

GENE LINKAGE IN MAN AND CHINESE HAMSTER STUDIED IN SOMATIC CELL HYBRIDS

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Aan mijn ouders
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INTRODUCTION

Genetic studies of higher organisms, including man, are based on the analysis of segregation and recombination events resulting from sexual reproduction. In 1962 Pontecorvo predicted, however, that cultured cells could also be employed for this purpose. He suggested that parasexual events, detected in certain fungi, might occur also in "in vitro" cultured cells. Now, ten years later, there are strong indications that this prediction will indeed come true.

Firstly Barski et al. (1960) discovered that cells from two mouse tumour lines could be fused to form one cell. The fusion was not restricted to cells from the same species, since also cells of different origin could form hybrids. An important improvement of the fusion technique was developed by Harris and Watkins (1965) who, building on Okada's data (1962), and using inactivated Sendai virus were able to increase the fusion frequency.

The second step forward in this field was the development by Littlefield (1964) of a selective system which made possible the selection of pure hybrid lines. The two parental cell lines used in the fusion were characterized by different mutations which rendered both lines sensitive to the selective medium. Hybrid cells, however, were able to grow in the selective conditions. During the last ten years an increasing number of these mutant lines became available. Many of them have been isolated from established cell lines, e.g. of mouse, hamster and human origin. Moreover there have been found also a few mutations, leading to genetic disorders in man, which appeared to be expressed in cultured cells and were suitable for fusion experiments.

The observation by Weiss and Green (1967) that human chromosomes are lost in man-mouse hybrids was the third important discovery in the field of somatic cell genetics. Chromosome loss, in fact, provides a mechanism by which segregation of genes can occur in a way comparable to the segregation occurring during meiosis. This observation showed that, in principle, cultured cells are suitable for gene localization, providing that identification of the chromosomes is possible.

Recently a fourth landmark was reached when it became possible to stain human chromosomes in such a way that characteristic staining patterns for each human chromosome were obtained (Caspersson et al., 1971^a; Summer et al., 1971; Rowley and Bodmer, 1971). At present, it is also possible to distinguish human chromosomes in a human-mouse hybrid cell (Caspersson et al., 1971^b).

Through all these developments human genetics has entered a new era in which previously limiting factors, as for instance a long generation time, are no longer an obstacle in the genetic analysis of man.

The two methods of studying human genetics, namely the classical pedigree analysis and the hybridization of somatic cells in culture can very well complement each other as has been proven by the studies on X-linked genes. Both methods have lead to the conclusion that the genes coding for glucose-6-phosphate dehydrogenase, hypoxanthine-guanine phosphoribosyl transferase and 3-phosphoglycerate kinase are located on the X-chromosome (Nabholz et al., 1969; Meera Khan et al., 1971).

The first autosomal linkage of human genes detected by cell fusion was described by Santachiara et al. (1970) and Ruddle et al. (1970^b) in relation to the genes coding for lactic dehydrogenase B and peptidase B. A second autosomal linkage involving the genes coding for 6-phosphogluconate dehydrogenase and phosphoglucomutase 1 is described in chapter 3 of this thesis.

A considerable limitation in the application of these methods in human genetics is that one can study only those human genes whose gene product in the hybrid cell can be distinguished from the homologous gene product of the other parent. This means that localization of characters determined by recessive alleles (many inborn errors in man) can not be accomplished by means of interspecific cell fusions.

An alternative way to carry out these studies could be the use of intra-specific hybrids, provided that chromosomal loss occurs and that changes in gene products can be measured quantitatively or qualitatively. The first indications that such an approach might be possible is described in the chapters 4 and 5 of this thesis.

The purposes of the studies presented in this thesis were the following:

- 1) the isolation of mutant cell lines from Chinese hamster cells to be used in cell fusion experiments with the Littlefield selective system (chapter 2);
- 2) the development of a second interspecific cell fusion system, analogous to the man-mouse one, in which linkage of human genes can be studied (chapter 3);
- 3) the establishment of a method to study linkage in intraspecific hybrids (chapter 4 and 5);
- 4) the study of the fusion process in intraspecific combinations by analysis of the proliferation kinetics of multinucleate cells (chapter 6).

The first chapter gives an introduction to the experimental part of the thesis. The relation between the literature concerning cell fusion and the data described in this thesis is also discussed.

(The references can be found at the end of Chapter 1.)

chapter 1

GENERAL INTRODUCTION TO THE EXPERIMENTS

Experimental work in the field of somatic cell genetics, study of gene linkage, localization and complementation of genes in animal cells requires well defined genetic markers, recognizable at the level of the cultured cell, and a system in which gene interaction and gene segregation can be studied.

In recent years the prerequisites for these studies have been fulfilled: several procedures have been developed to isolate mutant cell lines of different types. The discovery that cultivated cells can be fused producing proliferating hybrid cells has provided the tool for the study of gene interaction and segregation.

MUTANT CELL LINES

Mutant cell lines have been obtained following two different approaches. Firstly, genetically well defined mutant lines have been derived from skin biopsies of patients suffering from an inborn error of metabolism. Only those genetic diseases in which the mutation is expressed at the level of the cultured cell can be used for this purpose. The disappearance of specific functions, often observed in cells that are brought in tissue culture is, however, a limitation to this approach.

Secondly mutant lines have been isolated from diploid and heteroploid cell lines. The genetic basis of these mutant lines is in many cases unknown. The phenotypic alteration might be either the result of a change in the DNA code, or a permanent change in the differentiated state of a cell in tissue culture. However, the induction of these mutants by mutagenic agents (Chu et al., 1969; 1971), the demonstration of quantitative changes in the activity of the enzyme involved in the mutation (e.g. Kit et al., 1963) and their spontaneous occurrence in the absence of selective pressure (Atkins and Gartler, 1968)

make the hypothesis of a gene mutation very likely in most of the cases. In this thesis the term "mutation" will be used to indicate stable changes in the cellular phenotype, irrespective of the molecular mechanism involved at the genotype level.

Mutant cell strains derived from patients suffering from a genetic disease

The first cell strain carrying an inborn error was established by Krooth and Weinberg in 1961 from a skin biopsy of a galactosemia patient. Since then, an increasing number of genetic disorders have been identified in cultured cells of patients including errors in amino acid, carbohydrate and nucleic acid metabolism. These studies have been recently reviewed by Hsia (1970).

At present the recognition of genetic defects in cultured cells finds application in antenatal diagnosis, heterozygote detection and biochemical studies of the molecular basis of the disease.

Only a few of the available mutant cell strains can as yet be used in linkage studies of the type presented in this thesis. Especially when the mutation provides a selective system against the mutant cell, the inborn error strain can be used as parental cell in cell fusion experiments. Cells derived from patients suffering from the Lesch-Nyhan syndrome (Lesch and Nyhan, 1964) are very suitable in this respect. These cells are defective in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) (Seegmiller et al., 1967) as a result of a mutation on the human X-chromosome. A selective system which makes use of the presence or absence of that enzyme has been developed by Littlefield (1964).

As presented in chapter 3 a Lesch-Nyhan cell strain has been used in our human-Chinese hamster hybridization experiments.

Mutant cell lines isolated from established cell lines

Cell lines resistant to the thymidine analogue, 5-bromodeoxyuridine (5BUdR), the guanine analogue, 8-azaguanine (azg), the adenine analogue, 8-azaadenine (aza), or to several other antimetabolites have been isolated from cell cultures of different origin (man, mouse, hamster etc.). The resistance is maintained in the absence of the analogue. Several of these mutant cell lines are characterized by changes in the activity of a specific enzyme.

5-bromodeoxyuridine resistance

The first cell line lacking completely the enzyme thymidine kinase (TK) was isolated by Kit et al. (1963). These authors isolated a TK deficient mouse fibroblast line (LM TK⁻) by subculturing normal (wild type) LM cells in the

presence of 25 μg per ml of 5-BUdR. Littlefield (1965) isolated sublines of mouse fibroblasts at concentrations of 3-5 μg of 5-BUdR per ml which were partially resistant and had decreased levels of TK activities.

He suggested that the development of resistance to 5-BUdR was a two step mutation involving a progressive loss of TK activity. 5-BUdR resistance, however, is not always caused by a loss of the thymidine kinase activity. In 1969 Breslow and Goldsby described mutants of Chinese hamster cells in which the loss of the ability, to incorporate exogenous thymidine could be related to a membrane defect rather than to a loss of TK activity.

As described in chapter 2 we have isolated 5-BUdR resistant Chinese hamster cell lines in the presence of 100 μg 5-BUdR per ml. These mutants were found to be defective in TK activity.

8-azaguanine resistance

Szybalski and Smith (1959) were the first to isolate 8-azaguanine (azg) resistant cell lines from a human heteroploid cell line. Several discrete levels of resistance to azg have been described. Littlefield (1963) has studied two levels of resistance in murine lines of different origin and observed that cells resistant to 0,1 μg azg per ml had levels of HGPRT approximately 1/3 of that of the wild type cells, while cells resistant to 1,0 μg per ml had no detectable enzyme activity.

The Chinese hamster azg resistant cell lines described in chapter 2 have been isolated in two steps. These cells incorporate ^3H -hypoxanthine at less than 2% of the efficiency of wild type Chinese hamster cells.

8-azaadenine resistance

The third group of resistance markers which are suitable for hybridization experiments is the resistance to the adenine analogues 8-azaadenine and 2,6-diaminopurine. The biochemical basis of the resistance to these analogues is a loss of adenine phosphoribosyl transferase (APRT) activity (Lieberman and Ove, 1960; Blair and Hall, 1965). Using ^3H -adenine incorporation followed by autoradiography, Atkins and Gartler (1968) showed that APRT deficient mutants can arise spontaneously in cell cultures in the absence of the drug.

CELL HYBRIDIZATION

The process of cell fusion

Somatic cell fusion was first observed by Barski et al. (1960). Following cocultivation of two phenotypically and karyologically different mouse cell lines

they found hybrid cells showing characteristics of both parents. Other mouse-mouse (Yoshida and Ephrussi, 1967) as well as Syrian hamster-Syrian hamster hybrids (Littlefield, 1969) were also isolated by cocultivation of the parental cells. However, this technique is not very efficient since the occurrence of spontaneous fusion is extremely rare and sometimes absent (Yerganian and Nell, 1966).

Okada et al. (1962) improved the cell fusion procedure by using Sendai virus (a member of the parainfluenza group of myxoviruses) to fuse Ehrlich tumour cells. The virus is inactivated by irradiation with U.V. light but retains its ability to fuse cells. Under appropriate conditions the virus attaches to the cell membrane as can be seen with electron micrographs (Schneeberger and Harris, 1966), and the treated cells clump together. Following incubation at 37°C intercellular cytoplasmic bridges are formed; finally the cytoplasms of neighbouring cells coalesce and complete cellular fusion occurs. Harris and Watkins (1965) and Harris et al. (1966) applied this technique to show that both differentiated and undifferentiated cells from different species could be fused together to form viable multinucleate cells.

Characteristics of multinucleate cells

Two important aspects can be studied in multinucleate cells: 1) the interaction between nuclei of different origin and with different degree of specialization within the same cytoplasm, and 2) the interaction between nuclei in relation to the progression through the cell cycle (G1, S and G2) and to mitosis.

The interaction of nuclei has been extensively studied in multinucleate cells after fusion of proliferating cells (HeLa) with the highly differentiated chicken erythrocyte. In these multinucleate cells the inactive erythrocyte nucleus is stimulated by the HeLa nucleus to initiate RNA and DNA synthesis. The data obtained with this system (reviewed by Harris (1970)) provide new insights in the problems of nuclear differentiation and gene (in)activation.

More relevant to the work presented in this thesis is the problem of interaction of nuclei with respect to progression through the cell cycle (chapter 6). In recent years evidence has been accumulating that intraspecific fusions leading to homokaryons behave differently from interspecific fusions resulting in heterokaryons. Yamanaka and Okada (1966) and Johnson and Harris (1969^a) have shown that in the nuclei of a homokaryon of Ehrlich or HeLa cells DNA synthesis is synchronized in the first cell cycle after fusion.

An excellent analysis of this synchronization phenomenon in homokaryons was presented by Rao and Johnson in 1970. They fused HeLa cells in different

phases of the cell cycle and observed that DNA synthesis was induced in the G1 nucleus of a homokaryon containing a G1 and a S phase nucleus. On the other hand, DNA synthesis was not induced in the G2 nucleus of a G2+S homokaryon. In a homokaryon with a G1 and G2 phase nucleus, division of the G2 nucleus was delayed until the other one had reached mitosis; the two nuclei then divided synchronously. When mitotic HeLa cells are fused with interphase HeLa cells chromosome pulverization or condensation can be seen in the interphase nucleus (Johnson and Rao, 1970).

In heterokaryons formed by the fusion of HeLa and Ehrlich cells, Harris and Watkins (1965) observed that the nuclei do not synthesize DNA synchronously. Johnson and Harris (1969^b) actually observed in similar heterokaryons a form of antisynchrony, since most of the Ehrlich nuclei synthesized DNA whilst most of the HeLa nuclei did not.

In most of these studies the multinucleate cells have been followed during the first cell cycle after fusion. In chapter 6 of this thesis experiments are described in which multinucleate cells have been studied at different time intervals after cell fusion, to obtain more information on their ultimate fate in the hybridized cell population.

The process of nuclear fusion

The next step after cell fusion in the formation of a mononucleate hybrid cell should be the fusion of the nuclei in the multinucleate cell. Two possible mechanisms have been discussed: 1) fusion of the nuclei during interphase and 2) formation of a single spindle during mitosis resulting in the distribution of the chromosomes from the different nuclei into two mononuclear daughter cells (Harris, 1970). This latter mechanism requires a synchronous mitosis of the nuclei involved. In homokaryons, because of nuclear synchrony, this might be a frequent phenomenon. However, in the case of heterokaryons the nuclear asynchrony observed makes this fusion mechanism rather unlikely. Although this problem has not yet been solved, one can argue that also in the case of heterokaryons a small fraction of nuclei might enter cell division simultaneously by chance.

Isolation of hybrid cell lines

In cell fusion experiments, with or without Sendai virus, only a relatively small fraction of the population will consist of hybrid mononucleate cells. To isolate pure hybrid cell lines two requirements should be fulfilled:

- 1) the hybrid cell should be able to proliferate;
- 2) selection against the parental cells should be accomplished.

Since the first experiments of Barski (1960) several proliferating hybrid lines have been isolated in both intra- and interspecific hybridizations. Thus the presence of two genomes of very different origin within one cell is compatible with life. Furthermore, it has been proven that genes of both parental chromosome sets are functional in the hybrid cell. This is shown by the presence of isozymes (Weiss and Ephrussi, 1966^b), and antigenic markers (Weiss and Green, 1967), of both parental origin. As a consequence a hybrid cell obtained by fusion of two different mutant cells will be phenotypically wild type if the mutations are located in different genes (intergenomic complementation). Based on this complementation phenomenon Littlefield (1964) worked out a selective system for the isolation of hybrid cells. He fused HGPRT⁻ with TK⁻ mouse cells by simple cocultivation. The parental cells are not able to grow in medium supplemented with hypoxanthine, aminopterin and thymidine (HAT). Aminopterin, a folic acid antagonist, blocks the "de novo" synthesis of purines and pyrimidines and the mutant cells cannot bypass this block because they cannot incorporate the hypoxanthine or thymidine present in the medium. Hybrid cells obtained after fusion of TK⁻, HGPRT⁺ with TK⁺, HGPRT⁻ cells will be phenotypically wild type as a result of intergenomic complementation and will grow in HAT medium, producing clones that can be isolated and sub-cultured.

Modifications of this selective system have been developed; the most important one is the half selective system applied in the hybridization of a mutant cell line with lymphocytes from peripheral blood. The lymphocytes do not attach to the bottom of the culture dish and mostly do not proliferate, whereas the other parent is lost in HAT medium (Nabholz et al., 1969). Both selective systems have been applied in the hybridization experiments described in the chapters 3, 4 and 5.

Gene expression in hybrid cells

The first examples of expression of both parental genomes were obtained from mouse-mouse hybrid cells. In other types of intraspecific hybrids, for instance Syrian hamster (Marin, 1969; Littlefield, 1969) and Chinese hamster (Kao et al., 1969; this thesis chapter 4 and 5) the genes of both parental cells are also expressed.

Regulation of gene expression, as seen by the inactivation of one X-chromosome in female cells, has not been found in hybrid cells after fusion. Silagi et al. (1969) described the expression of two X- linked allelic genes in a human-human hybrid cell line. They fused diploid fibroblasts, obtained from a patient

having orotic aciduria, with heteroploid D98/AH-2 cells. The diploid cells were lacking the enzyme orotidine -5' -phosphate decarboxylase and orotidine -5' -phosphate pyrophosphorylase which play a role in the synthesis of uridylic acid (Krooth, 1964). The heteroploid partner was HGPRT deficient (Szybalski and Szybalska, 1962). Moreover, the glucose-6-phosphate dehydrogenase (G6PD) in the heteroploid cell line was of type B, whereas, the diploid cell strain had a G6PD of type A. The hybrid clone exhibited HGPRT activity as well as activity of both enzymes of the uridylic pathway as a result of intergenomic complementation.

Both types of G6PD (A and B) were also present in these cells. This finding demonstrated that the X-chromosome of both parental cells is active in the hybrid cell.

The first diploid-diploid human hybrid was isolated by Siniscalco et al. (1969). After fusion of HGPRT deficient male fibroblasts with G6PD deficient male fibroblasts, hybrid cells were obtained in the presence of azaserine, which kills specifically the HGPRT deficient cells. The hybrid cells were synthesizing HGPRT as well as G6PD indicating that the normal G6PD locus on one X and the normal HGPRT on the other X functioned simultaneously in the hybrid cells.

