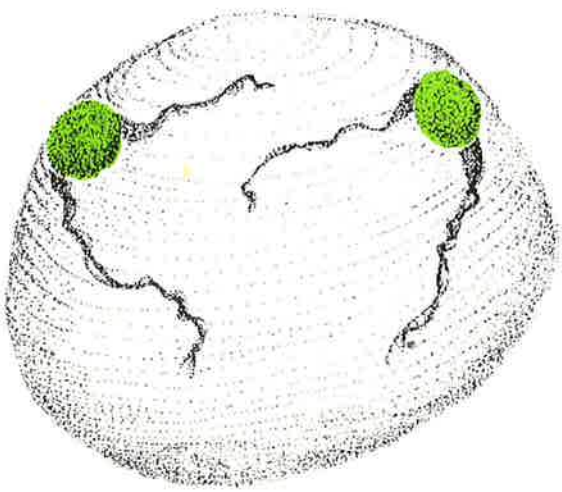
IN SITU HYBRIDIZATION AS A TOOL IN CYTOGENETICS

H. van Dekken



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STELLINGEN

1

Door het vermijden van hittedenaturatie met de Exonuclease-methode kan *in situ* hybridisatie een belangrijke techniek worden voor het celbiologisch onderzoek van kwetsbare structuren.

(Dit proefschrift)

2

Het is aannemelijk, dat binnen de intranucleaire architectuur van cellen uit het haemopoëtisch systeem een grote mate van flexibiliteit bestaat in de ruimtelijke organisatie van chromosomen.

(Dit proefschrift)

3

Interfase cytogenetica m.b.v. *in situ* hybridisatie, b.v. met een chromosoom Y-specifieke repetitieve DNA probe, leent zich uitstekend voor analyse met beeld- en flowcytometrische technieken.

(Dit proefschrift)

4

De hoge frequentie van afwijkingen van het chromosoom 1 in tumoren is niet gerelateerd aan de grootte van dit chromosoom.

(Atkin, Cancer Genet. Cytogenet. **21**: 279, 1986)

5

De bevinding van Lawrence et al., dat specifieke mRNA's via "kanaaltjes" in de celkern naar het cytoplasma gaan, onderschrijft de z.g. "gene gating" hypothese.

(Lawrence et al., Cell **57**: 493, 1989)

6

Interfase cytogenetica m.b.v. *in situ* hybridisatie is de aangewezen methode voor de cytogenetische bestudering van solide tumoren.

7

De behandeling van neutropenie met "recombinant human granulocyte colony-stimulating factor" (rhG-CSF), kan een alternatief worden voor beenmergtransplantatie bij congenitale en andere agranulocytosen.

(Bonilla et al., N. Engl. J. Med. **320**: 1574, 1989)

8

Het is te verwachten, dat de hoge resolutie van de "scanning tunneling microscope" (STM) en aanverwante apparaten een beter inzicht zal verschaffen in de complexe dynamiek van biologische oppervlaktestructuren, zoals de celmembraan.

9

De behandeling van solide tumoren met Interleukine-2 geactiveerde witte bloedcellen (LAK- en TIL-cellen) dient, ondanks enkele bemoedigende resultaten, op dit moment te worden beschouwd als palliatieve therapie.

10

Het lijkt nu wel zeker, dat de AIDS-epidemie 20-40 jaar geleden in Afrika is begonnen als een besmetting van aap naar mens.

(Hirsch et al., Nature **339**: 389, 1989).

11

Het moeilijke onderscheid tussen kanker ten gevolge van lage doses ioniserende straling op de arbeidsplaats en kanker door natuurlijke straling vereist duidelijke richtlijnen wat betreft wettelijke aansprakelijkheid.

(Jose, Radiat. Res. **117**: 181, 1989)

12

De betrouwbaarheid van voorspellingen van aardbevingen op de San Andreas breuk in Californië is omgekeerd evenredig aan de hoeveelheid research hiernaar.

13

De problemen geschapen door de Europese autoindustrie rond de invoering van de katalysator voor personenauto's staan in geen verhouding tot het milieu-maatschappelijk belang hiervan.

H. van Dekken
11 oktober 1989

IN SITU HYBRIDIZATION AS A TOOL IN CYTOGENETICS

PROEFSCHRIFT

ter verkrijging
van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus
Dr. J.J.M. Beenakker,
hoogleraar in de Faculteit der
Wiskunde en Natuurwetenschappen,
volgens besluit van het College van Dekanen
te verdedigen op
woensdag 11 oktober 1989
te klokke 16.15 uur

door

HERMAN VAN DEKKEN

geboren te Rotterdam in 1956

PROMOTIECOMMISSIE:

Promotoren:	Prof. dr. D.W. van Bekkum Prof. dr. M. van der Ploeg
Co-promotor:	Dr. J.G.J. Bauman (Erasmus Universiteit Rotterdam)
Referent:	Dr. A.H.N. Hopman (Katholieke Universiteit Nijmegen)
Leden:	Prof. dr. P. van Duijn
	Prof. dr. N. Nanninga (Universiteit van Amsterdam)

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CONTENTS

GENERAL INTRODUCTION

Non-isotopic *in situ* hybridization or hybridocytochemistry of DNA has made tremendous progress in the last decade. Introduced as a new but not too sensitive tool for the localization of specific nucleic acids in microscopic preparations, it developed rapidly into a fast, high-sensitivity, high-resolution detection procedure. It allows for the cytogenetic analysis of specific repetitive and single copy sequences on chromosomes as well as in cell nuclei for clinical and cell biological purposes.

OBJECTIVE OF THIS THESIS

The study, described in this thesis, was aimed at the development of a fluorescent *in situ* hybridization (FISH) procedure to detect DNA sequences in cells and cell nuclei in suspension. In this way quantitation of the hybridized target by flow cytometry is possible. Since the morphology of the objects was preserved throughout the procedure, computer-aided microscopy could be used to provide information concerning the spatial topography of the hybridized chromosome regions. An important factor was the sample preparation, in particular the fixation of the cells or cell nuclei. During the FISH method the objects undergo harsh chemical and physical treatment, e.g. heating, formamide. Therefore, the cells should be prepared such, that ideally, optimal accessibility of the probe to its target sequences is combined with preservation of the morphological properties of the nuclei.

In the first part of the thesis, the method itself is introduced, and the possibilities of FISH are outlined. The three-dimensional (3-D) analysis and reconstruction of hybridized nuclei in the first chapter were performed by the author of this thesis. An enzymatic technique for the production of ssDNA for FISH was developed. Due to its mild biochemical and physical characteristics, when compared with standard heat denaturation, this technique might preserve the morphology of the cells better. The feasibility and sensitivity of flow cytometric FISH was studied in human lymphocytes.

In the second part three-dimensional analysis by computer-aided fluorescence microscopy and confocal microscopy is applied to investigate the spatial organization of specific chromosome regions within cell nuclei. The enzymatic technique for ssDNA production was used for the simultaneous FISH to ribosomal RNA and centromeric DNA in suspended cells.

In the third part clinical applications for FISH are demonstrated in hybridizations on preparations on microscopic glass slides. The chromosome 1p centromere-telomere probe combination could be very useful for the study of the position of chromosome arms inside nuclei, e.g. the 3-D topography of centromeres and the corresponding telomeres. The detection of low numbers (below 1/100) of residual leukemic cells after sex-mismatched bone marrow transplantation by FISH with a chromosome Y-specific probe is at present beyond the sensitivity level of flow cytometric FISH, although its clear application.

THE DEVELOPMENT OF NON-ISOTOPIC *IN SITU* HYBRIDIZATION

The labeling of nucleic acids

The technique of hybridocytochemistry was introduced by Gall and Pardue and John et al. in 1969, using radioactively labeled probes. At that time it was called *in situ* hybridization (ISH), to emphasize the difference with the biochemical hybridization method, introduced in 1961 by Hall and Spiegelman. Since analysis by ISH in reality is not performed *in situ*, but on microscopic preparations, be it glass slides or cell suspensions, the name hybridocytochemistry would be more correct for this elegant method. This term, however, is rarely used in the literature. Using radioactive isotopes, the insulin gene was mapped to chromosome 11 by Harper et al. in 1981. The first reports on non-isotopic ISH came from Rudkin and Stollar in 1977. After hybridization of the 5S ribosomal RNA genes on Drosophila polytene chromosomes with a RNA probe, they used a rabbit-derived antibody against DNA-RNA hybrids for detection. The antibody was visualized by a second fluorochrome-labeled antibody against rabbit immunoglobulins. This technique was further explored by Van Prooijen-Knegt et al. (1982), but the difficulty in obtaining good anti-DNA-RNA antibodies impaired further development. A direct approach was used by Bauman et al. (1980), who applied fluorochrome-labeled RNA as a probe for the detection of kinetoplast DNA in the insect Crithidiae Luciliae and of 5S ribosomal RNA genes in Drosophila melanogaster.

The most important labeling techniques for ISH came with the introduction of enzymatically or chemically modified nucleic acid probes. In 1981 Langer et al. reported the first enzymatic synthesis of biotin-labeled polynucleotides. In 1982 the first applications were reported by using this technique for mapping genes on Drosophila chromosomes (Langer-Safer et al., 1982) and centromeric DNA on mammalian chromosomes (Manuelidis, 1982). The detection of the biotinylated hybrids is accomplished by anti-biotin antibodies or avidin, coupled to fluorochromes or enzymes. The first chemical introduction of a molecule, that could serve as a hapten for immunological detection, into the DNA was described by Tchen et al. and Landegent et al. (both in 1984). They used 2-acetylaminofluorene (AAF) to modify the guanine residues in the nucleic acids. This technique was further refined by Landegent et al. (1985), who reported the first detection of a single copy sequence by non-isotopic ISH. Using AAF-modified DNA probes and anti-AAF antibodies, these authors were able to localize part of the human thyroglobulin gene. Hopman et al. (1986) modified the cytosine bases within a genomic human DNA probe with mercuric acetate to achieve the simultaneous detection of mercurated human DNA and biotinylated mouse satellite DNA in a hybrid cell line, using different colors for visualization of the probes. The first triple color ISH was reported by

Nederlof et al. (1988), using a combination of biotinylated, mercurated and AAF-modified probes.
The latest modification procedures for ISH to be reported, were the use of sulfonated probes (Morimoto et al., 1987), and the incorporation of digoxigenin into the cytosine residues of the DNA (Manual Boehringer). In both cases the hybrids are detected by specific antibodies and labeling materials for both procedures are commercially available. However, the most frequently used labeling system is the biotin-avidin or streptavidin system, which is easy to use and widely available.

The sensitivity and application of hybridocytochemistry

When non-isotopic hybridocytochemistry was introduced, only large targets could be detected. This was in contrast with the radioactively labeled probes, which were able to detect smaller targets. But the higher speed of analysis and the more precise localization properties made non-isotopic ISH very attractive for cytogeneticists. Due to the absence of radioactive waste, laboratory space and equipment could be reduced. Two detection schemes are generally employed. The first uses a fluorochrome, such as fluorescein (FITC) or rhodamin (TRITC). The second uses enzymes, such as peroxidase or alkaline phosphatase. The first might yield a better resolution, the second a somewhat higher sensitivity and more permanent preparations. A rapid improvement came as a result of new and more sensitive labeling procedures. At first only repetitive DNA sequences (Cook and Hindley, 1979; Burk et al., 1985; Willard, 1985; Buroker et al., 1987) were used with targets, ranging from 100 Kb up to a few Mb's (megabase = 1000 Kb). Among those targets were the ribosomal RNA genes on the acrocentric chromosomes, the alpha-satellite DNA's on the centromeres and the repetitive sequences on the long arm of the Y chromosome (Bauman et al., 1981; Manuelidis, 1982; Pinkel et al., 1986b). As a consequence of these results, more scientists started to use hybridocytochemistry as an instrument to label specific DNA sequences. The refinement of the procedure ultimately led to the detection of single copy sequences. In 1985 Landegent et al. were able to detect a 22 Kb unique sequence using an AAF-modified probe. Recently, several investigators reported the detection of single copy genes, a few Kb in size (Garson et al., 1987; Ambros and Karlic, 1987; Lawrence et al., 1988). Lawrence et al. achieved to detect a 5 Kb unique sequence in interphase nuclei. This has made non-isotopic ISH suitable for gene mapping purposes and nearly as sensitive as radioactive ISH, but preferable to the latter with respect to the speed and resolution.
Non-isotopic ISH with chromosome specific repetitive DNA probes, mostly alpha satellite DNA, appeared to be very useful in detecting numerical chromosome aberrations in interphase nuclei (Pinkel et al., 1986b; Moyzis et al., 1987; Devilee et al., 1988). Hopman et al. (1988)

and Cremer et al. (1988a), who introduced the phrase "Interphase Cytogenetics" for this procedure, used bicolor double-target ISH to detect simultaneously numerical aberrations of both chromosomes 1 and 18, using biotin-, AAF- and Hg-labeled probes. The investigation of chromosome aberrations in interphase nuclei is of great importance to cancer research, since most tumors have low mitotic indexes (Sandberg and Turc-Carel, 1987). At present, satellite DNA probes for the majority of the human chromosomes are available (Willard and Waye, 1987). The latest development with respect to the detection of numerical and structural chromosome aberrations in interphase and metaphase cells came with the application of chromosome specific libraries as probes for ISH (Cremer et al., 1988b; Pinkel et al., 1988). By this method the whole chromosome is labeled, facilitating numerical aberration detection in interphase nuclei, and simplifying the identification of structural aberrations in metaphase figures.

Non-isotopic ISH in suspension

The first non-isotopic ISH to nuclei in suspension was reported by Trask et al. (1985). They hybridized mouse thymocyte nuclei with mouse satellite DNA and measured the probe related fluorescence on a flow cytometer. In this way the quantitation of target sequences by flow cytometry became possible. In a follow-up paper the detection of specific chromosomes within human cell line nuclei was described (Trask et al., 1988). Since the procedure was performed in suspension, the nuclei could also be used to determine the three dimensional localizations of the resulting specific hybridization signals by means of computer-aided fluorescence microscopy.

The first ISH to metaphase chromosomes in suspension was described by Dudin et al. (1987). They were able to visualize the presence of human chromosomes in a human–hamster hybrid cell line. They also used this procedure to isolate the human chromosomes, hybridized with biotinylated genomic DNA, with streptavidin-labeled magnetic beads (Dudin et al., 1988).

THE ORGANIZATION OF DNA INTO CHROMOSOMES AND NUCLEI

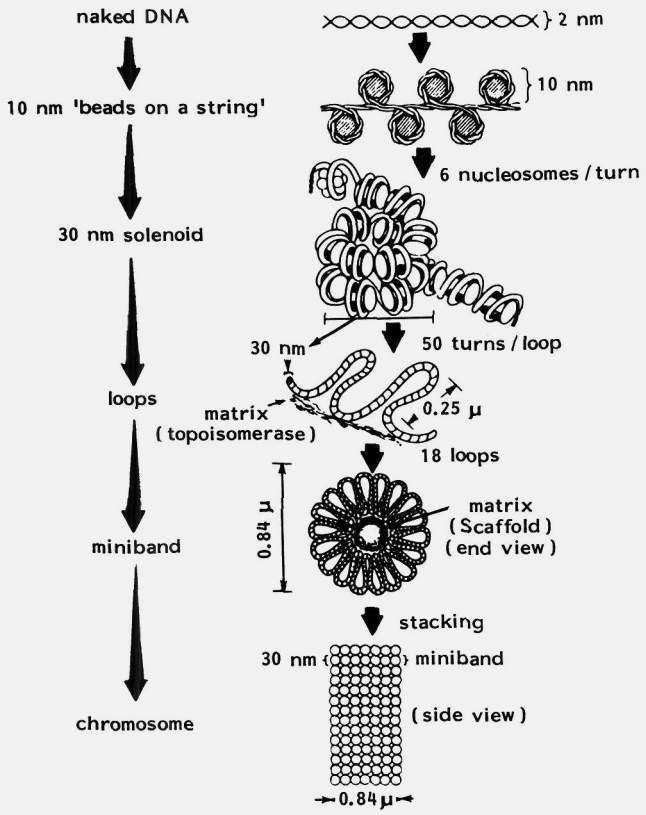
The organization of DNA in the nucleus

A mammalian diploid nucleus contains a total length of almost 200 cm of DNA. The packing of this amount of DNA into a nucleus of 10 μm diameter presents mammalian cells with a formidable topological problem: The total length of cellular DNA must be reduced about 50,000 fold to fit within the confines of a single nucleus (from Nelson et al., 1986). On the other hand, despite this packing ratio, the DNA should still be accessible and capable of performing a variety of biological activities.

Three higher-order levels of organization have been identified in the last decade (Nelson et al., 1986). The first one is the nucleosome, which is the folding of the 10 nm DNA fiber around the histone core. This is also known as the "beads on a string" configuration (fig. 1), which renders a packing ratio of 6-7. The second level of DNA organization is the 30 nm fiber or "solenoid", in which the 10 nm fiber is packed and twisted another 7 times. This 30 nm fiber is anchored to structural elements in the nuclear matrix, thus forming the third higher-order level of organization, the loop-domain (fig. 1). In the chromosome, these loops are positioned radially on the chromosome scaffold, forming the miniband, which approaches the smallest bands seen in cytological Giemsa bands of metaphase chromosomes. Altogether, the final packing order is 1.2 x 10,000, which is identical to the model, proposed for the mitotic chromosome (Paulson and Laemmli, 1977; Nelson et al., 1986). For extended reviews, see Nelson et al., 1986, and Newport and Forbes, 1987.

The organization of DNA in the mitotic chromosome

The introduction of electron microscopy (EM), combined with sophisticated digital contrast-enhancement techniques, facilitated the study of the organization of the 30 nm fiber in mitotic chromosomes. Bak et al. (1977) proposed from EM studies, that the 30 nm fiber was folding and coiling to form a hollow cylindrical structure with a diameter of 400 nm within mitotic chromosomes. Ten years later, the track of the of the 30 nm fiber could be visualized by transmission EM and computer reconstructions (Belmont et al., 1987). It was found, that the mitotic chromosomes of Drosophila melanogaster showed a defined, but complex pattern of chromatin structural domains with cross-sectional diameters of 12 to 100 nm, upto 300 nm in fully condensed metaphase chromosomes. Using a similar approach, Borland et al. (1988) found the human lymphocyte metaphase chromosome being organized in 100-350 nm diameter segments with an axial extent of about 200 nm. It can be expected, that the organization of DNA in mitotic chromosomes, at present not fully understood, will be elucidated, when more refined hard- and software has been developed to visualize the intricate structure.



base pairs per turn	packing ratio
10 b.p.	1
80 b.p. (160 b.p. per 2 turns)	6-7
1.200 b.p. (per turn)	40±
60.000 b.p. (per loop)	680
1.1×10^6 b.p.± (per miniband)	1.2×10^4
18 loops / miniband	1.2×10^4

Figure 1:

Schematic diagram of the organization of DNA in nuclei and metaphase chromosomes (reprinted from Nelson et al., 1986).

The dynamics of nuclear organization

The enclosure of DNA within in its own compartment, the nucleus, was a major event in evolution, that separated eukaryotic from prokaryotic organisms. The development of the nucleus was likely the result of increased organizational and regulatory problems, associated with the large genomes of more complex organisms (from Newport and Forbes, 1987). A major role in nuclear function is played by the nuclear matrix associated enzyme topoisomerase II (Earnshaw and Heck, 1985). This enzyme permits the densily packed DNA molecule to perform its biological activities by affecting the organization of the loop-domain (Berrios et al., 1985). How non-transcribed repetitive sequences (Schmid and Jelinik, 1982; Willard, 1985) and active transcribing genes fit in this system is largely unknown. In 1985 Blobel proposed the "gene gating" model. In this hypothesis, active (transcribing) genes move towards the nuclear pores (Maul et al., 1972) to release their product into the cytoplasm. The implication is a highly dynamic DNA organization, in which chromosome arms are very flexible. In this view active genes are more susceptible to DNAse treatment (Weisbrod, 1982), due to accessibility. The understanding of the dynamic structure of DNA organization requires a much more detailed insight of the structure and three dimensional architecture of chromosomes.

THE SPATIAL ORGANIZATION OF CHROMOSOMES IN THE NUCLEUS

Approaches to study metaphase and interphase cells

Already at the end of the last century scientists investigated the organization of chromosomes in nuclei. Speculations were made about chromosome domains and alignment of chromosomes within interphase nuclei (for reviews, see Comings, 1980; Hubert and Bourgeois, 1986). Rabl (1885) proposed the hypothesis, that the orientation of centromeres and telomeres, seen in a mitotic cell, is maintained in interphase. This clustering of centromeres and telomeres at opposite poles of the nucleus, known as the Rabl orientation, is still a matter of investigation and controversy. Boveri (1888) showed, that in Ascaris eggs, chromosomes had a relatively fixed position in the nucleus and remained as discrete entities in interphase. This was the first observation of chromosomal domains or territories, which suggests, that each chromosome occupies a confined space within the nucleus.

In the last decade much attention has been paid to the organization of chromosomes in interphase and metaphase cells. Using UV-microirradiation to mark chromatin territories, these domains were seen in interphase (Zorn et al., 1979), and during cell cycle (Hens et al., 1983). Hager et al. (1982) analyzed non-homologous chromatid translocations in patients with Fanconi's anemia and Bloom's syndrome to arrive at a pseudo 3-D model of intranuclear chromosome organization. In a study on the positions of Barr bodies in a XXX fibroblast cell line, the two Barr bodies appeared to be localized at the nuclear periphery without having a significant correlation with each other (Belmont et al., 1986). Moroi et al. (1981) saw the centromeres of mammalian interphase nuclei associated with the nuclear surface or with the nucleoli, using anti-centromere antibodies. Applying this same anti-serum, Hadlaczky et al. (1986) found non-random localization patterns of mammalian chromosomes in interphase nuclei. They observed pairing of homologues and signs of a Rabl configuration. Other investigators studied the distribution of chromosomes on the metaphase plate and found non-random patterns (Dolores Coll et al., 1980; Verma et al., 1988). Gleba et al. (1987) found evidence for the spatial separation of genomes in hybrid plant metaphase figures. However, all the data described above were derived from two-dimensional sources and were therefore difficult to fit in a three dimensional nuclear model.

Studies on 3-D architecture of chromosomes

The preservation of the morphology of the nucleus is required for the 3-D architecture of chromosomes within the nucleus. Avivi et al. (1982a,b) studied the spatial relationship of chromosomes within the three wheat genomes, combined in one species (allo-polyploid), of common wheat by measuring distances between marked chromosomes in root-tip cells with a

micrometric ocular. It was found, that within each genome the distances between homologues were significantly smaller than between non-homologues. Further, the three genomes tended to occupy different areas of the somatic nucleus. But rather than using this complex method to study the arrangement of chromosomes, investigators applied sectioning procedures in combination with 3-D computer modelling. In 1979, Murray and Davies used physical sectioning, electron microscopy and 3-D reconstruction of the images in mature newt erythrocytes and found each chromosome to have its own domain in the nucleus, attached to the nuclear envelope. The introduction of optical sectioning and 3-D computer analysis by John Sedat's group in San Francisco allowed these investigators to study the spatial chromosome organization in intact Drosophila melanogaster polytene nuclei (Agard and Sedat, 1983). They employed DAPI to stain the chromosomes within the nuclei and used the banding patterns to analyze the positions of the different polytene chromosomes. By stepping the microscope focus through each nucleus in small increments, successive planes of the sample were brought into view. These images were digitized, and per nucleus a stack of 24 images was stored in a computer memory and used for 3-D reconstruction. Extensive deconvolution algorithms were used to eliminate the "out-of-focus" fluorescence of DAPI, so that the banding patterns of the individual chromosomes could be recognized in the intact nucleus. In a series of papers their results were described (Mathog et al., 1984; Hochstrasser et al., 1986; Hochstrasser and Sedat, 1987). The most important observations were: In these nuclei the centromeres were always attached to the nuclear envelope, each polytene chromosome had its own domain and the chromosomes were aligned in the Rabl orientation. This configuration of Drosophila chromosomes in Rabl alignment was also observed by other investigators (Foe and Alberts, 1985). Kubai (1987) found, that in meiosis the maternally and paternally derived chromosome sets were non-randomly distributed in Sciara coprophila males. In this study serial section EM was used. A modified Rabl array with telomeres at the surface and centromeres inside, was seen in prometaphase mouse fibroblasts (Chaly and Brown, 1988). In Crepis capillaris root tip cells, however, no evidence was found for either non-random arrangements or homologues pairing of chromosomes (Rawlins and Shaw, 1988). They used optical sectioning and Sedat's computer deconvolution approach to analyze 88 sets of anaphase cells. In 1985, Brakenhoff et al. used a new type of computer-aided microscopy to study the 3-D chromatin distribution in neuroblastoma nuclei. With this system, called the confocal microscope, no time-consuming deconvolution procedures were required and the images were readily accessible for 3-D analysis.

Studies on interphase cells by hybridocytochemistry

The potentiality to label specific regions on chromosomes by use of non-isotopic hybridocytochemistry prompted several scientists to use this technique for the study of chromosome organization in cells. From *in situ* hybridization on slides it was known, that human centromeres, hybridized with chromosome specific centromeric DNA probes, occupy distinct regions within interphase nuclei (Pinkel et al., 1986b; Moyzis et al., 1987). It was also noted, that human chromosomes in hybrid cell line nuclei were visible as confined domains (Manuelidis, 1985; Schardin et al., 1985). Rappold et al. (1984) hybridized X and Y specific probes to interphase nuclei of two cell lines, one of the two carrying a X-Y translocation. By measuring the distances between the hybridization spots, they found, that in the cell line, carrying the translocation, the distance between the two chromosomes was significantly smaller. The first ISH data, derived from 3-D sources came from Manuelidis (1984). Using physical sectioning and 3-D image reconstruction, she found a non-random distribution of mouse satellite DNA in different cell types of nervous tissue. Pinkel et al. (1986a) applied optical sectioning after ISH to hybrid nuclei in suspension, thereby preserving the 3-D morphology, to demonstrate human chromosomal domains within 3-D reconstructions of human X hamster hybrid cells. In 1988 Trask et al. used this procedure to reconstruct the positions of centromeric regions in human nuclei. Manuelidis and Borden (1988) used optical sectioning to reconstruct centromeric and telomeric sequences in human CNS cells after ISH to brain tissue sections. They observed, that the chromosome specific regions occupied specific compartments in these nuclei. Using whole chromosome specific libraries as a probe to nuclei on slides, Pinkel et al. (1988) and Cremer et al. (1988b) saw, that within human nuclei, the hybridized chromosomes were organized as distinct domains.

In conclusion, much data, derived from 2-D as well as 3-D sources, provide indications for a defined intranuclear architecture of chromosomes. Although results by various investigators seem to be non-consistent, these differences might be caused by the various approaches used. Furthermore, tissue specificity and differentiation stage of the cells involved might be of great importance. Both ISH with whole and regional chromosome specific probes and immunocytochemical studies using antibodies against nuclear antigens wil help to elucidate the intriguing question, concerning the spatial intranuclear chromosome topography. One can speculate, that the results will be of great interest to the understanding of disease at the cytogenetic level. At present, many tumors are found to be associated with numerical and/or structural chromosome aberrations (Ohlsson and Pfeifer-Ohlsson; Sandberg and Turc-Carel, both 1987). The spatial topography of chromosomes, involved in translocations, which are leading to the rearrangement of oncogenes, are of particular interest.

Recently, Borden and Manuelidis (1988) reported the specific 3-D movement of the X chromosome in epileptic human cortex, as compared to normal brain tissue. We have started to investigate the intranuclear architecture of human chromosomes in the hemopoietic cell system (This thesis). In this system, the translocation and rearrangement of specific oncogenes is well described (Croce, 1986). The rapid progress in the field of microscopy and computer modelling will be of tremendous value in this field of research.

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Part I: METHODOLOGY

CHAPTER 1

CYTOGENETIC ANALYSIS BY IN SITU HYBRIDIZATION WITH FLUORESCENTLY LABELED NUCLEIC ACID PROBES

D. Pinkel, J.W. Gray, B. Trask, G. van den Engh,
J. Fuscoe and H. van Dekken

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ABSTRACT

The rapidly increasing availability of nucleic acid sequences homologous to specific chromosomal locations make *in situ* hybridization an exciting approach for identifying chromosomes in metaphase spreads and nuclei. Fluorescence detection of hybridized probes is advantageous because of the high spacial localization of the signal, ease of quantitative analysis, and the opportunity for simultaneous use of multiple probes, each with different fluorochrome. Hybridization can be accomplished on slides and in suspension, with probe binding analyzed using quantitative fluorescence microscopy or flow cytometry.

Genomic DNA can be used as a species-specific probe for the chromosomes of that species. Using human-hamster hybrids we demonstrate that the probe fluorescence intensities are proportional to the amount of target sequence in chromosomes and nuclei, that interspecies translocations can be detected with high sensitivity in metaphase spreads and that the 3-dimensional position of the human chromosomes can be determined in nuclei.

Human chromosome-specific repetitive probes for a growing number of chromosomes have been discovered. Binding of these probes is usually concentrated to limited regions of the chromosome, such as the centromeres, making them ideal for analysis of interphase cells. We demonstrate simultaneous detection of the binding of Y-specific and 9-specific probes in interphase nuclei using a different color for each probe, and the ability to determine the three dimensional localization of both homologues of chromosome 18 in nuclei using optical sectioning.

INTRODUCTION

The ability to detect cytogenetic abnormalities has increased as improved methods for chromosome identification have been developed. Banding techniques (Casperson et. al., 1968; Yunis, 1981) now allow identification of all human chromosomes as well as detection of a broad spectrum of structural and numerical chromosome aberrations. However, banding analysis has important limitations. It requires preparation of high quality metaphase spreads and the attendant cell culture, is not easily automated, and it requires time consuming work by skilled observers. These limitations affect the application of banding analysis in a variety of clinical and experimental settings. Prenatal detection of aberrations is limited by the time consuming nature of the cell culture and scoring process. Tumor cytogenetic analysis is limited by the difficulty of preparing high quality metaphase spreads that are representative of the tumorigenic cells in the population. Quantitative analysis of the frequency of translocated

chromosomes for biological dosimetry is limited by the time consuming nature of the scoring process.

A new approach to chromosome identification, *in situ* hybridization with chromosome-specific nucleic acid probes, promises to overcome some of the limitations of banding and offers new opportunities. Essential to this approach to chromosome staining, are nucleic acid probes that are homologous to DNA sequences located predominantly on specific chromosomes or chromosomal regions. These may be either probes for repetitive sequences or consist of collections of probes for unique sequences. Repetitive probes which bind predominantly to one chromosome type have been found for many of the human chromosomes (Yang et al. 1982; Graham et al., 1984; Jabs et al. 1984; Lau and Schonberg, 1984; Burk et al., 1985; Devine et al., 1985; Waye and Willard, 1985; Devilee et al. 1986; Moyzis et al., in preparation) and new ones are being discovered continually.

The use of chemically rather than radioactively modified probes (Langer et al., 1981; Landegent et al., 1984; Ruth and Bryan, 1984; Forster et al., 1985; Hopman et al., 1986) has advantages for chromosome labeling. Hybridization is detected with reagents that bind with high affinity to the chemically modified probe and carry various types of reporter molecules. Among the reporting systems that have proven useful are enzymes which form precipitates in the presence of substrate, fluorescent dyes, and gold particles. These allow localization of the probe binding with higher resolution than is possible with autoradiography of radioactive probes. In addition, discrimination of the hybridization of more than one probe to the same target becomes possible by using a different chemical modification and reporting molecule for each probe. We have focused on the use of fluorescent labeling because it is particularly suited for quantitative analysis through intensity measurement and high resolution imaging. We will call this procedure fluorescence hybridization.

In this paper we demonstrate the application of fluorescence hybridization to the: 1) rapid screening of interspecies hybrid cell lines for retained chromosomes and chromosomal rearrangements; 2) use of human chromosome-specific probes to label selected chromosomes in interphase nuclei; 3) study of the 3 dimensional chromosomal organization in nuclei; and 4) flow cytometric measurement of hybridization to human cell nuclei. In addition, we speculate on the utility of this technology to detection of clinically important aberrations in metaphase spreads, to detection of aneuploidy in human interphase nuclei, and to analysis of the architecture of interphase nuclei.

MATERIALS AND METHODS

Cell and chromosome preparations:

Methanol-acetic acid (3:1) fixed human chromosome spreads were prepared according to the procedure of Harper (Harper et al., 1981) using methotrexate synchronization. Similarly fixed metaphase spreads of human-hamster hybrid cells were prepared from mitotic cells collected during a 4 to 6 h colcemid block. Unstimulated human lymphocytes were separated from peripheral blood with Lymphocyte Separation Medium (Litton Bionetics, Kennsington MD), fixed in methanol-acetic acid, and dropped on slides. Sorted chromosomes were cyto-centrifuged onto slides and immersed in methanol-acetic acid. Slides containing cells or chromosomes were enclosed in sealed plastic bags containing nitrogen gas and stored at -20°C until used. The slides were baked in air at 65°C for 4 hours either prior to storage or hybridization to preserve chromosome morphology during hybridization. Nuclei for suspension hybridization were isolated, fixed in ethanol, and treated with 0.1 N HCl and 0.05% Triton X-100 (Trask et al., in preparation).

Probe modification:

DNA probes were chemically modified by nick translation with biotin-labeled deoxy-uridine triphosphate (dUTP; Langer et al., 1981) or by treatment with 2-acetylaminofluorene (AAF). DNA probes were biotinylated by nick translated according to the protocol of the supplier (Bethesda Research Laboratory) except that the amount of biotin-modified nucleotide was sometimes doubled. Approximately 10% to 30% of the thymidine in the probes was substituted with biotin-dUTP with this procedure. AAF modification was performed according the procedure of Landegent (Landegent et al., 1984)

In situ hybridization:

Hybridization on slides was carried out as described (Harper et al., 1981; Pinkel et al., 1986). Briefly, cells and chromosomes were treated with RNase (100 μg/ml, 37°C, 1 h) and denatured [70% formamide/2X SSC, 70°C, 2 min (1X SSC is 0.15 M NaCl/0.015 M sodium citrate)]. The hybridization mix (50% formamide, 2X SSC, 10% dextran sulfate, 1 mg/ml herring sperm DNA, and probe DNA) was applied, a coverslip was added and sealed with rubber cement, and the slide was placed at 37°C. Hybridizations using genomic DNA as a probe were typically accomplished during a 2 hour incubation at a probe concentration of 1 μg/ml. The RNase treatment was usually omitted in these preparations. Chromosome-specific repetitive probes were used at 0.1 to 0.5 μg/ml and the hybridization was allowed to take place overnight. After hybridization, slides were washed in 50% formamide/2xSSC (pH 7)

followed by 2xSSC and finally the same buffer in which the first cytochemical detection reagent was carried. All washes were at 45°C.

Hybridization to cells in suspension was basically similar to that described by Trask (Trask et al., 1985), with the addition of 10% dextran sulfate to the hybridization mixture. Briefly, fixed nuclei were suspended in the hybridization mix as above, heated 70°C for 10 minutes to denature both probe and target, and incubated overnight at 37°C. After hybridization the nuclei were washed (50% formamide, 2x SCC, for 10 minutes at 45°C, followed by 10 minutes of room temperature in 2x SCC). Dimethylsuberimidate-fixed mouse red blood cells were added to the first wash to minimize loss of nuclei. Finally the nuclei were suspended in the buffer appropriate for cytochemical detection.

