruth V, Matsuyama SS. Organic brain syndrome and aging. Arch Gen Psychiatry 1980; 37: 280-286.

Kipling D, Cooke H J. Hypervariable ultra-long telomeres in mice. 1990 Nature 347: 400-402.

Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Ooshima A, Yoshida H, Kawai C. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc Natl Acad Sci 1987; 84: 5928-5931.

Lanlois S, Kastelein JJP, Hayden MR. Characterization of six deletions in the low-density lipoprotein (LDL) receptor gene causing familial hypercholecterolemia (FH). Am J Hum Genet 1988; 43: 60-68.

Lehrman MA, Goldstein JL, Russel DW, Brown MS. Duplication of seven exons in LDL receptor gene caused by Alu-Alu recombination in a subject with familial hypercholesterolemia. Cell 1987; 48: 827-835.

Lohman PHM, Sankaranarayanan K, Ashby J. Choosing the limits to life. Nature 1992; 357: 185-186.

Masoro EJ. Retardation of aging processes by nutritional means. Ann N Y Acad Sci 1992; 673: 29-35.

Massie HR, Aiello VR, Iodice J. Changes with age in copper and superoxide dismutase levels in brains of C57BL/6J mice. Mech Age Dev 1979; 10: 93-99.

Osiewacz HD, Hermanns J, Marcou D, Triffi M, Esser K. Mitochondrial DNA rearrangements are correlated with a delayed amplification of the mobile intron (plDNA) in a long-lived mutant of Podospora anserina. Mutat Res 1988; 219: 9-15.

Paigen B, Nesbitt MN, Mitchell D, Albee D, LeBoeuf RC. *Ath-2*, a second gene determining atherosclerosis susceptibility and high density lipoprotein levels in mice. Genetics 1989; 122: 163-168.

Paigen B, Ishida BY, Verstuyft J, Waters RB, Albee D. Arteriosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. Arteriosclerosis 1990; 10: 316-326.

Riggins GJ, Lokey LK, Chastain JL, Leiner HA, Sherman SL, Wilkinson KD, Warren ST. Human genes containing polymorphic trinucleotide repeats. Nature Genet 1992; 2:18-191.

Ross R. The pathogenesis of atherosclerosis: An update. N Engl J Med 1986; 314: 488-500.

Saito F. A pedigree of homozygous familial hyperalphalipoproteinemia. Metabolism 1984; 33: 629-633.

Sell DR, Monnier VM. Structure elucidation of a senescence cross-link from human extracellular matrix. J Biol Chem 1990; 266: 21597-21602.

Sing CF, Boerwinkle E, Moll PP, Davignon J. Apolipoproteins and cardiovascular risk: Genetics and epidemiology. Ann Biol Clin 1985; 43: 407-417.

Slater TF, Block G. Antioxidant vitamins and β-carotene in disease prevention. Am J Clin Nutr 1991; 53: 189S-196S.

Uitterlinden AG, Slagboom PE, Mullaart E, Meulenbelt I, Vijg J. Genome scanning by two-dimensional DNA typing: The use of repetitive DNA sequences for rapid mapping of genetic traits. Electrophoresis 1991; 12: 119-134.

Van Leeuwen F, Van der Beek E, Seger M, Burbach P, Ivell R. Age-related development of a heterozygous phenotype in solitary neurons of the homozygous Brattleboro rat. Proc Natl Acad Sci USA 1989; 86: 6417-6420.

Woodruff RC. Transposable DNA elements and life history traits. Transposition of P DNA elements in somatic cells reduces the life span of Drosophila melanogaster. Genetica 1992; 86: 143-154.

Zurcher C, Slagboom PE. Basic Mechanisms of aging. In: Jones TC, Mohr U, Hunt, RD (eds.). Pathology of aging animals. ILSI Monographs on Pathology of Laboratory Animals. Springer Verlag Berlin Heidelberg New York. (1993) In press

CHAPTER 3

GENETIC INSTABILITY AND AGING: THEORIES, FACTS, AND FUTURE PERSPECTIVES

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Genome 1989; 31: 373-385.

24.2.3 Genetic instability and aging: theories, facts, and future perspectives

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The fundamental mechanisms involved in the physiological deterioration observed with age in mammalian organisms have not yet been elucidated. It appears that random alterations in informational biomolecules and in their synthesis could be the basis of such physiological changes. There is, however, a lack of knowledge with respect to the frequency and characteristics of changes introduced in the cellular molecular machinery. Moreover, the driving force initiating the generation of such alterations and the order of events in which they occur are unknown at present. In this article, data concerning the hypothesis that the aging process is associated with widespread genetic instability are reviewed in the context of the complex interactions between the three major informational biomolecules, DNA, RNA, and protein. We conclude that the results obtained to date do not rule out the possibility that genetic instability in a wide sense is a major causal factor in a number of age-related phenomena. However, it appears that new strategies based on a new technology are ultimately necessary to elucidate the alterations in the intricately interwoven patterns of molecular control that could underlie the various aspects of the aging process. A first attempt is made to formulate the problems in this field and to provide some solutions.

Key words: genetic instability, DNA and aging, gene expression, somatic mutations, DNA methylation, evolutionary aspects.

Slagboom, P. E., et Vijg, J. 1989. Genetic instability and aging: theories, facts, and future perspectives. Genome, 31 : 373–385.

Les mécanismes fondamentaux qui sont impliqués dans la détérioration physiologique observée avec l'âge dans l'organisme des mammifères n'ont pas encore été élucidés. Il semble que des altérations au hasard dans les biomolécules informationnelles et dans leur synthèse pourraient être à l'origine de tels changements physiologiques. Toutefois, il existe un manque de connaissances sur les changements qui sont introduits dans les mécanismes moléculaires des cellules. Bien plus, les forces agissantes qui initient la production de telles altérations et l'ordre dans lequel les évènements surviennent sont présentement inconnus. Dans cet article, les données concernant l'hypothèse que le processus du vieillissement est associé à une instabilité génétique très étendue sont passées en revue, dans le contexte des interactions complexes entre les trois biomolécules informationnelles principales : l'ADN, l'ARN et les protéines. La conclusion s'impose que les résultats obtenus à ce jour n'excluent pas la possibilité que l'instabilité génétique, au sens large, soit le facteur causal majeur d'un certain nombre de phénomènes liés au vieillissement. Cependant, il appert que de nouvelles stratégies fondées sur une nouvelle technologie sont ultimement nécessaires pur élucider les altérations des patterns complexes interreliés des contrôles moléculaires qui pourraient soustendre les divers aspects du processus de vieillissement. Une première tentive est donc faite de formuler les problèmes reliés à ce domaine et de proposer certaines solutions.

Mots clés : instabilité génétique, ADN et vieillissement, expression des gènes, mutations somatiques, méthylation de l'ADN, aspects évolutionnaire.

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Introduction

The basic similarity of biological processes in living systems pleads for a general mechanism underlying the aging process. Although there is no agreement on the nature of such a unifying mechanism of aging, changes in informational biomolecules are considered to play an important role in the etiology of age-related deteriorative processes. Conceptually, molecular biological theories of aging should first be assigned to the two fundamentally different schools of aging theories, according to which aging is regarded either as a species-specific genetically determined program or as a series of stochastic events (Schneider 1987).

With respect to program theories, Weissman proposed that the genetic control of senescence operates through a programmed limitation of the maximum number of mitotic cell divisions possible in a tissue cell lineage (cf. Sacher 1982). Hayflick and Moorhead (1961) provided suggestive evidence for this theory by demonstrating that human fibroblasts exhibit a limit on their replicative ability in tissue culture. The number of population doublings appeared to be inversely correlated with donor age (Martin et al. 1970) and positively correlated with the maximum life-span of the donor species (Röhme 1981).

Interesting from a molecular biological point of view is the work of Smith and Pereira-Smith (1989), who demonstrated in a series of elegant studies that senescent fibroblasts contain a protein factor capable of suppressing the replicative capacity of early-passage cells. These results point to an active rather than a passive mechanism of aging, which could be based on a genetic program. It should be noted, however, that the replicative capacity of cells in an adult organism covers a spectrum ranging from fixed postmitotic to continuously replicating cells. The precise relationship between replicative loss and differentiation is not yet fully understood and the interface between development, differentiation, and aging is still diffuse. Therefore, the loss of the replicative capacity of cells in vitro cannot be easily extrapolated to organismal senescence.

An important argument against the idea that aging is based on a genetic program is the necessity to consider group selection as the evolutionary mechanism by which the "aging genes" have evolved. In other words, aging and death should be of advantage to the group or species. However, group

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selection is rare in nature and the aged phenotype (the selectable trait) seldom becomes manifest among most species or animals living in the wild (for a recent review, see Kirkwood 1989).

Without completely ignoring adaptive aspects of aging (Mergler and Goldstein 1983), it now seems likely that organismal aging is caused by the late deleterious effects of (pleiotropic) genes that have beneficial effects early in life (Williams 1957). The main prediction of this concept, namely, that selection for increased life-span automatically occurs in populations that reproduce at an advanced age, has been demonstrated by successful selection for greater longevity in *Drosophila* (Clare and Luckinbill 1985).

The concept of pleiotropic "aging" genes was adopted by Kirkwood (1977) in his disposable soma theory. In this theory it is argued that the life-span of a species is determined by the balance between somatic maintenance and reproductive effort. Interestingly, this leads to the prediction that aging is due to an accumulation of unrepaired somatic defects. Thus, although the pleiotropic gene theory has often been considered as programmatic in nature (Sacher 1978), the above demonstrates that it can also provide the evolutionary background of stochastic theories (see also Kirkland 1989).

With respect to somatic maintenance, as opposed to the deteriorative effects of the pleiotropic aging genes, Sacher (1978) and Cutler (1979) independently postulated the existence of so-called longevity assurance (determinant) genes. Genes, such as those coding for DNA repair, anti-oxidant functions, etc., were considered responsible for longevity. In this regard it is interesting to realize that certain gene systems might both promote survival and simultaneously cause aging. For example, DNA repair has beneficial effects through its error-free components, whereas its error proneness would lead to an accumulation of mutations as a function of life-time (Vijg and Knook 1987).

In keeping with the theoretical framework provided above primary aging events could well be stochastic, as a direct or indirect consequence of cellular metabolism and environmental factors and expressed as an accumulation of errors in DNA, RNA, and proteins. The most well known theory in this respect is that of Orgel (1963, 1973), who postulated that cellular aging is due to the age-related accumulation of erroneous proteins, including those involved in DNA replication, repair, transcription, and translation. Later, Holliday and Kirkwood (1983) adopted this concept as part of their general error theory of aging.

A purely DNA-based theory is the somatic mutation theory, which attributes cell aging to the gradual accumulation of multiple mutations in the genetic material of somatic cells. Early proponents of this idea were Failla (1958), Szilard (1959), Curtis (1966), and Burnet (1974). Although sometimes carefully formulated, the lack of insight into the organization and structure of the genome and the lack of adequate techniques have thus far precluded any serious falsification of the theories mentioned above (see for example Kirkwood et al. 1984; Vijg and Uitterlinden 1987). Nevertheless, more recently a number of interesting studies on certain aspects of genetic instability and aging have demonstrated that these difficulties need no longer be prohibitive (see below).

On the following pages experimental results regarding genetic instability in its widest sense and aging are reviewed. Subsequently, an attempt is made to interpret these data in the context of the flow of genetic information and the patterns of influence between the (interconnected) informational biomolecules and their metabolism (Fig. 1). Finally, future prospects are discussed for elucidating the causes of aging at the molecular level in terms of technical requirements, research strategies, and model systems.

Age-related changes in gene expression

It seems reasonable to hypothesize that phenotypic alterations characteristic of the aging process somehow reflect age-related alterations in gene expression. If this is not the case, any further discussion of genetic instability and aging becomes futile. First, it should be emphasized that the relevance of observed age-related changes in gene expression for explaining the deteriorative aspects of the aging process is usually far from clear. It is, for example, very difficult to distinguish between adaptive alterations in gene expression and the kind of changes due to primary age effects on macromolecular synthesis. In addition to this basic problem, many conflicting data have been obtained, which could be a result of the lack of appropriate controls (parameters proven to remain unaltered during aging), the lack of appropriate techniques, and the choice of the age groups of the organism examined. Consequently, it has thus far not been possible to separate cause and effect with respect to the age-related alterations in gene expression observed to date.

Alterations at the protein level

Qualitative changes

The topic of protein alterations in relation to aging has been reviewed frequently (see for example Reff 1985). So far, most data have failed to indicate proteins with altered primary structure emerging during aging (Reff 1985; Rothstein 1987). Also, measurements on the accuracy of poly(U) translation, for example those involving brain cell extracts from 33-month-old rats, indicated no loss of translation fidelity (Filion and Laughrea 1985). These findings have been interpreted as evidence against the protein error catastrophe theory (Rothstein 1987; Dice and Goff 1987) from the point of view that, according to this theory, ultimately all proteins should become altered during aging as a result of a defective protein synthesis machinery. Rejection of this hypothesis, however, seems premature in view of the limitations of the detection level of the techniques used and the lack of insight as to what minimal level of erroneous proteins is required to cause an error catastrophe (for a discussion of this topic, see Shmookler Reis 1976; Kirkwood et al. 1984; Rosenberger and Kirkwood 1986).

Although there is no evidence for an accumulation of proteins with altered amino acid sequence, a number of specific proteins revealing aberrant heat stability, specific activity, and immune responsiveness have been observed in tissues of aged animals, varying from lower organisms as nematodes (Gershon and Gershon 1970; Reiss and Rothstein 1974) to rodents (Sharma and Rothstein 1980; Reiss and Sacktor 1983) and cattle (Ohrloff et al. 1980). These alterations are considered to result from a variety of posttranslational modifications (Rothstein 1987; Dice 1985). For example, observations of Oliver et al. (1987) indicate an increase in the level of oxidized key metabolic enzymes in fibroblast cultures from old human donors as compared with young donors. If enzymes involved in protein synthesis and degradation would be among such damaged proteins, this could have severe consequences for protein metabolism in general. Although the available data

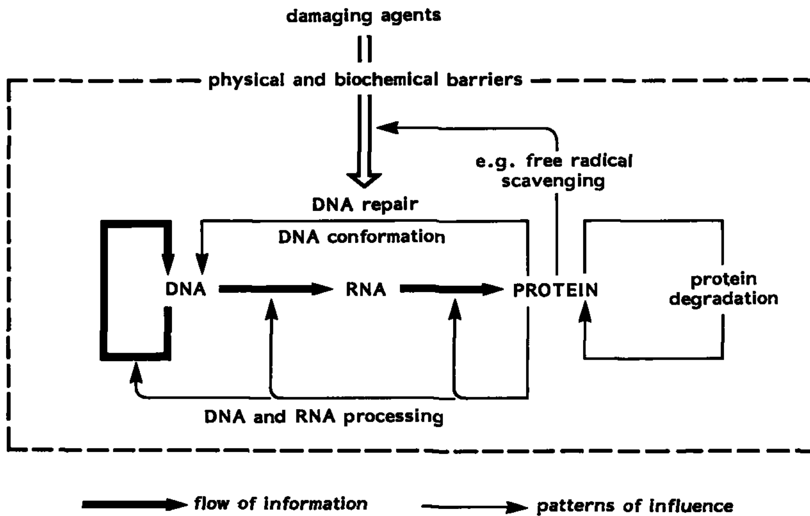

FIG. 1. Genetic instability and aging.

are somewhat contradictory, a progressive decline of overall protein synthesis has been observed in almost all aging systems (for reviews, see Makrides 1983; Richardson and Semsei 1987; see, however, Shmookler Reis 1981). This phenomenon has been attributed to the decreased activity of initiation and (or) elongation factors in translation (Castaneda et al. 1986), a decreased ribosome aggregation to mRNA (Egilmez and Rothstein 1985), or a decreased rate of protein degradation (Makrides 1983; Dice and Goff 1987).

Analogous to DNA repair, protein degradation systems appear to remove defective proteins. Thus, a lower rate of protein degradation could lead not only to a decrease in protein synthesis but also to an increased level of altered proteins (Rothstein 1987). It will be clear that due to the complex pattern of interrelationships between such events cause and effect can no longer be distinguished.

If DNA and RNA processing enzymes would become altered with time, this could theoretically lead to changes in the primary structures of the other two informational biomolecules, RNA and DNA. However, there is no convincing evidence for an increased error proneness of DNA polymerases (Silber et al. 1985) as yet, nor for considerable alterations in the activity of DNA repair systems with age (Vijg et al. 1985; Mullaart et al. 1989*a*, 1989*b*; for a recent review, see Hanawalt 1987).

An interesting phenomenon that might be explained by protein oxidation is the appearance with age of membrane-associated antigens that might be involved in cellular senescence. The presence of such membrane-associated proteins has been related to the senescent phenotype of erythrocytes (Kay 1985; Kay et al. 1986) and late-passage fibroblasts (Pereira-Smith et al. 1985; Smith and Pereira-Smith 1989). The relationship between the mechanisms underlying the limited proliferative life-span of dividing cells and the aging process in postmitotic tissue is still unclear.

Quantitative changes

Alterations in proper gene control could result in a quantitative deterioration of protein expression. Fleming et al. (1986) applied semi-automated, quantitative two-dimensional gel electrophoresis to the analysis of large numbers of [^{35}S]methionine-labeled individual polypeptides from *Drosophila,* which studies indicated a significant age-related heterogeneity in the quantitative distribution of the labeled proteins. The absence of age-associated molecular weight changes or charge alterations in the proteins analysed indicates once more that sequence alterations do not occur at a detectable level. The gradual quantitative alterations in the expression of a great number of these proteins observed in 28-day-old (middle aged) and 44-day-old (old) flies as compared with 10-day-old (young) flies might reflect alterations in the regulation of gene expression in aging *Drosophila*, an organism that consists entirely of postmitotic cells, except for the spermatocytes.

Chatterjee et al. (1981) demonstrated considerable age-related alteration in the levels of a number of proteins in rat liver tissue by in vitro translation of poly(A)$^+$ RNA isolated from young and old animals and subsequent analysis of the translation products by gel electrophoresis. One of these proteins was identified as a polymorphic form of $\alpha_{2\mu}$-globulin, and evidence has been provided that the gradual decrease in the levels of the isoelectric variants of $\alpha_{2\mu}$-globulin is differentially regulated as a function of age (Roy et al. 1983). The age-related up and down variation in the levels of individual proteins as observed in *Drosophila* by Fleming et al. (1986) and in additional studies on a great number of identified proteins (for a review, see Horbach et al. 1987) may reflect a general loss of control of gene expression. Some of these proteins, like the $\alpha_{2\mu}$-globulin family (Roy et al. 1983), are regulated by the action of growth factors or hormones. Therefore it should be noted once more that age-related alterations in gene expression could be intrinsic, adaptive, or a combination of the two.

Alterations at the RNA level

In view of the failure to detect proteins with altered amino acid sequences it is not likely that inaccurate transcription

interferes to a great extent with the transmission of information from the gene to the protein. The quantitative variations in protein levels discussed in the previous section may indicate, however, that the control of gene expression is affected during aging. Overall RNA synthesis, as measured by the incorporation of radiolabeled precursors into RNA, was found to decrease with age in human fibroblasts (Chen et al. 1980) and in various organs and tissues from experimental animals (for a review, see Richardson et al. 1983). In addition, the rate of mRNA synthesis appears to decrease with age (for a review, see Richardson and Semsei 1987). However, the total mRNA content in rat liver does not seem to change with increasing age (Horbach et al. 1984). These observations, which appear to be contradictory, can possibly be explained by a decline in turnover rate of mRNA molecules (Horbach et al. 1986).

One of the possible consequences of a decline in mRNA turnover could be the shortening of specific mRNAs "lingering" in the cytoplasm, as suggested by the age-related increase in poly(A)$^-$ albumin mRNA in the rat liver observed by Horbach et al. (1984). Indeed, Bernd et al. (1982) reported an age-related decrease in the size of poly(A) segments of the cell mRNA in quail oviduct, heart, and liver. However, this concept is in contrast with earlier observations suggesting that the presence of a poly(A) sequence is associated with mRNA stability (for a review, see Brawerman 1987).

There is no evidence that the decrease of mRNA synthesis is due to age-associated changes in the enzymes involved in RNA synthesis (Bolla and Denckla 1979). On the other hand, age-associated alterations in chromatin might affect transcription. Studies on the $\alpha_{2\mu}$-globulin gene family support such a concept, since it appears that transcriptional activation of this gene family at puberty and cessation of transcription at old age correlate with the association and dissociation of the gene domain with the nuclear matrix (Murty et al. 1988).

Alterations at the level of total mRNA synthesis will probably affect all proteins to the same extent and cannot explain various up and down regulations in individual protein levels. A number of studies have been conducted in which the level of specific mRNAs in tissues of aging animals were examined (Table 1). In these studies, the mRNA content was measured by different molecular hybridization techniques using radioactively labeled specific cDNA probes.

From those studies in which Northern blot analysis has been performed (Rutherford et al. 1986; Matocha et al. 1987; P. E. Slagboom, W. J. F. de Leeuw, and J. Vug, unpublished results; Richardson et al. 1985), it appears that the size of specific mRNAs remains unaltered during aging, indicating the absence of age-related alterations in posttranscriptional processing. Furthermore, no general trend can be derived as to the effect of age on the level of specific mRNAs. In some cases conflicting data have been presented, which might reflect differences between various animal species or strains, different definitions of an "old" animal, or the use of different controls to which mRNA levels were related. The use of albumin mRNA, for example, as an internal standard in the studies of Guigoz and Wellinger on tyrosine aminotransferase and tryptophan oxygenase mRNA levels in the rat liver is doubtful, since the albumin mRNA content in the liver changes with age (Table 1).

As expected, many age-related alterations in the level of individual proteins such as albumin, tyrosine aminotransferase, cytochrome P_{450}, and $\alpha_{2\mu}$-globulin were shown to be paralleled by quantitative changes at the mRNA level (Richardson and Semsei 1987; Horbach et al. 1987). The expression of the albumin gene has been studied by a number of investigators. Richardson et al. (1985) observed an increase in the albumin mRNA level in the liver of Ficher 344 rats between 6 and 29 months of age. Van Bezooijen et al. (1976) reported a decrease of albumin synthesis in rat hepatocytes from donors between 3 and 24 months of age followed by an increase between 24 and 36 months of age. In the same rat strain Horbach et al. (1984) and P. E. Slagboom, W. J. F. de Leeuw, and J. Vug (unpublished results) demonstrated increased levels of albumin mRNA in total liver with age. Interestingly, this increase was observed in all animals between 12 and 24 months of age, whereas the albumin mRNA level in old animals (36 months) appeared to be strongly dependent on the health status of the rats and thus shows a great deal of interindividual variability. An age-related increase in interindividual variability was also observed for the tyrosine aminotransferase and ornithine transcarbamoylase expression in these rats (P. E. Slagboom, W. J. F. de Leeuw, and J. Vug, unpublished results), suggesting that some loss of transcriptional control might occur with age, possibly associated with individual pathologies of senescence (Shmookler Reis 1981).

Another argument in support of an age-related loss of gene control is the decreased inducibility of a number of genes by hormones, growth factors, or cold stress, which has been observed in old animals (Dilella et al. 1982; Wellinger and Guigoz 1986).

Thus, it appears that both proteins and mRNAs in various tissues of experimental animals show a spectrum of quantitative variation with age. The possibility that at least part of these changes could reflect a general loss of transcriptional control is supported by experiments, the results of which indicate derepression of tissue-specific genes. Ono and Cutler (1978) demonstrated that significant amounts of α- and β-globin mRNA were present in mouse brain and liver tissue. Interestingly, the amount of α- and β-globin mRNA appeared to increase with age in C57Bl/6J mice, indicating derepression of this gene during aging. An age-related derepression was also shown for endogenous murine leukemia virus homologous sequences (Ono et al. 1985*a*) in brain and liver of both the C57Bl/6J and AKR mice. These data have been interpreted as evidence for the so-called dysdifferentiation theory of aging, that is, the age-dependent relaxation of proper gene regulation (for an extensive review, see Cutler 1985). This phenomenon, however, does not appear to be universal. The gene coding for casein, for example, which was found to be expressed at a low level in brain and liver of the C57Bl/6J strain (Dean et al. 1985), did not become further derepressed with age. Derepression of the α- and β-globin gene could not be observed in aging mice of the short-lived AKR strain as opposed to the long-lived C57Bl/6J mice (Ono et al. 1985*a*). A tissue-specific aspect of dysdifferentiation is suggested by studies on mouse mammary tumor virus (MMTV) homologous sequences which were expressed in the liver but not in brain tissue of aging C57Bl/6J mice (Dean et al. 1985).

Recently, interesting results were obtained by Wareham et al. (1987), who investigated the reactivation of the X-linked ornithine transcarbamoylase (OTC) gene in aging mice carrying an X-autosomal translocation (T(X;16)16H). These studies were facilitated by the fact that the normal X chromosome in these mice was consistently inactivated in a nonrandom manner. Reactivation of the intact OTC gene on this chromosome at the single-cell level was histochemically determined. Patches of

Table 1. Age-associated alterations in specific mRNA levels

Species	Strain*	Tissue†	Age (months)	Gene sequence‡	Change (%)§	Reference
Rat	F	L	3–12	Albumin	nc	Richardson et al. 1985
Rat	F	L	12–24	Albumin	−30	Richardson et al. 1985
Rat	F	L	24–29	Albumin	+93	Richardson et al. 1985
Rat	W/R	L	3–12	Albumin	nc	Horbach et al. 1984
Rat	W/R	L	12–24	Albumin	+83	Horbach et al. 1984
Rat	W/R	L	24–36	Albumin	nc	Horbach et al. 1984
Rat	W/R	L	6–24	Albumin	+69	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	L	24–36	Albumin	−67	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	F	L	6–29	Aldolase	nc	Richardson et al. 1985
Rat	F	L	6–29	P_{450}	−50	Richardson et al. 1985
Rat	F	L	6–29	$\alpha_{2\mu}$-globulin	−98	Richardson et al. 1985
Rat	S/D	L	10–24	TO	−23	Guigoz and Wellinger 1984
Rat	S/D	L	10–24	TAT	−34	Guigoz and Wellinger 1984
Rat	W/R	L	6–36	TAT	−66	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	L	6–36	OTC	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	S	6–36	GAPDH	+152	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	L	6–36	GAPDH	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	B	6–36	GAPDH	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	S	6–36	β-actin	+165	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	L	6–36	β-actin	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	B	6–36	β-actin	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	F	L	3–23	c-myc	+500	Matocha et al. 1987
Rat	F	B	3–23	c-myc	nc	Matocha et al. 1987
Rat	F	L	3–23	c-sis	nc	Matocha et al. 1987
Rat	F	B	3–23	c-sis	nc	Matocha et al. 1987
Rat	F	L	3–23	c-src	nc	Matocha et al. 1987
Rat	F	L	3–23	c-src	nc	Matocha et al. 1987
Rat	F	L	6–37	Compl. C3	−46	Rutherford et al. 1986
Rat	F	L	6–37	Compl. C4	−24	Rutherford et al. 1986
Rat	F	L	6–37	AGP	nc	Rutherford et al. 1986
Rat	F	L	6–37	Factor B	nc	Rutherford et al. 1986
Mouse	C	SG	12–27	EGF	−75	Gresik et al. 1986
Rat	F	L	6–37	SOD	−30	Semsei and Richardson 1986
Rat	F	L	6–37	Catalase	−30	Semsei and Richardson 1986

*F, Fischer 344; S/D, Sprague-Dawley; W/R, Wistar-derived WAG/Rij; C, C57Bl/6.
†L, Liver; B, brain; S, spleen; SG, submandibular gland.
‡P_{450}, cytochrome P_{450}; TO, tryptophan oxygenase; TAT, tyrosine aminotransferase; OTC, ornithil transcarbamoylase; GAPDH, glyceraldehyde phosphate dehydrogenase; compl. C3 and C4, immune complement C3 and C4; AGP, α1-glycoprotein; factor B, complement protein factor B; EGF, epidermal growth factor; SOD, superoxide dismutase.
§nc, No change; +, an increase; −, a decrease.

OTC-producing hepatocytes were 50-fold more frequent in old mice than in young mice, indicating an age-related decrease in the stability of mechanisms involved in the control of gene expression on the X chromosome. This phenomenon, which is at present the strongest evidence for dysdifferentiation, was suggested by the authors to be due to a reduction in the level of DNA methylation, which has been implicated in the maintenance of X-chromosome inactivation (Lock et al. 1987).

In a recent study of another X-linked locus, the hypoxanthine phosphoribosyltransferase (HPRT) gene in human fibroblasts, no age-related reactivation of the inactive gene could be detected (Migeon et al. 1988). In this respect, it has been suggested that age-related reactivation may have species, tissue, and locus-specific determinants. The possibility that methylation changes or other types of alterations at the DNA level are involved in the changes in gene expression observed during aging is discussed below.

Age-related changes in DNA

Alterations in DNA can be produced continuously as a result of the activity of damaging agents (Vijg and Uitterlinden 1987; Mullaart et al. 1988). However, in view of its unique role as the primary template, damaged DNA cannot be easily degraded and replaced like most RNAs and proteins. Damage in DNA can have a direct effect on genome functioning by interfering with transcription and replication. However, even when the induced damage is rapidly repaired by one of the many sophisticated DNA repair systems, sequence alterations can be induced as a consequence of mishandling of the lesions by error-prone repair enzymes. As discussed below, DNA damage and repair can initiate a number of changes in DNA modification, local configuration, and conformation.

Alterations in DNA methylation

There is strong evidence that the level and distribution of 5-methylcytosine (5-mC) residues is involved in the regulation of gene expression (for a recent review, see Cedar 1988). In general, hypermethylation has been found to be associated with transcriptional silence. Demethylation of tissue-specific genes at particular sites, usually near or at their 5′ ends, is observed in the tissue where the gene is expressed. In contrast, housekeeping genes generally contain CpG-rich "islands" at their 5′ or 3′ ends that are completely unmethylated (Bird 1986). Such genes appear to be transcriptionally active in all cell types. Interestingly, these so-called HTF islands are

methylated and nuclease insensitive on inactivated X chromosomes (Yang and Caskey 1987).

With respect to the mechanism by which methylation suppresses gene expression, there is evidence that the methyl groups generate local chromatin changes that inhibit binding of protein factors necessary for the initiation of transcription. As an exception to the general rule that hypomethylation is associated with gene expression, it should be noted that for some demethylated genes expression could not be measured, while on one occasion heavy methylation was found to be associated with expression (for a discussion of this topic, see Mays-Hoopes 1985).