The examples of complementation described so far deal with interaction of different genes located on the same or on different chromosomes (intergenomic complementation). Complementation within two alleles of the type found in microorganisms (interallelic complementation) is more difficult to detect in mammalian cells, but recently Nadler et al. (1970) presented the first evidence of this phenomenon. These investigators fused diploid fibroblasts originating from different patients suffering from galactosemia. In some combinations galactose-1-phosphate uridyl transferase activity was found in the hybrid cells, whereas this enzyme was lacking in the parental cell strains. These results indicate that human-human somatic cell hybrids might provide a new tool in the genetic classification of inborn errors of metabolism.

In the first interspecific cell lines (Weiss and Ephrussi, 1969^b), it was already found that both parental genomes were functional.

These cells contain many genetic markers, because frequently the homologous enzymes from the two parents differ in electrophoretic mobility. Weiss and Ephrussi made use of this difference by studying β -glucuronidase from rat and β -glucuronidase from mouse in rat-mouse hybrids. The enzymes from both parents were found to be present in these cells. Similar results were obtained in other interspecific combinations e.g. hamster-mouse (Scaletta et al., 1967)

The first interspecific hybridization involving human cells was carried out by

Weiss and Green (1967) who fused a TK deficient mouse line with a diploid human fibroblast strain. Human thymidine kinase as well as human and mouse surface antigens were present in these hybrid cells. We isolated human-Chinese hamster cell hybrids and found that they contained isozymes and species specific surface antigens of both parental cell lines (chapter 3).

Chromosome loss in hybrid cells

In many hybrid cell lines a gradual loss of chromosomes occurs during cultivation. In intraspecific cell hybrids this has a slow rate; for instance Littlefield (1966) has shown that in mouse-mouse hybrids about 9% of the chromosomes disappeared over a period of one year. Evidence for either preferential loss of telocentric chromosomes or formation of metacentric chromosomes by fusion of telocentric ones was presented by Yoshida and Ephrussi (1967) as well as Ruddle et al. (1970^a), who observed a preferential reduction of telocentric chromosomes in mouse-mouse cell hybrids.

In interspecific hybrid cells a specific loss of one of the parental genomes has been observed. Weiss and Ephrussi (1966^b) described a preferential loss of rat marker chromosomes in rat-mouse hybrid cells. A decrease in the number of mouse chromosomes in mouse-Chinese hamster cells was reported by Scaletta et al. (1967). The man-mouse hybrids are the most studied among the interspecific hybrids with respect to chromosome loss.

The man-mouse hybrid population, isolated by Weiss and Green (1967), showed a preferential reduction of human chromosomes; in some cases more than 95% of the human chromosome complement was lost. The loss of human chromosomes is apparently not always at random: Nabholz et al. (1969), in fact, observed that in mouse A9-human lymphocyte hybrids certain chromosome combinations were preferentially retained or lost.

Preferential loss of human chromosomes has also been found in the man-Chinese hamster hybrids described in this thesis (chapter 3).

At the present time the mechanisms of chromosome loss in hybrid cells are not well understood. The decrease in chromosome number in the intraspecific hybrid cells is most probably caused by mitotic non-disjunction (Ruddle, 1970^c). In interspecific hybrids more than one mechanism might be involved in the reduction of chromosomes. Evidence is presented that a rapid loss of chromosomes occurs within the first cell generations after fusion (Ruddle, 1970^c). This loss is sometimes followed by a gradual reduction in chromosome number comparable with that found in intraspecific hybrids.

There are several hypotheses which might explain the mechanism and the

specificity of the rapid chromosome loss in interspecific cell hybrids.

It has already been pointed out, that fusion of the nuclei in a multinucleate cell presumably occurs during mitosis (Harris, 1970), when the nuclei enter mitosis synchronously. However, the nuclei of a multinucleate cell are not always synchronized (Johnson and Harris, 1969^b). Johnson and Rao (1970) observed, after fusion of S phase cells with G2 phase cells, that the initiation of mitosis in the advanced nucleus resulted in condensation of the chromosomes in the lagging nucleus, which is a kind of atypical mitosis without the mitotic spindle. These authors suggested that this premature chromosome condensation is similar to the phenomenon firstly observed by Stubblefield (1964) in multinucleate Chinese hamster cells and later termed "chromosome pulverization" by other investigators (Takagi et al., 1969; Sandberg et al., 1970). It may be, that owing to this process only a small part of the pulverized nucleus is integrated in the chromosomal complement of the other nucleus, and that the rapid loss often observed in interspecific hybrids is caused by this condensation.

In this respect the work of Schwartz et al. (1971) is of great importance. They fused chick erythrocytes with mouse A9 (HGPRT⁻) cells and observed that the genome of the erythrocyte was pulverized in the heterokaryons when the A9 nucleus started to divide. They were then able to isolate hybrids with chick HGPRT, but without chick specific surface antigens. These results indicate, that a small part of the chick genome, bearing the HGPRT locus, was incorporated in the mouse cells.

Man-mouse hybrids loose exclusively human chromosomes (Weiss and Green, 1967). A preferential loss of one of the parental genomes has also been found in other interspecific combinations. This specificity might be caused by differences in the cell cycle of the partners concerned, assuming that no synchronization of the nuclei occurs in the multinucleate cells. The nucleus with the shortest cycle has the best chance to enter mitosis first, while the interphase nucleus will be pulverized giving origin to chromosomal configurations which might be taken up by the genome of the dividing nucleus.

It seems likely, that the selective system used in the isolation of the hybrid cells, plays a role in determining the specificity of the chromosome loss.

For instance, this might be the case after hybridization of a cell line which can only grow in suspension culture with a line growing in monolayer. By selecting hybrids that grow in monolayer, one isolates cells which have retained the characteristics of the monolayer parent and might have lost the genetic information originating from the other parental cell.

Recently Pontecorvo (1971) published two techniques which enabled him to predetermine before fusion which of the two sets of parental chromosomes

will be preferentially lost. One technique involves X- or γ -irradiation of the cells of one parent before fusion. In the other technique directional chromosome elimination can also be obtained by prelabeling the chromosomes of one parent with 5-BUDR followed by exposure to visible light after fusion. The 5-BUDR labeled chromosomes, being sensitive to visible light (Chu, 1965; Puck and Kao, 1967), will be lost in subsequent cell divisions.

The study of gene linkage in interspecific hybrid cells

The elimination of chromosomes in interspecific hybrids has provided an excellent tool for the study of genes in the species whose chromosomes are preferentially lost. The retention or loss of a given chromosome can be correlated with that of a specific genetic marker and the association of genetic markers can be established by their simultaneous retention or loss. The first approach has been followed by Migeon and Miller (1968) who obtained evidence for the localization of the human TK gene on a E group chromosome. They fused HGPRT deficient human cells with TK deficient mouse cells. In hybrid clones cultured in HAT medium a human E chromosome was present. By culturing the hybrid cell in 5-BUDR this chromosome could not be detected. The localization of the human TK on a E group chromosome, presumably E 17 was confirmed by Green (1969) and Ruddle (1970^c).

By using the second approach, simultaneous retention or loss of the human HGPRT and G6PD loci was observed (Nabholz et al., 1969). They fused HGPRT deficient mouse cells with normal human lymphocytes. Hybrids growing in HAT medium (requiring the presence of the human HGPRT⁺ locus) contained always the human G6PD isozyme. Human G6PD was lost after culturing the hybrids in the presence of azg, in which medium only the HGPRT⁻ cells are able to survive. These experiments have confirmed the linkage between the loci for G6PD and HGPRT, both being located on the X-chromosome in man.

About 20 different enzymes have now been introduced in these linkage studies using the species specific electrophoretic behaviour of these enzymes. The first autosomal linkage was detected by the simultaneous retention or loss of the genes coding for LDH B and peptidase B in man-mouse hybrids (Santachiara et al., 1970; Ruddle et al., 1970^b); the relative chromosomes have not yet been identified. Segregation was observed between the two genes involved in the production of lactic dehydrogenase (LDH A and LDH B) in man-mouse hybrids (Santachiara et al., 1970; Ruddle et al., 1970^b) and man-Chinese hamster hybrids (this thesis, chapter 3).

Evidence for a second autosomal linkage detected by cell hybridization is presented in our work on man-Chinese hamster hybrids. We found that the

loci coding for 6PGD and PGM1 were lost or retained together in the hybrid clones (Westerveld and Meera Khan, 1971; chapter 3 of this thesis). This finding has now been confirmed by family studies carried out by Renwick (1971).

By analysing the same man-Chinese hamster hybrids and several man-mouse hybrids we confirmed the X-linkage of the gene coding for 3-phosphoglycerate kinase (PGK), (Meera Khan et al., 1971). shown earlier by Valentine (1969) in family studies.

Techniques which might be extremely useful in the identification of the chromosomes involved in the autosomal linkage groups have recently been developed.

Caspersson (1971²) developed a technique for the characteristic staining of individual human chromosomes with quinacrine mustard.

An alternative to this fluorescent staining technique came from the work of Gall and Pardue (1970). They observed that after *in situ* DNA-DNA and DNA-RNA hybridization the hybridized chromosomes showed a darker colored area around the centromere when stained with Giemsa. Starting from this observation different groups (Summer et al., 1971; Rowley and Bodmer, 1971) worked out a technique for the identification of chromosomes based on differential Giemsa staining of denatured and renatured parts of the DNA.

The study of gene linkage in intraspecific hybrid cells

Generally, the loss of chromosomes in intraspecific hybrid cells is a slow process occurring in a non preferential and unpredictable way. Furthermore, the number of genetic markers in these hybrids is much less than in interspecific hybrids, although intraspecific enzyme polymorphisms are known to occur in mouse (Ruddle et al., 1969) and man (Harris, 1966). From this, it is clear that intraspecific somatic cell hybrids are less suitable for gene linkage studies in higher organisms, unless alternative procedures are available that make use of the characteristics of intraspecific hybrids.

In the chapters 4 and 5 of this thesis, experiments are described that were performed in order to study gene linkage in Chinese hamster-Chinese hamster hybrids. These studies were based on the existence of a relationship between the activity of an enzyme and the number of genes present in the cell and coding for that enzyme. By growing hybrid cells in media which select cells having lost particular genes (chromosomes), it was possible to obtain changes in gene dose and to establish a correlation with changes in the activities of particular enzymes. With this technique evidence has been obtained that the genes coding for HGPRT, G6PD and PGK are linked in Chinese hamster cells, as is the case in man.

Another approach to the study of gene linkage by means of intraspecific cell hybrids is presented in the work of Pontecorvo (1971), already mentioned in this chapter. The directional chromosome elimination accomplished by damaging the chromosomes of one parent provides a system in which gene segregation takes place. Pontecorvo pointed out that genetic defects known to be recessive (e.g. Xeroderma pigmentosum) can be used as markers in these experiments, since they will be expressed as soon as the dominant allele has been lost. In combination with techniques for chromosome identification or enzyme studies of the type presented in chapter 4 and 5 of this thesis, this technique of Pontecorvo might be of great value for the localization of mutations leading to inborn errors of metabolism in man.

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chapter 2

ISOLATION AND PROPERTIES OF CHINESE HAMSTER CELL LINES RESISTANT TO ANTIMETABOLITES

INTRODUCTION

In the past 15 years cell lines resistant to the guanine analogue 8-azaguanine (azg), the thymidine analogue 5-bromodeoxyuridine (5-BUDR), or the adenine analogue 8-azaadenine (aza), have been isolated (Szybalski and Smith, 1959; Kit et al., 1963; Atkins and Gartler, 1968; Breslow and Goldsby, 1969; and others).

In this chapter antimetabolite resistant cell lines, which have been isolated from the Chinese hamster DON cell line are described. The DON cells were chosen because they can be propagated in culture for long periods of time with only minor changes in chromosome number and structure (Ford and Yerganian, 1958). Unlike permanent mouse or human cell lines, they usually maintain a chromosome number at or near the diploid level.

MATERIALS AND METHODS

Cell lines and tissue culture conditions

The DON cells, a diploid Chinese hamster lung fibroblast line, were cultivated in F 12 medium (Ham, 1962), supplemented with 6% newborn calf serum and antibiotics (100 IU. of penicillin and 0,1 mg. of streptomycin per ml. medium). The special medium conditions required for growth of mutant cells are described in more detail in "RESULTS AND DISCUSSION". The cells were routinely cultivated in silicon rubber stoppered glass bottles. When Petri dishes were used the cultures were gassed with a humidified 95% air 5% CO₂ mixture to adjust the pH to 7.5. Cell lines were stored in liquid nitrogen after freezing in the presence of 4% dimethylsulphoxide.

Cloning procedures

Clonal lines were obtained by seeding 25 to 100 cells in Petri dishes containing the appropriate medium without feeder layer. After 6 days incubation, clones were isolated by trypsinization (0.25% trypsin) inside small glass rings placed over the clone and fixed to the bottom of the dish with sterilized silicone grease. The isolated clones were then cultured in glass bottles as described.

Survival experiments

In survival experiments, 200 to 400 cells (wild type or mutant) were seeded in Petri dishes and cultivated in increasing concentrations of the antimetabolite. After 6 days the cultures were fixed in Bouin's fluid for 20 min and stained with Mayer's haematoxylin.

In the absence of feeder layer cells and antimetabolites the wild type DON cell line has a cloning efficiency of approximately 50%.

Measurements of radioactivity in cells labeled with radioactive DNA and RNA precursors

Incorporation of radioactive precursors in the wild type and mutant cell lines was studied by autoradiography and by determining radioactivity in extracted nucleic acids and nucleotides.

For autoradiography, logarithmic growing cells were labeled with different concentrations of the nucleic acid precursors ^3H -thymidine (spec. act. 2.0 Ci/mmol), ^3H -hypoxanthine (spec. act. 3.14 Ci/mmol), or ^3H -adenine (spec. act. 4.3 Ci/mmol). The radioactive precursors were purchased from the Radiochemical Center, Amersham.

Afterwards the cells were washed three times with 0.9% NaCl and fixed in Bouin's fluid. The autoradiograms were prepared with Kodak AR 10 stripping film. After appropriate exposure times the preparations were developed (Kodak D19B), fixed and stained with Mayer's haematoxylin and eosin.

The nucleic acids were extracted in 5% trichloroacetic acid (TCA) at 4°C for 30 min. After centrifugation the TCA was removed by ether extraction and the supernatant was concentrated by evaporation to 0.1 ml. The nucleotides were separated by chromatography on polyethyleneimine-cellulose thin layer plates. The spots were scraped from the plates and the scrapings suspended in 0.5 ml water and the radioactivity counted in a liquid scintillation counter, after

addition of 13 ml of 5 volumes toluene with POPOP (0.1 g/l) and PPO (6 g/l) and one volume Triton X-100. The acid insoluble precipitate was washed once with 5% TCA and 96% ethanol, suspended in water and counted similarly.

Assays of enzyme activities in cell extracts

Thymidine - and deoxycytidine kinases (TK and CK respectively) were measured according to Mittermayer et al. (1968) with only minor modifications.

Chromosome analyses

Cultures were treated with 1.2 μ g colchicine per ml medium for 2 hr. The cells were then trypsinized and treated with 1% Na-citrate for 10 min at 37°C followed by fixation in methanol-acetic acid (3:1). Chromosome spreads were obtained by the conventional flame-dried technique.

RESULTS AND DISCUSSION

Isolation of 5-bromodeoxyuridine, 8-azaguanine and 8-azaadenine resistant cell lines

DON cells growing in monolayer were cultivated in F12 medium (without thymidine) containing 5-BUdR 100 μ g / ml. After one month of growth the monolayer of surviving cells could be subcultured once or twice a week. This 5-BUdR resistant population was cloned and the isolated clones (a3, a23 and a26) were cultivated for over one year in F12 medium without 5-BUdR and thymidine.

The azg and aza resistant cell cultures were obtained by growing DON cells in F12 medium without thymidine and hypoxanthine in the presence of increasing concentrations of 8-azaguanine (0.5 to 3.0 μ g per ml) or 8-azaadenine (3 to 20 μ g per ml). From these cultures the clones wg3-h (8-azaguanine resistant) and w-aza20 (8-azaadenine resistant) were isolated.

Double mutants were obtained by growing the 5-BUdR resistant cell lines a3 and a23 in the presence of azg or aza. The lines a3-azg6 and a23-azg6 were resistant to 5-BUdR+azg and the lines a3-aza20 and a23-aza20 to 5-BUdR+aza.

Resistance of mutant cell lines to antimetabolites

The antimetabolite resistance of the mutant cell lines was studied by

determining survival, in terms of clone formation, as a function of the concentration of the antimetabolite. The results are presented in the Figs 1, 2 and 3.

Three 5-BUdR resistant cell lines were investigated: a3, a23 and a26. Fig. 1 shows that two cell lines (a3 and a23) are very resistant to the antimetabolite (75% survives a treatment with 200 μ g 5-BUdR per ml medium). The line a26 shows an intermediate sensitivity to 5-BUdR. Since the slope of its survival curve might indicate the presence of a mixed population in terms of 5-BUdR sensitivity, this cell line has not been used in the genetic experiments described in the chapters 3, 4 and 5.