Probe detection:

Detection of the biotin labeled probes was accomplished with fluorescently labeled avidin. The fluorescence intensity was amplified, if desired, using biotinylated goat-anti-avidin followed by an additional layer of avidin (Pinkel et al., 1986). Up to three layers of avidin were used in some studies. (Avidin and anti-avidin were obtained from Vector Inc., Burlingame Ca.). AAF modified probes were detected with rabbit polyclonal or mouse monoclonal anti-AAF antibodies followed by a fluorescently labeled second antibody. For microscope observation the cells were stained for total DNA with either propidium iodide, Hoechst 33258, or DAPI. The DNA stain was carried in an antifade solution (Johnson and Aroujo, 1981) for microscopy. Hoechst 33258 was used as the DNA stain in the flow measurements.

Observation and measurement:

Fluorescence microscopy was performed using a Zeiss fluorescence microscope. Photographs were made with Ektachrome 400 color slide film. A SIT vidicon TV camera interfaced to an image analysis system (Trapix 55/64, Recognition Concepts Inc.) was used for quantitative microscope measurements. Images from cells labeled with fluorochromes which could not be simultaneously excited were obtained by digitally storing an image of each fluorochrome, assigning it a range of false color, and displaying the composite image on a color monitor. Three dimensional nuclear reconstructions were produced by collecting a series of images at 1μm focal intervals using a 100x, n.a. 1.3 objective (Mullikin et al., in preparation). The boundaries of the chromosomal domains in each image were defined by intensity thresholding. A stack of these thresholded images was made in computer memory and displayed with a contour program that allowed arbitrary image rotation and production of stereo image pairs. Additional contours were interpolated each 0.25 μm. Flow cytometric

measurements were performed with a dual beam flow cytometer. One beam was adjusted to emit in the ultraviolet to excite the Hoechst DNA stain and the other beam was adjusted to 488 nm to excite the fluorescein attached to the hybridized probe.

RESULTS

Species-specific chromosome staining in hybrid cell lines:

Human chromosomes in human-hamster hybrid cell lines were fluorescently stained by hybridizing with human genomic DNA, Fig. 1a (Durnam et al., 1985; Shardin et al., 1985; Manuelidis, 1985b; Pinkel et al., 1986). Specific hybridization to human chromosomes occurred because of the sequence differences between the repetitive DNA of the two species. The complete protocol could be accomplished easily in 3 hours, including a 2 hour hybridization incubation. However, chromosome-specific hybridization was visible after hybridizing for only a few minutes. Maximum intensity was reached after hybridizing for 6 to 8 hours. The human chromosomes were stained with high contrast both in metaphase spreads and nuclei. The existence of well defined human chromosome domains in interphase hybrid nuclei is a general feature.

The intensity of the probe fluorescence was proportional to the amount of target sequence present in cells and chromosomes. In a hybrid line containing one copy each of human chromosomes 8 and 12, which differ by only 8% in DNA content (Mendelsohn et al.,1973), the fluorescence was proportional to the number of chromosomal domains in the nuclei. Cells with 0, 1, or 2 domains were present due to karyotypic instability of the line. Cells with no human domain had probe fluorescence below the threshold of the measurement, cells with 1 domain had an intensity of 0.57 ± 0.04 (mean $\pm$ standard error of the mean), and cells with 2 domains had an intensity of 1.0 ± 0.1. In metaphase spreads of a hybrid line which contained human chromosomes 4, 8 and 21, the relative fluorescence intensities of these chromosomes were 0.56 ± 0.03, 0.34 ± 0.03 and 0.11 ± 0.01 respectively. The total intensity of these three chromosomes was normalized to 1.0 in each metaphase spread. The relative DNA contents of these three chromosomes are 0.50, 0.38 and 0.12 (Mendelsohn et al., 1973).

The high contrast species-specific labeling in hybrid cells is particularly suited for rapid cytogenetic screening for translocations. For example Fig. 1b shows that inter-species translocations can be detected simply by noticing the presence of distinctive bicolored chromosomes. Visual scoring can be accomplished using low magnification, 16-40X, dry optics. Once a spread is found, the presence of a translocation can be established in several seconds. Translocations that are too small to be readily detectable by banding are easily seen.

We have examined spreads at a rate of over 100 per hour to develop a dose response curve for the induction of interspecies translocations by radiation (Pinkel et al., 1986).

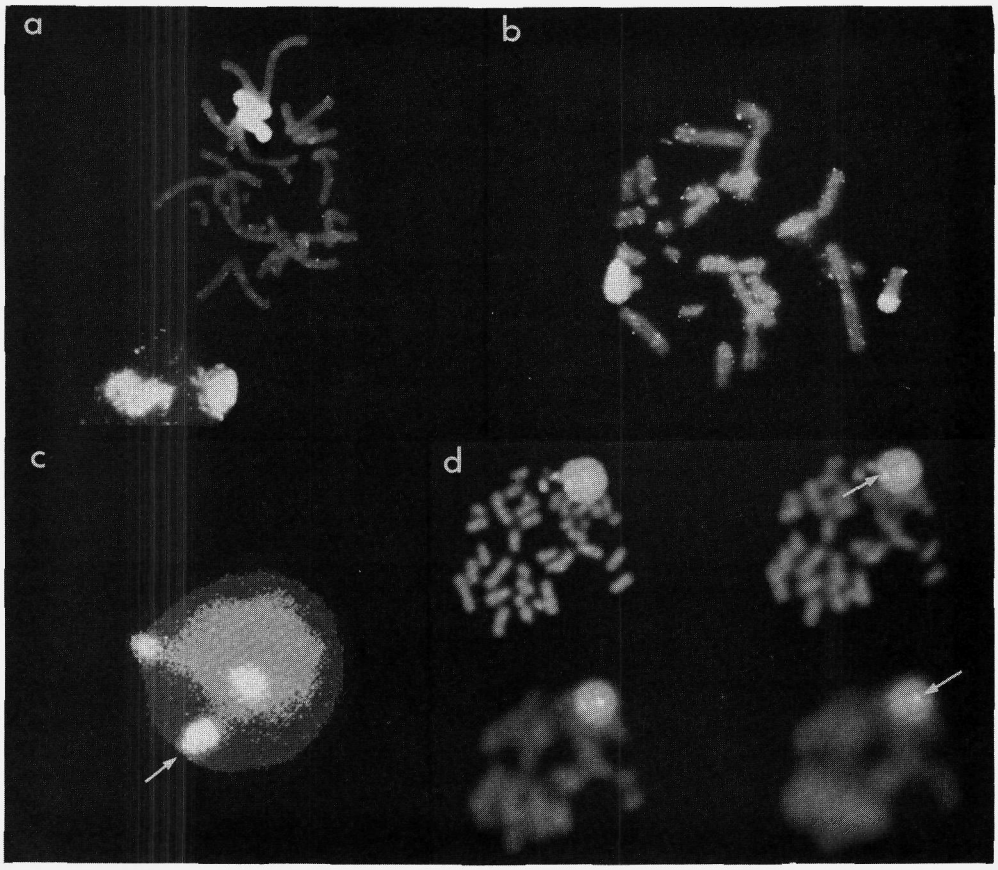

Figure 1:

a) Hybridization of human genomic DNA to human/hamster hybrid cells and chromosomes. Probe DNA was labeled with biotin and hybridized probe was detected with fluorescein-avidin (white). All of the DNA was stained with the red fluorescing dye propidium iodide (grey). One copy each of human chromosomes 8 and 12 are visible in the metaphase spread and interphase nucleus.

b) Detection of an inter-species translocation. Chromosomes were treated as in a). The translocation is indicated by the presence of a bi-colored chromosome.

c) Simultaneous hybridization of two chromosome-specific probes to a human cell. The binding of the AAF labeled probe for the Y chromosome was detected with FITC, rendered white in this image (arrow). The biotinylated 9-specific probe was detected with phycoerythrin and rendered two smaller grey-white spots in this image. The DNA was counterstained with DAPI. Each fluorophore was independently imaged with an image analysis system. The three images were then redisplayed in false color.

d) Positions of chromosome 18 in a human nucleus. Optical sectioning of a lymphocyte nucleus hybridized with a probe specific for the pericentric region of chromosome 18 shows the relative positions of the two homologues (arrows) in three dimensions.

Human chromosome-specific labeling:

The simultaneous use of chromosome-specific repetitive probes for human chromosomes 9 and Y to label human male lymphocytes is shown in in Fig. 1c. The Y-specific probe pY3.4 (Burk et al., 1985) was modified with AAF and detected with a fluorescein-labeled second antibody. The 9-specific probe pHuR98 (Moyzis et al., in preparation) was labeled with biotin and detected with avidin conjugated to phycoerythrin. The localization of the probe fluorescence to small regions within the nucleus is a general feature following hybridization with chromosome-specific repeat sequence probes and is an indication of the localization of chromosomes in human nuclei (Zorn et al., 1979).

Structure of interphase nuclei:

Hybridization to nuclei whose 3-dimensional structure has been preserved allows study of the spatial relationships of chromosomes. When fluorescent techniques are used, the interphase distribution of labeled chromosomes can be explored by optical sectioning (Agard and Sedat, 1983). The depth information comes from exploitation of the limited depth of field of high numerical aperture microscope objectives. Fig. 1d shows 4 views of a human metaphase spread and a cell nucleus to which has been hybridized a probe with predominant binding to chromosome 18 (Devilee et al., 1986). This nucleus has retained substantial thickness during preparation of the slide and the hybridization. Both copies of 18 in the metaphase spread are located near the nucleus. The chromosomes are blurry since focus has been adjusted to be at the bottom of the nucleus. In the other 3 panels, the focal plane has been sequentially raised. This is indicated by the progressive blurring of the chromosomes. The labeled regions of the 2 homologues of chromosome 18 are seen to be at different depths in the nucleus.

Fig. 2 shows 3 views, separated by 45 degree rotations, of a computer reconstruction of a human/hamster hybrid cell nucleus hybridized with human genomic DNA. This cell line contains 3 human chromosomes. The hybridization was done in liquid suspension to preserve nuclear morphology.

Flow cytometry:

Fig. 3 shows the quantitative measurement of bound probe by flow cytometry. Human cells were hybridized with AAF labeled human genomic DNA, an AAF labeled human chromosome 9 specific reagent, or with no probe, and counterstained with Hoechst 33258. A separate bivariate measurement of the probe-linked FITC fluorescence versus Hoechst fluorescence was made for each population, and the results combined into one histogram. The cells labeled with the 9-specific probe are clearly brighter than the cells in the control popula-

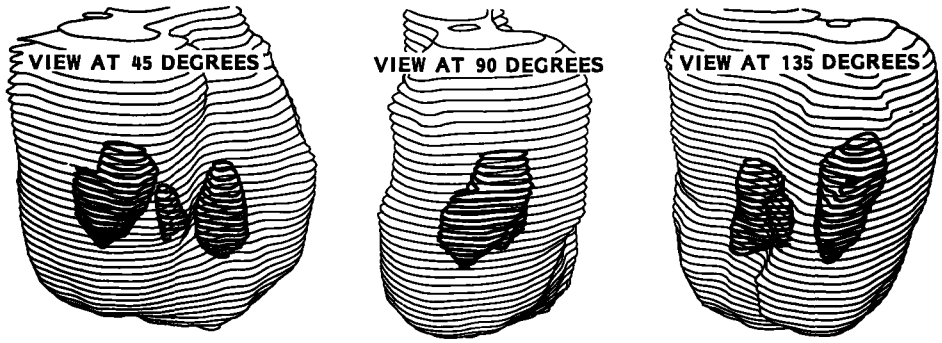

Figure 2:
Three dimensional reconstruction of the positions of human chromosomes in a human/hamster hybrid cell. The nucleus, which contains human chromosomes 4, 8, and 21, is shown from three different angles separated by 45 degrees.

tion which received no probe, and they are substantially less fluorescent than cells hybridized with human genomic DNA. The doubling in DNA content through the cell cycle is seen in both the intensity of the DNA stain and probe-linked stain. The relative intensity of the cells hybridized with genomic DNA and the 9-specific probe can not be interpreted quantitatively in this measurement since insufficient genomic probe was used to assure saturation of the target sites in the nuclei.

DISCUSSION

Hybridization with genomic DNA is a very powerful and rapid approach to cytogenetic analysis of interspecies hybrid cells. High contrast, species-specific, chromosome staining is possible using protocols that take only a few hours from start to finish. The number of chromosomes of each species present in metaphase spreads and inter-species exchanges of DNA are obvious in Figs. 1a and 1b. Translocations too small to be detected reliably by banding are distinctly visible. The ease of the analysis makes this technique ideal for studying the induction of chromosomal abnormalities by low doses of toxic agents. It is useful for establishing the species identity of presumed human chromosomes sorted from hybrid cell lines for production of chromosome-specific libraries and for determining the purity of the sort (van Dekken et al., in preparation). It may be used to determine the incorporation site in

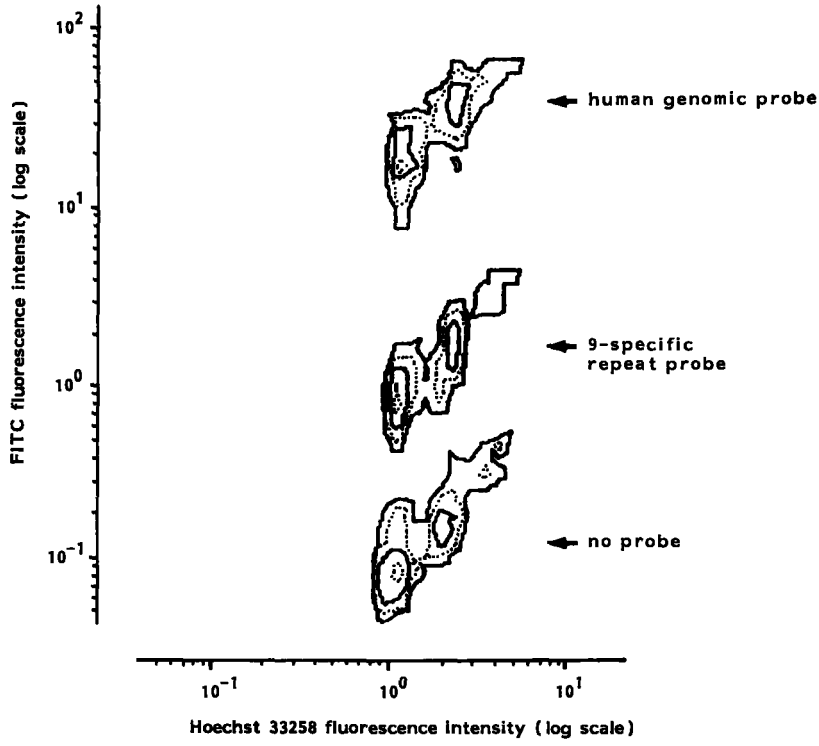

Figure 3:
Flow cytometric measurement of fluorescence hybridization. Human nuclei were hybridized with an AAF labeled probe specific for human chromosome 9, with AAF labeled genomic human DNA, or with no probe. Bound probe was rendered fluorescent with FITC. Nuclear DNA was counterstained with Hoechst 33258. The two peaks seen in the contour plots for each population correspond to the G1 and G2 phases of the cell cycle. The hybridization signal increases proportionately to the nuclear DNA content.

interspecies transfections if enough DNA is involved. In addition, fluorescence hybridization with species-specific probes also facilitates determination of the number of chromosomes of that species that have been retained and detection of spontaneous interspecies translocations in hybrid cells. If the number of chromosomes involved is small (e.g. if one is interested in whether or not there is a single chromosome of a given species present) then hybridization to nuclei may yield sufficient information. In any case, fluorescence-hybridization with species-specific probes may be preferable in many instances to banding analysis or G11 staining (Burgerhout, 1975) for hybrid cell cytogenetic analysis.

Repetitive probes specific for several human chromosomes have been discovered (Yang et al. 1982; Graham et al., 1984; Jabs et al. 1984; Lau and Schonberg, 1984; Burk et al., 1985; Devilee et al. 1985; Devine et al., 1985; Jabs and Perisco, 1985; Waye and Willard,

1985; Moyzis et al., in preparation), and it is possible that probes for all of them will eventually be found (Willard, 1985). The binding distribution of these probes is usually restricted to a small portion of the chromosome, such as the long arm of the Y or the pericentric region of the autosomes. The degree of chromosome specificity varies from probe to probe and depends in many cases on the stringency of the hybridization. However, these probes are useful for identifying chromosomes in metaphase spreads even if the binding is not perfectly chromosome specific. This allows rapid screening of spreads for specific aneuploidies. The currently known repetitive probes are not particularly useful for detecting translocations because the probability that the breakpoint will involve the labeled region of the chromosome is small, although it can happen (Lau et al., 1985).

Staining of a chromosome in a nucleus requires a probe of higher specificity than for staining in a spread since chromosome morphology can not be used to assist with the interpretation. Many of the repetitive probes are sufficiently specific for interphase analysis, as shown in Fig. 1c, where the Y and 9 have been labeled with different colors. We anticipate that these will prove extremely valuable for detecting aneuploidy directly in nuclei. There are several approaches to accomplish this.

First, it may be sufficient to simply count the number of spots in two dimensional images such as Fig. 1c. Our experience in analysis of lymphocytes, fibroblasts and amniocytes with probes for chromosomes 9 and Y suggests the feasibility of this approach. The binding of these probes is tightly localized so that the number of chromosome domains to which the probe binds can be counted. In addition, the two homologues of chromosome 9 are sufficiently separated on average so that overlaps are not common. This suggests that there may be some control of the position of this chromosome in these nuclei. However, other chromosomes and/or other cell types may demonstrate different behavior. Even if the homologues of a given chromosome are regularly separated in nuclei, spot counting in two dimensional images will not allow differentiation between a truly monosomic cell and one in which the two spots overlap due to the particular orientation of the cell on the slide. For homogeneous populations, where the abnormality is present in every cell, this will be not a major difficulty; but it will limit the ability to detect rare aneuploid cells. This may be overcome by the methods discussed below. Simultaneous hybridization with multiple probes, Fig. 1c, allows analysis of more than one chromosome at a time. In addition, the probes serve reciprocally as controls for the success of the hybridization and permit recognition of general aneuploidy of a cell. For example, when using probes for a sex chromosome and an autosome in male cells, detection of of the autosomal probe should insure that there was sufficient access for the hybridization reagents to a particular nucleus. Thus the absence of a signal from the sex chromosome could be interpreted as absence of that chromosome. Additionally, if two copies

of the sex chromosome are found, the number of spots from the autosomal probe should allow differentiation between an aneuploidy of the sex chromosome and a tetraploid cell.

A second approach to interphase aneuploidy detection is quantitative measurement of the intensity of probe-linked fluorescence. This method is not affected by the possible overlap of the labeled chromosomal domains. Both quantitative microscopy and flow cytometry have sufficient precision to differentiate populations differing by 50% in target sequence. In Fig. 2 the doubling of both DNA content and probe-linked fluorescence for a human chromosome 9 specific probe in human cells is clearly evident during the cell cycle. With the current measurement precision aneuploidy detection is limited to populations in which the proportion of abnormal cells is high.

The morphology of nuclei hybridized in suspension is well preserved, permitting three dimensional study of the chromosomal domains. This offers a third approach to the detection of aneuploidy because domain overlaps in two dimensional images are no longer a problem. Figure 3 shows the same nucleus viewed from three angles separated by 45 degrees. Two dimensional projections of these images would show 1 (center), 2 (right), or 3 (left) labeled domains.

Fluorescence hybridization with chromosome-specific probes to nuclei in suspension is a powerful approach to studies of the chromosomal organization in interphase nuclei (van Dekken et al., in preparation). It permits the use of optical sectioning for analysis of the organization in three dimensions at the light microscope resolution level, potentially tenths of a micrometer. Fig. 1d demonstrates optical sectioning of a human nucleus stained for chromosome 18 while Fig. 3 shows a full reconstruction of the human chromosome domains in a hybrid cell nucleus. By using multiple probes detected with different fluorophores, it may be possible to address such questions as the systematics of chromosome order in different cell types, the cell cycle dependence of the organization, the relation of the organization to cellular function, and mechanisms responsible for production of chromosome abnormalities.

The flow cytometric detection of the binding of a chromosome-specific repetitive probe demonstrates the ability to detect targets of several hundred kilobases with signals substantially above background. This represents sensitivity a thousand fold higher than reported previously (Trask et al., 1985) and opens the possibility of detecting the distribution of the amount of specific sequences, such as amplified genes, in a cell population. Hybridization to chromosomes in suspension, currently under development, would greatly facilitate chromosome discrimination and purification by flow cytometry. This would permit detection of rare translocations, signified by bi-colored chromosomes, at high rates.

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CHAPTER 2

ENZYMATIC PRODUCTION OF SINGLE STRANDED DNA AS A TARGET FOR FLUORESCENCE *IN SITU* HYBRIDIZATION

H. van Dekken, D. Pinkel, J. Mullikin and J.W. Gray

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ABSTRACT

This study demonstrates that Exonuclease III (Exo III) can be used to produce sufficient single stranded DNA (ssDNA) in chromosomes and cells to allow in situ hybridization. In this study, all of the probes were modified with biotin and the probe binding was visualized with fluorescein-labeled avidin. Exo III digestion starting at naturally occurring breaks in methanol-acetic acid preparations produced enough ssDNA for strong hybridization when human genomic DNA was used to probe human chromosomes. Pretreatment with the endonucleases Eco R1, Hind III and Bam H1 was used to produce more sites for initiation of Exo III digestion when using a chromosome-specific repetitive probe specific to a small chromosomal subregion near the telomere of human chromosome 1 (1p36). The fluorescence intensity following hybridization to Exo III treated targets was roughly equal to that following hybridization to thermally denatured targets, but background fluorescence was lower.

INTRODUCTION

Nucleic acid hybridization is becoming increasingly important as a cytogenetic tool. Among the applications are: 1) chromosomal localization of DNA probes (Harper et al., 1981) 2) assessment of the location of DNA sequences on nitrocellulose filters made from DNA blotted from agarose gels following electrophoresis (Southern, 1975), 3) assessment of the presence of a DNA sequence in chromosomes sorted onto nitrocellulose filters (Lebo et al., 1984) and 4) identification of specific chromosomes or chromosome segments in metaphase and/or interphase cells using chromosome-specific probes as labeling reagents (Manuelides and Ward, 1984, Pinkel et al., 1986, Trask et al., 1985). Nucleic acid hybridization typically requires that both the probe and target be single stranded. Thus, double stranded nucleic acids need to be denatured prior to hybridization. Normally this is accomplished by heating or by treatment at high or low pH. Unfortunately, these techniques suffer from some limitations: 1) The target DNA may partially reanneal as soon as the denaturing conditions are removed (e.g. after reducing the temperature to allow hybridization between probe and target) thereby reducing the extent of probe hybridization. 2) Substantial loss of DNA may occur during the denaturation process (Barbera et al. 1979, Raap et. al., 1986). 3) The denaturation procedures are harsh so that they disrupt the mechanical structure of cells and organelles.

We describe here an enzymatic method to produce ssDNA in cell nuclei and metaphase spreads that has some unique advantages. It uses Exonuclease III (Exo III) to remove one of the DNA strands starting at naturally occurring nicks and breaks in the DNA, or at sites

induced by cutting with specific restriction endonucleases. ExoIII catalyzes the 3' to 5' removal of 5' monophosphates. It is specific for double stranded DNA (dsDNA) that has a free 3' hydroxyl end or that has single strand breaks with 3' hydroxyl termini (Rogers and Weiss, 1980). We show that Exo III produces sufficient ssDNA to permit strong hybridization of both human genomic DNA and a cloned probe specific for the telomeric region (1p36) of human chromosome 1p (Buroker et al., 1987) to chromosomes and nuclei of human and human x hamster hybrid cells. The probes were modified with biotin and their binding was detected with fluorescein labeled avidin (Langer et al., 1981, Langer-Safer et al., 1982, Pinkel et al., 1986). Probe-linked fluorescence intensities and total nuclear DNA fluorescence after DAPI staining were compared for enzymatic and thermal denaturation using a quantitative fluorescence microscope system.

MATERIALS AND METHODS

Sample preparation:

Metaphase spreads of the human x hamster cell line UV20HL-21-27 (Thompson et al., 1985) were prepared as described previously (Pinkel et al, 1986) from cells shaken from monolayer cultures after treatment for 4 hours with Colcemid (0.1 μg/ml). This cell line (a gift from Dr. Larry Thompson, Lawrence Livermore National Laboratory) carries human chromosomes 4, 8 and 21. However chromosome 21 is missing in many of the cells in the cultures used in this work. Human lymphocyte chromosomes were prepared from methotrexate synchronized cultures (Harper et al. 1981). Metaphase and interphase cells were fixed in methanol/acetic acid (3:1; vol/vol) and dropped onto cleaned microscope slides. Slides were stored in a nitrogen atmosphere at -20°C prior to hybridization.

Preparation of single stranded DNA (ssDNA):

The slides were taken from the nitrogen and either denatured for 2 min. at 70°C in 70% formamide/2xSSC (final concentrations) at pH 7 or incubated for various times at 37°C with Exo III (Bethesda Research Laboratories) in 50 mM Tris-HCl; 5 mM $MgCl_2$; 10 mM dithioerythritol at pH 8. Some preparations were treated with the endonucleases Bam H1 (300 units/ml), Eco R1 (450 units/ml) and Hind III (200 units/ml) in buffer (100mM Tris-HCl; 50 mM NaCl; 10 mM $MgCl_2$; 2 mM DTE at pH 7.5) for 30 min at 37°C, prior to Exo III treatment. Slides treated with endonucleases were washed twice for 1 min. in 2xSSC at pH 7 at room temperature. All slides were dehydrated in an ethanol series (70%, 85% 100%) at room temperature before applying the probe.

***In situ* hybridization:**

Hybridization was performed as described previously (Harper et al., 1981; Pinkel et al., 1986). Briefly, the DNA probes (nick-translated with biotin-11-dUTP; Bethesda Research Laboratory), were denatured for 5 min. at 70°C in hybridization mixture (1-2 μg/ml probe DNA ; 500 μg/ml sonicated herring sperm DNA ; 50% formamide; 10% dextran sulfate; 1% Tween 20; 2xSSC; pH 7) and immediately cooled on ice. This mixture was applied to the slide under a glass coverslip (3 $\mu l/cm^2$) and sealed with rubber cement. After overnight incubation at 37°C, the slides were washed 3 times for two min. in 50% formamide/2xSSC/ pH 7 at 45°C, followed by two 1 min. washes in 2xSSC at pH 7. The slides were then immersed in PN buffer (0.1 M sodium phosphate; 0.05% Nonidet P-40 at pH 8).

Cytochemistry:

Detection of the biotin-labeled probes was accomplished with fluorescein-labeled avidin as described previously (Langer et al., 1981, Langer-Safer et al., 1982, Pinkel et al., 1986). The probe-linked fluorescence intensity was amplified by successive treatments with biotinylated goat-anti-avidin followed by fluorescein-labeled avidin (Avidin and goat-anti-avidin from Vector Labrotories Inc., Burlingame, California) The cells were stained for total DNA with either propidium iodide or DAPI (Sigma) for microscopic analysis. The DNA stains were carried in an antifade solution (Johnson and de C. Nogueiro Araujo, 1981) to preserve the fluorescein fluorescence intensity during extended microscopic analysis. The red-fluorescing DNA-specific dye propidium iodide was used to allow simultaneous observation of hybridized probe and total dsDNA. The fluorescein and propidium iodide were excited at 450-490 nm (Zeiss filter combination 487709). DAPI, a blue fluorescing DNA-specific stain, excited in the ultraviolet (Zeiss filter combination 487701) was used as the DNA specific stain in place of propidium iodide to allow quantitative analysis of biotin-labeled probe fluorescence. DAPI is not excited at 488 nm so that the presence of the DNA stain does not affect the measurement of probe-linked fluorescence intensity. Ektachrome ASA 400 color slide film and type R direct positive printing were used for color photographs.

Quantitative fluorescence microscopy:

A SIT vidicon camera interfaced to an image analysis system (Trapix55/64, Recognition Concepts Inc.; Lake Tahoe, CA) was used to record images from the fluorescing cells. The intensity of probe-linked fluorescence and the DAPI counterstain was determined by computer analysis of the images (Mullikin, manuscript in preparation). Small (G1- or early S-phase) nuclei were selected for the intensity measurements based on their size. The size standard was

set by an interactively drawn box displayed on the video screen. Measurements of non-specific background fluorescence were performed in the vicinity of several nuclei using a box of the same size.

RESULTS

Figure 1a shows fluorescence hybridization of whole genomic human DNA to the metaphase chromosomes from a human x hamster hybrid cell line. The ssDNA was produced by treating the spreads with Exo III alone. The fluorescence hybridization is intense and highly specific to the human chromosomes, four of which are visible in this photograph of the overlap region of two metaphase spreads. Figures 1b and 1c show hybridization to human metaphase spreads with a probe specific for human chromosome 1p36 (Buroker et al., 1987). Figure 1b shows hybridization to a spread treated only with Exo III. Telomeric hybridization is clearly visible on only one of the homologues and some binding to other chromosomes is present. Figure 1c shows hybridization with the same probe to a human metaphase spread treated with the endonucleases Eco R1, Hind III and Bam H1 prior to treatment with ExoIII. The telomeric hybridization on both homologues is now clearly visible. Figure 1d shows hybridization of the same probe to a thermally denatured human metaphase spread. The intensity of hybridization to 1p is comparable to that in Figure 1c, but there is less "non-specific" hybridization on the other chromosomes.

The ssDNA exposure can be controlled by the concentration of the Exo III. Table 1 shows the changes in the probe-linked fluorescence intensity (a measure of the amount of ssDNA exposed) and the intensity of DAPI staining (a measure of the relative amount of dsDNA remaining) for human nuclei treated with various concentrations of ExoIII and hybridized to whole genomic human DNA. The probe-linked fluorescence intensity increases to a maximum when the Exo III concentration reaches 300 units/ml (incubation time 30 min). The maximum probe-linked fluorescence intensity achieved is about the same as that obtained with thermal denaturation (Table 1). The DAPI fluorescence does not begin to decrease until the the Exo III concentration is increased to about 30000 units/ml. Table 1 also shows that at an Exo III concentration of 3000 units/ml the maximum exposure of ssDNA is reached within a few minutes.

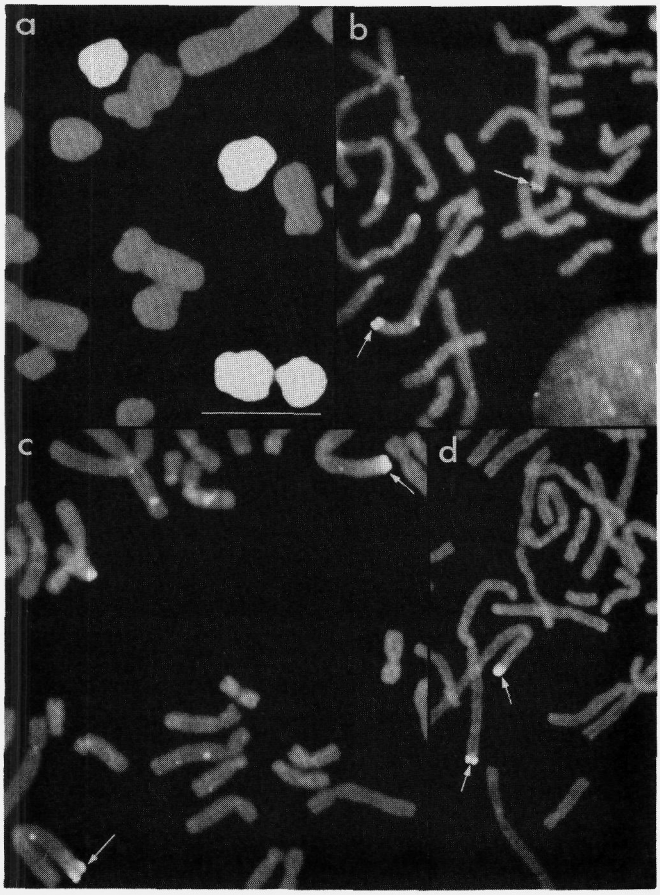

Figure 1:
Photomicrograph showing fluorescence hybridization of biotin labeled probe DNA to metaphase spreads. The hybridized probe was detected with fluorescein-labeled avidin. The metaphase spread was counterstained with propidium iodide. The regions to which probe hybridized appear white in the photomicrograph. The regions to which the probe did not bind appear grey. Panel a. Hybridization of whole genomic human DNA to a human x hamster hybrid cell metaphase chromosomes treated with Exo III only. Panel b. Hybridization of a probe to human chromosome 1p36 to a human metaphase spread treated with Exo III alone. Panel c. Hybridization with the probe to 1p36 to a human metaphase spread treated with three endonucleases and Exo III. Panel d. Hybridization with the probe to 1p36 to a human metaphase spread following thermal denaturation. A 10 μm scale bar for all photographs is shown in panel a.

TABLE 1

INTENSITY MEASUREMENTS OF DAPI AND PROBE-LINKED FLUORESCENCE OF HUMAN NUCLEI FOLLOWING FLUORESCENCE IN SITU HYBRIDIZATION WITH TOTAL HUMAN DNA

The table compares the results of variations in the DNA denaturation conditions.

Denaturation Condition	DAPI Fluorescence Intensity	Probe-linked Fluorescence Intensity
No denaturation	17.2	----
Heat denaturation only	6.6	27.5
30 U/ml, 30 min Exo III	17.6	10.3
300 U/ml, "	18.5	30.0
3000U/ml, "	18.1	25.3
30000U/ml, "	8.6	26.5
1 min Exo III, 3000U/ml	19.4	23.3
5 min Exo III, "	13.5	27.6
10 min Exo III, "	17.6	29.6
15 min Exo III, "	16.0	34.6
30 min Exo III, "	18.1	25.3
60 min Exo III, "	11.1	33.2

Ten nuclei were measured for each denaturation condition. The fluorescence intensities are corrected for background fluorescence.

One advantage of the use of ExoIII to expose ssDNA for hybridization, compared to thermal denaturation, appears to be a decrease in the amount of probe binding to regions of the microscope slide not containing nuclei or chromosomes (called background fluorescence). This is illustrated in Fig. 2. Table 2 shows quantitative fluorescence measurements of the intensities of specific and background fluorescence obtained with thermal and enzyme production of ssDNA in in human fibroblast nuclei.