It is conceivable that the age-related changes in gene expression described in the previous section stem from age-related changes in the genomic pattern of 5-mC. Indeed, the age-related increase in X-chromosome reactivation events observed by Wareham et al. (1987) was suggested to be due to loss of 5-mC (Holliday 1987*a*). Age-related loss of 5-mC can have multiple causes, varying from imperfect transmission during replication (Holliday 1987*b*), incomplete remethylation after excision–repair (Kastan et al. 1982), and deamination of 5-mC to thymine. This last mechanism is particularly interesting, since it results in a C-T transition mutation. CpG dinucleotides have been suggested as potential hot spots for mutation (Youssoufian et al. 1986).

Obviously, a part of the alterations in DNA methylation patterns that may arise randomly with age could affect gene expression. Recently, a loss of 5-mC from genomic DNA of various organs has been observed during aging of the mouse (Wilson et al. 1987). The same authors also found a loss of 5-mC from the DNA of bronchial epithelial cells of old human donors as compared with young ones (Wilson et al. 1987).

Since overall reductions in 5-mC DNA methylation with age cannot be easily extrapolated to specific cellular functions, a number of attempts have been made to analyse the methylation status of specific genes. Thus, a number of individual genes have been examined by molecular hybridization analysis, using the isoschizomeric restriction enzymes *Hpa*II and *Msp*I. This method is characterized by the fact that although both these enzymes recognize the same sequence (5′-CCGG-3′), *Hpa*II does not cut the site when the internal cytosine is methylated, while *Msp*I will not cut sequences methylated at the external 5-mC.

The results of these studies are summarized in Table 2. They indicate that age-associated alterations in DNA methylation occur in some genes, but not in all. No general pattern as to specific categories of genes that might undergo methylation changes can be deduced. Of the tissue-specific genes studied at both the mRNA and DNA level no changes in methylation pattern were observed that parallel the observed age-related changes in mRNA level (for example albumin and TAT in Tables 1 and 2). Tissue-specific age-associated alterations in DNA methylation were observed for the c-myc sequence in mice, indicating hypermethylation of these sequences in the liver and hypomethylation in the spleen (Ono et al. 1986; Table 2). These results do not suggest a role for DNA methylation in the increased c-myc mRNA level that was observed in the liver of old rats, although a species difference could be involved here.

Thus, a role for DNA methylation changes in the age-related loss of transcriptional control has not yet been confirmed. However, it should be noted that the specific sites which may be of functional importance in the regulatory role of DNA methylation for the expression of the genes studied, may not have been identified yet.

Changes in DNA sequence organization

The possibility that continuous exposure to environmental mutagens could be responsible for the increased age-associated incidence of cancer in man and experimental animals initially was a major argument in favour of the somatic mutation theory of aging (Curtis 1966). Later it was realized that (*i*) any putative age-related accumulation of somatic mutations was more likely to be due to a species-specific level of error proneness of DNA processing enzymes (Burnet 1974) and (*ii*) endogenous DNA-damaging factors are far more important than environmental ones (Ames 1983). In spite of the inherent logic of ascribing a great number of age changes to the continuous induction of irreversible mutational alterations in DNA, it has thus far not been possible to experimentally verify somatic mutational hypotheses of aging. It has also been concluded that the mutation rate might be too low to allow for the age changes commonly observed (Maynard Smith 1962). More recently, techniques have been developed to measure the mutation frequency in some cells directly. The use of these techniques in the study of mutation accumulation with age will be discussed below.

Gene mutations

Analogous to the studies on age-related changes in RNA and protein, low-frequency sequence alterations in DNA are difficult to detect. This is especially the case for the in vivo situation in view of a lack of selectable traits that allow for the rapid detection of mutant cells. The method that is at present commonly used for mutation studies on somatic cells in vivo employs the X-linked locus hypoxanthine–guanine phosphoribosyltransferase (HPRT) as the selectable gene, using 6-thioguanine (TG) as a selective agent. Cells with an inactivated HPRT gene are resistent to TG. Results of studies on the *frequency of spontaneous somatic mutations in relation to* aging are listed in Table 3. With the exception of Strauss and Albertini (1979), all authors found an age-related increase in the mutation frequency of the HPRT locus in human T cells. However, the most recent data from Albertini and co-workers (R. Albertini, personal communication) also indicated an age-related increase in HPRT mutants of 2×10^{-6} to 8×10^{-6}. Therefore, there seems to be no doubt as to whether the number of HPRT mutant T cells increases with age; it is evident that donor age is the strongest single risk factor in this respect (B. Bridges, personal communication). It should be noted, however, that cell types other than T lymphocytes might not show the same characteristics (Horn et al. 1984; B. Bridges, personal communication).

A considerable interindividual variation with respect to the frequency of TG-resistent T cells from donors of the same age group was generally observed. It should be noted that although somatic mutations may be generated randomly in a population of cells, differential selection in vivo against or in favour of mutated cells might affect the mutation frequencies measured and could explain the variation between individuals. An analysis of the T lymphocyte subpopulations by cell surface monoclonal antibodies indicated that different TG-resistent clones can stem from the same in vivo mutated T cell (Featherstone et al. 1987). Thus the observed mutant frequencies may not be representative of in vivo mutant frequencies. Moreover, the mutant nature of the TG-resistent cells has not always been determined.

TABLE 2. DNA methylation patterns of specific genes in relation to aging

Species	Strain*	Tissue†	Age (months)	Gene sequence‡	Change§	Reference
Mouse	C/J	L	6–24	IAP	Hypomethylation	Mays-Hoopes et al. 1983
Rat	C/J	L	3–30	L1Md	Hypomethylation	Mays-Hoopes et al. 1986
Rat	W/R	L	24–45	c-ras	nc	Vijg et al. 1984
Mouse	C/J	L	2–26	c-myc	Hypermethylation	Ono et al. 1986
Mouse	C/J	S	2–26	c-myc	Hypomethylation	Ono et al. 1986
Mouse	C/J	B	2–26	c-myc	nc	Ono et al. 1986
Rat	W/R	L	6–45	MHC	nc	Uitterlinden et al. 1985
Rat	W/R	S	6–45	MHC	nc	Uitterlinden et al. 1985
Rat	W/R	L	6–36	TAT	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	S	6–36	TAT	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	B	6–36	TAT	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	L	6–36	OTC	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	S	6–36	OTC	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	B	6–36	OTC	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	L	6–36	Albumin	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	S	6–36	Albumin	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	B	6–36	Albumin	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	L	6–36	GAPDH	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	S	6–36	GAPDH	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	B	6–36	GAPDH	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Mouse	C/J	L	2–26	Actin	nc	Ono et al. 1986
Mouse	C/J	S	2–26	Actin	nc	Ono et al. 1986
Mouse	C/J	B	2–26	Actin	nc	Ono et al. 1986
Rat	W/R	L	6–36	β-actin	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	S	6–36	β-actin	Hypomethylation	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Rat	W/R	B	6–36	β-actin	nc	P. E. Slagboom, W. J. F. de Leeuw, and J. Vijg, unpublished results
Mouse	C/J	L	2–26	DHFR	nc	Ono et al. 1986
Mouse	C/J	S	2–26	DHFR	nc	Ono et al. 1986
Mouse	C/J	B	2–26	DHFR	nc	Ono et al. 1986
Rat	W+F	L	5–28	Coll I	Hypomethylation	Gershon et al. 1985
Rat	W+F	L	5–28	Albumin	nc	Gershon et al. 1985

*C/J, C57Bl/6J; W+F, Wistar and Fischer 344; W/R, Wistar-derived WAG/Rij.
†L, liver; B, brain; S, spleen.
‡IAP, intracisternal A particle; L1Md, long interspersed repeated sequence; MHC, major histocompatibility complex; TAT, tyrosine aminotransferase; OTC, ornithil transcarbamoylase; GAPDH, glyceraldehyde phosphate reductase; DHFR, dehydrofolate reductase, Coll I, collagen type I.
§nc, no change.

The results presented in Table 3 are contradictory with respect to the issue whether a linear (Evans and Vijayalaxmi 1981) or an exponential (Morley et al. 1982) frequency of mutant cells is associated with aging. A progressive increase in the rate of accumulation of somatic mutations could indicate an error catastrophe process underlying the aging phenotype. Such a concept, however, is not supported by the results of studies on the accumulation of altered proteins, which could not be detected (see above). A linear increase of mutations, occurring at a constant low rate, is likely to have much less severe physiological effect on the aging individual.

Qualitative analysis of HPRT and adenine phosphoribosyltransferase (APRT) mutant T cell clones by Southern hybridization revealed a variety of structural alterations including many DNA rearrangements (Turner et al. 1985; Bradley et al. 1987; Nalbantoglu et al. 1987). Since it is known that many DNA rearrangements are mediated by homologous sequences, so-called repetitive elements, an interesting point of discussion that can only partly be addressed by using the HPRT method is whether mutations occur at random or at certain "mutation hot spots" in the genome. Conceptually, patterns of genetic instability, for example, involving families of certain recom-

Table 3. Accumulation of gene mutations with age

Species	N	Age	Tissue*	Mutation frequency, young–old ($\times 10^{-5}$)	Reference
Human	63	11–75 years	T lymphocytes	13.0–13.0	Strauss and Albertini 1979
Human	26	16–82 years	T lymphocytes	8.0–40.0	Evans and Vijayalaxmi 1981
Human	37	9–95 years	T lymphocytes	0.5–0.5	Morley et al. 1982
Human	117	0–86 years	T lymphocytes	0.5–1.0	Trainor et al. 1984
Mouse	52	3–32 months	Spleen T	0.9–1.5	Inamizu et al. 1986
Mouse	200	29–43 months	Kid/musc	5.0–5.0	Horn et al. 1984

*Spleen T, splenic T cells; Kid/musc, kidney and muscle cells

bination-prone repetitive sequences, could affect the control of gene expression in a consistent manner (Vijg et al. 1985).

DNA rearrangements

Chromosome rearrangements were the first DNA sequence alterations found to be associated with aging in a number of cytogenetic studies (Curtis 1966; Brooks et al. 1973; Martin et al. 1985). Several studies have been performed on human individuals of varying ages. The most extensive of these studies are those of Marlhens et al. (1986) and Prieur et al. (1988), involving more than 1000 metaphases from four young and two old individuals. In these studies an increase in the number of chromosome breaks and chromatid-type lesions in aged individuals (75 years old) was demonstrated. Furthermore, a progressive accumulation of chromosome rearrangements was found to be correlated with donor age.

In human peripheral blood lymphocytes a selective loss of specific chromosomes was observed with aging (for a review, see Schneider 1985). In most of the studies this loss concerns the X chromosomes in females and the Y chromosome in males. Evidence has been presented indicating that the age-related aneuploidy of the X chromosome preferentially involves the inactive X chromosome, perhaps as a consequence of an increased propensity of this chromosome for mitotic errors (Abruzzo et al. 1985). Apart from chromosome rearrangements and aneuploidy, an increase in micronuclei has been observed in lymphocytes from young versus old human donors (Fenech and Morley 1986).

From the above it can be concluded that rearrangements at the level of the whole chromosome do occur with age. It is not inconceivable that also on a micro scale, spontaneous DNA rearrangements occur. The effect of such events on gene expression depends on whether genes, or regions that have a function in regulating gene expression, are involved in the rearrangement. Apart from the striking rearrangements during immunoglobulin gene ontogeny, there has not been much evidence so far that DNA rearrangements are involved in other developmental processes in higher organisms. Studies on a number of genes in aging mice did not reveal any alterations in primary DNA sequence (Ono et al. 1985*b*). This could partly be due to the limitations of the Southern hybridization technique, used in these studies, in detecting low-frequency alterations in the DNA sequence organization. The use of histochemical procedures in this respect might be very informative. This is illustrated by the study of Van Leeuwen et al. (1988), who applied immunocytochemistry to demonstrate the activation of the defective neurophysin gene in 20 of 4500 neuronal cells from the hypothalamus of homozygous diabetes insipidus Brattleboro rats, which carry a frameshift mutation in this gene. The number of immunoreactive cells showed an age-dependent increase. This age-related activation of the neurophysin gene has been suggested by the authors to indicate somatic gene conversion events between this gene and the strongly homologous oxytocin gene.

The enormous amount of repetitive DNA that is present in the coding as well as in the noncoding fraction of the mammalian genome could be of major importance in the control of gene expression (Davidson and Britten 1979). It is possible that subtle gene rearrangements could be mediated by reiterated sequences in the genome through homologous recombination. Evidence for the occurrence of such processes in mammalian cells has been provided by Reis and co-workers (Shmookler Reis et al. 1980), who demonstrated that replicative aging of human fibroblasts is associated with a depletion from the genome of various repetitive sequences, including centromeric alphoid tandem repeats and other highly repetitive DNA sequences. The authors provided evidence indicating that this phenomenon was not due to the loss of chromosomes. Instead, they suggested that nonreciprocal processes, like excision, incomplete replication, or unequal recombination were responsible for the observed DNA instabilities.

Another observation, which has been interpreted as evidence for the occurrence of DNA instabilities with age, is the appearance of extrachromosomal covalently closed circular DNA (cccDNA) in a wide variety of eukaryotic tissues and cell culture systems. Several lines of evidence suggest a chromosomal origin for most of these cccDNA elements and their appearance has been associated with developmental processes (Fujimoto et al. 1985; Fujimoto and Yamagishi 1987).

An age-related increase in the abundance of cccDNA has been observed in mouse and rat lymphocytes and in human fibroblast cultures during in vivo and in vitro aging (Yamagishi et al. 1985; Kunisada et al. 1985). Analysis of cccDNA in heart, brain, and liver tissue from aging mice, however, revealed similar amounts and size distributions of the circular DNAs at all ages except for the slightly larger molecules that were observed in heart preparations of the oldest (24-month-old) mice tested (Flores et al. 1988). In comparison with genomic DNA the cccDNA species contained an overrepresentation of various types of repetitive sequences, including intracisternal A particle genes. The abundance of these sequences in cccDNA appeared to decrease in the oldest animals.

The overall structures of the cccDNA segments often bear a resemblance to that of typical transposons (Fujimoto et al. 1985). It has been proposed that, owing to their characteristics, dispersed repetitive sequences could yield extrachromosomal DNA intermediates by reverse transcription, whereas for tandem repeats unequal recombination followed by excision from the genome was considered a more likely mechan-

ism. An interesting finding in this regard is the excision of the *Sau*3A alphoid tandem repeat from the genome of HeLa cells (Okumura et al. 1987). This sequence has been reported to be unstable in human DNA, causing a high degree of restriction fragment length polymorphism among individuals.

An important issue with respect to the role of cccDNA in the aging process is whether such elements are being generated continuously and subsequently degraded or whether there is an equilibrium of excision from and integration into the genome. In summary, the role if any of cccDNA in aging is unclear at present. However, it seems likely that cccDNA includes intermediates of recombinational events, the location of which might be directed by repetitive sequences.

In *Podospora*, a filamentous fungus, senescence is thought to occur through excision, amplification, and ligation of short sequences (plDNAs or senDNAs) from the mitochondrial genome. These plDNAs are rearranged in the mitochondria of senescence-resistent mutants (Osiewacz et al. 1989). Thus far there is no evidence for an equivalent DNA rearrangement phenomenon in mammalian mitochondria. An age-related loss of mitochondria, however, has been observed in insects (Massie et al. 1975) and rat liver (Stocco and Hutson 1978). It has been suggested that the mitochondria of postmitotic cells play a major role in the aging process because of damage induced by free radicals produced in the organelles during respiration (for a review see Fleming et al. 1985).

Strehler (1986) proposed that aging results from the loss of ribosomal and other DNA sequences containing internal complementarity. This loss has been attributed to DNA strand shifts and removal of nonpaired strands (Strehler 1986). Experimental data supporting the age-related loss of ribosomal cistrons have been obtained from human, dog, and mouse tissue (Johnson and Strehler 1972; Johnson et al. 1975). These observations were confirmed by the studies of Gaubatz and Cutler (1978), who examined rDNA content in mouse liver and brain. These authors, however, suggested that the decreased hybridization in DNA obtained from old donors is due to residual protein, bound to ribosomal DNA regions, and not to a loss of rDNA sequences from the genome.

Obviously, alterations in the DNA sequence organization do occur in somatic tissue with time. The question is whether these alterations interfere with the control of gene expression. In this respect, high-frequency alterations might be more likely to be involved in the aging process than random errors. The identification and characterization of such mutational hot spots appear to be opportune.

General conclusions and future perspectives

From the overview provided above, it can be concluded that genetic instability could be of major importance to the physiological changes associated with the aging process. It appears that errors in the DNA of somatic cells do occur, although the frequency in postmitotic tissue in vivo has thus far not been established. The quantitative variation in both RNA and proteins that has been demonstrated in relation to aging could be due to alterations in the DNA affecting the control of transcription and translation. Thus far there is no evidence for an accumulation with time of sequence changes in either RNA or protein molecules. Because of technical difficulties, however, it has not been possible to detect low-frequency alterations. On the other hand, it has become clear that random errors in biomolecules can originate from different sources. This is illustrated by the age-related accumulation of proteins that have been altered by posttranslational processes. Such abnormal proteins might affect the functioning of all biomolecules. The branched interactions between biomacromolecules, which undoubtedly have only partly been revealed, are a major difficulty in determining the nature and order of events contributing to the aging process. In spite of the abundant evidence for the increase in genetic instability with age, it has thus far not been possible to disprove any of the molecular aging theories discussed in the introduction. This could have the following reasons.

(*i*) Understanding of the complexity of the genome is limited. Recently, the insight in the structure and organization of the genetic material has been greatly increased. The somatic stability of the genome with respect to its primary sequence organization seems to be dependent on the sequence studied and the location of this sequence in the genome. DNA sequences that act as mutational hot spots in germ cells (Jeffreys et al. 1988; Hyrien et al. 1988) should be investigated with respect to their genetic activity in somatic tissue. Such regions could be different from coding DNA with respect to the induction and repair of DNA damage (Hanawalt 1987), which might influence their stability. The noncoding fraction of the mammalian genome containing large amounts of repetitive DNA sequences that were initially regarded as "junk" DNA is gradually revealing its relevance to cell functioning. Many different *cis*-acting DNA elements that play a role in the regulation of gene expression by their interaction with *trans*-acting protein factors now become identified (Sassone-Corsi and Borelli 1986). It seems important to determine the somatic stability of such DNA regions in relation to age. Apart from instability at the primary sequence level, alterations in the secondary structure of DNA might affect gene regulation through an altered signal recognition and activity of DNA-binding proteins (Wang and Glaever 1988).

(*ii*) Uncertainties exist as to the most suitable model systems for molecular biological studies on aging. Although material from humans should be employed where possible, for practical reasons animal model systems like rats and mice are indispensible. There is evidence that, provided their health status and husbandry is optimal, rodents age much in the same way as humans do (Burek 1978). For studying certain fundamental processes, such as the occurrence of various types of DNA rearrangement, lower organisms and cell lines can also be employed. Various aspects of mammalian development and differentiation have been revealed in such model systems, which could facilitate the interpretation of observed phenomena and their relevance to the aging process. However, in these cases results obtained cannot be extrapolated directly to the human situation with respect to physiological consequences.

(*iii*) Existing aging theories are imperfect with respect to their predictions on the longer term. Many aging theories are vaguely formulated and the definitions used vary from theory to theory. Attempts should be made to reformulate aging theories; eliminate logical errors; reduce them to a network of causal relationships; and test each relationship for its consistency with the literature. In this regard, the use of artificial intelligence may be of great help.

(*iv*) The technical approaches being used do not allow the sensitive detection of quantitative or qualitative alterations in molecular biological macromolecules. With respect to the detection of alterations at the DNA level almost all studies are concerned with mitotic somatic tissue. One of the experi-

mental strategies for the analysis of somatic mutations arising at low frequency in postmitotic tissues is based on the use of transgenic experimental animals harbouring the sequence of interest (the target gene for mutagenesis) as a recombinant DNA vector integrated in the DNA of all their cells. Somatic mutations in these sequences can be analysed after the DNA has been rescued from the tissues of these animals (Vijg and Uitterlinden 1987; Vijg et al. 1989). The rapid qualitative analysis of somatic mutations can be performed by direct sequencing, following enzymatic amplification of the target gene sequences (Simpson et al. 1988; Wong et al. 1987). In addition to the use of Southern analysis for the detection of alterations in DNA primary sequence, sequence alterations that do not affect the length of DNA restriction fragments can be detected by denaturing gradient analysis (Vijg and Uitterlinden 1987; Uitterlinden et al. 1989). The high resolution of this technique allows for the analysis of high copy number repetitive sequences.

The variable degree at which the expression of specific genes appears to alter during aging in various tissues raises the question whether gene expression in different cell types or in different cells of a population is differentially affected during the aging process. The use of hybridization histochemistry should provide an answer to such an issue. Recently it has been demonstrated that somatic mutations of an X-linked gene can be measured on the single-cell level by using histochemistry (Griffiths et al. 1988). The role of specific gene functions in the aging process is difficult to establish. The introduction of specific candidate genes into the germ line of experimental animals, in combination with the inactivation of the endogenous gene, offers a possibility to study its regulation during the aging process and its effect on the life-span of the animal.

In summary, the various alterations that have been observed in the organization and expression of the genome during organismal aging have been described. A picture emerges in which aging appears to be associated with an accumulation of alterations in all processes involved in the functioning of informational macromolecules. These processes include the safeguarding of expression and repair of functioning molecules and the removal of dysfunctioning molecules. The complex interaction between macromolecules and the random character of the errors induced might explain the difficulty in distinguishing cause from effect. The rapid development of recombinant DNA technology will be highly beneficial to aging research. Once a great number of age-related alterations have been described in detail, an integrative study of the causative factors that could initiate such alterations and their relevance with respect to the deteriorative aspects of aging can be performed. Finally, such alterations should be correlated with the variation in life-span and age-related pathology of the organism studied.

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Abruzzo, M. A., Mayer, M., and Jacobs, P. A. 1985. Aging and aneuploidy: evidence for the preferential involvement of the inactive X chromosome. Cytogenet. Cell Genet. **39**: 275–278.

Ames, B. N. 1983. Dietary carcinogens and anti-carcinogens. Science (Washington, D.C.), **221**: 1256–1264.

Bernd, A., Batke, E., Zahn, R. K., and Müller, W. E. G. 1982. Age-dependent gene induction in quail oviduct. XV. Alterations of the poly(A)-associated protein pattern and of the poly(A)-chain length of mRNA. Mech. Ageing Dev. **19**: 361–377.

Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. Nature (London), **321**: 209–213.

Bolla, R., and Denckla, W. D. 1979. Effect of hypophysectomy on liver ribonucleic acid synthesis in aging rats. Biochem. J. **184**: 669–674.

Bradley, W. E. C., Gareau, J. L. P., Seifert, A. M., and Messing, K. 1987. Molecular characterization of 15 rearrangements among 90 human in vivo somatic mutants show that deletions predominate. Mol. Cell. Biol. **7**: 956–960.

Brawerman, G. 1987. Determinants of messenger RNA stability. Cell, **48**: 5–6.

Brooks, A., Mead, D., and Peters, R. 1973. Effect of aging on the frequency of metaphase chromosome aberrations in the liver of Chinese hamsters. J. Gerontol. **28**: 452–454.

Burek, J. D. 1978. Pathology of aging rats. CRC Press, West Palm Beach, FL.

Burnet, F. M. 1974. Intrinsic mutagenesis: a genetic approach to aging. J. Wiley and Sons, New York.

Castaneda, M., Vargas, R., and Galvan, S. 1986. Stagewise decline in the activity of brain protein synthesis factors and relationship between this decline and longevity in two rodent species. Mech. Ageing Dev. **36**: 197–210.

Chatterjee, B., Nath, S. J., and Roy, A. 1981. Differential regulation of the messenger RNA for three major senescence marker proteins in the male rat liver. J. Biol. Chem. **256**: 5939–5941.

Cedar, H. 1988. DNA methylation and gene activity. Cell, **53**: 3–4.

Chen, J. J., Brot, N., and Weissbach, H. 1980. RNA and protein synthesis in cultured human fibroblasts derived from donors of various ages. Mech. Ageing Dev. **13**: 285–295.

Clare, M. J., and Luckinbill, L. S. 1985. The effects of gene–environment interaction on the expression of longevity. Heredity, **55**: 19–29.

Curtis, H. J. 1966. Biological mechanisms of aging. C. C. Thomas, Springfield, IL.

Cutler, R. G. 1979. Evolution of human longevity: a critical overview. Mech. Ageing Dev. **9**: 337–354.

——— 1985. Dysdifferentiative hypothesis of aging: a review. *In* Molecular biology of aging: gene stability and expression. *Edited by* R. S. Sohal, L. S. Birnbaum, and R. G. Cutler. Raven Press, New York. pp. 307–340.

Davidson, E. H., and Britten, R. J. 1979. Regulation of gene expression: possible role of repetitive sequences. Science (Washington, D.C.), **204**: 1052–1059.

Dean, R. G., Socher, S. H., and Cutler, R. G. 1985. Dysdifferentiative nature of aging: age-dependent expression of mouse mammary tumor virus and casein genes in brain and liver tissues of the C57Bl/6J mouse strain. Arch. Gerontol. Geriatr. **4**: 43–51.

Dice, J. F. 1985. Cellular theories of aging as related to the liver. Hepathology, **5**: 508–513.

Dice, J. F., and Goff, A. 1987. Error catastrophe and aging: future directions of research. *In* Modern biological theories of aging. *Edited by* H. R. Warner, R. N. Butler, R. L. Sprott, and E. L. Schneider. Raven Press, New York. pp. 155–168.

Dilella, A. G., Chiang, J. Y., and Steggles, A. W. 1982. The quantitation of liver cytochrome P450-LM_2 mRNA in rabbits of different ages and after phenobarbital treatment. Mech. Ageing Dev. **19**: 113–125.

Egilmez, N. K., and Rothstein, M. 1985. The effect of aging on cell-free protein synthesis in the free-living nematode *Tubatrix aceti*. Biochim. Biophys. Acta, **840**: 355–363.

Evans, H. J., and Vijayalaxmi. 1981. Induction of 8-azaguanine resistance and sister chromatid exchange in human lymphocytes exposed to mitomycin C and X rays in vivo. Nature (London), **292**: 601–605.

FAILLA, G. 1958. The aging process and carcinogenesis. Ann. N.Y. Acad. Sci. 71: 1124–1135.

FEATHERSTONE, T., MARSHALL, P. D., and EVANS, H. J. 1987. Problems and pitfalls in assessing human T-lymphocyte mutant frequencies. Mutat. Res. 179: 215–230.

FENECH, M., and MORLEY, A. A. 1986. Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low dose X-irradiation. Mutat. Res. 161: 193–198.

FILION, A. M., and LAUGHREA, M. 1985. Translation fidelity in the aging mammal: studies with an accurate *in* vitro system on aged rats. Mech. Ageing Dev. 29: 125–142.

FLEMING, J. E., MIQUEL, J., and BENSCH, K. G. 1985. Age dependent changes in mitochondria. *In* Basic life sciences. Vol. 35. *Edited by* A. D. Woodhead, A. D. Blackett, and A. Hollaender. Plenum Press, New York. pp. 143–156.

FLEMING, J. E., QUATTROCKI, E., LATTER, G., MIQUEL, J., MARCUSON, R., ZUCKERKANDL, E., and BENSCH, F. G. 1986. Age-dependent changes in proteins of *Drosophila melanogaster*. Science (Washington, D.C.), 231: 1157–1159.

FLORES, S. C., SUNNERHAGEN, P., MOORE, T. K., and GAUBATZ, J. W. 1988. Characterization of repetitive sequence families in mouse heart small polydisperse circular DNAs: age-related studies. Nucleic Acids Res. 16: 3889–3906.

FUJIMOTO, S., and YAMAGISHI, H. 1987. Isolation of an excision product of T-cell receptor α-chain gene rearrangements. Nature (London), 327: 242–243.

FUJIMOTO, S., TSUDA, T., TODA, M., and YAMAGISHI, H. 1985. Transposon-like sequences in extrachromosomal circular DNA from mouse thymocytes. Proc. Natl. Acad. Sci. U.S.A. 82: 2072–2076.

GAUBATZ, J. W., and CUTLER, R. G. 1978. Age-related differences in the number of ribosomal RNA genes of mouse tissues. Gerontology, 24: 179–207.

GERSHON, H., and GERSHON, V. 1970. Detection of inactive enzyme molecules in ageing organisms. Nature (London), 227: 1214–1217.

GERSHON, D., KOHNO, K., MARTIN, G. R., and YAMADA, Y. 1985. Studies on gene structure and function in aging: collagen types I and II and the albumin genes. *In* Interrelationships among aging, cancer and differentiation. *Edited by* B. Pullman et al. D. Reidel Publishing Company. pp. 143–148.

GRESIK, E. W., WENK-SALAMONE, K., ONETTI-MUDA, A., GUBITS, R. M., and SHAW, P. A. 1986. Effect of advanced age on the induction by androgen or thyroid hormone of epidermal growth factor and epidermal growth factor mRNA in the submandibular glands of C57Bl/6 male mice. Mech. Ageing Dev. 34: 175–189.

GRIFFITHS, D. F. R., DAVIES, S. J., WILLIAMS, G. T., and WILLIAMS, E. D. 1988. Demonstration of somatic mutation and colonic crypt clonality by X-linked enzyme. Nature (London), 333: 461–463.

GUIGOZ, Y., and WELLINGER, R. 1984. Tyrosine aminotransferase mRNA and tryptophan oxygenase mRNA induction by physiological stress or by dexamethasone in adult and senescent rats. *In* Pharmacological, morphological and physiological aspects of liver aging. *Edited by* C. F. A. van Bezooijen. EURAGE, Rijswijk. pp. 25–31.

HANAWALT, P. C. 1987. On the role of DNA damage and repair processes in aging: evidence for and against. *In* Modern biological theories of ageing. *Edited by* H. R. Warner, R. N. Butler, R. L. Sproth, and E. L. Schneider. Raven Press, New York. pp. 183–198.

HAYFLICK, L., and MOORHEAD, P. S. 1961. Recent advances in the cell biology of aging. Exp. Cell Res. 25: 585–621.

HOLLIDAY, R. 1987*a*. X-chromosome reactivation. Nature (London), 327: 661–662.

——— 1987*b*. The inheritance of epigenetic defects. Science (Washington, D.C.), 238: 163–170.

HOLLIDAY, R., and KIRKWOOD, T. B. L. 1983. Theories of cell ageing: a case of mistaken identity. J. Theor. Biol. 103: 329–330.