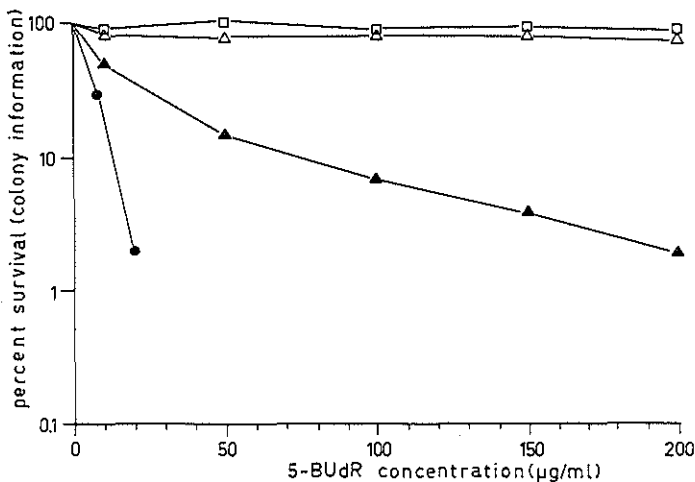


Fig. 1 Cloning efficiency of wild type and mutant cell lines in different concentrations of 5-bromodeoxyuridine.

●—● DON; □—□ a3; △—△ a23; ▲—▲ a26.

Survival experiments were performed with three azg resistant cell lines, wg3-h, a3-azg6 and a23-azg6. The first two cell lines were found similar in sensitivity to azg (Fig 2).

The aza resistance was tested in w-aza20 and the double mutants a3-aza20 and a23-aza20. Fig. 3 shows that the first two cell lines were very resistant to aza (80 to 100% survives a treatment with 80 μ g of the antimetabolite), whereas a23-aza20 shows an intermediate sensitivity.

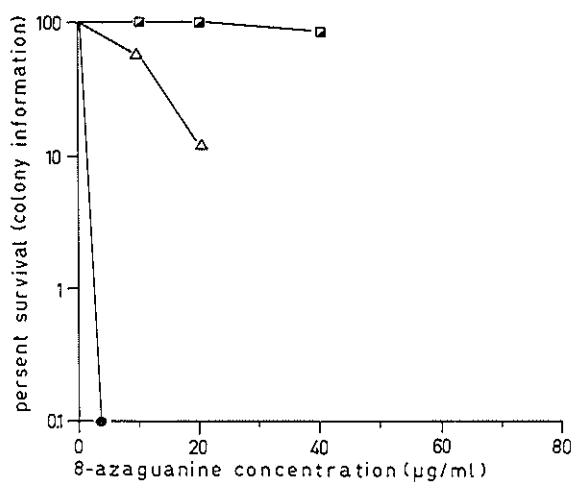


Fig. 2 Cloning efficiency of wild type and mutant cell lines in different concentrations of 8-azaguanine.

● — ● DON; ■ — ■ wg3-h and a3-azg6; Δ — Δ a23-azg6.

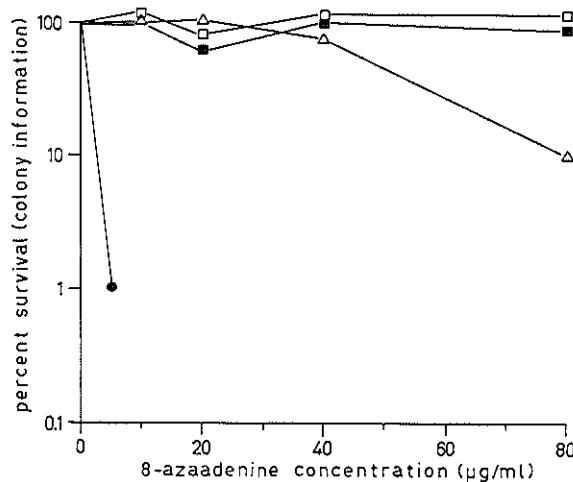


Fig. 3 Cloning efficiency of wild type and mutant cell lines in different concentrations of 8-azaadenine.

● — ● DON; □ — □ a3-aza20; ■ — ■ w-aza20; Δ — Δ a23-aza20.

The enzymatic defect in the mutant lines, studied by autoradiography and enzyme analysis

The enzymatic defect underlying the 5-BUdR resistance in the a3, a23 and a26 cells and in the double mutants a3-azg6 and a23-azg6 was studied in two ways:

1) by autoradiography and by measuring the radioactivity in extracts of labeled

cells after ^3H -thymidine incorporation ;2) by assaying the activity of the enzyme TK in cell extracts of some of the 5-BUdR resistant cell lines.

In the autoradiographic experiments cells in log. phase were labeled with $0.5\ \mu\text{Ci}\ ^3\text{H}$ -thymidine per ml medium for 24 hr. To be sure that all the cells had passed through S phase, parallel cultures were labeled with ^3H -deoxycytidine ($0.5\ \mu\text{Ci}/\text{ml}$). After an exposure time of one week the preparations were developed and the number of silver grains above the nuclei was counted.

The number of grains per nucleus in the a3 and a23 cell lines varied between 5 and 10 in different experiments. The wild type DON cells were found to be heavily labeled under these conditions. About half a year after isolation 41% of the a26 cell population was heavily labeled with an intensity comparable with that of the wild type DON cells. The survival curve of the a26 cell line presented in Fig. 1 was obtained at that time. The labeling experiment, therefore, supports the idea that the a26 line consists of a 5-BUdR resistant and 5-BUdR sensitive population. The double mutants a3-azg6 and a23-azg6 resistant to 5-BUdR and azg, showed the same labeling intensity as the parental cells a3 and a23 after labeling with ^3H -thymidine (^3H -TdR).

After TCA extraction of ^3H -TdR labeled cells, it was found that the amount of ^3H -TdR incorporated by the 5-BUdR resistant cells was about 5% of that incorporated by wild type cells. The radioactivity of the thymine nucleotides (TMP, TDP and TTP) from these cells was also 5% of that found in wild type cells.

The direct assay of TK was performed on extracts from the DON wild type and the a3 and a23 cells. The TK activities in a3 and a23 was found to average between 2 and 5% of the level in wild type cells. The CK activity of the 5-BUdR resistant cells was comparable with the activity of the wild type cells, indicating the same rate of DNA synthesis in the tested cell lines.

From these results it is concluded that the 5-BUdR resistance is caused by a mutation which influences the TK activity. A defect in the thymidine transport system as described by Breslow and Goldsby (1969) can be excluded.

The azg resistance in the wg3-h, a3-azg6 and a23-azg6 cell lines was studied by autoradiography and by determination of the radioactivity in cell extracts after labeling the cells with ^3H -hypoxanthine ($2.0\ \mu\text{Ci}/\text{ml}$) for 24 hr. From the work of Subak-Sharpe and coworkers (1969) it is known that in the case of cellular contact between a wild type and an azg resistant cell, the latter one is able to incorporate ^3H -hypoxanthine (metabolic cooperation). To exclude metabolic cooperation in our experiments labeling was performed on cells growing in discrete colonies instead of in monolayer.

Colonies obtained from the wild type DON cell line were heavily labeled. All the clones obtained from the wg3-h and a3-azg6 lines were unlabeled or only

very slightly labeled. The a23-azg6 population produced heavily and slightly labeled clones, indicating that this line is a mixture of cells with different degrees of azg resistance.

The radioactivities of the nucleic acids and the guanine nucleotides were measured after labeling wild type and wg3-h cells with ^3H -hypoxanthine. The radioactivities found in the wg3-h cell line were about 2% of that in wild type cells.

In Table 1 the results obtained by labeling the aza resistant lines a3-aza20, a23-aza20 and w-aza20 with ^3H -adenine for 0.5 hr ($2\mu\text{Ci/ml}$), 2 hr ($2\mu\text{Ci/ml}$) or 24 hr ($0.5\mu\text{Ci/ml}$) are presented. Following labeling for 30 min or 2 hr, the aza resistant cells were weakly labeled in comparison with the wild type and the a3 cells. Due to the high grain densities the differences could not be determined after labeling for 24 hr.

Table 1 ^3H -adenine incorporation in wild type and mutant cells

cell line	number of grains per cell				
	0.5 hr	s_m	2 hr	s_m	24 hr*
DON	n.c.		n.c.		n.c.
a3	n.c.		n.c.		n.c.
w-aza20	13	1.7	20	2.5	n.c.
a3-aza20	11	0.9	18	4.0	n.c.
a23-aza20	36	6.2	n.c.		n.c.

Average number of grains per cell in DON, a3, w-aza20, a3-aza20 and a23-aza20 cells following incubation with ^3H -adenine for 0.5 hr ($2\mu\text{Ci/ml}$), 2hr ($2\mu\text{Ci/ml}$) or 24 hr ($0.5\mu\text{Ci/ml}$). About 20 cells were analyzed in each sample.

n.c. = number of grains too high for counting

*no differences in grain densities between the cell lines are detectable

s_m = standard error of the mean.

The radioactivity present in the nucleic acid fractions and in the adenine nucleotides was determined after labeling with ^3H -adenine for varying periods. The results are presented in Table 2. The decreased level of radioactivity observed in w-aza20 cells compared to DON cells after short autoradiography periods was also found in the nucleic acids and adenine nucleotides extracts of the w-aza20 cells. This difference is leveled off at longer incubation times.

Table 2 ^3H -adenine incorporation in wild type and mutant cells

Incubation time min	AMP+ADP+ATP counts/min			DNA+RNA counts/min		
	DON	w-aza20	$\frac{\text{w-aza20}}{\text{DON}} \times 100$	DON	w-aza20	$\frac{\text{w-aza20}}{\text{DON}} \times 100$
5	27,903	1,658	6	8,517	748	9
15	58,262	4,777	8	16,595	3,325	20
45	60,064	9,110	15	19,207	3,924	20
75	65,725	18,171	27	22,562	6,086	27

Incorporation of ^3H -adenine (1 $\mu\text{Ci}/\text{ml}$) in growing DON cells and w-aza20 cells, measured in the acid soluble fraction (AMP, ADP and ATP) and in the acid insoluble fraction (DNA+RNA) of the cells after TCA extraction.

This can be explained by postulating that in the w-aza20 cells the adenine is converted to hypoxanthine which can then be incorporated, since the aza resistant cells contain the enzyme hypoxanthine-guanine phosphoribosyl transferase. Another explanation of this phenomenon can be a saturation effect of the adenine nucleotide pool in the DON cells.

Chromosome complement in mutant cells

The karyogram of the wg3-h cell line is shown in Fig. 4. It differs from the normal diploid Chinese hamster chromosome set by having four abnormal chromosomes. The first two in group E might be the result of a translocation between an A and a C group chromosome. An acrocentric and a small telocentric chromosome are also present in many of the wg3-h cells. The distribution of chromosomal numbers in wg3-h cells is presented in Fig. 5. This figure indicates a modal value of 22 chromosomes with only a few deviations to lower or higher chromosome numbers.

A metaphase of an a3 cell having 51 chromosomes is presented in Fig. 4. This metaphase contains 5 chromosomes which can not be identified as Chinese hamster chromosomes. As indicated by this metaphase, a greater or smaller number of chromosomes than the tetraploid number can be present in the A, B, C, or D group. Fig. 6 shows the chromosome distribution of this a3 cell line. The modal value in this case is about 44 chromosomes. The aza resistant clone isolated from the tetraploid a3 line, however, showed a normal modal value of 22 chromosomes (Fig. 7).

The 5-BudR resistant a23 cells have 24 to 26 chromomes (Fig. 8). A karyogram of an a23 cell with an extra telocentric (C group), a small submetacentric (B group) and an acrocentric (E group) chromosome is also shown in Fig. 4. From this line an azg resistant cell line was derived having a chromosome number at about the tetraploid level (Fig. 9). An aza resistant cell line obtained from the a23 had 22 to 26 chromosomes (Fig. 10).

The chromosome distribution in the a26 cell line is shown in Fig. 11. Its mixed character in terms of 5-BUdR sensitivity, discussed earlier, is apparently not reflected in two clearly distinguished populations with different chromosome numbers.

The chromosome distribution in the aza resistant cell line w-aza20 resembles that of the wg3-h cell line (Fig. 12).

The chromosome distribution of the different clonal cell lines remained stable during the period of cultivation. In the case of the a3, a23 and wg3-h cell lines, this period lasted for more than one year, during which karyograms were prepared and analyzed at various time intervals.

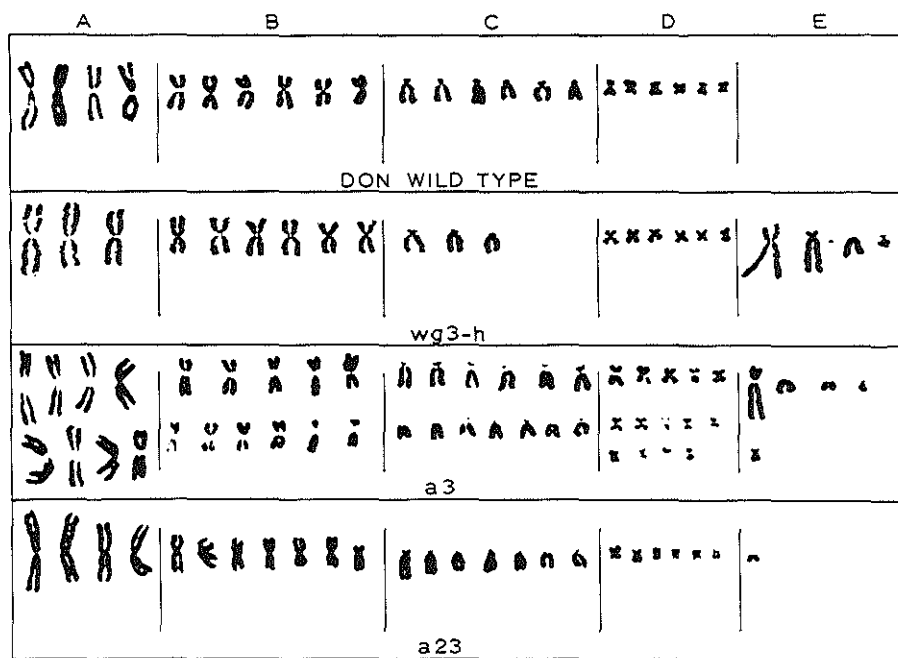


Fig. 4 Karyotype of wild type DON cells and the mutant cell lines wg3-h, a3 and a23. The groups A, B, C and D contain characteristic Chinese hamster chromosomes. Group E contains chromosomes with altered morphology.

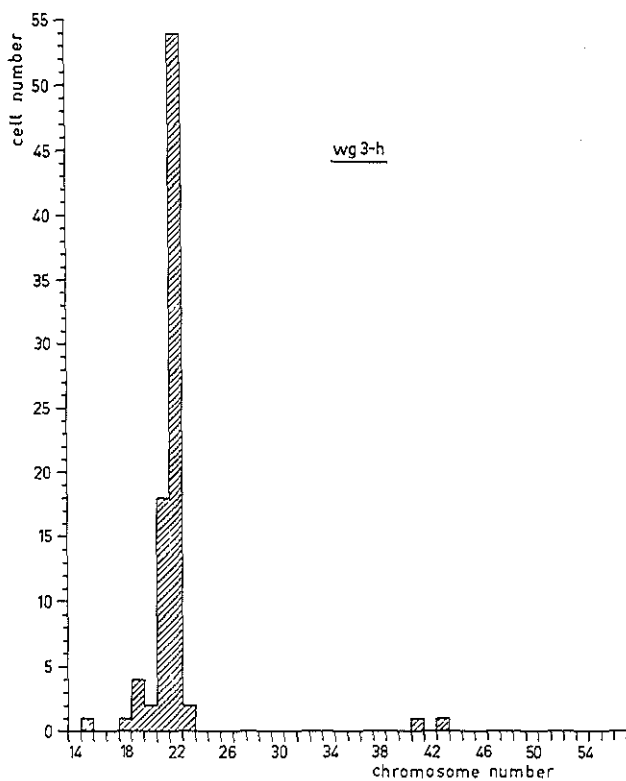


Fig. 5 Histogram of chromosome number distribution of the wg3-h cell line.

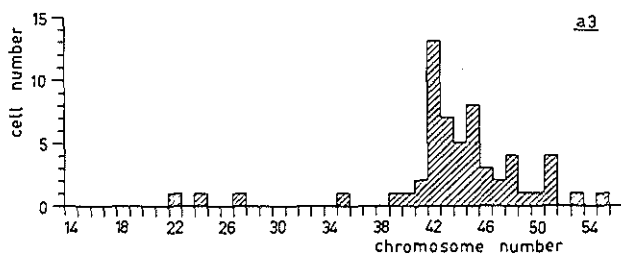


Fig. 6 Histogram of chromosome number distribution of the a3 cell line.

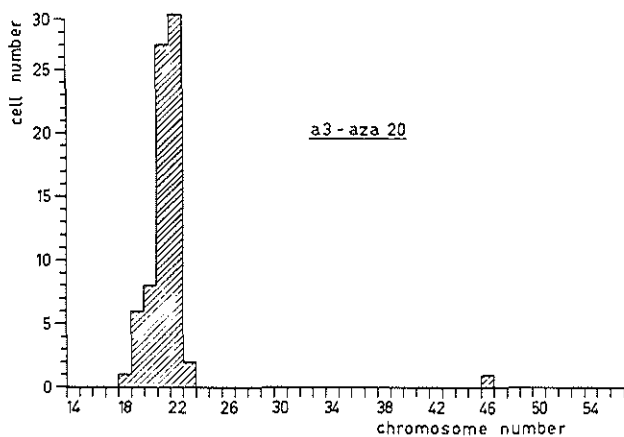


Fig. 7 Histogram of chromosome number distribution of the a3-aza20 cell line.

