1994

Identification of the chromosomal origin of three supernumerary marker chromosomes ascertained at amniocentesis

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Order Number 1358156

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IDENTIFICATION OF THE CHROMOSOMAL ORIGIN OF THREE SUPERNUMERARY MARKER CHROMOSOMES ASCERTAINED AT AMNIOCENTESIS

A Thesis
Presented to
the Faculty of the Department of Biological Sciences
San Jose State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts

by
Terence Anderson Black
May, 1994

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ABSTRACT

IDENTIFICATION OF THE CHROMOSOMAL ORIGIN OF THREE SUPERNUMERARY MARKER CHROMOSOMES ASCERTAINED AT AMNIOCENTESIS

by Terence Anderson Black

The technique of fluorescent in situ hybridization (FISH) was used to identify the origin of supernumerary, structurally abnormal marker chromosomes in amniotic fluid samples obtained for prenatal diagnosis in 1991 at the San Jose Cytogenetics Laboratory of the Northern California Kaiser Permanente Medical Group. Various non-hybridization staining techniques indicated that the marker chromosomes observed in three patients were derived from acrocentric chromosomes. Alphoid repetitive DNA probes specific for the pericentromeric regions of either chromosomes 13 and 21 or chromosomes 14 and 22 were used to identify the origin of each marker chromosome. A fourth amniotic fluid sample with a marker chromosome was also analyzed. This marker was present in 8% of the amniotic fluid cells examined and did not appear to be derived from an acrocentric chromosome. Although FISH analysis with various centromeric-specific probes was employed, the origin of this marker chromosome remains unclear.

Acknowledgments

This thesis is dedicated to my parents, who provided me with two of the most important elements in any research: funding and encouragement. Any success I have had in my education is due to the fact that they taught me how to focus my curiosity into a desire to find things out for myself.

I would like to thank Dr. Susan Berlani at Santa Clara University, who first sparked my interest in molecular biology as an undergraduate.

I also wish to thank my thesis committee: Steve White, Robert Fowler, and Lauren Jenkins. In his lectures and in his review of my thesis work, Dr. White expanded my understanding of the scientific method and my appreciation for the rigorous evaluation of information obtained by experimentation. Dr. Fowler's kind and patient assistance in developing my research plan and in fulfilling the requirements of the graduate program has been invaluable. I am especially grateful to Lauren, as well as to Leann Bros and the others at Kaiser Permanente who helped me find my way around a cytogenetics laboratory.

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INTRODUCTION

Marker chromosomes are structurally abnormal chromosomes. While some well-characterized abnormal chromosomes are often called marker chromosomes in the literature, the term is generally reserved for unidentified structures (Hook and Cross, 1987). In human cytogenetics, marker chromosomes, or simply markers, generally refer to those abnormal chromosomes which are present in addition to the normal complement of chromosomes.

The appearance of a marker chromosome at prenatal diagnosis presents a considerable dilemma for genetic counselors and medical practitioners because of the heterogeneity of these chromosomes, and the various clinical outcomes that have been associated with marker cases. Fluorescent in situ hybridization, using chromosome-specific DNA sequence probes, was used to identify markers in amniotic fluid samples from four patients ascertained at prenatal diagnosis. The long term goal of such research is to contribute to the body of literature concerning marker chromosomes, in an attempt to discern patterns in the phenotypes associated with particular markers. If successful correlations between karyotype and phenotype can be made, this information may be used to predict the clinical consequence of these markers when found at prenatal diagnosis.

LITERATURE REVIEW

Normal and Structurally Abnormal Human Chromosomes

In the field of cytogenetics, chromosomes are generally studied during early metaphase of the mitotic cell cycle. During metaphase, eukaryotic chromosomes become most condensed, assuming conformations that are characteristic for the karyotype of a particular species (Therman, 1986). All human chromosomes have a short arm and a long arm, designated the p and q arms, respectively, which are separated by a primary constriction indicating the location of the centromere (Therman, 1986; Sandberg, 1990). The centromere is the point of attachment between the chromatids and between the chromosome and the spindle fibers (Sandberg, 1990). Variable regions are also present. These are locations on some chromosomes other than the centromeres where the chromatin is sparse and despiralized. These regions are most evident on chromosomes 1, 3, 9, 16, and Y, but are variable in size and appearance even between homologs. Small, spherical structures termed satellites are characteristic of the short arms of the acrocentric chromosomes. Satellites are attached to the short arms of these chromosomes by stalks, thin chromatin fibers which are apparently the loci of the ribosomal RNA genes (Sandberg, 1990).

A chromosome may be described in terms of the location of the centromere along its length. Thus a metacentric chromosome contains a

centromere located at its middle, and in an acrocentric chromosome, the two arms are quite unequal in length, with the centromere placed near one end of the chromosome (Therman, 1986). Chromosomes which fall between these two extremes are termed submetacentric. Before the advent of techniques which were capable of staining chromosomes selectively for the purposes of identification, individual chromosomes were described by the total length of the chromosome and by the relative length of each arm (Sandberg, 1990). The London Conference (1963) classified the human complement of chromosomes into seven groups based on these parameters (Therman, 1986; Sandberg, 1990):

Group A (chromosomes 1-3): large, approximately metacentric

Group B (chromosomes 4 and 5): large, submetacentric

Group C (chromosomes 6-12 and X): medium-sized, submetacentric

Group D (chromosomes 13-15): medium-sized, acrocentric

Group E (chromosomes 16-18): short, metacentric (chromosome 16) or submetacentric

Group F (chromosomes 19 and 20): short, approximately metacentric

Group G (chromosomes 21 and 22): very short, acrocentric

Chromosome Y is not included in any of these groups, since the length of its long arm varies among individuals (Sandberg, 1990). The variable size of the Y is an inherited polymorphism and is not associated with any phenotypic consequence.

Although human chromosomes can be divided into groups in this manner, it is generally not possible to identify individual chromosomes in groups B, C, D, F, and G on morphological grounds alone (Therman, 1986; Sandberg, 1990; Verma and Babu, 1989). In 1970, Caspersson et al. showed that the fluorescent dye quinacrine mustard could form patterns of dark and bright bands along the lengths of human metaphase chromosomes under ultraviolet light (Therman, 1986; Verma and Babu, 1989; Figure 1). Quinacrine dihydrochloride may also be used to produce these Q-bands, which form specific patterns for individual chromosomes (Sandberg, 1990). The most brightly fluorescent Q-bands are located on the distal Y chromosome, and bands of variable length are located at the centromeres of chromosomes 3 and 4 and the centromeres and satellites of the acrocentric chromosomes (Sandberg, 1990; Therman, 1986). Although the mechanism producing Q-bands is still unclear, it is known that adenine-thymine base pairs enhance while guanine-cytosine base pairs quench fluorescence, and that AT pairs must stretch uninterrupted for a certain length in order to produce fluorescence (Therman, 1986). Histone and non-histone chromosomal proteins have also been implicated in the banding mechanism (Therman, 1986). The Q-bright bands represent chromosomal regions which are replicated late in the S phase of the cell cycle and may contain fewer genes than Q-dark bands (Therman, 1986).

The development of Q-banding was followed by a variety of banding methods employing either fluorescent dyes or non-fluorescent stains. Perhaps pre-eminent among these techniques is G-banding, which is routinely used to examine chromosomes in most clinical laboratories