HORBACH, G. J. M. J., PRINCEN, H. M. G., VAN DER KROEF, M., VAN BEZOOIJEN, C. F. A., and YAP, S. H. 1984. Changes in the sequence content of albumin mRNA and its translational activity in the rat liver with age. Biochim. Biophys. Acta, 783: 60–66.

HORBACH, G. J. M. J., VAN DER BOOM, H., VAN BEZOOIJEN, C. F. A., and YAP, S. H. 1986. Molecular aspects of age-related changes in albumin synthesis in rats. *In* Liver drugs and aging. Topics in aging research in Europe. Vol. 7. *Edited by* C. F. A. van Bezooijen, F. Miglio, and D. L. Knook. EURAGE, Rijswijk. pp. 121–126.

HORBACH, G. J. M. J., VAN BEZOOIJEN, C. F. A., and KNOOK, D. L. 1987. Age-related changes in the sythesis of individual liver-specific proteins. Rev. Biol. Res. Aging, 3: 485–494.

HORN, P. L., TURKER, M. S., OGBURN, C. E., DISTECHE, C. M., and MARTIN, G. M. 1984. A cloning assay for 6-thioguanine resistance provides evidence against certain somatic mutational theories of aging. J. Cell. Physiol. 121: 309–315.

HYRIEN, O., DEBATISSE, M., BUTTIN, G., and DE SAINT VINCENT, B. R. 1988. A hotspot for novel amplification joints in a mosaic of Alu-like repeats and palindromic A+T-rich DNA. EMBO (Eur. Mol. Biol. Org.) J. 6: 2401–2408.

INAMIZU, T., NOBUHISA, N., CHANG, M., and MAKINODAN, T. 1986. Frequency of 6-thioguanine-resistant T cells is inversely related to the declining T-cell activities in aging mice. Proc. Natl. Acad. Sci. U.S.A. 83: 2488–2491.

JEFFREYS, A. J., ROYLE, N. J., and WONG, Z. 1988. Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature (London), 332: 378–381.

JOHNSON, R., and STREHLER, R. L. 1972. Loss of genes coding for ribosomal RNA in ageing brain cells. Nature (London), 240: 412–414.

JOHNSON, L. K., JOHNSON, R., and STREHLER, B. L. 1975. Cardiac hypertrophy, aging and changes in cardiac ribosomal RNA gene dosage in man. J. Mol. Cell. Cardiol. 7: 125–133.

KASTAN, M. B., GOWANS, B. J., and LIEBERMAN, M. W. 1982. Methylation of deoxycytidine incorporated by excision–repair synthesis of DNA. Cell, 30: 509–516.

KAY, M. M. B. 1985. Aging of cell membrane molecules leads to appearance of an aging antigen and removal of senescent cells. Gerontology, 31: 215–235.

KAY, M. M. B., BOSMAN, G. J. C. G. M., SHAPIRO, S. S., BENDICH, A., and BASSEL, P. S. 1986. Oxidation as a possible mechanism of cellular aging: vitamin E deficiency causes premature aging and IgG binding to erythrocytes. Proc. Natl. Acad. Sci. U.S.A. 83: 2463–2467.

KIRKLAND, J. L. 1989. Evolution and ageing. Genome. This issue.

KIRKWOOD, T. B. L. 1977. Evolution of aging. Nature (London), 270: 301–304.

——— 1989. DNA, mutations and aging. Mutat. Res. 219: 1–7.

KIRKWOOD, T. B. L., HOLLIDAY, R., and ROSENBERGER, R. F. 1984. Stability of the cellular translation process. Int. Rev. Cytol. 92: 93–132.

KUNISADA, T., YAMAGISHI, H., OGITA, Z., HIRAKAWA, T., and MITSUI, Y. 1985. Appearance of extrachromosomal circular DNAs during in vivo and in vitro ageing of mammalian cells. Mech. Ageing Dev. 29: 89–99.

LOCK, L. F., TAKAGI, N., and MARTIN, G. R. 1987. Methylation of the HPRT gene on the inactive X occurs after chromosome inactivation. Cell, 48: 49–56.

MAKRIDES, S. C. 1983. Protein synthesis and degradation during aging and senescence. Biol. Rev. Cambridge Philos. Soc. 58: 343–422.

MARLHENS, F., ACHKAR, W. AL., AURIAS, A., COUTURIER, J., DUTRILLAUX, A. M., GERBAULT-SEREAU, M., HOFFSCHIR, F., LAMOLIATTE, E., LEFRANCOIS, D., LOMBARD, M., MULARIS, M., PRIEUR, M., PROD'HOMME, M., SABATIER, L., VIEGAS-PEQUIGNOT, E., VOLOBOUEV, V., and DUTRILLAUX, B. 1986. The rate of chromosome breakage is age dependent in lymphocytes of adult controls. Hum. Genet. 73: 290–297.

MARTIN, G. M., CURTIS, A., SPRAGUE, B. S., and EPSTEIN, C. J. 1970. Replicative life-span of cultivated human cells. Lab. Invest. 23: 86–92.

MARTIN, G. M., SMITH, A. C., KETTERER, D. J., OGBURN, C. E., and DISTECHE, C. M. 1985. Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice. Isr. J. Med. Sci. 21: 296–301.

MASSIE, H. R., BAIRD, M. B., and MCMAHON, M. M. 1975. Loss of mitochondrial DNA with aging. Gerontologia, 21: 231–238.

MATOCHA, M. F., COSGROVE, J. W., ATACK, J. R., and RAPOPORT, S. I. 1987. Selective elevation of c-myc transcript levels in the liver of the aging Fischer-344 rat. Biochem. Biophys. Res. Commun. 147: 1–7.

MAYNARD SMITH, J. 1962. The causes of ageing. Proc. R. Soc. London Ser. B. 157: 115–127.

MAYS-HOOPES, L. L. 1985. DNA methylation: a possible correlation between aging and cancer. *In* Molecular biology of aging: gene stability and gene expression. *Edited by* R. S. Sohal, L. S. Birnbaum, and R. G. Cutler. Raven Press, New York. pp. 49–65.

MAYS-HOOPES, L. L., BROWN, A., and HUANG, R. C. C. 1983. Methylation and rearrangement of mouse intracisternal A particle genes in development, aging and myeloma. Mol. Cell. Biol. 3: 1371–1380.

MAYS-HOOPES, L. L., CHAO, W., BUTCHER, H. C., and HUANG, R. C. C. 1986. Decreased methylation of the major mouse long interspersed repeated DNA during aging and in myeloma cells. Dev. Genet. 7: 65–73.

MERGLER, N. L., and GOLDSTEIN, M. D. 1983. Why are there old people. Hum. Dev. 26: 72–90.

MIGEON, B. R., AXELMAN, J., and BEGGES, A. H. 1988. Effect of ageing on reactivation on the human X-linked HPRT locus. Nature (London), 335: 93–96.

MORLEY, A. A., COX, S., and HOLLIDAY, R. 1982. Human lymphocytes resistant to 6-thioguanine increase with age. Mech. Ageing Dev. 19: 21–26.

MULLAART, E., BOERRIGTER, M. E. T. I., BROUWER, A., BERENDS, F., and VIJG, J. 1988. Age-dependent accumulation of alkali-labeled sites in DNA of post-mitotic but not in that of mitotic rat liver cells. Mech. Ageing Dev. 45: 41–49.

MULLAART, E., BOERRIGTER, M. E. T. I., LOHMAN, P. H. M., and VIJG, J. 1989*a*. Age-related induction and disappearance of carcinogen–DNA adducts in livers of rats exposed to low levels of 2-acetylaminofluorene. Chem. Biol. Interact. In press.

MULLAART, E., ROZA, L., LOHMAN, P. H. M., and VIJG, J. 1989*b*. The removal of UV-induced pyrimidine dimers from DNA of rat skin cells in vitro and in vivo in relation to aging. Mech. Ageing Dev. In press.

MURTY, C. V. R., MANCINI, M. A., CHATTERJEE, B., and ROY, A. 1988. Changes in transcriptional activity and matrix association of $\alpha_{2\mu}$-globulin gene family in the rat liver during maturation and aging. Biochim. Biophys. Acta, 949: 27–34.

NALBANTOGLU, J., PHEAR, G., and MEUTH, M. 1987. DNA sequence analysis of spontaneous mutations at the *aprt* locus of hamster cells. Mol. Cell. Biol. 7: 1445–1449.

OHRLOFF, C., LANGE, G., and HOCKWIN, O. 1980. Post-synthetic changes of glutathione peroxidase (EC 1.11.1.9) and glutathione reductase (EC 1.6.4.2) in the ageing bovine lens. Mech. Ageing Dev. 14: 453–458.

OKUMURA, K., KIYAMA, R., and OISHI, M. 1987. Sequence analysis of extrachromosomal *Sau*3A and related family DNA: analysis of recombination in the excision event. Nucleic Acids Res. 15: 7477.

OLIVER, C. N., AHN, B., MOERMAN, E. J., GOLDSTEIN, S., and STADTMAN, E. R. 1987. Age-related changes in oxidized proteins. J. Biol. Chem. 262: 5488–5491.

ONO, T., and CUTLER, R. G. 1978. Age-dependent relaxation of gene expression: increase of endogenous murine leukemia virus-related and globin-related RNA in brain and liver of mice. Proc. Natl. Acad. Sci. U.S.A. 75: 4431–4435.

ONO, T., DEAN, R. G., CHATTOPADHYAY, S. K., and CUTLER, R. G. 1985*a*. Dysdifferentiative nature of aging: age-dependent expression of MuLV and globin genes in thymus, liver and brain in the AKR mouse strain. Gerontology, 32: 362–372.

ONO, T., OKADA, S., KAWAKAMI, T., HONJO, T., and GETZ, M. J. 1985*b*. Absence of gross change in primary DNA sequence during aging process of mice. Mech. Ageing Dev. 32: 227–234.

ONO, T., TAWA, R., SHINYA, K., HIROSE, S., and OKADA, S. 1986. Methylation of the c-myc gene changes during aging process of mice. Biochem. Biophys. Res. Commun. 139: 1299–1304.

ORGEL, L. 1963. The maintenance of the accuracy of protein synthesis and its relevance to aging. Proc. Natl. Acad. Sci. U.S.A. 49: 517–521.

——— 1973. Ageing of clones of mammalian cells. Nature (London), 243: 441–445.

OSIEWACZ, H. D., HERMANNS, J., MARCOU, D., TRIFFI, M., and ESSER, K. 1989. Mitochondrial DNA rearrangements are correlated with a delayed amplification of the mobile intron (plDNA) in a long-lived mutant of *Podospora anserina*. Mutat. Res. 219: 9–15.

PEREIRA-SMITH, O. M., FISCHER, S. F., and SMITH, J. R. 1985. Senescent and quiescent cell inhibitors of DNA synthesis. Exp. Cell Res. 160: 296–306.

PRIEUR, M., ACHKAR, W. A. I., AURIAS, A., COUTURIER, J., DUTRILLAUX, A. M., FLURY-HERARD, A., GERBAULT-SEUREAU, M., HOFFSCHIR, F., LAMOLIATTE, E., LEFRANCOIS, D., LOMBARD, M., MULERIS, M., RICOUL, M., SABATIER, L., and VIEGAS-PEQUIGNOT, E. 1988. Acquired chromosome rearrangements in human lymphocytes: effects of aging. Hum. Genet. 79: 147–150.

REFF, M. E. 1985. RNA and protein metabolism. *In* Handbook of the biology of aging. *Edited by* C. E. Finch and E. L. Schneider. Van Nostrand Reinhold Co., New York. pp. 225–254.

REISS, U., and ROTHSTEIN, M. 1974. Isocitrate lyase from the free-living nematode *Tubatrix aceti*: purification and properties. Biochemistry, 13: 1796–1800.

REISS, U., and SACKTOR, B. 1983. Monoclonal antibodies to renal brush border membrane maltase: age-associated antigenic alterations. Proc. Natl. Acad. Sci. U.S.A. 80: 3255–3259.

RICHARDSON, A., and SEMSEI, I. 1987. Effect of aging on translation and transcription. *In* Review of biological research in aging. Vol. 3. *Edited by* M. Rothstein. A. R. Liss, New York. pp. 467–483.

RICHARDSON, A., BIRCHENALL-SPARKS, M. C., and STAECKER, J. L. 1983. Aging and transcription. *In* Review of biological research in aging. Vol. 1. *Edited by* M. Rothstein. A. R. Liss, New York. pp. 275–294.

RICHARDSON, A., RUTHERFORD, M. S., BIRCHENALL-SPARKS, M. C., ROBERTS, M. S., WU, W. T., and CHEUNG, H. T. 1985. Levels of specific messenger RNA species as a function of age. Aging, 29: 229–236.

RÖHME, D. 1981. Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo. Proc. Natl. Acad. Sci. U.S.A. 78: 5009–5013.

ROSENBERGER, R. F., and KIRKWOOD, T. B. L. 1986. Errors and the integrity of genetic information transfer. *In* Accuracy in molecular processes. *Edited by* T. B. L. Kirkwood, R. F. Rosenberger, and D. J. Galas. Chapman and Hall, London. pp. 17–35.

ROTHSTEIN, M. 1987. Evidence for and against the error catastrophe theory. *In* Modern biological theories of aging. *Edited by* H. R. Warner, R. N. Butler, R. L. Sprott, and E. L. Schneider. Raven Press, New York. pp. 139–154.

ROY, A. K., NATH, T. S., MOTWANI, N. M., and CHATTERJEE, B. 1983. Age-dependent regulation of the polymorphic forms of 2-globulin. J. Biol. Chem. 258: 10 123 – 10 127.

RUTHERFORD, M. S., BAEHLER, C. S., and RICHARDSON, A. 1986. Genetic expression of complement factors and α^1-acid glycoprotein by liver tissue during senescence. Mech. Ageing Dev. 35: 245–254.

SACHER, G. A. 1978. Longevity, aging and death: an evolutionary perspective. Gerontologist, 18: 112–119.

——— 1982. Evolutionary theory in gerontology. Perspect. Biol. Med. 25: 339–353.

SASSONE-CORSI, P., and BORRELLI, E. 1986. Transcriptional regula-

tion by trans-acting factors. Trends Genet. 2: 215–219.
SCHNEIDER, E. L. 1985. Cytogenetics of aging. *In* Handbook of the biology of aging. *Edited by* C. E. Finch and E. L. Schneider. Van Nostrand Reinhold Co., New York. pp. 357–373.
——— 1987. Theories of aging: a perspective. *In* Modern biological theories of aging. *Edited by* H. R. Warner, R. N. Butler, R. L. Sprott, and E. L. Schneider. Raven Press, New York. pp. 1–4.
SEMSEI, I., and RICHARDSON, A. 1986. Effect of age on the expression of genes involved in free radical protection. Fed. Proc. Fed. Am. Soc. Exp. Biol. 45: 217.
SHARMA, H. K., and ROTHSTEIN, M. 1980. Altered phosphoglycerate kinase in aging rats. J. Biol. Chem. 255: 5043–5050.
SHMOOKLER REIS, R. J. 1976. Enzyme fidelity and metazoan aging. Interdiscip. Top. Gerontol. 10: 11–23.
——— 1981. Ribosomes from aging mice are not generally deficient in cell-free protein synthesis. Mech. Ageing Dev. 17: 311–320.
SHMOOKLER REIS, R. J., and GOLDSTEIN, S. 1980. Loss of reiterated DNA sequences during serial passage of human diploid fibroblasts. Cell, 21: 739–749.
SILBER, J. R., FRY, M., MARTIN, G. M., and LOEB, L. A. 1985. Fidelity of DNA polymerases isolated from regenerated liver chromatin of *mus musculus*. J. Biol. Chem. 260: 1304–1310.
SIMPSON, D., CROSBY, R. M., and SKOPEK, T. R. 1988. A method for specific cloning and sequencing of human HPRT cDNA for mutation analysis. Biochem. Biophys. Res. Commun. 151: 487–492.
SMITH, J. R., and PEREIRA-SMITH, O. M. 1989. Altered gene expression during cellular aging. Genome. This issue.
STOCCO, D. M., and HUTSON, J. C. 1978. Quantitation of mitochondrial DNA and protein in the liver of Fischer 344 rats during aging. J. Gerontol. 33: 802–809.
STRAUSS, G. H., and ALBERTINI, R. J. 1979. Enumeration of 6-thioguanine-resistent peripheral blood lymphocytes in man as a potential test for somatic cell mutations arising in vivo. Mutat. Res. 61: 353–379.
STREHLER, B. 1986. Genetic instability as the primary cause of human aging. Exp. Gerontol. 21: 283–319.
SZILLARD, L. 1959. On the nature of the aging process. Proc. Natl. Acad. Sci. U.S.A. 45: 35–45.
TRAINOR, K. J., WIGMORE, D. J., CHRYSOSTOMU, A., DEMPSEY, J. L., SESHADRI, R., and MORLEY, A. A. 1984. Mutation frequency in human lymphocytes increase with age. Mech. Ageing Dev. 27: 83–86.
TURNER, D. R., MORLEY, A. A., HALIANDROS, M., KUTLACA, R., and SANDERSON, B. J. 1985. In vivo somatic mutations in human lymphocytes frequently result from major gene alterations. Nature (London), 315: 343–345.
UITTERLINDEN, A. G., VIJG, J., GIPHART, M. J., and KNOOK, D. L. 1985. Variation in restriction fragment length and methylation pattern of rat MHC class I genes. Exp. Clin. Immunogenet. 2: 215–222.
UITTERLINDEN, A. G., SLAGBOOM, P., KNOOK, D. L., and VIJG, J. 1989. Two-dimensional DNA fingerprinting of human individuals. Proc. Natl. Acad. Sci. U.S.A. In press.
VAN BEZOOIJEN, C. F. A., GRELL, T., and KNOOK, D. L. 1976. Albumin synthesis by liver parenchymal cells isolated from young adult and old rats. Biochem. Biophys. Res. Commun. 71: 513–519.
VAN LEEUWEN, F. W., BURBACH, J. P. H., and IVELL, R. 1988. Evidence for gene conversion between the oxytocin and vasopressin genes of Brattleboro rats. Soc. Neurosci. 14. In press.
VIJG, J., and KNOOK, D. L. 1987. DNA repair in relation to the aging process. J. Am. Geriatr. Soc. 35: 532–541.
VIJG, J., and UITTERLINDEN, A. G. 1987. A search for DNA alterations in the aging mammalian genome: an experimental strategy. Mech. Ageing Dev. 41: 47–63.
VIJG, J., UITTERLINDEN, A. G., and KNOOK, D. L. 1984. Arrangement and methylation state of ras oncogenes in liver hyperplastic nodules. *In* Pharmacological, morphological and physiological aspects of liver aging. *Edited by* C. F. A. van Bezooijen. EURAGE, Rijswijk. pp. 49–55.
VIJG, J., UITTERLINDEN, A. G., MULLAART, E., LOHMAN, P. H. M., and KNOOK, D. L. 1985. Processing of DNA damage during aging: induction of genetic alteration. *In* Molecular biology of aging: gene stability and gene expression. *Edited by* R. S. Sohal, L. S. Birnbaum, and R. G. Cutler. Raven Press, New York. pp. 155–171.
VIJG, J., GOSSEN, J. A., SLAGBOOM, P. E., and UITTERLINDEN, A. G. 1989. New methods for the detection of DNA sequence variation. *In* Early human retroviruses. UCLA symposia on molecular and cellular biology. New Series, 123. *Edited by* M. Clegg and S. O'Brien. A. R. Liss, New York. In press.
WANG, J. C., and GLAEVER, G. N. 1988. Action at a distance along a DNA. Science (Washington, D.C.), 240: 300–304.
WAREHAM, K. A., LYON, M. F., GLENISTER, P. H., and WILLIAMS, E. D. 1987. Age-related reactivation of an X-linked gene. Nature (London), 327: 725–727.
WELLINGER, R., and GUIGOZ, Y. 1986. The effect of age on the induction of tyrosine aminotransferase and tryptophan oxygenase genes by physiological stress. Mech. Ageing Dev. 34: 203–217.
WILLIAMS, G. C. 1957. Pleiotropy, natural selection, and the evolution of aging. Evolution (Lawrence, Kans.), 11: 398–411.
WILSON, V. L., SMITH, R. A., MA, S., and CUTLER, R. G. 1987. Genomic 5-methyldeoxycitidine decreases with age. J. Biol. Chem. 262: 9984–9951.
WONG, C., DOWLING, C. E., SAIKI, R. K., HIGUCHI, R. G., EHRLICH, H. A., and KAZAZIAN, H. H., JR. 1987. Characterization of β-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. Nature (London), 330: 384–386.
YAMAGISHI, H., KUNISADA, T., and TAKEDA, T. 1985. Amplification of extrachromosomal small circular DNAs in a murine model of accelerated senescence. A brief note. Mech. Ageing Dev. 29: 101–103.
YANG, T. P., and CASKEY, C. 1987. Nuclease sensitivity of the mouse HPRT gene promotor region: differential sensitivity on the active and inactive X chromosomes. Mol. Cell. Biol. 7: 2994–2998.
YOUSSOUFIAN, H., KAZAZIAN, H. H., JR., PHILLIPS, D. G., ARONIS, S., TSIFTIS, G., BROWN, V. A., and ANTONARAKIS, S. E. 1986. Recurrent mutations in haemophilia A give evidence for CpG mutation hotspots. Nature (London), 324: 380–382.

CHAPTER 4

MESSENGER RNA LEVELS AND METHYLATION PATTERNS OF GAPDH AND β-ACTIN GENES IN RAT LIVER, SPLEEN AND BRAIN IN RELATION TO AGING

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MESSENGER RNA LEVELS AND METHLATION PATTERNS OF GAPDH AND β-ACTIN GENES IN RAT LIVER, SPLEEN AND BRAIN IN RELATION TO AGING

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SUMMARY

Messenger RNA levels and methylation patterns of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and β-actin genes were studied in spleen, liver and brain of 6-, 24- and 36-month old female inbred rats. In the spleen, the mRNA levels of both housekeeping genes significantly increased between 24 and 36 months. No age-related alterations in the expression of GAPDH or β-actin mRNA were observed in brain or liver. A considerable intertissue and interindividual variation was observed in the mRNA levels of these genes in all age-groups as compared to the level of 28 S rRNA, which was used as an internal control. In this respect the interindividual variation in the level of GAPDH mRNA paralleled the variation observed in the β-actin mRNA level in the three tissues studied. The methylation pattern of β-actin was found to be tissue-specific in contrast to that of GAPDH, which was identical in all three tissues. No significant age-related alterations were observed in the GAPDH methylation pattern, whereas β-actin appeared to become slightly demethylated with age in the spleen at the CpG site for which tissue-specificity was observed.

Key words: DNA methylation; mRNA; Gene expression; Aging; β-Actin; GAPDH

INTRODUCTION

Loss of epigenetic control has been proposed as a major causal factor in the

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aging process [1]. A direct way to test this hypothesis is by studying levels of specific mRNAs as a function of age and the factors involved in their regulation. At the level of individual mRNAs, age-related alterations were observed for a number of genes [for a review, see 2] among which the *c*-myc oncogene [3] the tyrosine aminotransferase [4] albumin [5,6], and tryptophane oxygenase [4] genes. Most of these genes have in common that they are inducible and have a tissue-specific expression pattern. As yet it is not clear whether the transcription of constitutively expressed genes is also affected in aging organisms. One might expect that the mRNA levels of these genes reflect primary alterations in genetic control rather than the effect of endocrine factors, which might be the underlying cause for age-related changes in the expression level of inducible genes [7].

One of the mechanisms that could be involved in transcriptional deregulation with age involves alterations in the non-random distribution of DNA methylation. In general, hypermethylation has frequently been associated with transcriptional silence, whereas demethylation at particular sites is associated with derepression [8,9]. An age-related loss of 5-methylcytosine (5 mC) residues was observed at the level of the whole genome in various experimental animals and in human epithelial cells [10]. The same was found in transcriptionally active and inactive genomic DNA isolated from lymphocytes of young and old human donors [11]. Alterations in the methylation pattern of individual genes, for example, the *c*-myc gene in mice [3] and the collagenase I gene in the rat [12] have also been observed.

In the present study we have investigated the mRNA levels and methylation patterns of two housekeeping genes that are frequently used as internal controls in comparative studies, the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene and the β-actin gene in various tissues of 6-, 24- and 36-month old rats. We found that between 24 and 36 months of age the mRNA levels of both housekeeping genes are elevated significantly in the spleen. No age-related changes in the mRNA level of these genes were observed in liver or brain, although a considerable interindividual variation was observed in the mRNA levels of both genes in all tissues. The methylation pattern of β-actin showed an age-related demethylation in the spleen, at a CpG site for which tissue-specific hypomethylation was also observed.

MATERIALS AND METHODS

Animals

Organs were dissected from female inbred Wistar-derived WAG/Rij rats aged 6, 24 and 36 months. Animals were fed *ad libitum* and maintained under clean conventional conditions at the colony of the TNO Institute for Experimental Gerontology [13]. Under these circumstances the animals have a maximum life-

span of about 4 years. All animals sacrificed for this study were subjected to complete gross and microscopic examination. Tissues affected by a well defined disease process, e.g. tumors etc., were excluded from this study.

DNA probes

Plasmids pRGPDN5 [14] and pAct-1 [15] contain cDNAs corresponding to the GAPDH and the β-actin gene, respectively. The 2 kb BglI-EcoRI fragment of plasmid pHR28.1 [16], which corresponds to 28 S rDNA was used as a control. All hybridization experiments were performed with the purified insert fragments of these plasmids.

Dot blot and Northern blot hybridization analysis

Total RNA was extracted from whole liver, brain and spleen using the frozen tissue/LiCl procedure [17]. The concentration of each RNA preparation was measured by photospectometry and by the chemical orcinol method [18]. Dot blot analysis of equal amounts of total RNA (0.1 μg RNA denatured for 1 h at 50°C in 50% (v/v) formamide and 6.5% formaldehyde) was performed using a dot blot apparatus (Bio-Rad) according to the manufacturer's specifications. For Northern analysis, equal amounts (20 μg) of total RNA were size-fractionated by electrophoresis in 1.5% agarose gels containing formaldehyde [19]. Electrophoresis was performed for 16 h at 25 V/15 mA. The gels were stained with ethidium bromide to assess the integrity of the different RNA preparations and then subjected to Northern blotting onto Gene Screen Plus nylon membranes (NEN Research Products) as described by Thomas [20]. After transfer, membranes were baked for 2 h at 80°C. Hybridization was carried out in the presence of 7% SDS (w/v) as described by Church and Gilbert [21], using random primed ^{32}P-labeled probes with a specific activity of 5×10^8 cpm/μg [22]. Autoradiography was performed at -80°C using Kodak X-omat AR2 films and X-omatic intensifying screens. The hybridization intensity of the dots and bands was measured by densitometric scanning using a Model 620 Video Densitometer (Bio-Rad). For rehybridization the membranes were stripped for 10 min at 100°C in 1% SDS, 1.8 mM NaCl, 0.1 mM NaH_2PO_4, 0.01 mM EDTA.

Southern blot hybridization analysis

Frozen liver, spleen and brain tissue were homogenized at 0°C and incubated overnight at 65°C in 100 mM EDTA, 50 mM Tris-HCl (pH 7.5), 1% SDS and 200 μg/ml proteinase K. After mixing with 1/5 vol. of 8 M KAc the solution was kept at 0°C for 30 min and subsequently extracted with one volume of chloroform. DNA was ethanol-precipitated and solubilized in 10 mM Tris—HCl (pH 7.5), 0.1 mM EDTA. High molecular weight DNA was digested for 3 h at 37°C with a 5-fold excess of Msp I or Hpa II restriction enzyme according to the manufacturer's specifications (BRL) The samples were mixed with 1/5 vol.

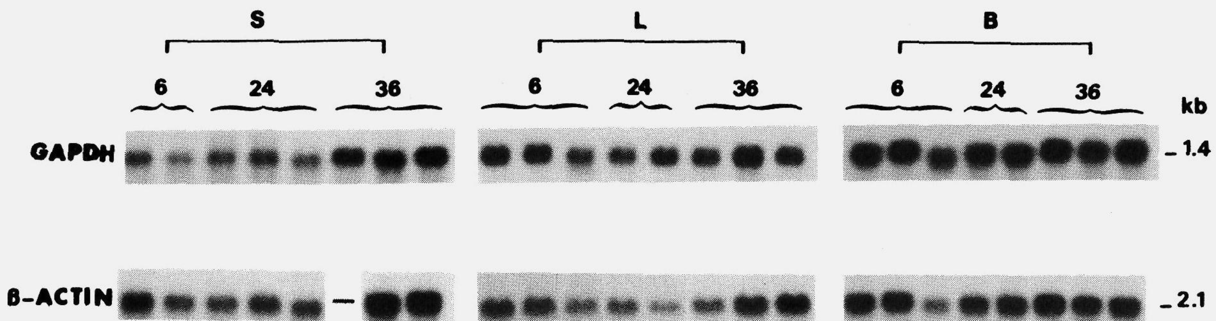


Fig. 1. Northern hybridization patterns of GAPDH and β-actin in 20 μg of total RNA isolated from spleen (S), liver (L) and brain (B) of female WAG/Rij rats aged 6, 24 and 36 months (different autoradiographic exposure times). The same filters were sequentially hybridized, stripped and rehybridized with the cDNA probe homologous to the GAPDH and β-actin gene. Size markers are presented in kilobases (kb).

of 8 M Kac, kept at 0°C for 30 min and subsequently chloroform-extracted. DNA was recovered after precipitation with ethanol, solubilized in sterile water and redigested under the aforementioned conditions. To quantify the digested DNA, subfractions of the samples were denatured in 4.5 ml 0.06 M NaOH, 0.02 M EDTA (pH 12.6) and neutralized and stained with 0.8 ml 4 M NaCl, 0.6 M NaOH, 1.0 M NaH_2PO_4 and 0.5 mg/ml Hoechst 33258 dye [23]. The fluorescence was measured at 430 nm by excitation at 370 nm in a Pye Unicam LC-FL detector. Equal amounts of DNA (5 μg) were electrophoresed in 0.8% agarose gels. The separation patterns were transferred to Gene Screen Plus nylon membranes (NEN Research Products) and hybridized as described in the previous section for Northern hybridization analysis. To check for complete Hpa II digestion all filters were rehybridized with total rat mitochondrial DNA which is known to be completely demethylated [24].