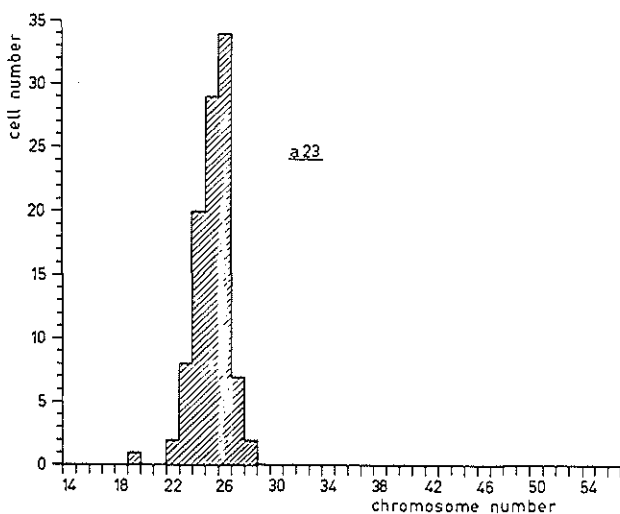


Fig. 8 Histogram of chromosome number distribution of the a23 cell line.

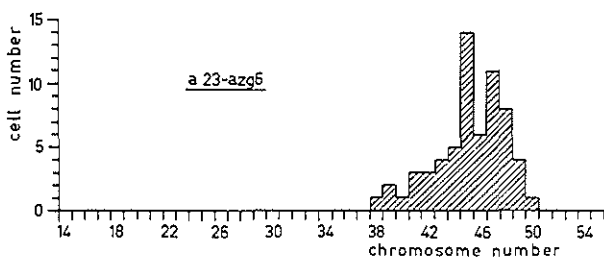


Fig. 9 Histogram of chromosome number distribution of the a23-azg6 cell line.

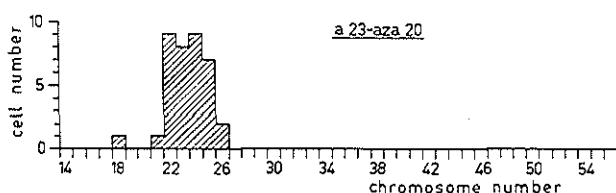


Fig. 10 Histogram of chromosome number distribution of the a23-aza20 cell line.

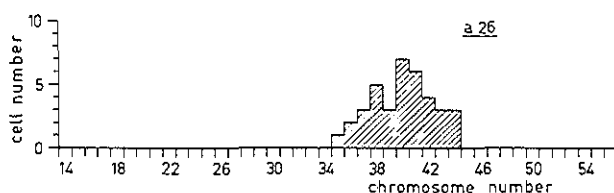


Fig. 11 Histogram of chromosome number distribution of the a26 cell line.

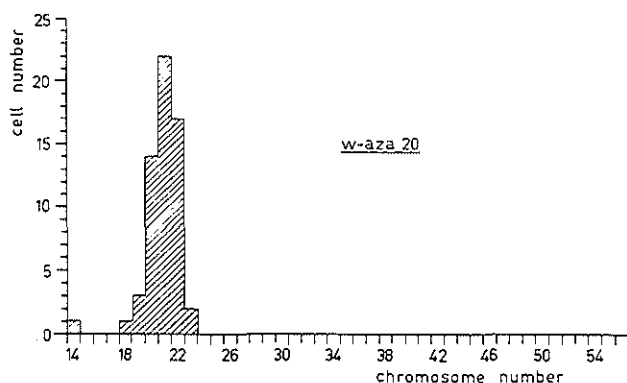


Fig. 12 Histogram of chromosome number distribution of the w-aza20 cell line.

CONCLUSIONS AND SUMMARY

From the Chinese hamster cell line DON we isolated a number of mutant cell lines resistant to the antimetabolites 5-BUDR, 8-azaguanine or 8-azaadenine. The resistant cells showed no or a decreased incorporation in the nucleic acids of ^3H -thymidine, ^3H -guanine or ^3H -hypoxanthine and ^3H -adenine respectively.

The resistance to 5-BUDR is caused by inactivity or absence of thymidine-kinase.

The resistance to 8-azaguanine and 8-azaadenine can be caused by inactivity

or absence of the enzymes HGPRT and APRT respectively as well as by a defect in the transport system.

The karyograms of the cell lines were stable during the period of observation.

No revertants were encountered in the 8-azaguanine resistant cell line wg3-h and the 5-BUdR resistant cell lines a3 and a23 during the observation period of over one year.

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chapter 3

LOSS OF HUMAN GENETIC MARKERS IN MAN-CHINESE HAMSTER SOMATIC CELL HYBRIDS*

Man-mouse somatic cell hybrids have been shown to be useful for the study of genetic linkage in man, because the human chromosomes are preferentially lost and a great majority of murine and human forms of homologous isozymes are clearly distinguishable ^{1,2,3}. However, certain limiting factors in this system, call for the introduction of other interspecific somatic cell hybrids:

1. Not all the enzyme phenotypes of man and mouse are easily distinguishable by means of conventional electrophoretic procedures.
2. It has been argued that groups of human chromosomes can be preferentially retained or lost in the man-mouse hybrids ⁴. We do not know whether such groups form regular patterns and are constant for a given type of hybrid.
3. Also the frequency and types of chromosomal rearrangements, the occurrence of which has been reported in man-mouse hybrids, might be different in other human interspecific hybrids.

We have isolated a number of man-Chinese hamster somatic cell hybrids and noticed a preferential loss of human genetic markers. The isozymes which were studied could be distinguished between the two species with one possible exception (LDH B).

PARENTAL AND HYBRID CELL LINES

Four different somatic hybrid lines were obtained by fusing three mutant cell lines derived from the Chinese hamster DON line with a mutant human diploid fibroblastic strain and with normal human lymphocytes (see Fig. 1).

The three Chinese hamster mutant lines were derived from the diploid Chinese

* By A. Westerveld, P. Meera Khan, R.P.L.S. Visser, and D. Bootsma (1971), Nature (in press).

hamster DON cell line. Two lines a3 and a23, were selected in the presence of 100 μ g 5-bromodeoxyuridine and the third (wg3-h) by stepwise selection in increasing concentrations (0.5-3.0 μ g/ml) of 8-azaguanine.

The thymidine kinase (TK) activity in a3 and a23 cells, as studied in cell extracts, was less than 2% of that in normal DON cells. The a3 line was found to be tetraploid ($4n = 44$) and a23 hyperdiploid ($2n + 2 = 24$).

Survival experiments showed that the diploid wg3-h cells were resistant to 20 μ g 8-azaguanine per ml F12 medium. The activity of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) was about 2% compared to normal DON cells.

No revertants were encountered in the three mutant cell lines during the growth for over a year in F12 medium in the absence of the appropriate anti-metabolite.

The diploid human parental fibroblast strain ms2 (HGPRT⁻) was derived from a male patient with the Lesch-Nyhan syndrome⁵. The human lymphocytes, from a male donor, were stimulated by phytohaemagglutinine and grown for three days before fusion.

All the hybrid lines were selected in F12 medium containing 4×10^{-7} M aminopterin, 1.7×10^{-5} M thymidine, 3.0×10^{-5} M hypoxanthine and 2.0×10^{-4} M glycine (F12 HAT medium). F12 HAT medium was also used for cloning and subcloning. These selection procedures were identical to those described by Littlefield⁶. The fusion was performed with UV-inactivated Sendai virus⁷. The parental cells were mixed in a ratio of 1 : 1 (a3/ms2 and a23/ms2) or 1 : 3 (wg3-h/lymphocytes).

The hybrid nature of all the clones and subclones was confirmed by a mixed agglutination or cytotoxicity test using horse anti human lymphocyte serum (ALS).

The subsequent steps for the isolation of the hybrid clones are summarized in Fig. 1 (a, b, c and d).

Fig. 1a

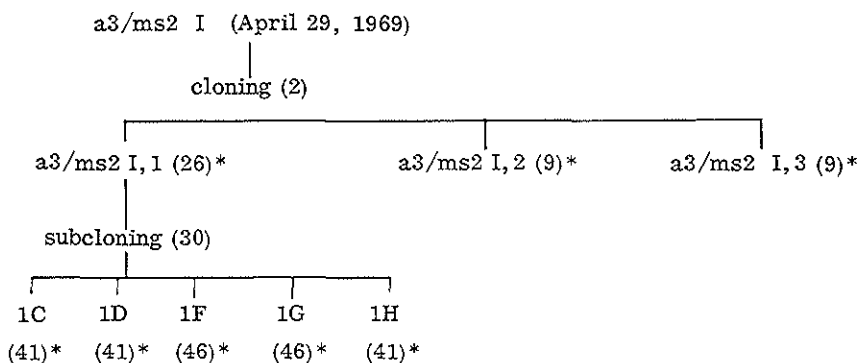


Fig. 1b

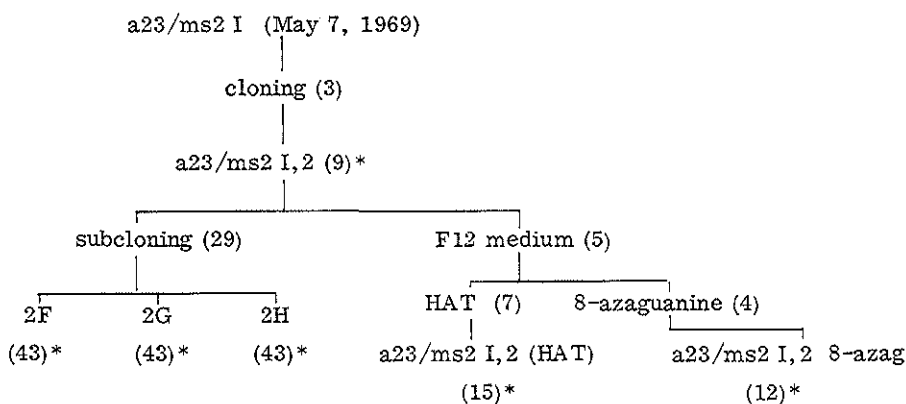


Fig. 1c

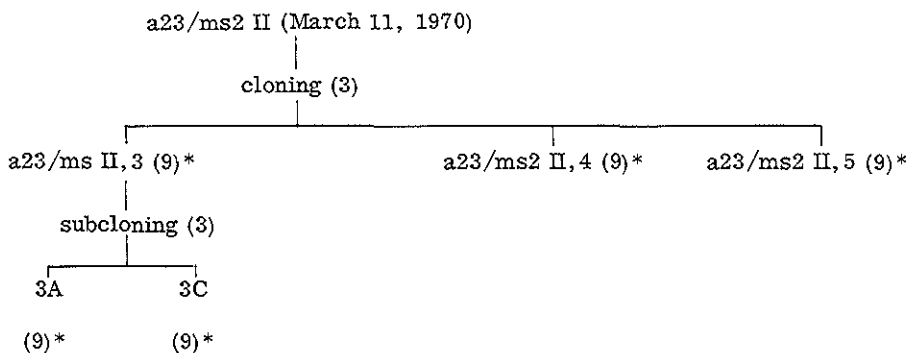


Fig. 1d

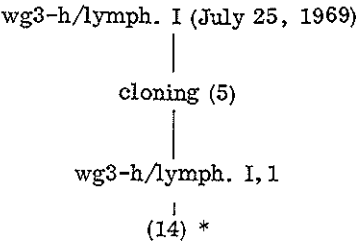


Fig. 1 Genealogical relationship and cultural histories of the different hybrid cell lines. If not stated otherwise the cells were grown in normal F12 medium. Cloning and sub-cloning were performed in F12 HAT medium. The numbers mentioned after cloning and subcloning indicate the time of growth of the parental line before (sub)cloning (in weeks).

(..) * = the number of weeks between fusion and electrophoresis.

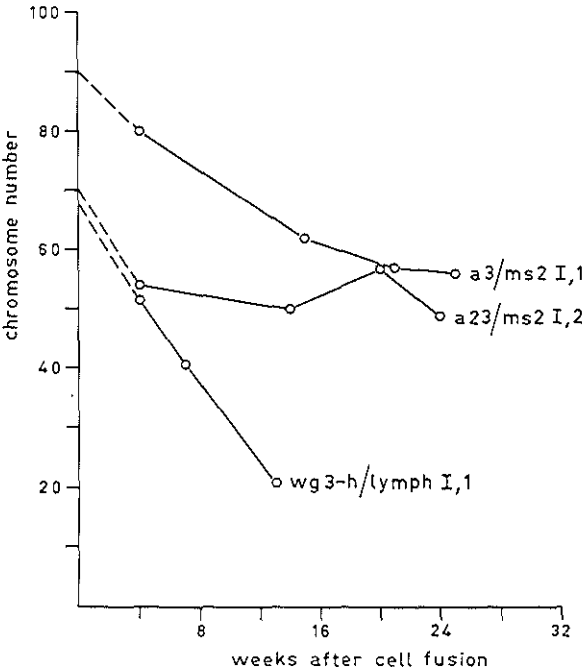


Fig. 2 Karyotype evolution in three different man-Chinese hamster hybrid cell lines.

KARYOTYPIC EVOLUTION OF HYBRID LINES

Karyotypic analysis was performed at regular intervals during growth of three of the clonal hybrid populations. The change in the average chromosome number, determined from the analysis of 20-50 cells of each cell line is described in Fig. 2. From the graph it appears that the chromosomal loss was a general phenomenon though the rate and extent of loss appeared to be different in different hybrid lines. Though only the B, C and G groups (Denver classification) of human chromosomes are distinguishable from the Chinese hamster chromosomes, we feel confident, that like the man-mouse hybrid also the human-Chinese hamster hybrids undergo preferential loss of human chromosomes. This conclusion is born out from the analysis of the hybrid metaphases, since it was always possible to reconstruct a complete chromosomal complement of a Chinese hamster (Fig. 3) but never that of man. Moreover, in one hybrid clone there was apparent loss of the entire human genome : the hybrid wg3-h/lymph I,1 (Fig. 2). Obviously these observations do not unequivocally prove that the lost chromosomes were exclusively human. Until new methods^{2, 8} become available to distinguish human from Chinese hamster chromosomes, the use of the man-Chinese hamster hybrid system for the determination of gene-chromosomal associations on a morphological basis will be restricted to the human chromosome groups B, C and G only.

Indirect evidence for preferential loss of human chromosomes in the man-Chinese hamster somatic cell hybrids comes from the enzyme studies at the single cell level (Table 1) and from the study of zymogram patterns of various cell lines (Tables 2 and 3).

TK AND HGPRT ACTIVITIES IN THE HYBRID CELLS

The same hybrid cell lines which were analysed for their chromosomes were employed also for the detection of TK and HGPRT activities, at the level of a single cell, at various periods of their propagation in normal medium. Petri dishes (inner diameter 4.5 cm) were seeded with 400 cells in F12 medium without thymidine and hypoxanthine. After 6 days of growth the small colonies of cells were labelled with ³H-thymidine (0.5 μ Ci/ml ; spec. act. 2.0 Ci/mmole) or ³H-hypoxanthine (1.0 μ Ci/ml ; spec.act. 3.14 Ci/mmole) for 24 hours. Control cultures from the a3, a23 and wg3-h parental lines were treated in the same way. Parallel cultures were labelled with ³H-deoxycytidine (0.5 μ Ci/ml ; spec.act. 3.16 Ci/mmole) to make sure that all the cells had been in S phase during the 24 hours labelling period. The results of autoradiography are presented in Table 1. It is clear from this table that 4 weeks after

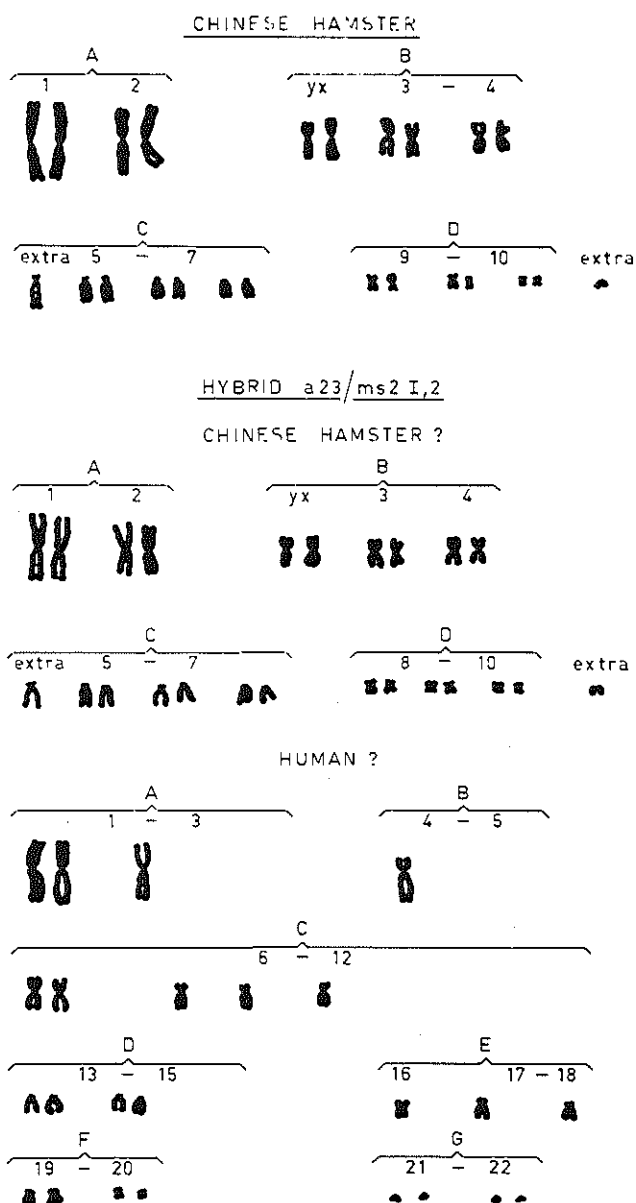


Fig. 3 Chromosome complement in a Chinese hamster parental cell line (a23) and in a hybrid clone a23/ms2 I,2, 16 weeks after cell fusion.