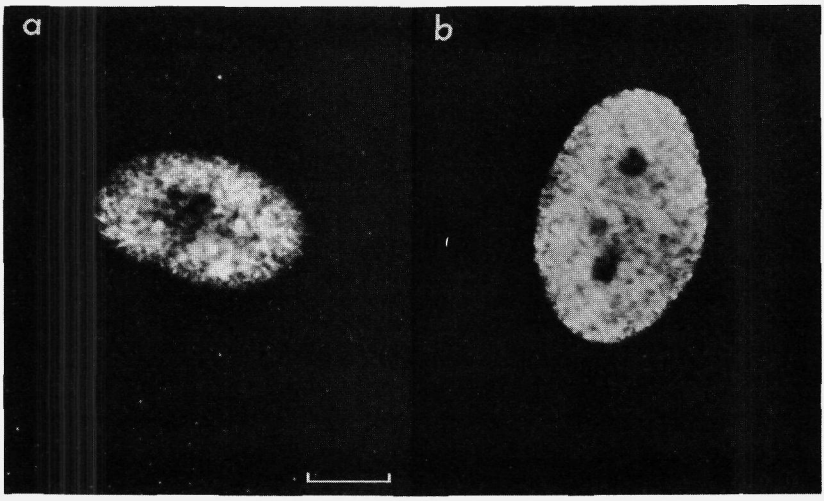

Figure 2:
Comparison of the hybridization of biotin-labeled whole genomic DNA to human nuclei. The nuclei were counterstained with DAPI. The photomicrographs show the probe-linked fluorescence intensity induced by excitation at 480 nm. Panel a. Hybridization following thermal denaturation. Panel b. Hybridization following treatment with Exo III. Note the higher frequency of backround spots in panel a. A 10 μm scale bar for both photographs is shown in panel a.

TABLE 2

PROBE-LINKED FLUORESCENCE INTENSITY OF HUMAN NUCLEI FOLLOWING HYBRIDIZATION WITH WHOLE GENOMIC HUMAN DNA

Denaturation	Nuclei[a]	Background[b]
Thermal	7.1 ± 1.9	0.9 ± 0.2
ExoIII	13.3 ± 3.2	0.1 ± 0.2

[a]from measurements of 30 different nuclei

[b]from measurement of 10 different background areas

DISCUSSION

This study demonstrates that significant amounts of ssDNA can be produced by Exo III digestion of methanol-acetic acid fixed chromosomes and nuclei. This is consistent with the studies of Dolbeare and Gray (manuscript in preparation) who showed that BrdUrd in ssDNA could be exposed to antibodies against BrdUrd in ssDNA using Exo III and a panel of endonucleases and with the study of Adolph and Hameister (1985) who showed that chromosomes could be nick-translated in situ using Kornberg polymerase. There were sufficient naturally occurring or induced nicks and/or breaks in the DNA of our preparations for Exo III to produce enough ssDNA so that probes which contain highly repetitive elements, such as genomic DNA (Schmid and Jelinek, 1982), could be hybridized efficiently, Fig. 1a. The maximum probe-linked fluorescence intensity signal was obtained using an Exo III concentration of 300 units/ml, and remained approximately constant as the Exo III concentration was increased to 30000 units/ml (Table 1). The production of ssDNA by Exo III was nearly complete after treatment for a few minutes. Thus, the ability of Exo III to produce ssDNA in these targets reached a plateau in both incubation time and enzyme concentration. Since the DAPI fluorescence under these conditions is essentially the same as for undenatured nuclei, we imagine that only a relatively small proportion of the DNA is digested by the enzyme. This is consistent with the view that the enzyme starts at the available nicks and breaks and moves along the DNA until it encounters an obstacle such as remaining protein. Double stranded regions delimited by such blocking structures and containing no start sites for the enzyme will not be rendered single stranded. Since Exo III removes bases at rates of several hundred per minute, it can rapidly process the available DNA. The drop in DAPI fluorescence at the longest incubation times and highest enzyme concentration may indicate that the nuclei lose DNA under those conditions but we have not investigated this fully. When probes with smaller target sizes were used, it was necessary to introduce additional initiation sites by the use of endonucleases (see Fig. 1b,c). Presumably, this allows a greater portion of the target to be made single stranded.

An important feature of the enzymatic production of ssDNA is the reduction of background fluorescence of the slides compared to that obtained with thermal denaturation of the target, Fig. 2 and Table 2. Thermal denaturation causes a substantial loss of DNA from the target (Barbera et. al. 1979, Raap et. al., 1986). It may be that some of this attaches as fragments to the surface of the slides and is detected by the probe. While we have no general body of quantitative data, we have noticed qualitatively that with thermal denaturation the amount of background hybridization increases with increasing loss of DNA from the targets. We postulate that ExoIII results in fewer DNA fragments attatched randomly over the slide

since the removed DNA strand is digested. The enzymatic production of ssDNA also has the potential advantage that production of ssDNA can be targeted to locations starting at the cutting sites of a restriction endonuclease used to induce Exo III initiation sites. If the probe has been produced by cloning DNA cut with the same endonuclease, the exposed target will be biased toward the proper binding site(s). This may reduce non-specific hybridization to the target.

The enzymatic denaturing procedure is more complex than other techniques and therefore may not be as convenient for routine use. However in some situations, such as optimizing production of ssDNA at cloning sites or hybridizing to targets whose structure is easily disrupted, it may be advantageous.

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CHAPTER 3

FLOW CYTOMETRIC QUANTITATION OF CHROMOSOME SPECIFIC REPETITIVE DNA SEQUENCES BY SINGLE AND BICOLOR FLUORESCENT *IN SITU* HYBRIDIZATION TO HUMAN LYMPHOCYTE INTERPHASE NUCLEI

H. van Dekken, G.J.A. Arkesteijn, J.W.M. Visser and J.G.J. Bauman

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ABSTRACT

Fluorescent *in situ* hybridization allows for the rapid and precise detection of specific nucleic acid sequences in interphase and metaphase cells. We applied fluorescent *in situ* hybridization to human lymphocyte interphase nuclei in suspension to determine differences in amounts of chromosome specific target sequences amongst individuals by dual beam flow cytometry. Biotinylated chromosome 1 and Y specific repetitive satellite DNA probes were used to measure chromosome 1 and Y polymorphism amongst eight healthy volunteers. The Y probe fluorescence was found to vary considerably in male volunteers (mean fluorescence 169, s.d. 35.6). It was also detectable in female volunteers (mean fluorescence 81, s.d. 10.7), because 5-10% of this repetitive sequence is located on autosomes. The Y probe fluorescence in males was correlated with the position of the Y chromosome cluster in bivariate flow karyotypes. When chromosome 1 polymorphism was studied, one person out of the group of eight appeared to be highly polymorphic, with a probe fluorescence 26% below the average. By means of fluorescent *in situ* hybridization on a glass slide and bivariate flow karyotyping, this 26% difference was found to be caused by a reduction of the centromere associated satellite DNA on one of the homologues of chromosome 1.

The simultaneous hybridization to human lymphocyte interphase nuclei of biotinylated chromosome 1 specific repetitive DNA plus AAF-modified chromosome Y specific DNA was detected by triple beam flow cytometry. The bicolor double hybridized nuclei could be easily distinguished from the controls. When the sensitivity of this bicolor hybridization will be improved, this approach could be useful for automatic detection of numerical chromosome aberrations, using one of the two probes as an internal control.

INTRODUCTION

In tumour cytogenetics, the analysis of chromosomes is hampered by the difficulty of preparing high-quality metaphase spreads which are representative of the tumorigenic cells. Cytogenetic analysis in the interphase nucleus ("interphase cytogenetics") would overcome this problem. Rapid chromosome labeling by fluorescent *in situ* hybridization (FISH) might allow for the fast identification and quantitation of certain chromosome aberrations by flow cytometry.

The introduction of non-isotopic *in situ* hybridization during the last decade has enabled the labeling of specific nucleic acid sequences with high speed and precise localization (6, 24, 26, 34). In recent years the refinement of the method led to the localization and detection of single copy sequences (2, 3, 15, 25, 29). The fact that non-isotopic *in situ* hybridization now is

of equal sensitivity as the routinely used radioactive method, makes it the method of choice because of its speed and localization properties.

The ability to label specific chromosomes by FISH allows for the rapid detection of numerical aberrations. For this purpose a large target sequence would be preferable, since it facilitates detection. Such targets are provided by some families of repetitive DNA (10, 12, 44). The family of alpha satellite DNA probes is most widely used. These satellite DNA's are AT-rich, are found predominantly at the centromeres of specific chromosomes and are polymorphic amongst individuals (14, 21, 32, 43). Using such a probe, numerical abnormalities were detected in metaphase and interphase cells by means of FISH (13, 32, 34, 41).

Recently, the quantitation of FISH by flow cytometry was reported. Satellite DNA probes were hybridized to human cell line interphase nuclei and the amount of hybridized probe was measured by flow cytometry (33, 38). The detection of extra copies of chromosome 17 in a tetraploid human cell line, using a chromosome 17 specific repetitive DNA probe, was described by Trask et al. (39).

We have studied chromosome 1 centromeric and chromosome Y specific repetitive satellite DNA sequences, using a satellite DNA probe specific for chromosome 1 (11), and a Y specific repetitive DNA probe (12). Quantitative differences between male and female volunteers and polymorphism of chromosome 1 were detected. The amount of hybridized targets was determined by dual beam flow cytometry after FISH in suspension to isolated peripheral lymphocyte interphase nuclei. Results were confirmed by FISH on glass microscope slides both in metaphase as well as interphase cells. The probes were labeled with biotin (26) and detected by fluoresceinated avidin. The polymorphism of chromosome 1 and Y was also demonstrated by bivariate flow karyotyping. To further improve the possibilities to quantify chromosome aberrations by flow cytometry of interphase nuclei, we detected two differently labeled (biotin, AAF; 20, 24) repetitive DNA probes by triple beam flow cytometry.

MATERIALS AND METHODS

DNA probes:

Human genomic DNA was isolated from peripheral blood lymphocytes. Hamster genomic DNA was isolated from a new born Chinese hamster fibroblast cell line. The human chromosome 1 specific centromeric probe pUC1.77, 1770 basepairs in pUC18, was kindly supplied by H. Cook (MRC Edinburgh, UK). The human Y chromosome specific repeat pY2.45 was obtained commercially (Amersham Int., Amersham, UK). For *in situ* hybridization, genomic and complete plasmid DNA was labeled with biotin-11-dUTP using the

BRL nick translation kit (BRL, Gaithersburg, MD) according to the manufacturers directions. In the double labeling experiment, pY2.45 was modified with 2-acetylaminofluorene (AAF) as described by Landegent et al. (24).

Fluorescent *in situ* hybrization in suspension:

Fresh lymphocytes were isolated from 20 ml peripheral blood with a lymphocyte separation medium (Organon, Durham, NC). Nuclei were isolated according to the Hepes-$MgSO_4$ method (42) and fixed in 70% ethanol for 10 min at 4 °C, centrifugated (each centrifugation: 10 min, 200 g.) and resuspended in 100% ethanol for 10 min at 4 °C. The FISH method for nuclei in suspension was derived from the protocol, described by Trask et al. (39). After centrifugation protein was extracted by resuspending the nuclei in 0.1 M HCl, 0.05% Triton X-100 for 10 min at room temperature. Then a fixation in 1% paraformaldehyde in isolation buffer (5 mM Hepes, 50 mM KCl, 10 mM $MgSO_4$, 0.05% Tween 20, pH 8) for 1 min at room temperature was applied. Nuclei were washed once in isolation buffer and resuspended in this buffer at a concentration of 5 x 10^4/µl. Nuclei and probe were denatured seperately: 2 µl nuclei suspension was added to 8 µl of hybridization mix (final concentrations: 50% formamide, 2xSSC, 10% dextran sulphate, 1% Tween 20, pH 7) and denatured for 10 min at 70 °C. Formamide (BRL) was de-ionised by treatment with a mixed-bed ion-exchange resin (Dowex AG501-X8; Bio-Rad Labs, Richmond, CA) and thereafter stored at -20°C. Probe DNA (2 µg/ml) and sonicated herring sperm carrier DNA (250 µg/ml; Sigma) were denatured for 10 min at 70 °C in hybridization mix. Both mixtures were quickly cooled on ice and 20 µl probe mix was added to the 10 µl nuclei in an Eppendorf tube. The sample was mixed and incubated for 14-18 hours at 37 °C in a dry incubator. Then nuclei were washed in 1 ml 50% formamide, 2xSSC, pH 7 for 10 min at 42 °C and prior to centrifugation 100 µl DMS (Aldrich, Steinheim, FRG) fixed erythrocytes (10^8/ ml in isolation buffer) were added to serve as a carrier (38). After centrifugation the pellet was resuspended in 1 ml 2xSSC, pH 7 for 10 min at room temperature and washed again in 1 ml PN buffer (0.1 M sodium phosphate, 0.05% Nonidet P40, pH 8) for 10 min at room temperature. After resuspending the pellet in 100 µl PNM buffer (5% non-fat dry milk {Safeway,USA} in PN buffer; non-fat dry milk was added to prevent non-specific binding of the avidin (34)) for 5 min at room temperature, the biotinylated probe was stained by adding an equal volume of staining buffer (10 µg avidin-FITC DCS Grade {Vector, Burlingame, CA}/ml PNM). The sample was mixed and incubated for 30 min at 37 °C in a dry incubator. For the double labeling experiment the nuclei were incubated for 30 min at 37 °C in a dry incubator with rabbit anti-AAF (a gift from Dr. R. Baan, MBL Rijswijk, The Netherlands; diluted 1:100 in PNM), washed once, blocked in 100 µl PNM, and incubated with staining buffer (10 µg/ml avidin-TRITC D {Vector} plus FITC-

conjugated goat anti-rabbit {Nordic, Tilburg, The Netherlands} at a 1:40 final dilution in PNM). After centrifugation the pellet was washed in 1 ml PN for 10 min at room temperature and finally resuspended in 400 µl isolation buffer containing 5 µM DAPI (Sigma, St. Louis, MO). Before measurement, the nuclei were forced three times through a 23G needle to reduce the number of clumps in the sample.

Fluorescent *in situ* hybridization on slides:

Metaphase and interphase cells were obtained from peripheral lymphocytes as described by Harper et al. (18) and prepared according to conventional cytogenetic techniques. Briefly, after colcemid arrest in metaphase, the cells were incubated for 12 min at 37 °C in 75 mM KCl and fixed three times in methanol/acetic acid (3:1; vol/vol) before being dropped on ethanol cleaned microscope slides. The *in situ* hybridization and fluorescent detection protocol followed that of Pinkel et al. (34). The hybridization mixture and probe concentrations were as described above. After overnight hybridization at 37°C the slides were washed three times for 3 min in 50% formamide, 2xSSC, pH 7 at 45 °C and subsequently two times for 5 min in 2xSSC, pH 7 at 45 °C. Then the slides were incubated with fluoresceinated avidin DCS grade (5 µg/ml in PNM). In some experiments the probe-linked fluorescence was amplified (34) by incubation with biotinylated goat-anti-avidin-D (Vector; 5 µg/ml in PNM) followed by fluoresceinated avidin DCS. Finally, the DNA of the cells was counterstained with propidium iodide (Sigma) to allow simultaneous observation of total DNA and hybridized probe. The DNA stain was carried at a concentration of 1 µg/ml in an antifade solution containing p-phenylenediamine dihydrochloride (Sigma) to preserve the fluorescein fluorescence during extended microscopy (22).

Dual and triple bean flow cytometry:

Flow analysis of the hybridized nuclei was performed on the RELACS-3 flow cytometer (Van den Engh and Stokdijk, manuscript in preparation). For dual beam flow cytometry, the first laser (Spectra Physics, Series 2000, Mountain View, CA) was set at the ultraviolet mode (351-363 nm lines, 300 mW) to excite the DAPI, and the blue DAPI fluorescence was measured through 408 nm long pass (Schott Glaswerke, Mainz, FRG) and 450 nm short pass (Corion Corp, Holliston, MA) filters. In this way the DMS-fixed carrier erythrocytes were effectively gated out on absence of DAPI (DNA) fluorescence. The second laser (Coherent Innova 90, Palo Alto, CA) was set at 488 nm (1 W) to excite the FITC, and the green FITC fluorescence was measured through a 530 nm band pass filter (Corion). For triple beam operation a third laser (Coherent Innova 70) was added, tuned at 515 nm (1 W) to excite the TRITC, and the red TRITC fluorescence was measured through two 550 nm long pass filters (Schott). In the triple laser set-up a 520 nm long pass filter (Schott) was added to the 530 nm band pass filter in order

to block scattered 515 nm laser light. Nuclei were measured at a rate of 50-100 nuclei per sec. The resulting fluorescence data from 5,000-10,000 nuclei were read into the memory of a Hewlett Packard Series 9000 computer and stored in list mode. Univariate and bivariate fluorescence distributions were constructed using the ELDAS software package (23). The green and red fluorescence signals were amplified logarithmically. To calculate the mean linear fluorescence intensity from the log histograms, channel 1 on the log scale was taken to be 1.0 arbitrary units on the linear scale and 60 channels difference on a 256 channel histogram represented a 10 fold difference in signal intensity. Using this conversion, CV's from log histograms were defined as 0.425 x FWHM/ mean.

Bivariate flow karyotyping:

Chromosome suspensions were made from cultured peripheral blood lymphocytes and bivariate flow karyotypes were recorded using a dual beam flow cytometer as described by Arkesteijn et al. (4). Briefly, isolated lymphocytes were cultured for 72 houres at 37 °C in the presence of 1% phytohemagglutinin (PHA; Wellcome Reagents, Dartford, UK) and treated with colcemid to accumulate cells in mitosis. After swelling the mitotic cells in hypotonic buffer (final concentrations: 10 mM NaCl, 4 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5), the chromosomes were released by lysing the cells through the addition of detergent Triton X-100 (0.4% final concentration), followed by gentle shearing through an Eppendorf pipet. Chromosomes were stained directly afterwards in this buffer with DAPI (2.7 μM) and chromomycin A3 (26 μM; both were from Sigma) and measured on the RELACS-3 flow cytometer, using UV (351-363 nm, 0.4 W) and 458 nm (0.2 W) excitation.

RESULTS

In situ hybridization in suspension:

Detection of DNA probes:

To assess the specificity of the fluorescent *in situ* hybridization procedure, isolated human peripheral lymphocyte nuclei were hybridized in suspension with genomic hamster DNA, genomic human DNA, the chromosome 1 specific repetitive DNA probe pUC1.77 and the chromosome Y specific repetitive DNA probe. Genomic hamster DNA served as a negative control, genomic human DNA served as a positive control. The biotinylated probes were labeled with avidin-FITC and all of the DNA was counterstained with DAPI. The results of hybridization of nuclei with the chromosome 1 specific probe pUC1.77, with genomic hamster

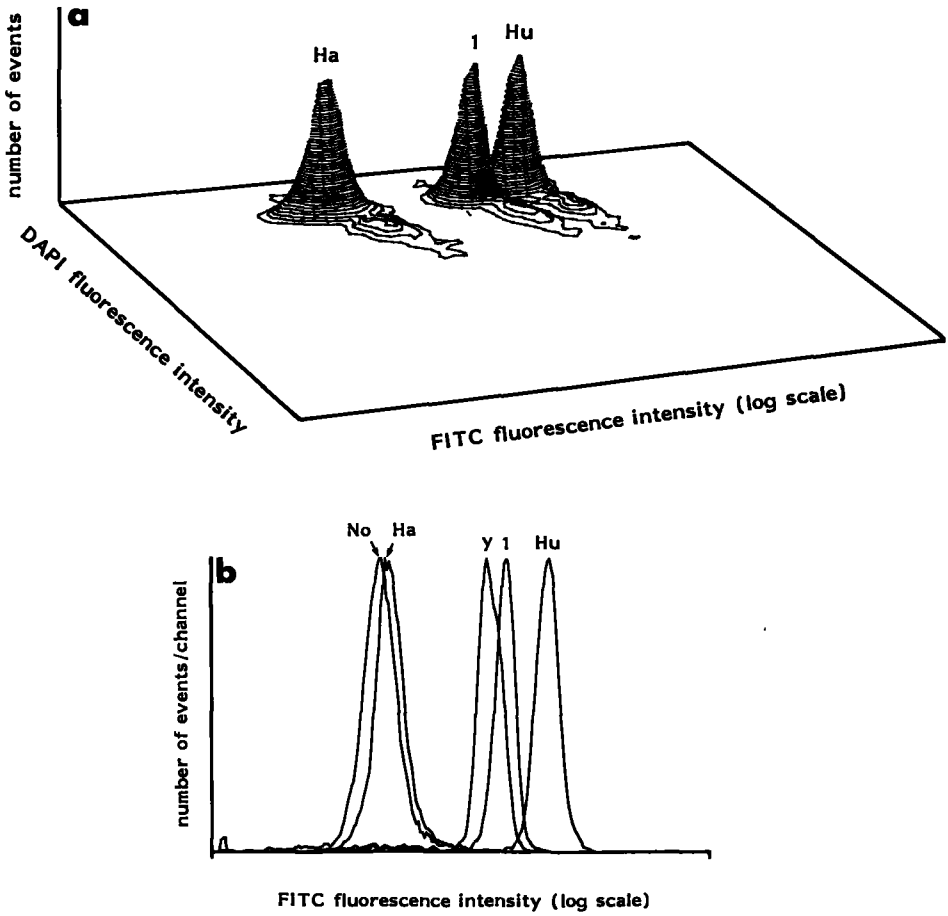


Figure 1:

Flow cytometric fluorescent *in situ* hybridization (FISH) of human lymphocyte interphase nuclei with biotinylated genomic hamster DNA (Ha), genomic human DNA (Hu), chromosome 1 specific repetitive DNA probe pUC1.77 (1) or chromosome Y specific repetitive DNA probe pY2.45 (Y). A: Dual parameter 64 x 64 channel stacked contour plot of DAPI vs. FITC (probe) fluorescence from 10,000 nuclei, hybridized with either genomic hamster, pUC1.77 or genomic human DNA. B: Univariate FITC (probe) fluorescence histograms from gated G0/G1 nuclei, hybridized with either genomic hamster, pUC1.77, pY2.45 or genomic human DNA. Also a histogram of nuclei, that followed the procedure without any biotinylated DNA (No) is included. Coefficients of variation (in %) of the histograms are: No, 30; Ha, 23; Y, 23; 1, 16 and Hu, 23.

DNA or with genomic human DNA, respectively, are displayed in one plot. The DAPI fluorescence is plotted against the FITC (probe) fluorescence as a stacked contour plot. The pUC1.77 probe fluorescence appeared to be less strong than the genomic human DNA probe fluorescence, but much stronger than the genomic hamster DNA probe fluorescence (fig. 1a). From the DAPI fluorescence it was concluded, that the peripheral lymphocytes appeared to be predominantly in G0/G1. Therefore, the univariate probe fluorescence histograms of nuclei in G0/G1 after hybridization with the above mentioned probes are plotted together (fig. 1b). A histogram of nuclei, which had followed the entire procedure without any biotinylated DNA, is also included. The mean linear fluorescence intensities of these histograms were calculated and expressed relative to the genomic hamster DNA fluorescence. The ratios obtained were as follows: for the chromosome 1 specific probe pUC1.77 9.1, for the chromosome Y specific probe pY2.45 6.8, and for genomic human DNA 21.3. The ratio between genomic hamster DNA and no probe was minimal: 0.83. To assess the reproducibility of the procedure within one experiment, three identical samples hybridized with the Y probe were measured. The mean linear fluorescence intensities were very similar (mean fluorescence 244 a.u., s.d. 5).

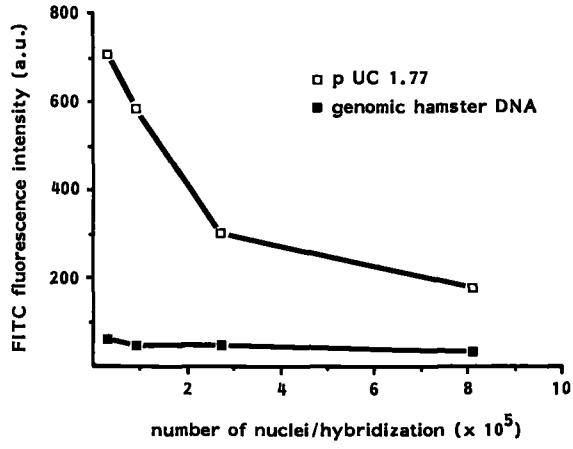


Figure 2:

The effect of the number of nuclei in the hybridization mixture on the resulting probe (FITC) fluorescence. Open squares indicate pUC1.77 DNA probe, closed squares indicate genomic hamster DNA. The nuclei concentrations were respectively 0.3, 0.9, 2.7 and 8.1 x 10^5 per 30 µl hybridization mixture.

Effect of target amount:

To assess the effect of variations in number of nuclei in the hybridization reaction on the resulting probe fluorescence, different numbers of nuclei were hybridized with the same amount of pUC1.77 or genomic hamster DNA (2 μg/ml final concentration). In fig. 2 the mean linear fluorescence intensity of G0/G1 nuclei is plotted against the number of nuclei, ranging from 0.3 to 8.1 x 10^5. It is clear, that after hybridization with the pUC1.77 probe, the fluorescence is strongly dependent on the concentration of these nuclei. Only a small effect is seen for genomic hamster DNA. The pUC1.77 probe obviously did not saturate its target at the lowest nuclei concentration. If the counting accuracy is in the order of 10%, a fluorescence difference of only 3% is expected at 10^5 nuclei as can be calculated from the slope of the curve. Since the fixed nuclei samples contained a significant number of clumps, forcing through a 23G needle of the aliquot that had to be counted, appeared necessary to perform an accurate count.

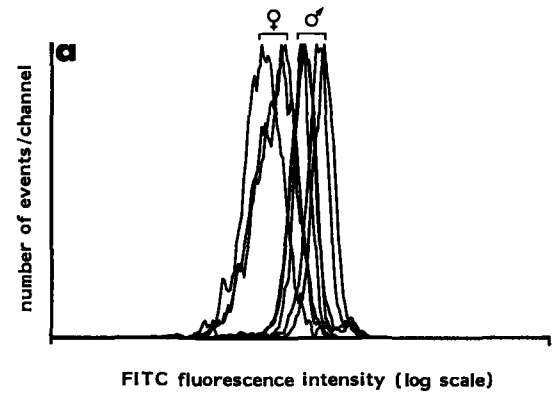
Interindividual Y probe variation:

Eight healthy volunteers (5 males, 3 females) were studied with respect to the quantitative differences of pY2.45 fluorescence in isolated lymphocyte nuclei. Genomic hamster DNA served as a negative control. All samples were measured in one run sequence after calibrating the flow cytometer with fluorescent beads. Laser power and amplifier settings were not changed during one run. The fluorescence histograms of the G0/G1 nuclei, hybridized with pY2.45 or hamster control DNA are shown separately in two plots (figs 3a and 3c). To quantitate these measurements, the mean linear fluorescence values were calculated from both pY2.45 and genomic DNA fluorescence histograms (table 1). A strong variation in pY2.45 mean linear fluorescence was seen in male volunteers (mean fluorescence 169 a.u., s.d. 35.6). In females also a significant pY2.45 fluorescence was found, but the variation was less apparent (mean 81 a.u., s.d. 10.7). The average of the means of the histograms was 2.1 fold higher for male than for female, and 7.2 fold higher than the mean of the hamster control values. The ratio between the mean linear female fluorescence and the mean hamster control was 3.4. The hamster control mean linear values were very similar (mean 23.5 a.u., s.d. 3.2) except for volunteer H, who had a much higher mean linear fluorescence value (table 1).

Polymorphism of centromere regions of chromosome 1:

In situ hybridization in suspension:

Isolated human peripheral lymphocyte nuclei of eight healthy volunteers (5 males, 3 females) were hybridized in suspension with pUC1.77 and with genomic hamster DNA as a negative control. Staining and flow cytometric measurement were performed as described above. The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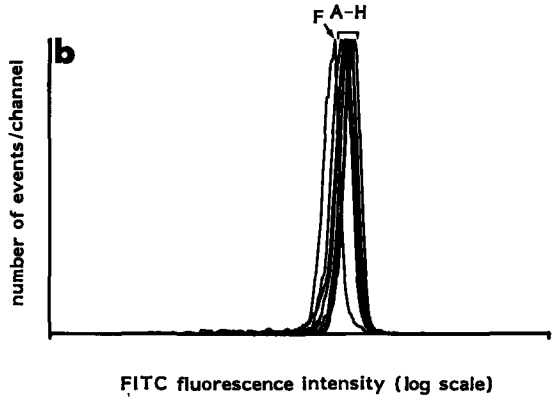
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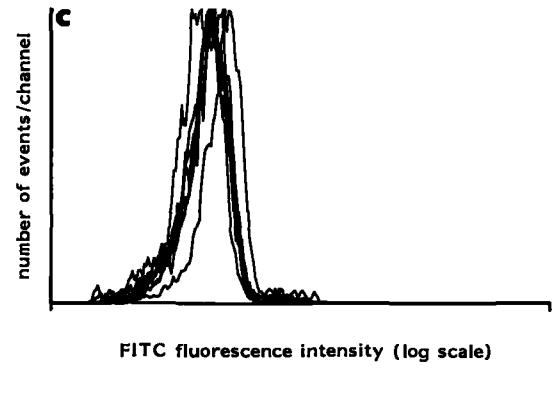
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TABLE 1

THE MEAN LINEAR FLUORESCENCE INTENSITY (A.U.) OF THE HISTOGRAMS OF G0/G1 LYMPHOCYTE NUCLEI AFTER FLOW CYTOMETRIC FISH. BETWEEN BRACKETS THE COEFFICIENT OF VARIATION (IN %) OF THE FLUORESCENCE HISTOGRAM PEAK IS GIVEN.

		DNA probe			
				pY2.45	
		hamster	pUC1.77	male	female
Volunteer	A:	23 (43)	366 (13)	136 (17)	
	B:	20 (37)	376 (11)	151 (22)	
	C:	23 (25)	322 (25)		69 (32)
	D:	23 (28)	384 (13)		88 (23)
	E:	23 (28)	334 (14)		87 (33)
	F:	22 (18)	258 (12)	146 (20)	
	G:	23 (29)	317 (15)	192 (21)	
	H:	31 (28)	347 (14)	220 (17)	
mean:		23.5 (29.5)	338 (14.6)	169 (19.4)	81 (29.3)
s.d.:		3.2	40.6	35.6	0.7
n:		8	8	5	3

Figure 3:

Combined probe (FITC) fluorescence histograms of eight healthy volunteers. Histograms were constructed from 4,000-5,000 G0/G1 gated lymphocyte nuclei after flow cytometric FISH. A: Chromosome Y specific DNA probe pY2.45. B: Chromosome 1 specific DNA probe pUC1.77. C: Genomic hamster control DNA probe.

fluorescence histograms of the G0/G1 nuclei, hybridized with pUC1.77 are shown in one plot (fig. 3b). The mean linear fluorescence values were calculated from both pUC1.77 and genomic hamster DNA fluorescence histograms (table 1). The mean pUC1.77 value was 14.4 fold higher, than the mean hamster control value. It appeared, that one person (volunteer F) had a clearly lower pUC1.77 log fluorescence profile, as compared with the others. The difference between the mean linear pUC1.77 signal from F and the mean value of the seven others was 26%.

In situ hybridization on glass slides:

Interphase and metaphase cells of peripheral blood lymphocyte cultures of the volunteers were fixed and dropped on glass slides. After *in situ* hybridization with pUC1.77 the resulting hybrid was labeled with avidin-FITC and the DNA was counterstained with propidium iodide to allow simultaneous detection of both probe and total DNA. The results are shown in figure 4. The pUC1.77 satellite DNA targets were localized on the centromeres of the chromosomes 1 and had about the same size both in interphase and metaphase cells (fig. 4a). Metaphase chromosomes of volunteer F appeared to have one normal hybridization signal on one chromosome 1 and a very small signal on its homologue. This pattern could be distinguished both in interphase as well as metaphase cells (fig.4b).

Bivariate flow karyotyping:

Bivariate flow karyotypes were constructed of DAPI-Chromomycin A3 double stained lymphocyte metaphase chromosomes of all volunteers except H (not available). Bivariate histograms were made of the linear signals of both Chromomycin A3 and DAPI fluorescence and the chromosome peaks were numbered based on quantitative cluster analysis. Volunteer D, assigned as "normal" after ISH on a glass slide (fig. 4a) also appeared to have normal chromosomes 1 in a bivariate flow karyotype (fig. 5a). Volunteer F, who appeared to have very little satellite DNA on one of the chromosomes 1 (fig. 4b), showed clear polymorphism of chromosome 1 in the bivariate flow karyotype (fig. 5b). In this karyotype both homologues can be distinguished as separate peaks. The 1b peak has a 5.5% lower DAPI (AT-binding) fluorescence and a 0.5% lower Chromomycin A3 (GC-binding) fluorescence, when compared with homologue peak 1a. In another flow karyotype of volunteer F, the differences between the homologue peaks were 7.7% for DAPI and 1.1% for Chromomycin A3, respectively (table 2). The bivariate histograms of the volunteers (except H) were used to study the relationship between pUC1.77 fluorescence intensity and flow karyotype position of the #1 chromosomes. In order to compare the #1 chromosomes related fluorescence intensities of DAPI and Chromomycin A3, the chromosome 10-12 peak position in the flow karyotype was taken as a

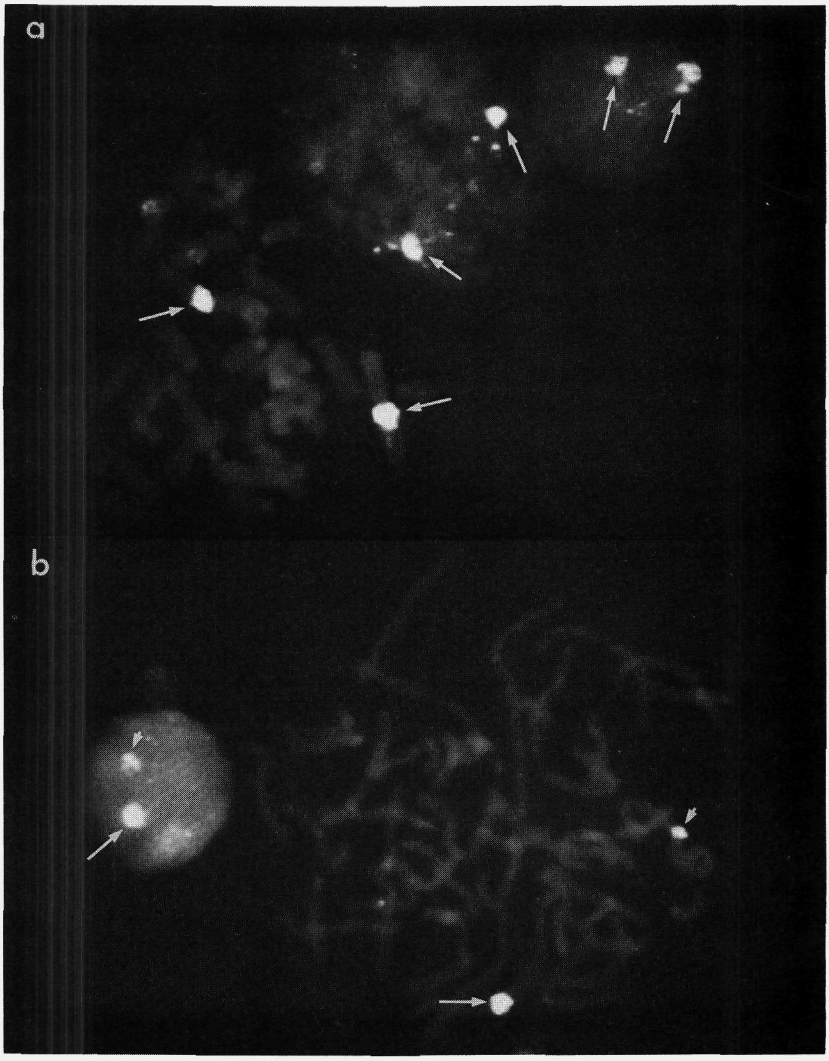

Figure 4:

Photomicrographs demonstrating FISH of biotinylated chromosome 1 specific satellite DNA probe pUC1.77 to lymphocyte metaphase and interphase cells. The hybridized probe was detected with fluoresceinated avidin. All of the DNA was counterstained with propidium iodide. The regions to which probe hybridized appear white in the photomicrograph. The regions of DNA to which the probe did not bind appear grey. A: Hybridization of pUC1.77 to normal chromosomes 1. B: Hybridization of pUC1.77 to polymorphic chromosomes 1. Arrows indicate normal pUC1.77 targets, arrowheads indicate polymorphic pUC1.77 targets.