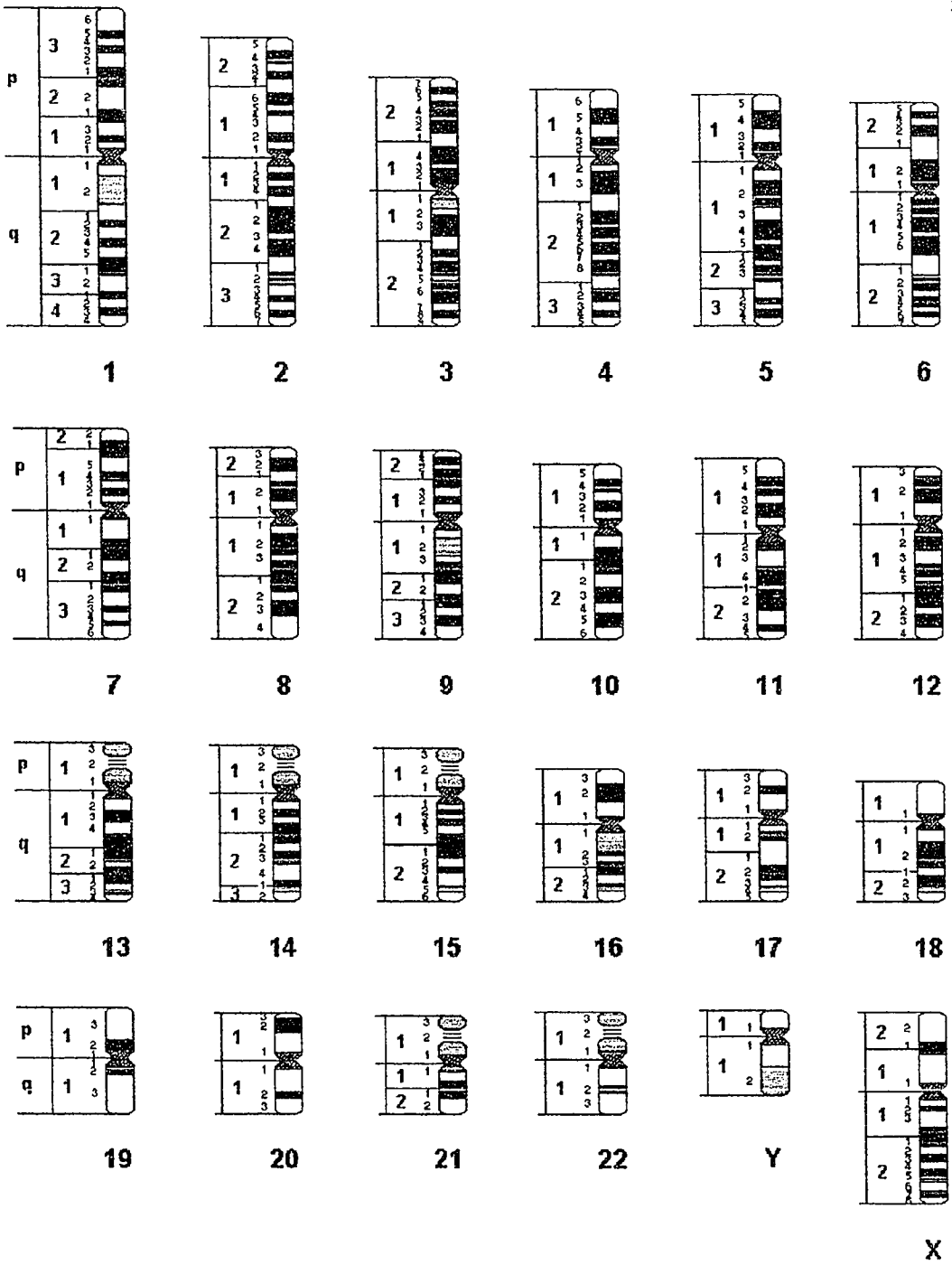

Figure 1. Ideogram of the human karyotype showing the pattern produced by G-, Q-, and R-banding (from ISCN 1985). G-dark, Q-bright, and R-dull bands are represented by black bands, and G-light, Q-dull, and R-bright bands are represented by white bands. Gray areas are polymorphic regions containing variable amounts of heterochromatin.

(Verma and Babu, 1989). In this method, chromosomes are first trypsinized and then stained with Giemsa dye, producing a pattern of light and dark bands that is roughly similar to the Q-band pattern (Verma and Babu, 1989), with intensely stained dark bands corresponding to Q-bright bands and poorly stained light bands corresponding to Q-dark bands (Figures 1 and 2). Some researchers have suggested that the G-banding pattern is produced by the distribution of proteins and DNA along the length of the chromosome, while others believe that G-dark bands are produced by regions rich in protein disulfides, while G-light bands are rich in sulfhydryls (Verma and Babu, 1989).

A banding pattern that is the reverse of the G- or Q-band patterns can be produced by heat treatment of chromosomes followed by staining with acridine orange or Giemsa or treatment with fluorescent dyes such as chromomycin A_3 or olivomycin (Verma and Babu, 1989). Thus, these reverse bands, or R-bands, stain intensely in chromosome regions that fluoresce or stain weakly with the Q- or G-banding procedures (Figure 1). The high temperatures involved in the R-band procedure denature chromosomal proteins and AT-rich DNA, leaving the intensely stained GC-rich sequences intact (Verma and Babu, 1989). R-banding offers the advantage of clearly delineating and enhancing the ends (telomeric regions) of several chromosomes which, in general, are poorly resolved by Q- or G-banding methods (Verma and Babu, 1989).

These differential staining techniques have been invaluable in identifying individual human chromosomes (Verma and Babu, 1989), and may be used to designate chromosomal locations. A nomenclature

Figure 2. Normal male G-banded karyotype from an amniotic fluid sample.

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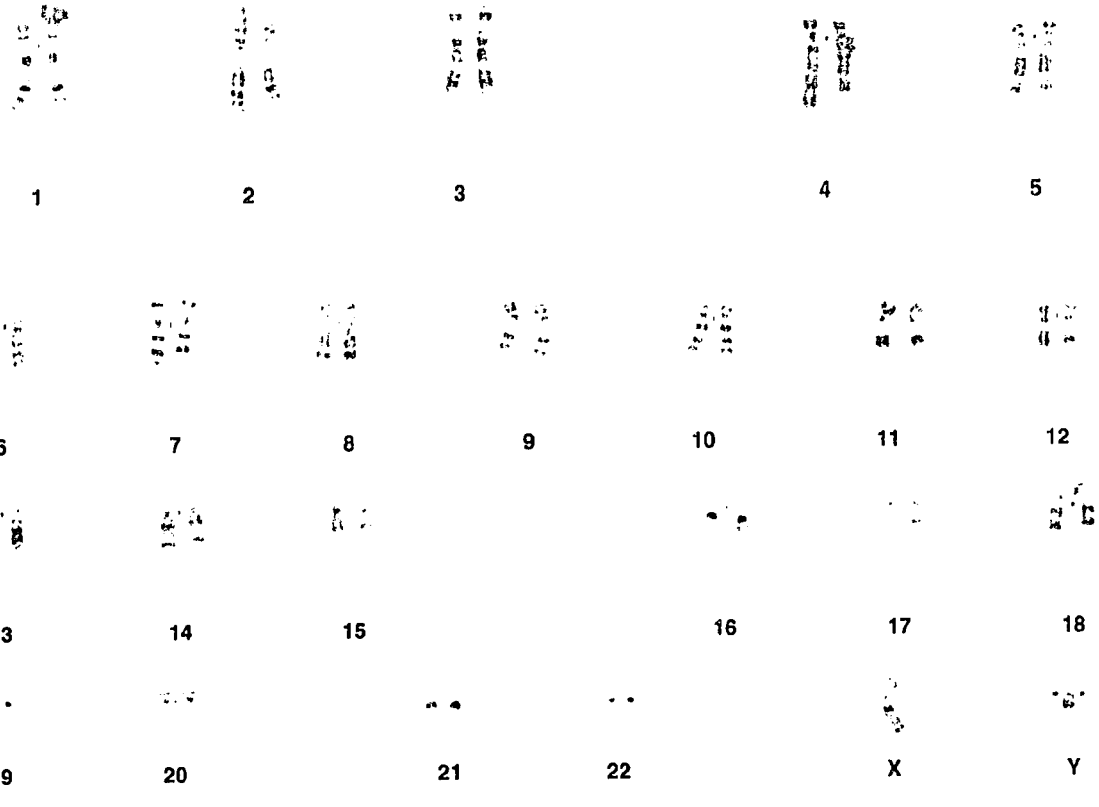
KARYOTYPE

KARYOTYPE	CUT	DATE	LABEL
46, XY	TAB	4/10/94	
	CHECK	LAB NO / VESSEL: Amniotic fluid	
	CHECK	PRINT IDENTIFICATION	



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established at the Paris Conference (1972) is based on bands chosen as landmarks from the G-, Q-, and R-band patterns of all human chromosomes (Sandberg, 1990). These bands define regions, further subdivided into individual bands, which are numbered distally from the centromere on each arm of a chromosome (Sandberg, 1990; see Figure 1). Individual chromosome bands are designated by chromosome number, chromosome arm (p or q), region, and band (Sandberg, 1990). Thus, 1p33 designates the third band in the third region on the short arm of chromosome 1.