RESULTS

GAPDH and β-actin mRNA levels

By using Northern hybridization analysis, we have measured the GAPDH and β-actin mRNA levels in total RNA isolated from spleen, liver and brain obtained from rats aged 6, 24 and 36 months (Fig. 1). The transcript size of the mRNAs, which is 1.4 kb and 2.1 kb for the GAPDH and the β-actin gene, respectively, as measured by Northern hybridization analysis, remained unaffected during aging. The mRNA levels of these genes in different tissues are compared in Fig. 2, showing that the GAPDH mRNA level is higher in liver

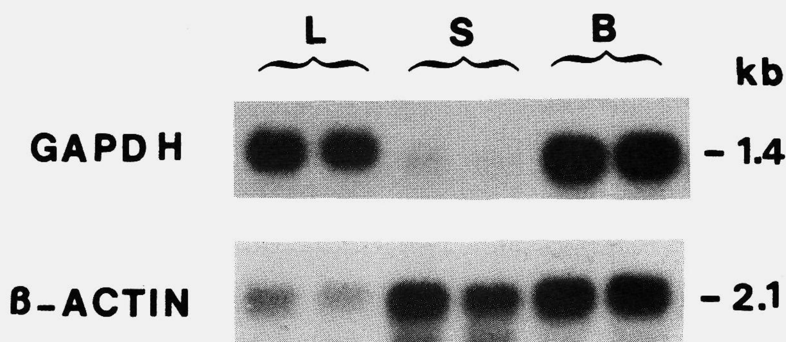


Fig. 2. Northern hybridization patterns of GAPDH and β-actin in 20 μg of total RNA isolated from liver (L), spleen (S) and brain (B) of two different 6-month-old female WAG/Rij rats using the GAPDH and β-actin cDNA probes (identical autoradiographic exposure times). Size markers are presented in kilobases (kb).

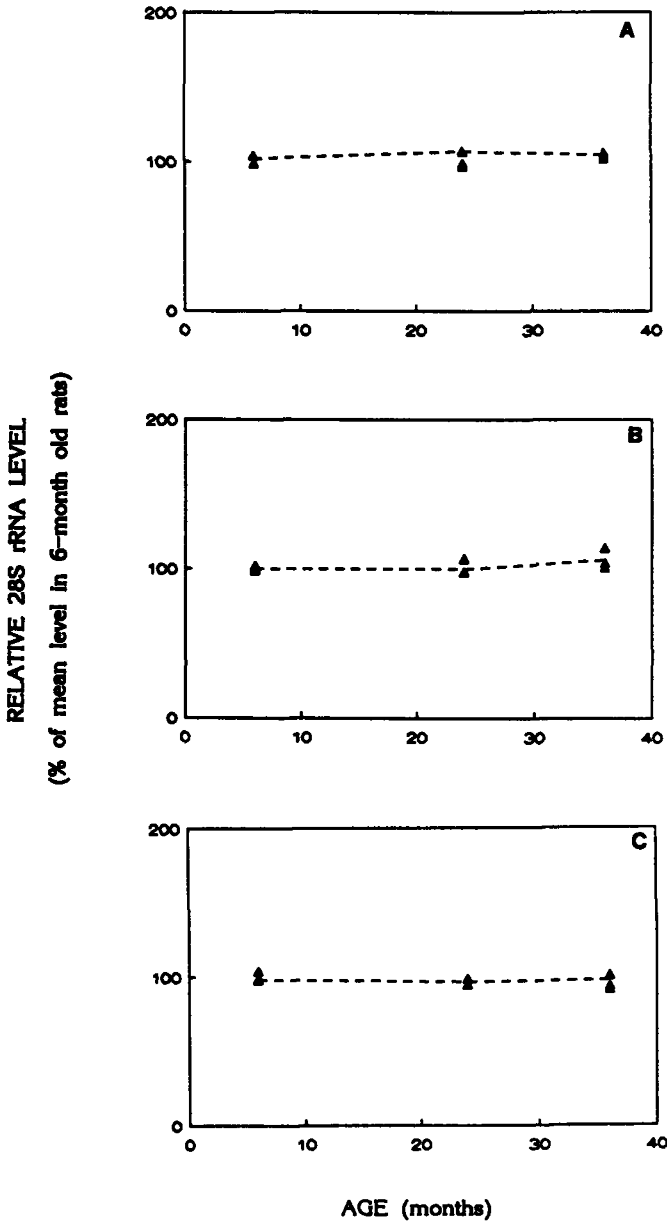


Fig. 3. Expression levels of 28 S rRNA in spleen (A) liver (B) and brain (C) as a function of age. Data were obtained by densitometric scanning of dot blot hybridization signals. Each point represents the result of a determination on one individual animal and is expressed as the percentage of the average value in 6-month-old rats (100%).

and brain than in spleen, whereas the β-actin mRNA level is the highest in brain and only minimal in liver.

For quantitative comparison, the levels of GAPDH and β-actin mRNA were normalized to the level of 28 S rRNA in the same samples as measured by dot blot hybridization analysis. Hybridization signals were quantified by densitometric scanning. Interindividual or age-related variations in the ribosomal RNA concentrations were not observed (Fig. 3) while also the amount of total RNA isolated per gram tissue did not change with age (results not shown).

The relative mRNA levels of the two housekeeping genes in each tissue, as a function of age are shown in Fig.4. The levels of GAPDH and β-actin mRNA

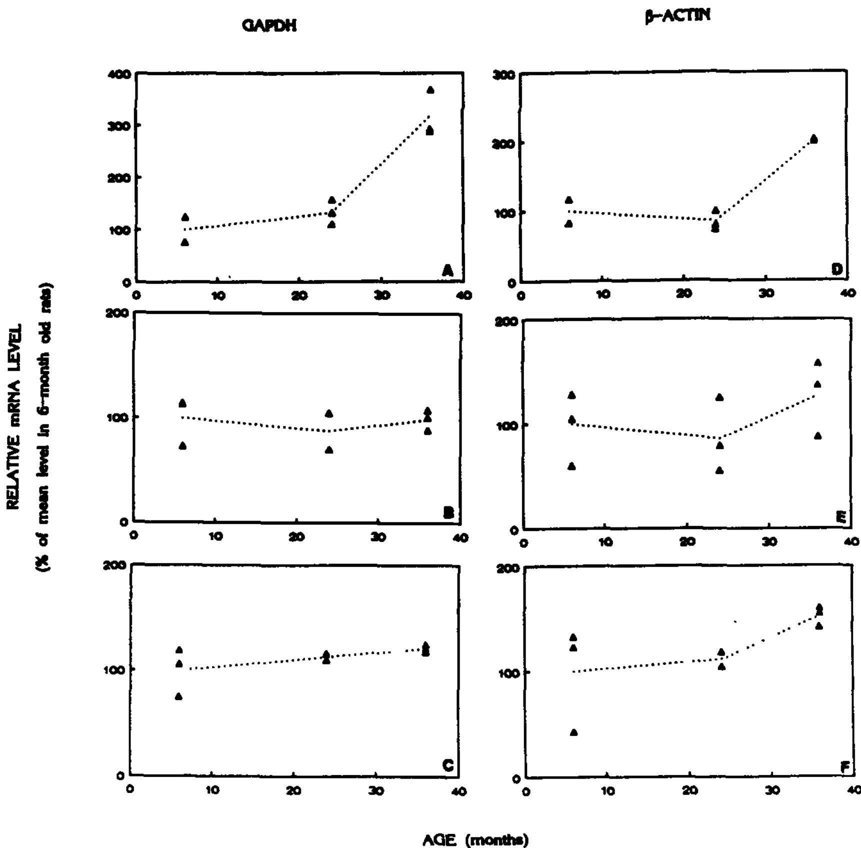


Fig. 4. Expression levels of GAPDH and β-actin mRNA in the spleen (A,D), liver (B,E) and brain (C,F) as a function of age. The data, obtained by densitometric scanning of the specific bands shown in Fig. 1, were normalized to the relative 28 S rRNA concentration of the samples as measured by dot blot analysis (Fig. 3). Each point represents the result of a determination on one individual animal and is expressed as the percentage of the average value in 6-month-old rats (100%). Repeated determinations on one sample indicated an experimental variation of less than 10%.

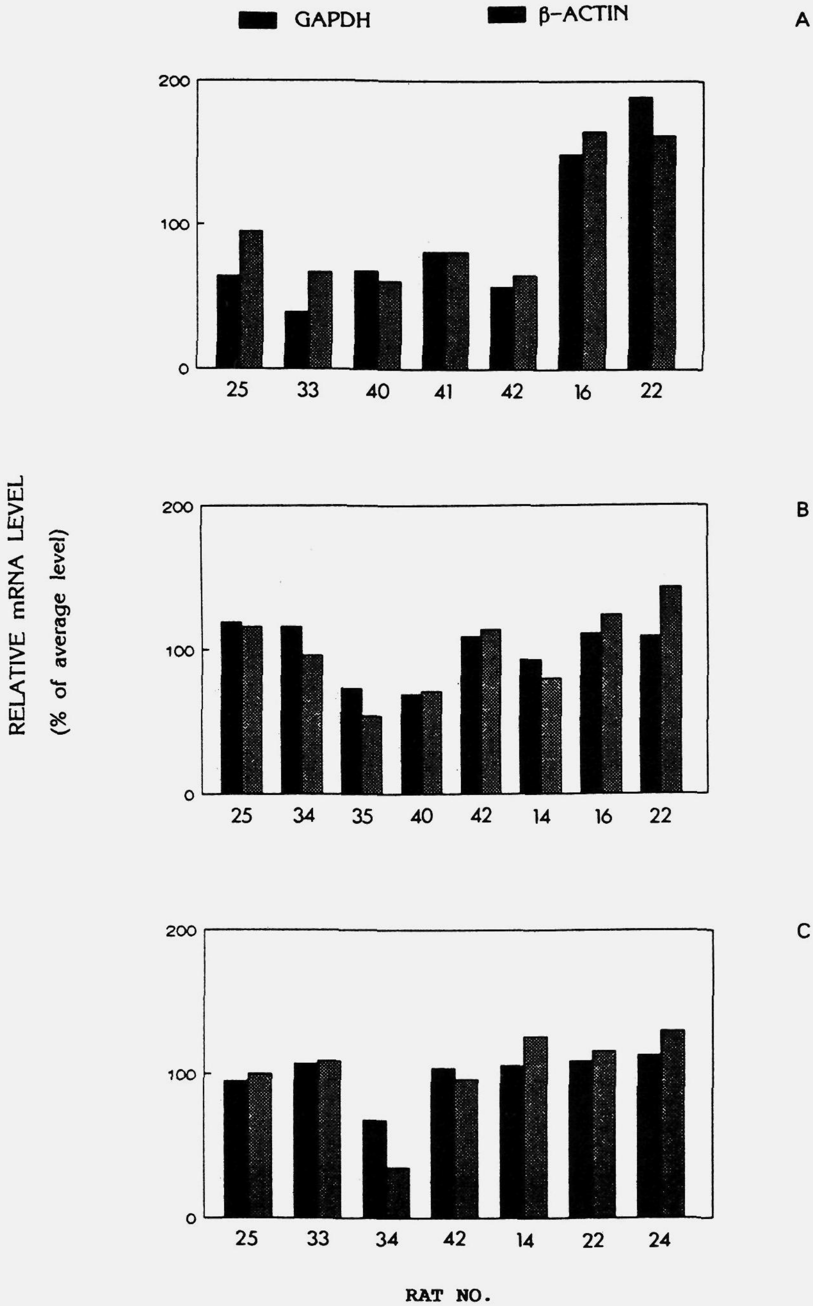


Fig. 5. Expression levels of GAPDH and β-actin mRNA in the spleen (A), liver (B) and brain (C) of different individual animals. Data were obtained by densitometric scanning of the bands shown in Fig.1. Relative mRNA levels are presented as a percentage of the average mRNA level in all animals.

in the spleen were increased 200% and 100% (Figs. 4A and D), respectively, in 36-month-old animals as compared to 6- and 24-month-old rats. In liver and brain, however, no age-associated alterations in the level of these mRNAs were observed (Figs. 4B and E, and 4C and F, respectively). A considerable interindividual variation in the mRNA levels of both genes was observed in all tissues, but most strikingly for the β-actin gene in the liver (Fig. 4E). In the tissues studied GAPDH and β-actin mRNA levels appeared to show a paralleled interindividual variation (Fig. 5) i.e. when the GAPDH level of one tissue was high in a particular animal, the same was true for β-actin. However, relatively high levels of GAPDH and β-actin mRNA in one tissue were sometimes accompanied by relatively low levels in another tissue from the same animal (Fig. 5, rat no. 25, 42 and 22), indicating organ-specificity.

Methylation patterns of GAPDH and β-actin genes

The methylation patterns of cytosine residues within and in the vicinity of the genes under study were examined by Southern hybridization analysis using the methylation sensitive restriction enzymes Hpa II and Msp I. Both enzymes recognize the 5′-CCGG-3′ site, but Hpa II will not cut when the internal cytosine is methylated. The same is true for Msp I when the external cytosine is methylated [25].

Figure 6 shows the result of Southern analysis of the 5′-CCGG-3′ sites detected by the GAPDH and β-actin cDNAs. Since the GAPDH cDNA does not contain 5′-CCGG-3′ sites [26] all sites studied were present in intron sequences, at the 3′ and 5′ ends of the gene and in the pseudogenes. The large number of sites that gave rise to 32 bands upon digestion with Msp I, remained completely methylated during aging in all tissues (Fig. 6A) which is indicated by the fact that none of these fragments is generated following digestion with Hpa II. No tissue-specific variations in the GAPDH methylation pattern were observed.

With respect to β-actin, it should first be noted that some of the weakly hybridizing fragments of the Msp I digestion pattern shown in Fig. 6B, may be derived from the τ-actin gene, due to cross-hybridization [15]. A number of 5′-CCGG-3′ sites were found to be methylated in a tissue-specific manner, which is illustrated by comparison of the Hpa II digestion patterns of β-actin in the three tissues (Fig. 6B) Hypomethylation of the sites resulting in a 0.95 kb β-actin fragment upon Hpa II digestion, for example, was found in brain as compared to liver DNA. Interestingly, the degree of hypomethylation of these sites corresponded with the different mRNA levels of the β-actin gene in these tissues (compare Fig. 2 with Fig. 6B) Furthermore, demethylation of the β-actin gene was observed as a function of age in spleen DNA (Figs. 6B and 7A), especially at the 0.95 kb band for which also tissue-specificity was found. Densitometric scanning of the hybridization signal of this band in Fig. 7A showed that the

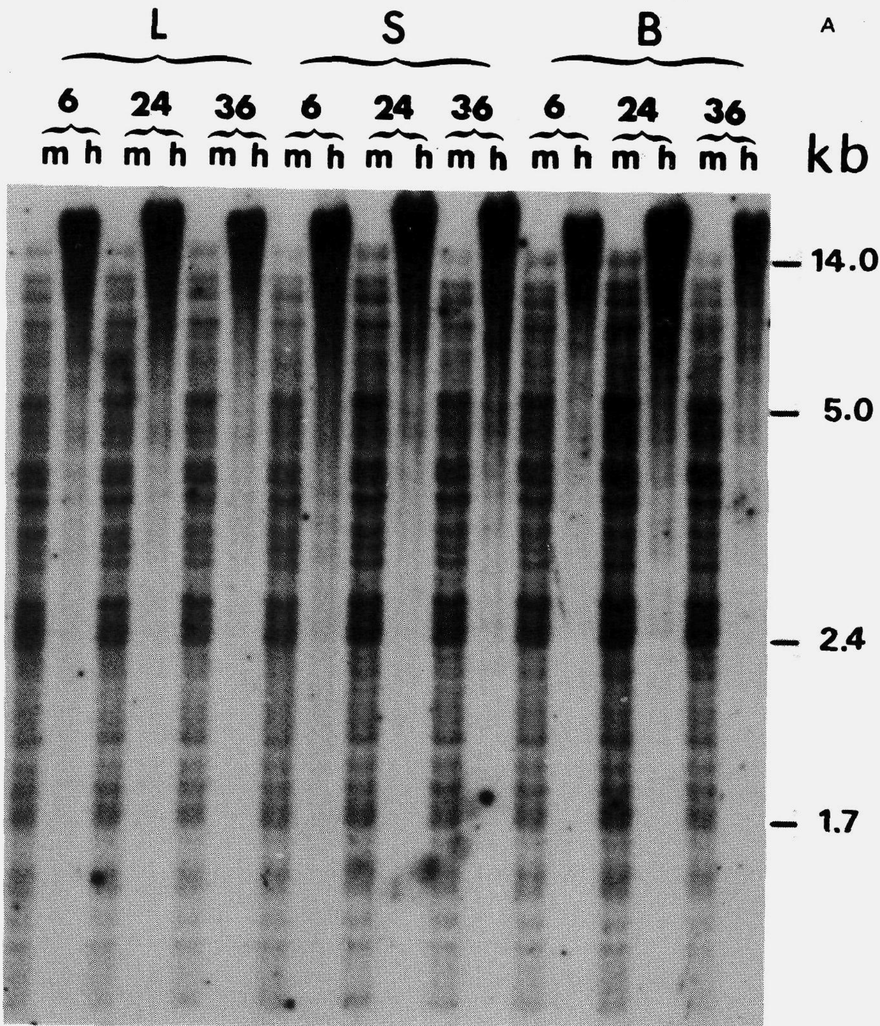


Fig. 6. Southern hybridization analysis of sequences homologous to the GAPDH (A) and *β-actin* (B) cDNAs in genomic DNA isolated from liver (L), spleen (S) and brain (B) of rats of different ages (6, 24 and 36 months) and digested with the restriction enzymes Msp I (m) and Hpa II (h) Due to the electrophoretic conditions used, four LMW *β*-actin fragments are not included in the Msp I digestion pattern shown here. For each age group three animals were studied; the results obtained with one representative per age group are shown. Fragment sizes were estimated from lambda × Hind II and ϕ X174 × Hae III markers (kb).

CpG sites involved, underwent a significant demethylation between 6 and 24 months of age (Fig. 7B). No age-related variation in the *β*-actin methylation patterns was observed in brain and liver DNA. Finally, we found no age-related alterations in the primary sequence of these genes; the Msp I digestion patterns were identical in all samples tested.

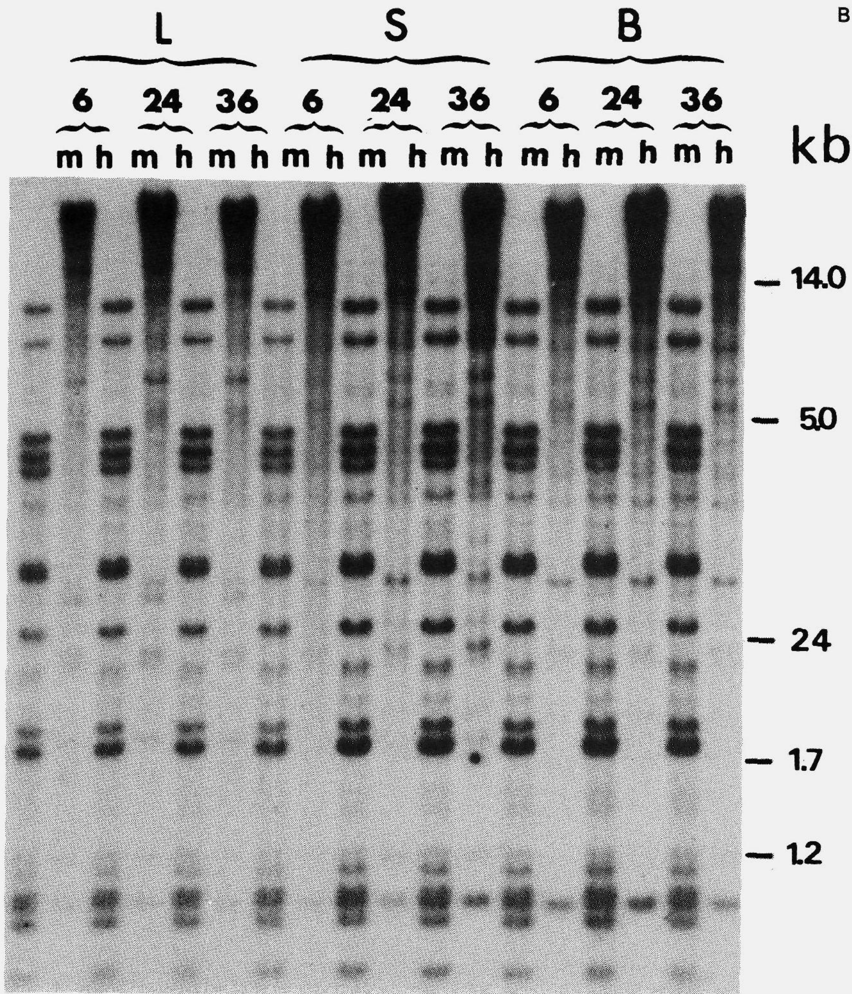


Fig. 6B.

DISCUSSION

We have investigated the mRNA levels and methylation patterns of two constitutively expressed genes, GAPDH and β-actin, in spleen, liver and brain of 6-, 24- and 36-month-old inbred rats. The mRNA levels of these genes were determined by Northern hybridization analysis of total RNA using ribosomal RNA as an internal standard. The transcript sizes of the β-actin and GAPDH mRNAs, as measured by Northern hybridization analysis, were not affected during the aging process in any of the tissues tested (Fig. 1) Neither did the transcription levels of these genes show significant quantitative alterations in the

6 24 36

h m h m h m h m h m h m h m h m h m Kb

-14.0

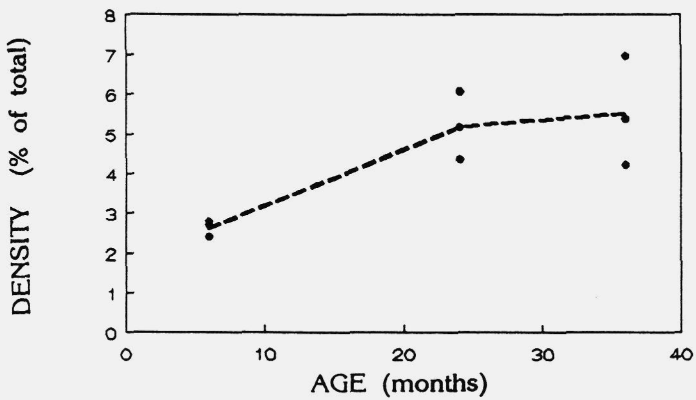
-5.0

-2.4

-1.7

-1.2

A



B

liver and brain as a function of age (Fig. 3). In the spleen of 36-month-old rats, however, the mRNA level of GAPDH and β-actin showed a 200% and 100% increase, respectively, as compared to the 6-and 24-month-old animals.

These observations could indicate an altered control of gene expression in the spleen of old rats. On the other hand, the phenomenon could be due to age-related changes in the relative proportions of the different cell types present in the spleen. Indeed, histopathological examination of the rats used in our study clearly indicated the occurence of extramedullary hemopoiesis during aging of the spleen (results not shown). However, extramedullary hemopoiesisthis was already observed in the spleens of 24-month-old rats in which elevated levels of GAPDH and β-actin mRNA did not occur and was sometimes not present in 36-month-old rats in which highly increased levels of these mRNAs were always found. Therefore, we conclude that there is no correlation between the increased mRNA levels observed in the spleens of 36-month-old rats and alterations in cell composition observed by histopathology.

In spite of the fact that the rat strain used in our studies is highly inbred, we found a considerable interindividual variation in the mRNA levels of the two genes in all tissues (Fig. 4), but especially for β-actin in the liver. The β-actin and GAPDH mRNA levels varied in a parallel fashion when the same organs from different animals were compared (Fig. 5). Since this variation was also observed intra-individually, this might reflect organ-specific variation in tissue structure, mitotic activity or cell composition. These results demonstrate that β-actin and GAPDH are not suitable as internal standard in comparative studies. In this regard, the level of total 28 S rRNA is a much better control [26]; interindividual and/or age-related variation in ribosomal RNA concentrations were negligible (Fig. 3).

The DNA methylation patterns of the β-actin and GAPDH genes were analysed by Southern hybridization using the methylation-sensitive restriction enzymes Hpa II and Msp I (Fig. 6). The GAPDH coding region does not contain any Msp I/Hpa II recognition sites [27]. Therefore, all the Msp I sites that gave rise to the large number of restriction fragments hybridizing to the GAPDH cDNA probe, are located at the 5′ and 3′ ends of the gene and in its intron sequences and its 400 pseudogenes [28]. Data on the exact location of these sites are not available yet. Tissue-specific or age-related alterations in the methylation pattern of the GAPDH gene were not observed.

The Msp I/Hpa II sites detected upon hybridization with the β-actin cDNA are partly originating from β-actin-homologous sequences in the τ-actin gene. The β-actin gene contains 20 Msp I/ Hpa II sites, 14 of which are present in

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Fig. 7. (A) Southern hybridization analysis of β-actin sequences at Msp I (m) and Hpa II (h) sites generating the 0.95 kb band (Fig. 6) in spleen DNA in rats of different ages (6, 24 and 36 months). (B) Densitometric analysis of the 0.95 kb band, presented as the fraction of the total hybridization signal in one lane and plotted as a function of age.

intron sequences [28]. Some of the sites have a tissue-specific methylation pattern (Fig. 6B). However, the low intensity of the corresponding hybridizing bands (for example, the 0.95 kb band) suggests that only a small number of cells is involved in this specific methylation pattern. Interestingly, the extent of hypomethylation at the 0.95 kb band corresponded to the variation in mRNA level among different tissues (Fig. 2). The sites that gave rise to this band are present in exon 2 and 4 of the β-actin gene. Considerable age-related demethylation of these sites was observed in the spleen of 24-month-old rats as compared to 6-month-old animals (Fig. 7). In summary our findings do not support the concept of a general demethylation in relation to the aging process, although demethylation at specific sites can occur as was demonstrated for the β-actin gene. The latter could be mechanistically related to the age-related increase in β-actin mRNA level observed in the spleen.

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REFERENCES

1 R. Holliday, The inheritance of epigenetic defects. *Science, 238* (1987) 163—170.
2 P.E. Slagboom and J. Vijg, Genetic Instability and Aging: Theories, Facts and Future Perspectives. *Genome, 31* (1989) 373—385.
3 T. Ono, N. Takahashi and S. Okada, Age-associated changes in DNA methylation and mRNA level of the c-myc gene in spleen and liver of mice. *Mutat. Res., 219* (1989) 39—50.
4 R. Wellinger and Y. Guigoz, The effect of age on the induction of tyrosine aminotransferase and tryptophan oxygenase genes by physiological stress. *Mech. Ageing Dev., 34* (1986) 203—217.
5 A. Richardson, M.S. Rutherford, M.C. Birchenall-Sparks, M.S. Roberts, W.T. Wu and H.T. Cheung. Levels of specific messenger RNA species as a function of age. *Aging, 29* (1985) 229—236.
6 G.J.M.J. Horbach, H. van der Boom, C.P.A. van Bezooijen and S.H. Yap, Molecular aspects of age-related changes in albumin synthesis in rats. In C.P.A. van Bezooijen *et al.* (eds.) *Liver Drugs and Aging. Topics in Aging Research in Europe.* Vol. 7, Eurage, Rijswijk, 1986, pp. 121—126.
7 B. Chatterjee, S.J. Nath and A. Roy, Differential regulation of the messenger RNA for three major senescence marker proteins in the male rat liver. *J. Biol. Chem., 256* (1981) 5939—5941.
8 A. Bird, DNA methylation — how important in gene control. *Nature, 307* (1984) 503—504.
9 H. Cedar, DNA methylation and gene activity. *Cell, 53* (1988) 3—4.
10 V.L. Wilson, R.A. Smith, S. Ma and R.G. Cutler, Genomic 5-methyldeoxycitidine decreases with age. *J. Biol. Chem., 262* (1987) 9948—9951.
11 R.D. Drinkwater, T.J. Blake, A.A. Morley and D.R. Turner, Human Lynphocytes in vivo

have reduced levels of methylation in transcriptionally active and inactive DNA. *Mutat. Res.* *219* (1989) 29—37.

12 D. Gershon, K. Kohno, G.R. Martin and Y. Yamada, Studies on gene structure and function in aging: Collagen types I and II and the albumin genes. In B. Pullman (ed.), *Interrelationship Among Aging, Cancer and Differentiation,* D. Reidel Publishing Company., 1985, pp. 143—148.

13 M.J. van Zwieten, *The Rat as Animal Model in Breast Cancer Research,* Nijhoff, The Hague, 1984.

14 M. Piechaczyk, J.M. Blanchard, L. Marty, C. Dani, F. Panabieres, S.E. Sabouty, P. Fort and P. Jeanteur, Posttranscriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. *Nucl. Acids Res.* *12* (1984) 6951—6963.

15 H.J. Dodemont, P. Soriano, W.J. Quax, F. Ramaekers, J.A. Lenstra, M.A.M. Groenen, G. Bernardi and H. Bloemendal, The genes coding for the cytoskeletal proteins actin and vimentin in warm-blooded vertebrates. *EMBO J.,* *1* (1982) 167—171.

16 R. Krumlauf, M. Jeanpierre and B. Young, Construction and characterization of genomic libraries from specific human chromosomes. *Proc. Natl. Acad. Sci. USA,* *79* (1982) 2971—2975.

17 C. Auffray and F. Rougeon, Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumour RNA. *Eur. J. Biochem.,* *107* (1980) 303—314.

18 W.C. Schneider, Determination of Nucleic Acids in tissues by pentose analysis. In S.P. Colowick and N.V. Kaplan (eds.), *Methods in Enzymology,* Vol. 3, Academic Press, New York, 1956, pp. 680—684.

19 H. Lehrach, D. Diamond, J. Wozney and H. Boedtker, RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry,* *16* (1977) 4743—4751.

20 P.S. Thomas, Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA,* *77* (1980) 5201—5205.

21 G.M. Church and W. Gilbert, Genomic sequencing. *Proc. Natl. Acad. Sci. USA,* *81* (1984) 1991—1995.

22 P. Feinberg and B. Vogelstein, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.,* *132* (1983) 6—13.

23 E. Mullaart, M.E.T.I. Boerrigter, A. Brouwer, F. Berends and J. Vijg, Age-dependent accumulation of alkali-labile sites in DNA of post-mitotic but not in that of mitotic rat liver cells. *Mech. Ageing Dev.,* *45* (1988) 41—49.

24 S. Goldstein and R.J. Shmookler Reis, Methylation patterns in the gene for the alpha subunit of chorionic gonadotropin are inherited with variable fidelity in clonal lineages of human fibroblasts. *Nucl. Acids Res.,* *13* (1985) 7055—7065.

25 C. Waalwijk and R.A. Flavell, Msp I, an isoschizomer of Hpa II which cleaves both unmethylated and methylated Hpa II sites. *Nucl. Acids. Res.,* *5* (1978) 3232—3236.