TABLE 2

THE RELATION BETWEEN THE MEAN LINEAR FLUORESCENCE INTENSITIES (a.u.) OF DNA PROBE PUC1.77 AFTER FLOW CYTOMETRIC FISH TO LYMPHOCYTE NUCLEI AND OF DAPI/CHROMOMYCIN A3 DOUBLE STAINED #1 CHROMOSOMES AFTER BIVARIATE FLOW KARYOTYPING.

DAPI/Chromomycin A3 fluorescence intensity values are relative to those of the 10-12 peak in the flow karyotype. Between brackets are given the values, normalized to the highest value in each group.

		Flow cytometric FISH	Bivariate flow karyotyping	
		pUC1.77	DAPI	Chromomycin A3
Volunteer	A:	366 (95%)	185 (98%)	178 (97%)
	B:	376 (98%)	172 (91%)	177 (96%)
	C:	322 (84%)	178 (94%)	179 (97%)
	D:	384 (100%)	189 (100%)	184 (100%)
	E:	334 (87%)	171 (90%)	174 (95%)
	F:	258 (67%)	176 (93%)*	178 (97%)*
	- peak 1a:		183 (97%)	179 (97%)
	- peak 1b:		169 (89%)	177 (96%)
	G:	317 (83%)	183 (97%)	180 (98%)

*Here, the mean value of the two homologue clusters is given.

reference point (17). The variation in pUC1.77 fluorescence did not demonstrate a direct correlation with the DAPI (and Chromomycin A3) fluorescence values of the #1 chromosomes in the flow karyotype (table 2). Although the lower #1 peak of volunteer F showed the lowest DAPI value obtained (89%), the mean of the DAPI values of both homologue peaks (93%) fell within the observed range of DAPI variation (90-100%; table 2). From this we conclude, that the variation of the position of the chromosome 1 cluster in the flow karyotype is related only to a minor extent to differences in satellite DNA content. But, when extreme polymorphism was seen between the two homologues of one individual with respect to satellite DNA (fig. 4b), the flow karyotype did demonstrate this homologue polymorphism.

TABLE 3

THE RELATION BETWEEN THE MEAN LINEAR FLUORESCENCE INTENSITIES (a.u.) OF DNA PROBE PY2.45 AFTER FLOW CYTOMETRIC FISH TO LYMPHOCYTE NUCLEI AND OF DAPI/CHROMOMYCIN A3 DOUBLE STAINED Y CHROMOSOMES AFTER BIVARIATE FLOW KARYOTYPING.

DAPI/Chromomycin A3 fluorescence intensity values are relative to those of the 10-12 peak in the flow karyotype. Between brackets are given the values, normalized to the highest value in each group.

		Flow cytometric FISH	Bivariate flow karyotyping	
		pY2.45	DAPI	Chromomycin A3
Volunteer	A:	136 (71%)	67 (86%)	26 (84%)
	B:	151 (79%)	67 (86%)	30 (97%)
	F:	146 (76%)	68 (87%)	29 (94%)
	G:	192 (100%)	78 (100%)	31 (100%)

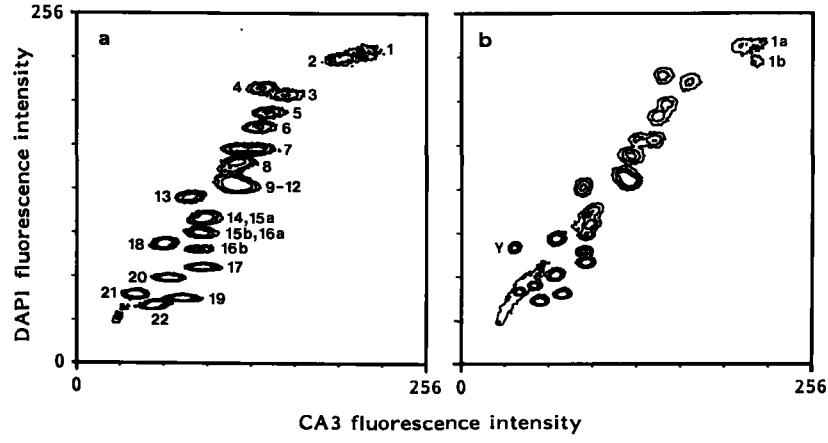

Figure 5:

Bivariate flow karyotypes of Chromomycin A3/DAPI double stained lymphocyte metaphase chromosomes of female (A) and male (B) healthy volunteers. Fluorescent signals from 100,000 chromosomes were recorded to construct a high-resolution 256 x 256 channel matrix. The chromosome peaks were numbered based on quantitative cluster analysis.

The bivariate histograms of male volunteers A, B, F and G were used to study the relationship between pY2.45 fluorescence intensity and flow karyotype position of the Y chromosome (table 3). Again, the chromosome 10-12 peak position in the flow karyotype was taken as a reference point. The strong variation in pY2.45 fluorescence was found to correlate with the DAPI (and Chromomycin A3) fluorescence of the Y chromosome in the flow karyotype. Volunteer G, who showed a strong pY2.45 fluorescence, when compared with subjects A, B and F, also appeared to have accordingly higher fluorescence values for DAPI (and Chromomycin A3).

Bicolour double probe flow cytometric fluorescent *in situ* hybridization:

The results described above indicated that it would be useful to have an internal control in each measurement. Such a control could be a second probe. To investigate, whether two target sequences could be detected simultaneously by two color flow cytometric FISH, isolated human peripheral lymphocyte nuclei were hybridized *in situ* in suspension simultaneously with the biotinylated probe pUC1.77 and the AAF-modified Y probe. As a control each probe was also hybridized separately. Biotin was detected with avidin-TRITC (red) and AAF was detected

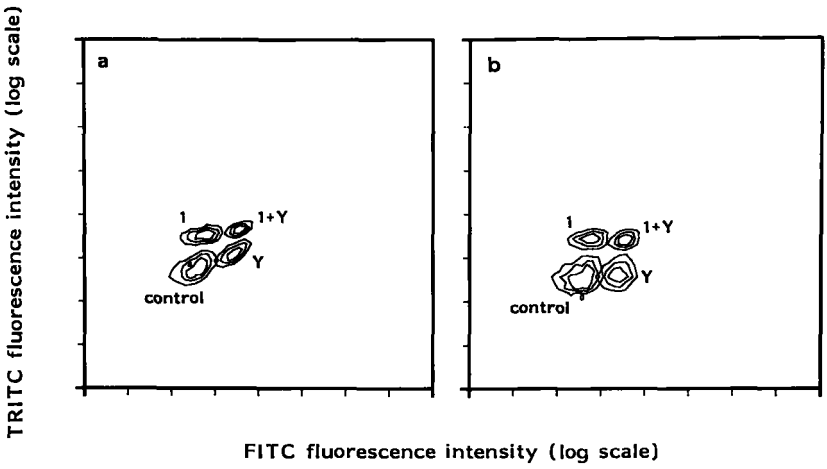

Figure 6:

Bivariate 64 x 64 channel FITC vs. TRITC plots from 4,000-5,000 gated G0/G1 human lymphocyte nuclei, hybridized with no probe, biotinylated pUC1.77 only, AAF-modified pY2.45 only or both probes simultaneously. The biotinylated pUC1.77 DNA probe was detected with avidin-TRITC, the AAF-modified pY2.45 DNA probe was detected with FITC-labeled second antibody. A: Combination plot. B: Combination plot after software correction for FITC fluorescence spillover into the TRITC log fluorescence.

that were hybridized with both DNA probes, carried two red fluorescing chromosome 1 spots and one green fluorescing chromosome Y spot (not shown). The samples were also analyzed by triple beam flow cytometry. The results are plotted as contour lines in a 64 x 64 channel matrix. by indirect immunofluorescence with a FITC-labeled (green) second antibody. After hybridization the samples were examined by fluorescence microscopy. The lymphocyte nuclei, The results of the separate experiments are combined in one plot. The bivariate plots of no probe control, pUC1.77 only, Y probe only and both probes simultaneously are found clearly in four different sectors (fig. 6a). It can be seen, that the TRITC log fluorescence signal was enhanced by spectral overlap from the FITC fluorescence due to significant excitation of FITC by 515 nm laser light and this was corrected by software (fig. 6b; 23). The weaker TRITC fluorescence spillover into the FITC log fluorescence was not considered for correction. The ratio between the negative control and the pUC1.77 TRITC fluorescence as well as the chromosome Y hybrid FITC fluorescence was a factor 3 in both simultaneous and separate hybridization with these probes.

DISCUSSION

The ability to perform fluorescent *in situ* hybridization to cell nuclei in suspension enabled us to determine the amount of target sequences by flow cytometry. The reliability of the measurements depends strongly on the sensitivity and the reproducibility of this FISH method. The probe related fluorescence can be influenced by several variables, such as probe and target amount (fig. 2), size and labeling of the probe, and hardware fluctuations. Within one experiment these variables can be adequately controlled. The most important variables might be the difference in accessibility and aspecific binding of the DNA in different cells. In order to compare aspecific binding of different samples, we used a hamster DNA probe as a negative control. To validate specific signals, we compared flow cytometric FISH with FISH on slides and bivariate flow karyotyping.

The sensitivity of the measurements can be estimated by comparing results of the genomic hamster negative control and the chromosome 1 and Y specific DNA probes. The ratio in fluorescence intensity was approx. 10 fold (fig. 1a,b). The ratio between the chromosome specific probes and the genomic human DNA probe was only 2-3. With a genome of roughly 10^3 megabase (Mb) and probes with targets of several Mb, we would expect at least a factor 100. However, we calculated, that a shortage of genomic DNA probe could be expected. In each sample 10^5 nuclei were used, representing 0.6 µg of DNA (6 pg/cell). The amount of probe added per sample for all probes used was 0.06 µg, leading to a 10-fold shortage of

genomic probe DNA. Consequently the chromosome specific probes were hybridized under near-saturating conditions (fig. 2). In case of the chromosome specific probes, the whole (biotinylated) plasmid was used for hybridization. Under our hybridization conditions this caused network formation of the probe, leading to increasing fluorescence signals (28). The genomic probe was not cloned in plasmids, and therefore the formation of networks is not expected. Furthermore one might expect, that under the conditions used, mainly the repetitive sequences will hybridize. For human DNA this accounts for 15-20% of the total amount of DNA. This would reduce the amount of target for hybridization. Taken this altogether, the genomic probe signals can be expected to be considerably lower.

Until now large targets, in the megabase range, are required for reliable quantitation of FISH by flow cytometry. Those are provided by most repetitive DNA probes, especially the satellite DNA probes. Using these probes the accuracy is excellent. However, when different experiments were compared, there was a slight variation in reproducibility of the relative signal intensities. The fluorescence intensity for pUC1.77 relative to hamster control was a factor 9.1 in fig. 1b, a factor 14.4 in fig. 3 for pUC1.77. For pY2.45 this was 6.7 in fig. 1b and 7.2 in fig. 3. This is most likely due to the variation of the efficiency of biotin-labeling of the probe (34). The amount of aspecific binding of the genomic hamster probe appeared to be minimal, when compared with no probe (fig. 1b), indicating that the procedure causes little aspecific binding of DNA.

Chromosome polymorphism in normal individuals is frequently seen (7, 16). Harris et al. (19) investigated the flow karyotypes of healthy volunteers and found a high frequency of polymorphism of, especially, chromosomes 1, 9, 16 and Y. The big differences in pY2.45 fluorescence (fig. 3a) in the male volunteers represent the naturally occurring variation within the large block of heterochromatin, found on the long arm of the Y chromosome. In this region the chromosome Y satellite DNA sequences are located. In the bivariate flow karyotype, we are able to analyze the chromosomes with respect to their DNA content, and the AT to GC ratio, as determined by their DAPI and Chromomycin A3 fluorescence, respectively. For the study of pericentromeric heterochromatin variation of chromosomes 1, 9, 16 and Y, DAPI/Chromomycin flow karyotyping appeared to be very useful (31; Arkesteijn et al., manuscript in preparation). When compared with Hoechst/Chromomycin staining, these chromosomes show stronger AT-related fluorescence, probably due to higher AT-specificity of DAPI. In these flow karyotypes, polymorphism between individuals of the Y chromosome can be seen as a remarkable shift in DAPI or Hoechst fluorescence (17, 27). This polymorphism most likely reflects the underlying variation in satellite DNA, which is AT-rich. This is consistent with our results (table 3). The relatively high pY2.45 fluorescence intensities of the females (fig. 3a) can be partly explained by the fact, that 5-10% of the Y-satellite sequences are

located elsewhere in the genome (12). Using a comparable Y specific repetitive DNA probe (9), Smith (cited in (39)) found by Southern blot analysis, that average male cells contained sixfold more target for this probe than female cells. We attribute the lower value we found to differences in target saturation by the probe. In males probe pY2.45 hybridizes under non-saturating conditions (compare pUC1.77 in fig. 2), whereas in females the probe most likely can saturate its smaller autosomal target.

No direct correlation was found between the pUC1.77 values and the DAPI (and Chromomycin A3) fluorescence intensities (table 2), whereas pY2.45 fluorescence was found to correlate clearly with the flow karyotype position of the Y chromosomes (table 3). This is likely due to the proportion of satellite DNA on both chromosomes, being high for the Y chromosome and much smaller for chromosome 1. Therefore, variations in satellite DNA content of the Y chromosome, as measured by *in situ* hybridization (fig. 3), will result in significant differences in total DNA of the Y chromosome. For chromosome 1, this effect on the position of the #1 peaks in the flow karyotype will be less obvious, and can be "masked" easily by differences between individuals in total DNA content of this chromosome (table 2).

A statistical evaluation of the pUC1.77 and hamster control fluorescence data was carried out to check the significance of this experiment. Therefore, the mean linear fluorescence values were calculated. Testing the mean linear fluorescence value of subject F (258) against the distribution of the pUC1.77 values for the other volunteers (mean fluorescence 349, s.d. 26.5), the former value appeared to be an outlier (level of significance < 0.002). Likewise, at the same level of significance the hamster control fluorescence of volunteer H is higher than the others. This was not correlated with a different pUC1.77 fluorescence value (table 1). For the higher control value no explanation was found. The 26% difference in pUC1.77 fluorescence was found to be due to one chromosome 1 with a very small hybridization spot (fig. 4). We estimate, that this difference, which we were able to measure by flow cytometry, is in the order of a few Mb.

The results of the flow karyotypes (figs 5) match with the results of both pUC1.77 hybridization studies. In our study, we were able to assign this chromosome 1 polymorphism to a loss of satellite DNA on a homologue of chromosome 1. In order to connect the results of the hybridization studies with the flow karyotypes, we estimated the relative mass of pUC1.77 target on chromosome 1. This was performed by measuring the length of both chromosome 1 and hybridized target on photographs of metaphase chromosomes after FISH on glass slides. From 12 equally hybridized chromosomes 1 we estimated the pUC1.77 target to be approx. 13% of the total chromosome 1.

A 26% decrease of pUC1.77 fluorescence was measured after FISH in suspension to normal diploid nuclei. This was attributed to one homologue by conventional FISH (fig. 4b),

which implicates an approx. 50% loss of pUC1.77 DNA on that chromosome. The amount of pUC1.77 satellite DNA on chromosome 1 being estimated to be 13% of the total chromosome DNA, this would be 6.5% on the polymorphic homologue. It means, that we expect a 6.5% decrease of the total DNA content of this chromosome 1. Since satellite DNA is very rich in AT basepairs, we would expect a decrease in DAPI fluorescence, rather than in Chromomycin A3 fluorescence in the bivariate flow karyotype. This was indeed the outcome of the karyotype; a 5.5% decrease in DAPI fluorescence and a 0.5% decrease in Chromomycin A3 fluorescence in one of the chromosome 1 peaks (fig. 5b), which is in close agreement with the expected 6.5%.

The fluorescence intensities in the double labeling experiment (figs 6) are lower, when compared to single labeled nuclei (figs 1 and 3). For the pUC1.77 TRITC fluorescence, it can be explained by the inefficient excitation of this fluorochrome at 515 nm. The resolution of the measurement can be improved by using a dye laser and replacing TRITC by XRITC or Texas Red. Such a setup will also solve the spectral overlap problem. The Y probe related FITC fluorescence was probably significantly lowered through the addition of a 520 nm long pass filter to the 530 nm band pass filter. The 520 nm filter was used to block scattering 515 nm laser light. A dye laser for TRITC or XRITC excitation would make this filter unnecessary.

Some aspects of the procedure can be considered for improvement. The fixation, which prevents the nuclei from disintegrating during denaturation and hybridization, might reduce the accessibility of the DNA target sequences. The thermal denaturation causes a significant loss of DNA (5, 35), which might also affect the amount of target DNA. When less harsh denaturation protocols are used, such as enzymatic procedures (40), more DNA might be left for hybridization and less stringent fixation protocols might be required, leading to better accessibility.

Since satellite DNA polymorphism is inherited, it is possible to trace its origin. Thus it could be used in family studies for prenatal detection of chromosome 1 related diseases (30). A more important application of FISH in suspension is the quantitation of numerical aberrations in tumour cells and cell lines. Strong polymorphism, as shown in this paper, could reduce the validity of these measurements. Therefore, it is important to view the hybridized nuclei under the fluorescence microscope before flow cytometry. When strong polymorphism is seen, the results should be interpreted with caution. Bicolor hybridizations using one probe as a reference might be useful in this respect. This is probably preferable to the method, described by Trask et al. (39), who hybridized mixtures of different cell lines as internal controls. These different cell lines might exhibit different hybridization qualities, due to differences in chromatin structure.

An advantage of FISH in suspension is the possibility to perform the investigation on interphase nuclei instead of metaphase figures, since most solid tumours have a very low mitotic index (36). Flow cytometry allows for the discrimination of subpopulations, based on

parameters like light scatter and DNA content. The combination of interphase cytogenetics and flow cytometry could improve the detection of tumorigenic populations with numerical chromosome aberrations within tumours. FISH might be used to quantitate gene amplification, when the sensitivity has improved. This gene amplification is correlated with clinical and prognostic parameters in various cancers (37, 45).

By performing the entire procedure in suspension, the morphology of the nuclei is preserved (33). It opens the way to the investigation of the three-dimensional intranuclear architecture of chromosomes by optical sectioning and 3-D reconstruction (1, 8). By labeling specific regions (centromeres, telomeres) with chromosome specific DNA probes, a map could be constructed of the interior of the cell nucleus (33; Van Dekken et al., in preparation).

The applicability of the flow cytometric cytogenetic analysis described in this paper for the detection of chromosome aberrations within human tumour cells and cell lines is presently being investigated.

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Part II: THREE-DIMENSIONAL ANALYSIS OF CHROMOSOMES IN CELL NUCLEI

CHAPTER 4

THREE DIMENSIONAL ANALYSIS OF THE ORGANIZATION OF HUMAN CHROMOSOME DOMAINS IN HUMAN AND HUMAN-HAMSTER HYBRID INTERPHASE NUCLEI

Herman van Dekken, Daniel Pinkel, Jim Mullikin, Barbara Trask,
Ger van den Engh and Joe Gray

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ABSTRACT

This report describes the intranuclear organization of chromosomes in human-hamster hybrid nuclei and in human cell nuclei. The target chromosomes were stained using *in situ* hybridization with biotinylated, chromosome-specific DNA probes. Bound probe was detected with fluorescein-avidin. Hybridizations were performed to fixed nuclei in aqueous suspension in order to preserve their three dimensional morphology. Total nuclear DNA was stained with DAPI. Three dimensional information about the organization of DNA and probe within the nucleus was obtained by optical sectioning.

The human chromosomes in human-hamster hybrid nuclei were found to be confined to domains" that were maintained during the cell cycle". Different spatial localization patterns of the human chromosomes were seen in interphase nuclei of two different hybrid cell lines. The positions of chromosome-specific repetitive sequences in human fibroblast interphase nuclei were also studied using probes for 1p36, 9q12 and Yq12. These studies showed that the two 1p36 loci (near the telomere of the short arm of chromosome 1) are located near the nuclear surface. The 9q12 loci are similarly located. Simultaneous hybridization of the 1p36 probe (target size ~200 kb) and the Yq12-specific probe (target size >2Mb), demonstrate that the binding sites of the two probes can be distinguished in the same nucleus based on domain size.

INTRODUCTION

Interest in the organization of chromosomes in interphase nuclei began as soon as chromosomes were first visualized (Rabl, 1885; Boveri, 1888). Some evidence for consistent spatial relationships among interphase chromosomes has been inferred from the statistical associations of chromosomes types in metaphase spreads (Comings, 1980 and references therein), and from the relative frequencies of reciprocal translocations (Hager et al, 1982). In the last decade UV-microbeam irradiation of small portions of nuclei indicated that chromosomes were confined to compact domains rather than being dispersed throughout the nucleus (Zorn et al, 1979). However, all of these studies have been limited by the inability to visualize individual chromosomes in interphase nuclei.

This situation has now changed as a result of the development of chromosome-specific staining by *in situ* hybridization with non-radioactively labeled chromosome specific nucleic acid probes. In these procedures (Langer et al, 1981; Landegent et al, 1984; Hopman et al, 1986) the probes are chemically modified, and after hybridization, the modifications in the bound probe are detected with affinity reagents such as antibodies, that carry fluorochromes or

enzymes. The main advantages of these techniques over auto-radiographic detection of radioactively labeled probes are: 1) Speed. Hybridization can be accomplished in one day. 2) Spatial resolution. The reporting molecules are located very close to the bound probe. 3) Probe stability. Chemically modified probes are stable for many months.

Genomic DNA can be used as a species-specific probe to identify chromosomes in human-rodent hybrid cell lines (Schardin et al, 1985; Manuelidis, 1985; Pinkel, 1986b). In addition many DNA sequences that can be used to identify specific chromosomes within a species have been cloned. Probes for chromosome-specific repeated sequences on over half of the human chromosomes are now available (Trask et al, 1988 and references therein). These typically hybridize to restricted regions of the target chromosome, most frequently near the centromere. In addition, techniques for use of multiple probes distributed along the length of a chromosome that permit hybridization to the entire chromosome have been developed (Pinkel et al, 1988, Fuscoe et al, 1988).

Application of these techniques to analysis of the organization of human and hybrid interphase nuclei has already shown that chromosomes are located in discrete domains (Manuelidis, 1985; Schardin et al, 1985; Pinkel et al, 1986; Rappold et al, 1984). In addition, studies of polytene chromosomes in Drosophila (Agard and Sedat, 1983; Mathog et al, 1984) and human neural cells (Manuelidis, 1984 and Manuelidis and Borden, 1988) have demonstrated the non-random arrangement of chromosomes in interphase.

We report here, further evidence of organization in interphase nuclei. In this study, chromosomes were stained using fluorescence *in situ* hybridization with chromosome specific probes (Pinkel et al, 1986b). The three dimensional (3-D) morphology of the nuclei was preserved by performing the entire procedure in suspension (Trask et al, 1985; 1988), thus allowing optical sectioning and 3-D reconstruction. These techniques were applied to analysis of the organization of human chromosomes in human-hamster hybrid nuclei and to the organization of the 1p36, 9q12 and Yq12 loci in human fibroblast nuclei.

MATERIALS AND METHODS

Cell lines:

Human-hamster cell lines UV20HL21-27 and UV20HL21-29 were developed by Larry Thompson at the Lawrence Livermore National Laboratory. They contain human chromosomes 4, 8 and 21; and 8 and 12, respectively. This was confirmed by isozyme and banding analysis (Thompson et al, 1985), and by flow karyotyping (Gray et al, 1986). However, UV20HL21-

27 loses chromosome 21 during extended culture. The human fibroblast cell strain 761 (46,XY) was established from cells isolated from normal human foreskin (Gray et al, 1979).

Preparation of isolated nuclei:
Nuclei were isolated according to the Hepes-$MgSO_4$ method (Van den Engh et al, 1985) and fixed in 70% ethanol for 10 min. at 4°C, centrifuged (each centrifugation: 10 min, 100 g) and resuspended in 100% ethanol for 10 min. at 4°C. Isolated nuclei were stained with the DNA specific dye Hoechst 33258 (2 mg/ml; Sigma) in isolation buffer and sorted with the Livermore High Speed Sorter (Peters et al, 1985; Gray et al, 1987). Up to $5x10^6$ nuclei in G1, S and G2 + M were sorted according to Hoechst 33258 fluorescence. Isolation buffer was used as the sheath fluid to preserve nuclear morphology after sorting. Human fibroblast nuclei predominantly in the G0/G1 phase of the cell cycle were obtained by growing the cells to confluency in T-150 flasks (Corning) in 5% CO_2 at 37°C in minimum essential medium-alpha containing 20% fetal calf serum.

Probes:
Human genomic DNA was isolated from peripheral blood lymphocytes. The human 1p36 probe (p1-79) is a 900 basepair DNA sequence cloned in pSP65 (Buroker et al, 1987). This probe was supplied by Dr. M. Litt (University of Oregon). The human 9q12 probe, pHuR98, is a 160 basepair sequence cloned in pBR322 (Moyzis et al, 1987). This probe was provided by Dr. R. Moyzis (Los Alamos National Laboratory). The human Yq12 probe, pY431 A, is a 800 basepair sequence cloned in pBR322 (Burk et al, 1985). This probe was supplied by Dr. K. Smith (Howard Hughes Medical Institute, Johns Hopkins University.) Probe DNA was labeled by nick-translation with biotin-11 dUTP (Bethesda Research Laboratory). For the cloned probes, the complete plasmid was nick-translated and used for *in situ* hybridization.

Fluorescence *in situ* hybridization:
Protein was extracted from isolated nuclei by resuspending them in 0.1 M HCl + 0.5% Triton X100 for 10 min at room temperature. The nuclei were then fixed in 1% paraformaldehyde in isolation buffer (5 mM Hepes; 50 mM Kcl; 10 mM $MgSO_4$; 0.05% Tween 20; pH 8) for 1 min. at room temperature. Nuclei were washed once in isolation buffer and resuspended in this buffer at a concentration of 10^5/ml. Approximately $2x10^5$ nuclei in 2 µl of isolation buffer were added to 8 µl hybridization mix (final concentrations: 50% formamide; 2x SSC; 10% dextran sulphate; 1% Tween 20; pH 7) and denatured for 10 min. at 70°C. Probe DNA (1-3 µg/ml) and carrier DNA (250 µg/ml herring sperm DNA) were denatured for 10 min. at 70°C in hybridization mix. Both mixtures were quickly cooled on ice and 20 µl probe mix was added to

the nuclei in an Eppendorf tube. The sample was mixed and incubated for 10-18 hours at 37°C. The hybridized nuclei were washed in 1 ml 50% formamide; 2x SSC; pH 7 for 10 min. at 42°C. 100 µl dimethyl–suberimidate-fixed erythrocytes (10^8/ml in isolation buffer) were added to serve as a carrier (Trask et al, 1985; 1988). The nuclei and erythrocytes were centrifuged and resuspended in 1 ml 2x SSC; pH 7 for 10 min. at room temperature and washed again in 1 ml PN buffer (0.1 M sodium phosphate; 0.05% Nonidet P40; pH 8) for 10 min. at room temperature. The cells were then incubated in 100 µl PNM buffer (5% non-fat dry milk [Carnation] in PN) for 5 min. at room temperature to prevent non-specific binding of the avidin. The nuclei were stained by adding an additional 100 µl of staining buffer (10 µg/ml avidin-FITC DCS [Vector Laboratories, Burlingame, CA] in PNM) and incubating for 30 min. at 37°C. The stained nuclei were washed in 1 ml PN for 10 min. at room temperature and resuspended in 100 µl isolation buffer after vigorous mixing. They were counterstained for DNA using 100 µl DAPI (1 µg/ml; Sigma) in an antifade solution (1 mg/ml p-phenyl–enediamine dihydrochloride (Sigma) in 90% glycerol; 10% PBS; pH 8 (v/v); (Johnson and Nogueria, 1981). Twenty µl of the mixture was put on a glass microscope slide under a 22 x 22 mm glass coverslip for microscopic analysis. The viscosity of the antifade solution prevents the nuclei from moving during microscopy.

Optical sectioning and 3-D reconstruction:

Optical sectioning was performed using a Leitz microscope equipped with a 100X, 1.3 numerical aperture objective. Images from the microscope were acquired using a SIT vidicon TV camera interfaced to an image analysis system (Trapix 55/64, Recognition Concepts Inc.) controlled by a microvax computer (Digital Equipment Corp.). The microscope focus was adjusted by a stepping motor, also controlled by the microvax. This system allowed automatic acquisition of serial images (optical sections) at regularly spaced planes of focus through a nucleus. Typically, the spacing between optical sections was 0.5 µm or 1.0 µm. Two sets of up to 50 images were collected for each nucleus. One set was acquired using filters that passed green in fluorescence from fluorescein to define the chromosomal boundaries. The other set was acquired using filters that passed blue fluorescence from DAPI to define the nuclear boundary. Individual optical sections were composed of 128x128 pixels, each containing 8 bits of intensity information. The images were transferred to a VAX 750 computer where nuclear and chromosome domain boundaries were determined by interactive intensity thresholding. The stacks of nuclear and chromosome boundaries were then displayed at a user-defined orientation using an isometric display program. Image interpolation was used as necessary so that all reconstructions appeared to have sections separated by 0.5 µm.

RESULTS

Human chromosomes in hybrid cell nuclei:

Optical sections were recorded for interphase nuclei from two different human-hamster hybrid cell lines following hybridization in suspension with human genomic DNA probe. Flow cytometric analysis of the DNA content of the hybrid cell nuclei following Hoechst staining indicated that the majority of these interphase cells were in G1 phase of the cell cycle. Three dimensional reconstructions of over 20 nuclei of each hybrid were made and analyzed and many more nuclei were viewed under the microscope. Figure 1 a-f show reconstructions of 6 nuclei from hybrid UV20HL 21-29 and Figure 2 a-f show reconstructions of 6 nuclei from hybrid

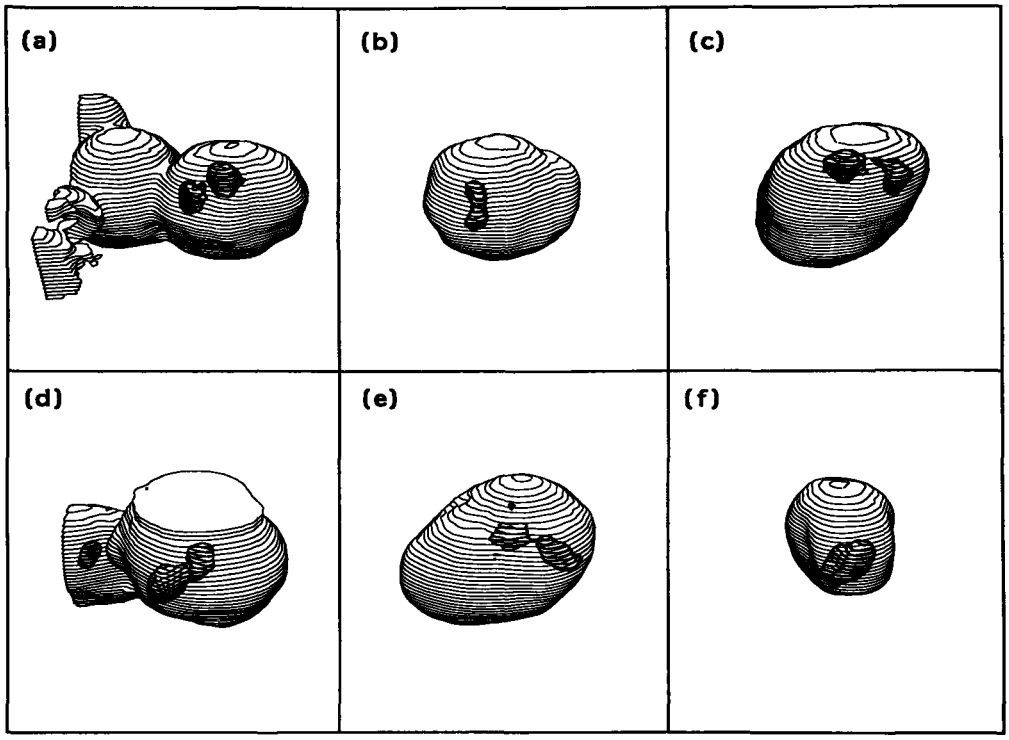

Figure 1:
Reconstructions of the positions of human chromosomes in interphase nuclei from the human-hamster hybrid cell line UV20HL21-29. This line contains human chromosomes 8 and 12. Panels a-f show 3-D reconstructions of six different nuclei.

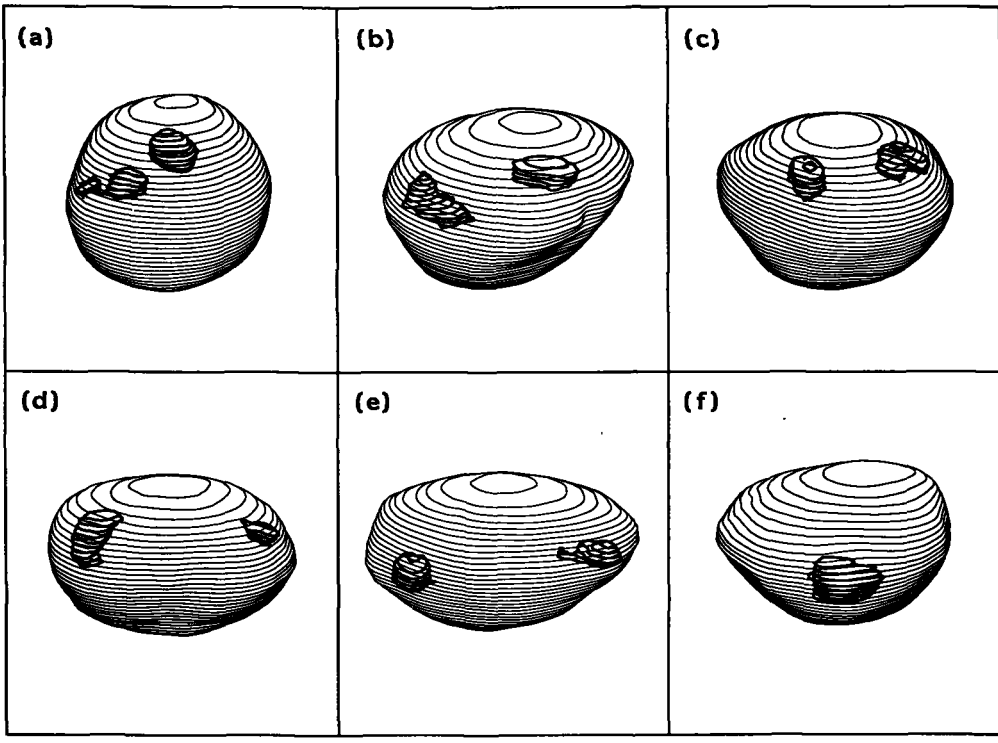

Figure 2:
Reconstruction of the positions of human chromosomes in interphase nuclei from the human-hamster hybrid line UV20HL21-27, containing human chromosomes 4, 8 and occasionally also 21. Panels a-f show 3-D reconstructions of 6 different nuclei.