The chromosomes of the hybrid have been arranged in order to reconstruct a complete Chinese hamster complement (see text).

the fusion and the isolation in HAT medium, all the hybrid cells were normal in TK and HGPRT activities as expected. However, after long culturing in normal medium, i.e. when normal activity of these enzymes was no longer necessary for survival, colonies of cells deficient in one or the other of these enzymes started to appear. Thus at week 17, TK⁻ colonies were present in the TK⁻Chinese hamster x HGPRT⁻ human hybrid lines and their number increased progressively. There were no HGPRT⁻ cells in these lines. The HGPRT⁻ Chinese hamster x HGPRT⁺ human hybrid, which returned to the diploid Chinese hamster chromosome complement (Fig. 1) was found to be also HGPRT deficient. The HGPRT⁻ cells did not appear in the parental lines a3 and a23, nor TK⁻ cells in the wg3-h line. These data indicate a preferential loss of human chromosomes in man-Chinese hamster hybrids at least in so far as the loci determining TK and HGPRT are concerned.

Table 1 Autoradiographic determination of TK and HGPRT activities in hybrid cell lines following ³H-thymidine and ³H-hypoxanthine incorporation for 24 hours. The hybrid cell lines were seeded in small densities, 6 days before the labelling, to facilitate the determination of the percentage of unlabelled colonies

A : hybrid cell line a3/ms2 I, 1 (TK⁻Chinese hamster-HGPRT⁻human)
 B : hybrid cell line a23/ms2 I, 2 (TK⁻Chinese hamster-HGPRT⁻human)
 C : hybrid cell line wg3-h/lymph. I, 1 (HGPRT⁻Chinese hamster-normal human lymphocytes)

Weeks after cell fusion*	Percentage of TK deficient colonies**			Percentage of HGPRT deficient colonies**		
	A	B	C	A	B	C
4	0	0	0	0	0	0
12	nt	nt	0	nt	nt	97
17	1	5	nt	0	0	nt
19	12	nt	nt	0	nt	nt
24	46	81	nt	0	0	nt
28	66	95	nt	0	0	nt

*Following fusion the hybrids were selected in HAT medium for 2-5 weeks and maintained in F12 medium thereafter.

** At least 100 colonies were counted.

nt = not tested

Table 2 Distribution of human enzyme activities in hybrid clones

	HGPRT	G6PD	PGK	LDH A	LDH B	6PGD	PGM ₁	IPO	MDH	IDH
a3/ms2 I, 1	-	+	+	+	+	+	+	+	-	-
a3/ms2 I, 2	-	-	-	-	+	-	-	-	-	-
a3/ms2 I, 4	-	-	-	+	-	-	-	+	-	-
a23/ms2 I, 2	-	+	+	-	+	-	-	+	-	-
a23/ms2 I, 2 8-azag.	-	+	+	-	+	-	-	+	-	-
a23/ms2 I, 2 HAT	-	-	-	+, and -	+	-	-	-	-	-
a23/ms2 II, 3	-	-	-	-	+	-	-	-	-	-
a23/ms2 II, 4	-	-	-	+, and -	+	-	-	-	-	-
a23/ms2 II, 5	-	-	-	-	+	-	-	-	-	-
wg3-h/lymph. I, 1		+	+	+	-	+	+	-	-	-

Table 3 Distribution of human enzyme activities in subclones derived from hybrid clones

	HGPRT	G6PD	PGK	LDH A	LDH B	6PGD	PGM ₁	IPO	MDH	IDH
a3/ms2 I, 1C	-	+	+	+	+	+	+	+	-	-
a3/ms2 I, 1D	-	-	-	+	+	-	-	-	-	-
a3/ms2 I, 1F	-	+	+	+	+	-	-	+	-	-
a3/ms2 I, 1G	-	+	+	+	+	-	-	-	-	-
a3/ms2 I, 1H	-	+	+	+	+	+	+	+	-	-
a23/ms2 I, 2F	-	+	+	-	+	-	-	+	-	-
a23/ms2 I, 2G	-	+	+	-	+	-	-	+	-	-
a23/ms2 I, 2H	-	+	+	-	+	-	-	+	-	-
a23/ms2 II, 3A	-	-	-	-	+	-	-	-	-	-
a23/ms2 II, 3C	-	-	-	-	+	-	-	-	-	-

ISOZYME PATTERNS IN HYBRID CLONES AND SEGREGATION OF HUMAN GENETIC MARKERS

We have determined the zymogram patterns of the following enzymes : hypoxanthine-guanine phosphoribosyl transferase (HGPRT), glucose-6-phosphate dehydrogenase (G6PD), 3-phosphoglycerate kinase (PGK), lactate dehydrogenase (LDH A and LDH B), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucumutase 1 (PGM 1), indophenol oxidase (IPO), NAD-dependent soluble malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH). Cellulose acetate gel (Cellogel) was used as supporting medium for performing the electrophoresis of these enzymes as described elsewhere^{3,9,10,11}.

Intermediate isozyme bands are noticed in the hybrid cells for the following enzymes : G6PD, LDH A, LDH B, 6PGD and IPO. The presence of at least one of these interspecific heteropolymeric enzyme molecules in all the hybrid clones tested (Tables 2 and 3) is an unequivocal proof of their hybrid nature. The human MDH and IDH appeared to have been lost before the cloning of the four hybrid cell lines was performed (Table 2).

Bodmer and colleagues^{4,1} and Ruddle and colleagues^{12,2} using human-mouse hybrids have presented evidence that the human genes for LDH A and LDH B are not linked. Our results obtained with the Chinese hamster-human system led to the same conclusion. The LDH zymogram of the Chinese hamster DON line shows only the LDH 5 band consisting of Chinese hamster A subunits¹³ (Fig. 4, channel 2). The LDH B subunits, though present in some organs *in vivo*, have so far not been found in cultured Chinese hamster cells.

Fig. 4 shows the LDH patterns of 8 hybrid clones, the ms2 strain, the DON line and of a mixture of both parental cells. The probable subunit composition and the relative positions of the respective isozymes in three different types of zymograms (the channels 6, 7 and 8) are shown in Fig. 5. All the hybrids seem to have kept the Chinese hamster LDH. The clones of channels 4, 5 and 6 have evidently kept the Chinese hamster LDH and both the human types of LDH and consequently possess the whole series of interspecific and intraspecific polymers. The hybrid clone a3/ms2 I, 2 (Fig. 4, channel 7) has only the heteropolymers between Chinese hamster LDH A and human LDH B, thus suggesting the loss of the human gene for the LDH A subunit. The reverse of this situation has happened in hybrid clone a3/ms2 I, 4 (Fig. 4, channel 8) where the gene coding for the human LDH B seems to be lost. Clone a23/ms2 I, 2 (Fig. 4, channel 10) which was grown in HAT (Fig. 1b) seems to consist of a mixture of hybrid cells having retained only the human LDH A or the human LDH B, the latter forming the majority of the population. The loss of human LDH A is found in a3/ms2 hybrids as well as in a23/ms2 hybrids obtained

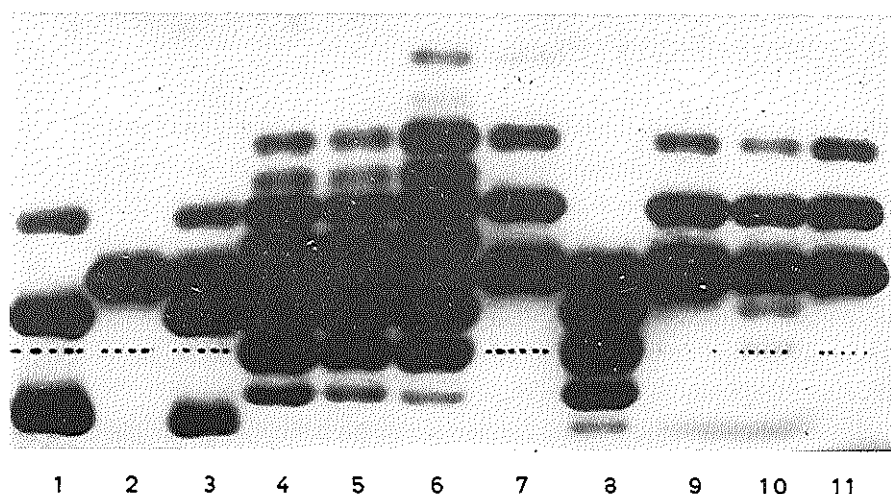


Fig. 4 LDH zymograms of hybrids and parental cells. Channel (1); human ms2 strain; (2) Chinese hamster a3 line; (3) mixture of ms2 and a3; (4) hybrid clone a3/ms2 I, 1C; (5) a3/ms2 I, 1D; (6) a3/ms2 I, 1H; (7) a3/ms2 I, 2; (8) a3/ms2 I, 4; (9) a23/ms2 I, 2 grown in 8-azaguanine; (10) a23/ms2 I, 2 grown in HAT medium; (11) a23/ms2 I, 2. Electrophoresis on cellulose acetate gel (Celloigel).

in two independent experiments. Human LDH B was lost in a3/ms2 and wg3-h/ lymphocytes hybrids (Tables 2 and 3). This lack of correlation in the loss or retention of the human LDH genes, on repeated and independent observations supports the hypothesis that the two loci are located on different human chromosomes, and makes very unlikely the alternative explanation of linkage and segregation by chromosome fragmentation.

The possibility that the varying patterns of LDH in man-mouse hybrids were due to the presence of a regulatory gene for the expression of LDH B, has been discussed by Santachiara et al.¹ and Ruddle et al.². The presence of a regulatory gene for LDH B cannot be excluded with certainty even in our experiments. The difference in electrophoretic mobility between LDH 1 of man and Chinese hamster is too small (own unpublished observations on the Chinese hamster diaphragm and heart tissue extracts) to be able to distinguish the isozymes built up by Chinese hamster LDH A and B subunits from those consisting of Chinese hamster LDH A and human LDH B subunits.

The number of clones that have been analysed so far is too little to justify conclusions about positive linkage relationships between the genetic markers that have been utilized. There are, however, two interesting points which are worth emphasizing. Firstly we did not find a positive correlation between the human 6PGD and the other human markers except PGM 1. Experiments to

mutants with human lymphocytes and the isolation of the hybrid cells in the appropriate minimal medium, may lead eventually to the discovery and localization of new genes in the humane genome.

We thank Miss Maria Freeke and Mr A.M. Bogaart for their skillful technical assistance, Dr M. Siniscalco for his stimulating discussions and advice and Dr Henry Harris for introducing one of us (A.W.) to the hybridization technique. We feel very much obliged to the Radiobiological Institute of the Health Organization TNO at Rijswijk (ZH) (Dr H. Balner) for providing the antihuman lymphocyte serum.

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chapter 4

EVIDENCE FOR LINKAGE BETWEEN GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE LOCI IN CHINESE HAMSTER CELLS*

SUMMARY

G6PD and 6PGD activities were determined in diploid, hyperdiploid, tetraploid and hybrid cells all originating from the same Chinese hamster cell line (the DON line). A relationship between gene multiplicity and enzyme activity has been observed. The same enzymes were studied in hybrid cells cultivated in selective media. Selection was carried out against and for the HGPRT⁺ locus. The differences in G6PD and 6PGD activities between the cell lines found under these conditions indicate a positive linkage of the G6PD and HGPRT locus and negative linkage of the 6PGD and the HGPRT locus in these Chinese hamster cells.

INTRODUCTION

Differences in electrophoretic mobility between isozymes of different species and specific loss of human chromosomes facilitate the study of gene linkage in interspecific human-mouse (Ruddle et al., 1970a and Santachiara et al., 1970) and human-Chinese hamster (Westerveld et al., 1971) somatic cell hybrids.

In intraspecific hybrids the situation is different. Electrophoretically detectable markers are rare. In several cases a decrease in the number of chromosomes has been found (Yoshida and Ephrussi, 1967; Ruddle et al., 1970b and Marin, 1969). These hybrids bear a different number of chromosomes, and therefore they may possess different gene multiplicities coding for a particular enzyme. A relationship between gene multiplicity and enzyme activity has been

*By A. Westerveld, R.P.L.S. Visser and M.A. Freeke (1971), *Biochem. Genet.* 5, 591-599.

observed by Puck and Kao (1968) in Chinese hamster cells in culture. They reported that the proline synthesizing capacity increases with increasing multiplicity of the genes coding for proline synthesis in the cell. This phenomenon has also been observed in cells from patients with an inborn error. For example, in patients suffering from galactosemia, the activity of the galactose-1-phosphate-uridyl transferase in cells of the heterozygous parents is about half the value of the activity in the homozygous normal controls (Hsia, 1970).

In this paper a relationship is described between enzyme activity and gene multiplicity in Chinese hamster cells with different chromosome numbers and in Chinese hamster somatic cell hybrids. The activity of glucose-6-phosphate dehydrogenase (G6PD) and 6-Phosphogluconate dehydrogenase (6PGD) was determined in diploid, hyperdiploid, tetraploid and hybrid Chinese hamster cells. By using this gene dose relationship and the Littlefield (1964) selection system in the case of the hybrid cell lines, evidence is presented for the linkage between the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and G6PD loci in Chinese hamster cells. 6PGD was found not to be linked to these loci.

MATERIALS AND METHODS

Cell lines

Several mutant cell lines were derived from a Chinese hamster fibroblast cell line (DON). Two thymidine kinase deficient (TK^-) cell lines, a3 and a23, were obtained by cultivating DON cells in F12 medium (Ham, 1965) containing 100 μ g 5-bromodeoxyuridine (5-BUDR) per ml. Most of the a3 cells are tetraploid ($2n = 22$) whereas the a23 cells are hyperdiploid (24-26 chromosomes). The in vitro thymidine kinase (TK) activity (determined according to Mittermayer, 1968) of the a3 and a23 cells was 2% of the activity in wild type cells.

An 8-azaguanine resistant cell line, wg3-h, was selected stepwise by growing DON cells in increasing concentrations (0.5 - 3 μ g per ml) of 8-azaguanine. This line, mostly diploid, is resistant to doses up to 20 μ g 8-azaguanine per ml when tested for survival. In 40 μ g 8-azaguanine per ml, the cloning efficiency was 80% of the normal value. Following 3H -guanine incorporation in the cells, the extraction of the nucleic acids was performed in trichloroacetic acid. The nucleotides were separated by chromatography on polyethyleneimine cellulose thin layer plates. The radioactivities in the nucleotides and of the acid insoluble precipitate were counted in a liquid scintillation counter. It was found that the activities of the wg3-h cells were 2% of that in normal cells.

After isolation, the clones a3, a23 and wg3-h were cultivated for over one year in modified F12 medium without the antimetabolites. Revertants were not found during this period of growth.

The morphology of the Chinese hamster hybrid cells does not differ from the morphology of the mutant cells a3 and a23. The hybrid cells, like the parental cells, show no contact inhibition. By pulse labeling with ^3H -thymidine and scoring the fraction of labeled mitoses as function of time after labeling, the cell cycles of the parental cells a3, a23 and wg3-h and hybrid cells were determined. The cell cycle of these tested cell lines was 12-15 hr, and no significant difference was observed in the proportion of cells in G_1 , S and G_2 phases between the cell lines studied.

Culture media

The cell lines a3 and a23 were maintained routinely in F12 medium without thymidine, wg3-h cells were cultivated in F12 medium lacking thymidine and hypoxanthine. The hybrids were routinely cultivated in normal F12 medium. All the media were supplemented with 6% newborn calf serum and 100 IU penicillin and 0.1 mg streptomycin per ml.

The hybrid cell lines were isolated in F12 HAT medium (Littlefield, 1964) containing 4.0×10^{-7} M aminopterin, 2.0×10^{-4} M glycine, 1.7×10^{-5} M thymidine, and 3.0×10^{-5} M hypoxanthine.

HGPRT⁻ hybrids were selected in F12 medium lacking thymidine and hypoxanthine but supplemented with 3 μg 8-azaguanine (azg) per ml.

Cell fusion

The Sendai virus was propagated, isolated, and purified as described by Harris and Watkins (1965). The virus was diluted to 1000 haemagglutination units (HAU) per ml and inactivated by ultraviolet irradiation (7200 ergs). In the fusion experiments, 3×10^6 cells of each cell line were mixed in 10 ml Hank's balanced salt solution (BSS) without glucose, in order to assure random inter-cellular contact. The cells were collected by centrifugation and resuspended in 1 ml BSS. One ml of inactivated virus was added to this suspension. The mixture was kept at 4 C for 5 min and then incubated at 37 C for 20 minutes. After this procedure the cells were seeded in Petri dishes and incubated at 37 C. Afterwards, the hybrid cells were maintained as monolayer cultures in silicon-rubber stoppered glass bottles. For selection of the hybrids, the hybridized

population was cultivated in F12 HAT medium for about 6 weeks.

Preparation of extracts

Cells growing in monolayer for 3 or 4 days after seeding were removed from the glass by trypsinization, counted, washed once in 10 ml medium containing 6% serum and twice in 10 ml. 0.9% NaCl solution. Thereafter, the tubes were thoroughly drained and the pellets were resuspended in 0.5 ml of the appropriate buffer for the enzyme determination. The cells were disrupted by sonication (80 sec, 20 KC/sec in a M.S.E. sonicator). The sonicate was then mixed with 0.5 ml carbontetrachloride and centrifuged at $17,000 \times g$ for 30 min at 4 C. The supernatant was recovered and stored in ice. Normally, the extracts were prepared from 20 to 50×10^6 cells.