26 W.J.F. de Leeuw, P.E. Slagbloom and J. Vijg, Quantitative comparison of messenger RNA levels in mammalian tissues — 28S ribosomal RNA level as an accurate internal control. Nucl. Acids Res.,. 17 (1989) 10137—10138.

27 P. Fort, L. Marty, M. Pieckhaczyk, S. El Sabrouty, C. Dani, P. Jeanteur and J.M. Blanchard, Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigene familie. *Nucl. Acids. Res.,* *13* (1985) 1431—1442.

28 J.Y. Tso, X. Sun, T. Kao, K.S. Reece and R. Wu, Isolation and characterization of rat and human glyceraldehyde-3-phosphate-dehydrogenase cDNAs; genomic complexity and molecular evolution of the gene. *Nucl. Acids. Res.,* *13* (1985) 2485—2502.

29 U. Nudel, R. Zakut, M. Shani, Z. Levy and D. Yaffe, The nucleotide sequence of the rat β-actin gene. *Nucl. Acids. Res.* *11* (1983) 1759—1771.

CHAPTER 5

mRNA LEVELS AND METHYLATION PATTERNS OF THE THYROSINE AMINOTRANSFERASE GENE IN AGING INBRED RATS

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mRNA levels and methylation patterns of the tyrosine aminotransferase gene in aging inbred rats

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We have examined the mRNA levels and methylation patterns of the liver-specific tyrosine aminotransferase (TAT) gene in inbred female rats aged 6, 24 and 36 months. Northern hybridization analysis of total RNA showed a 65% decrease in the steady state transcript level of TAT in the liver of 24- and 36-month old rats as compared to 6-month old animals. The TAT gene as studied by Southern hybridization analysis using the isoschizomers Hpa II and Msp I was found to be hypomethylated in the liver as compared to spleen and brain at six CpG sites within the gene. Methylation at these sites remained unchanged during aging.

Tyrosine aminotransferase; Aging; DNA methylation; mRNA

1. INTRODUCTION

Aging of metazoa has been attributed to the loss of epigenetic control [1]. There is strong evidence that DNA methylation of specific CpG sites is associated with the control of tissue and stage specific gene transcription [2], cell differentiation [3] and X-chromosome inactivation [4]. Alterations in DNA methylation have also been associated with the aging process. A progressive decline of the 5-methylcytosine (5mC) level in total genomic DNA was observed with age in various systems, including human peripheral blood lymphocytes [5], various organs and tissues of the mouse [6], and in cultured fibroblasts during replicative senescence [7]. However, it is not clear whether this loss of 5mC from the genome involves the methylation patterns of individual genes and how it affects gene transcription. Both de- and repression of gene transcription has been associated with aging. In only a limited number of studies, however, a relationship has been found between such changes and alterations in the methylation status of specific CpG sites (for a review, see [8]).

We have studied the mRNA levels and methylation patterns of the liver-specific tyrosine aminotransferase (TAT) gene in various tissues of the aging rat. The TAT mRNA level showed a 65% decrease in the 24- and 36-month-old rats as compared to 6-month-old animals. We found a liver-specific hypomethylation of six CpG sites within the TAT gene. The methylation pattern at these or other sites did not alter during aging.

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Abbreviations: kb, kilobasepairs; OTC, ornithine transcarbamoylase (EC 2.1.3.3); TAT, tyrosine aminotransferase (EC 2.6.1.5); 5mC, 5-methylcytosine

2. MATERIALS AND METHODS

2.1. *Animals*

Organs were dissected from female inbred Wistar-derived WAG/Rij rats aged 6, 24 and 36 months. Animals were fed ad libitum and maintained under clean conventional conditions at the colony of the TNO Institute for Experimental Gerontology [9]. Under these circumstances the animals have a median lifespan of 33 months. All animals sacrificed for this study were subjected to complete gross and microscope examination. Tissues affected by a well defined disease process, e.g. tumors etc., were excluded from this study.

2.2. *DNA probes*

For the rat TAT gene, three probes, derived from the lambda genomic clone rTAT1 [10] were used. Their inserts correspond to exons B and C (pUTAT0.94), exons F, G, H and part of exon I (pUTAT2.45) and exons K and L (pUTAT1.05). Plasmid pHR28.1 [11] and pR021 [12], both used as a control, contain genomic fragments from the human 5.8S and 28S rRNA genes and the rat OTC cDNA respectively. Only the 2 kb *BglI-EcoRI* fragment corresponding to 28S rDNA was used as a probe. All hybridization experiments were performed with purified insert fragments.

2.3. *Northern, Southern and dot blot hybridization analysis*

Total RNA was extracted from whole liver, brain and spleen using the frozen tissue/LiCl procedure [13]. The amount of total RNA isolated per gram liver, brain or spleen did not change with age. Dot blotting was performed as described [14]. For Northern analysis equal amounts of total RNA (20 μg) were size-fractionated by electrophoresis for 16 h at 25 V/15 mA in 1.5% agarose gels containing formaldehyde. The gels were then subjected to Northern blotting [15]. Total genomic DNA was isolated as described earlier [16]. Genomic DNA was digested for 3 h at 37°C with a 5-fold excess of *MspI* or *HpaII* restriction enzyme according to the manufacturer's specifications (BRL). Then, the samples were chloroform-extracted, ethanol-precipitated, solubilized and re-digested under the aforemen-

tioned conditions. After quantification of the digested samples equal amounts of DNA (5 μg) were electrophoresed in 0.8% agarose gels and subjected to Southern blotting [16]. Hybridization of Northern, Southern and dot blots was carried out as described by Church and Gilbert [17], using random primed ^{32}P-labeled probes with a specific activity of 5×10^8 cpm/μg [18]. Autoradiography was performed at −80°C using Kodak X-omat AR2 films and X-omatic intensifying screens. The hybridization intensity of the dots and bands was measured by densitometric scanning, using a Model 620 Video Densitometer (Bio-Rad). For rehybridization the membranes were stripped for 10 min at 100°C in 1% SDS, 1.8 mM NaCl, 0.1 mM NaH_2PO_4, 0.01 mM EDTA.

3. RESULTS AND DISCUSSION

3.1. *mRNA levels*

Northern analysis of total rat liver RNA revealed a 2.4 kb TAT mRNA in all three age groups with no additional bands present (Fig. 1). As a control for the presence of comparable mRNA concentrations on the Northern blot, the same filter was rehybridized with a cDNA probe for the liver-specific rat OTC gene (Fig. 1B). The OTC mRNA levels vary interindividually but not with age.

For quantitative comparison of the TAT mRNA level in the three age groups, the hybridization signals obtained after autoradiography of the Northern blot were measured by densitometric scanning (Fig. 2A) and normalized to the hybridization signals obtained after dot-blot analysis of the same RNA samples using a 28S rRNA probe (Fig. 2B) as described earlier [14]. The TAT mRNA level in the rat liver showed a 65% decline when the 24- and 36-month-old rats were compared with the 6-month-old animals. Using the Northern hybridization analysis we found no expression of TAT mRNA in tissues other than the liver (results not shown).

A decrease in TAT enzyme activity combined with a 35% decline in mRNA level has been previously observed in the liver of 25-month-old Sprague-Dawley rats as compared to 10-month-old adults [19]. The results obtained in our present study on WAG/Rij rats confirm this and indicate that no further changes in the TAT mRNA level occur at old age, that is, between 24 and 36 months. It is important to note that results from Horbach et al. [20] as well as our own unpublished data indicate that the mRNA level of another liver-specific gene, albumin, was elevated 80% in 24-month-old WAG/Rij rats as compared to 6-month-old animals. Such early age-associated alterations in the expression of inducible genes are likely to reflect regulatory changes rather than an age-related loss of transcriptional control.

3.2. *Methylation pattern*

In order to establish whether the age-related alteration in TAT mRNA level is associated with methylation changes, a number of CpG sites within the TAT gene were examined in liver, spleen and brain of 6-, 24- and

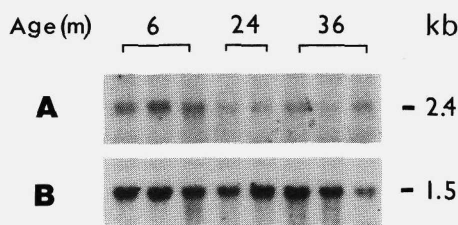

Fig. 1. Northern hybridization patterns of TAT (A) and OTC (B) mRNAs in 20 μg of total RNA isolated from the liver of female WAG/Rij rats aged 6, 24 and 36 months. The same filter was sequentially hybridized, stripped and rehybridized with a mixture of pUTAT0.94, pUTAT2.45 and pUTAT1.05 (A) and pR021 (B). Size markers are presented in kb.

36-month-old rats by Southern hybridization analysis (Fig. 3A) using the isoschizomers *Hpa*II and *Msp*I [21]. As a control for the presence of equal DNA concentrations in each lane, the same filter was rehybridized with the OTC cDNA probe (Fig. 3B). A liver-specific methylation pattern was observed for 6 CpG sites within the TAT gene. Among the 5 hybridizing *Msp*I restriction fragments, three bands, representing 1.5 kb, 1.2 kb and 0.8 kb fragments were also generated

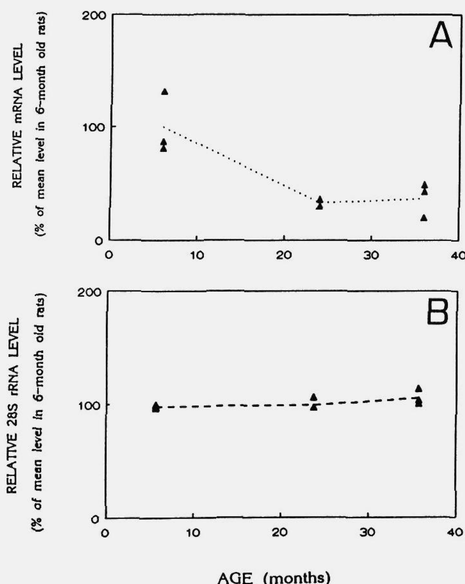

Fig. 2. Expression level of TAT mRNA in rat liver in relation to aging (A). The data, obtained by densitometric scanning of the specific bands shown in Fig. 1, were normalized to the relative 28S rRNA concentration of the samples as measured by dot blot analysis (B). Each point in both figures represents the result of a determination on one individual animal and is expressed as the percentage of the average value in the 6-month-old rats (100%). Repeated determinations on one sample indicated an experimental variation of less than 10%.

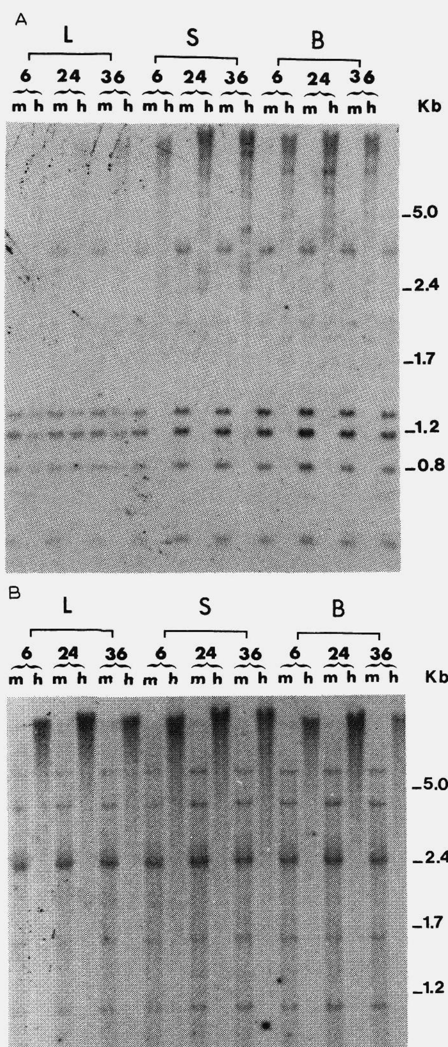

Fig. 3. Southern hybridization analysis of the TAT (A) and OTC (B) genes in genomic DNA isolated from liver (L), spleen (S) and brain (B) of rats of different ages (6, 24 and 36 months) and digested with the isoschizomeric restriction enzymes *Msp*I (m) and *Hpa*II (h). The probes used were the genomic (A) and cDNA (B and C) probes mentioned in Fig. 1. For each age group results obtained with only one animal are shown; among the animals studied per age group (at least 3) no differences were observed. Fragment sizes were estimated from lambda × *Hin*dIII and ϕX174 × *Hae*III markers (kb).

following *Hpa*II digestion of liver DNA; in spleen and brain DNA these fragments were absent (Fig. 3A). This indicates that in most liver cells the *Hpa*II/*Msp*I recognition sites generating these fragments are hypomethylated. To our knowledge this has not been reported before.

It can be concluded that the age-related decrease of the TAT mRNA level is not associated with methylation changes of any of the sites examined. In spite of the previously reported random loss of 5mC with age and the age-related methylation changes at CpG sites for which tissue-specificity was observed (e.g. the c-myc gene [22]), such alterations were not detected within the TAT gene.

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REFERENCES

[1] Holliday, R. (1985) in: Basic Life Sciences, 35th edn (Woodhead, A.D., Blackett, A.D. and Hollander, A. eds) Plenum, New York, pp. 269–283.
[2] Adams, R.L.P. and Burdon, R.H. (1985) in: Molecular Biology of DNA methylation, Springer, New York, pp. 115–161.
[3] Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N. and Cedar, H. (1984) Proc. Natl. Acad. Sci. USA 81, 2275–2279.
[4] Sanford, J.P., Chapman, V.M. and Rossant, J. (1985) Trends Genet. 1, 89–93.
[5] Drinkwater, R.D., Blake, T.J., Morley, A.A. and Turner, D.R. (1989) Mutation Res. 219, 29–37.
[6] Wilson, V.L., Smith, R.A., Ma, S. and Cutler, R.G. (1987) J. Biol. Chem. 262, 9948–9951.
[7] Wilson, V.L. and Jones, P.A. (1983) Science 220, 1055–1056.
[8] Slagboom, P.E. and Vijg, J. (1989) Genome 31, 373–385.
[9] Van Zwieten, M.J. (1984) in: The Rat as Animal Model in Breast Cancer Research, Nijhoff, The Hague.
[10] Shinomiya, T., Scherer, G., Schmid, W., Zentgraf, H. and Schütz, G. (1984) Proc. Natl. Acad. Sci. USA 81, 1346–1350.
[11] Krumlauf, R., Jeanpierre, M. and Young, B. (1982) Proc. Natl. Acad. Sci. USA 79, 2971–2975.
[12] Horwich, A.L., Kraus, J.P., Williams, K., Kalousek, F., Konigsberg, W. and Rosenberg, L.E. (1983) Proc. Natl. Acad. Sci. USA 80, 4258–4262.
[13] Auffray, C. and Rougeon, F. (1980) Eur. J. Biochem. 1070, 303–314.
[14] De Leeuw, W.J.F., Slagboom, P.E. and Vijg, J. (1989) Nucleic Acids Res. 17, 10137–10138.
[15] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[16] Slagboom, P.E., De Leeuw, W.J.F. and Vijg, J. (1990) Mech. Ageing Dev. (in press).
[17] Church, G.M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991–1995.
[18] Feinberg, P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[19] Wellinger, R. and Guigoz, Y. (1986) Mech. Ageing Dev. 34, 203–217.
[20] Horbach, G.J.M.J., Van der Boom, H., Van Bezooijen, C.F.A. and Yap, S.H. (1986) in: Liver Drugs and Aging. Topics in Aging Research in Europe, 7th edn (Van Bezooijen et al., ed.) EURAGE, Rijswijk, pp. 121–126.
[21] Waalwijk, C. and Flavell, R.A. (1978) Nucleic Acids Res. 5, 3231–3236.
[22] Ono, T., Takahashi, N. and Okada, S. (1989) Mutat. Res. 219, 39–51.

CHAPTER 6

TWO-DIMENSIONAL DNA FINGERPRINTING OF HUMAN INDIVIDUALS

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Two-dimensional DNA fingerprinting of human individuals

(DNA polymorphisms/minisatellites/variable number of tandem repeat sequences/denaturing gradient gel electrophoresis/gene mapping)

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ABSTRACT **The limiting factor in the presently available techniques for the detection of DNA sequence variation in the human genome is the low resolution of Southern blot analysis. To increase the analytical power of this technique, we applied size fractionation of genomic DNA restriction fragments in conjunction with their sequence-dependent separation in denaturing gradient gels; the two-dimensional separation patterns obtained were subsequently transferred to nylon membranes. Hybridization analysis using minisatellite core sequences as probes resulted in two-dimensional genomic DNA fingerprints with a resolution of up to 625 separated spots per probe per human individual; by conventional Southern blot analysis, only 20–30 bands can be resolved. Using the two-dimensional DNA fingerprinting technique, we demonstrate in a small human pedigree the simultaneous transmission of 37 polymorphic fragments (out of 365 spots) for probe 33.15 and 105 polymorphic fragments (out of 625 spots) for probe 33.6. In addition, a mutation was detected in this pedigree by probe 33.6. We anticipate that this method will be of great use in studies aimed at (*i*) measuring human mutation frequencies, (*ii*) associating genetic variation with disease, (*iii*) analyzing genomic instability in relation to cancer and aging, and (*iv*) linkage analysis and mapping of disease genes.**

The possibility to identify DNA sequence heterogeneity is of major importance for the analysis of genetic diseases and genomic instabilities. This identification depends on the availability of probes that detect variable sites in the genome and on the resolution of electrophoretic separation techniques for the analysis of DNA restriction fragments.

The discovery of hyperpolymorphic VNTR (variable number of tandem repeat) DNA sequences or minisatellites has greatly facilitated studies of genetic variation in the human population (1–6). It has been demonstrated that so-called core probes derived from minisatellites can be used to simultaneously detect a large number of hyperpolymorphic VNTR loci, dispersed in the genome, to provide genetic fingerprints of human individuals (7, 8). Core sequences have been successfully applied in the analysis of tumors for genetic instability (9) and in linkage analysis of genetic diseases (3). Such applications rely on the resolution of Southern blot hybridization analysis, which is based on the gel electrophoretic separation of genomic DNA restriction fragments according to size (10). One-dimensional separation of DNA fragments allows only ≈30 hypervariable minisatellite fragments to be resolved (ref. 3; this publication).

It has been demonstrated that by combining ordinary size separation with sequence separation in denaturing gradient gels, all restriction fragments in an *Eco*RI digest of the *Escherichia coli* genome can be resolved (11–13). To investigate whether this principle can be applied to the analysis of DNA sequence variation in the mammalian genome, we separated genomic DNA restriction fragments of human individuals according to size and base-pair composition by neutral and denaturing gradient polyacrylamide gel electrophoresis, respectively. By subsequent transfer of the electropherograms to nylon membranes and hybridization with radiolabeled minisatellite core sequences as probes, high-resolution DNA fingerprints were obtained. We show that many polymorphic DNA restriction fragments can be detected in the molecular size region of fragments <3 kilobase pairs (kbp), which is not accessible for conventional Southern hybridization analysis. We demonstrate the applicability of this technique in genetic studies on humans by showing that a large number of transmitted polymorphic spots can be simultaneously followed in a two-generation human pedigree of three members.

MATERIALS AND METHODS

DNA Isolation and Restriction Enzyme Digestion. Genomic DNAs, isolated from peripheral blood lymphocytes according to standard procedures, were a kind gift from E. Bakker. DNAs were digested with the restriction endonucleases *Hae* III or *Hin*fI (BRL) under conditions recommended by the manufacturer.

Electrophoretic Separations. Agarose gel electrophoresis of 5 μg of DNA restriction fragments was performed in a horizontal 1.2% gel in 1× TAE (40 mM Tris·HCl, pH 7.4/20 mM sodium acetate/1 mM NaEDTA) at 65 V for 14–30 hr. Gels were stained for 10 min in a solution containing ethidium bromide (EtdBr) at 0.1 μg/ml followed by destaining for at least 30 min. Two-dimensional separations of 5 μg of DNA restriction fragments were performed in 1-mm-thick polyacrylamide gels (acrylamide/bisacrylamide, 37:1) using a gel apparatus that was essentially the same as the one described by Fischer and Lerman (12). The first dimension was run in a neutral 6% gel at 50°C for 2 hr at 250 V in 1× TAE. The separation patterns were visualized by staining the gel in the dark with EtdBr (0.1 μg/ml) for 10 min, followed by destaining for at least 30 min. The 0.34- to 2.8-kbp region (probe 33.15) or the 0.54- to 10-kbp region (probe 33.6) of the lane was quickly cut out of the gel and applied to a 6% polyacrylamide gel containing a 10–75% linear concentration gradient of denaturant (100% denaturant = 7.0 M urea/40% formamide) parallel to the direction of electrophoresis. This gradient was found to give optimal separation patterns for the VNTR sequences. Gels were poured by mixing two solutions, containing the desired boundary denaturant concentrations, in a linear gradient maker with a peristaltic pump. After electrophoresis for 12 hr at 225 V and 60°C, the gel was stained with EtdBr as described above.

Transfer of Separation Patterns. DNA separation patterns in agarose gels were capillary transferred to a nylon membrane (Nytran 13N, Schleicher & Schuell; Zetaprobe, Bio-Rad) in 0.4 M NaOH/0.6 M NaCl for 12 hr. After transfer, the filter was rinsed in 2× SSC (1× SSC = 150 mM NaCl/15 mM

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Abbreviations: EtdBr, ethidium bromide; VNTR, variable number of tandem repeats.

sodium citrate), air dried, baked for 1 hr at 80°C, and irradiated with 302-nm UV light (Transilluminator, UVP Products, San Gabriel) for 45 s, which was found to be optimal for cross-linking DNA to the filter (results not shown). DNA separation patterns in denaturing gradient polyacrylamide gels were fragmented by irradiating the gel with 302-nm UV light (Transilluminator, UVP Products) for 4 min, which was found to be optimal (results not shown). Before transfer, the gel was boiled for 5 min in 1× TBE (89 mM Tris·borate, pH 8.0/89 mM boric acid/2 mM NaEDTA) and subsequently placed in an identical solution at room temperature. Transfer to a nylon membrane (Nytran 13N, Schleicher & Schuell; Zetabind, Bio-Rad) was achieved by semidry electroblotting at 400 mA (6–28 V) between horizontal graphite plates. Electrophoresis was performed twice for 45 min between 10 Whatman 3MM paper sheets, which were soaked in fresh 1× TBE between the two transfers. After transfer, the filter was rinsed in 2× SSC, air-dried, baked for 1 hr at 80°C and irradiated for 45 s with 302-nm UV light to cross-link the DNA fragments to the filter.

Probe Preparation and Labeling. The probe was prepared by using T4 kinase (Boehringer Mannheim) to individually phosphorylate the 5′-hydroxyl groups of two partially complementary and overlapping oligonucleotides, each representing the complete 33.15 (5′-AGAGGTGGGCAGGTGG-3′ and 5′ CCACCTCTCCACCTGC-3′) or 33.6 (5′-AGGGCTGGAGG-3′ and 5′-AGCCCTCCTCC-3′) minisatellite core sequence (7). Subsequently, the two 33.15 or 33.6 oligonucleotides were mixed and allowed to anneal at 57°C for 1 hr, followed by ligation according to standard procedures (14). The synthetic probes thus prepared had an average length of 500 bp or more. Occasionally, T4 kinase phosphorylation and ligation were repeated to increase the average length of the probe. The ligation products (20 ng) were [α-^{32}P]dCTP-labeled either by the random-primer oligolabeling method (Boehringer Mannheim) or by self-priming, after boiling for 5 min and reannealing at 37°C in the presence of 1 unit of Klenow enzyme (Boehringer Mannheim)/2 μM dNTP/50 mM Tris·HCl, pH 7.2/10 mM $MgCl_2$. Specific activities of 3×10^8–8×10^8 cpm/μg were obtained.

Hybridization Analysis. Filters were prehybridized in 5× SSC/20 mM sodium phosphate, pH 7.2/1% SDS/1 mM NaEDTA/heparin (50 μg/ml) for 2 hr at 65°C. After adding denatured probe at a concentration of 1×10^6 cpm/ml, hybridization was performed for 12 hr at 65°C. The filter was washed three times for 5 min at room temperature and three times for 20 min at 65°C in 2.5× SSC/0.1% SDS. Autoradiography was performed for 12–48 hr at −80°C using fine intensifying screens and XAR-5 film (Kodak). For subsequent rehybridizations of the filters, the probe was removed by boiling the filter for 20 min in a solution containing 0.01× SSC and 0.1% SDS. Filters were rinsed in 2.5× SSC and hybridization analysis was performed as described above. Two-dimensional spot patterns were interpreted by eye examination, using grids to quantitate the spots and to match individual two-dimensional fingerprints.

RESULTS

In Fig. 1, Southern blot autoradiographs are shown of a human pedigree (of six members) obtained after prolonged electrophoresis in agarose gels and by using the synthetic probes derived from minisatellite core sequences 33.15 and 33.6 (7) on *Hae* III- and *Hin*fI-digested genomic DNA. A

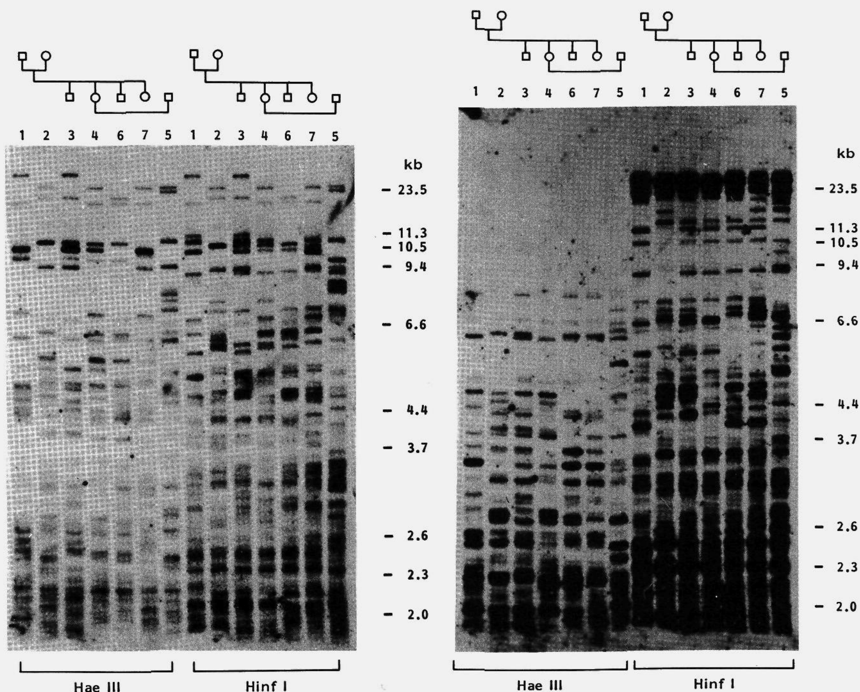


FIG. 1. Southern blot hybridization analysis of DNAs isolated from six members of a human pedigree (DL63; schematically depicted above the lanes in the autoradiographs) and one from an unrelated individual (DL 63.5), digested with *Hae* III or *Hin*fI, and using 35.15 (*Left*) or 33.6 (*Right*) minisatellite core sequences as probe. The autoradiograph on the *Right* was obtained by rehybridizing the filter that was used to obtain the autoradiograph on the *Left*.

number of simultaneously transmitted polymorphic bands were detected in this pedigree in the high molecular weight area of restriction fragments. For the restriction enzyme *Hin*fI, this number was 28 for probe 33.15 and 33 for probe 33.6. With *Hae* III, the number was 28 for probe 33.15 and 22 for probe 33.6. After *Hae* III digestion, the size range of bands detected by probe 33.6 was found to be smaller than after digestion with *Hin*fI, which could be an indication for the presence of *Hae* III recognition sites in the minisatellites homologous to this probe.

When genomic DNA was digested with *Hae* III and subjected to a two-dimensional separation (on the basis of size and base-pair sequence in neutral and denaturing gradient polyacrylamide gels, respectively), clusters of restriction fragments were observed in the EtdBr-stained two-dimensional gel as shown in Fig. 2 (*Left*). A large cluster is seen in the upper part of the gel, containing low concentrations of denaturant, and a smaller cluster is located near the bottom of the gel, which contains the highest concentration of denaturant.

Hybridization analysis of VNTR sequences detected with probe 33.6 resulted in a more or less evenly spread spot pattern. The total number of spots observed for probe 33.6 under the stringency of washing applied here (2.5× SSC) was 545 for this individual (DL 63.4 from the pedigree shown in Fig. 1). Slightly smaller numbers were obtained when the stringency was increased to 1× SSC (results not shown).

We subsequently analyzed three members of the pedigree described above (mother, father, and a son) by two-dimensional DNA fingerprinting. For optimal comparisons a 30-cm-wide version of the gel apparatus originally described by Fischer and Lerman (13) was constructed and used in these experiments so that up to six individuals could be compared on one denaturing gradient gel. Close inspection and comparison of individual spot patterns of the two parents obtained with probes 33.6 (Fig. 3) revealed a total number of 569 spots for the father, 607 for the mother, and 625 for the son. Between the two parents, 150 spot polymorphisms were observed, 105 (70%) of which were transmitted to the son (52 of maternal and 53 of paternal origin). Details of the separation patterns are shown in Fig. 3 (*Lower Left*). Using probe 33.6, we detected a fragment in the son that was not present in the mother or the father (Fig. 3 *Lower Right*) and four fragments, common to the parents, could not be detected in the son (two examples are shown in Fig. 3 *Lower Right*). With probe 33.15, considerably less VNTR-containing fragments were detected than with probe 33.6 (Fig. 4). Among the 372, 290, and 365 spots for the father, mother, and son, respectively, 50 spot polymorphisms could be detected. Among the 37 transmitted spot polymorphisms (74%), 17 were of paternal and 20 were of maternal origin. Some of these spot polymorphisms are shown in detail in Fig. 4 (*Lower*).

DISCUSSION

With the two-dimensional DNA fingerprinting system presented here, we were able to distinguish up to 625 spots per individual for probe 33.6 and 372 for probe 33.15. Since we did not observe severe clustering of spots, it is likely that we have resolved all VNTR sequences belonging to the sets of sequences detected with these probes. The difference between the two probes in number of spots detected could therefore be due to a different copy number of these sets of repetitive sequences. In addition to 33.15 and 33.6, we have also used other core sequences (5) as probes in two-dimensional DNA fingerprinting and obtained comparable results. In this respect, VNTR sequences appear to exhibit an exceptional distribution of spots over a considerable part of the denaturing gradient in the second-dimension gel. By contrast, as illustrated in Fig. 2 (*Left*), total genomic DNA digests have a tendency to cluster in the second dimension.