UV20HL 21-27. These reconstructions were rotated about the Z axis so that the maximum separation of the human chromosome domains could be judged. In hybrid UV20HL 21-29, containing human chromosomes 8 and 12, the two chromosomal domains were generally in close proximity. However, in hybrid UV20HL 21-27, containing human chromosomes 4, 8 and occasionally also 21, the human chromosomal domains were generally well separated. Three domains were visible in ~25% of the nuclei from UV20HL 21-27.

Cell cycle effects in hybrid cell nuclei:

The spatial localization of the human chromosomal domains was analyzed for cells in the G1, S and G2 + M phases of the cell cycle. Nuclei for these studies were selected according to cell cycle phase by flow sorting. In general, the domains appeared more diffuse and the hybridization intensity was weaker in the sorted cells (perhaps due to the presence of Hoechst 33258 during hybridization). As a result, only a few nuclei were suitable for 3-D

reconstruction and the 3-D images were of lower resolution. In spite of this, the human chromosome domains were clearly visible and intact in G1, S and G2+M phase nuclei (Figure 3). Furthermore, as expected, the nuclear and chromosomal domains were larger in G2+M phase nuclei than in G1 phase nuclei as were the nuclei themselves.

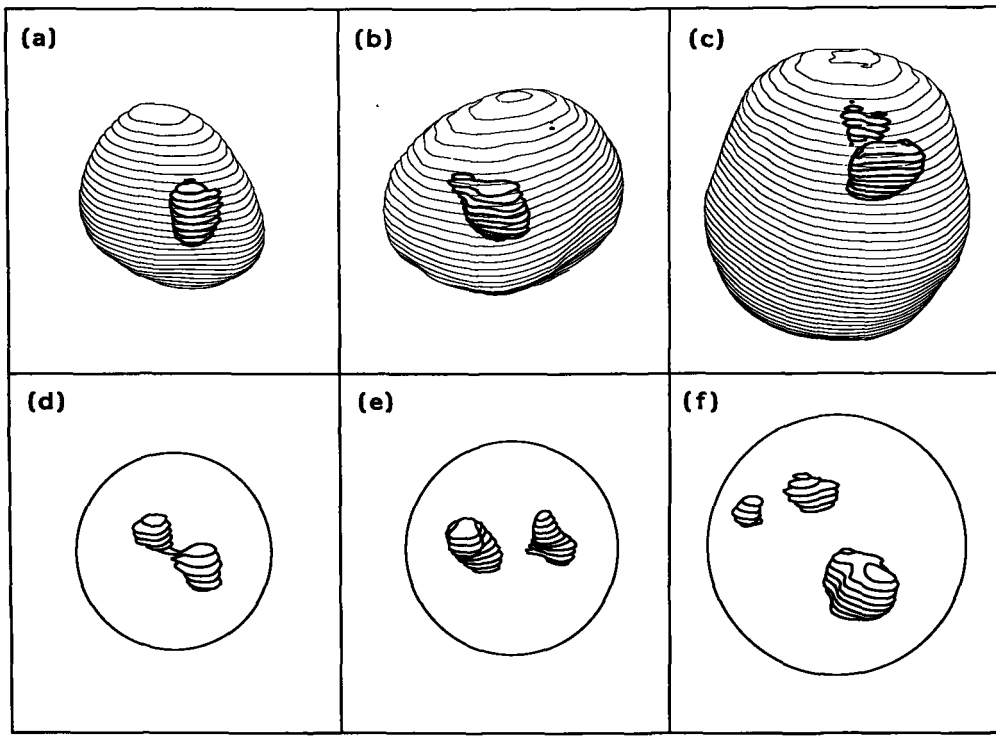

Figure 3:
Three dimension reconstructions of the locations of human chromosomes in nuclei from human hamster hybrids separated according to DNA content to be in the G1-, S- and G2M-phases of the cell cycle. Panels a-c show nuclei from cell line UV20HL21-29 in the G1-, S- and G2M-phases of the cell cycle, respectively. Panels d-f show nuclei from cell line UV20HL21-27 in the G1-, S- and G2M-phases of the cell cycle. The nuclear boundaries were not measured in this study and are thus indicated symbolically by circles.

Human interphase cells:

Normal human fibroblast interphase cells were hybridized in suspension with chromosome-specific DNA probes in order to study the spatial localization of specific chromosome regions. Ten 3-D reconstructions were made and analyzed for each probe and many more were viewed under the microscope. All nuclei were sectioned at 0.5 μm intervals. Figure 4 shows a typical 3-D reconstruction of a human nucleus hybridized with a probe for 1p36. The 1p36 loci are located near the nuclear boundary, opposite of each other. This appeared to be the predominant pattern. Figure 5 shows a reconstruction of a human nucleus hybridized with a probe for 9q12.

A comparable pattern is seen here, though occasionally one of the centromeres was more centrally located (not shown).

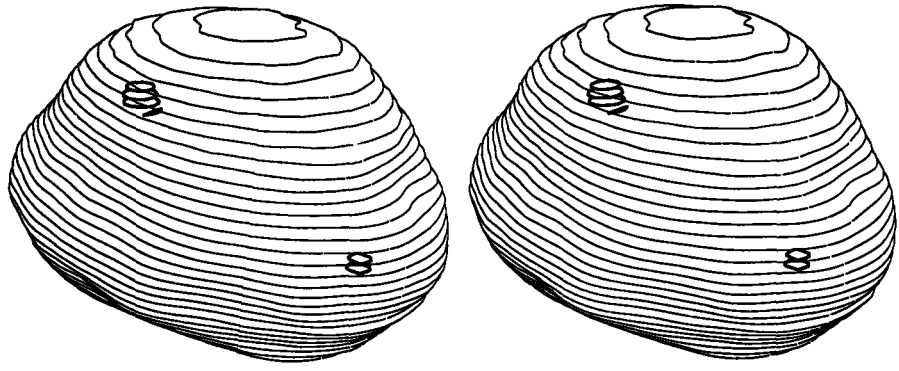

Figure 4:
Stereo reconstruction of the hybridization domains in an interphase nucleus of human fibroblast cell line 761 (46,XY) after hybridization with a DNA probe that hybridizes to 1p36.

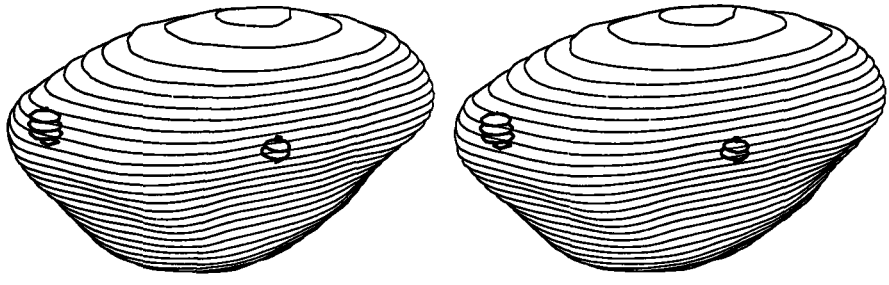

Figure 5:
Stereo reconstruction of the hybridization domains in an interphase nucleus of human fibroblast cell line 761 (46,XY) after hybridization with a DNA probe that hybridizes to 9q12.

Double hybridization to human cells:

Interphase nuclei from normal human fibroblasts were hybridized in suspension with two biotinylated, chromosome-specific probes in order to determine the feasibility of identifying specific chromosome loci by hybridization domain size. The probes for 1p36 and Yq12 with target sizes of a few hundred kilobases and ~6 megabases, respectively, were used in this

study. The 3-D reconstruction in Figure 6 shows that the two small 1p36 domains can be easily distinguished from the large Yq12 domain.

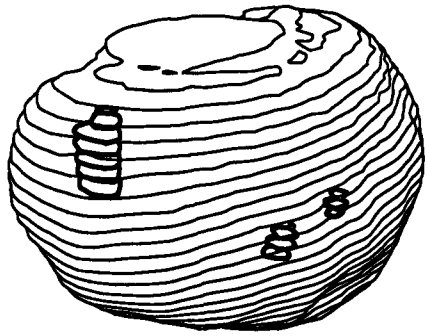

Figure 6:
Reconstruction of the hybridization domains in an interphase nucleus of human fibroblast cell line 761 (46,XY) after simultaneous hybridization with probes that hybridize to Yq12 and to 1p36. The hybridization domain for the Yq12 probe is significantly larger than the hybridization domain for the 1p36 probe.

DISCUSSION

Several reports have documented the presence of compact human chromosomal domains in hybrid interphase cells (Manuelidis, 1985; Schardin et al, 1985; Pinkel et al, 1986b). However, the organizational details remained unclear because of the nuclear distortion (e.g. flattening) that occurs when the cells were fixed, dropped on microscope glass slides and hybridized. Thus, in this study, we employed a suspension hybridization procedure to minimize nuclear distortion (Trask et al, 1985, 1988). The procedure was modified by adding a paraformaldehyde step since this was found to result in more intense hybridization and tighter domain structure.

Two general conclusions are apparent from this study: 1) The individual chromosomes are localized in domains that are small compared to the size of the nucleus. Furthermore, these domains are maintained throughout the cell cycle, even during DNA synthesis. 2) Some regularity of chromosome organization appears to exist. For example, the average proximity of the human chromosome domains in the hybrids UV20HL 21-29 and UV20HL 21-27 appear to

be different. In addition, the 1p36 loci appear to be located near the nuclear surface of human fibroblast nuclei, often opposite each other. The 9q12 locus also seems to be located predominantly near the nuclear membrane although one 9q12 locus was sometimes located in the nuclear interior. Interestingly, the localization of the 1p36 and 9q12 loci appears to be tissue specific. Manuelidis and Borden (1988), for example, found for large neurons that the 1p36 loci are in the nuclear interior. Also one 9q12 locus was in the interior near the nucleolus and the other 9q12 locus was either on the nuclear membrane or near the nucleolus.

These observations do not support the hypothesis of somatic pairing of homologues (see Comings, 1980). Further, we did not see the alignment of chromosomes suggested by Rabl (Rabl, 1885; Mathog et al, 1984); that is centromeres clustered at one pole of the nucleus and with the telomeres at the opposite side. However, a definitive analysis of this phenomenon will require simultaneous hybridization with centromere and telomere specific DNA probes and an algorithm to measure the distance between hybridization domains.

Analysis of the hybridization patterns produced by multiple probes requires a procedure for chromosomal identification of the hybridization domains. This may be accomplished either by labeling the probes with different chemical modifications and detecting with reagents that have different spectral properties (Hopman et al, 1986; Nederlof et al, 1989; Trask et al, 1988) or by identifying the domains according to their size. As preliminary study of the latter possibility, we hybridized with probes specific for the 1p36 and Yq12 loci and distinguished between the probes according to the size of the hybridization domain.

In summary, we have used fluorescence in situ hybridization in suspension to allow 3-D analysis of the chromosomal organization of interphase nuclei using optical sectioning. We found regularity in the 3-D proximity of human chromosomes in two different hybrid cell lines. We also found that the compact chromosomal domains seem to be maintained during cell cycle. Finally, we showed that in human fibroblast nuclei, spatial patterns can be distinguished using DNA probes that label specific regions on chromosomes. These data indicate that some, but not all, aspects of the intranuclear organization of chromosomes are constant from cell to cell. Additional information about the degree of organizational constancy in nuclei from various tissues should come from analysis of the locations of other chromosomes using repeat sequence probes (Bauman et al., 1989, and references therein) or composite probes (Pinkel et al, 1988; Fuscoe et al, 1988) for other chromosomes. The use of confocal microscopy also should facilitate nuclear organization studies (Brakenhoff et al, 1985).

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CHAPTER 5

SPATIAL TOPOGRAPHY OF CENTROMERE 1 DNA AND RIBOSOMAL RNA SEQUENCES IN HAEMOPOIETIC CELLS STUDIED BY *IN SITU* HYBRIDIZATION AND CONFOCAL MICROSCOPY.

H. van Dekken, A. van Rotterdam, R. Jonker, H.T.M. van der Voort, G.J. Brakenhoff, and J.G.J. Bauman

Submitted for publication

ABSTRACT

A fluorescent *in situ* hybridization procedure with a chromosome 1-specific (1q12) repetitive satellite DNA probe was used to label the centromeres of the chromosomes 1 in haemopoietic cell nuclei. The entire procedure was performed in suspension to preserve nuclear morphology. The result was studied by three-dimensional analysis, as provided by a scanning laser confocal microscope. The centromeres of chromosome 1 were measured to be closely associated with the nuclear enveloppe in isolated nuclei of unstimulated diploid human lymphocytes. The relative positions to each other in the periphery of these spherical nuclei could not be distinguished from a random distribution pattern. In the diploid and tetraploid polymorphic nuclei of cells of the promyelocytic leukaemia cell line HL60 the centromeres 1 were also associated with the nuclear surface.

As a framework for the study of the 3-D localization of genes and gene transcripts, we studied the simultaneous *in situ* hybridization of ribosomal RNA in the cytoplasm and the centromeres 1 DNA in the nucleus of HL60 cells. The intracellular positions of both cytoplasmic rRNA and intranuclear centromere 1 DNA could easily be distinguished.

INTRODUCTION

In 1885, Rabl proposed a model of intranuclear chromosome topography, that is still under investigation. He assumed, that the alignment of centromeres and telomeres, present in the mitotic cell, is preserved throughout cell cycle ("Rabl orientation"). A few years later (1888), Boveri showed, that in Ascaris eggs chromosomes occupied a discrete territory in the interphase nucleus, the chromosome "domain". Almost a century later, the understanding of the intranuclear architecture of chromosomes has not very much increased, despite various efforts (for reviews on this subject see Comings, 1980, and Hubert and Bourgeois, 1986). A Rabl orientation was seen in mammalian cells (Cremer et al. 1982) and in Drosophila (Foe and Alberts, 1985).

An important technique with respect to the study of the spatial organization of chromosomes within nuclei was introduced by Agard and Sedat (1983). They applied optical sectioning of intact cells by sampling images of successive planes, while moving the microscope focus stepwise through the DAPI-stained nucleus. After using deconvolution algorithms to eliminate the out-of-focus fluorescence, three-dimensional reconstructions were made to analyze the DAPI banding patterns of polytene chromosomes in intact Drosophila nuclei. It was found, that the polytene chromosomes were in Rabl alignment and that each occupied a confined domain (Mathog et al., 1984). Using anti-centromere antibody serum, Moroi et al. (1981) found the

centromeres in mammalian nuclei atthe periphery, while Hadlaczky et al. (1986) observed non-random chromosome patterns, e.g. somatic pairing of homologues.

The introduction of non-isotopic *in situ* hybridization (ISH) enabled investigators to tag specific nucleic acid sequences with high speed and high visual resolution on chromosomes and in cell nuclei with a fluorescent or enzymatic label (Bauman et al., 1980; Langer et al., 1981; Landegent et al., 1985). This ISH method appeared to be very suitable to detect numerical chromosome aberrations in interphase nuclei (Pinkel et al., 1986b; Hopman et al., 1988; Van Dekken and Bauman, 1989). For this purpose repetitive satellite DNA probes were used, which hybridize to the pericentromeric heterochromatin on specific chromosomes (Cook and Hindley, 1979;; Willard, 1985). Recently, several investigators reported the detection of target sequences on metaphase chromosomes as small as 1 to 5 kb (Ambros and Karlic, 1987; Garson et al., 1987; Lawrence et al., 1988b), indicating the high sensitivity achievable with non-isotopic ISH.

Using ISH with genomic human DNA as a probe, human chromosomes were seen as discrete domains in interphase nuclei of hybrid cell lines (Manuelidis et al.; Schardin et al., 1985). In earlier work, we employed optical sectioning of human X hamster hybrid cell nuclei (Pinkel et al. 1986a), hybridized in suspension (Trask et al., 1985, 1988) with genomic human DNA. The human chromosomes within these nuclei could be seen as distinct three-dimensional domains (Van Dekken et al., manuscript submitted). Manuelidis and Borden (1988) described the specific spatial distribution of specific chromosome domains in 3-D models of neuron cells after ISH to CNS tissue sections with chromosome specificrepetitive DNA probes. In these cells no Rabl orientation or somatic pairing was seen.

With the advent of confocal microscopes, accurate 3-D analysis of fluorescently labeled biological structures became possible without the necessity of performing computer time consuming deconvolution procedures (Brakenhoff et al., 1985; White et al., 1987). In such a set-up the images are readily accessible for measurement and reconstruction purposes (Brakenhoff et al., 1989; Van der Voort et al., 1989).

In the present work, we have applied fluorescent *in situ* hybridization (FISH) to haemopoietic cells and cell nuclei in suspension to analyze the spatial distribution of specific nucleic acid sequences by confocal scanning laser microscopy. Quantitative spatial measurements were performed, using the 3-D image data sets. The spatial distribution of the centromeres of chromosome 1 was investigated within near-spherical lymphocyte and polymorphic HL60 nuclei after FISH with a chromosome 1-specific satellite (1q12) DNA probe (Cook and Hindley, 1979). These centromere 1 sequences appeared to be organized in compact domains, which were localized in the nuclear periphery in a random distribution pattern.

Intracellular mechanisms underlying the regulation of gene expression of specific mRNA's can be studied using FISH approaches. The non-homogeneous intracellular distribution of

specific mRNA's has been described in chicken muscle and human fibroblast cells (Singer and Ward, 1982; Lawrence et al., 1988a). To address questions related to gene localization and gene expression (Blobel, 1985), we studied thefeasibility to detect simultaneously specific DNA and RNA sequences. For this purpose, the simultaneous FISH of cytoplasmic rRNA and nuclear 1q12 sequences to cells in suspension was explored. A recently described FISH method to RNA in suspended cells was employed (Bauman and Bentvelzen, 1988). This approach was combined with the enzymatic production of ssDNA for FISH to DNA targets in order to preserve the cytoplasmic RNA's (Van Dekken et al., 1988).

RESULTS

Localization of centromeres 1 in human lymphocytes

Isolated nuclei of unstimulated human peripheral lymphocytes were hybridized *in situ* in suspension with biotin-labeled probes. Hybridization was visualized with fluoresceinated avidin. Total nuclear DNA was counterstained with propidium iodide. The hybridized nuclei were carried in a viscous solution to avoid movements of the nuclei during microscopy. The solution contained an antifading component to prevent bleaching of the fluorochromes. A scanning confocal microscope, equipped with a krypton laser, was applied to generate 16 x-y planes of each nucleus at successive z intervals. The laser was tuned at 482.5 nm to excite both FITC and propidium iodide. The laser output was kept as low as possible (10mW) to minimize bleaching of the fluorochromes.

In an initial experiment, the accessibility of the nuclei was investigated, using biotin-labeled genomic human DNA as a probe. After FISH with this probe, the entire nucleus appeared to be hybridized (Fig. 1). Here, the nucleus was not counterstained with propidium iodide.

In another experiment lymphocyte nuclei were hybridized with a chromosome 1-specific repetitive satellite DNA probe. Hybridization to the pericentromeric heterochromatin of chromosomes 1 (1q12) in the interphase nuclei resulted in two brightly fluorescing compact domains. A total of 87 lymphocyte nuclei, from three hybridization experiments, were scanned and the spatial relationships of the hybridized centromericregions with respect to each other and with respect to their position within the nucleus were quantified. Only 3-D images with intact nuclear morphology and diploid number of FISH spots were used for analysis (Fig. 2). For these reasons, 9 nuclei were excluded from measurements.

First, the position of the center of mass of the hybridized region was defined as a percentage of the radius of a nuclear plane. The particular optical section of the nucleus, in which the center of mass of one of the hybridized centromere 1 spots was localized, was determined on a

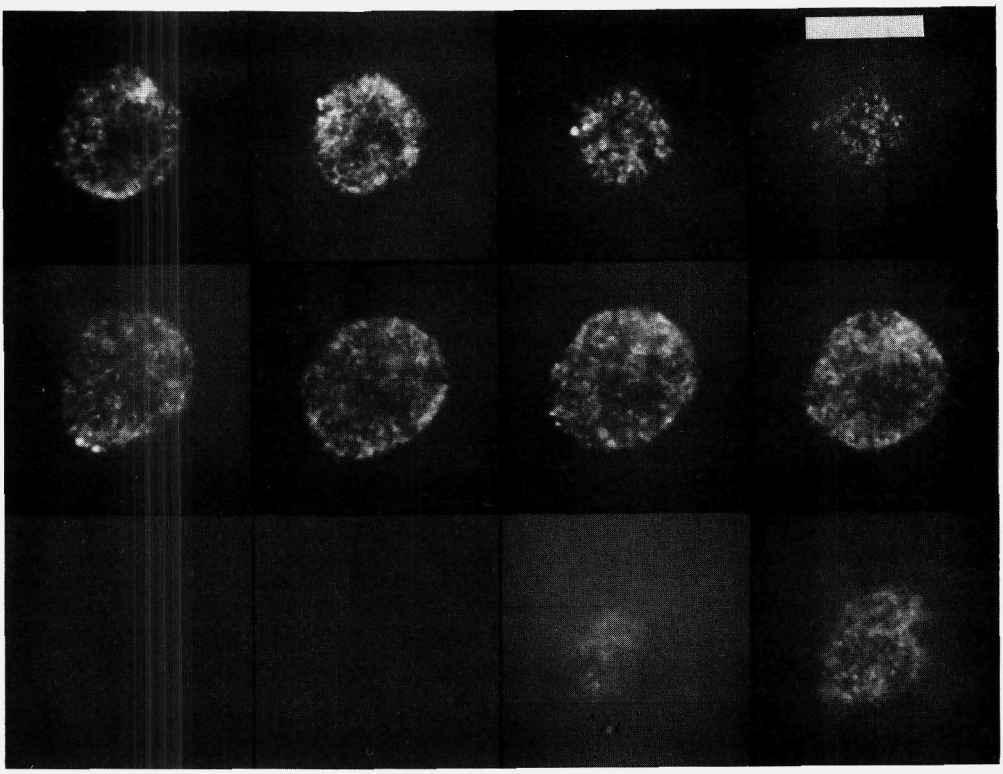
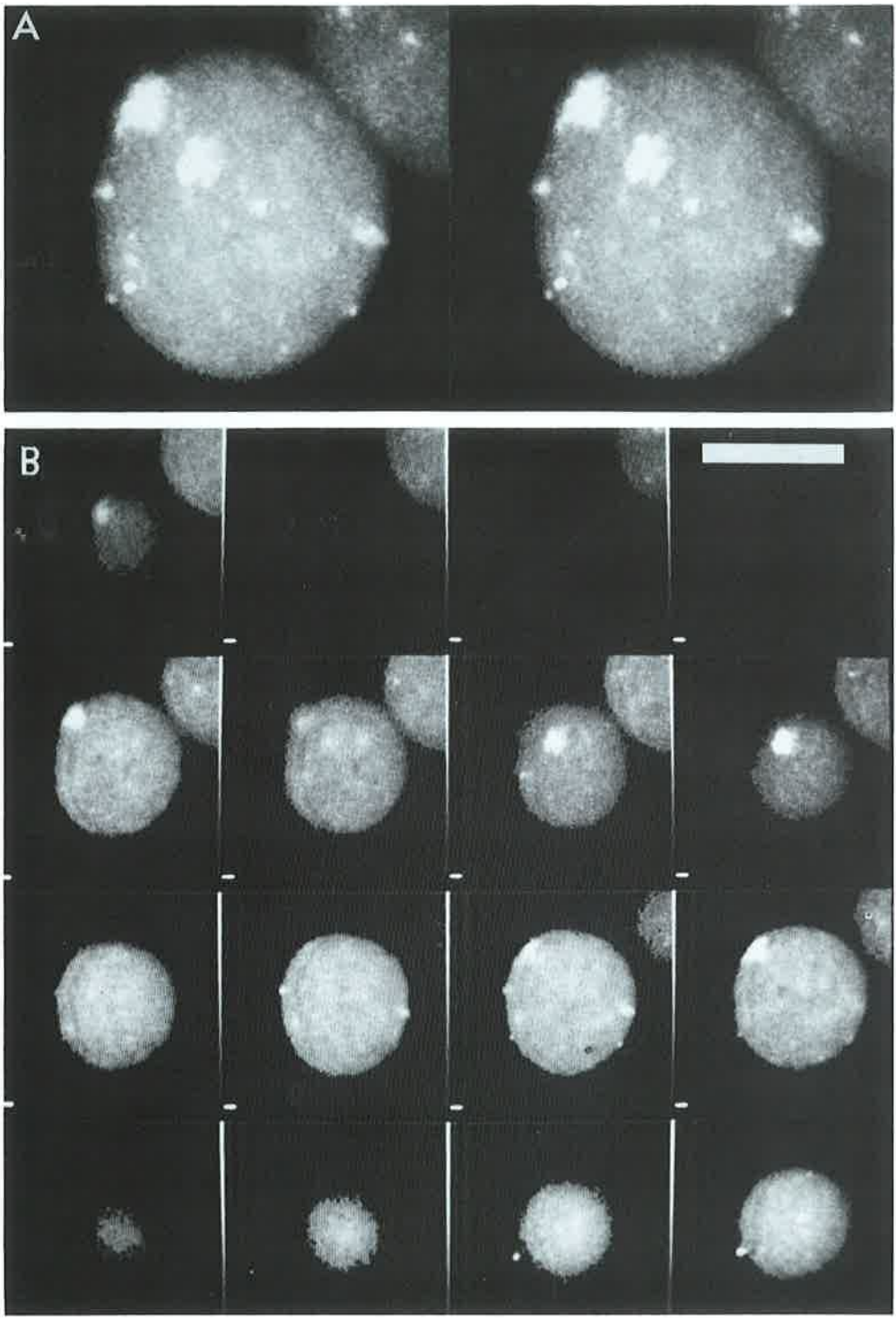


Figure 1:
Photomicrographs demonstrating twelve optical sections, obtained by confocal microscopy, of a peripheral human lymphocyte nucleus after fluorescent *in situ* hybridization (FISH) with biotinated genomic human DNA to suspended human peripheral lymphocyte nuclei. The hybridized probe was detected with fluoresceinated avidin, appearing white in the photomicrograph. The nuclear DNA was not counterstained with propidium iodide. Sections were typically obtained at sampling intervals of 1 μm. Bar is 10 μm.

video screen, displaying all the sections of a 3-D image. In this section, the center of the nucleus, the center of the FISH spot, and the nuclear edge were marked interactively with the cursor on the video screen. This 2-D measurement within a 3-D image was employed, since in this way the analysis could be performed simply in spherical as well as polymorphic nuclei (See Experimental Procedures). A set of 24 3-D images, representing one recording session, was used for this analysis. Fourteen FISH spots in these 24 cells were localized in the outer x-y planes and could not be used in the analysis, since the center and edge of the section of the nucleus could not be well defined in these planes. Of the remaining 34 FISH spots the distance to the center of the section was determined, and the results were plotted in a frequency histogram (Fig. 3). The vast majority of the spots was found at 80-90% of the radius. For statistical evaluation, the frequency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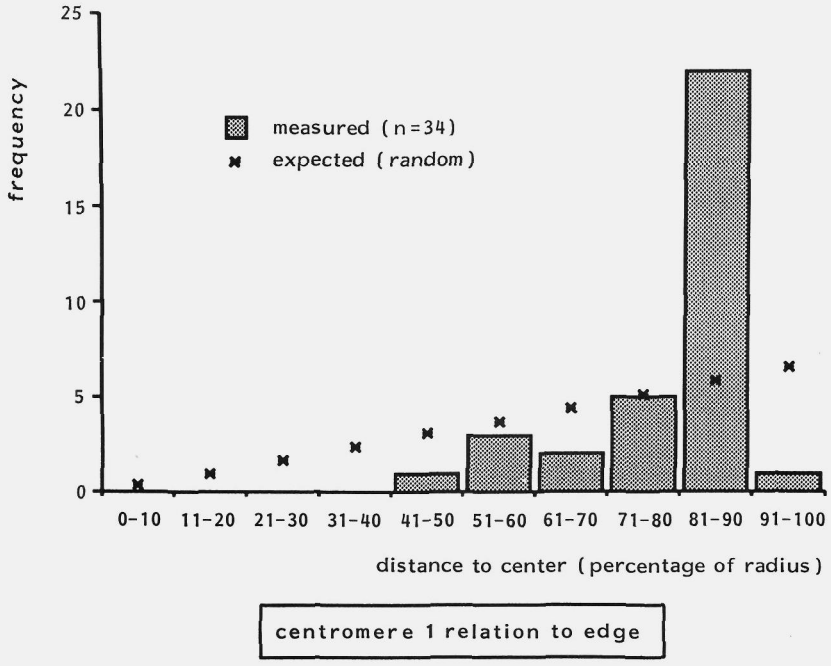


Figure 3:
The position of the 1q12-FISH region in 24 diploid human lymphocyte nuclei, determined by confocal microscopy. The position of the 1q12 region was defined as a percentage of the radius in the x-y plane, in which the center of mass of the FISH-spot was localized; 0 represents the center of the nuclear plane, 100 represents the nuclear boundary. Of 48 1q12-FISH regions, 34 could be used for this measurement (see text). The values, indicated expected random distribution, are marked with asterisks.

Figure 2:
Photomicrographs demonstrating confocal scanning microscopy of FISH with the biotinated 1q12 satellite DNA probe to suspended diploid human peripheral lymphocyte nuclei. Hybridized probe was detected with fluoresceinated avidin and all of the DNA was counterstained with propidium iodide. The regions to which probe hybridized appear white in the photomicrograph. The regions of DNA to which the probe did not bind appear grey. A: Stereoscopic representation of a lymphocyte nucleus, hybridized with the 1q12 probe. B: Sixteen optical sections of the same nucleus. Bar is 10 μm.

distribution was compared with a random distribution (Fig. 3). For this purpose, the Kolmogorov-Smirnov test (K-S test) was applied (Young, 1977). A p-value of <0.01 was calculated. This indicated, that the distribution of the centromere 1regions was different from a random distribution at a highly significant level.

Secondly, the smallest angle between the two 3-D vectors of both centromere 1 regions and the center of mass of the nucleus was analyzed. The centers of mass of the FISH spots and of the nucleus were determined interactively with the cursor on a video screen, displaying the 16 x-y planes. For this analysis, three-dimensional images of all 78 nuclei were used. In a few nuclei only one big hybridization spot, probably due to merging of the two individual spots, was seen. In that case the angle was taken to be 0. The distribution of angles is given as a frequency histogram in Fig. 4. The distribution pattern of the centromeres 1 on the surface of the spherical lymphocyte nuclei could not be distinguished from a random distribution, with respect to this angle (K-S test: p>0.20).

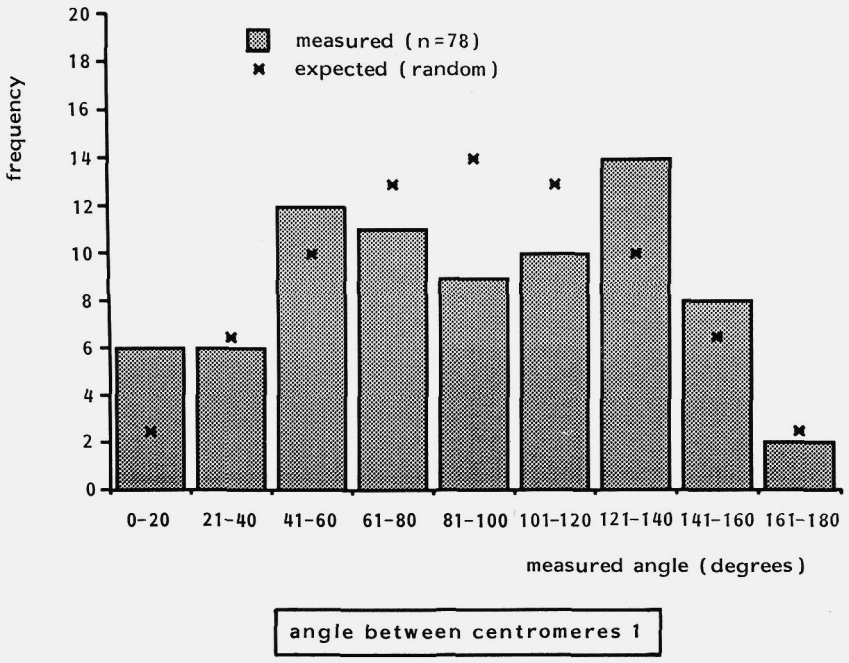


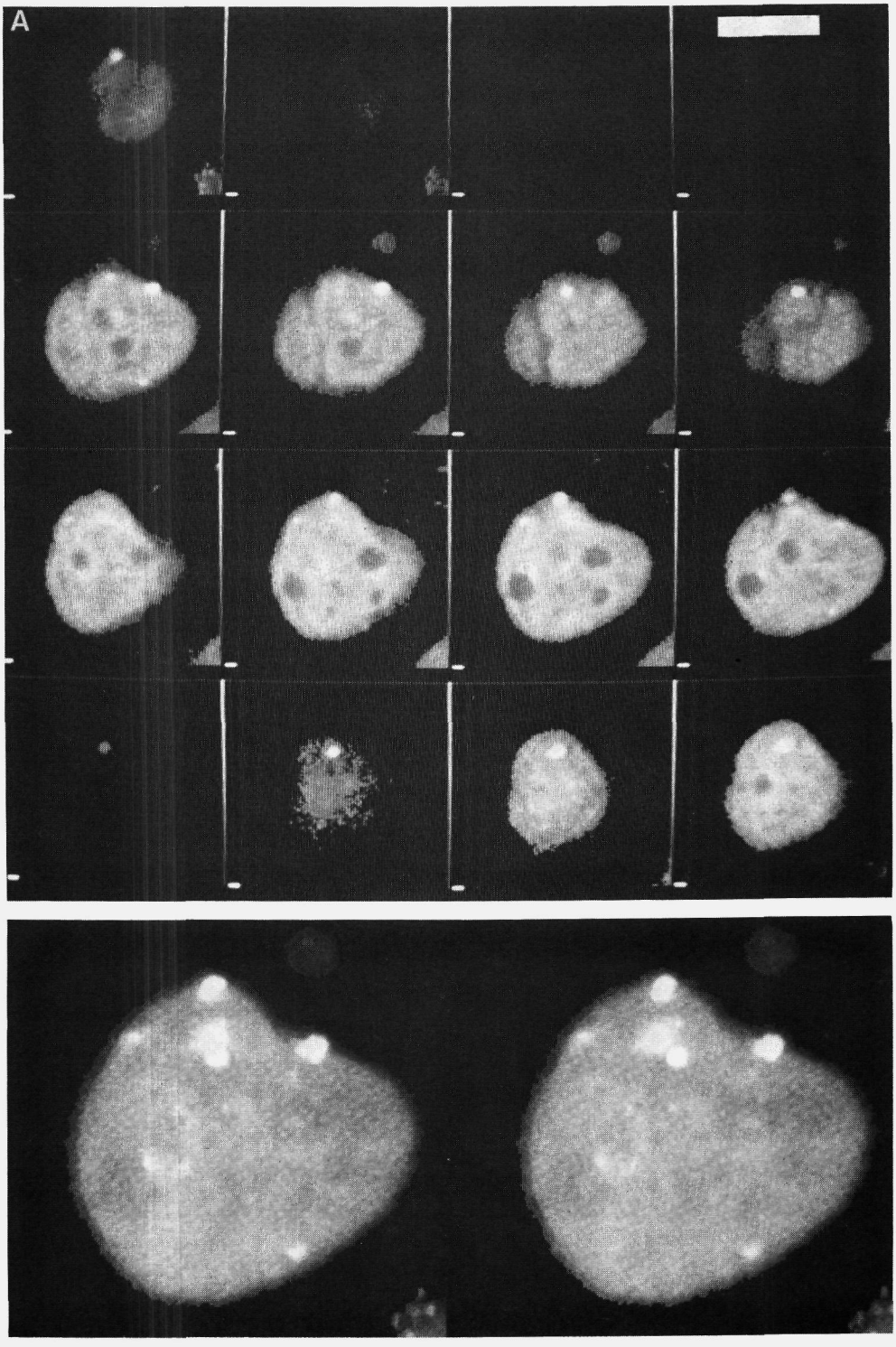
Figure 4:
The spatial relation between the two 1q12-FISH regions on the chromosome 1 homologues in diploid human lymphocyte nuclei, determined by confocal microscopy of 78 nuclei. Since these nuclei are spherical and the 1q12-spots were found at the nuclear periphery (Fig. 2), the relationship between the FISH-spots was measured as the smallest angle between the two 3-D vectors of both 1q12-spots and the center of mass of the nucleus. Asterisks indicate the expected random distribution values.