While the differential staining techniques stain the entire length of a chromosome, other methods may be used to selectively stain certain chromosome structures. C-banding stains constitutive heterochromatin, which is primarily located at the centromeric regions of all human chromosomes and contains repeated DNAs, such as the satellite, alphoid, and beta families of sequences (Verma and Babu, 1989; Therman, 1986; Figure 3). The C-bands on chromosomes 1, 9, and 16 are variable in size (Verma and Babu, 1989). Treatment in an acid such as HCl followed by an alkali such as $Ba(OH)_2$ denatures and breaks down chromosomal DNA, leaving the heterochromatin in C-bands intact to be stained by Giemsa.

The nucleolus organizer regions may be stained by silver impregnation (Ag-NOR staining). Silver staining is localized to the stalk regions of the acrocentric chromosomes (band p12: see Figure 1), and appears as a black dot-like deposition that may extend into the space around a stained acrocentric short arm (Verma and Babu, 1989). The target chromosome structures for silver impregnation are NOR-specific proteins in active NORs, that is, those NORs containing ribosomal RNA

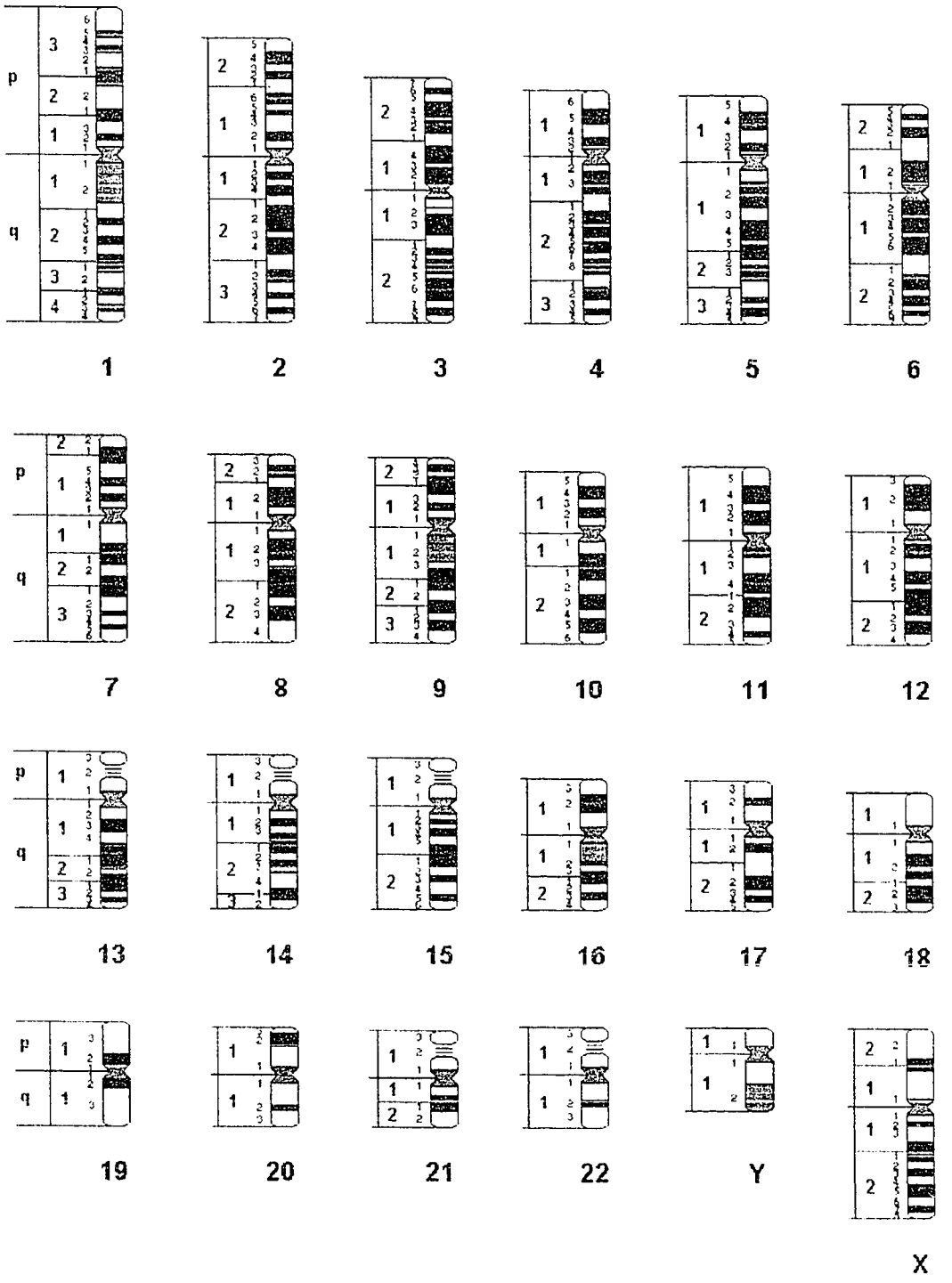


Figure 3. Schematic diagram of the pattern produced by C-banding. Chromosomal regions stained by C-banding are highlighted in red.

genes transcribed during the previous interphase (Verma and Babu, 1989). Thus not all NOR regions are likely to be evident by silver staining of a particular metaphase.

When human chromosomes are stained with 4'-6-diamidino-2-phenylindole (DAPI), the pericentromeric secondary constrictions of chromosomes 1 and 16 show bright fluorescence, while the remaining chromosomes exhibit a pattern similar to that of Q-banding (Verma and Babu, 1989). Treatment of chromosomes with the antibiotic distamycin A (DA), a non-fluorescent counterstain, before DAPI produces bright fluorescence at the variable regions of chromosomes 1, 9, and 16, the proximal short arm of chromosome 15, and the distal long arm of the Y (Verma and Babu, 1989; Figure 4). Some other chromosomes show fluorescence of varying intensities with DA/DAPI, but most other chromosomes show only faint fluorescence (Verma and Babu, 1989). The mechanism behind the DA/DAPI procedure involves binding of DA and DAPI directly to DNA at similar but not identical sites, and competitive binding between the two ligands may produce the differential fluorescence pattern (Verma and Babu, 1989).

One of the great benefits offered by the differential and selective staining techniques is the ability to characterize structural abnormalities of chromosomes. The breaks involved in forming structurally aberrant chromosomes occur during and after DNA replication. In deleted chromosomes, a single break in one chromosome results in the loss of chromosomal material (Therman, 1986). Translocations result from the breakage of different chromosomes and the subsequent transfer of

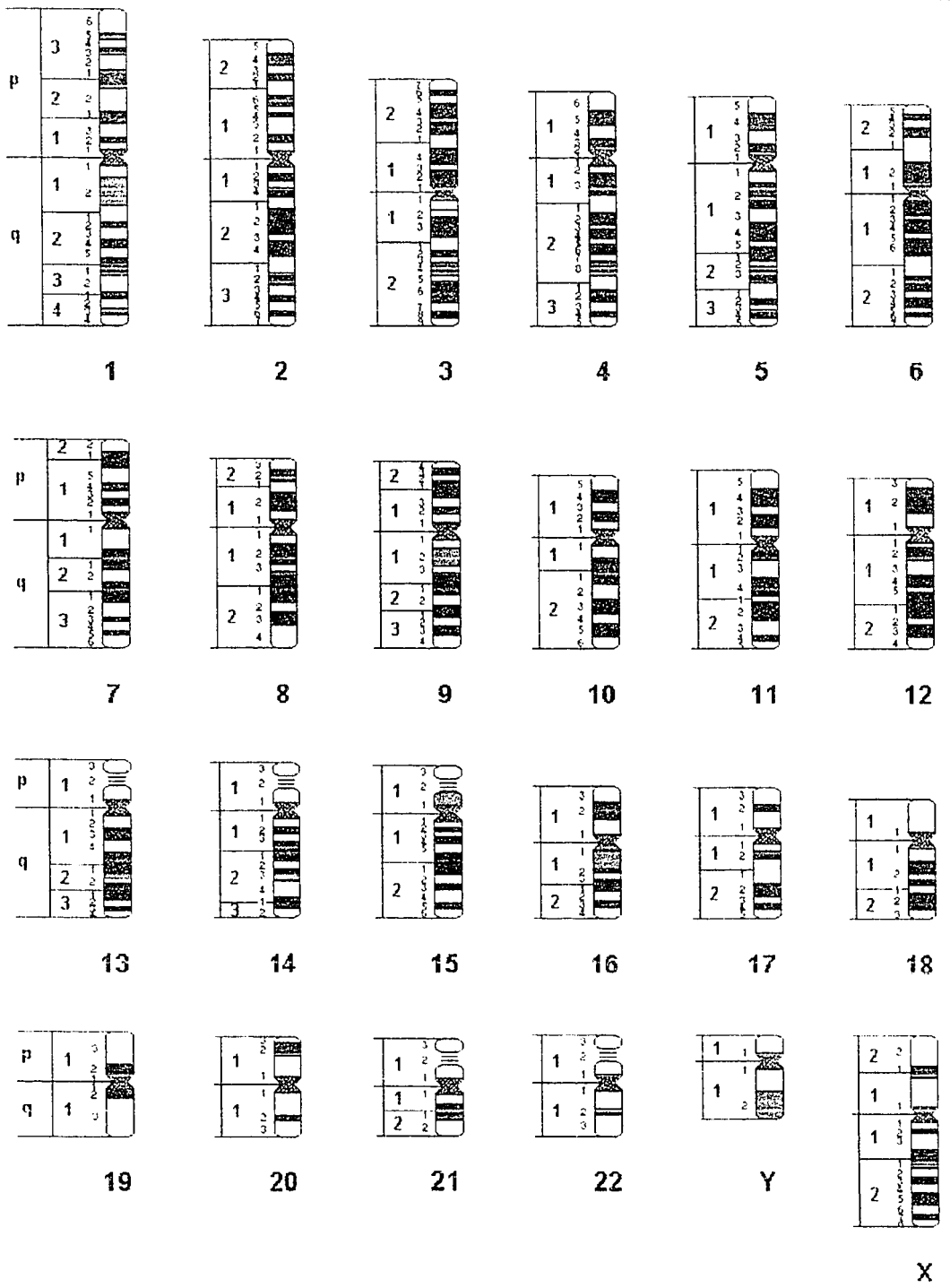
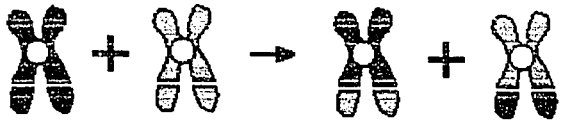