Most spots were found in the 20–50% denaturant range and should therefore have a high to medium AT/GC ratio of their lowest melting domain. Although the number of repeat units of any VNTR locus is high enough to generate a melting domain, both 33.15 and 33.6 are G+C-rich. Therefore, the gradient level of the majority of homologous restriction fragments in the two-dimensional pattern cannot be determined by the VNTR sequences themselves. Instead, the position of most of the spots in the gradient will be determined by sequences adjacent to the VNTR regions. Virtually all VNTR alleles with a particular locus are therefore iso-

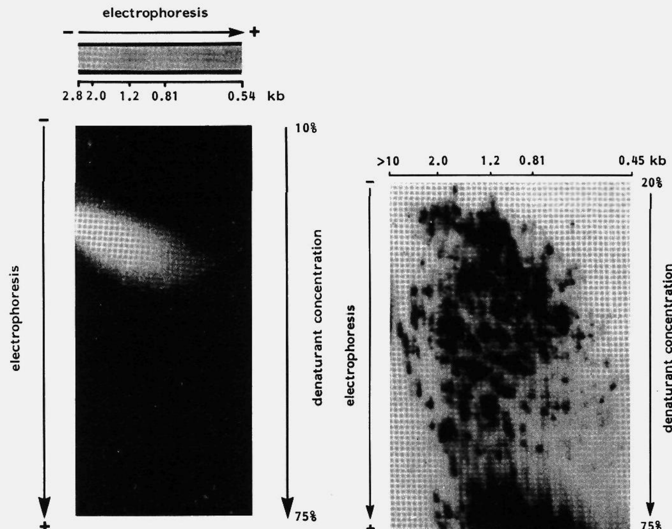
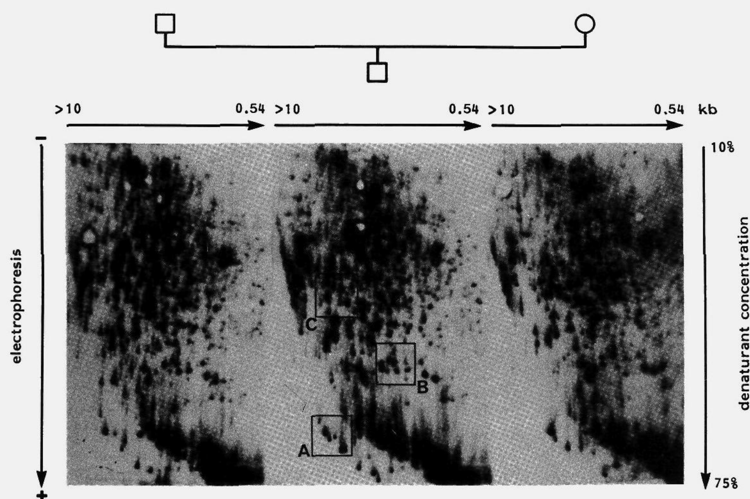


FIG. 2. Two-dimensional DNA fingerprint analysis of a human individual (DL 63.4). The two-dimensional separation pattern is shown after EtdBr staining (*Left*) and after hybridization analysis of the nylon replica filter with probe 33.6 (*Right*).



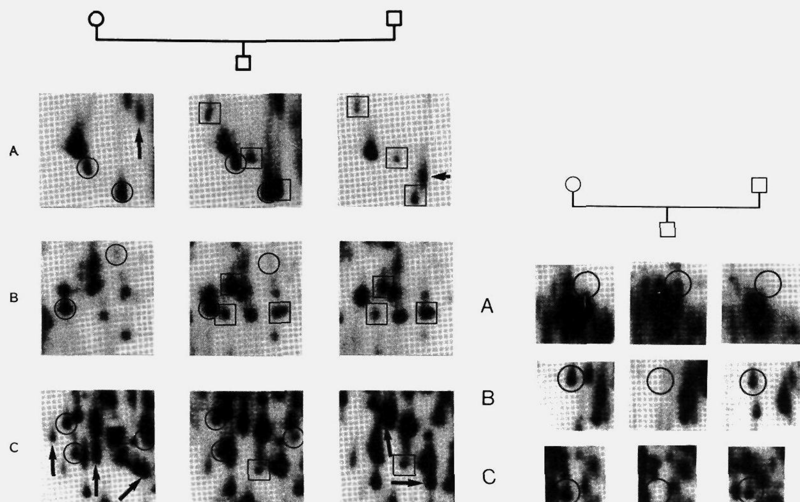


FIG. 3. (*Upper*) Two-dimensional DNA fingerprint analysis of *Hae* III-digested genomic DNA from three members of the human pedigree DL63 (DL63.1, -2, and -3), using probe 33.6. (*Lower Left*) Details from three different areas indicated in *Upper*, showing the transmission of particular spot polymorphisms. ○, Maternal fragments; □, paternal fragments. Arrows indicate nontransmitted polymorphic fragments. (*Lower Right*) Details from *Upper*, demonstrating the presence of a VNTR fragment in the son that is absent from the parents (circles in row A) and two cases in which both parents share a spot that is absent in the son (circles in rows B and C).

thermal—i.e., reach identical positions in the denaturing gradient. This characteristic and the fact that in the two-dimensional system virtually no sequences detected with a particular core probe are lost in a smear allows one to identify them.

When individuals from a human pedigree were analyzed by two-dimensional DNA fingerprinting, the majority of spots detected with both probes were found to be monomorphic (75–85%). However, a large number of spot polymorphisms (up to 150 for probe 33.6) were observed. Evidence for heterozygosity of the parents at particular VNTR loci detected by probe 33.6 was provided in four cases in which the parents shared a spot that could not be demonstrated in the son (Fig. 3 *Lower Right*). The percentage of spot polymorphism transmitted to the son was found to be 70% for both probes 33.6 and 33.15. This phenomenon could be due to clustering in the genome of VNTR loci or to the presence of one or more *Hae* III restriction sites in the minisatellites themselves. In the latter case, several polymorphic spots could stem from the same minisatellite locus, thereby resulting in a number of spots being cotransmitted as minisatellite haplotypes (3). This possibility is supported by the data in Fig. 1 (*Right*), which indicate the presence of *Hae* III sites in large alleles detected by probe 33.6 in these individuals. The spot present in the son but not in the parents is likely to have arisen by unequal exchange between two VNTR regions rather than by point mutation in a VNTR region (6).

On the basis of the results obtained with VNTR core sequence probes, we anticipate that two-dimensional DNA fingerprinting can be applied in a number of different areas in genetic research. The method should be useful in the analysis of large parts of the genome for measuring mutation frequencies (15) and for detecting putative changes in VNTR loci or other unstable DNA regions during tumor induction and growth (9, 16) or during aging (17).

Two-dimensional DNA fingerprinting can be used in association studies and to extend and improve linkage analysis and gene mapping. In this respect, it should be noted that the introduction of the denaturing gradient separation principle offers a solution to the problem of not being able to identify the same VNTR locus in different pedigrees, which effectively constrains widespread application of one-dimensional

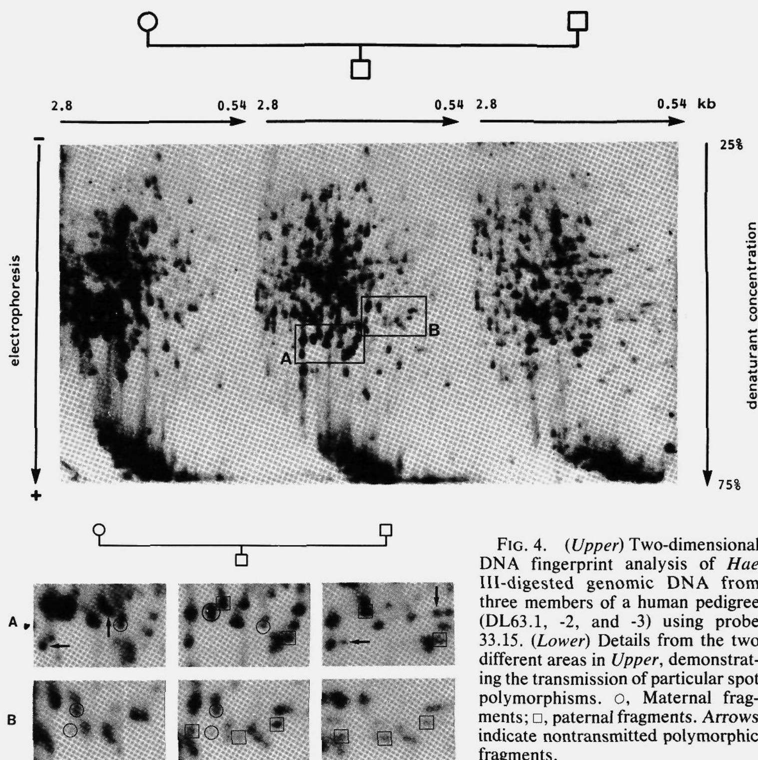


FIG. 4. (*Upper*) Two-dimensional DNA fingerprint analysis of *Hae* III-digested genomic DNA from three members of a human pedigree (DL63.1, -2, and -3) using probe 33.15. (*Lower*) Details from the two different areas in *Upper*, demonstrating the transmission of particular spot polymorphisms. ○, Maternal fragments; □, paternal fragments. *Arrows* indicate nontransmitted polymorphic fragments.

DNA fingerprinting to genetic analysis. Sequence-specific separation allows one to identify alleles with a particular locus in different pedigrees on the basis of their position on the same isotherm (see above). Furthermore, by using locus-specific probes in parallel experiments, each VNTR core homologous spot in a two-dimensional gel can be identified by comparison.

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1. Wyman, A. R. & White, R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6754–6758.
2. Reeders, S. T., Breuning, M. H., Davies, K. E., Nicholls, R. D., Jarman, A. P., Higgs, D. R., Pearson, P. L. & Weatherall, D. J. (1985) *Nature (London)* **317,** 542–544.
3. Jeffreys, A. J., Wilson, V., Thein, S. L., Weatherall, D. J. & Ponder, B. A. J. (1986) *Am. J. Hum. Genet.* **39,** 11–24.
4. Donis-Keller, H., Green, P., Helms, C., *et al.* (1987) *Cell* **51,** 319–337.
5. Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. & White, R. (1987) *Science* **235,** 1616–1622.
6. Jeffreys, A. J., Royle, N. J., Wilson, V. & Wong, Z. (1988) *Nature (London)* **332,** 278–281.
7. Jeffreys, A. J., Wilson, V. & Thein, S. L. (1985) *Nature (London)* **314,** 67–73.
8. Jeffreys, A. J., Wilson, V. & Thein, S. L. (1985) *Nature (London)* **316,** 76–79.
9. Thein, S. L., Jeffreys, A. J., Gooi, H. C., Cotter, F., Flint, J., O'Connor, N. T. J., Weatherall, O. J. & Wainscoat, J. S. (1987) *Br. J. Cancer* **55,** 353–356.
10. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
11. Fischer, S. G. & Lerman, L. S. (1979) *Cell* **16,** 191–200.
12. Fischer, S. G. & Lerman, L. S. (1979) *Methods Enzymol.* **68,** 183–191.
13. Fischer, S. G. & Lerman, L. S. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1579–1583.
14. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
15. Delehanty, J., White, R. L. & Mendelsohn, M. L. (1986) *Mutat. Res.* **167,** 215–232.
16. Colb, M., Yang-Feng, T., Francke, U., Mermer, B., Parkinson, D. R. & Krontiris, T. G. (1986) *Nucleic Acids Res.* **14,** 7929–7937.
17. Vijg, J. & Uitterlinden, A. G. (1987) *Mech. Ageing Dev.* **41,** 47–63.

CHAPTER 7

SOMATIC MUTATIONS AND CELLULAR AGING: TWO-DIMENSIONAL DNA TYPING OF RAT FIBROBLAST CLONES

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Somatic mutations and cellular aging: two-dimensional DNA typing of rat fibroblast clones

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Summary

Aging may be explained, to some extent, as a stochastic process of macromolecular damage. The rate of such a process should then determine longevity and be genetically controlled, as can be derived from the species specificity of maximum lifespan. The genome of the somatic cell is a major candidate to study for loss of DNA sequence integrity during aging. Unfortunately, a lack of adequate techniques has thus far hampered progress in testing the aging genome for changes in its DNA sequence content. Here we discuss recently developed sophisticated technology for studying spontaneous somatic mutations in relation to aging. More specifically, we describe the use of a novel two-dimensional DNA typing technique for the analysis of fibroblast clones derived from primary cultures established from skin biopsies of rats of different ages. Preliminary data are presented indicating the occurrence of DNA sequence changes in mini- and microsatellite regions of the rat genome at an average frequency of 2.7×10^{-3} per analyzed DNA fragment. Age-related variations in the somatic mutation frequency of these genomic regions were not observed.

In spite of the fact that somatic mutation theories of aging have been formulated more than 30 years ago (Failla, 1958; Szilard, 1959), the role, if any, of somatic instabilities in the etiology of aging and age-related disease has not been revealed yet. In order to properly investigate this hypothesis four aspects should be studied for each species and each cell and tissue type separately: (1) the frequency at which somatic mutations occur spontaneously; (2) the type of mutations and their location in the genome; (3) the effect of mutations on (the regulation of) gene expression; and (4) the relation of spontaneous mutagenesis to the rate of the aging process and the pattern of age-related pathology. Here we will mainly focus on the estimation of somatic mutation frequencies in relation to aging (for reviews, see Slagboom and Vijg, 1989; Vijg, 1990).

There is substantial evidence that DNA sequence variations accumulate during aging. An age-related increase of gross chromosomal aberrations has been detected by cytogenetic analysis

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of metaphase chromosomes in a number of studies. A sevenfold increase in chromosomal aberrations was found in mouse liver cells with age (Curtis, 1966), while Martin et al. (1985) detected a fivefold increase of chromosomal aberrations in mouse kidney cells. In a number of studies of peripheral blood lymphocytes of human donors, the age-related increase in chromosomal aberrations varied from two- to ninefold (Hedner et al., 1982; Prieur et al., 1988). The variation among these data may be due to possible artefacts of culture conditions and to the limited number of cells and individuals screened in these elaborate experiments. In any case it can be concluded that spontaneous chromosomal abnormalities occur at a considerable rate in young individuals and that this level increases with age.

Also at the submicroscopical level an increase in spontaneous somatic mutation frequency has been found with age. Mutation analysis of endogenous DNA sequences in the human genome have been performed on the selectable hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene in T lymphocytes using 6-thioguanine or other purine analogs as a selective agent. Due to the selection procedures associated with these systems there is some uncertainty as to the interpretation of the data (Featherstone et al., 1987). The general consensus, however, is that the spontaneous somatic mutation frequency of HPRT sequences in the human genome varies from 0.6×10^{-6} in newborns through 6×10^{-6} in young adults to 16×10^{-6} in aged twins (Carrano, 1989). With respect to rodents it was shown that the somatic mutation frequency in the adenine phosphoribosyl transferase (APRT) gene in hamster increases with age from 10^{-6} to 10^{-5} (Inamizu et al., 1986). Since the HPRT and APRT mutation assays are based on selection, only mutations that abolish gene activity are detected. This suggests that the somatic mutation frequency is likely to be higher than indicated by these data.

The mutation frequency as measured in mitotically active blood lymphocytes probably differs from that in postmitotic cells. Mutations in the latter, however, are very difficult to measure. Merely by coincidence an age-related increase in reverse mutation events was observed by van Leeuwen et al. (1989) in the hypothalamic neurons of Brattleboro rats which are homozygous for a single-base deletion in the vasopressin gene. The reversion was suggested to result from gene conversion of the mutated vasopressin and the highly homologous oxytocin gene. The number of neurons which reversed to normal vasopressin production increased with age from 0.1% to 3%, indicating the presence of a rare mutational hot spot.

Another more generally applicable experimental strategy for the analysis of somatic mutations arising in postmitotic tissues during aging is provided by recently developed transgenic mouse systems. These transgenic mice harbor a bacterial target gene for mutagenesis as a recombinant DNA vector integrated in the genome of all their cells. Somatic mutations in the target sequences can be analyzed after the recombinant bacteriophage lambda vector DNA has been rescued from the tissues of these animals (Gossen et al., 1989).

Recently, somatic instability of human telomeric DNA has been associated with aging both in vivo and in vitro. The ends of human chromosomes consist of tandem arrays of the (TTAGGG)n repetitive motif (for a review, see Blackburn, 1991). A reduction in the average size of telomeric repeat arrays (TRAs) has been found associated with serial passage and donor age of human fibroblasts (Harley et al., 1990). Furthermore, it has been demonstrated that average TRA sizes in human mitotic tissues (blood and colorectal mucosa) are shorter in old than in young individuals, corresponding to a rate of telomere loss of 33 bp per year (Hastie et al., 1990). Telomere size in humans at young age is, to a large extent, genetically determined (Slagboom et al., unpublished results). As yet it is not clear whether the rate of telomere shortening with age is also genetically determined.

Although the data discussed above indicate an age-related increase in DNA sequence variation in different somatic cell types, the rate of such a process and its functional relevance are as yet unclear. An important aspect that should be taken into consideration in studies on the aging somatic genome is the intragenomic variation in genetic instability. Mutation frequencies vary between genes, gene regions and coding vs. non-coding

genomic regions. The rate of spontaneous mutations in the germ line varies from 10^{-8}/nucleotide/generation at coding DNA (Neel et al., 1986) to 4×10^{-3} at minisatellite loci (Jeffreys et al., 1988). Even the mutation rate at the latter type of sequence varies depending on the locus. Mutation-prone hot spots are also a common phenomenon among the characterized human disease loci (Cooper and Clayton, 1988). For example, such hot spots susceptible to genetic instability have been found at the human insulin gene (Chakravarti et al., 1986), around the fragile X (Oberlé et al., 1985) and Duchenne muscular dystrophy (Davies et al., 1985) loci. Recently Gossen et al. (1991) observed high mutation frequencies at a bacterial *lacZ* gene that was integrated near the pseudoautosomal region of the mouse X chromosome. Variations in the mutation frequencies of different loci can be a consequence of both the DNA sequence (i.e., the presence of highly mutable CpG sites or the degree of sequence repetitiveness) and the genomic architecture of that region (Cooper and Krawezak, 1990).

Today there is no evidence for the occurrence of consistent DNA sequence alterations with age at the somatic cell level, like those observed in cancer (Thein et al., 1987), due to the clonal nature of the tissue outgrowth. Interestingly, the incidence of somatic mutations at minisatellite loci in tumors varies from locus to locus with the same loci showing the highest level of germ line and somatic instability (Armour et al., 1989). This may be explained by the fact that most of the germ line variations observed at minisatellite loci have been ascribed to mitotic mutation events during maturation of the germ cells rather than meiotic mutation events (Jeffreys et al., 1990). Also for a number of human disease loci, mosaicism of the germ line resulting from mitotic mutations has been observed (for a review, see Hall, 1988). The above illustrates that high somatic mutation frequencies can be expected at germ line mutation hot spots. When such hot spots occur in functional sequences their detailed analysis as a function of age would be highly relevant.

Thus far, techniques were lacking to scan the genome for hot spots of DNA sequence variation. Recently, a novel genome scanning technique has been developed, based on the separation in two dimensions of restriction enzyme digested genomic DNA followed by hybridization analysis, using repetitive DNA sequence motifs as probes (Uitterlinden et al., 1989). This 2-D DNA typing system lends itself well to the random analysis of the aging genome for hot spots of somatic instability and for the detection of low frequency mutations. Here we provide some preliminary results of 2-D DNA typing of fibroblast clones derived from young and old rats.

Materials and methods

Cell isolation and culture

Rat primary fibroblasts were obtained from 10-mm surgical ellipse biopsies from the skin of the back of female inbred Wistar and BN derived rats (WAG/Rij and BN/BiRij, respectively). The cells were isolated and cultured as described earlier (Vijg et al., 1986). DNA was isolated from 10^6 cells per clone (passage 15–20). At this point most of the clones were immortalized.

DNA isolation and restriction enzyme digestion

Fibroblast cells were rinsed with phosphate-buffered saline (PBS), drained, and stored in PBS at −80 °C. High-molecular-weight genomic DNA was isolated essentially as described (Slagboom et al., 1990). DNAs were digested with restriction endonuclease *Hae*III (BRL) under conditions recommended by the manufacturer.

Preparation of Southern blots

For Southern hybridization analysis (Southern, 1979) 5 μg of digested DNA was fractionated in a 1% agarose gel in 1 × TAE buffer (40 mM Tris–HCl, pH 7.4, 20 mM NaAc, 1 mM NaEDTA) at 1700 Vhr. After ethidium bromide staining the DNA separation patterns were transferred to a nylon membrane (Hybond N^+, Amersham) by vacuum blotting (VacuGene, LKB) in 0.4 M NaOH, 0.6 M NaCl for 1 h.

Preparation of two-dimensional DNA typing blots

Two-dimensional separation of 5 μg of *Hae*III-digested genomic DNA was carried out as described (Uitterlinden et al., 1989). Briefly, this

method is based on size fractionation of DNA restriction fragments in a neutral 6% polyacrylamide gel followed by electrophoresis in the second dimension in a 6% polyacrylamide gel containing a 10–75% linear concentration gradient of denaturant (100% denaturant = 7.0 M urea/ 40% formamide) parallel to the direction of electrophoresis (Fischer and Lerman, 1979). Under the conditions used, the 0.1–5 kb molecular weight range was fully resolved in a 2-D gel. After transfer of the genomic separation patterns to a nylon membrane (Hybond N^+) by semidry electroblotting at 400 mA (Uitterlinden et al., 1989), the membrane bound DNA fragments were denatured by incubating the filters in 0.4 N NaOH for 10 min. Filters were then rinsed in 2 × SSC (1 × SSC = 150 mM NaCl, 15 mM $NaHCO_3$), dried for 30 min at 80 °C and irradiated for 45 s with 302-nm UV light to cross-link the DNA fragments to the filter.

Probe preparation and labeling

Oligonucleotides for the core sequences $(TTAGGG)_5$, 33.15 (5'-AGAGGTGGGCAG-GTGG-3') and 33.6 (5'-AGGGCTGGAGG-3') (Jeffreys et al., 1985) and the complementary oligonucleotides were chemically synthesized. Probes were prepared essentially as described earlier (Uitterlinden et al., 1991). Thus oligonucleotide polymers were obtained with an average length between 400 and 2000 bp. In case the probe length was less than 400 bp, the chemically synthesized oligos were subjected to 20 cycles of a polymerization reaction in an automated thermocycler (Biorad). 1–3 μg of DNA was dissolved in 50 mM KCl, 10 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 200 μM dTTP, 200 μM dATP, 200 μM dGTP, 200 μM dCTP and 1 unit of Taq polymerase (BRL) in a total volume of 50 μl. The PCR cycles consisted of 2 min denaturation at 95 °C, 2 min annealing at 55–60 °C and 2-min extension at 72 °C. 20–100 ng of the probe was labeled by the random-primer oligolabeling method (BRL) or by self-priming after boiling for 5 min and annealing at room temperature in the presence of Klenow enzyme (Boehringer), 2 μM dNTP and 10 mM $MgCl_2$. Specific activities of 5×10^8 cpm/μg DNA were obtained.

Hybridization analysis

All membranes were hybridized essentially according to Church and Gilbert (1985) in 7% SDS/0.5 M Na phosphate, pH 7.2/1 mM NaEDTA at 65 °C for 2–12 h and washed twice in 2.5 × SSC/0.1% SDS or 1 × SSC/0.1% SDS at 65 °C for 30 min. Filters were then exposed to Kodak XAR films in cassettes with intensifying screens for 4–48 h. For rehybridization, the membranes were stripped for 10 min in 50% formamide, 0.5 × SSC, 10 mM $NaHPO_4$, pH 7.2, 25 μg/ml heparin, 0.5 mM NaEDTA, 0.5% SDS at 65 °C. After dehybridization membranes were washed for 20 min in 0.1 × SSC, 0.1% SDS.

Results

Fibroblast primary cultures were established from skin biopsies of young and old animals of two different inbred rat strains (WAG/Rij and BN/BiRij). Both the primary cultures and a number of clones derived from those cultures were studied for putative DNA sequence changes during in vivo and in vitro aging. Fig. 1 shows the Southern hybridization patterns obtained with the minisatellite core probe 33.6. The presence of one additional band at 11 kb in the 33.6 hybridization pattern of clone 1d of a WAG/Rij rat fibroblast clone indicated very limited genomic instability in the clones examined. We found no variations in the (TTAGGG)n or 33.15 hybridization patterns of the same fibroblast clones (results not shown).

We then analyzed a limited number of clones by two-dimensional DNA typing using the same probes. Fig. 2 shows the complete 2-D DNA typing pattern of WAG/Rij fibroblasts for the three probes used. The great number of spots in Fig. 2 illustrates the power of 2-D DNA typing in scanning the higher animal genome for the occurrence of DNA sequence alterations. The small number of fragments in the high-molecular-weight range that cannot be resolved (Fig. 2) is covered by regular Southern hybridization (Fig. 1).

Table 1 shows the number of spots and bands that were detected for each clone. The 2-D DNA typing patterns of BN/BiRij genomic DNA were comparable to those obtained from WAG/Rij

rats in number of spots, but differed for up to 40% of the sites detected (Slagboom et al., unpublished results).

To guarantee maximum reproducibility the DNA samples to be compared were run on one and the same gel as much as possible. For this purpose we ran the second dimension gels in two different size ranges: 5–0.7 kb and 0.7–0.1 kb. This is illustrated in Fig. 3 which shows 2-D DNA hybridization patterns of a primary culture and

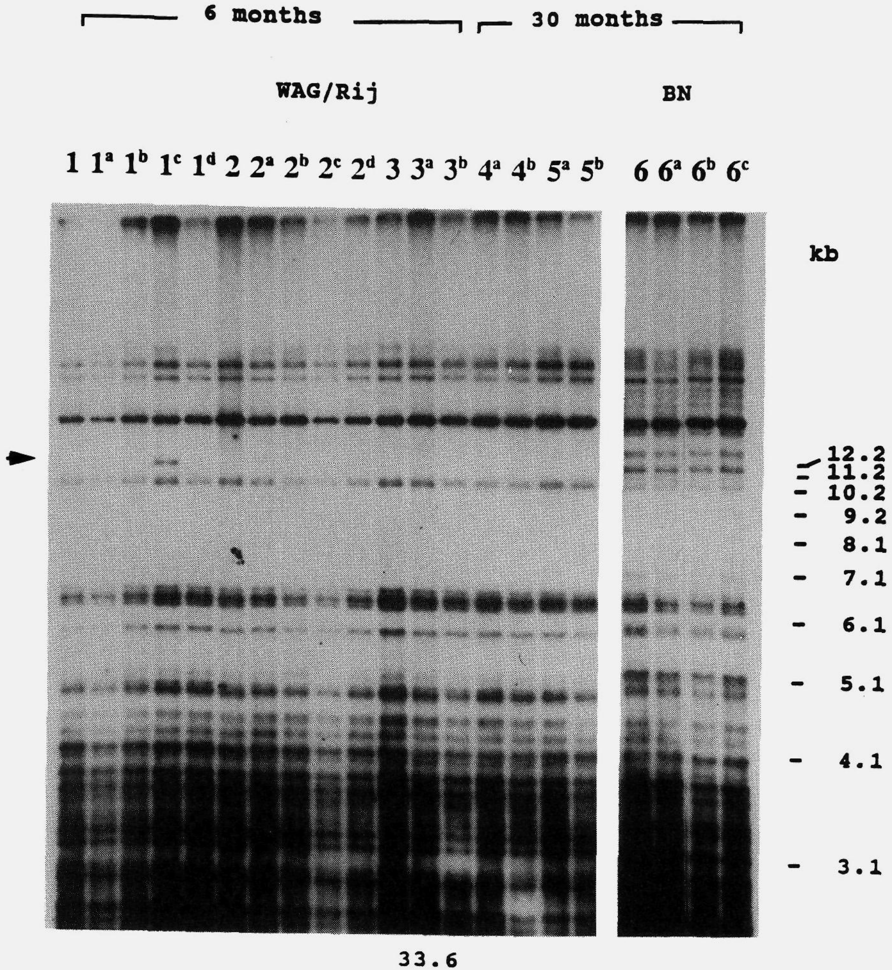

Fig. 1. Southern hybridization analysis of *Hae*III-digested genomic DNA isolated from the primary fibroblast cultures (1, 2, 3, and 6) and various clones derived from three 6-month-old (1a–1d, 2a–2d and 3a–3b), two 30-month-old (4a–4b and 5a–5b) WAG/Rij rats, and one 30-month-old (6a–6c) BN/BiRij rat, using minisatellite core probe 33.6.

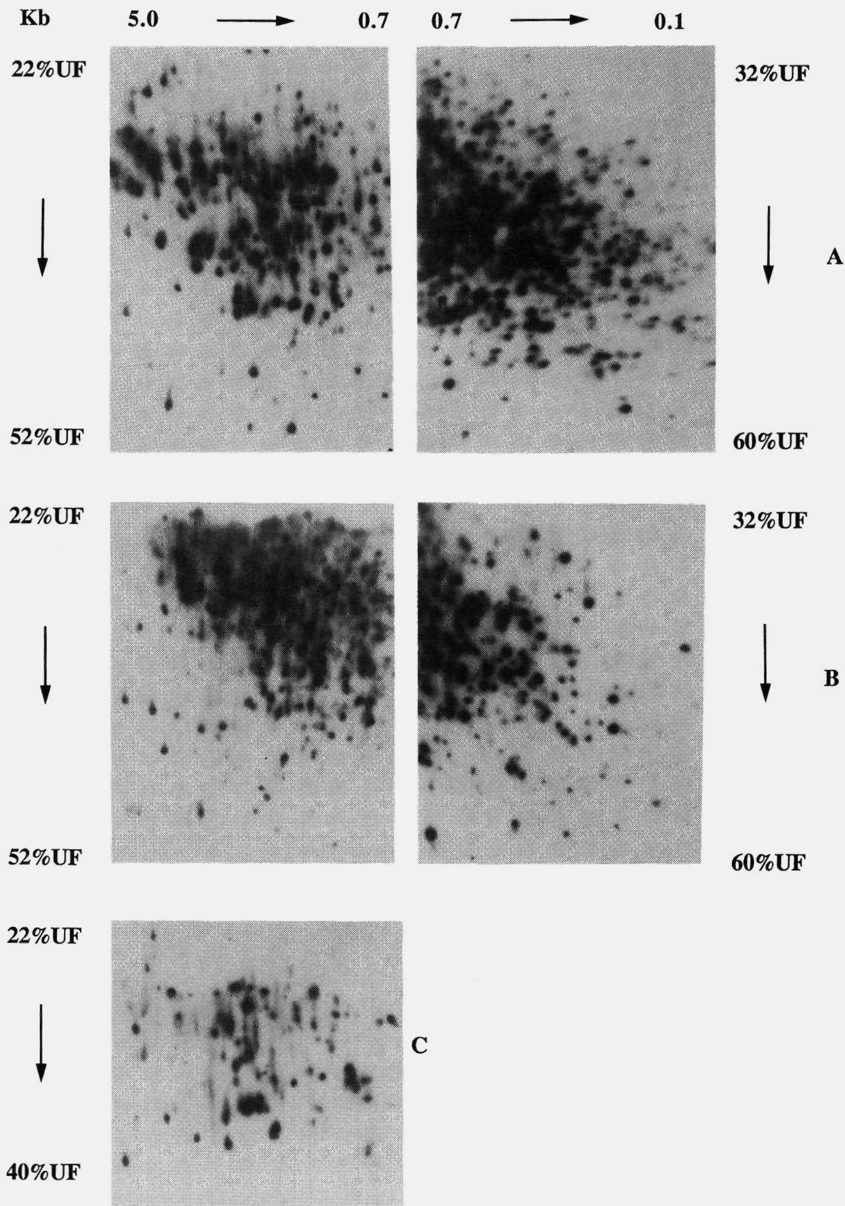

Fig. 2. 2-D DNA typing patterns of *Hae*III-digested genomic DNA isolated from a fibroblast clone obtained from the skin of a young WAG/Rij rat using minisatellite core probes 33.15 (A), 33.6 (B) and the simple sequence probe (TTAGGG)n (C). Note the absence of spots in the low-molecular-weight range of the TTAGGG hybridization pattern.