Localization of centromeres 1 in HL60 cells

To investigate the spatial localization of the centromere 1 regions in aneuploid polymorphic cell nuclei, whole fixed HL60 cells were hybridized in suspension with the biotin-labeled repetitive 1q12 DNA probe. Visualization of probe and nuclear DNA, as well as confocal scanning microscopy were performed as descrived above.

In this experiment, a comparison was made between FISH of heat denatured cell nuclei and FISH of cell nuclei, that were made single-stranded enzymatically by restriction enzymes and exonuclease III (Fig. 5). The latter method was applied to investigate, whether the two denaturation protocols caused differences in localization patterns. Sincethese nuclei were polymorphic and (mostly) tetraploid, only the spatial localization of the FISH spots within the nucleus was examined. The localization of centromeres 1 was calculated as a percentage of the radius, as described above. Three heat denatured (tetraploid) and five enzyme treated (4 tetra-, 1 diploid) cells were analyzed (Fig. 6). The centromere 1 regions were also found to be associated with the nuclear surface (Fig. 6). K-S testing versus a random distribution rendered p-values of <0.05 for the heat denatured cells and <0.20 for the enzyme treated cells. To assess the effectivity of the enzyme treatment with respect to ssDNA production, FISH with biotin-labeled genomic human DNA was performed to HL60 cells. Hybridization signals could be found throughout the entire nucleus (Fig. 7). Here, propidium iodide was omitted as a DNA stain. The darker regions in the nuclei, visible in Figs. 5 and 7 (also in Fig. 1), represent nucleoli, devoid of DNA.

Figure 5:
Stereoscopic representations and optical sections, obtained by confocal microscopy, of HL60 cell line nuclei after FISH with the 1q12 satellite DNA probe. For details on visualization, see Figure 2. A: Heat-denatured tetraploid polymorphic HL60 nucleus. B: Tetraploid polymorphic HL60 nucleus, of which the DNA was made single-strandedfor FISH by the enzyme treatment (See text). Bar is 10 μm.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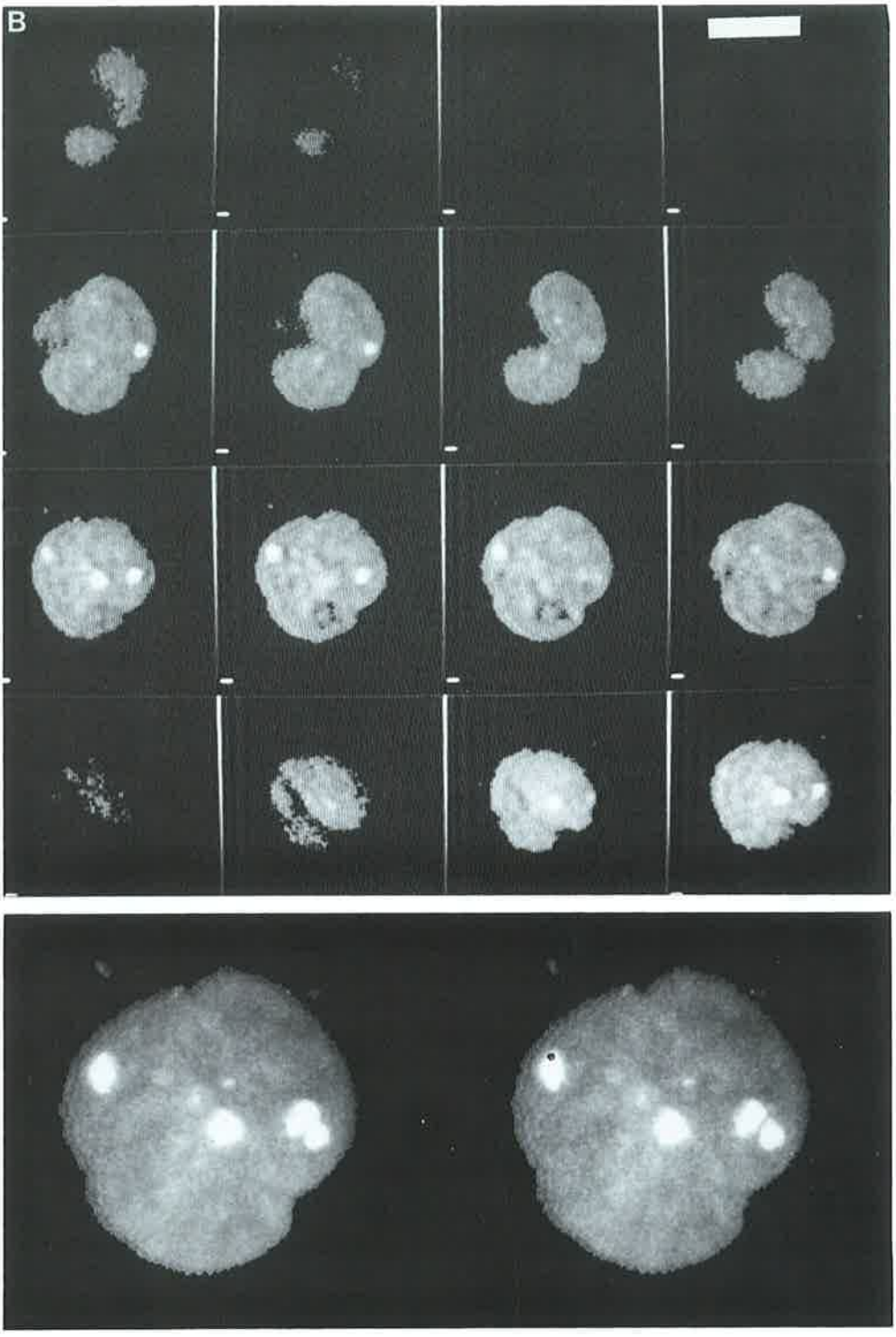
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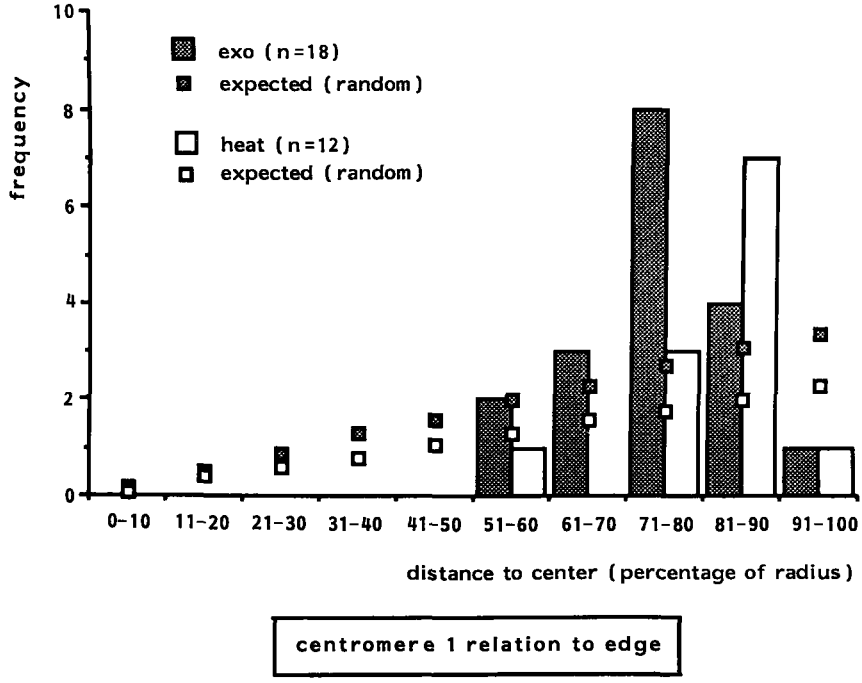


Figure 6:
The position of the 1q12-FISH regions in 8 HL60 cell line nuclei, determined by confocal microscopy. For details on the measurement, see figure 3. Grey bars indicate enzyme-treated nuclei (4 tetra-, 1 diploid), white bars indicate heat-denatured nuclei (3 tetraploid). Little squares indicate the expected random distribution values.

Localization of centromeres 1 and ribosomal RNA in HL60 cells

To investigate, whether it is possible to demonstrate the spatial distribution of both specific DNA and RNA sequences in the same cell, the detection of ribosomal RNA was combined with the detection of centromere 1 DNA using enzymatic production of ssDNA.

Whole fixed HL60 cells were hybridized *in situ* in suspension with biotin-labeled ribosomal antisense RNA transcripts. This was followed by hybridization with the biotin-labeled repetitive 1q12 DNA probe. In between both hybridizations the DNA in the nucleus was made single-stranded by enzyme treatment instead of heat denaturation, topreserve the rRNA hybrids in the cytoplasm and allow the 1q12 probe to hybridize its target. Visualization of the probes and confocal microscopy were performed as described above. The nuclei were not counterstained in this experiment.

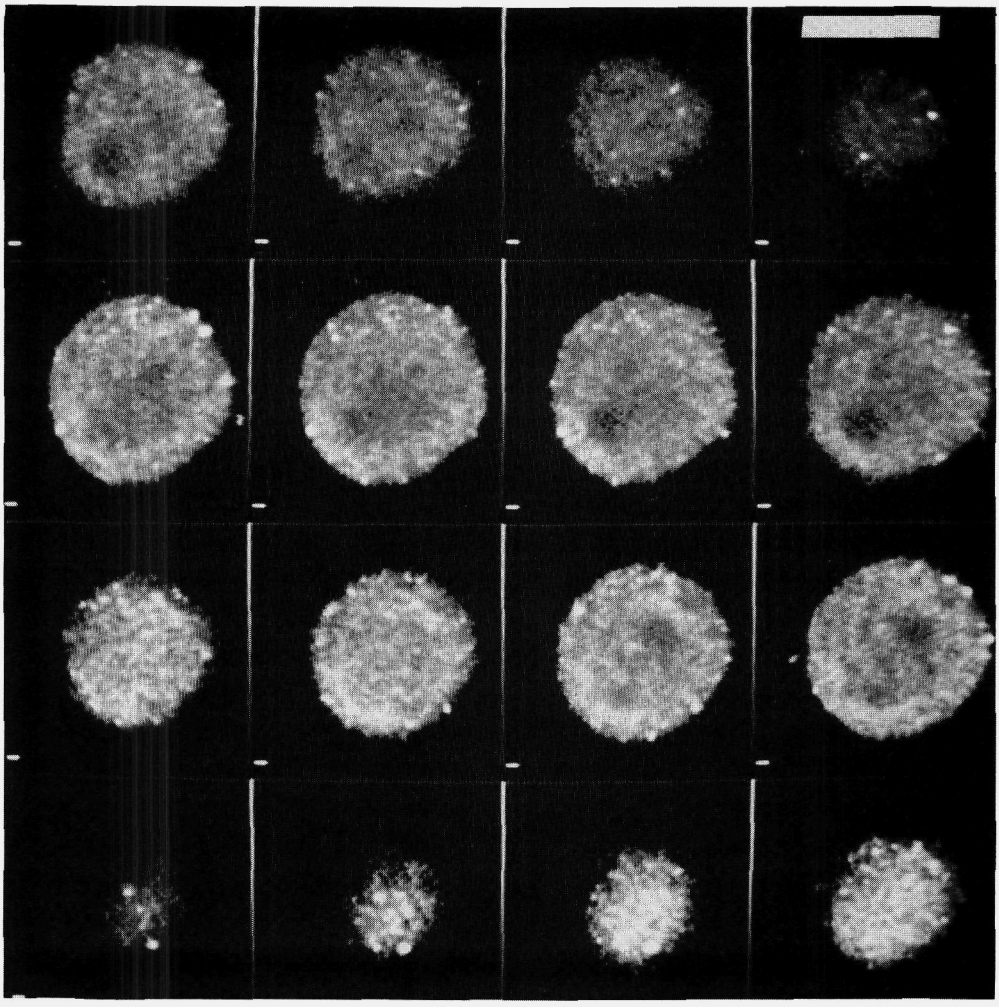


Figure 7:
Sixteen optical sections, obtained by confocal microscopy, of an HL60 cell line nucleus after FISH with biotinated genomic human DNA as a probe. The DNA was made single-stranded for FISH by the enzyme treatment. For details on visualization, see figure 1. Bar is 10 μm.

FISH with the rRNA probe alone resulted in complete staining of the cytoplasm (Fig. 8), with some dim regions, where the Golgi apparatus can be expected. No FISH inside the nucleus was found, although due to the polymorphic properties of the nucleus thin cytoplasmic "threads" in nuclear folds could be distinguished (arrow in Fig. 8). The rRNA-1q12DNA double-FISH image of two cells is represented in Fig 9. A diploid and a tetraploid cell can be distinguished.

This is evident from the number of centromere 1 spots, as well as from the size of the cells (Fig. 9a). The rRNA sequences were localized in the cytoplasm (Fig. 9b). The resolution of the RNA fluorescence in the cytoplasm in this image is lower, when compared with Fig. 8, due to the low concentration of the rRNA probe, used in this experiment. The low rRNA probe concentration was used to distinguish the centromere 1 FISH regions in the periphery of the nucleus from the brightly fluoresceing rRNA probe. The centromere 1 FISH spots were associated, as expected, with the nuclear surface. They were localized near or at the nuclear boundary, which is visualized in Figs 9c and 9d.

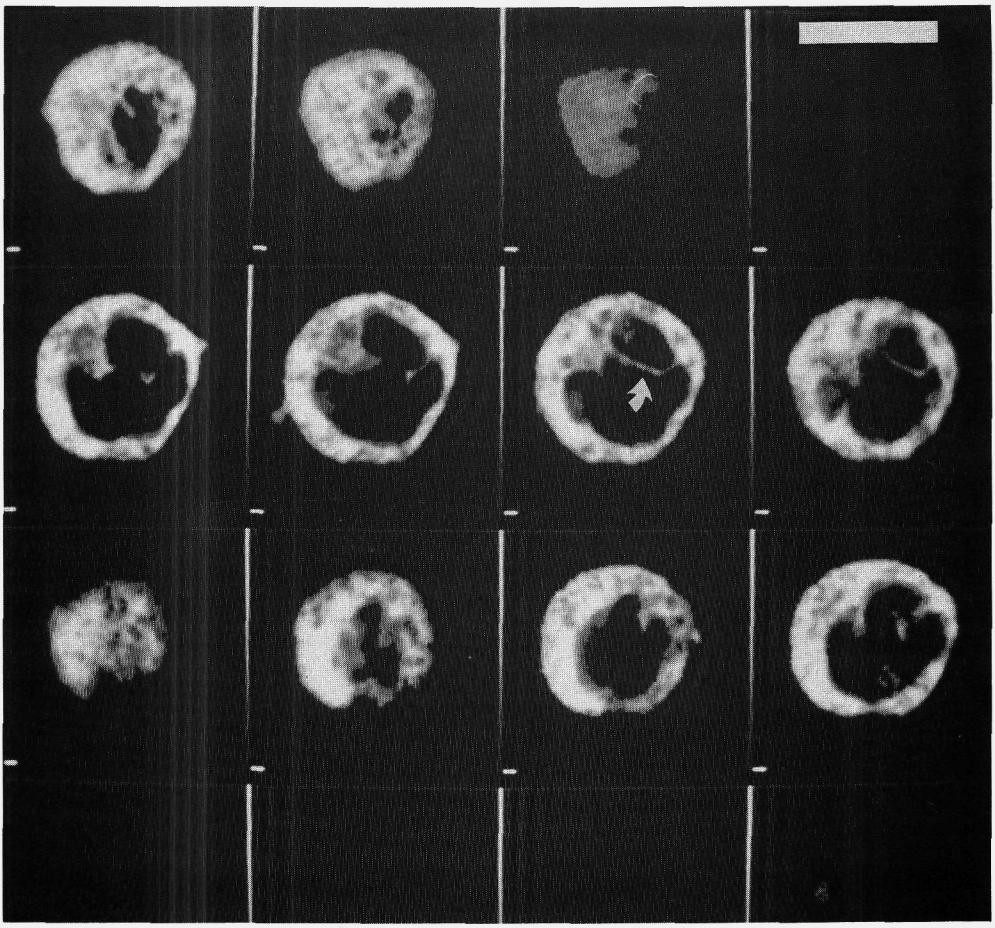
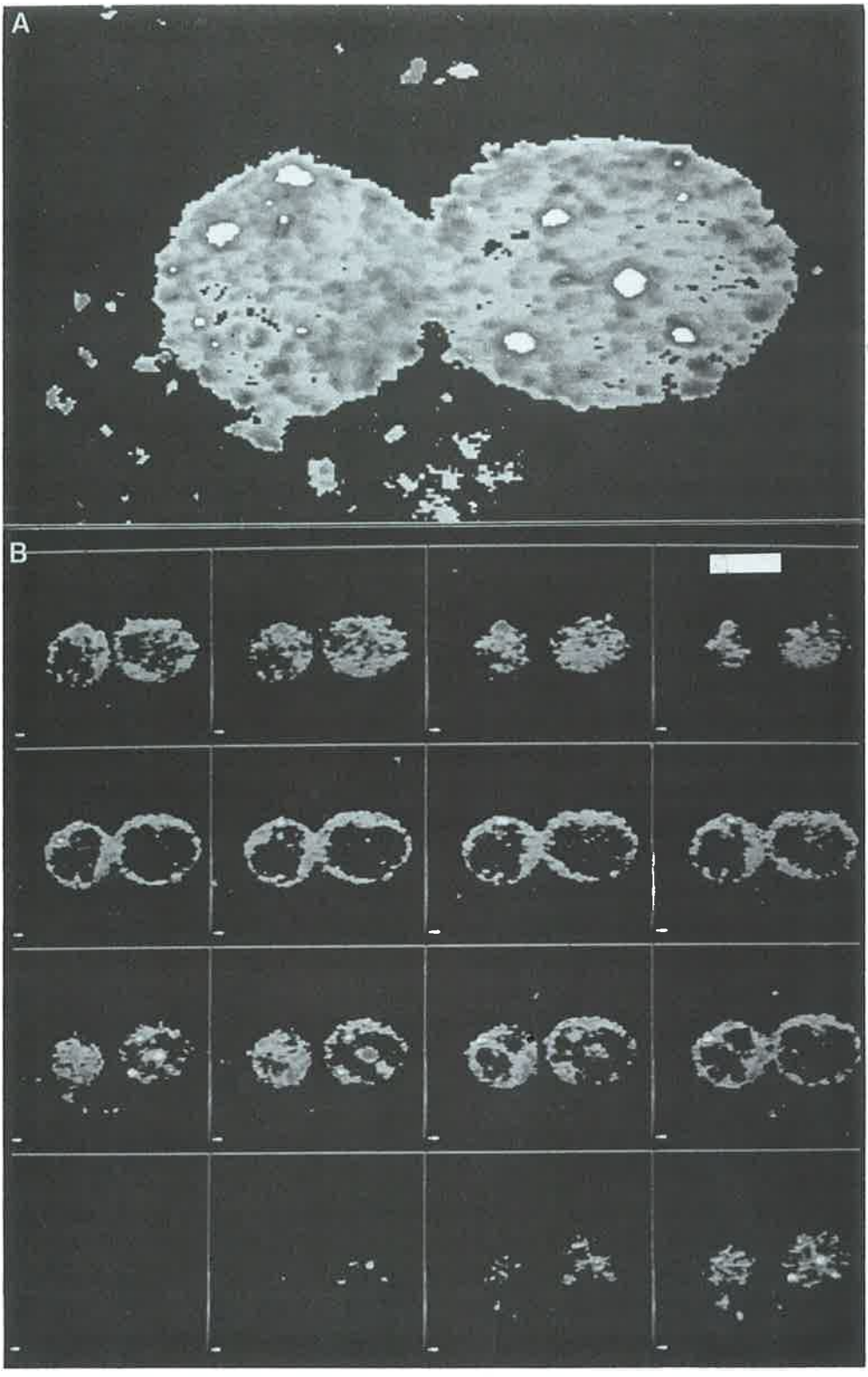


Figure 8:
Sixteen optical sections, obtained by confocal microscopy, of an HL60 tumour cell after FISH with biotinated 28S ribosomal antisense RNA transcripts. For details on visualization, see Figure 1. Note the absence of hybridization within the nuclear compartment. Arrow indicates hybridized rRNA sequences in, presumably, a nuclear fold of this polymorphic nucleus. Bar is 10 μm.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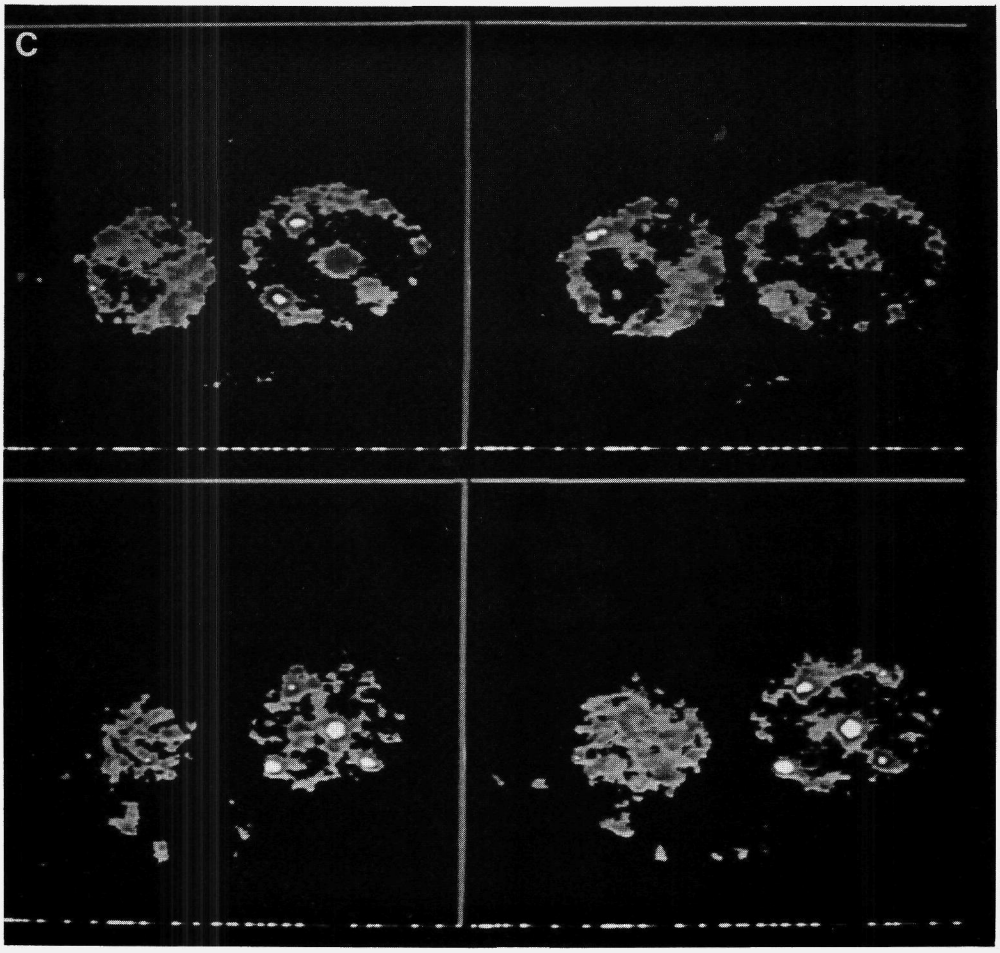
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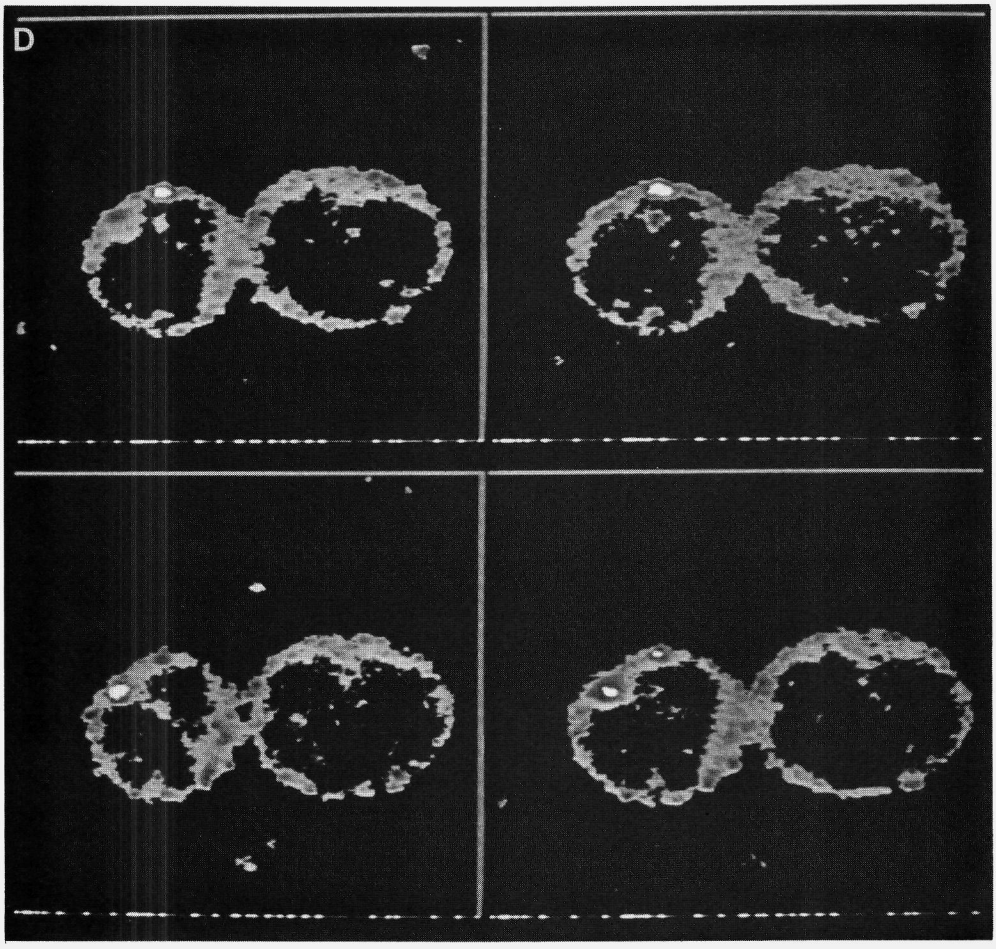

Figure 9:
Photomicrographs, showing confocal microscopy of two HL60 tumourcells (1 diploid, 1 tetraploid) after FISH with biotinated 28S ribosomal antisense RNA transcripts (low probe concentration; see text), followed by the enzyme treatment and FISH with the biotinated 1q12 satellite DNA probe. For details on visualization, see figure 1. A: Photomicrograph, demonstrating the superimposed 16 sections. Note the size difference of the diploid and the tetraploid cell. B: Sixteen optical sections, demonstrating localization of the 1q12-spots in the nuclear periphery of the cells. Bar is 10 um. C: Four successive optical sections, showing the spatial topography of the 1q12-FISH regions in the tetraploid HL60 cell. D: Four successive optical sections, showing the spatial topography of the 1q12-FISH regions in the diploid HL60 cell.

DISCUSSION

Three-dimensional analysis: Confocal microscopy

To study the spatial relationship between the pericentromeric regions of the chromosomes 1 in haemopoietic cells, we applied a fluorescent *in situ* hybridization (FISH) procedure to suspended cells and cell nuclei to specifically label centromere 1 regions within nuclei and rRNA sequences in the cytoplasm. For this purpose, we used biotinated repetitive 1q12 satellite DNA and antisense rRNA transcripts as probes. The FISH procedure we used was such, that the 3-D properties of the cells and nuclei were excellently conserved. This allowed us to apply high-resolution confocal scanning microscopy in order to analyze the spatial characteristics of these specific targets. The 3-D images, containing all the necessary x-y-z information, were used to determine interactively the spatial relationships of the centromere 1 FISH spots in spherical and polymorphic cell nuclei. In this way, quantification and statistical evaluation of the topographic patterns of the centromeres became feasible. Furthermore, by performing simultaneous FISH of cytoplasmic rRNA and nuclear centromere 1 DNA, it was demonstrated, that specific RNA and DNA sequences could be visualized at the same time in individual cells, opening the way to the analysis of the localization of gene and gene transcript.

The confocal microscope is becoming increasingly important as an apparatus to analyze the 3-D topography of cellular objects. Main reasonsare the high-resolution optical sectioning capacity, the non-invasiveness, which leaves the objects intact, and the imaging capabilities (Brakenhoff et al., 1989). Combining such a machine with a flow cytometer creates a powerful tool for the study of spatial and quantitative aspects of fluorescent objects. The quantitation by flow cytometry of hybridized ribosomal RNA sequences (Bauman and Bentvelzen, 1988), as well as repetitive DNA probes (Trask et al., 1988; Van Dekken et al., 1989) in suspended cells has been described.

Three-dimensional analysis: Statistical significance

Algorithms were developed to investigate quantitative aspects of the intranuclear architecture of chromosomes. We investigated the position of the centromeric region of chromosomes 1 and found an association of these repetitive sequences with the nuclear boundary. The analytical approach, we have chosen, is more realistic than those, employing studies on flattened nuclei, dropped and hybridized on glass slides (Rappold et al., 1984; Lawrence et al., 1988). This is because the 3-D morphology is preserved by performing the entire procedure in suspension, thereby eliminating uncertainties in the extrapolation of data from distorted "2-D" objects to 3-D models. From the results of figs 3 and 6, obtained by calculating the positions of centromere 1 relative to the center of the nuclear DNA, we concluded, that the centromeres 1 were

associated with the nuclear surface. In both lymphocytes and HL60 cells, we found the majority of the centromeres 1 in the area outside 70% of the nuclear radius. No centromere 1 was found inside the area, determined by 40% of the nuclear radius. We performed our measurement in the optical sections, containing the center of mass of the centromere 1 FISH spot. However, in a circular plane, the area outside 70% of the radius, represents 51% of the total area. Therefore we statistically evaluated the data of figs 3 and 6, using the Kolmogorov-Smirnov test (K-S test). The distribution of the centromeres 1 in lymphocytes was found to be significantly different from a random distribution ($p<0.01$, $n=34$). In HL60 cells, this difference was less significant ($p<0.05$ and $p<0.20$ for heat and enzyme treatment, respectively; Fig. 6). This is likely due to a low sample size. If we combine both lymphocyte and HL60 data, the association with the nuclear enveloppe in these cell types is clear (K-S test: $p<0.01$). Since the measurements were performed within sections, 14 out of 48 spots had to be excluded from the measurement, depicted in Fig. 3. However, these FISH spots were in the outer x-y planes, and therefore would have significantly contributed to the peripheral localization pattern of the spots. The distribution of the two centromeres 1 near the surface of lymphocyte nuclei appeared to be random (Fig. 4; K-S test: $p>>0.20$). This was determined by measuring the smallest angle between the two 3-D vectors in 78 3-D images. The centromeric spots were found to be rather large, when compared to the nuclear radius (See Figs 2, 5, 9). It can be explained by the target size of the 1q12 probe (several megabases), as well as the signal amplification, that was used for better visualization of the probe.

Three-dimensional analysis: Biological significance

What is the biological significance of the localization patterns of pericentromeric DNA of the chromosomes 1, as described by us? I.e., Why are the centromeres 1 associated with the nuclear boundary, but randomly distributed in the peripheral area of the nucleus?

The localization of centromeres in the nuclear periphery is also described by other investigators (Moroi et al., 1981; Haaf and Schmid, 1989). They employed anti-kinetochore antiserum to visualize centromeres in human and rodent cell lines. Using *in situ* hybridization on human neurons and glia cells, Manuelidis and Borden (1988) showed with the same 1q12 probe in only a small number of 3-D images, that at least one of the centromeres was associated with the nuclear periphery. Borden and Manuelidis (1988) also described a change in the localization of the X chromosome in neurons of epileptic brain tissue, as compared with normal cells. These results suggest a more or less defined and fixed intranuclear topography of chromosomes in neurons and glia cells of the central nervous system, which is known to be highly differentiated and non-proliferating.

For undifferentiated cells, the localization of centromeres associated with the nuclear surface would make sense for organizational reasons. The position of the centromere might be

variable to give rise to a great flexibility of chromosome arms, capable of having contact with any defined region within the nucleus. A highly dynamic intranuclear organization, as hypothesized by Blobel (1985) would be the implication. In haemopoietic cells, such as promyelocytes and unstimulated peripheral lymphocytes, a less defined 3-D arrangement of chromosomes withinnuclei might be required. In contrast to neurons and glia cells, haemopoietic cells are premature with respect to differentation and are still capable of proliferation. A flexible chromosome topography would facilitate the rapid changes, needed in proliferating and differentiating cells. In our study, we did not find signs of centromere-telomere alignment (Rabl orientation) e.g. clustering of centromeres, or somatic pairing of homologues (Mathog et al., 1985; Hadlaczky et al., 1986).

With the introduction of whole chromosome specific libraries as probes for FISH (Pinkel et al., 1988), it will be possible to rapidly investigate the question, whether chromosomes each occupy a confined domain or territory in the human cell nucleus. Cremer et al. (1988) performed FISH with a number of chromosome specific libraries to study the localization in interphase nuclei, attached on glass slides. The chromosome specific library probes indeed hybridized to a confined domain in these *in situ* hybridizations to nuclei. When FISH can be performed in suspended cells with intact morphology, more detailed questions, regarding spatial topography, can be answered adequately.