Figure 4. Schematic diagram of the pattern produced by DA/DAPI staining. Chromosomal regions stained brightly fluorescent by this method are highlighted in red.

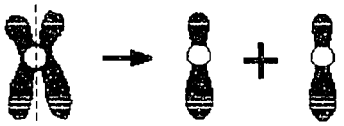
chromosome material between them (Figure 5A; Sandberg, 1990). Isochromosomes are metacentric chromosomes with two identical arms which may result from the misdivision of the centromere of a single chromosome in a transverse plane (Figure 5C; Sandberg, 1990; Therman, 1986). Misdivision of the centromere may involve the breakage and improper reunion of chromatids within the centromeric region (Daniel, 1988). Thus, in a submetacentric chromosome, the short arm chromatids recombine together to form a short metacentric chromosome, and the long arm chromatids recombine to form a long metacentric chromosome (cf. Figures 5B and C). Isodicentric chromosomes are symmetric chromosomes containing two centromeric regions which may be formed by breaks at identical points in homologous chromosomes (Figure 5D; Sandberg, 1990; Daniel, 1988). Alternatively, these chromosomes may result from a break in one arm and reunion between the chromatids of a single chromosome (Figure 5E; Sandberg, 1990; Daniel, 1988). Dicentric chromosomes may also be formed by two non-homologous chromosomes (Daniel, 1988). A special class of translocations, the Robertsonian translocations, have been thought to be formed by the fusion of long arms from two acrocentric chromosomes at the centromeric region (Figure 5F; Daniel, 1988; Therman, 1986). However, recent molecular cytogenetic research suggests that sequences from the centromeres of both acrocentric chromosomes forming the Robertsonian may be present in the derivative chromosome (Callen et al., 1992). Thus, this type of translocation chromosome may actually be dicentric, with breakpoints in the proximal short arms of the two parent acrocentrics rather than within the centromeres (Figure 5G). If these

Figure 5. Mechanisms for the formation of structurally abnormal chromosomes. (A) Translocation chromosomes. (B) Normal separation of chromatids during anaphase involves the longitudinal division of the centromere. (C) Abnormal transverse misdivision of the centromere involved in the formation of isochromosomes. (D) Formation of isodicentric chromosomes by breakage and reunion of homologous non-sister chromatids. (E) Formation of isodicentric chromosomes by breakage and reunion of sister chromatids. (F) Formation of Robertsonian translocation chromosomes by centric fusion between two acrocentric chromosomes. (G) Formation of Robertsonian translocation chromosomes by breakage and reunion between the short arms of acrocentric chromosomes.

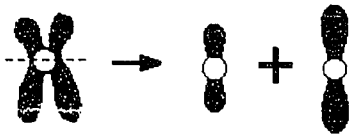
A



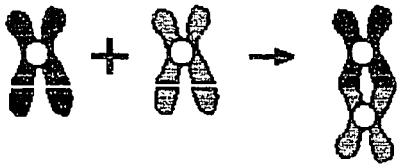
B



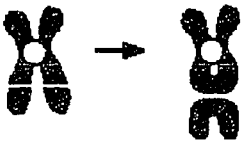
C



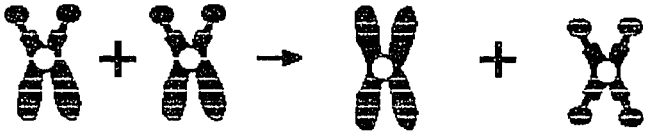
D



E



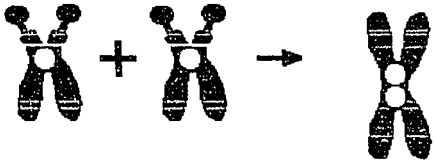
F



Robertsonian translocation chromosome

bisatellited reciprocal product

G



breakpoints are close enough to each centromere, the Robertsonian chromosome may appear to have a single centromere.

All the structurally abnormal chromosomes described here are formed by improper reunion between the broken ends of chromosomes or chromatids. In the case of isochromosomes and isodicentric chromosomes, a "U-type exchange" results from fusion between both proximal ends of breaks in chromatids rather than between a proximal and a distal end as occurs in translocations or in restitution of the normal chromosome structure after a break (Daniel, 1988). Another aspect of the formation of dicentric chromosomes involves the behavior of the two centromeric regions. If the centromeres of the derivative chromatid are adjacent to one another, they may function as a single centromere during anaphase (Therman, 1986; Daniel, 1988). If they are separated by any significant distance, one of the centromeres may have to be inactivated in order for the dicentric chromatid to survive anaphase intact, since the centromeres are more likely to be drawn to opposite spindle poles (Daniel, 1988). Inactivation is apparent as the absence of one primary constriction, although a C-band will still mark the position of the inactivated centromere (Therman, 1986; Daniel, 1988). Alternatively, the segregation of a dicentric chromosome can be determined by the fact that the centromeres often move toward opposite poles at anaphase with different relative strengths (Daniel, 1988). Thus the dicentric chromosome may be drawn to only one pole without breakage of the chromosome (Daniel, 1988).

When determining the effects of an abnormal chromosome, the possibility of mosaicism must be considered in addition to the genomic

content of the chromosome. Mosaicism describes the presence of two or more genetically distinct cell lines within a tissue sample or between tissues of an individual. Mosaicism in which an abnormal chromosome is found in a percentage of cells examined results from the clonal development of a cell line containing extra or missing chromosomes or parts of chromosomes (Hall, 1988). Any harmful effects of a chromosomal abnormality on the organism may be suppressed or extenuated by mosaicism (Benn and Hsu, 1984). The extent of mosaicism may vary in different tissues, possibly reflecting the relative tolerance for the abnormal cell line in these tissues (Hall, 1988). For example, isochromosome 12p mosaicism may be found in skin fibroblasts, but the abnormal chromosome is generally not found in lymphocytes (Hall, 1988; Ohashi, 1993). In general terms, then, mosaicism may have from no effect to a quite substantial effect on the phenotypic expression of an abnormal chromosome in different individuals (Hall, 1988).