TABLE 1

THE NUMBER OF DNA FRAGMENTS DETECTED BY SOUTHERN BLOT ANALYSIS (SBA) AND 2-D DNA TYPING (2DDT) OF RAT FIRBOBLASTS

Probe	Number of bands per clone (SBA)	Number of spots per clone (2DDT)
33.15	27	539
33.6	24	337
(TTAGGG)n	25	88
Total	76	964

two clones for the two size ranges using minisatellite core probe 33.15. Once a difference was found confirmation was obtained by repeated determinations. A number of the observed spot variants are depicted in detail in Fig. 4. The results are summarized in Table 2.

The frequency of spot variants detected in 14 fibroblast clones and four primary cultures is 2.7×10^{-3} per DNA fragment. The variation frequency detected with the (TTAGGG)n probe was the highest (Table 2). The variation frequency of

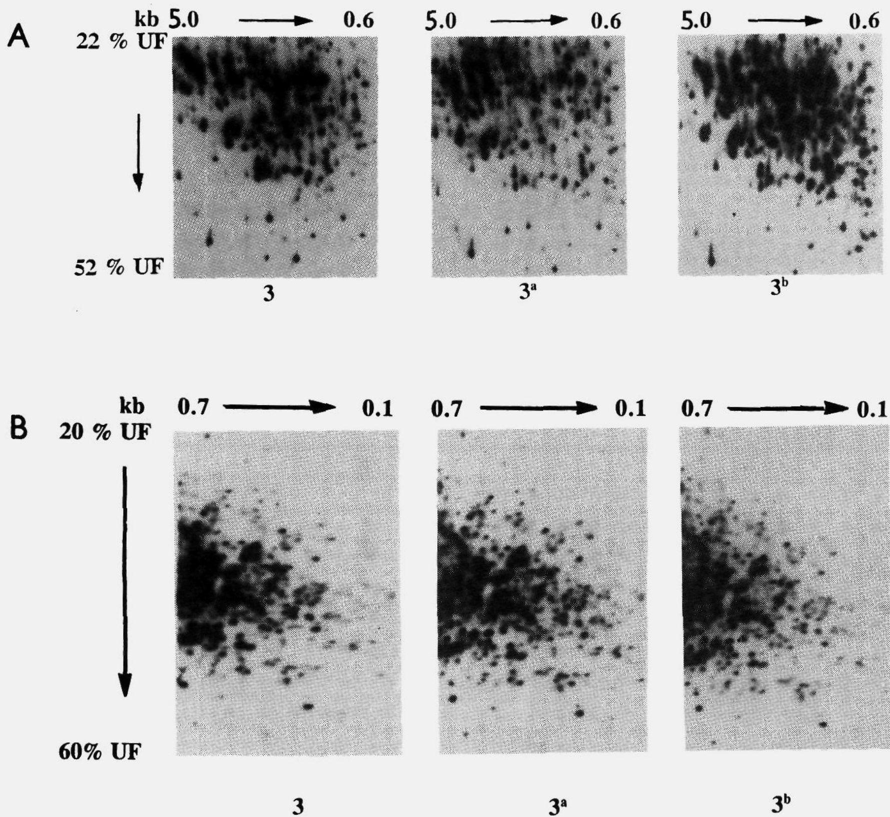

Fig. 3. The high- (A) and low-molecular-weight (B) range of the 33.15 2-D DNA typing pattern of *Hae*III-digested genomic DNA isolated from the primary culture and different clones from a young WAG/Rij rat (No. 3 in Fig. 1).

the clones derived from young rats did not differ significantly from those derived from the old animals (Table 2).

Discussion

In order to measure random low frequency DNA sequence alterations during the aging process, we have analyzed mini- and microsatellite regions in the genome of fibroblast clones derived from skin biopsies from young and old inbred rats.

With Southern hybridization analysis, using three probes only one variation in the form of an extra 11-kb band was observed in the 33.6 hybridization pattern. The number of DNA fragments that could be analyzed by 2-D DNA typing using the same probes increased 12-fold as compared to Southern hybridization analysis (Table 1). Using the Southern hybridization analysis, however, more DNA samples can be analyzed simultaneously. For this reason and for the fact that the two methods together provide for the analysis of both high- and low-molecular-weight fragments, a combination of both techniques was used.

By using multilocus core probes the number of hybridizing DNA fragments (spots and bands) does not necessarily correspond directly to the number of genomic loci, since various hybridizing DNA fragments may originate from the same locus. The variation frequency in Table 2 is therefore expressed per DNA fragment and not per locus. Spot variants were detected in 14 fibroblast clones and four primary cultures at an average frequency of 2.7×10^{-3} per DNA fragment and 1.5×10^{-3} per DNA fragment per clone, for the clones exhibiting the largest number of spot variants (primary culture 3 and clones therefrom, Table 2). This frequency is 100 times higher than the somatic mutation frequency of the human HPRT gene and the mouse APRT gene (Inamizu et al., 1986). Although such a high mutation frequency may be expected from repetitive DNA, we do not know what proportion of the variation we detected was generated in vitro. The calculated variation frequencies may therefore not completely represent the somatic mutation frequency in vivo.

The mean mutation rate of minisatellite and simple sequence loci in the germ line is 4×10^{-3} per DNA fragment per gamete (Jeffreys et al., 1988) and for the most unstable locus 5×10^{-2} per gamete. It has been suggested that most of the germ line mutations at minisatellite loci arise through slippage replication during mitosis (sperm maturation includes 400 postzygotic cell divisions). Somatic mutation events at minisatellite and simple sequence loci have been found in tumor cell populations (Thein et al., 1987; Armour et al., 1988) and early in mouse development (Kelly et al., 1989). In the tumor studies no variation was measured in normal tissue by Southern hybridization analysis. In cultured human T cells no DNA

TABLE 2

THE NUMBER OF SPOT VARIANTS DETECTED BY 2-D DNA TYPING IN RAT FIBROBLASTS

Primary culture	Number of clones	Donor age (months)	33.15		33.6		(TTAGGG)n		Total spot v./N	Frequency ($\times 10^{-3}$)
			N	spot v.	N	spot v.	N	spot var.		
1	3	6	1617	2	1115	5	455	3	10/3187	3.1
2	3	6	1200	3	–	–	168	1	4/1368	2.9
3	3	6	1131	3	895	5	159	2	10/2185	4.5
4	2	30	1010	1	730	3	78	0	4/1818	2.2
5	3	30	1380	0	1216	2	153	0	2/2749	0.7
Total			6337	9	3955	15	1013	6	30/11307	
Frequency ($\times 10^{-3}$)				1.4		3.8		5.9		2.7

N, average number of spots analyzed in all clones derived from the same rat; spot v., number of spot variants observed in all clones derived from the same rat; Frequency, the number of spot variants divided by the number of fragments analyzed.

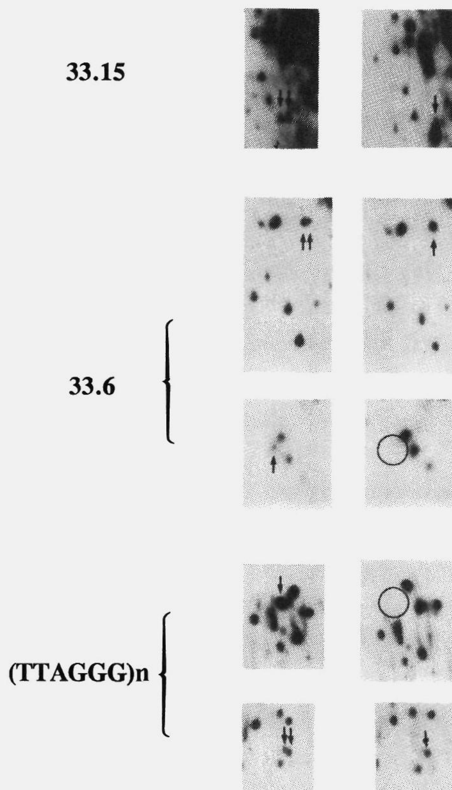

Fig. 4. Details of a part of the spot variations detected by 2-D DNA typing of various fibroblast clones listed in Table 2. Spot variants are marked by arrows and circles.

sequence alterations were observed in the Southern hybridization analysis of the (CAC)5/(GTG)5 simple sequence probe (Nürnberg et al., 1989). Recently, 2-D DNA typing was applied for the analysis of T cell clones revealing a very low number of variations (B. Morolli, personal communication).

A comparison of the spot variation frequencies (Table 2) with respect to the age of the animals is possible if we assume that the mutation frequency in vitro of clones from young and old animals is about the same. We did not observe significant age-related alterations in the spot variation frequency (Table 2). We conclude that the somatic mutation frequency at the mini- and microsatellite genomic regions that were investigated here does not change with age, either in vivo or in vitro.

In summary, our present data indicate that the somatic mutation frequency at mini- and microsatellite loci studied here in the rat fibroblast genome is high. This observation confirms and extends (due to the large number of loci tested) earlier findings. Furthermore our data suggest that no increase in genomic instability occurs with age at the loci investigated. This is somewhat surprising in view of the many data from the literature suggesting a general increase in genomic instability during cellular aging. These contradictory observations may be explained in three ways. (1) Cells with high mutation frequencies are negatively selected for in tissue culture. Although this is certainly expected to occur such was also the case in previous studies considering the mutation frequencies of human HPRT genes in which an age-related increase could still be detected. (2) We did not investigate enough minisatellite loci. Somatic mutations may accumulate during aging at some loci but not at others. Our results indicate that even at loci that are highly variable in germ cells and in somatic cells (as confirmed by our data) accumulation of mutations does not necessarily occur with age. In order to evaluate the somatic mutation theories of aging many different types of DNA sequences must be analyzed. The investigation of the same fibroblast clones as the ones examined here by using a panel of additional mini- and microsatellite probes is still in progress. In addition it might be interesting to determine the mutation frequency at DNA sequences in coding DNA that are known mutation hot spots in the germ line.

Finally, these results illustrate the usefulness of the 2-D DNA typing method to scan the higher animal genome using repetitive sequences as anchor points. Besides the mini- and microsatellite sequences used in this study, the 2-D DNA typing system can be used to analyze a wide range of non-functional and functional sequences, e.g., transcriptional control elements, protein binding DNA sites, coding and controlling sequences from "key genes" (DNA repair genes, phosphorylating

genes), etc. We anticipate the use of this system as a first screen for the rapid identification of sequences that are most unstable during aging. Once found, such sequences can be further analyzed by regular techniques, among which the polymerase chain reaction for studies in small numbers of cells.

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References

Armour, J.A.L., I. Patel, S.L. Thein, M.F. Fey and A.J. Jeffreys (1989) Analysis of somatic mutations at human minisatellite loci in tumors and cell lines, Genomics, 4, 328–334.

Blackburn, E.H. (1991) Structure and function of telomeres, Nature, 350, 569–572.

Carrano, A.V. (1989) Summary of the workshop on mammalian in vivo somatic mutation, Genome, 31, 458–459.

Chakravarti, A., S.C Elbein and M.A. Permutt (1986) Evidence for increased recombination near the human insulin gene: implication for disease association studies, Proc. Natl. Acad. Sci. (U.S.A.), 83, 1045–1049.

Church, G.M., and W. Gilbert (1984) Genomic sequencing, Proc. Natl. Acad. Sci. (U.S.A.), 81, 1991–1995.

Cooper, D.N., and J.F. Clayton (1988) DNA polymorphism and the study of disease association, Hum. Genet., 78, 299–312.

Cooper, D.N., and M. Krawezak (1990) The mutational spectrum of single base-pair substitutions causing human genetic disease: patterns and predictions, Hum. Genet., 85, 55–74.

Curtis, H.J. (1966) Biological Mechanisms of Aging, C.C. Thomas, Springfield, IL.

Davies, K.E., A. Speer, F. Hermann, A.W.J. Spiegler, S. McGlade, M.H. Hofker, P. Brainet, R. Hanke, R. Schwartz, V. Steinbicker, R. Szibor, H. Korner, D. Sommes, P.L. Pearson and C. Coutelle (1985) Human X chromosome markers and Duchenne muscular dystrophy, Nucleic Acids Res., 13, 3419–3426.

Failla, G. (1958) The aging process and carcinogenesis, Ann. NY Acad. Sci., 71, 1124–1135.

Featherstone, T., P.D. Marshall and H.J. Evans (1987) Problems and pitfalls in assessing human T-lymphocyte mutant frequencies, Mutation Res., 179, 215–230.

Fischer, S.G., and L.S. Lerman (1979) Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis, Cell, 16, 191–200.

Gossen, J.A., C.H.T. Tan, P.H.M. Lohman, F. Berends, D.L. Knook, E.C. Zwarthoff and J. Vijg (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a new model for studying mutations in vivo, Proc. Natl. Acad. Sci. (U.S.A.), 86, 7971–7975.

Gossen, J.A., W.J.F. de Leeuw, A. Verwest, P.H.M. Lohman and J. Vijg (1991) High somatic mutation frequencies in a *lacZ* transgene integrated on the mouse X-chromosome, Mutation Res., 250, 423–429.

Hall, J.G. (1988) Somatic mosaicism: observations related to clinical genetics, Am. J. Hum. Genet., 43, 355–363.

Harley, C.B., A.B. Futcher and C.W. Greider (1990) Telomeres shorten during aging of human fibroblasts, Nature, 345, 458–460.

Hastie, N.D., M. Dempster, M.G. Dunlop, A.M. Thompson, D.K. Green and R.C. Allshire (1990) Telomere reduction in human colorectal carcinoma and with ageing, Nature, 346, 866–868.

Hedner, K., B. Högstedt, A.M. Kolnig, E. Mark-Vendel, B. Strömbeck and F. Mitelman (1982) Sister chromatid exchanges and structural chromosome aberrations in relation to age and sex, Hum. Genet., 62, 305–309.

Inamizu, T., N. Nobuhisa, M. Chang and T. Makinodan (1986) Frequency of 6-thioguanine-resistant T cells is inversely related to the declining T-cell activities in aging mice, Proc. Natl. Acad. Sci. (U.S.A.), 83, 2488–2491.

Jeffreys, A.J., N.J. Royle, V. Wilson and Z. Wong (1988) Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA, Nature, 332, 278–281.

Jeffreys, A.J., R. Neumann and V. Wilson (1990) Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis, Cell, 60, 473–485.

Jeffreys, A.J., V. Wilson and S.L. Thein (1985) Hypervariable "minisatellite" regions in human DNA, Nature, 314, 67–73.

Kelly, R., G. Bulfield, A. Collick, M. Gibbs and A.J. Jeffreys (1989) Characterization of a highly unstable mouse minisatellite locus: evidence for somatic mutation during early development, Genomics, 5, 844–856.

Martin, G.M., A.C. Smith, D.J. Ketterer, C.E. Ogburn and C.M. Disteche (1985) Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice, Isr. J. Med. Sci., 21, 296–301.

Neel, J.V., C. Satoh, K. Goriki, M. Fujita, N. Takahashi, J. Asakawa and R. Hazama (1986) The rate with which spontaneous mutation alters the electrophoretic mobility of polypeptides, Proc. Natl. Acad. Sci. (U.S.A.), 83, 389–393.

Nürnberg, P., L. Roewer, H. Neitzel, K. Sperling, A. Pöpperl, J. Hundrieser, H. Pöche, C. Epplen, H. Zischler and J.T. Epplen (1989) DNA fingerprinting with the oligonucleotide probe $(CAC)_5/(GTG)_5$: somatic stability and germline mutation, Hum. Genet., 84, 75–78.

Oberlé, I., D. Drayna, G. Camerino, R. White and J.L. Mandel (1985) The telomeric region of the human X chromosome long arm: presence of a highly polymorphic

DNA marker and analysis of recombination frequency, Proc. Natl. Acad. Sci. (U.S.A.), 82, 2824–2828.
Prieur, M., W. Al Achkar, A. Aurias, J. Couturier, A.M. Dutrillaux, B. Dutrillaux, A. Flury-Herard, M. Gerbault-Seureau, F. Hoffschir, E. Lamoliatte, D. Lefrancois, M. Lombard, M. Muleris, M. Ricoul, L. Sabatier and E. Viegas-Pequignot (1988) Acquired chromosome rearrangements in human lymphocytes: effect of aging, Hum. Genet., 79, 147–150.
Slagboom, P.E., and J. Vijg (1989) Genetic instability and aging: theories, facts and future perspectives, Genome, 31, 373–385.
Slagboom, P.E., and J. Vijg, 2-D DNA typing of small simple sequence and minisatellite alleles in humans, rats and mice, in preparation.
Slagboom, P.E., W.J.F. de Leeuw and J. Vijg (1990) mRNA levels and methylation patterns of the tyrosine aminotransferase gene in aging inbred rats, FEBS Lett., 269, 128–130.
Slagboom, P.E., D. Boomsma and J. Vijg (1991) Genetic determination of telomere size: a twin study, submitted for publication.
Southern, E.M. (1979) Gel electrophoresis of restriction fragments, Methods Enzymol., 68, 152–176.
Szilard, L. (1959) On the nature of the aging process, Proc. Natl. Acad. Sci. (U.S.A.), 45, 35–45.
Thein, S.L., A.J. Jeffreys, H.C. Gooi, F. Cotter, J. Flint, N.T.J. O'Connor, D.J. Weatherall and J.S. Wainscoat (1987) Detection of somatic changes in human cancer DNA by DNA fingerprint analysis, Br. J. Cancer, 55, 353–356.
Uitterlinden, A.G., P.E. Slagboom, D.L. Knook and J. Vijg (1989) Two-dimensional DNA fingerprinting of human individuals, Proc. Natl. Acad. Sci. (U.S.A.), 86, 2742–2746.
Uitterlinden, A.G., P.E., Slagboom, E. Mullaart, I. Meulenbelt and J. Vijg (1991) Genome scanning by two-dimensional DNA typing: the use of repetitive DNA sequences for rapid mapping of genetic traits, Electrophoresis, 12, 119–134.
van Leeuwen, F., E. van der Beek, M. Seger, P. Burbach and R. Ivell (1989) Age-related development of a heterozygous phenotype in solitary neurons of the homozygous Brattleboro rat, Proc. Natl. Acad. Sci. (U.S.A.), 86, 6417–6420.
Vijg, J. (1990) DNA sequence changes in aging: how frequent, how important?, Aging, 2, 105–123.
Vijg, J., E. Mullaart, F. Berends, P.H.M. Lohman and D.L. Knook (1986) UV-induced DNA excision repair in rat fibroblasts during immortalization and terminal differentiation in vitro, Exp. Cell Res., 167, 517–530.

CHAPTER 8

GENETIC DETERMINATION OF TELOMERE SIZE IN HUMANS A TWIN STUDY OF THREE DIFFERENT AGE GROUPS

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Submitted for publication.

Summary

Reduction of telomere length has been postulated to be a causal factor in cellular aging. Human telomeres terminate in tandemly arranged repeat arrays consisting of the TTAGGG motif. The length of these arrays in cells from human mitotic tissues is inversely related to the age of the donor, indicating telomere reduction with age. In addition to differences of telomere length between different age groups, a considerable variation is present even among individuals of the same age group. To investigate whether this is due to genetic influences, we have measured the size of telomeric repeat fragments in human mono- and dizygotic twin pairs aged between 2 and 95 years. Statistical analysis and model-fitting of our data indicate a heritability of 60% in infants and 85% in adolescents and adults. The individual differences in mean telomere length in blood within groups of the same age, therefore, is to a large exent genetically determined.

Introduction

The ends of human chromosomes consist of tandem arrays of the (TTAGGG)n repetitive motif (Moyzis et al., 1988). Telomeric repeats are lost during each cell cycle, when small DNA fragments remain uncopied at the discontinuously replicating DNA strand (Olovnikov 1973). In germ cells, this mechanism is compensated by the activity of the telomerase enzyme, a ribonucleoprotein capable of elongating telomeres de novo, thereby overcoming the loss of telomeric repeats (Morin, 1989; Blackburn, 1991).

Telomere reduction occurs progressively with serial passage of human fibroblasts in culture. Moreover, the initial telomere length of these cells predicts their replicative capacity (Harley et al. 1990; Allsopp et al. 1992). The average telomere size was found to be shorter in peripheral blood cells and colorectal mucosa epithelia from old than from young human individuals, corresponding with a rate of telomere loss of 33 bp per year (Hastie et al., 1990).

It was proposed that loss of telomeric DNA ultimately leads to cell-cycle exit and senescence (Harley 1991). There is an increase in terminal chromosomal rearrangements with age (Bender et al. 1989). This is especially interesting since the subtelomeric regions are extremely gene-rich (Saccone et al. 1992). Several lines of evidence suggest that telomere shortening beyond a critical length is involved in such chromosomal instability. Some immortalized SV40 transformed human embryonic kidney cells in culture express telomerase activity (Counter et al. 1992). Progressive telomereshortening and accumulation of dicentric chromosomes, which occurs in normal cells in cuture, is arrested in these immortalized cellines.

A considerable variation of telomeresize in peripheral blood lymphocytes from human subjects is present even among individuals of the same age (Hastie et al. 1990). An important question is therefore, how the individual rate of telomere loss is determined as a function of age. To study the possibility that this can be attributed to genetic influences, the length of

telomeric restriction fragments (TRFs) was measured in peripheral blood cells from mono- and dizygotic human twins of ages between 2 and 95 years. TRF length was determined by Southern hybridization analysis. The mean TRF length in blood DNA from twins aged between 2 and 95 years decreased in a range from 10 to 5 Kb. Based on statistical analysis and model fitting of the data, a heritability for telomere size of 60% was found in infants and of 85% in adolescents and adults. These results indicate that telomere size variation is genetically determined to a large extent.

Subjects, Material, and Methods

Telomersizes were measured in monozygotic (MZ) and dizygotic (DZ) twins of 4 different age groups. Average age of the youngest group was 4.15 years (sd = 1.40), 17.1 years (sd = 2.40) in adolescent twins, 43.7 (sd = 5.79) in adult twins, and 79 years (sd = 7.82) in the oldest group. Blood samples were obtained from healthy Dutch twins between 2 and 95 years of age, who are registred with the Netherlands Twin Register (Boomsma et al, 1992, 1993). DNA was isolated from white blood cells as described in Meulenbelt et al., (1993). Twin zygosity was determined by DNA fingerprint analysis (Jeffreys et al., 1985). In the youngest group there were 16 MZ and 15 DZ pairs; there were 10 MZ and 12 DZ adolescent twin pairs, and 28 MZ and 23 DZ adult pairs. The oldest group contained 4 MZ and 4 DZ pairs.

Analysis of telomere length

Telomeric restriction fragments (TRF) containing only a small portion of DNA proximal to the repeat array are generated when genomic DNA is digested with HaeIII, because of the absence of HaeIII recognition sites within the (TTAGGG)n tandem repeat arrays. Digested genomic DNA (5 μg) was electrophoresed for 1050Vh in 1% agarose gels containing 13 twin pairs per gel (batch) and subjected to Southern hybridization analysis. The blots were subsequently hybridizaed to a random primed ^{32}P labeled (TTAGGG)n and the 33.15 minisatellite core probe (Jeffreys et al. 1985). For preparation of the probe, chemically synthesized $(TTAGGG)_7$ and its complementary oligo were kinased, ligated and subjected to 20 rounds of a polymerization reaction (95°C, 2 min; 45°C, 2 min; 74°C 2 min). The product of this reaction was used as a probe. Hybridization was performed in 7% SDS/ 0.5M NaH_2PO4 pH 7.2, 1 mM NaEDTA at 65°C for 1 h and washed twice in 2.5x SSC, 0.1% SDS at 65°C for 30 min. Filters were exposed for 3-7h. For rehybridization, the membranes were stripped for 10 min in 50% formamide, 0.5 x SSC, 10 mM NaH_2PO4 pH 7.2, 25 μg/ml heparin, 0.5 mM NaEDTA, 0.5% SDS at 65°C and washed 20 min in 0.1 x SSC, 0.1% SDS.

Autoradiographs exposed within the linear range of signal response were analysed by computer assisted image analysis using the ACE (vd Hofstede et al, 1993) programme adapted for quantification of the smeared telomere hybridization patterns. The position (Kb) of the mean integrated signal was determined.

Statistical Analysis

Genetic analyses were carried out on data from the first 3 age groups (average ages 4, 17 and 43 years). Data from the oldest group were excluded from the genetic analysis because there were too few twin pairs in this group. To analyze the resemblance between MZ twins, who are genetically identical, and DZ twins, who share 50% of their genes on average, we used a pedigree-based maximum likelihood method developed by Lange et al. (1988). For a given pedigree of n individuals (n = 2 in our case) a vector of observations (x) is defined and a vector of expected values (Ex), that can depend on measured variables such as age, and, as in our case, batch (gel). In a pedigree, the distribution of Ex is expected to be multivariate normal. The expected covariance matrix (Σ) for a pedigree depends on the relationship between pedigree members and on the genetic model that is specified for the observations. For a given Ex and Σ, the log-likelihood of obtaining the observation vector x is:

$$L = -1/2 \ln | \Sigma | -1/2 (x-E(x))'\Sigma^{-1}(x-E(x))+c$$

The joint log-likelihood of obtaining all pedigrees is the sum of the log-likelihood of the separate pedigrees. Estimation involves selection of parameter values under a specific model which maximizes the joint likelihood of all pedigrees. There is no overall test of the goodness-of-fit of the model using this approach (Lange et al. 1976.), but the likelihoods obtained for different models can be compared with the chi-squared (χ^2) difference tests, where χ^2 is twice the difference between the general and the more restricted model. The FISHER package (Lange et al.1988) was used for genetic modeling. The genetic models that were tested specified twin resemblance to be due to additive genetic factors or to the effects of a shared family environment. Submodels were tested if the effect of these factors was the same in all 3 age groups. All models included age and batch effects on the phenotype.

Results

The TRF length as a function of age was assessed by Southern hybridization analysis (Fig. 1A). The smeared hybridization pattern indicates considerable intra-tissue heterogeneity of TRF size in the blood sample from each individual. For zygosity determination and as a control for the concentration and quality of DNA and gel, the filters were rehybridized with a minisatellite probe (Fig. 1B). Telomeresize reduction when different age groups are compared is illustrated in Fig. 2. The mean TRF length in white blood cells of 112 twin pairs aged 2 to 95 years, corresponded to a loss of 31.0 bp per year ($P << .005$; $r = -0.71$) (Fig. 3). The mean TRF length in the four age groups was 8.3 (sd = 0.64) for the youngest twins, 7.8 (sd = 0.56) for adolescents, 7.3 (sd = 0.76) for the adults and 5.6 (sd = 0.40) for the oldest. In addition to these differences between age groups, a considerable variation of telomeresize was present in all age-groups. In general, TRF length variation among pairs, i.e. unrelated individuals, was found to be higher than the variation within twin pairs. The variation within monozygotic twin pairs was found to be the smallest.

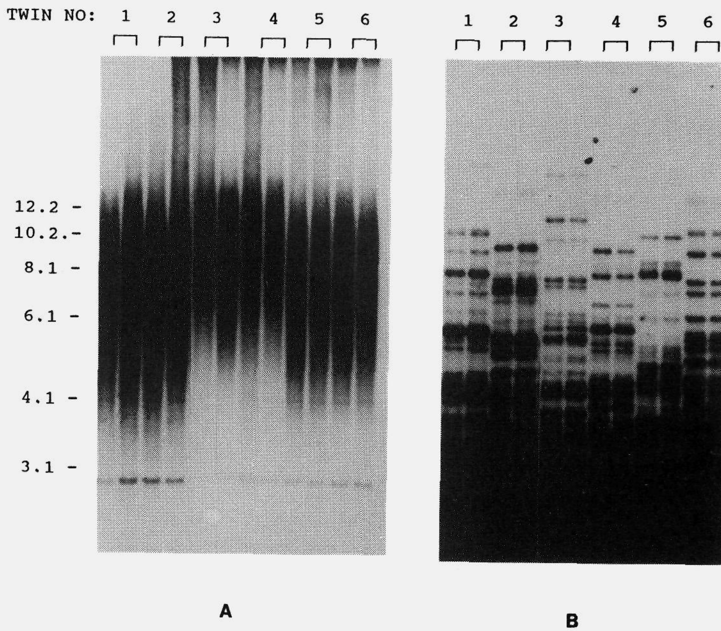

Figure 1. Southern hybridization analysis of monozygotic twin pairs by the (TTAGGG)n polymer (A) and the 33.15 minisatellite core probe 33.15 (Jeffreys et al., 1985) for zygosity determination and as a control for the quality of DNA and gel (B).