Determination of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities

The reactions catalysed by G6PD and 6PGD can be followed by measuring the increase of the optical density at 340 nm due to the formation of NADPH. The conversion of $0.16 \mu\text{mole}$ per ml glucose-6-phosphate gives a reading of 1.0 optical density unit for 1 cm light path.

For the determination of the G6PD activity the following reaction-mixture modified after Greenhouse et al. (1969) was used : 1 ml tricinebuffer (0.1 M, pH 7.5), 1.5 ml glucose-6-phosphate solution, and 0.1 ml NADP solution (2.5 mg/ml).

The reaction-mixture for the determination of the 6PGD activity contained 1 ml glycylglycine (0.2 M, pH 7.5), 1.5 ml 6-phosphogluconate solution, 0.1 ml NADP solution (2.5 mg/ml). Calculation of the enzyme activity was made at the optimal substrate concentration. The reaction was started by adding 0.05 ml of the cell extract and performed at 38 C.

RESULTS

Karyology

The hybrid populations were maintained for about one year in F12 medium. At various intervals karyograms were prepared as described by Evans (1965), and the mean number of chromosomes was determined in at least 25 metaphases.

In the hybrid populations a3 x wg3-h and a23 x wg3-h a gradual loss of chromosomes was observed during the first two months of culture (in the a3 x wg3-h hybrid cells the number of chromosomes decreased from the

expected value of 66 to about 50 ; in a23 x wg3-h there was a decrease from the expected number of 46 to about 42). During the following period of observation no detectable loss was apparent.

TK and HGPRT activities in the hybrid cells

If continuous loss of genetic information occurs an increase in the fraction of TK⁻ and HGPRT⁻ cells can be expected. The TK and HGPRT activities were measured autoradiographically at the single cell level in small cell clones labeled for 24 hr with ³H-thymidine or ³H-hypoxanthine. By cloning the hybrid population at different time intervals after fusion of the a3 x wg3-h hybrid cells a low number of TK⁻ (1-4%) and HGPRT⁻ (3-10%) clones was found. The hybrid cell population a23 x wg3-h showed an increase with time in the percentage of TK⁻ cells (from 3 to 60%), whereas the fraction of HGPRT⁻ clones remained at the constant level of 0.5%.

G6PD and 6PGD activities in parental and hybrid cells

To investigate whether the tested enzymes in the mutant and hybrid cells were still identical with the enzymes of normal DON cells the Michaelis-Menten constant (K_m) of all the tested cell lines was determined by means of a Lineweaver-Burk plot. The K_m value of G6PD related to G6P as the substrate was 1.6×10^{-4} M, and the K_m value of 6PGD related to 6PG as the substrate was 8.3×10^{-5} M in all the cell lines studied.

The G6PD and 6PGD activity per cell was determined in the parental cell lines wg3-h (2n), a3 (4n), and a23 (2n + 2 to 4 chromosomes extra) and in the hybrid cell populations a3 x wg3-h and a23 x wg3-h cultivated in F12 HAT medium or in F12 azg medium. The relative enzyme activities per cell for G6PD and 6PGD are compared with the theoretically expected relative activities if the loci for these enzymes are linked or not with the HGPRT locus (Table I).

It appears from Table I that the parental cells a3 and a23 had twice the G6PD activity of the diploid wg3-h parental cells. The hybrid cells which were cultivated in the F12 HAT medium contained the sum of the enzyme activities present in the parental cells. However, the hybrids grown in the presence of 8-azaguanine showed the same level of G6PD activity as the wg3-h cells.

The 6PGD activities of the wg3-h and a23 cells were comparable, whereas the tetraploid a3 cell exhibited twice this activity. The 6PGD activity of the hybrid cells cultivated either in F12 HAT or in F12 azg medium, equals the sum of

Table I G6PD (IA) and 6PGD (IB) Activities per Cell ^a

IA

Cell line	experiment no.					mean relative		relative
						activity		activity expected
	1	2	3	4	5	exp.	exp.	if linked with
						1-3	4-5	HGPRT
wg3-h	1.0(1.0)	0.8(1.0)	0.9(1.0)	0.8(1.0)	0.8(1.0)	1.0	1.0	1
a3	2.2(2.2)	2.1(2.6)	2.3(2.6)			2.5		2
a3 x wg3-h azg	1.4(1.4)	0.9(1.1)	0.9(1.0)			1.2		1
a3 x wg3-h HAT	2.4(2.4)	3.6(4.5)	3.5(3.9)			3.6		3
a23				2.0(2.5)	2.0(2.5)		2.5	1 or 2
a23 x wg3-h azg				1.0(1.3)	0.9(1.1)		1.2	1
a23 x wg3-h HAT				3.6(4.5)	3.1(3.9)		4.2	2 or 3

IB

Cell line	experiment no.				mean relative		relative
					activity		activity expected
					exp.	exp.	if not linked with
	6	7	8	9	6-7	8-9	HGPRT
wg3-h	0.4(1.0)	0.6(1.0)	0.6(1.0)	0.6(1.0)	1.0	1.0	1
a3	0.7(1.8)	1.3(2.2)			2.0		2
a3 x wg3-h azg	1.1(2.8)	1.8(2.7)			2.8		1
a3 x wg3-h HAT	1.1(2.8)	1.8(3.0)			2.9		3
a23			0.5(0.8)	0.6(1.0)		0.9	1 or 2
a23 x wg3-h azg			1.3(2.2)	1.1(1.8)		2.0	2 or 3
a23 x wg3-h HAT			1.2(2.0)	1.1(1.8)		1.9	2 or 3

^a Measured in $\frac{\Delta E}{\Delta T}$ units $\times 10^{-5}$ in extracts prepared from wg3-h, a3, a23 and the hybrids a3 x wg3-h and a23 x wg3-h cultivated in F12 azg or F12 HAT medium. The numbers in parentheses indicate the relative activity related to wg3-h. The last column represents the theoretically expected relative activities in the case of positive or negative linkage with the HGPRT locus.

the activities of the parental cells.

In the enzyme assays, different number of cells were used. In control experiments, a linear relationship between cell number and enzyme activity was observed.

A relationship between enzyme activity and the stage of the growth curve was studied by determining the activities of G6PD and 6PGD at different time intervals after seeding of the cultures. The results are presented in Table II.

Table II G6PD and 6PGD Activities per Cell^a

Days after seeding	wg3-h		a3		a3 x wg3-h azg	
	G6PD	6PGD	G6PD	6PGD	G6PD	6PGD
1	0.8	0.5	1.6	1.1	0.7	1.9
2	0.7	0.7	1.6	1.2	0.5	1.9
3	1.1	0.6	1.7	1.2	1.0	1.9
4	1.0	0.6	2.1	1.2	0.8	1.8

^a Measured in $\frac{\Delta E}{\Delta T}$ units $\times 10^{-5}$ at successive days after seeding of wg3-h, a3 and the hybrid a3 x wg3-h azg

The results from Table II show that the differences in enzyme activities between the tested cell lines of 100% and more, mentioned in Table I, are not due to possible variations during the growth of the cultures.

The influence of inhibitors (for instance ATP) or activators was tested by adding a known quantity of pure G6PD or 6PGD from yeast to the reaction mixture. Afterwards the amount of added enzyme was subtracted. The calculated values were compared with those obtained without addition of the yeast enzyme (Table III). The table shows that the measured $\frac{\Delta E}{\Delta T}$ of the G6PD and 6PGD in the cell extract equals the value of the measured $\frac{\Delta E}{\Delta T}$ of the cell extract plus the $\frac{\Delta E}{\Delta T}$ value of the added yeast enzyme.

During the measurement, NADPH is formed in two reactions. To eliminate the influence of the NADPH formed in the reaction by 6PGD, a proper amount of 6PG was added to reach maximal velocity of 6PGD. The 6PG formed by the reaction glucose-6-phosphate $\longrightarrow$ ribulose-5-phosphate cannot contribute to the 6PGD activity, since this enzyme is already operating at the maximal velocity. By subtracting the $\frac{\Delta E}{\Delta T}$ value of this maximal activity from the $\frac{\Delta E}{\Delta T}$ of the reaction mixture containing G6P and 6PG, the remaining activity is the $\frac{\Delta E}{\Delta T}$ value of the NADPH formed by the G6PD activity only.

It was found that the calculated sum of the $\frac{\Delta E}{\Delta T}$ values of the reactions mixtures measured with G6P and 6PG separately, equals the measured $\frac{\Delta E}{\Delta T}$ value of the reaction mixture containing both G6P and 6PG.

Table III G6PD (IIIA) and 6PGD (IIIB) Activities^a

IIIA	Concentration G6P (mM)	$\frac{\Delta E}{\Delta T}$ extract	$\frac{\Delta E}{\Delta T}$ added enzyme	$\frac{\Delta E}{\Delta T}$ extract + added enzyme	
				measured	calculated
	0.1	50	46	90	96
	0.3	58	46	108	104
	0.5	62	26	90	88
	1.0	64	26	90	90

IIIB	Concentration 6PG (mM)				
	0.2	36	16	50	52
	0.5	36	16	56	52
	1.0	32	16	50	48
	2.0	36	16	52	52

^aMeasured in $\frac{\Delta E}{\Delta T}$ units of 0.05 ml cell extract with and without addition of yeast G6PD or 6PGD enzyme in wg3-h cells.

DISCUSSION

After the first two months following fusion no progressive loss of genetic information was observed in the hybrids, as has been shown by karyogram analysis and study of the TK and HGPRT activities. The exception of the a23 x wg3-h hybrid, which showed an increase in TK⁻ clones, might be explained by assuming that the TK⁻ cells have overgrown the phenotypically wild type hybrid cells.

Comparison of the G6PD and 6PGD activities in diploid wg3-h with tetraploid a3 cells indicates a relationship between enzyme activity expressed per cell

and the number of genes coding for the enzymes. The hyperdiploid a23 cell line instead, shows the diploid level of 6PGD activity but the tetraploid level of G6PD activity, indicating the presence of a double set of chromosomes or chromosome parts carrying the G6PD locus.

The differences in enzyme activities between the cell lines mentioned in Table I cannot be attributed to variable factors like a different K_m value for G6PD or 6PGD, differences in cell cycle and growth phase, inhibitors or activators, or number of cells used for the determination. It was ascertained that the 6PGD activity does not contribute to the measured $\frac{\Delta E}{\Delta T}$ in the reaction of the G6PD measurement.

Hsia (1970) reviewed a comparable gene dose effect for fibroblasts derived from skin biopsies of patients with hereditary metabolic diseases. However, this seems not to be a general phenomenon, as has been shown by Nitowski and Grunfeld (1967) in fibroblast cell cultures from patients suffering from type II glycogenosis. In those studies cell strains were compared which were genetically different, and therefore interaction between genes might have masked differences in gene dose. The Chinese hamster cell lines used in the present experiments, and derived from the same parental DON cell line, provide a system in which all the cells have the same genetic information. The only difference is the multiplicity of some or all of the chromosomes. This might be the reason why the enzyme activities correlate with the gene dose. A similar gene dose effect is present in the hybrid cells cultivated in F12 HAT medium in which the activity of the hybrid cells is the sum of the activities found in the parental cells. These results support the idea that the gene dose-enzyme activity relationship can be used for linkage studies.

The $TK^- \times HGPRT^-$ hybrids grown in the presence of 8-azaguanine can only survive if they have lost the HGPRT activity. This can be achieved by a loss of a chromosome or a part of it carrying the HGPRT locus, a mutation of the $HGPRT^+$ locus or by some kind of inactivation of this locus. At the present time we have no evidence in favour of either one of these possibilities. However, our observations concerning the G6PD and the 6PGD activity in these hybrid cells present evidence that the loss of the HGPRT activity is accompanied by the loss of the genetic information coding for G6PD, whereas the 6PGD activity is not influenced. This phenomenon can be explained by assuming a linkage of the G6PD and HGPRT loci in Chinese hamster while 6PGD is not linked with these loci. As shown by Meera Khan et al. (1971) in man, G6PD is linked to HGPRT and 3-phosphoglycerate kinase (PGK). Experiments are in progress to study PGK and other Chinese hamster enzymes in this respect.

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chapter 5

EVIDENCE FOR LINKAGE BETWEEN 3-PHOSPHOGLYCERATE KINASE AND HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE LOCI IN CHINESE HAMSTER CELLS*

SUMMARY

The PGK activity was assayed in diploid, hyperdiploid, tetraploid and hybrid cells all originating from the same Chinese hamster cell line (DON line). A relationship between gene multiplicity and enzyme activity was observed. Selective pressure on the HGPRT locus by growth of hybrid cells in the presence of 8-azaguanine resulted in decreased levels of PGK activity. Growth of these hybrids in the presence of 5-BuDR did not influence the enzyme activity. It was concluded that the genes coding for HGPRT and PGK are linked in Chinese hamster. The TK locus seems neither to be linked to the PGK and G6PD loci nor to the 6PGD locus.

INTRODUCTION

In a previous paper (Westerveld et al., 1971) a relationship was described between the activities of the two enzymes glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) and the number of genes coding for these enzymes in Chinese hamster cells. This relationship was obtained by comparing the enzyme activities in cell lines with different numbers of chromosomes. Evidence was presented that the loci coding for G6PD and hypoxanthine-guanine phosphoribosyl transferase (HGPRT) are linked, whereas the gene coding for 6PGD is located on another chromosome.

The same mutant cell lines and intraspecific somatic cell hybrids have now

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been used to study the linkage between the HGPRT and 3-phosphoglycerate kinase (PGK) loci in Chinese hamster.

The results of these experiments suggest that in Chinese hamster the loci for G6PD, HGPRT and PGK are linked as it is in man.

MATERIALS AND METHODS

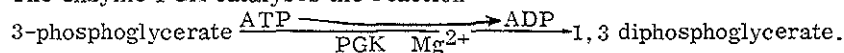
Cell lines and tissue culture techniques

The parental cell lines in the experiments were the 5-bromodeoxyuridine (5-BUDR) resistant cell lines a3 (about 44 chromosomes) and a23 (24 to 26 chromosomes) and the 8-azaguanine (azg) resistant cell line wg3-h (2n = 22). All mutant cell lines were isolated from the Chinese hamster cell line DQn. The details for the isolation procedure of the mutant cell lines as well as the techniques of tissue culture, cell hybridization and selection of cell hybrids have been described previously (Westerveld et al., 1971).

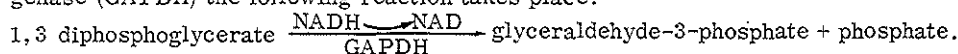
Thymidine kinase deficient (TK⁻) hybrid cells were selected from TK⁺ hybrids by growing the latter in F12 medium supplemented with 50 µg 5-BUDR per ml and lacking thymidine and hypoxanthine.

Enzyme assays

The enzyme PGK catalyses the reaction



In the presence of the muscle enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the following reaction takes place:



By using an excess of this enzyme the 1,3 diphosphoglycerate formed by the PGK reaction is converted instantly to glyceraldehyde-3-phosphate. The formation of NAD from NADH is a measure for the activity of PGK, because the amount of 1,3 diphosphoglycerate formed, is the rate limiting factor in the reaction catalyzed by GAPDH. The formation of NAD can be followed by measuring the decrease of the optical density at 340 nm. The conversion of 0.16 µmole/ml 3-phosphoglycerate gives a decrease of 1.0 optical density for 1 cm light path. The determinations of the enzyme activities were performed with various substrate concentrations and the activity was calculated at the optimal concentration. The Michaelis-Menten constant (Km) was obtained from a Lineweaver-Burk plot.

The composition of the reaction mixture was:

2.48 ml 0.1 M triethanolamine, pH 7.6 ; 0.1 ml EDTA (10 mg/ml) ; 0.5 ml NADH (10 mg/ml); 0.2 ml ATP (10 mg/ml) ; 0.04 ml 0.1 M MgSO_4 ; 0.01 ml glyceraldehyde-3-phosphate dehydrogenase (10 mg/ml) (Boehringer). To this reaction mixture was added 0.2 ml of 3-phosphoglycerate (3PG) in the final concentrations 0.5 ; 1.0 ; 2.0 ; 4.0 ; 6.0 ; 8.0 ; 10.0 mM. The reaction was initiated by the addition of 0.02 ml of the cell extract and performed at 25 C.

The assays for G6PD and 6PGD have been described elsewhere (Westerveld et al., 1971).

RESULTS

PGK activity in parental and hybrid cells

The PGK activity was determined in the parental cell lines and in the HGPRT⁺ and HGPRT⁻ hybrid cell lines. The HGPRT⁺ hybrids were selected and continuously grown in F12 HAT medium. From these hybrids HGPRT⁻ cells were selected in F12 medium supplemented with 3 μg 8-azaguanine per ml. To be able to survive, these hybrid cells must have lost the HGPRT activity. The results of these PGK assays are presented in Table I. The relative enzyme activities related to the activities of wg3-h cells are calculated to rule out variations between the different experiments.

Table I shows that the tetraploid a3 cells have about twice the PGK activity found in the diploid wg3-h cells. The a23 cells, differing from the wg3-h cells in only a few extra chromosomes, exhibit also twice the PGK activity of the diploid cells. The hybrid cells which were cultivated in F12 HAT medium contained the sum of the activities present in the parental cells. The PGK activities in the hybrids, cultivated in F12 azg are comparable with those found in wg3-h cells.