At present, chromosome specific centromeric sequences for almost all human chromosomes are available (Willard, 1985). With the approach, described in this paper, a map of specific regions within the nucleus could be constructed. With multicolor labeling (Hopman et al., 1988; Nederlof et al., 1989) and a refinement of the method with regard to sensitivity and 3-D image analysis, the 3-D topography of the nucleus and the localization of gene and gene transcripts can be ultimately studied. As a framework for these kind of studies, we explored the simultaneousFISH of RNA and DNA sequences (Figs 9). We intend to investigate these topics systematically within the haemopoietic system. With respect to proliferation, unstimulated and stimulated peripheral lymphocytes will be analyzed. As a model system for differentiation, the HL60 cell line offers the unique opportunity of targeted stimulation and differentiation.

EXPERIMENTAL PROCEDURES

Probes and probe labeling: Human genomic DNA was isolated from peripheral blood lymphocytes. The human chromosome 1q12 specific repetitive satellite DNA probe pUC1.77, 1770 basepairs in pUC18, was kindly supplied by Dr. H. Cook (MRC, Edinburgh, UK). For *in situ* hybridization, complete plasmid DNA was labeled with biotin-11-dUTP by nick translation

(BRL, Gaithersburg, MD) according to the manufacturers' directions. Single-stranded RNA (ssRNA) probes were prepared as described previously (Bauman and Bentvelzen, 1988). Briefly, a 2.1 kb fragment of the human 28S ribosomal gene (a gift from Dr. B.D. Young, St. Bartolomews Hospital, London, UK), was cloned into plasmid pGEM2 (Promega Biotec, Madison, WI). Labeled antisense rRNA transcripts were prepared according to the manufacturers' directions using SP6 DNA-dependant RNA polymerase (Promega Biotec) and biotin-11-UTP (BRL). The ssRNA probe was degraded before use by controlled limited alkaline hydrolysis to about 100-150 nucleotides in size. Probe labeling, probe size and specificity of the antisense transcript were checked as described (Bauman and Bentvelzen, 1988). All probes were stored at -20 °C.

Sample preparation: Fresh lymphocytes were isolated from 20 ml peripheral blood with a lymphocyte separation medium (Organon, Durham, NC). The promyelocytic cell line HL60 was cultured in RPMI 1640 Medium(Gibco Ltd., Paisley, UK), containing 10% foetal calf serum. In culture, this cell line displayed a tendency to become tetraploid, resulting in a mixture of diploid and tetraploid cells (Nowell et al., 1983).

Nuclei were isolated according to the Hepes-MgSO4 method (Van den Engh et al., 1985) and fixed in 70% ethanol for 10 min at 4 °C, centrifugated (each centrifugation: 10 min, 200 g.) and resuspended in 100% ethanol for 10 min at 4 °C. After centrifugation protein was extracted by resuspending the nuclei in 0.1 M HCl, 0.05% Triton X-100 for 10 min at room temperature. Then a fixation in 1% paraformaldehyde in isolation buffer (5 mM Hepes, 50 mM KCl, 10 mM MgSO4, 0.05% Tween 20, pH 8) for 1 min at room temperature was applied. Nuclei were washed once in isolation buffer and resuspended and stored in this buffer at 4 °C at a concentration of 5 x 10^7/ml.

Whole cells, used for *in situ* hybridization, were washed once in phosphate buffered saline (NPBI, Emmer-Compascum, The Netherlands) and fixed in 70% and 100% ethanol as described above. Then a fixation in 0.5% formalin in PBS for 5 min at 4 °C was applied. Cells were washed once in PBS, once in PBS plus 0.1% Triton X-100 and stored in 70% ethanol at -20 °C at a concentration of 10^7/ml. When cells were used for hybridization of DNA only, the cells, after fixation stored in 70% ethanol, were washed once in PBS, and protein extraction was carried out by resuspending the cell pellet in 0.1 M HCl, 0.05% Triton X-100 at 4 °C for 15 min After centrifugation, the cells were washed once in PBS and resuspended in PBS at 4 °C at a concentration of 5 x 10^7/ml.

FISH to DNA in suspended nuclei or cells: Isolated cells, and probe were denatured by heat, but separately: 2 µl cell suspension was added to 8 µl of hybridization mix

(final concentrations: 50% formamide, 2xSSC, 10% dextran sulphate, 1% Tween 20, pH 7) and denatured for 10 min at 70 °C. Formamide (BRL) was de-ionised by treatment with a mixed-bed ion-exchange resin (Dowex AG501-X8; Bio-Rad Labs, Richmond, CA) and thereafter stored at -20°C. Probe DNA (2 μg/ml) and sonicated herring sperm carrier DNA (250 μg/ml; Sigma) were also denatured for 10 min at 70 °C in hybridization mix. For details on the enzymatic production of ssDNA, see the next paragraph. Now, both mixtures were quickly cooled on ice and 20 μl probe mix was added to the 10 μl cells in an Eppendorf tube. The sample was mixed and incubated for 14-18 hours at 37 °C in a dry incubator. Then cells were washed in 1 ml 50% formamide, 2xSSC, pH 7 for 10 min at 42 °C and prior to centrifugation 100 μl DMS (Aldrich, Steinheim, FRG) fixed erythrocytes (10^8/ ml in isolation buffer) were added to serve as a carrier (Trask et al., 1985). After centrifugation the pellet was resuspended in 1 ml 2xSSC, pH 7 for 10 min at room temperature and washed again in 1 ml PN buffer (0.1 M sodium phosphate, 0.05% Nonidet P40, pH 8) for 10 min at room temperature. After resuspending the pellet in 100 μl PNM buffer (5% non-fat dry milk {Safeway,USA} in PN buffer; non-fat dry milk was added to prevent non-specific binding of the avidin (Pinkel et al., 1986b)) for 5 min at room temperature, the biotinated probe was stained by adding an equal volume of staining buffer (10 μg/ml avidin-FITC DCS Grade (Vector, Burlingame, CA)/ml PNM). The sample was mixed and incubated for 30 min at 37 °C in a dry incubator. After centrifugation the pellet was washed in 1 ml PN for 10 min at room temperature. The probe-linked fluorescence intensity was amplified by successive treatments with biotinated goat-anti-avidin (Vector; 10 μg/ ml in PNM) and avidin-FITC, as described previously (Pinkel et al., 1986b; Van Dekken et al., 1988), and finally resuspended in 50 μl PN buffer. Now 50 μl of an antifade solution (1 mg/ ml p-phenylenediamine dihydrochloride (Sigma) in 90% glycerol/ 10% PBS/ pH 8 (v/v); Johnson and de C. Nogeira Araujo, 1981) was added, *containing 1 μg/ml propidium iodide* (Sigma, St. Louis, MO). Due to the presence of glycerol the solution is very viscous, which prevents the cells from movements during microscopy. Now 5 μl of the mixture was put on a glass microscope slide under a 18 x 18 mm glass coverslip. Special care was taken to avoid pressure on the coverslip, which could affect the morphology of the cells and nuclei.

FISH to RNA and DNA in suspended cells: The *in situ* hybridization of RNA in suspended cells was carried out as described by Bauman and Bentvelzen (1988). Endogenous RNAse activity in fixed cells was blocked before rehydration by 0.2% di-ethyl-pyrocarbonate (DEPC, 2 μl of a 10% stock solution of DEPC in ethanol per 100 μl fixed cell suspension in 70% ethanol). In addition, all reagents and equipment were kept free of RNAse. After 15 min at room temperature, cells were centrifuged as desribed above (10 min, 200 g.), and resuspended in PBS plus 0.5% Tween 20 at a final cell concentration of about 10^7/ml. After 5 min at room

temperature, one volume 20xSSC and, after mixing, two volumes deionized formamide were added. After mixing the sample, approximately 100,000 cells were transferred from this suspension to the required number of Eppendorf tubes. The cells, now in 50% formamide and 5xSSC, were centrifuged and the pellet was resuspended in 10 µl of RNA hybridization mixture (50% formamide, 5xSSC, 0.5 mg/ml E.coli tRNA, 0.5% SDS). If FISH to rRNA only was performed, the final probe concentration in the RNA hybridization mixture was 2.5 µg/ml. In the RNA-DNA double-FISH the rRNA probe was used in a final concentration of 0.05 µg/ml, to avoid masking of the 1q12 DNA probe-related fluorescence after cytochemistry by the strong cytoplasmic rRNA probe-linked fluorescence. Incubation for 90 min was performed at 45 °C in an oven with forced ventilation. While hybridizing, the tubes were agitated by a vibrating device. Excess probe was removed after hybridization by adding 100 µl RNA hybridization mixture without probe and further incubation was carried out at 45 °C for 15 min, prior to washing in PBS.

In the RNA-DNA double-FISH experiments, protein extraction was carried out by resuspending the cell pellet after centrifugation in 0.1 M HCl, 0.05% Triton X-100 for 15 min at 4 °C. Enzymatic production of ssDNA was performed basically, as described by Van Dekken et al. (1988). After centrifugation, the cells were washed once in 100 µl. React 2 buffer (BRL; React 2 is 50 mM Tris-HCl, 10 mM MgCl2, 50 mM NaCl, pH 8). Next, after centrifugation, the cell pellet was resuspended in 25 µl React 2, 10 mM dithioerythritol (DTE), containing Exonuclease III (3000 U/ml), HindIII (200 U/ml) and EcoRI (450 U/ml). The cells were incubated at 37 °C for 60 min, before 100 µl DNA hybridization mixture was added. After 5 min at room temperature, centrifugation was performed, and the cells were resuspended in 25 µl DNA hybridization mixture, containing the heat denatured DNA probe (see above). The sample was mixed and incubated for 14-18 hours at 37 °C. Washing and cytochemistry were carried out as described above. The cells were brought into the antifade solution, without DNA fluorochrome, and 5 µl suspension was put under a 18 x 18 mm coverslip on a glass microscope slide, avoiding pressure on the coverslip.

Confocal scanning microscopy and image analysis: The confocal scanning laser microscope (Brakenhoff et al., 1985, 1989) was used to perform 3-D analysis on the hybridized cells. A krypton laser (Spectra Physics Series 2000, Mountain View, CA), tuned at 482.5 nm (10mW), was used to excite both the probe-linked FITC as well as the total nuclear DNA stain propidium iodide. The resulting green and red fluorescence, respectively, was measured through a 500 nm long pass filter (Corion Corp., Holliston, MA). The specimen was scanned mechanically along three axes through the confocal point under microprocessor control to produce a 3-D image. Typically, the sampling interval in this image is 1 µm along the optical axis and 90

nm in the lateral plane. The fluorescence intensity data of the objects were stored in a 256x256x16 8-bit memory array as a function of specimen position.

Image processing and generation of stereoscopic pairs was performed as described by Van der Voort et al. (1989). Preprocessing of the images was done by means of a median filtering procedure with a 7-voxel window. Briefly, this implicates, that the 8-bit grey level value of each voxel (volume element) becomes the median of itself and the six perpendicularly adjacent voxels. This noise reducing filter results in images with more defined contours, surrounding areas of different fluorescence intensities.

Two different spatial measurements were performed. The first type of measurement was performed on the spherical lymphocyte nuclei. The angle between the two centromere 1 FISH spots near the nuclear surface was determined, the center of mass of the nucleus being the origin. The positions of the center of mass of the nucleus, and the centers of mass of centromeres A and B were determined interactively with the cursor on a video screen, containing the 16 x-y planes of the image. The cursor was moved to the appropriate image and a mark was put on the above named spots. The following algorithm was used to define the angle phi: phi=arccos (a.b /|a||b|), in which a.b is the dot product of the two 3-D vectors between the point of weight of the nucleus and hybridization spots A and B; phi is the smallest angle between the two 3-D vectors. The expected random distribution for the K-S test was obtained by computer simulation with 10,000 randomly chosen values (Monte Carlo procedure). The random values in Fig. 4 were normalized to the number of 3-D images, that was analyzed.

The second type of measurement was the calculation of the relative position of the centromere FISH spots on the radius of the nucleus. Therefore the center and edge of the nuclear plane in one optical section, as defined by propidium iodide staining, and the hybridization spot withinthe same section were determined interactively with the cursor on the video screen. The measurement was performed on the image plane, in which the center of mass of the FISH spot was located. The position of the centromere 1 was expressed as a percentage of the radius, 0 being the center of the nuclear section. Likewise, 100% would implicate the center of mass of centromere 1 being at the nuclear edge itself. This 2-D measurement within one optical section was applied as a simple determination of the location of the hybridization spot, since a center of mass in sets of images of the polymorphic nuclei of HL60 cells was difficult to appoint. The expected random distribution, used in Figs. 3 and 6 was derived from a Monte Carlo simulation, and the value for each concentric area was calculated. E.g., the area between 60 and 70% of the radius represents |0.36-0.49|= 13% of the total area. Subsequently, these values were normalized to the number of FISH spots, involved in the particular measurement.

Photography: Pictures were taken directly from the video screen. Agfapan 25 film was used for black and white photography.

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Part III: CLINICAL APPLICATIONS

CHAPTER 6

A NEW APPLICATION OF FLUORESCENT *IN SITU* HYBRIDIZATION: DETECTION OF NUMERICAL AND STRUCTURAL CHROMOSOME ABERRATIONS WITH THE COMBINATION OF A CENTROMERIC AND A TELOMERIC DNA PROBE

H. van Dekken, and J.G.J. Bauman

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ABSTRACT.

Fluorescent *in situ* hybridization provides a fast method for detection of specific nucleic acid sequences. We have used high-resolution unicolor fluorescent *in situ* hybridization with the combination of a centromeric and a telomeric DNA probe, specific for chromosome 1, to investigate the simultaneous assessment of numerical and structural chromosome aberrations. The K562 leukemia cell line served as a model.

INTRODUCTION

In recent years non-isotopic *in situ* hybridization techniques were introduced as a fast method for the detection of specific nucleic acid sequences in cell nuclei and on intact chromosomes (Bauman et al., 1981; Langer-Safer et al., 1982; Landegent et al., 1985). Some investigators reported the detection of numerical chromosome aberrations by fluorescent *in situ* hybridization, using repetitive DNA probes (Pinkel et al., 1986; Moyzis et al., 1987). These probes bind to large, easy-to-detect targets, both in metaphase and interphase cells. The majority of these repetitive DNA probes consists of satellite DNA, which is present mainly at the centromeres of chromosomes. Recently, a DNA probe was described, detecting repetitive sequences at the 1p36 telomeric region of the short arm of chromosome 1 (Buroker et al., 1987). By combining such a probe with a centromeric satellite DNA probe, a powerful instrument is created for the detection of structural abnormalities on a specific chromosome arm by non-isotopic *in situ* hybridization (Lucas et al., 1987). An advantage with respect to conventional cytogenetic analysis is the speed of analysis of the hybridized target sequences.

In this study, we used a chromosome 1 specific centromeric satellite DNA probe (Cook and Hindley, 1979) in combination with the chromosome 1p specific telomeric repetitive DNA probe to investigate both numerical and structural aberrations of chromosome 1 in the leukemia cell line K562. Both probes were biotin-labeled (Langer-Safer et al., 1982) and, after hybridization, visualized with fluoresceinated avidin. Application of the centromeric probe only revealed a trisomy of chromosome 1 in the K562 cell line, which could be detected in both metaphase and interphase cells. When the telomeric probe was also added to the hybridization mixture, it was possible to visualize translocations or deletions of the short arm of chromosome 1.

MATERIALS AND METHODS

Metaphase and interphase cells were obtained from the K562 cell line and from peripheral lymphocytes and prepared according to conventional cytogenetic techniques. Briefly, after colcemid arrest in metaphase, the cells were incubated for 12 min. at 37 °C in 75mM KCl and fixed three times in methanol/acetic acid (3:1; vol:vol) before being dropped on ethanol cleaned microscope slides. The human 1p specific telomeric probe p1-79, 900 basepairs in pSP65, was obtained from M. Litt (University of Oregon, USA). The human chromosome 1 specific centromeric probe pUC1.77, 1770 basepairs in pUC18, was obtained from H. Cook (MRC Edinburgh, UK). For *in situ* hybridization, complete plasmid DNA was labeled with biotin-11-dUTP by nick translation (Bethesda Research Laboratories, USA) according to the manufacturers' directions. In view of the similarities of both plasmid vectors used, it cannot be excluded, that network formation between both plasmids contributed to the hybridization signal of each probe. However, when the same fluorochrome is used for visualization of the hybrids, this is not a limitation of the technique. The specificity of binding is in each case caused by the base sequence of the inserted fragment.

The *in situ* hybridization and fluorescent detection protocol followed that of Pinkel et al. (1986). The hybridization mixture contained 1 μg/ml probe DNA, 250 μg/ml sonicated herring sperm DNA, 1% Tween 20, 10% dextran sulphate and 2xSSC in 50% formamide at pH 7. When the probes were used simultaneously, the concentration was 0.2 μg/ml for the centromeric probe and 0.8 μg/ml for the telomeric probe, respectively. After overnight hybridization at 37 °C the slides were washed three times for 3 min. in 50% formamide, 2xSSC, pH 7 at 45 °C and subsequently two times for 5 min. in 2xSSC, pH 7 at 45 °C. Then the slides were incubated with fluoresceinated avidin (5 μg/ml). In some experiments the probe-linked fluorescence was amplified by incubation with biotinylated goat-anti-avidin followed by fluoresceinated avidin (Pinkel et al., 1986; avidin and anti-avidin from Vector Laboratories Inc., Burlingame CA, USA). Finally, the DNA of the cells was counterstained with propidium iodide (1 μg/ml; Sigma) to allow simultaneous observation of total DNA and hybridized probe. The DNA stain was carried in an antifade solution to preserve the fluorescein fluorescence during extended microscopy (Johnson and de C. Nogueira Araujo, 1981).

RESULTS AND DISCUSSION

Normal human lymphocyte metaphase and interphase cells were hybridized with the chromosome 1 specific centromere-telomere probe combination and hybridized probe was

detected as described above. Propidium iodide was used as a DNA counterstain. The hybridization spots on both centromere and p-telomere of the two homologues of chromosome 1 are clearly visible (Fig. 1a). In fig. 1b a metaphase plus interphase cell of leukemia cell line K562 is shown, hybridized with the centromeric probe only. Three centromeres 1 are seen in

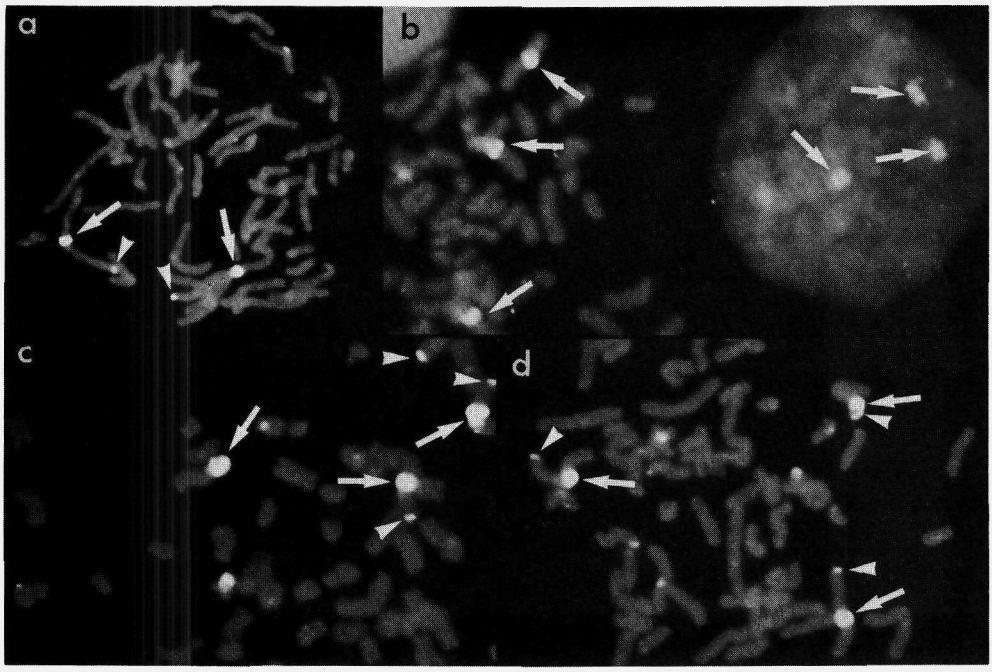

Figure 1:
Photomicrographs showing fluorescent *in situ* hybridization of biotin-labeled probe DNA to metaphase and interphase cells. The hybridized probe was detected with fluoresceinated avidin. All of the DNA was counterstained with propidium iodide. The regions to which probe hybridized appear white in the photomicrograph. The regions of DNA to which the probe did not bind appear grey. Panel a. Hybridization of the chromosome 1 specific combination of a centromeric and a telomeric DNA probe to normal human lymphocyte metaphase chromosomes. Arrows indicate centromeric hybridization, arrowheads indicate telomeric hybridization. Panel b. Hybridization of the chromosome 1 specific centromeric DNA probe to an interphase nucleus and metaphase chromosomes of leukemia cell line K562. Panels c and d. Hybridization of the chromosome 1 specific centromere-telomere DNA probe combination to metaphase chromosomes of leukemia cell line K562.

the metaphase as well as in the interphase cell. The weaker spot present is considered to be a minor binding site on the centromere of another chromosome (Cook and Hindley, 1979). The appearence of minor binding sites is dependent on the stringency of the hybridizaton procedure and the quality of the slides. However, after hybridization with the combination of the chromosome 1 centromeric and the 1p-telomeric probe also a translocation or a deletion of one

of the short arms of chromosome 1 is revealed. Of 20 randomly selected metaphase figures roughly 2/3 expressed the translocation, the rest showed the 1p deletion. In fig. 1c the short arm of one of the three chromosomes 1 is missing (centre). In the upper right corner the translocated fragment, showing telomere hybridization, is visible. A deletion of a large part of the short arm of chromosome 1 is demonstrated in fig. 1d. Here, the hybridized chromosome in the upper right corner exhibits a shortened 1p arm, when compared with the other two chromosomes 1. Thus, in one hybridization a numerical and structural aberration is detected by "spanning" the short arm of chromosome 1 with a centromeric plus a telomeric DNA probe.

Information about numerical aberrations can be derived simply from interphase cells only (fig. 1b; Moyzis et al., 1987). An advantage is, that interphase cells can be easily obtained. However, structural abnormalities can in most cases not be visualized in interphase nuclei. In this situation, metaphase figures are required for target localization on specific chromosomes.

The technique described above, can be a powerful tool in the study of radiobiological effects on human chromosomes. Pinkel et al. (1986) investigated the effect of radiation on human-hamster hybrid cells by scoring the number of human-hamster translocations in metaphase cells after *in situ* hybridization with genomic human DNA as a probe. Using the chromosome 1 specific centromere-telomere probe combination, this technique can now be applied to human blood (Lucas et al., 1987) and tumor cells and cell lines. Further, this chromosome 1 specific combination is of special interest because of the increased frequency of chromosome 1 aberrations in cancer (Atkin, 1986). Another application, in combination with conventional cytogenetic analysis, can be the localization of a chromosome fragment in translocations, where more than two chromosomes are involved. Interpretation of these translocations is often difficult using conventional banding techniques. The combination of conventional cytogenetic analysis and *in situ* hybridization might reduce this difficulty. It is expected, that centromeric satellite DNA probes for all human chromosomes will come available in the near future, so that karyotyping might be performed by multicolor multiprobe hybridizations (Hopman et al., 1986). Furthermore, when more telomere or other non-centromere probes will be isolated, a rapid and sensitive analysis of ultimately all chromosome translocations will be possible. This opens the way to automatic karyotyping plus detection of numerical and structural chromosome aberrations without the necessity to perform an analysis based on chromosome banding patterns.

In this report, we demonstrated a new application of fluorescent *in situ* hybridization. A chromosome 1p specific combination of a centromeric and a telomeric DNA probe was used to detect numerical as well as structural chromosome aberrations in a leukemia cell line. It can be concluded, that this technique provides a fast means of targeted cytogenetic analysis.

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CHAPTER 7

DETECTION OF HOST CELLS FOLLOWING SEX-MISMATCHED BONE MARROW TRANSPLANTATION BY FLUORESCENT IN SITU HYBRIDIZATION WITH A Y-CHROMOSOME SPECIFIC PROBE

H. van Dekken, A. Hagenbeek, and J.G.J. Bauman

Submitted for publication.

SUMMARY

Fluorescent *in situ* hybridization (FISH) with a biotinylated Y-chromosome specific repetitive DNA probe was applied to detect Y-bearing cells in blood and bone marrow samples from patients with haemopoietic malignancies after a sex-mismatched bone marrow transplantation. The sensitivity of this method is in the order of 0.1% Y-bearing nuclei in male recipients, transplanted with female marrow. In female recipients of male marrow, the detection of low numbers of non-Y-bearing nuclei is less sensitive. The presence of host cells in blood and bone marrow of seven patients (4 males, 3 females) was investigated with respect to successful engraftment or recurrence of the disease. The results obtained were compared with cytology (all 7 cases) and with conventional cytogenetics (5 cases). In 5 patients, the results of Y-FISH and cytology were identical. In 2 patients, low numbers of male host cells were detected in the marrow by Y-FISH, whereas cytology indicated complete remission of the disease. In 3 patients Y-FISH and cytogenetic data were similar, but in 2 patients Y-FISH revealed the presence of 0.2% and 7% male host cells, respectively, in bone marrow, whereas cytogenetics indicated a 100% female marrow in both cases. Because the hybridization was performed *in situ* , the morphology of the nuclei was preserved. To differentiate between normal and leukaemic cells, the size of the blast cell nuclei appeared to be a very useful indicator. Our data suggest, that fluorescent *in situ* hybridization with a Y-chromosome specific probe is a fast and sensitive technique to identify the host cells after sex-mismatched bone marrow transplantation, in particular in case of male recipient and female donor combinations.

INTRODUCTION

Increasing numbers of patients with haemopoietic disorders and malignancies are being treated with bone marrow transplantation (BMT) in recent years (1, 2). The analysis of the bone marrow with respect to successful engraftment, and in a later stage, the recurrence of host cells, is of great importance to the final outcome of the treatment. At the moment, the detection level of residual leukaemic cells in bone marrow by standard cytological tests is generally held to be 5%. In most cases, standard cytology cannot discriminate between blast cells of donor or recipient origin. When a sex-mismatched BMT case is analyzed, this can be studied by determination of the karyotype of the transplant (3). This approach is of low sensitivity, because the method is laborious and therefore restricted to a limited number of metaphases. The use of dot blot analysis of DNA with a Y-specific DNA probe was

investigated to detect the occurrence of host cells in the graft (4). The lower detection limit achieved, was approximately 10% male cells in a sample.

Fluorescent *in situ* hybridization (FISH) with chromosome specific repetitive DNA probes is becoming increasingly important as a tool for the detection of specific numerical and structural chromosome aberrations in both metaphase and interphase cells (5, 6, 7, 8). Bicolor double-target FISH was used for the simultaneous assessment of both chromosome 1 and 18 numerical abnormalities in interphase nuclei ("Interphase Cytogenetics") of tumour cells and cell lines (9, 10).

Recently, the use of FISH with chromosome specific probes was demonstrated to monitor the fate of the host cells during leukaemia therapy (11). The advantage of FISH is the speed of the analysis, due to both the fastness of the procedure and to the detectability of the probe in interphase nuclei, which allows one to analyze hundreds of nuclei rapidly using fluorescence microscopy. Furthermore, the cells are readily available for *in situ* hybridization on interphase nuclei. Culturing is not required to obtain cells in metaphase for chromosome analysis. This circumvents the possible selection of cell clones by culture conditions.

Following this approach, we applied FISH to study the presence of host cells in blood and bone marrow samples of patients after allogeneic sex-mismatched BMT. For this purpose a biotin-labeled (12) Y-chromosome specific repetitive DNA probe was used (13). The purpose of the study was to examine the feasibility of this method to detect residual host cells in the bone marrow of patients, grafted with bone marrow of the opposite sex. The sensitivity level and applicability in patient material (four male patients, three female patients) was investigated. Further, the results obtained by FISH with the Y-specific probe, were compared with cytological techniques and conventional cytogenetics.

MATERIALS AND METHODS

Sample preparation: Mononuclear cells were isolated from peripheral blood and bone marrow aspirates with a lymphocyte separation medium (Organon, Durham, NC) and cytological preparations were performed according to conventional cytogenetic techniques. In some cases also the granulocyte fraction was recovered and prepared for fixation. Per sample 1-5 million cells were incubated for 12 min at 37 °C in 75mM KCl and fixed once for 30 min in methanol/acetic acid (3:1; vol:vol) before being dropped on ethanol cleaned microscope slides. Air-dried slides were stored in a nitrogen atmosphere at -20°C prior to hybridization.

Probe and probe labeling: The human Y-chromosome specific repetitive satellite DNA probe, 2450 basepairs in pSP65, was obtained commercially (Amersham Int., Amersham, UK). This sequence is repeated approximately 2000 times on the long arm of the Y chromosome, but 5-10% is located elsewhere in the human genome (13). For *in situ* hybridization, complete plasmid DNA was labeled with biotin-11-dUTP by nick translation (BRL, Gaithersburg, MD) according to the manufacturers' directions.

In situ hybridization: Hybridization was performed as described previously (5, 14). Prior to hybridization, slides were baked in air for 2-4 hours at 75°C to improve preservation of the morphology during the ISH procedure. This treatment also reduces the loss of DNA, which occurs during denaturation (15). When a strong variation in probe-related signal intensity was seen, proteinase K (Boehringer, Mannheim, FRG) treatment was applied to remove matrix and histone proteins and to improve accessibility of the nuclei for the probe. This treatment resulted in more uniform FISH to the nuclei on the slide. In these cases the slides were immersed in digestion buffer (3 µg proteinase K in 50 ml buffer: 20 mM Tris-HCl, 2 mM CaCl2 at pH 7.5) for 7.5 min at 37°C, followed by two 1 min washes in 2xSSC, pH 7 at room temperature. Slides were denatured for 2 min at 70°C in 70% formamide, 2xSSC at pH 7. Formamide (BRL) was deionized by treatment with a mixed-bed ion-exchange resin (Dowex AG501-X8; Bio-Rad Labs, Richmond, CA). The slides were dehydrated in an ethanol series (70%, 83%, 96%) and air-dried at room temperature before applying the probe. The probe was denatured for 5 min at 70°C in the hybridization mixture. This mixture contained 2 µg/ml probe DNA, 500 µg/ml sonicated herring sperm DNA, 0.1% Tween 20, 10% dextran sulphate and 2xSSC in 50% formamide at pH 7. The hybridization mixture (12.5 µl) was put under a 22x22 mm glass coverslip and the edges were sealed with rubber cement. The *in situ* hybridization was performed overnight at 37°C. However, after 3 hours of hybridization also sufficient binding of the probe was observed. The rubber cement was removed with a forceps, and coverslips were removed by immersing the slides briefly in 2xSSC. Next, the slides were washed three times for 3 min in 50% formamide, 2xSSC, pH 7 at 45 °C and subsequently two times for 5 min in 2xSSC, pH 7 at 45 °C. The slides were then immersed in PN buffer (0.1 M sodium phosphate, 0.05% Nonidet P40, pH 8).

Cytochemistry: Detection of the biotinylated probe was accomplished with fluorescein-labeled avidin as described previously (12, 14). The slides were incubated with fluoresceinated avidin DCS Grade (5 µg/ml in PNM buffer: 5% non-fat dry milk in PN buffer; non-fat dry milk was added to prevent non-specific binding of the avidin (5)) for 20 min at 37°C. The slides were then washed two times for 5 min in PN buffer at 45°C. The probe-linked fluorescence was

amplified by incubation with biotinylated goat-anti-avidin (5 μg/ml in PNM; 20 min at 37°C), washed in PN buffer and again incubated for 20 min with fluoresceinated avidin ((5), avidin DCS Grade and anti-avidin from Vector Laboratories Inc., Burlingame, CA). The signal amplification was performed to facilitate detection of the probe at lower microscope magnifications. After the last wash in PN buffer, the DNA of the cells was counterstained with propidium iodide (1 μg/ml; Sigma, St. Louis, MO) to allow simultaneous observation of total DNA and hybridized probe. The DNA stain was carried in an antifade solution containing p-phenylenediamine dihydrochloride (Sigma) to preserve the fluorescein fluorescence during extended microscopy (16).

RESULTS

Sensitivity of male cell detection:

To estimate the accuracy of the method, a 10-fold dilution series, ranging from 1:10 to 1:1,000 (XY:XX), was made of unstimulated, fixed male and female peripheral blood lymphocytes from two healthy volunteers. The undiluted male and female samples served as positive and negative controls, respectivily. The interphase cells on the slides were hybridized with the Y-chromosome specific DNA probe, and hybridized probe was detected with fluoresceinated (FITC) avidin. Propidium iodide was used as a DNA counterstain. Of each preparation a random sample of 1,000 nuclei was counted and scored for probe-related FITC fluorescence, and the percentage of Y-bearing cells was determined. The criteria for scoring a cell as Y-bearing (male) were as follows. A nucleus should contain only one bright solid yellow-green (FITC) dot. Cells with a vague or small dot were not considered positive. Cells with low propidium iodide fluorescence intensity, indicating DNA loss, overlapping cells and incomplete cells were excluded from analysis. Using these criteria, approximately 5-10% of the cells were not suitable for scoring. The countings were performed on coded slides. The results are given in table 1. The percentages of Y-bearing nuclei obtained, were very similar to the dilution factor. In the 1:1,000 (XY:XX) dilution, 2 male cells were scored (0.2%). In the male control only 6/1,000 cells did not contain a fluorescent Y-spot. No cells with two or more Y-probe related spots were observed. In the female control no cells with solid Y-spots were seen in 1,000 nuclei.

TABLE 1

NUMBER OF Y-BEARING CELLS IN MIXTURES OF NORMAL MALE AND FEMALE LYMPHOCYTES (XY:XX), DETERMINED BY FISH WITH A Y-CHROMOSOME SPECIFIC PROBE.

	Number of Y-bearing cells*
Mixture 1:10	110
Mixture 1:100	12
Mixture 1:1000	2
Male control	994
Female control	0

*Of each sample 1,000 cells were analyzed. Cells with low propidium iodide fluorescence intensity, overlapping cells and incomplete cells were excluded from analysis. Only nuclei with one bright solid FITC-spot were scored as "Y-bearing".