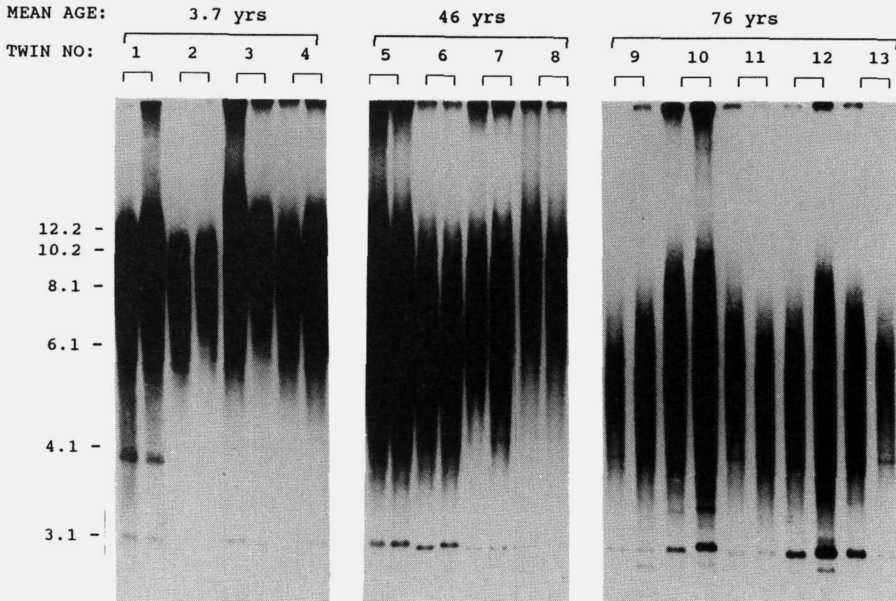

Figure 2. Southern hybridization analysis of a subset of twin pairs of different age groups using the (TTAGGG)n polymer as a probe. Average donor age and size markers (Kb) are indicated. The bands appearing at ± 2.7 Kb are non-telomeric repeat units homologous to the probe.

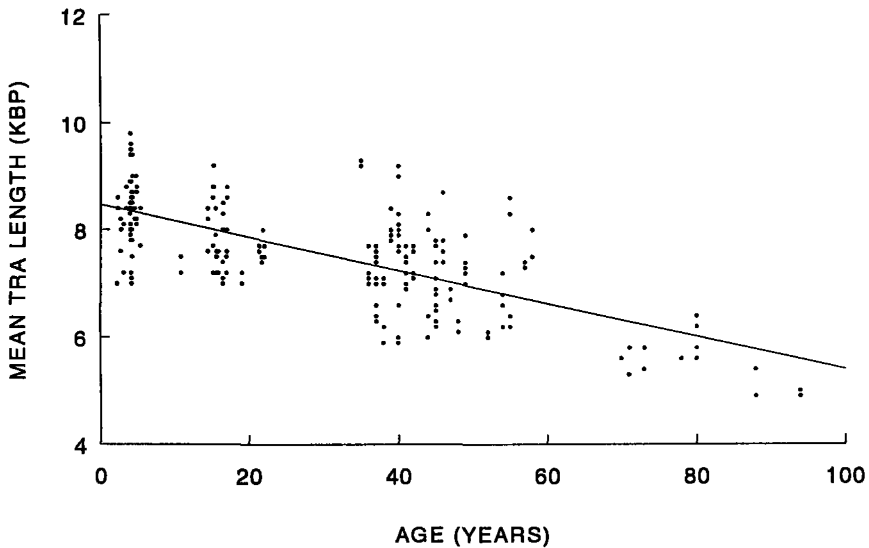

Figure 3. Mean telomerelength as a function of donor age as measured in genomic DNA from peripheral blood lumphocytes as described in subjects, materials and methods. The slope (-31 bp/year) of the linear regression line is significantly different from 0 ($p << 0.005$).

Statistical analysis of the data is presented in Table 1. Improvement or deterioration in fit due to addition or deletion of parameters are judged by likelihood ratio tests. Table 1 shows the different models that were evaluated, their log-likelihoods and the χ^2 test statistics used to compare the goodness-of-fit of the more restricted models versus the more general model. Twice the difference between the log-likelihoods of these models is distributed as χ^2. The most general model allowed for the effects of age and batch on the phenotype and estimated 6 different correlations for the 6 age x zygosity groups. Estimates from the general model are shown in Table 2. The amount of variance explained by age differences was 27% and by batch effects 32%. For the part of the phenotypic variance that was not explained by age or batch, the pattern of estimated twin correlations suggests that individual differences in telomersizes are to a large extent genetically determined. The second model in Table 1 sets all batch effects equal to each other and clearly shows a marked decrease in fit. The third model is a test of the additive genetic model, in which the MZ correlation is exactly twice the DZ correlation. This model fits the data as well as the general model (χ^2 difference is 1.95 with 3 df). The next 2 models show that the MZ and DZ correlations in adolescents and adults may be equated, but that resemblances in 4-year olds are significantly lower. Model F tests if the MZ and DZ correlations may be equated to each other, this is a test of the shared environment model. It is clear that shared family environment cannot explain twin resemblance as good as an additive genetic model. Heritabilities based on the final model are 60% in 4-year olds, and 85% in 17- and 44-year olds.

Table 1. Genetic model fitting using FISHER (Lange, Weeks and Boehnke, 1988).

Model	Likelihood	tested against against	difference df	χ^2
A 15 batch effects age regression total variance 3 MZ and 3 DZ correlations	56.429			
B as A, no batch effects	17.902	A	14	77.05*
C as A, r(MZ) = 2 r(DZ)	55.453	A	3	1.95
D as C, twin correlations two oldest groups equal	55.389	C	1	0.13
E twin correlations all three groups equal	52.263	D	1	6.25*
F r(MZ) = r(DZ); correlations two oldest groups equal	50.832	D	2	9.11*
G as D, no age regression	49.800	D	1	11.18*

* significant deterioration in fit

A: most general model against which more restricted models are tested
B: all batch effects equal (allowing for age regression)
C: test of the additive genetic model
D+E: test of equality of twin correlations across 3 age groups
F: test of the shared environmental model

Table 2. Maximum likelihood parameter estimates of twin correlations for general and final model.

	Correlations free	rMZ = 2rDZ and r(17year) = r(44year)
MZ 4 year	0.594	0.598
DZ 4 year	0.324	0.299
MZ 17 year	0.859	0.846
DZ 17 year	0.669	0.423
MZ 44 year	0.838	0.846
DZ 44 year	0.532	0.423

Discussion

The most striking finding of our study is that individual differences in mean telomeresize of DNA from white blood cells are to a large extent genetically determined. Heritabilities based on model-fitting of the data are 60% in 4-year olds, and 85% in 17- and 44-year olds. Regarding the fact that the total variance also includes error variance (heritability = genetic variance/total variance), the high heritability suggests that individual diferences are almost entirely genetic in origin. The somewhat lower heritability in the 4-year olds as compared to the other age groups, may be due to the poor resolution of classical electrophoretic separation in the high molecular weight range resulting in higher errors in mean TRF length assessment of larger fragments.

Considerable differences were observed between the means of assay batches (gels). This could be due to unnoticed variations in electrophoretic conditions (although the amount of Vhrs was equal for all gels) and/or concentration differences between DNA samples (with the smallest variation among samples of a single gel) and/or autoradiographic exposure times. The quantification of smeared autoradiographic patterns and estimation of the midpoint position of the integrated signal could possibly be improved by phosphorimaging detection. The only way around a batch effect would be a randomization of all individuals in the study across all batches. For comparison of the DNA fingerprint patterns in the zygosity test, however, twin pair samples were run on the same gel.

The differences in telomeresize among individuals of the same age groups could reflect TRF length differences in the germ line even in the presence of telomerase; differences in the amount of telomeric DNA lost per cell doubling or differences in turnover rate of cells *in vivo*. The genetic basis for this variation, as indicated by our data, may be found in the presence of telomerase gene variants with variable activity in germ line and/or stem cell pools or in a genetically determined variation of immunological response to antigenic exposure. In this respect, TRF length should also be measured in subpopulations of white blood cells and in tissues other than blood. Human telomeres are very similar to the telomeres of other organisms. Genetic control of telomere size has also been suggested to explain strain-specific variation in telomere size of mice (Kipling and Cooke, 1990). The length and stability of yeast telomeres is controled by at least four genes (Carson and Hartwell, 1985; Lundblad and Szostak, 1989). A mutation in one of these loci, the *est1* locus causes gradual loss of telomeric repeats, aneuploidy and senescence (Lundblad and Szostak 1989).

Telomere shortening in humans might eventually lead to chromosomal instability, DNA sequence and gene expression changes in subtelomeric regions (Biesmann and Mason, 1992) and in subsequent decrease of cellular functions. Data on transformed human fibroblasts indicate that instabilities of the telomeric regions and cell death occur when a critical minimum telomere length of 1.5 kb is passed (Counter et al. 1992). However, telomere loss *in vivo*, as measured in blood DNA of random individuals, occurs at a rate of 31-90 bp/year (Hastie et al., 1990, Vaziri et al., 1993 and this study) making it unlikely that essential coding sequences would be lost or damaged during an individual's lifespan. Indeed, mean

TRF length of less than 5 Kb was not observed in blood of the oldest individuals in our study. However, a group of old individuals may represent a selected sample and hence reveal telomeres above the critical length of 1.5 Kb. Furthermore, it cannot be ruled out that a few chromosome ends in subsets of cells may have reached the critical TRF length in these blood samples. The large variation of mean TRF length among twins in the younger age-groups may indicate that the critical telomere length may be reached in blood of some individuals. Telomere shortening may well be involved in cell-cycle control and cellular senescence. Our data indicate that such a process *in vivo* is largely determined by genetic influences.

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References

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci 89: 10114-10118.

Bender MA, Preston RJ, Leonard RC, Pyatt BE, Gooch PC (1989) Chromosomal aberration and sister-chromatix exchange frequencies in peripheral blood lymphocytes of a large human population sample. Mutat Res 212: 149-154.

Biessmann H, Mason JM (1992) Genetics and molecular biology of telomeres. Adv Genet 30: 185-233.

Blackburn EH (1991) Structure and function of telomeres. Nature 350: 569-573.

Boomsma DI, Koopmans JR, Doornene LJP van, Orlbeke JF (1993) Genetic and social influences on starting to smoke: A study of Dutch adolescent twins and their parents. Brit J of Addiction, in press.

Boomsma DI, Orlebeke JF, Baal GCM van (1992) The Dutch Twin register: Growth data on weight and height. Behavior Genet 22: 247-251.

Carson MJ, Hartwell L (1985) CDC17: an essential gene that prevents telomere elongation in yeast. Cell 42: 249-257.

Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bachetti S (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11: 1921-1929.

Harley CB (1991) Telomereloss: mitotic clock or genetic time bomb? Mutat Res 256: 271-282.

Harley CB, Futcher AB, Greider CW (1990) Telomeres shorten during ageing of human fibroblasts. Nature 345: 458-460.

Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC (1990) Telomere reduction in human colorectal carcinoma and with ageing. Nature 346: 866-868.

Hofstede JW van de, Harıng S, Slagboom PE (1993) ACES: Automated comparison and evaluation of spot images. Medical Informatics Conference: Communication and Integration. in press.

Jeffreys AJ, Wilson V, Thein SJ (1985) Hypervariable "minisatellite" regions in human DNA. Nature 314: 67-73.

Kipling D, Cooke HJ (1990) Hypervariable ultra-long telomeres in mice. Nature 347: 400-402.

Lange K, Weeks D, Boehnke M (1988) Programs for pedigree analysis: Mendel, Fisher and dGene. Genet Epidem 5: 471-472.

Lange K, Westlake J, Spence MA (1976) Extension to pedigree analysis III. Variance components by the scoring method. Ann Hum Genet 39: 485-491.

Lundblad V, Szostak JW (1989) A mutant with a defect in telomere elongation leads to senescence in yeast. Cell 57: 633-643.

Meulenbelt I, Williams CJ, Giessen vd GC, te Koppele JM, Slagboom PE (1993) Linkage disequilibrium analysis of restriction fragment length polymorphisms (RFLPs) of the procollagen type II gene (COL2A1) in the Dutch popultaion. submitted to Hum Genet.

Morin GB (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 59: 521-529.

Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratcliff RL, Wu J (1988) A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci USA 85: 6622-6626.

Olovnikov AM (1973) A theory of Marginotomy. J Theor Biol 41: 181-190.

Pluta AF, Zakian VA (1990) Recombination occurs during telomere formation in yeast. Nature 337: 429-433.

Saccone S, De Sario A, Della Valle G, Bernardi G (1992) The highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes. Proc Natl Acad Sci 89: 4913-4917.

Vaziri H, Schächter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB (1993) Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet 52: 661-667.

SAMENVATTING

Veroudering wordt wel omschreven als een proces van voortgaande nadelige veranderingen binnen een organisme, die leiden tot een toenemend risico om dood te gaan. In principe kunnen alle nadelige fysiologische veranderingen optredend vanaf het moment van conceptie tot aan de dood van het organisme een rol spelen bij veroudering. In een verouderende populatie, zoals die van de mens in de huidige Westerse maatschappij, zijn drie hoofdgroepen te onderscheiden. De eerste groep bestaat uit individuen die op jonge leeftijd overlijden, bijvoorbeeld door trauma en ernstige genetische aandoeningen; deze personen zijn bepalend voor de 'Initial Mortality Rate' (IMR; het aantal personen per jaar dat als eerste deel van de populatie overlijdt). De tweede groep bestaat uit individuen die overlijden aan verouderingsgerelateerde ziekten na de reproduktieve periode; deze groep is bepalend voor de 'Mortality Rate Doubling Time' (MRDT; de tijd waarin het dubbele aantal mensen per jaar komt te overlijden). Ten derde zijn er individuen die zo succesvol verouderen dat veelvoorkomende verouderingsziekten hen lange tijd, en soms zelfs volledig, bespaard blijven. Eén individu tenslotte bepaalt de maximale levensduur van de populatie.

De maximale levensduur en de MRDT zijn veelal vergelijkbaar in verschillende populaties van één soort, terwijl verschillende species hierin sterk variëren. Dit wordt algemeen gezien als een sterke aanwijzing dat de genetische constitutie de belangrijkste determinant van het verouderingsproces is. Verschillende typen levensduur-regulerende genen kunnen worden onderscheiden.

1. Veroudering kan **geprogrammeerd** zijn analoog aan vroege ontwikkelings-stadia van het organisme. Levensduur-regulerende genen kunnen door hun geprogrammeerde expressie aanleiding geven tot de dood van het organisme.
2. Veroudering kan worden beschouwd als een bijprodukt van natuurlijke selectie. Veroudering zou het gevolg kunnen zijn van zich ophopende mutaties in de geslachtscellijn met een neutraal of positief effect op de reproduktie van een individu en een nadelig neveneffect op latere leeftijd. De genen met dergelijke mutaties worden **pleiotrope** of 'late-acting deleterious' genen genoemd.
3. Veroudering kan worden veroorzaakt doordat tijdens de evolutie steeds een bepaalde balans bestaat tussen de hoeveelheid energie die geïnvesteerd wordt in reproduktie en in het onderhoud van het organisme (het somatisch weefsel). Soorten met een hoog risico om dood te gaan bereiken op jonge leeftijd de reproduktieve fase, hebben een hoge reproduktie-snelheid, een relatief slecht lichaamsonderhoud en een korte levensduur (zelfs in afwezigheid van risico bepalende factoren). De genen die de lengte van het leven bepalen volgens deze '**disposable soma**' theorie hebben een functie bij het somatisch onderhoud. De snelheid van veroudering is dan in hoge mate genetisch bepaald door de kwaliteit van de netwerken waarin deze genen opereren (die nooit 100% is). Veroudering bij deze hypothese wordt veroorzaakt door een tijdsafhankelijke ophoping van schade aan biomacromoleculen (lipiden, eiwitten en nucleinezuren) in somatisch weefsel.

Aangezien de meeste macromoleculen zowel determinant als lijdend voorwerp van de voorspelde veranderingen zijn (als er bijvoorbeeld mutaties in DNA-herstel-genen optreden) is het mogelijk dat een sneeuwbal-effect optreedt ('error-catastrophe') waarvan de snelheid bepalend is voor de levensduur van de soort. In het experimentele gedeelte van dit proefschrift ligt de nadruk op de vraag of de voorspelde ophoping van somatische defecten detekteerbaar is op RNA en DNA nivo. Voor het bepalen van genomische instabiliteit tijdens veroudering werden cross-sectionele studies verricht in genetisch homogeen materiaal: weefsels en celcultures van ingeteelde ratten en witte bloedcellen van humane tweelingen van verschillende leeftijden.

Er zijn bij hogere diersoorten geen aanwijzingen gevonden dat het verouderingsproces wordt ingeleid door de geprogrammeerde expressie van één of meerdere specifieke genproducten in meerdere weefsels. De meest universele verandering bleek een afname van totale eiwit en RNA synthese, een verminderde afbraak van afwijkende eiwitten en een toename van post-translationeel gemodificeerde eiwitten (glycosylering, 'cross-linking' en oxydatie). De hoeveelheid van individuele mRNA's en eiwitten neemt in sommige gevallen toe, in andere gevallen af (Hoofdstukken 1 en 3). Het mRNA nivo van de 'housekeeping'-genen ß-actine en glyceraldehyde-fosfaat-dehydrogenase (GAPDH) in de rattemilt neemt toe met veroudering (Hoofdstuk 4), terwijl dat van het lever-specifieke tyrosine aminotransferase (TAT) gen afneemt (Hoofdstuk 5). Naast veranderingen in constitutionele mRNA nivo's is voor de expressie van veel genen de induceerbaarheid en de repressie vertraagd en/of verminderd met veroudering. Deze intrinsieke veranderingen, die de basis voor een verminderde homeostase capaciteit zouden kunnen vormen, worden veelal toegeschreven aan verlaagde nivo's van regulerende moleculen zoals hormonen, groei- en transcriptie-factoren en hun receptoren. Anderzijds zijn deze veranderingen mogelijk het gevolg van derepressie van ontwikkelingsprogramma's (dedifferentiatie). Het verlies van DNA methylering op willekeurige plaatsen in het genoom met veroudering zou hieraan kunnen bijdragen. Zo gaat de toename van de hoeveelheid ß-actine mRNA gepaard met demethylering op een specieke CpG plaats in het gen (Hoofdstuk 4). Om ondersteuning te verkrijgen omtrent het belang van random demethylering voor veroudering, is additioneel onderzoek naar de relatie tussen veranderde gen expressie en functionele CpG plaatsen noodzakelijk.

In het nucleaire en mitochondriale genoom werd een toename van het aantal DNA-beschadigingen en mutaties met veroudering gevonden. De somatische mutatie frequentie in het tot nu toe onderzochte coderend DNA was te laag om een basis voor veroudering te kunnen vormen. Het is inmiddels echter bekend dat er een grote variatie bestaat in de mutatiefrequentie van verschillende loci (zowel in somatische als geslachtscellen). Minisatelliet en 'simple sequence' loci leken bij aanvang van het eigen onderzoek een geschikt type DNA sequenties waarin at random de frequentie van somatische mutaties zou kunnen worden bepaald. Deze repetitieve structuren zijn hypervariabel en liggen verspreid over het genoom.

Met behulp van Southern-hybridisatie-analyse en twee dimensionale (2D) DNA typering werd een groot aantal repetitieve loci in het genoom van rat en mens onderzocht. De 2D-DNA typering werd ontwikkeld om laagmolekulaire fragmenten, die bij de Southern-

hybridisatie-analyse niet kunnen worden onderzocht, te kunnen scheiden op basis van zowel fragmentgrootte als basecompositie (Hoofdstuk 6). In fibroblastenklonen verkregen uit 'single'-cel-klonering vanuit huidbiopten van jonge en oude ratten werd een hoge frequentie van spot-polymorfismen gevonden; deze was echter gelijk in klonen afkomstig van jonge en oude dieren. (Hoofdstuk 7). De gevonden spot-polymorfismen suggereren dat DNA-veranderingen veelvuldig ontstaan maar zich niet ophopen met veroudering. Anderzijds kunnen deze DNA-veranderingen in kweek zijn ontstaan. Daarom werden analoog aan deze studie homologe repetitieve loci onderzocht in DNA uit bloed van oude humane monozygote tweelingen (Hoofdstuk 2). Er werden slechts 3 polymorfismen gevonden. Vooralsnog geven de data geen enkele ondersteuning dat somatische mutaties accumuleren tot een hoeveelheid waardoor veroudering kan worden verklaard. Het grootste probleem bij deze studies is echter dat een toename van geheel random optredende veranderingen met de huidige methoden niet kan worden gemeten, zelfs niet als deze exponentieel is. Het optreden van mutaties zal echter niet volledig willekeurig gebeuren. Voor verder onderzoek naar de rol van somatische mutaties bij veroudering zouden mutatie 'hot spots' in genen kunnen worden onderzocht in cellen met een sleutelfunctie in het lichaam, zoals het onderzoek naar mitochondriale mutaties in verschillende gebieden van de hersenen.

Naast het verlies van ontwikkelingsgereguleerde genexpressie-patronen en het stochastisch optreden van somatische mutaties lijkt aan de telomeren bij de mens een min of meer geprogrammeerde genetische instabiliteit te ontstaan. Aan het uiteinde van ieder chromosoom ligt bij de mens en veel diersoorten een groot aantal repetitieve DNA-sequenties kop aan staart georiënteerd. Bij elke replicatie ronde verliest één van de DNA strengen een klein DNA fragment (31-90 bp). Het verlies van DNA aan de telomeren speelt mogelijk een rol bij het uittreden van cellen uit de delingscyclus en het intreden van cellulaire veroudering. De telomeren in delend weefsel van oude individuen zijn korter dan bij jonge personen. De telomeerlengte van jonge individuen vertoont echter aanzienlijke spreiding. Uit telomerenonderzoek in het bloed van humane mono- en dizygote tweelingen van diverse leeftijdsgroepen bleek dat telomeerlengte in hoge mate genetisch bepaald is (Hoofdstuk 8). Indien cellen *in vivo* zo vaak delen dat de kritische telomeerlengte wordt overschreden waarop de integriteit van het chromosoom-uiteinde en aangrenzende gensystemen verloren gaat, zou reductie van telomeren bij kunnen dragen aan veroudering. Telomeerlengte zou dan een genetisch bepaalde marker van veroudering kunnen zijn.

Bij mens en dier treden verouderingsgerelateerde veranderingen op, lang voordat er sprake is van ziekte. Ondanks het feit dat de gemiddelde maximale levensduur in geïndustrialiseerde landen toeneemt, lijkt het niet mogelijk door het wegnemen van momenteel veel voorkomende verouderingsziekten de maximale levensduur van de mens als soort te verlengen. De ene doodsoorzaak wordt daarbij slechts vervangen door een andere doodsoorzaak waarbij de levensduur nauwelijks wordt verlengd. Levensduur verlenging zou mogelijk kunnen zijn indien netwerken van somatische onderhoudsfuncties kunnen worden verbeterd. Zinniger lijkt het echter om subpopulaties met een verhoogd risico op verouderingsziekten te herkennen en daarin het vervroegd intreden van die ziekten te vertragen.

ABBREVIATIONS

A	adenine
APRT	adenosine phosphoribosyl transferase
ATP	adenosine triphosphate
bp	basepair
C	cytosine
cM	centimorgan
CR	caloric restriction
2-D	two-dimensional
DMD	Duchenne muscular dystrophy
DNA	desoxy nucleic acid
EF	elongation factor
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPA	glycophorin A
HLA	human lymphocyte antigen
HPLC	high performance liquid chromatography
HPRT	hypoxanthine phosphoribosyl transferase
IgG	Immunoglobulin G
IMR	initial mortality rate
kb	kilobasepairs
LDL	low density lipoprotein
LOH	loss of heterozygosity
5-mC	5-methylcytosine
MRDT	mortality rate doubling time
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
OCT	ornithine carbamoyl transferase
8OH-dG	8-hydroxy-2'-deoxyguanosine
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PLA	phospholipase
RI	recombinant inbred
RNA	ribonucleic acid
SCA	senescent cell antigen
SCE	sister chromatid exchange
SOD	superoxide dismutase
T	thymine
TAT	tyrosine aminotransferase
TRF	telomeric restriction fragment

LIST OF PUBLICATIONS

Lohman, P.H.M., J. Vijg, A.G. Uitterlinden, P. Slagboom and F. Berends. DNA methods for dectecting and analysis mutations *in vivo*. In: Notani, N.K. and Chauhan, P.S. (eds.): Environmental mutagenesis and carcinogenesis. Bombay, Bhabha Atomic Research Centre (1986) 51-60.

Devilee, P., P. Slagboom, C.J. Cornelisse and P.L. Pearson. Sequence heterogeneity within the human alphoid repetitive DNA family. Nucleic Acids Res. 14 (1986) 2059-2073.

Devilee, P., T. Cremer, P. Slagboom, E. Bakker, H.P. Scholl, H.D. Hager, A.F.G. Stevenson, C.J. Cornelisse and P.L. Pearson. Two subsets of human alphoid repetitive DNA show distinct preferential localization in the pericentric regions of chromosomes 13, 18 and 21. Cytogen. Cell Genet. 41 (1986) 193-202.

Slagboom, P., A.G. Uitterlinden and J. Vijg. Screening for age-related changes in gene expression in the rat liver. In: C.F.A. van Bezooijen, F. Miglio en D.L. Knook (eds.). Topics in Aging Research in Europe; Liver, Drugs and Aging, EURAGE, Rijswijk, 7 (1987) 127-133.

Lohman, P.H.M., J. Vijg, A.G. Uitterlinden, P. Slagboom, J.A. Gossen and F. Berends. DNA methods for detecting and analyzing mutations *in vivo*. Mutation Res., 181 (1987) 227-234.

Slagboom, P. and J. Vijg. Genetic instability and aging: theories, facts, and future perspectivs. Genome 31 (1989) 373-385.

Slagboom, P.E. and R.J. Shmookler Reis. Genetics of aging: longevity- and senescence-determining genes. A symposium report on the XVIth International Congress of Genetics, Toronto, August 1988, Mutation Res. 219 (1989) 135-137.

Uitterlinden, A.G., E. Slagboom, T.E. Johnson and J. Vijg. The caenorhabditis elegans genome contains monomorphic minisatellites and simple sequences. Nucleic Acids Res. 17 (1989) 9527-9530.

Uitterlinden, A.G., P.E. Slagboom, D.L. Knook and J. Vijg. Two-dimensional DNA fingerprinting of human individuals. Proc. Natl. Acad. Sci. USA 86 (1989) 2742-2746.

De Leeuw, W.J.F., P. E. Slagboom and J. Vijg. Quantitative comparison of mRNA levels in mammalian tissues: 28S ribosomal RNA level as an accurate internal control. Nucleic Acids Res. 17 (1989) 10137-10138.

Slagboom, P.E. The aging genome: determinant or target? Mutation Res. 237 (1990)183-187.

Slagboom, P.E., W.J.F. de Leeuw and J. Vijg. Messenger RNA levels and methylation patterns of GAPDH and ß-actin genes in rat liver, spleen and brain in relation to aging. Mech. Ageing Dev. 53 (1990) 243-257.

Slagboom, P.E., W.J.F. de Leeuw and J. Vijg. mRNA levels and methylation patterns of the tyrosine aminotransferase gene in aging inbred rats. FEBS lett. 269 (1990) 128-130.

Slagboom, P.E., E. Mullaart, A.G. Uitterlinden and J. Vijg. Two-dimensional DNA typing as a tool for detecting somatic instability in aging rats. In: From gene to man. C.F.A. van Bezooijen, R. Ravid and A.A.J. Verhofstad (eds.) Stichting Gerontologie en Geriatrie, Rijswijk (1990) 345-348.

Vijg, J., J.A. Gossen, P. Slagboom and A.G. Uitterlinden. New methods for the detection of DNA sequence variation: applications in molecular genetic studies on aging. In: Molecular biology of aging. Proceedings of a UCLA Colloquium, Santa Fe, New Mexico, March 4-10, 1989. C.E. Finch and T.E. Johnson (eds.) Wiley-Liss, New York (1990) 103-119.

Vijg, J., J.A. Gossen, W.J.F. de Leeuw, E. Mullaart, P. Slagboom and A.G. Uitterlinden. New methods for detecting DNA sequence variation in relation to aging. In: Molecular mechanisms of aging. K. Beyreuther and G. Schettler (eds.) Springer-Verlag (1990) 77-90.

Vijg, J., J.A. Gossen, W.J.F. de Leeuw, E. Mullaart, P.E. Slagboom, A.G. Uitterlinden DNA processing, Aging and Cancer: The impact of new technology. Annals NY Acad. Sci. 621 (1991) 53-66.

Slagboom, P.E., A.G. Uitterlinden and J. Vijg. Methylation status of cKi-ras and MHC genes in rat pituitary glands during aging and tumorigenesis. Aging 18 (1991) 1-6.

Slagboom, P.E., E. Mullaart, S. Droog and J. Vijg. Somatic mutations and cellular aging: Two-dimensional DNA typing of rat fibroblast clones. Mutation Res. 256 (1991) 311-321.

Uitterlinden, A.G., P.E. Slagboom, E. Mullaart, I. Meulenbelt and J. Vijg. Genome scanning by two-dimensional DNA typing: The use of repetitive DNA sequences for rapid mapping of genetic traits. Electrophoresis 12 (1991) 119-134.

Slagboom, P.E. and J. Vijg. Dynamics of genome organization and expression during the aging process. New York Acad. Sci. 673 (1992) 673: 58-69.

Zurcher, C., and P.E. Slagboom. Basic Aspects of aging. In: Jones TC, Mohr U, Hunt, RD (eds.). Pathology of aging animals. ILSI Monographs on Pathology of Laboratory Animals. Springer Verlag Berlin Heidelberg New York. (1993) In press.

Slagboom, P.E. and Zurcher C. Molecular Mechanisms of aging. J. of Zoology (1993) In press.

Te Koppele, J.M., B. Beekman, I. Meulenbelt, R.A. Bank en P.E. Slagboom. Artrose en veroudering van gewrichtskraakbeen. In: D.L. Knook and W.J.A. Goedhard (eds.) In beweging blijven .... artrose bij ouderen. Bohn Stafleu Van Loghum, Houten (1993) in press.

Meulenbelt, I., C.J. Williams, G.C. vd Giessen, J.M. te Koppele and P.E. Slagboom. Linkage disequilibrium analysis of restriction fragment length polymorphisms (RFLPs) of the procollagen type II gene (COL2A1) in the Dutch popultaion. (1993) submitted to Hum. Genet.

van der Hofstede, J.W., S. Haring, P.E. Slagboom. ACES: Automatic Comparison and Evaluation of spots images. Medical Information Conference. Communication and integration of informative design to medical practice (1993).

Slagboom, P.E., S. Droog and D. Boomsma. Genetic determination of telomere size: a twin study. Submitted to Am. J. Hum. Genet. (1993).

Slagboom, P.E. and J.M. te Koppele. Collagen genes and skeletal disorders. Lancet 390 (1993) in press.

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