In the hybrid and the parental cell lines the Michaelis-Menten constant (K_m) of PGK for 3PG as the substrate was determined. The K_m value of this enzyme was 1.8×10^{-3} M in all the cell lines studied. These results indicate that the measured differences in enzyme activities between the cell lines were not due to a mutation in the PGK locus.

The experiments mentioned in Table I have been carried out with different numbers of cells. In control experiments a linear relationship was found between the number of cells used and the enzyme activity. The PGK activities shown in Table I have been determined at the third or fourth day after seeding of the cultures. In control experiments we tested the enzyme activity as a

Table I PGK Activity per Cell^a

Cell line	Experiment no.					Mean relative activity Exp. 1-5
	1	2	3	4	5	
wg3-h	4.3 (1.0)	3.9 (1.0)	5.9 (1.0)	4.1 (1.0)	4.6 (1.0)	1.0
a3	10.6 (2.5)	9.3 (2.4)	9.9 (1.7)	10.6 (2.6)	10.4 (2.3)	2.3
a3 x wg3-h azg	n t	4.4 (1.1)	5.4 (0.9)	4.0 (1.0)	4.6 (1.0)	1.0
a3 x wg3-h HAT	n t	14.4 (3.7)	14.5 (2.5)	18.8 (4.6)	15.8 (3.9)	3.7

	Experiment no.				Exp. 6-9
	6	7	8	9	
wg3-h	3.9 (1.0)	4.3 (1.0)	5.2 (1.0)	3.4 (1.0)	1.0
a23	9.0 (2.3)	9.4 (2.2)	11.2 (2.2)	7.8 (2.3)	2.3
a23 x wg3-h azg	5.0 (1.3)	6.0 (1.4)	5.1 (1.0)	4.8 (1.4)	1.3
a23 x wg3-h HAT	13.4 (3.4)	n t	17.7 (3.4)	15.7 (4.6)	3.8

^aExpressed in $\frac{\Delta E}{\Delta T}$ units $\times 10^{-5}$. The hybrids and parental cells were cultivated in F12 8-azaguanine (azg) or F12 HAT medium. In parentheses the relative activity related to the activity of wg3-h cells
nt is not tested.

function of time after seeding of the cultures. The results obtained with two parental lines and a hybrid line are presented in Table II.

Apparently the length of the period of growth after seeding is of some importance, but it is clear that this parameter cannot be responsible for the large differences in enzyme activities between the tested cell lines, shown in Table I.

The influence of inhibitors or activators of PGK present in the cell extract was studied by determining the PGK activities before and after addition of pure PGK (Boehringer) to the reaction mixture (Table III).

Table II PGK Activity per Cell^a

Days after seeding	wg3-h	a3	a3 x wg3-h azg
1	5.5	11.0	6.8
2	5.5	10.0	6.3
3	4.5	10.1	4.8
4	4.7	10.0	4.2

^aExpressed in $\frac{\Delta E}{\Delta T}$ units $\times 10^{-5}$ at different time intervals after seeding of the cultures wg3-h, a3 and the hybrid a3 x wg3-h cultivated in 8-azaguanine.

Table III PGK Activity^a

Concentration 3PG (mM)	$\frac{\Delta E}{\Delta T}$ extract	$\frac{\Delta E}{\Delta T}$ added enzyme	$\frac{\Delta E}{\Delta T}$ extract expected	+ added enzyme measured
2	20	30	50	48
4	34	30	64	61
6	44	30	74	70
8	64	30	94	94
10	62	30	92	94

^aExpressed in $\frac{\Delta E}{\Delta T}$ units of 0.02 ml cell extract of wg3-h cells with and without addition of pure PGK.

Table IV PGK (IVa), G6PD (IVb) and 6PGD (IVc) Activities per Cell^a

IVa cell line	Experiment no.			mean relative activity
	1	2	3	
wg3-h	5.4 (1.0)	4.9 (1.0)	4.6 (1.0)	1.0
a3	9.1 (1.7)	10.6 (2.2)	9.5 (2.0)	2.0
a3 x wg3-h azg BUdR	5.1 (0.9)	4.7 (1.0)	4.4 (1.0)	1.0
a3 x wg3-h HAT BUdR	15.1 (2.8)	15.6 (3.2)	15.3 (3.3)	3.1
a23 x wg3-h azg BUdR	4.9 (0.9)	4.6 (0.9)	5.1 (1.1)	1.0
a23 x wg3-h HAT BUdR	16.4 (3.0)	14.7 (3.0)	13.6 (3.0)	3.0
IVb	4	5	6	
wg3-h	0.9 (1.0)	0.8 (1.0)	0.7 (1.0)	1.0
a3	2.3 (2.6)	1.8 (2.3)	nt	2.5
a3 x wg3-h azg BUdR	1.0 (1.1)	1.0 (1.3)	0.9 (1.3)	1.2
a3 x wg3-h HAT BUdR	3.1 (3.4)	2.6 (3.3)	2.9 (4.1)	3.6
a23 x wg3-h azg BUdR	1.0 (1.1)	1.0 (1.3)	0.9 (1.3)	1.2
a23 x wg3-h HAT BUdR	3.2 (3.6)	2.6 (3.3)	2.7 (3.9)	3.6
IVc	7	8	9	
wg3-h	0.5 (1.0)	0.4 (1.0)	0.5 (1.0)	1.0
a3	1.2 (2.4)	0.9 (2.3)	nt	2.4
a3 x wg3-h azg BUdR	1.6 (3.2)	1.5 (3.8)	1.5 (3.0)	3.3
a3 x wg3-h HAT BUdR	1.8 (3.6)	1.3 (3.3)	1.8 (3.6)	3.5
a23 x wg3-h azg BUdR	1.1 (2.2)	1.0 (2.5)	1.2 (2.4)	2.4
a23 x wg3-h HAT BUdR	1.5 (3.0)	1.0 (2.5)	1.5 (3.0)	2.8

^aExpressed in $\frac{\Delta E}{\Delta T}$ units $\times 10^{-5}$ in the 5-BUdR resistant hybrids and in the parental cells. In parentheses the relative activities related to the activity of wg3-h cells.

nt is not tested

If inhibitors or activators are present the PGK activity after addition of the enzyme will not be equal to the sum of the activities in the extract and the added activity. The results of Table III show that the measured and expected values are in good agreement, indicating the absence of inhibitors or activators in the cell extract.

PGK, G6PD and 6PGD activities in hybrid cells after selective pressure on the thymidine kinase locus

A decrease in the PGK activities down to the level of the parental HGPRT⁻ cell wg3-h was observed in the hybrid cells cultured in 8-azaguanine. This decrease has also been found for the G6PD activities, whereas the 6PGD activities were not influenced by selective pressure on the HGPRT locus.

In order to study whether this decrease in enzyme activity only occurs in this selective system the relationship between gene dose and enzyme activity was also investigated after selective pressure on the TK locus. Cultivation of the HGPRT⁺ and HGPRT⁻ hybrids in 5-bromodeoxyuridine (5-BUDR) medium selects for thymidine kinase deficient (TK⁻) cells. The PGK, G6PD and 6PGD activities were studied in these hybrid cell lines. The results of these experiments are presented in Table IV.

The table learns that while selective pressure on the TK locus is present, the PGK, G6PD and 6PGD activities of the HGPRT⁺ TK⁻ hybrids (a23 x wg3-h HAT BUDR ; a3 x wg3-h HAT BUDR) are still equal to the sum of the parental cells as was also seen in these hybrids when cultivated in medium without 5-BUDR (Table I ; Westerveld et al., 1971).

DISCUSSION

The PGK activity expressed per cell in tetraploid cells was found to be twice the activity in the diploid cells. The PGK activity in hybrid cells cultivated in HAT medium is equal to the sum of the activity in the parental cells. Several factors that might influence enzyme activity and could attribute to the large differences found between the cell lines have been ruled out in control experiments. Moreover, their common origin and identical cell cycle (Westerveld et al., 1971) support the assumption that a linear relationship exists between enzyme activity and the number of genes coding for this enzyme. In experiments with the same cell lines this relationship has also been found for the enzymes G6PD and 6PGD (Westerveld et al., 1971). In the hyperdiploid cell line a23 the PGK activity is of the level present in the tetraploid cell line a3.

The G6PD activity in the a23 cell line is also comparable with that found in the tetraploid cell line a3, whereas 6PGD exhibits the activity present in the

diploid wg3-h cells (Westerveld et al., 1971). These results suggest that the genes coding for G6PD and PGK are located on those chromosomes of the a23 cell of which extra copies are present. The genetic information for 6PGD seems not to be located on these chromosomes.

The X-linkage of G6PD and HGPRT loci in man is well known and has been confirmed by somatic cell hybridization (Nabholz et al., 1969). The X-linkage of PGK has been recently demonstrated by pedigree analyses (Valentine et al., 1969) and cell hybridization studies (Meera Khan et al., 1971).

In a previous paper (Westerveld et al., 1971) evidence was presented for the linkage between the G6PD and HGPRT loci in Chinese hamster cells using intraspecific cell hybrids. In the present study it was shown that after cultivation in 8-azaguanine the loss of the HGPRT locus always was accompanied by a decrease in the PGK activity to the level of the parental wg3-h cell. The loss of the HGPRT locus is presumably caused by chromosome loss. The decreased PGK activity in these 8-azaguanine resistant hybrid cell lines can be explained by assuming a localization of the PGK locus on the chromosome which bears the HGPRT locus and is lost by the selection for HGPRT deficient hybrid cells.

Selective pressure on the TK^+ allele, performed by growing the $HGPRT^+$ hybrids in the presence of 5-BUdR, does not alter the PGK, G6PD and 6PGD activity.

These results underline the specificity of the changes in enzyme activity that have been found after selective pressure on the $HGPRT^+$ allele.

From these experiments it is concluded that in Chinese hamster the genes coding for the enzymes PGK, HGPRT and G6PD seem to be located on the same chromosome as is the case in man. Since the loss of the TK^+ allele does not influence the activities of PGK, G6PD and 6PGD in the $HGPRT^+$ hybrids, it seems likely that the thymidine kinase locus is neither linked to the PGK, G6PD and HGPRT loci nor to the 6PGD locus.

In male Chinese hamster cells the X-chromosome can be distinguished by 3H -thymidine labelling pattern during S phase (Taylor, 1960). To study the possible X-linkage of G6PD, HGPRT and PGK, experiments are in progress in which the number of X-chromosomes in parental and hybrid cell lines is related to the activities of these enzymes.

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chapter 6

CELL CYCLE OF MULTINUCLEATE CELLS AFTER CELL FUSION*

SUMMARY

The cell cycle of binucleate and multinucleate cells was studied after fusion of synchronized cells. A prolongation of the S phase in the nuclei of the binucleate cells was observed. Mitosis was not always synchronized in the homokaryons. The duration of the G1 phase in binucleate S/G1 cells is shortened. The binucleate cells and multinucleate cells are able to progress through the cell cycle for more than one generation.

INTRODUCTION

Okada et al. (1) were able to induce the formation of multinucleate cells by fusing together single cells in the presence of Sendai virus. Harris & Watkins (2). Harris (3) and Harris et al. (4) showed that both differentiated and undifferentiated cells from different species could successfully be fused together to form viable heterokaryons. In these multinucleate cells DNA synthesis has been extensively studied.

In heterokaryons formed by the fusion of Ehrlich and HeLa cells the nuclei do not synthesize their DNA synchronously (2). -Johnson & Harris (5) observed in these HeLa-Ehrlich heterokaryons even a form of antisynchrony. Most of the Ehrlich nuclei synthesized DNA whilst most of the HeLa nuclei did not.

In contrast to these observations with heterokaryons it was found that when homokaryons (cells containing genetically similar nuclei) are fused together, DNA-synthesis in the first cell-cycle after cell fusion is synchronized (6, 7).

* By A. Westerveld and Maria A. Freeke (1971), *Exptl. Cell Res.* 65, 140-144.

Recently Rao & Johnson (8) showed an induction of DNA-synthesis in nuclei of multinucleate HeLa cells produced by fusion between cells in different phases of the cell cycle. In the heterophasic G1/S cells DNA-synthesis was induced in more than 50% of the G1 nuclei within 2 h after cell fusion. DNA-synthesis was not induced in G2 nuclei after fusion of S phase cells with G2 phase cells.

In the present study DNA synthesis and the duration of the phases of the cell cycle during several cell generations after hybridization of genetically identical cells was investigated.

MATERIALS AND METHODS

A heteroploid cell line of human kidney origin (T cell) (9) was used in the experiments. These cells have a cell cycle of 24 h (G1, 12h; S, 8 h; G2, 4 h) (10). The cells were cultivated in F12 medium without thymidine, supplemented with 6% newborn calf serum and antibiotics. The cells were synchronized with the excess thymidine method (11, 12).

The technique used for inducing cell fusion was essentially similar to that described by Okada (13). For the fusion procedure a Sendai virus suspension with 500-1 000 haemagglutination units (HAU)/ml was used. After the cell fusion or the synchronization procedure the cells were seeded in Petri dishes containing 4 ml F12 medium and a sterile coverslip. These cultures were incubated at 37°C in a CO₂ incubator.

The percentage of nuclei synthesizing DNA in the multinucleate cells was determined after pulse labelling for 20 min with 1 or 2 μ Ci/ml ³H-thymidine (spec. act. 2.0 Ci/mmol). Cells cultivated for a certain period after labelling were maintained in medium containing 10 μ g/ml TdR to prevent further incorporation of ³H-TdR. The labelled cells were fixed with Bouin fluid and autoradiograms were prepared with Kodak AR10 stripping film. To determine the percentage of labelled nuclei, 500 nuclei were counted in each preparation. The mitotic index was obtained by counting 500-1 000 cells. The percentage of mitotic figures in multinucleate cells was obtained by counting 500 nuclei.

RESULTS AND DISCUSSION

Randomness of fusion with respect to the phases of the cell cycle was indicated by two findings: Firstly, 24 h after fusion of a ³H-thymidine pulse labelled asynchronous cell population the same percentage of labelled nuclei was found in multinucleate cells as was present in mononucleate cells before fusion (30%). Secondly, after fusion of prelabelled synchronized cultures the mononucleate

and the multinucleate cell population contained the same fraction of labelled nuclei (table 1). These results confirm the work of Yamanaka & Okada (6) and Johnson & Harris (7) who studied virus induced fusion of KB cells and HeLa cells respectively.

Table 1. The percentages of labelled nuclei in mononucleate and multinucleate cells 24 h after the fusion of ^3H -TdR pulse-labelled cells 2, 6 or 15 h after synchronization

Time of fusion after excess TdR treatment (hours)	Percentage of labelled nuclei	
	in mononucleate cells	in multinucleate cells
2	79	80
6	69	68
15	11	11

After fusion of randomly dividing cells it was found that in binucleate cells which contain a S phase and a G phase nucleus, DNA synthesis is induced in a certain percentage of the latter ones. To get more information about this induction of DNA synthesis and the duration of the S phase in binucleate cells, synchronized cells were hybridized. DNA synthesis in the binucleate and mononucleate cells was studied as a function of time after fusion by pulse labelling with ^3H -TdR. The frequency distribution of the S phase nuclei in the binucleate cells 4 h after fusion was found not to be different from the calculated value at the time of fusion (fig. 1).

From 4 up to 8 h after cell fusion an increase of the fraction of binucleate S/S cells (fig. 2b) was accompanied by a decrease of binucleate S/G cells (fig. 2c). This suggests an induction of DNA synthesis of G nuclei, apparently G1 nuclei, (because G2 nuclei are not present immediately after synchronization) in binucleate cells with a G phase and a S phase nucleus. This result is in agreement with the work of Rao & Johnson (3) who also observed an induction of DNA synthesis in G1 nuclei of HeLa cells after fusing with S phase cells. From 8 h up to 18 h after cell fusion an increase of the fraction of S/G cells was observed (fig. 2c). This finding can be expected because the G1 nucleus induced to synthesize DNA started this synthesis later than the nucleus that was already in S phase, and will also complete it at a later time. The proportion of binucleate S/G cells started to increase at 10 h after fusion (fig. 2d). In fig. 2 the theoretical values concerning binucleate cells with no, one and two nuclei labelled, as calculated from the observed DNA synthesis in mononucleate cells at that time (fig. 2a) were plotted. The S phase of the nuclei in S/S cells was

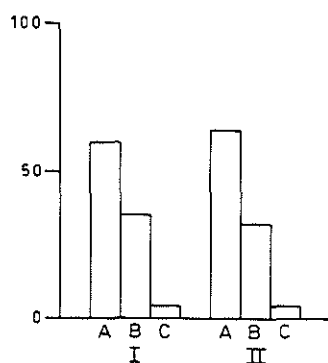


Fig. 1 Ordinate: percentage of nuclei.

The observed patterns (I) of nuclear labelling of binucleate cells 4 h after cell fusion compared with the calculated random patterns (II) expected when at the time of fusion 80% of the cells were in S phase.

A, both nuclei labelled; B, one nucleus labelled and one nucleus unlabelled; C, both nuclei unlabelled.