Marker Chromosomes

Markers appear in 0.2-1.5/1000 fetuses and in 0.1-0.5/1000 newborns (Djalali et al., 1990; Rauch et al., 1992; Winsor and Van Allen, 1989; Kaffe and Hsu, 1988; Warburton, 1984; Yip et al., 1982; Hook and Cross, 1987; Verschraegen-Spee et al., 1993; Brondum-Nielsen, 1991). The incidence of marker chromosomes is elevated in some populations; markers appear with frequencies of about 3.5/1000 in the mentally retarded, 1.7-2.0/1000 in the subfertile, and 2.9/1000 in patients with abnormal sexual development

(Plattner et al., 1991; Yip et al., 1982). The reported effects of marker chromosomes include mental retardation and sterility, as well as non-specific behavioral and neurological problems (Buckton et al., 1985; Warburton, 1984). Marker chromosomes may be associated with dysmorphic facies or with a malformation syndrome, and they are also found in normal individuals (Warburton, 1984; Schmid et al., 1986).

Several large surveys of amniocentesis results have attempted to establish the risk for abnormality of marker chromosomes found at prenatal diagnosis. Routine cytogenetic analysis of these markers has been limited to staining techniques, so the abnormal accessory chromosomes in these surveys had to be classified in broad categories. For example, marker chromosomes may be inherited from a parent who also carries the marker in somatic cells, or may appear de novo in the fetus alone. De novo markers are more likely to cause concern than markers also found in a healthy parent or sibling of the proband (Buckton et al., 1985). Familial and de novo markers are found in roughly equal proportion (0.20-0.77/1000 and 0.27-0.46/1000 amniocenteses, respectively: Benn and Hsu, 1984; Warburton, 1991; Kaffe and Hsu, 1988; Hook and Cross, 1987). Familial markers are usually associated with a normal phenotype. Since any accompanying abnormalities have frequently been assumed to be coincidental (cf. Benn and Hsu, 1984), the prenatal risk of these markers has not been assessed in surveys (cf. Warburton, 1984; Hook and Cross, 1987; Warburton, 1991). This is unfortunate, since much recent research has shown that, due to the phenomena of imprinting and isodisomy, paternal or maternal inheritance may greatly influence the behavior of

genetic material in the offspring (Hall, 1990). In contrast to familial markers, de novo marker chromosomes are associated with an elevated risk of pre- or postnatal abnormalities. In two large surveys of amniocentesis results, the overall risk of abnormality for all de novo markers was calculated to be either 13% or 18.2% (Warburton, 1991, and Warburton, 1984, respectively). These figures represent a statistically significant increase in the risk of serious congenital abnormality for fetuses with de novo markers. The common estimate of the rate of abnormalities in all births is 2-3% (Warburton, 1991). The rate of stillbirth or spontaneous abortion among fetuses with de novo marker chromosomes was not found to represent a significantly increased risk (Warburton, 1991).

Since marker chromosomes may be identified as satellited or non-satellited by NOR staining, and possibly by G- or Q-banding, many reports of prenatal diagnosis cases have attempted to ascertain the phenotypic risk represented by de novo markers falling into either of these categories. Satellited and non-satellited marker chromosomes appear in roughly equal proportions (Crolla et al., 1992), although Buckton et al. (1985) reported that 37 out of 44 probands had satellited markers. About half of all satellited markers have been reported to be derived from chromosome 15 based on DA/DAPI staining results (Buckton et al., 1985; Stetten et al., 1992; Schmid et al., 1986). The risk of abnormality associated with de novo satellited markers has been calculated to be as low as 8.3%, while the risk for non-satellited markers may be as high as 26.9% (Warburton, 1984; see also Warburton, 1991). Although these numbers certainly suggest a

lower risk of abnormality for satellited versus non-satellited markers, the difference was not found to be statistically significant (Warburton, 1984).

These surveys suggest that the appearance of de novo marker chromosomes at prenatal diagnosis may represent a significantly increased risk for congenital abnormalities (Warburton, 1991). However, several factors complicate the interpretation of data from these surveys. Mosaicism may be present in a significant proportion of marker cases. Buckton et al. (1985) reported that 27% of their probands with markers were mosaics. Warburton (1991) found mosaicism in 70% of cases with non-satellited markers and in 39% of cases with satellited markers. In a large survey conducted by Hook and Cross (1987), the ratio of mosaics to non-mosaics among cases with de novo markers was 1.4-1.9, while the ratio among cases with familial markers was 0.4-0.8; these figures represent a significant difference. No difference in the overall risk of congenital abnormalities has been found between mosaics and non-mosaics (Warburton, 1984; Verschraegen-Spee et al., 1993). This is surprising, since, as noted earlier, the phenotypic effects of the marker chromosome may be expected to be suppressed or modified by mosaicism in some probands. However, some marker chromosomes are very small and may be easily missed during cytogenetic analysis. False negatives in which the marker is not found at all or which underestimate the extent of mosaicism for the marker would bias estimates of the phenotypic risk of the marker (Benn and Hsu, 1984; Buckton et al., 1985). The presence of mosaicism in a fetus or a carrier parent also raises the question of the importance of dosage in marker chromosome effects (Benn and Hsu, 1984).

Adding to the difficulty of predicting clinical outcome is the lack of long-term follow-up of live-born probands with marker chromosomes found during prenatal diagnosis (Warburton, 1991; Crolla et al., 1992). There is very little information about delayed development, neurological disorders, and other problems which may not be apparent in early infancy. Other considerations must also be taken into account when determining the significance of a particular marker chromosome, such as whether the observed phenotype is due to the presence of the marker, or is coincidental (Buckton et al., 1985).

The heterogeneity of marker chromosomes emphasizes the need to study the content of the chromatin forming these markers. A landmark paper by Steinbach et al. (1983) sought to define phenotypic risk according to the amount of euchromatin contained in a particular marker chromosome. Bisatellited markers were classified into three categories:

AI: markers with a single C-band

AII: markers with a bipartite C-band, containing interstitial C-negative chromatin which does not have a discernible G- or R-banding pattern

AIII: markers containing euchromatin with a G-light or R-bright bands between two clearly separated C-bands (Steinbach et al., 1983).

From the results of their own prenatal screening results, Steinbach et al. found a low risk of phenotypic abnormality for probands with an AI or AII marker, while AIII markers were associated with a high risk of abnormality (Steinbach et al., 1983; Djalali, 1990).

Despite the positive correlation between the amount of euchromatin contained in a marker and risk of abnormality, the variety of marker chromosomes and associated phenotypes which have been reported suggests that it may be important to identify the normal chromosome from which a particular marker was derived in order to correlate marker genotype and phenotype. Until recently, the identification of marker chromosomes was limited to the use of non-hybridization staining techniques. Only a few marker chromosomes that can be identified by their staining characteristics are associated with a recognizable syndrome. An isochromosome formed from the short arms of chromosome 18, the i(18p), is involved in a syndrome characterized by mental retardation and postnatal dysmorphic features (Verschraegen-Spee et al., 1993). In the Pallister-Killian syndrome, a marker isochromosome derived from chromosome 12, the i(12p), is associated with symptoms such as a "coarse" face, sparse hair, linear pigmented stigmata on the skin, and mental retardation (Ohashi et al., 1993). Marker chromosomes formed from the short arm, centromere, and proximal long arm of chromosome 15 are found in patients with varying degrees of mental and developmental retardation, infertility, and severe autism or seizures, as well as in normal individuals (Plattner et al., 1991; Crolla et al., 1992; Stetten et al., 1981). Patients with this type of marker may also show symptoms of Prader-Willi syndrome, which include neonatal hypotonia, short stature, hypogonadism, hyperphagia, obesity, and acromicria (Wisniewski et al., 1980). Partial tetrasomy for the proximal long arm of chromosome 22 in the form of an isodicentric chromosome is associated with the cat-eye syndrome, characterized by imperforate anus,

abnormal ears, and coloboma of the iris (Rosenfeld et al., 1984). The syndrome presents a variable clinical picture, and some patients may also have abnormalities of the heart, large blood vessels, kidney, and urinary tract, and cleft palate and mental retardation may also be present (Liehr et al., 1992).

Although these four marker chromosomes have been extensively studied using non-hybridization techniques, chromosomal material is difficult to identify with staining procedures in most marker chromosome cases. In situ hybridization of chromosome-specific DNA probes to markers can be used to identify the chromosomal origin of these abnormal chromosomes. Variations of the technique using single copy sequence probes for individual loci or spanning the length of an entire chromosome or chromosome segment, may yield further structural information about individual marker chromosomes. Clearly markers must be identified by stricter criteria than gross morphological staining characteristics in order to discern useful patterns of phenotypic risk among these markers.