STUDIES ON MALE PATIENTS

Mononuclear cells from blood and bone marrow aspirates of four male patients (2 acute myelocytic leukaemia (AML), 1 acute lymphocytic leukaemia (ALL), 1 Non-Hodgkin's lymphoma (NHL)), transplanted with female marrow were hybridized with the Y-specific DNA probe. Y-bearing cells were scored as described above with the exception, that, when more than 10 male cells were seen, the analysis was terminated as soon as 200 cells were counted. The results of the Y-FISH measurements are presented in table 2. The cytological and conventional cytogenetic data of the material are also given. In patients 1 and 2 (AML and ALL, respectively) no Y-spot containing nuclei were found in 1,000 counted cells. In addition, no Y-bearing cells were observed by superficial inspection of the whole slide (containing 10^4-10^5 nuclei). Patient 3 (NHL) appeared to have 0.2% (2/1,000) male cells in the bone marrow aspirate (fig. 1a). This was not detected by analysis on metaphase preparations. According to cytology, this patient showed no signs of NHL in the bone marrow. Patient 4 had a relapse of AML after BMT. This was also evident from the presence of large male nuclei in the marrow, presumably blast cells (fig. 1b). After chemotherapy this patient was cytologically in complete

remission. Cytogenetics indicated a 100% female bone marrow aspirate. However, by Y-FISH 7% (14/200) male nuclei were found, which is illustrated in fig. 1c. In this figure the Y-spot containing nuclei are also larger in size, suggesting blast cell origin. After receiving a second BMT 0.7% (7/1,000) large male nuclei were found by Y-FISH in the bone marrow of this patient (fig. 1d). This correlated rather well with the percentage of residual leukaemic cells, found by immunophenotyping. Here 0.3% of the marrow cells showed positivity for both TdT and CD33 (MY9), which was specific for this particular case of AML.

TABLE 2

COMPARISON OF RESULTS, OBTAINED BY CYTOLOGY, CONVENTIONAL CYTOGENETICS AND FISH WITH THE Y-CHROMOSOME SPECIFIC PROBE IN THE BONE MARROW OF PATIENTS AFTER RECEIVING A SEX-MISMATCHED BONE MARROW TRANSPLANTATION.

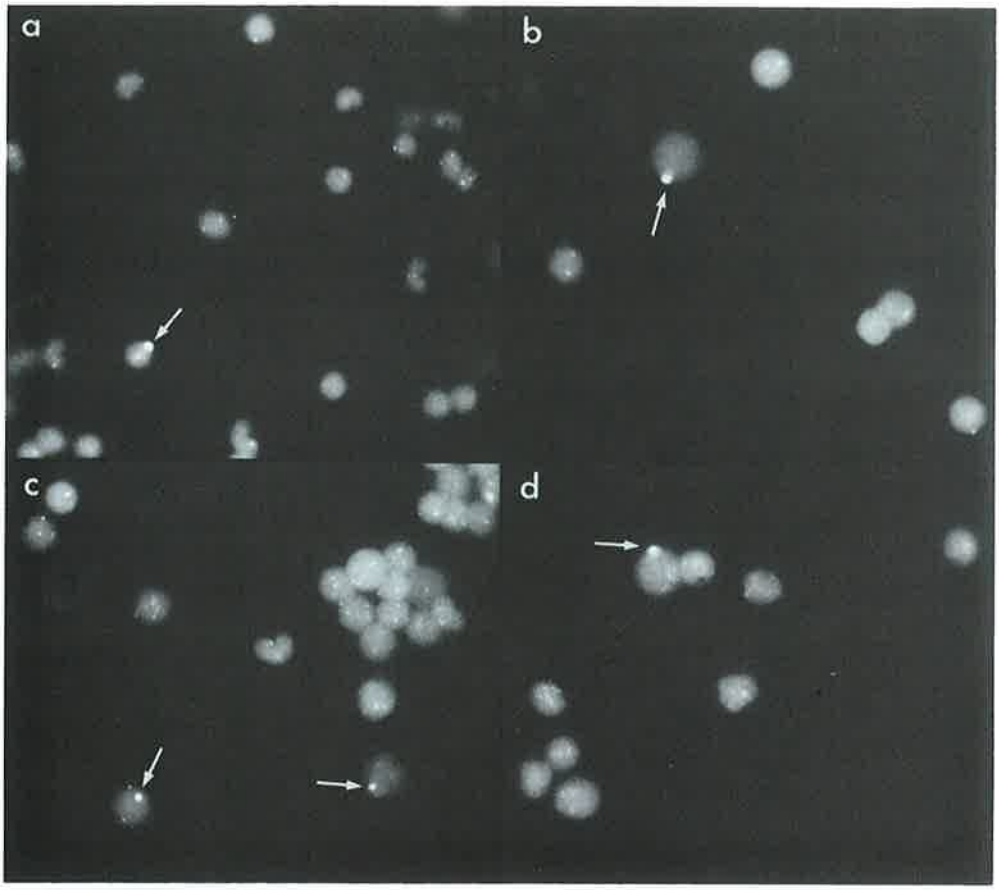
patient (sex)	diagnosis	tissue	Y-FISH[1]	cytology	cytogenetics[2]
pat. 1 (male)	AML	BM	0.0	CR	100% XX
2 (male)	ALL	BM	0.0	CR	100% XX
-		PB	0.0	N	-
3 (male)	NHL	BM	0.2	CR	100% XX
4 (male)	AML	BM1	17.0	22% BC	-
-		BM2	18.0	17% BC	-
-		PB2	6.0	-	-
-		BM3	7.0	CR	100% XX
-		BM4	0.7	CR[3]	-
5 (female)	AML	BM	-	CR	100% XY
-		PB	99.6	N	-
6 (female)	MM	BM	99.3	N	-
7 (female)	CML	PBm	68.5	20% BC	-
-		PBg	99.1	-	-

[1]Y-FISH data indicate the percentage of Y-bearing cells, typically, obtained after analyzing 1,000 nuclei.

[2]Cytogenetic data were usually derived from 25-40 metaphase figures.

[3]Specific immunophenotyping revealed the presence of 0.3% leukaemic cells; for details, see Results.

Abbreviations used: ALL, acute lymphocytic leukaemia; AML, acute myelocytic leukaemia; BC, blast cells; BM, bone marrow, 1-2-3-4 refers to subsequent sampling times; CML, chronic myelocytic leukaemia; CR, complete remission; N, no abnormalities; NHL, non-Hodgkin's lymphoma; MM, multiple myeloma; PB, peripheral blood; PBg, peripheral blood granulocyte fraction; PBm, peripheral blood mononuclear cell fraction.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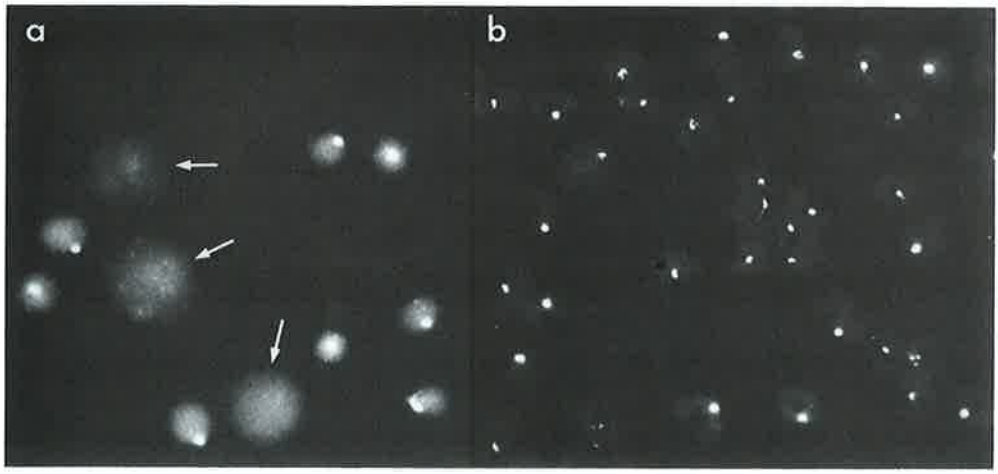
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Figure 1:

Photomicrographs (40X objective) demonstrating fluorescent *in situ* hybridization (FISH) with the biotinylated Y-chromosome specific repetitive DNA probe to mononuclear bone marrow cells of male patients after transplantation with female marrow. The hybridized probe was detected with fluoresceinated avidin. All of the DNA was counterstained with propidium iodide. The regions to which probe hybridized appear white in the photomicrograph. The regions of DNA to which the probe did not bind appear grey. A: Detection of low amounts of host cells in the bone marrow of patient 3 (table 2). B: Recurrence of AML cells in the bone marrow of patient 4 (table 2). Note the larger size of the host nuclei in this patient. C: Bone marrow of patient 4 after chemotherapy. D: Bone marrow of patient 4 after second BMT. Arrows indicate male host cells.

Figure 2:

Photomicrographs (40X objective) demonstrating FISH of the biotinylated Y-chromosome specific repetitive DNA probe to blood cells of a female CML patient after transplantation with male marrow, now suffering from blast crisis. For details on visualization of probe and nuclei, see fig. 1. A: Mononuclear blood cells of patient 7 (table 2). Recurrence of female host cells (arrows) with very large nuclei. B: Granulocytes of the same patient. No female host cells are detectable.

STUDIES ON FEMALE PATIENTS

Mononuclear cells and granulocytes from blood and bone marrow aspirates of three female patients (1 AML, 1 chronic myelocytic leukaemia (CML), 1 multiple myeloma), transplanted with male marrow were hybridized with the Y-specific DNA probe. In all cases 1,000 cells were counted as described above. Patients 5 and 6 (AML and multiple myeloma, respectively) both had more than 99% male cells in the graft (table 2), which is similar to the male control (table 1). The cytology and conventional cytogenetics were without abnormalities (table 2). Patient 7, suffering from blast crisis during CML, had 31.5% (63/200) Y-spot negative cells in the mononuclear peripheral blood fraction. Cytology indicated the presence of 20% blast cells in peripheral blood. All of these female nuclei were extremely large (fig. 2a). The granulocyte fraction was also investigated for the presence of female cells. Here, 99.1% (991/1,000) granulocytes carried a Y-spot.

DISCUSSION

This study demonstrates the feasibility of the FISH method with a Y-chromosome specific DNA probe to detect low numbers of host cells in sex-mismatched bone marrow transplant recipients. The method is simple and fast. If preferred, the whole procedure can be accomplished in one day, using 3 hours for *in situ* hybridization of the probe, instead of overnight incubation. After completion of the FISH procedure, it takes about 15 min to analyze 1,000 cells. The accuracy of the method can be assessed by the experiment, shown in table 1. After Y-FISH to the male control, 994/1,000 cells carried a clear Y-spot, leaving about 1% spotless.

The sensitivity of the detection of male cells in a female graft is in the order of 0.1%. This is far more sensitive than chromosome analysis, based on banding patterns. One of the most important aspects is the fact, that this methodology provides a means of interphase cytogenetics. No metaphase cells are required for cytogenetic analysis. Thus, there is no need for culturing or growth stimulation of the cells, which might lead to the selective growth (and analysis) of specific cell subsets. This can be the reason of the discrepancy between the Y-FISH and conventional cytogenetic data, found in patient 4-BM3 (table 2). Also the low number of metaphase figures (table 2), that is usually studied by conventional cytogenetics, because there are (in most cases) no more available, might be the cause. In a sex-mismatched graft, the origin of the blast cells is disclosed, which in most cases is not possible by cytological procedures. This was illustrated in patient 4 (BM3 and BM4), who was in "complete remission", according to the cytology. However, the blast cells appeared to be of the host type, suggesting residual leukaemia.

The sensitivity with respect to the detection of female cells in male grafts is lower, due to the nature of the probe. A solid fluorescent spot in a nucleus indicates the male karyotype. In most female nuclei some FITC fluorescence was seen, caused by the fact, that 5-10% of the repetitive sequence is located on autosomes (13). A weak fluorescent spot may indicate female origin, but can also be caused by weak hybridization in a male cell. The results, obtained with the mixtures (table 1), confirmed our assumption, that only a solid bright spot could be judged as "male". We considered more than 99% Y-bearing nuclei in a male graft as 100% male (table 1). Sometimes, a strong variation in probe fluorescence intensity was seen. In such cases, proteinase K treatment appeared to be necessary for good and homogeneous hybridization in all cells on the slide. To increase the sensitivity of detecting low amounts of female cells, double hybridization in combination with an X-chromosome specific repetitive DNA probe (17, 18) might be useful. Acetylaminofluorene-modification (AAF, (19)) or Hg-labeling (9) can tag the second probe with a different colour. However, in this case the detection of low

amounts of female host cells is hampered by the observation, that only 75% of diploid cells show two spots, when hybridized with chromosome specific alfoid repetitive DNA probes (7, 9).

The size of the nuclei appeared to be a useful indicator of the recurrence of leukaemic blast cells. These nuclei were larger in size than the graft cell nuclei and were found to be of host origin (fig.'s 1b, 1c, 1d and 2a). Granulocyte nuclei retained their polymorphic appearance during the procedure and could be easily recognized by this property. When the mononuclear and granulocyte fractions of blood and bone marrow samples would be separated even further, it should be possible to follow the pathway of the recurring host cells in the different blood cell compartments (fig. 2).

The technique described in this paper is limited to the detection of host cells in grafts of patients after sex-mismatched bone marrow transplantation. Other DNA diagnostic techniques might be of use in sex-matched cases. Restriction fragment length polymorphism (RFLP, (20)), if found between donor and acceptor, might be of use in both sex-matched and sex-mismatched transplantation studies. The polymerase chain reaction technique (PCR) is extremely sensitive in detecting a specific DNA sequence, but it is difficult to quantitate the percentage of cells involved (21). It further requires a disease-specific oligonucleotide marker, such as the breakpoint cluster region in Ph+ CML (22). Both RFLP and PCR can only be applied to lysates of cells, which implies, that information about nuclear morphology is lost.

The technique described here should be eligible for computer-aided analysis. Automated screening of large numbers of cells after FISH could be used to reveal low frequencies (below 0.1%) of cells with specific numerical chromosome properties. This would improve the diagnosis and treatment of diseases, such as the relapse of haemopoietic malignancies after bone marrow transplantation.

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GENERAL DISCUSSION

The objective of this thesis was to explore the possibilities of fluorescent *in situ* hybridization (FISH) as a tool for studies with cell biological or clinical interest. Advanced technology was applied to investigate these features. Computer-aided microscopy, such as confocal microscopy, was used to investigate the three-dimensional topography of specific hybridized regions within intact cell nuclei. Two and three laser flow cytometry was used to quantitate the fluorescent *in situ* hybrids after FISH to cells in suspension. In the clinical studies standard fluorescence microscopy was applied to evaluate the result of the hybridization. In the next part of this discussion the results, that were obtained, will be discussed, and future prospects will be mentioned.

Flow cytometric FISH

In Chapter 3 the flow cytometric measurements after FISH to isolated lymphocyte nuclei in suspension are described. We were able to measure quantitatively differences in repetitive DNA sequences between individuals after FISH with biotinylated chromosome Y and 1-specific satellite DNA probes. The sensitivity achieved was in the order of 1 megabase. As a preliminary experiment we performed bicolor FISH with differently labeled Y and #1 probes.

It appeared, that the probe related fluorescence intensity was correlated strongly with the nuclei concentration. This was the result of the amount of probe used, which was non-saturating. Therefore accurate counting of the samples before hybridization was required. In practice, this turned out to be very difficult, since isolated nuclei render lots of clumps in suspension. Shearing of the sample through a needle before counting worked satisfactory. A better solution to this problem might be the use of fixed whole cells instead of nuclei. These cells appear to produce good single cell suspensions (See Chapter 5). As a control for hybridization we used Chinese hamster DNA. This probe should hybridize to the same degree to lymphocyte nuclei of different persons, which allows the comparison of the results with chromosome specific probes. Although this worked nicely, it would be preferable to add a second, differently labeled, probe to check for differences in hybridization and autofluorescence properties of different cell types. Especially, when aneuploid tumour cell nuclei have to be measured, this could be useful, since the autofluorescence increases with the size. As a second probe, genomic human DNA or the probe, described by Mitchell et al. (1985), could be of use. The latter hybridizes to the same extent to the centromeres of all human chromosomes, providing a nice hybridization control. In experiments with centromeric satellite DNA, this probe would not interfere with the satellite DNA hybridization, since its target on the centromeres is much smaller.

The sensitivity of the suspension hybridization method is rather low, when compared with FISH on microscope glass slides. We estimated this to be about 1 megabase, which is comparable with the results of Trask et al. (1988). Sofar only repetitive DNA targets can be measured. This is likely mainly due to autofluorescence. But also the stringent fixation, that is required for the maintenance of the nuclei during the whole procedure in suspension, could interfere with sufficient accessibility of the probe fragments. The enzymatic procedure for ssDNA production for FISH, described in Chapter 2, might require less stringent fixation, leading to increased accessibility. Autofluorescence, a well known phenomenon in flow cytometry, is difficult to circumvent, although software corrections, using control samples, could help to diminish this problem. This could make detection by flow cytometry of single copy and amplified genes possible.

3-D microscopy and FISH

In Chapters 4 and 5 the three-dimensional architecture of chromosomes within cell nuclei is investigated by means of FISH with chromosome specific DNA probes to suspended cells and nuclei. By performing the whole procedure in suspension, the nuclear morphology is preserved, thus enabling 3-D analysis. In Chapter 4 computer-aided fluorescence microscopy was applied to optically section the nuclei. The contours of hybridized regions and of the nuclear boundary were defined by intensity thresholding. Specific patterns of human chromosomes were found in human-hamster hybrid and human fibroblast cell line nuclei. In Chapter 5 confocal scanning microscopy was used to analyze the chromosomal topography in cell nuclei of the haemopoietic system. Measurements within the nuclei were performed interactively by marking specific points in the sections with the cursor on a video screen. The distribution patterns of centromeres 1 in peripheral lymphocyte and HL60 cell line nuclei were evaluated statistically. It was found, that the centromeres 1 were associated with the nuclear envelope. The relation between the two centromeres in peripheral lymphocyte nuclei appeared to be random.

The advantage of confocal microscopy is the resolution of the images. This is due to the confocal principle, which results in complete suppresssion of out-of-focus fluorescence "glare" (Brakenhoff et al., 1985). Therefore, the association of the centromeres with the nuclear boundary could be defined. In the set-up, described in Chapter 4, this is very difficult to perform, because the borders are set by intensity thresholding and are therefore rather arbitrary. These images were evaluated by eye with respect to specific chromosome patterns after rotating them into a standard position. In this way, comparisons could be made, but the quantification and statistical analysis, as is possible in confocal microscopy, could not be applied.

The sensitivity level at present is comparable with the flow cytometric FISH method. Only repetitive sequences with big targets can be visualized, due to autofluorescence and fixation conditions (See previous paragraph). New fixation methods, as described in Chapter 5, could pave the way to simultaneous detection of specific genes and their corresponding mRNA's, or proteins and surface markers. Therefore, multicolor labeling would be required, using biotinated, sulfonated, AAF-modified and mercurated probes (Nederlof et al., 1989). It would be interesting to combine the centromeric probes with the telomeric probe, described by Moyzis (1988). This could finally elucidate the issue of the existence of the centromere-telomere alignment of chromosomes in interphase nuclei, the socalled Rabl orientation (Chapter 5). With the advent of the chromosome specific library probes (Pinkel et al., 1988), that stain complete chromosomes, the organization of chromomal domains can be investigated simply.

The spatial organization of chromosomes in cell nuclei is probably dependent on the state of proliferation and /or differentiation of the specific cell type. It can be imagined, that different cell functions need different (or a preferable) 3-D situation. It appears, that in central nervous

system cells, the 3-D topography of chromosomes is rather fixed (Manuelidis and Borden, 1988), whereas in haemopoietic cells, such as unstimulated peripheral lymphocytes and promyelocytes, a more flexible pattern is found (Chapter 5). CNS cells are fully differentiated cells, that do not divide anymore, whereas haemopoietic cells are rather undifferentiated and fully proliferating. These results suggest, that chromosome topography can be related to cell type and function.

Clinical studies and FISH

In Chapter 6 and 7 clinical studies are described using FISH to cell preparations on slides to detect cells with specific chromosome properties, such as numerical aberrations and translocations (Chapter 6), and sex-chimerism (Chapter 7).

For the detection of translocations of the #1p chromosome arm, a combination of a #1 specific centromeric (1q12) probe and a telomeric (1p36) repetitive DNA probe was used. This fruitful combination is, however, rare. Chromosome specific repetitive telomeric sequences, apart from 1p36, have not been found so far. Another aspect is, that metaphase spreads are required for the analysis. This is a major drawback in tumour cytogenetics, because most (solid) tumours have low mitotic indices. So, it would be advantageous to perform the analysis on interphase nuclei. This cannot be done by conventional cytogenetics, based on chromosome banding patterns. However, when alpha-satellite DNA probes are used, numerical aberrations can easily be detected by FISH in interphase nuclei. At present, for the majority of the human chromosomes alphoid probes are available (Willard and Waye, 1987). Using single and bicolor labeling, Hopman et al. (1988) and Devilee et al. (1988) applied FISH with alphoid probes to detect numerical chromosome aberrations in solid tumour cell line interphase nuclei. This approach was called "Interphase Cytogenetics". Although this technique is very promising, one aspect requires attention. Only 70-80% of normal diploid nuclei displayed two spots, when hybridized with the alphoid probes. Most of the remainder showed one or no spot, due to loss of DNA or impaired accessibility. Some nuclei did show three or more spots. This phenomenon would make the analysis of tumours containing cell subsets with different ploidy or numerical aberrations very difficult, especially when low numbers of aberrant cells are involved. This problem could be decreased by improving the conditions for fixation and denaturation of the cell preparations.

The availability of the chromosome specific library probes can result in an increased significance of interphase cytogenetics. Cremer et al. (1988) and Pinkel et al. (1988) demonstrated the applicability of this technique with respect to the detection of numerical chromosome aberrations and translocations in interphase nuclei. Cremer et al. found, that 50-60% of diploid nuclei showed two spots, when hybridized with a chromosome specific library. It can be expected, that this procedure will improve rapidly. Then, the investigation of numerical as

well as structural chromosome aberrations within solid tumours might be possible in the interphase nucleus.

It can be concluded, that FISH to interphase nuclei can and will be used for clinical and cell biological purposes. Interesting new developments, such as new probe labeling procedures for multicolor hybridizations, chromosome specific library probes, or new microscopical techniques, like the confocal microscope, can rapidly improve the detection level and possibilities of *in situ* hybridization. Finally, the investigation of the 3D topography of chromosomes within nuclei might lead to a better understanding of the relationship between disease and chromosome aberrations.

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SUMMARY

The purpose of the study, described in this thesis, was the investigation of fluorescent in situ hybridization as a cytogenetic tool in cell biology and clinical genetics. For this reason various procedures were applied and developed, such as in situ hybridizations to microscopic preparations on glass slides, and in suspension. For analysis fluorescence microscopy, 3-D image reconstruction, confocal microscopy and flow cytometry were used. In situ hybridization or hybridocytochemistry allows for the labeling of specific nucleic acids in microscopic preparations. For this purpose the DNA has to be made single-stranded or denatured, before the probe is applied. We used biotin-labeled and AAF-modified repetitive DNA probes to localize the target sequences in fixed nuclei and metaphase chromosomes. In situ hybridizations to suspended nuclei were performed to study the spatial relationships of the hybridized regions, and the reliability of the interphase-suspension method was evaluated. The probe-linked fluorescence intensity was also measured by flow cytometry in order to determine differences among individuals with respect to the amount of hybridized sequences. In situ hybridizations to chromosomes and nuclei, fixed on glass slides, were done to search for new clinical applications in hybridocytochemistry. In Chapter 1 an outline of the possibilities of fluorescent in situ hybridization is given.

In Chapter 2 a new method for producing single-stranded DNA (ssDNA) for hybridocytochemistry is described. It employs Exonuclease III in combination with restriction endonucleases to generate stretches of ssDNA for *in situ* hybridization. The method appeared to be nearly as effective as the routinely used heat denaturation with respect to probe detection. Due to the mild chemical and physical properties, this method can be of great use in hybridocytochemistry of targets, that are easily disrupted, such as nuclei in suspension (Chapter 5).

The flow cytometric quantification of fluorescent *in situ* hybrids in human lymphocyte nuclei in suspension after *in situ* hybridization with chromosome specific repetitive satellite DNA probes, is described in Chapter 3. This approach appeared to allow the detection of polymorphisms in individuals with respect to chromosome 1 and Y heterochromatin variation. The sensitivity achieved, was in the megabase range. In this Chapter also the double-target bicolor hybridization with chromosome 1 and Y specific probes is demonstrated. Double hybridizations, with one probe as a reference for hybridization efficiency, might be useful, e.g. when different tumour cells or cell lines are compared in order to measure numerical chromosome aberrations.

Chapter 4 deals with the three dimensional investigation by computer-aided fluorescence microscopy of the localization of specific chromosomes within nuclei with preserved morphology. For this purpose fluorescent *in situ* hybridization to hybrid and human cell line nuclei in suspension was applied. Genomic human DNA, and chromosome specific centromeric and telomeric sequences were used as probes. In different human-hamster hybrid cell lines, the hybridized human chromosomes within the nuclei tended to show typical spatial patterns. In human fibroblast nuclei, centromeric and telomeric sequences were visualized near the nuclear boundary, often in specific positions.

A follow-up of this study is described in Chapter 5, where confocal microscopy was applied to quantitatively delineate the spatial relationship between hybridized centromere 1 regions in spherical lymphocyte and polymorphic HL60 cell line nuclei. It was measured, that the centromere 1 regions were associated with the nuclear surface. The positions of these regions with respect to each other appeared to be random. Using the enzymatic procedure, described in Chapter 2, simultaneous fluorescent *in situ* hybridization was performed to chromosome 1 specific satellite DNA and ribosomal RNA in the nucleus and the cytoplasm, respectively, of HL60 cells. This set-up provides a means for the three dimensional study of gene and gene products.

Clinical cytogenetics is the topic of Chapters 6 and 7. In Chapter 6 the detection of numerical and structural chromosome aberrations in metaphase figures by hybridocytochemistry is demonstrated. By using the combination of a chromosome 1 specific centromeric probe in combination with a 1p specific telomeric DNA probe, numerical aberrations of chromosome 1 as well as translocations and deletions of 1p were observed in a leukaemia cell line. Interphase cytogenetics with a Y-chromosome specific probe was used to detect low numbers of residual or recurring host cells in blood and bone marrow of patients with hematological malignancies after sex-mismatched bone marrow transplantation (Chapter 7). Percentages of male host cells as low as 0.2% and 0.7% could be detected by *in situ* hybridization to marrow cells of patients. These residual host cells were not detectable by conventional cytogenetics or cytology.

It is concluded, that fluorescent *in situ* hybridization or hybridocytochemistry can serve as a cytogenetic tool, in cell biological studies, as wel as for diagnostic purposes. The advent of interphase cytogenetics has made it possible to study the cytogenetics of cells and tumours with low mitotic indexes. The introduction of the confocal scanning microscope has facilitated and improved the investigation of the interior of cells and cell nuclei. Flow cytometry could be useful in the rapid assessment of the presence of numerical chromosome aberrations within tumours and tumour cell subsets, when *in situ* hybridization to suspended interphase cells is applied.

SAMENVATTING

Het doel van de studie, beschreven in dit proefschrift, betrof het onderzoeken van de waarde van *in situ* hybridisatie met een fluorescerend eindproduct als instrument in de celbiologie en klinische genetica. Hiervoor werden diverse technieken toegepast en geoptimaliseerd, zoals in situ hybridisatie op microscopische preparaten op glas, maar ook in suspensie. Fluorescentie microscopie, 3-D beeld analyse, confocale microscopie en flow cytometrie werden gebruikt om de resultaten te analyseren. In situ hybridisatie of wel hybridocytochemie verschaft de mogelijkheid tot het aantonen van specifieke nucleinezuren in microscopische preparaten. Voordat de probe kan worden toegepast, moet het DNA in enkelstrengsvorm worden gebracht of wel gedenatureerd. Wij hebben biotine- en AAF-gemerkte repetitieve DNA probes gebruikt om de specifieke doelsequenties in de gefixeerde kernen en metafase chromosomen te lokalizeren. *In situ* hybridisatie op kernen in suspensie werd gebruikt om de ruimtelijke samenhang van de gehybridiseerde gebieden te kunnen bestuderen, maar ook om na te gaan, of deze via interfase hybridisatie verkregen resultaten dezelfde betrouwbaarheid hebben, als die afkomstig van metafase *in situ* hybridisaties. De probe-gerelateerde fluorescentie werd ook via flow cytometrie bepaald met het oog op verschillen tussen individuen wat betreft de hoeveelheid gehybridiseerde sequenties. Nieuwe klinische toepassingen van hybridocytochemie werden onderzocht door middel van *in situ* hybridisatie op chromosomen en kernen, die gefixeerd waren op microscoop glaasjes. In Hoofdstuk 1 wordt een overzicht gegeven van de mogelijkheden van fluorescerende *in situ* hybridisatie.

In Hoofdstuk 2 is een nieuwe methode beschreven om enkelstrengs DNA (ssDNA) te genereren voor hybridocytochemie. Deze methode maakt gebruik van Exonuclease III in combinatie met restrictie enzymen om stukjes ssDNA te produceren voor *in situ* hybridisatie. Deze procedure bleek nagenoeg even effectief te zijn, wat betreft probe detectie, als de routinematig gebruikte hitte denaturatie. De Exo-methode kan van belang zijn bij hybridisaties van kwetsbare structuren, bijvoorbeeld kernen in suspensie, omdat de fysisch-chemische condities relatief mild zijn (Hoofdstuk 5).

De flow cytometrische bepaling van fluorescerende *in situ* hybriden in humane lymfocyten kernen in suspensie na *in situ* hybridisatie met chromosoomspecifieke repetitieve satelliet DNA probes, is beschreven in Hoofdstuk 3. Deze aanpak bleek gevoelig genoeg te zijn om verschillen in hoeveelheid heterochromatine bij chromosomen 1 en Y in proefpersonen aan te tonen. De gevoeligheid was in de orde van een tot enkele megabasen. Ook dubbel-hybridisatie met verschillend gemerkte en gekleurde #1 en Y probes wordt in dit Hoofdstuk beschreven. Dubbel-

hybridisaties, waarbij een probe dient als referentie voor de hybridisatie-efficientie, kunnen belangrijk zijn bij de bepaling van numerieke chromosoomafwijkingen in bijvoorbeeld verschillende tumoren of tumor cellijnen.

Hoofdstuk 4 handelt over de 3-dimensionale analyse via computer-gesteunde fluorescentie microscopie van de lokalisatie van bepaalde chromosoomgebieden in kernen met intacte morfologie. Voor dit doel werd fluorescerende *in situ* hybridisatie op hybride en humane cellijnkernen toegepast. Als probes zijn genomisch humaan DNA, en chromosoomspecifieke centromeer en telomeer sequenties gebruikt. De gehybridiseerde humane chromosomen in verschillende mens-hamster hybride cellijnen bleken cellijn-specifieke ruimtelijke patronen te vertonen. In humane fibroblasten kernen werden de centromeer en telomeer gebieden vaak in een karakteristieke distributie langs de kernmembraan aangetroffen.

Een vervolg op deze studie is beschreven in Hoofdstuk 5. In dit Hoofdstuk werd confocale microscopie toegepast om kwantitatieve ruimtelijke metingen te verrichten op gehybridiseerde centromeer 1 gebieden in sferische lymfocyten- en polymorfe HL60 cellijnkernen. Gevonden werd, dat de centromeer 1 gebieden geassocieerd waren met de nucleaire membraan. Ten opzichte van elkaar bleken deze gebieden een random relatie te hebben. Gebruikmakend van de enzymatische methode, beschreven in Hoofdstuk 2, werden simultaan chromosoom 1 specifiek satelliet DNA en ribosomaal RNA in respektievelijk kern en cytoplasma van HL60 cellen gevisualiseerd. Deze opzet zou als raamwerk kunnen dienen voor de 3-dimensionale studie van genen en genprodukten.

Klinische cytogenetica is het onderwerp van Hoofdstuk 6 en 7. In Hoofdstuk 6 wordt de detectie van numerieke en strukturele chromosoomafwijkingen door hybridocytochemie gedemonstreerd. Door middel van de combinatie van een chromosoom 1 specifieke centromeer probe plus een 1p specifieke telomeer DNA probe, werden numerieke afwijkingen plus translocaties en deleties van de korte arm van chromosoom 1 gezien in metafase preparaten van een leukemie cellijn. Interfase cytogenetica met een chromosoom Y specifieke probe werd aangewend ter detectie van lage aantallen achtergebleven of recidiverende gastheercellen in bloed en beenmerg van patienten met hematologische maligniteiten na een sex-mismatched beenmergtransplantatie (Hoofdstuk 7). Percentages in de orde van 0,2 en 0,7% konden worden gevonden na *in situ* hybridisatie op beenmergkernen van deze patienten. Deze residuele gastheercellen waren niet meer aantoonbaar met conventionele cytogenetica of cytologie.

Men kan concluderen, dat fluorescerende *in situ* hybridisatie of hybridocytochemie geschikt is om als een cytogenetisch instrument te dienen in celbiologische studies en diagnostische procedures. Door de introductie van de interfase cytogenetica is het mogelijk geworden om de cytogenetica van cellen en tumoren met een lage mitotische index te bestuderen.

De introductie van de confocale scanning microscoop heeft het onderzoek van het cel- en celkerninterieur aanzienlijk vereenvoudigd en verbeterd. Flow cytometrie zou daarnaast bijzonder waardevol kunnen blijken bij de snelle screening op de aanwezigheid van numerieke chromosoomafwijkingen in tumoren en tumorcel-subsets. In dit geval dient *in situ* hybridisatie op cellen in suspensie te worden toegepast.

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H. van Dekken, A. van Rotterdam, R. Jonker, H.T.M. van der Voort, G.J. Brakenhoff, and J.G.J. Bauman. Confocal microscopy as a tool for the study of the intranuclear topography of chromosomes. J. Microsc. (submitted).

H. van Dekken, A. van Rotterdam, R. Jonker, H.T.M. van der Voort, G.J. Brakenhoff, and J.G.J. Bauman. Spatial topography of centromere 1 DNA and ribosomal RNA sequences in hemopoietic cells studied by in situ hybridization and confocal microscopy. (submitted).

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 10 februari 1956 te Rotterdam geboren. In Alkmaar bezocht hij het Murmellius Gymnasium, alwaar in 1974 het diploma Gymnasium-B werd behaald. In datzelfde jaar werd een aanvang gemaakt met de studie Geneeskunde aan de Gemeentelijke Universiteit van Amsterdam. Van 1978 tot 1980 werd een Candidaats-assistentschap Biochemie vervuld op het B.C.P. Jansen Instituut van de Gemeentelijke Universiteit van Amsterdam. Het doctoraalexamen Geneeskunde haalde hij in 1981, het artsexamen in februari 1984. In de loop van dat jaar werd hij aangesteld als onderzoeksmedewerker, in dienst van IKR, op de afdeling Flow Cytometrie van het Radiobiologisch Instituut TNO te Rijswijk (Projectleider: Dr. G.J. van den Engh). Van oktober 1985 tot februari 1989 werkte hij op deze afdeling onder leiding van Dr. J.G.J. Bauman en Dr. J.W.M. Visser aan het in dit proefschrift beschreven onderzoek. Tijdens dit door NWO-Medigon gefinancierde project verbleef hij van november 1985 tot december 1986 in de Verenigde Staten op de Biomed Department van het Lawrence Livermore National Laboratory (Projectleiding: Dr. D. Pinkel en Dr. J.W. Gray). In 1989 werd het onderzoek afgerond met een proefschrift (Promotores: Prof.Dr. D. van Bekkum en Prof. Dr. M. van der Ploeg). Momenteel vervult hij een post-doc positie bij Prof. Dr. M. Melamed op de afdeling Pathologische Anatomie van het Memorial Sloan-Kettering Cancer Center in New York.

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