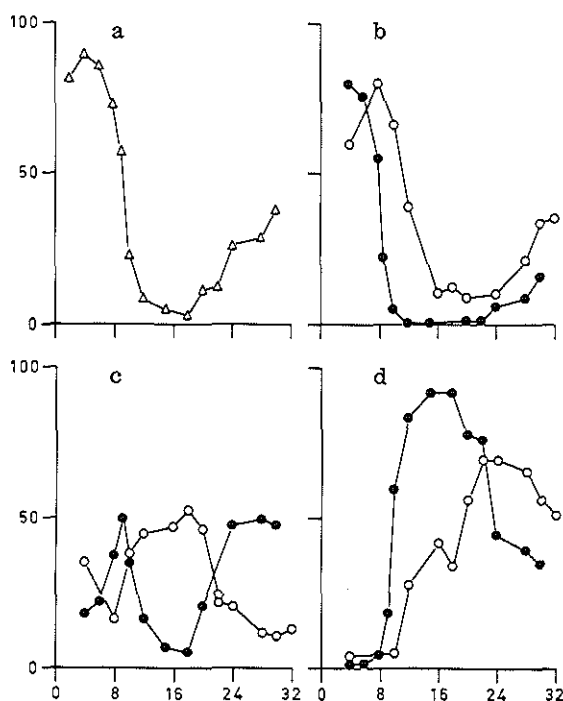


Fig. 2 Abscissa: hours; ordinate: percentage of cells.

(a) Percentage of mononucleate cells synthesizing DNA; (b) binucleate cells with both nuclei labelled (c) one nucleus labelled and one unlabelled and (d) both unlabelled following pulse labelling with $^3\text{H-TdR}$ at various time intervals after cell fusion of synchronized T cells. O—O, observed values; ●—●, expected values calculated from the random binomial expansions of the labelled mononucleate cells observed in the culture from 2 up to 30 hr after cell fusion.

lengthened by about 4 h as compared with the theoretical values (fig. 2b).

A relatively high proportion of S/G cells was observed from 10 h up to 20 h after cell fusion (fig. 2c). The calculated and observed data depicted in fig. 2 demonstrate that DNA synthesis in most of the binucleate cells with two S phase nuclei during the first hour after the cell fusion is not exactly synchronized. Starting about 23 h after cell fusion an increase of the percentage of the expected and observed binucleate S/S cells was present (fig. 2b). The increase of the observed and calculated value at the same time indicates that the duration of the G1 phase in the second cycle after the cell fusion in binucleate S/G1 cells is shortened in comparison with the duration of the G1 phase of the mononucleate cells.

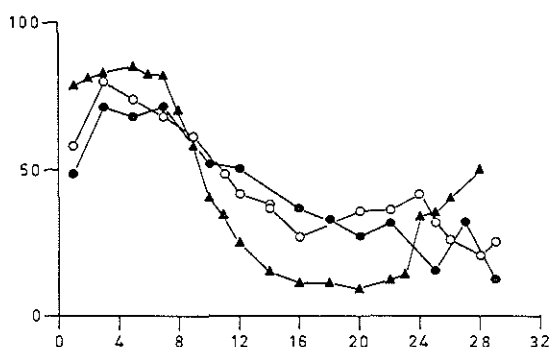


Fig. 3 Abscissa: hours; ordinate: percentage of labelled nuclei. DNA synthesis of nuclei in cells with 1 (▲—▲), 2 (O—O) or 3 (●—●) nuclei following pulse labelling with ^3H -TdR at various time intervals after synchronization with excess thymidine. The thymidine treatment was started 72 h after cell fusion.

The mitotic peak of the mononucleate cells was present about 8 h earlier than the mitotic peak of the multinucleate cells. It was found that mitosis was not synchronized in about 20% of the binucleate and in 45% of the tri- and tetra-nucleate cells.

Three days after cell fusion multinucleate cells and some binucleate cells are still present in the cultures. To get information whether these binucleate and multinucleate cells taking part in the cell cycle, the hybridized cell population was treated with excess thymidine for 24 h. Fig. 3 shows that 80% of the mononucleate cells synthesized DNA after the excess thymidine treatment. Seventy to 80% of the nuclei in binucleate and trinucleate cells were found to be synthesizing DNA indicating that the nuclei of the multinucleate cells had been synchronized by the excess thymidine treatment. This synchrony is also substantiated by the decrease in the labelling index reaching 30% after 14 h. The

duration of the S phase of the nuclei in the binucleate cells and trinucleate cells seems to be the same. The duration of the S phase of the mononucleate cells was shorter than that of the bi- and trinucleate cells. Since the slope for the accumulation function of the mononucleate cells in S phase is steeper than those for bi- and trinucleate cells (fig. 3) it is suggested that there is greater variation in the length of the S phase in bi- and trinucleate cells.

Three hours after the TdR treatment it was found that in 75% of the multinucleate cells all nuclei were synthesizing DNA (fig. 4). Starting 3 h after the TdR treatment an increase was found in the proportion of multinucleate cells with one or more nuclei in G phase. From 11 h after synchronization an increase of multinucleate cells with only G phase nuclei was observed. These results indicate that the nuclei of a large proportion of multinucleate cells progress through the cell cycle 4 days after fusion.

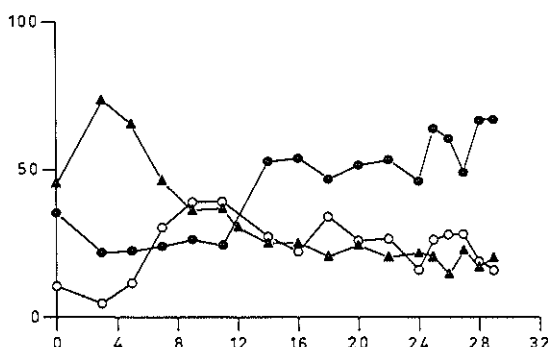


Fig. 4 Abscissa: hours; ordinate: percentage of multinucleate cells.

DNA synthesis in multinucleate cells following pulse labelling with ^3H -TdR at various time intervals after synchronization with excess thymidine. The thymidine treatment was started 72 h after cell fusion. ▲-▲, all nuclei labelled; ●-●, no nucleus labelled; ○-○, one or more nuclei labelled.

CONCLUSIONS

The results show that there is no preference of S, G1 or G2 cells participating in the cell fusion. Within a few hours of the fusion an induction of DNA synthesis of G nuclei, apparently G1 nuclei, can be seen in binucleate cells with a G phase and a S phase nucleus. The S phase of a relative large fraction of the nuclei in the binucleate cells seems to be lengthened. This is indicated by an increase in the time during which both nuclei can be labelled (fig. 2b) and the high fraction of binucleate cells with a labelled and an unlabelled nucleus. This latter observation suggests that the S phase in binucleate cells is not exactly

synchronized. The mitotic peak of the mononucleate cells is present about 8 h earlier than the mitotic peak of the multinucleate cells. Mitosis in multinucleate cells is not always synchronized. Binucleate cells formed by the fusion between two cells in S phase appear to complete this first cell cycle and, in the next cycle, the nuclei enter another round of synchronous DNA synthesis. The duration of the G1 phase in the second cycle after the fusion seems to be shortened. The binucleate and multinucleate cells present in the culture 4 days after cell fusion do still take part in the cell cycle, although these experiments do not prove that these multinucleate and binucleate cells are still present in the beginning of the fusion.

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SUMMARY

The purposes of the studies presented were the following: 1) the isolation of mutant lines from Chinese hamster DON cells; 2) the development of a interspecific cell fusion system, in which linkage of human genes can be studied; 3) the establishment of a method to study linkage in intraspecific hybrids; 4) the study of the fusion process in intraspecific combinations by analysis of the proliferation kinetics of multinucleate cells.

Properties of cell lines resistant to antimetabolites

From the Chinese hamster cell line DON a number of mutant lines were isolated, resistant to the antimetabolites, 5 BUdR, or 8-azaguanine, or 8-azaadenine. The resistant cells showed no/or a decreased incorporation in the nucleic acids of the compounds ^3H -thymidine, ^3H -guanine or ^3H -hypoxanthine and ^3H -adenine respectively. The resistance to 5-BUdR is caused by inactivity or absence of thymidine kinase. The karyograms of the cell lines were stable during the period of observation. No revertants were encountered in the 8-azaguanine resistant cell line wg3-h and the 5-BUdR resistant cell lines a3 and a23 during the observation period of over one year in the absence of the appropriate antimetabolite.

Linkage studies in man using man-Chinese hamster hybrid cell lines

Hybrid lines were obtained by fusing Chinese hamster mutant cell lines with a mutant human diploid cell strain or with human lymphocytes. The hybrids undergo preferential loss of human chromosomes.

The zymogram patterns of the following enzymes: HGPRT, G6PD, PGK, LDHA and LDHB, 6PGD, PGM 1, IPO, NAD-MDH and IDH were studied. The human IDH and MDH appeared to have been lost before the cloning of the hybrid cells lines. We found a positive linkage relationship between the human genes for 6PGD and PGM 1. Furthermore, a positive correlation was observed between the human HGPRT, G6PD and PGK loci. No positive correlation was found between the other human markers.

Linkage studies in Chinese hamster with Chinese hamster-Chinese hamster hybrid cell lines using a relationship between gene multiplicity and enzyme activity

G6PD, PGK and 6PGD activities were determined in diploid, hyperdiploid, tetraploid and hybrid cell lines all originating from the Chinese hamster cell line DON. A relationship between gene multiplicity and enzyme activity was

observed. The same enzymes were studied in the hybrid cells cultivated in selective media. Selective pressure on the HGPRT locus by growth of hybrid cells in the presence of 8-azaguanine resulted in decreased levels of G6PD and PGK activities. Growth of the HGPRT⁺ hybrids in the presence of 5-BUdR did not influence the activities of G6PD, PGK or 6PGD. It was concluded that the genes coding for HGPRT, G6PD and PGK are linked in Chinese hamster. The TK locus seems neither to be linked to the G6PD and PGK loci nor to the 6PGD locus.

Cell cycle of multinucleate cells after cell fusion

The cell cycle of binucleate and multinucleate cells was studied after fusion of synchronized cells. A prolongation of the S phase in the nuclei of the binucleate cells was observed. Mitosis was not always synchronized in the homokaryons. The duration of the G1 phase in binucleate S/G1 cells is shortened. The binucleate and multinucleate cells are able to progress through the cell cycle for more than one generation.

SAMENVATTING

Doel van het onderzoek was: 1) met mutantcellijnen van mens en Chinese hamster een interspecies hybridisatie systeem te ontwikkelen waarin koppeling van menselijke genen bestudeerd kan worden; 2) het ontwikkelen van een methode voor het onderzoek van koppeling in intraspecies hybride cellen; 3) het bestuderen van het fusieproces in intraspecies combinaties door de proliferatie kinetiek van meerkernige cellen na fusie te analyseren.

Eigenschappen van cellijnen die resistent zijn tegen antimetabolieten

Uit de Chinese hamster fibroblasten cellijn DON ($2n=22$) afkomstig van longweefsel werden mutant cellijnen geïsoleerd die resistent waren tegen de antimetabolieten 5-bromodeoxyuridine (5-BUdR) of 8-azaguanine (azg) of 8-azaadenine (aza). Aangetoond werd, dat deze resistentie gepaard ging met een sterk verminderde inbouw in de nucleinezuren van respectievelijk de radioactieve verbindingen ^3H -thymidine, ^3H -guanine of ^3H -hypoxanthine en ^3H -adenine. De resistentie tegen 5-BUdR werd veroorzaakt door afwezigheid van de activiteit van het enzym thymidinekinase. Chromosoomtellingen wezen uit, dat de karyogrammen van deze lijnen stabiel zijn.

Aantonen van koppeling van genen bij de mens met behulp van mens-Chinese hamster hybriden

Uit fusie van menselijke cellen (lymfocyten zowel als fibroblasten) met Chinese hamster fibroblasten werden een aantal hybride cellijnen geïsoleerd. In deze hybride cellijnen werd een verlies van menselijke chromosomen waargenomen. Dit verlies werd bevestigd door de waarneming, dat in de hybride cellen mens (HGPRT⁻) - Chinese hamster (TK⁻), het TK verloren ging, terwijl in de hybriden menselijke lymfocyten - Chinese hamster (HGPRT⁻), het HGPRT verloren ging.

Het verlies van menselijke genetische informatie werd ook vastgesteld door elektroforetisch onderzoek van de enzymen HGPRT, G6PD, PGK, LDH A en LDH B, G6PD, PGM1, IPO, NAD-MDH, en IDH.

Het menselijke IDH en MDH waren tijdens de eerste test, ongeveer vier weken na het fuseren van de oudercellen al niet meer aantoonbaar. Aanwijzingen werden verkregen, dat de loci coderend voor de enzymen 6PGD en PGM1, op hetzelfde chromosoom zijn gelegen. Verder werd de koppeling tussen de loci voor HGPRT, G6PD en PGK bevestigd. Geen koppeling werd aangetoond tussen de overige geteste menselijke markers.

Het aantonen van koppeling van genen met behulp van intraspecies Chinese hamster-Chinese hamster hybriden

Na fusie van de diploide HGPRT deficiënte Chinese hamster cellijn met een hyperdiploide en een tetraploide cellijn, beiden deficiënt voor het enzym thymidine kinase, werden in een selectie medium met hypoxanthine (H), aminopterine (A) en thymidine (T) hybride cellijnen geïsoleerd. TK of HGPRT deficiënte oudercellen zijn dan aangewezen op de "de novo" synthese van de nucleïnezuren. Aminopterine remt deze synthese van de nucleïnezuren waardoor de oudercellen weggeselecteerd worden, terwijl de hybride cellen in dit HAT selectie medium kunnen groeien.

In deze hybride cellen werd gedurende de eerste maanden na de fusie slechts een gering verlies van chromosomen waargenomen. De oudercellen verschillen onderling in chromosomenaantal en aangetoond werd, dat er een relatie bestaat tussen de activiteit van de enzymen G6PD, PGK en 6PGD en het aantal in de cel aanwezige genen dat voor deze enzymen codeert.

In de hybride cellen, gekweekt in HAT medium, waren de G6PD en PGK activiteiten gelijk aan de som van de respectievelijke activiteiten in de oudercellen. Ook werden de hybride cellen gekweekt in 8-azaguanine bevattend medium. De cellen die in dit medium kunnen groeien moeten HGPRT deficiënt zijn. In deze cellen bleken de G6PD en PGK activiteiten dezelfde te zijn als in de HGPRT⁻ oudercellijn. De 6PGD activiteit bleek zowel in de hybriden gekweekt in 8-azaguanine als HAT medium, gelijk te zijn aan de som van de activiteiten in de oudercellen. Uit bovenstaande gegevens werd de conclusie getrokken dat in de Chinese hamster de loci voor G6PD, HGPRT en PGK op hetzelfde chromosoom gelegen zijn.

Het kweken van deze hybriden in 5-BUDR bevattend medium beïnvloedde de G6PD, PGK en 6PGD activiteiten niet. Hieruit werd geconcludeerd dat het TK locus niet gekoppeld is met de loci voor HGPRT, G6PD, PGK en 6PGD.

De celcyclus van meerkernige cellen na celfusie

De celcyclus van twee- en meerkernige cellen werd bestudeerd na fusie van met overmaat thymidine gesynchroniseerde menselijke cellen (T-cellijn). De S fase van de tweekernige cellen is verlengd in vergelijking met de S fase van de eenkernige cellen. Ook werd aangetoond, dat de eenkernige cellen na synchronisatie eerder in mitose gaan dan de meerkernige cellen. In 20% van de tweekernige cellen was de mitose niet gesynchroniseerd. In de drie- en vierkernige cellen bedroeg deze asynchroniteit 45%. De G1 fase in de tweekernige cellen met een S- en G1 fase kern is verkort. De twee- en de meerkernige cellen nemen waarschijnlijk langer dan één generatie deel aan de celcyclus.

ABBREVIATIONS

ADP	adenosine diphosphate
ALS	horse anti human lymphocyte serum
AMP	adenosine monophosphate
APRT	adenine phosphoribosyl transferase
ATP	adenosine triphosphate
aza	8-azaadenine
azg or 8-azag	8-azaguanine
BSS	Hank's balanced salt solution
5-BUdR	5-bromodeoxyuridine
CK	deoxycytidine kinase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
G1 phase	interphase period in between mitosis and DNA synthesis
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G2 phase	interphase period in between S phase and mitosis
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
HAT	hypoxanthine aminopterin thymidine
HAU	hemagglutination units
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
IDH	isocitrate dehydrogenase
IPO	indophenol oxidase
Km	Michaelis-Menten constant
LDH	lactate dehydrogenase
NAD	nicotinamide-adenine-dinucleotide
NADH	reduced form of nicotinamide-adenine-dinucleotide
NAD-MDH	NAD-dependent soluble malate dehydrogenase
NADP	nicotineamide-adenine-dinucleotide phosphate
NADPH	reduced form of nicotinamide-adenine-dinucleotide phosphate
3PG	3-phosphoglycerate
6PG	6-phosphogluconate
6PGD	6-phosphogluconate dehydrogenase
PGM 1	phosphoglucomutase 1
PGK	3-phosphoglycerate kinase
POPOP	2,2'-p-phenylene-bis-(4-methyl-5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazol
RNA	ribonucleic acid
S phase	interphase period during which DNA is replicated

TCA	trichloroacetic acid
TDP	thymidine diphosphate
TdR	thymidine
TMP	thymidine monophosphate
TTP	thymidine triphosphate
TK	thymidine kinase
UV	ultraviolet light

NAWOORD

Op verzoek van de faculteit der Geneeskunde volgen hier enkele persoonlijke gegevens.

Ik bezocht van 1952 tot 1957 het Christelijk Lyceum te Hogeveen. Na het afleggen van eindexamen H.B.S.-B begon ik in 1957 met de studie biologie aan de Vrije Universiteit te Amsterdam. In 1960 werd het kandidaatsexamen afgelegd; het doctoraal examen met als hoofdvak endocrinologie en bijvakken dierfysiologie en plantenfysiologie in 1965.

Tijdens het vervullen van de militaire dienstplicht werd ik gedetacheerd op het Medisch Biologisch Laboratorium TNO te Rijswijk. Medio 1967 werd begonnen met het beschreven onderzoek.

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