Fluorescence In Situ Hybridization

For the past two decades, the identification and characterization of metaphase chromosomes has been accomplished primarily by the use of staining techniques such as G- and Q-banding. While these procedures can produce banding patterns characteristic for each human chromosome, the differences between patterns are subtle and the interpretation of banding results requires much skill and experience. Interpreting abnormal

karyotypes is especially difficult, and minor structural changes in chromosomes or the complex aneuploidy of tumor cells may be impossible to identify. Staining is not specific for DNA sequence, but instead recognizes chemical or structural differences among various types of chromatin.

During the past 20 years, cytogenetic procedures have been augmented by in situ hybridization techniques. These techniques allow the identification of chromosomes based on the DNA sequences they contain. Unlike other hybridization techniques such as Southern blotting, in situ hybridization allows the direct localization of DNA sequences within the genome by directly placing these sequences, known as probes, in the chromatin of metaphase chromosomes spread on a microscope slide. Labeled probe sequences and target chromosomes are denatured, and complementary sequences in both probe and chromosome are allowed to reanneal on the slide. Originally radioisotopes were used to label probes, but this strategy has several drawbacks. Radioactive in situ hybridization has limited sensitivity and resolution (Oncor, 1990). The proper location of probe sequences must be determined by a statistical examination of signal distribution to account for non-specific binding of probes (Van Hemel et al., 1992). Probes become damaged due to radiolysis by their own labels, and autoradiography causes a long delay in completing experiments (Cherif et al., 1989).

The development of non-radioactive in situ methods in the late 1970's and early 1980's offered an attractive alternative to radioactive hybridization. Instead of radioisotope labeling, probe sequences may first

be labeled with a reporter molecule. After hybridization, the position of the probe sequence is located by incubation with a labeled detector molecule, which binds to the reporter molecule. Davidson et al. cross-linked biotin to RNA with cytochrome C or polyamine, and used these complexes as in situ hybridization probes (Langer et al., 1981). Several immunological, histochemical, or affinity detector systems have been developed using nucleotide analogs modified with reporter molecules incorporated into probe sequences. Hybridization was visualized under the microscope as binding of avidin modified with ferritin or methacrylate spheres to biotin molecules (Langer et al., 1981). Langer et al. synthesized biotin-labeled UTP or dUTP derivatives as a reporter system. They developed a detection system using rabbit antibiotin antibodies and fluorescently-labeled goat anti-rabbit IgG.

Fluorescent in situ hybridization (FISH) is perhaps the most commonly used non-isotopic hybridization method, but the general strategy used in this technique is similar to that used in the other in situ procedures, including the radioisotope method (Figure 6). Cells are fixed on slides according to methods used for banding analysis. Slides are incubated in a solution containing a high concentration of formamide at high temperature (typically 70% formamide at 70° C: Trask, 1991) to denature DNA. Denaturation must not be allowed to proceed for too long, or the denaturing agent formamide will begin to degrade chromosome structure (Tucker et al., 1988). Immediate immersion of the slides in cold ethanol after denaturation prevents strand reannealing before the addition of the probe (Trask, 1991).

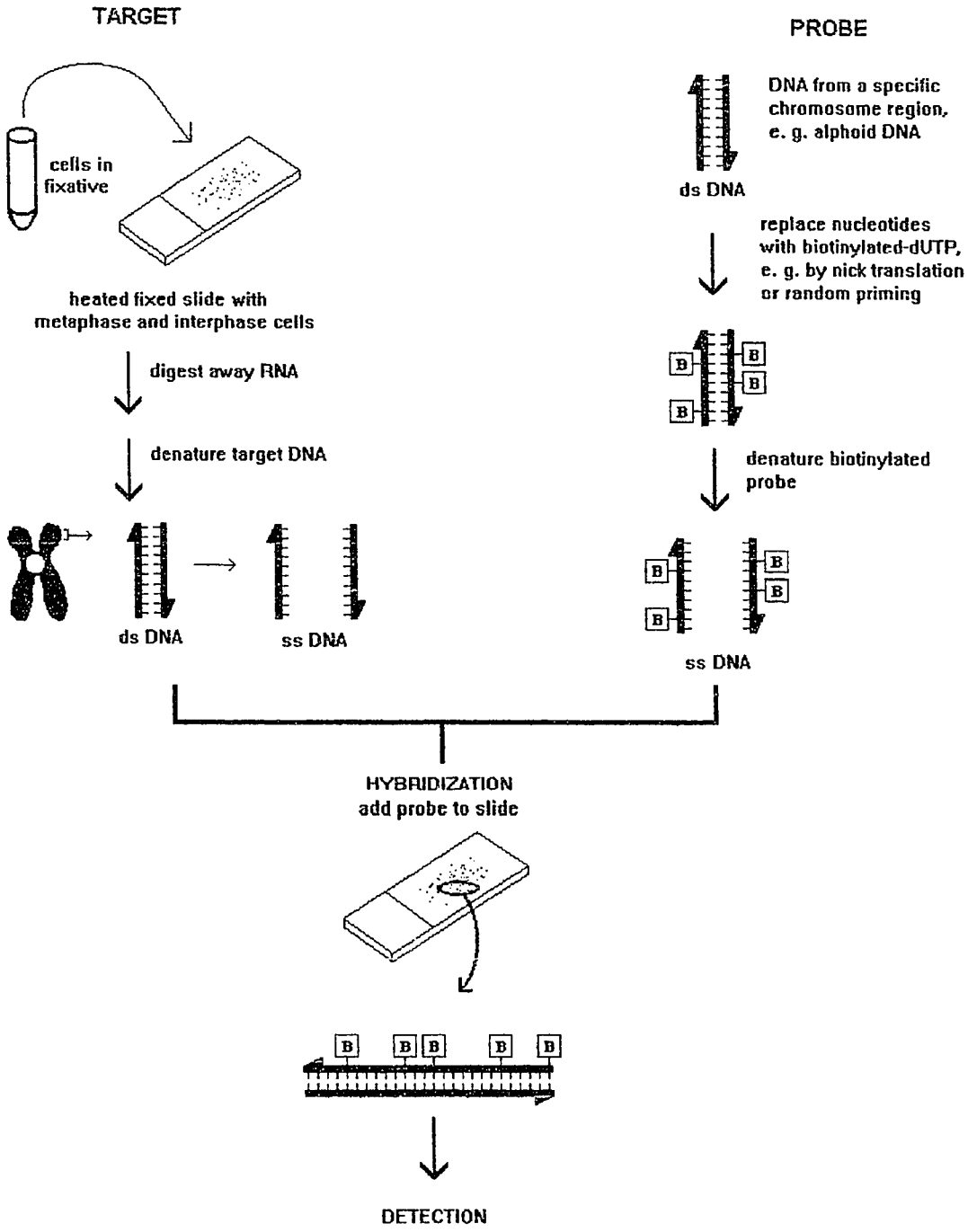


Figure 6. Diagram of the fluorescent in situ hybridization technique.

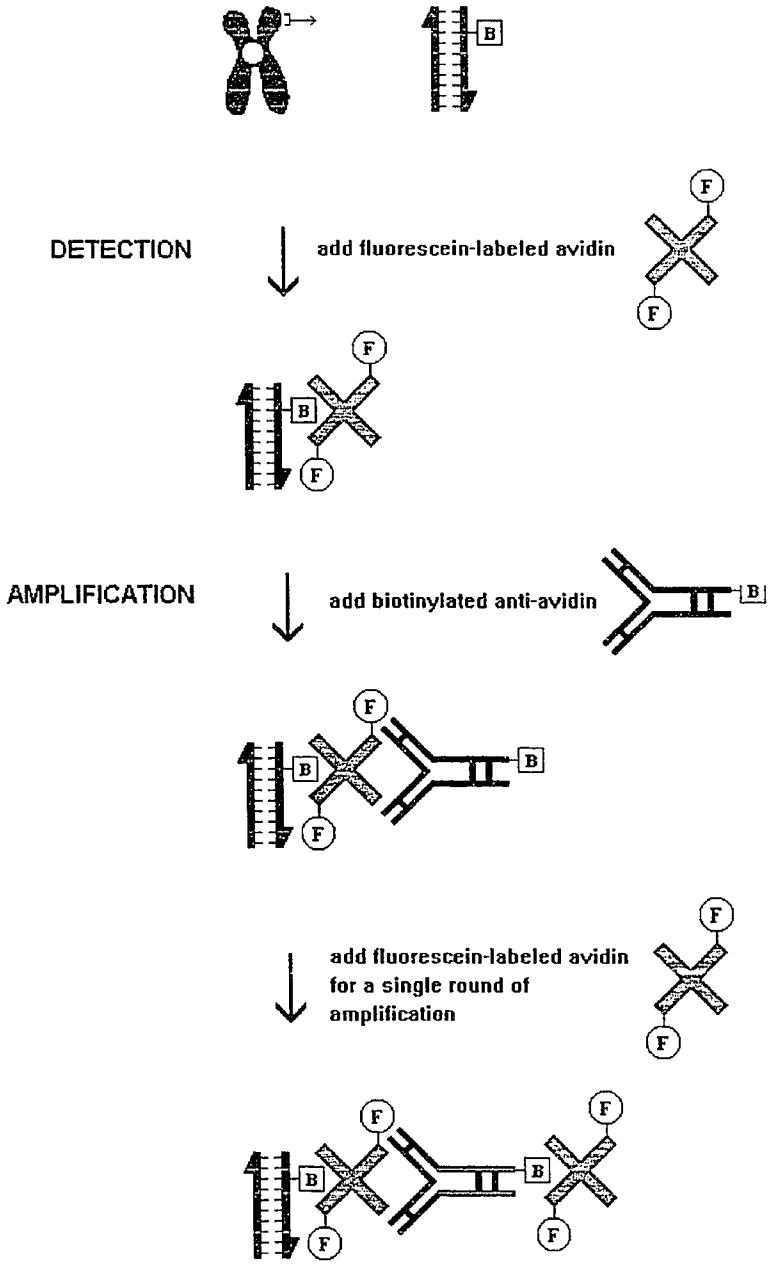


Figure 6. (continued)

For the identification of individual chromosomes, hybridization probes containing tandemly arranged repetitive DNA sequences, particularly from the alphoid DNA family, are generally used (Kiechle-Schwarz et al., 1991). Such sequences make ideal probes, since they are clustered at specific chromosome structures (primarily at or near the centromere). Sequences may be used which are specific for individual chromosomes. In this way tandemly repeated sequences nucleate intense, distinct, and highly focused fluorescent signals. After the appropriate sequences are isolated from cloning inserts or by other means, they are homogenized into 200-400 bp fragments. For repetitive sequence probes, this fragment size seems to maximize hybridization specificity and efficacy, and reduces non-specific background fluorescence (Trask, 1991). While the direct conjugation of fluorescent molecules to probe DNA is possible, generally a reporter molecule is attached first. After hybridization of the probe to target DNA, these reporter molecules will bind fluorescently labeled detector molecules. Nick translation or random priming may be used to enzymatically replace nucleotides within probe sequences with modified nucleotides conjugated to reporter molecules. In the experiments reported here, biotin was used as the reporter molecule, but several other ligands may be used, including digoxigenin, dinitrophenyl (DNP), aminoacetylfluorene (AAF), mercury, and sulfonate. Biotin, digoxigenin, and DNP may be incorporated into probe sequences as labeled nucleotides, while AAF, mercury, and sulfonate must be chemically attached to DNA.

Hybridization of probes to chromosomes is carried out in a buffer containing the probe, formamide, and salt. Dextran sulfate may also be

added to increase the rate of hybridization by promoting the formation of DNA networks between probe sequences and chromatin DNA (Mattei et al., 1985). The mixture containing the double-stranded DNA probe is denatured and then added to the slides containing the denatured target chromosomes. It is common practice to allow hybridization to take place overnight, although hybridization times as low as 15 minutes at 37° C may be sufficient for experiments using repetitive sequence probes (Trask, 1991).

After hybridization, the slides are washed with formamide and salt to remove mismatched or unhybridized probe sequences. A quality of washing and hybridization, called the stringency of these procedures, may be raised by increasing the concentration of formamide or decreasing the concentration of salt used, or by raising the temperature of the hybridization and washing solution (Cremer et al., 1986). At high stringency, denaturation conditions are severe enough to suppress reannealing except between probe and chromosome sequences sharing the most homology, This ensures that hybridization events occur at a high degree of specificity.

Washing is followed by incubation of the slides with reagents containing fluorescently labeled detector molecules, which bind to reporter molecules and produce fluorescent signals at the sites of probe hybridization. In the experiments presented here, avidin labeled with the ligand fluorescein isothiocyanate (FITC) was used to detect the presence of biotin. The affinity reaction between avidin and biotin makes an extremely effective probe system. Avidin, a tetrameric, 68 kilodalton

glycoprotein from egg white, and biotin have one of the highest binding constants known (dissociation constant $K_d = 10^{-15}$ M: Langer et al., 1981; Weber et al., 1989). This essentially irreversible binding event allows avidin, which can be coupled to a variety of indicators such as fluorescent dyes, electron-dense proteins, enzymes, or antibodies, to detect small amounts of biotin (Langer et al., 1981).

In other detection systems, mercury-labeled probes are detected with an indicator carrying a sulfhydryl group and the hapten DNP. Digoxigenin, DNP, AAF, and sulfonate are detected by antibodies and then fluorescently labeled anti-immunoglobulins (Trask, 1991). In addition to FITC, the most common fluorescent labels include rhodamine and Texas Red dyes. After labeled detector molecules have been applied to slides, fluorescent signals may be immediately observed through a fluorescence microscope.

Fluorescence microscopes are primarily of two types, those that use transmitted light illumination and those that use incident light illumination (epi-illumination)(Figure 7). Most fluorescence microscopes currently in use are epi-illumination instruments, which are easier to use and provide brighter, clearer images than transmission microscopes. An epi-fluorescence microscope was used in the experiments reported here, and the discussion which follows will describe the components forming the optical train of these microscopes.

The excitation lamp emits the light which causes fluorophores to fluoresce. The lamp must emit light within the absorption peaks of the fluorophores, and while brighter excitation intensity produces brighter

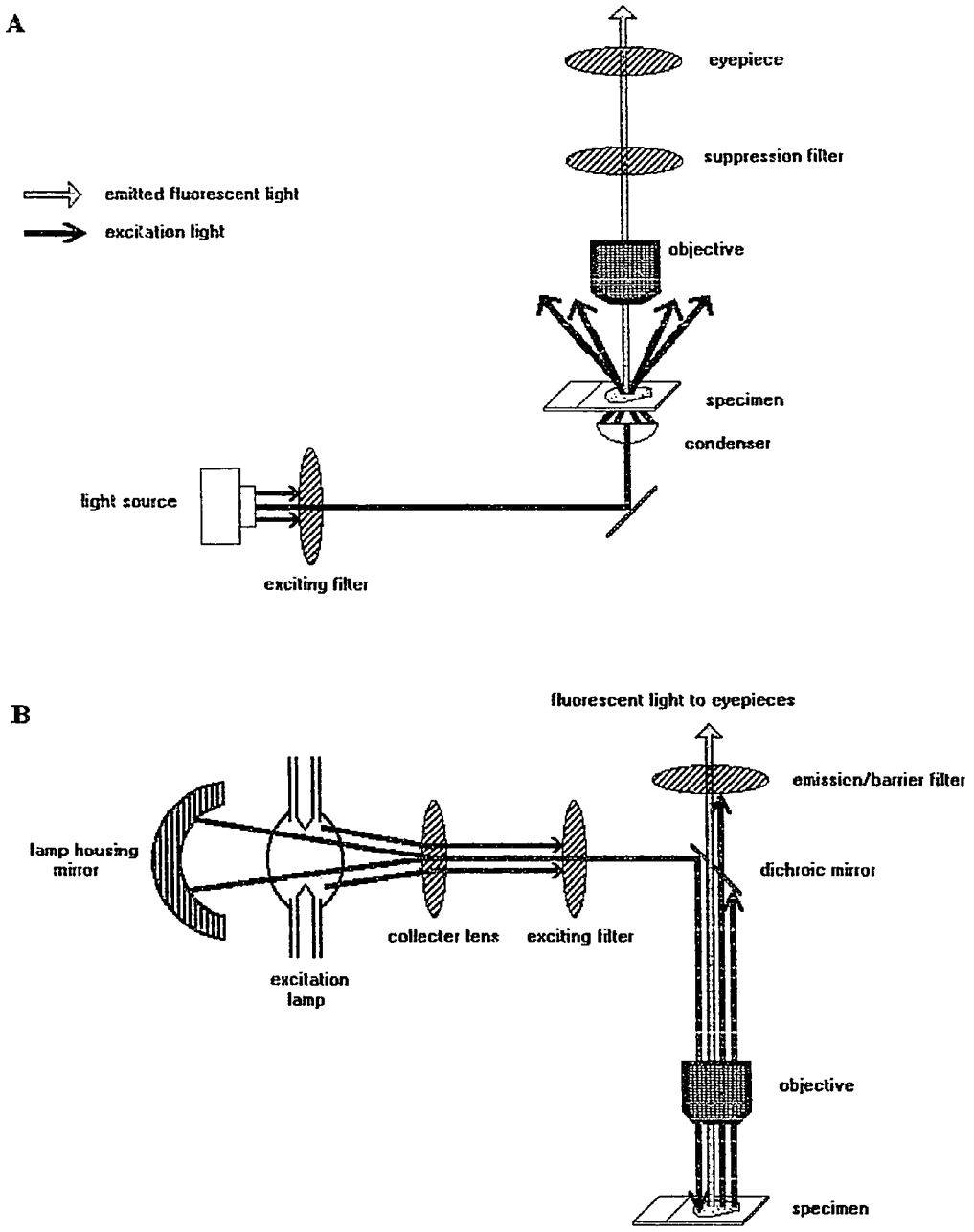


Figure 7. Schematic diagram of transmitted light (A) and incidence light (B) fluorescence microscopes. Adapted from Oncor 1992-93 Catalog and Imagenetics SpectrumCEP Direct Chromosome Enumeration System instructional booklet.

fluorescent signals from labeled DNA, it also hastens photo-oxidizing and fading of the fluorophores. High pressure mercury lamps are generally considered to be best for fluorescence microscopy, since they produce a number of emission peaks within the absorption range of many fluorescent molecules used as labels. High pressure xenon lamps may also be used, but they do not emit light as strongly at the fluorophore absorption wavelengths.

The objectives magnify the image of the specimen and act as the condenser for excitation light leaving the microscope. In order to make them better suited for fluorescence microscopy, objectives have been developed which are made with low self-fluorescent glass, large aperture, and few lens corrections. These modifications are critical for high light transmission and greatly influence the overall quality of the fluorescent image.

The transmission of excitation light and the resultant fluorescent light through the microscope is accomplished by filters. Filter sets are designed for specific fluorochromes, either individually or in combination (Figure 7). The excitation filter passes only the wavelengths from the lamp needed to excite the fluorescent molecule used. A dichroic mirror reflects excitation light down to the slide, and passes the fluorescent light emitted by the hybridized specimen while blocking excitation light reflected back up from the slide. Finally, an emission or barrier filter passes only the emission wavelengths of light for specific fluorophores through to the eyepieces.

Photography of in situ hybridizations is generally performed with a 35 mm camera mounted on the microscope, using high speed film. The type of film and camera settings used will depend largely on the quality of photographs desired by the researcher, and must be determined experimentally.

The FISH detection systems represent a marked improvement over isotopic methods. Long delays are introduced into radioactive in situ hybridization experiments by the autoradiographic detection of probe signals, which can require weeks or months to complete. The FISH procedure, including photography of slides, may be performed in two working days. Furthermore, fluorescent signals may be amplified by the addition of layers of reporter and detector molecules at the site of hybridization. For example, in the biotin-avidin system, anti-avidin antibodies labeled with biotin may be applied after FITC-avidin, followed once again by avidin, in each amplification step. The number of amplification steps may be selected for signal intensity and for the best signal-background ratio (cf. Cherif et al., 1989), since each step may be observed under the microscope. Pinkel et al. (1986) reported the effects of amplifying probe signals twice (to form three layers of fluorescein-labeled avidin), and estimated a six-fold increase in fluorescence intensity with each amplification. Since each avidin molecule usually carries six fluorescein molecules, a two-fold amplification should result in the presence of about 200 fluorescein molecules for each biotin molecule at the site of hybridization.

The previous discussion has focused on the use of repetitive sequence probes. Unique sequences may also be used as probes, and review of the literature will find a number of reports describing the use of FISH to localize individual genes to particular chromosomes (Berge-Lefranc et al., 1984; Bhatt et al., 1988; Landegant et al., 1986; Taviaux and Demaille, 1993). Identification of individual chromosomes may also be made without the use of repetitive sequence probes. With a procedure known as chromosomal in situ suppression hybridization (CISS), or chromosome painting, part or almost the entire length of specific chromosomes can be made fluorescent. Chromosome painting makes use of mixtures of probes which are made from DNA libraries derived from whole chromosomes or specific chromosomal regions. Painting probes may be made from flow-sorted chromosome-specific DNA libraries or libraries from somatic hybrid cell lines. One recent innovation of the technique produces painting probes after laser microdissection of specific chromosome regions (Deng et al., 1992). In order to produce mixtures of probes that are chromosome-specific, highly repetitive sequences that may be shared among chromosomes must be suppressed. After a probe solution has been denatured, these sequences are allowed to reanneal before hybridization of the probe solution to target chromosomes. The middle-repetitive, low copy number, and unique sequences which remain dissociated in the probe solution after this reannealing period have been found to provide chromosome-specific signals (Lichter et al., 1988).

PATIENTS AND METHODS

Patients

Samples of amniotic fluid from four patients were obtained for prenatal diagnosis in 1991 at the San Jose Cytogenetics Laboratory of the Northern California Kaiser Permanente Medical Group. Although the probands for study were the fetuses of these patients and the samples primarily used for the experiments reported here were composed of fetal cells from amniotic fluid, the patients' initials are used throughout to designate the patients or the fetuses of these patients.

Patient SD: The indications for amniocentesis were advanced maternal age as well as a history of four spontaneous abortions, including one stillbirth. The patient's age was 37 years, and the father's age was 39 years. Down syndrome was present in the patient's sister. The patient's family history was negative for any other birth defects. The patient had three normal male children. Fetal anatomy and size were normal by ultrasound. Amniocentesis was performed at 16.0 weeks gestational age, and chromosome studies of amniotic fluid samples found a female karyotype including a supernumerary marker chromosome (47,XX,+mar). Chromosome studies of the parents showed a normal female karyotype for the mother (46,XX), but the father's karyotype included a supernumerary marker chromosome (47,XY,+mar) identical to the marker found in amniotic

fluid samples. Because the fetal marker was inherited from the father, blood samples from the father were used for the FISH studies using the probes for chromosome 15 and chromosome 13/21, and the generic probe for all human centromeres. There was no report of abnormality in the newborn child.

Patient NH: The indication for amniocentesis was an advanced maternal age of 46 years. The father was 53 years old. The patient's pregnancy history included one therapeutic abortion and no spontaneous abortions. The patient had no history of infertility and nor children with birth defects. The patient's family history was negative for any known birth defects. Ultrasound was performed at 12 weeks gestational age, and fetal anatomy and size were normal. Amniocentesis was performed at 16.5 weeks gestational age, and chromosome studies revealed a mosaic female karyotype: 46,XX/47,XX,+marker. The marker chromosome was detected in 89% of amniocytes examined. Chromosome studies on both parents showed normal female and male karyotypes. The newborn child was small (4 lbs., 11 oz. at full-term birth), and exhibited a number of minor anomalies. The left side of the face, including the orbit, was larger than the right. The ears were low set and posteriorly rotated, and the nipples were widely spaced. Additional abnormalities included tight joints, mildly contracted elbows and knees, and hyperextensible fingers. The child also had bilateral

clinodactyly of the fifth fingers, and the thumbs were held in adduction.

Patient CL: The indication for amniocentesis was low maternal serum alpha-fetoprotein (MSAFP) but the AFP concentration in amniotic fluid was within normal limits. Low MSAFP is an indication for Down syndrome, while high MSAFP is an indication for neural tube defects. The patient's age was 33 years, and the father's age was 37 years. Amniocentesis was performed at 18.9 weeks gestational age, and chromosome studies of the amniotic fluid samples found a female karyotype including a supernumerary marker chromosome (47,XX,+mar). Chromosome studies of the parents showed a normal female karyotype for the patient (46,XX), but the father's karyotype included a supernumerary marker chromosome identical to the marker found in the fetus (47,XY,+mar). A normal child was born at term, and at six months of age showed normal development, motor skills, and physical features.

Patient MP: The indication for amniocentesis was low MSAFP, but the AFP concentration in amniotic fluid was within normal limits. The patient's age was 31 years, and the father's age was 32 years. Amniocentesis was performed at 18.8 weeks gestational age, and chromosome studies of amniotic fluid samples found a mosaic female karyotype: 46,XX/47,XX,+mar. Chromosome studies showed a normal male karyotype for the father (46,XY), but the mother's

karyotype included a dicentric translocation chromosome with breakpoints in the proximal regions of the short arms of chromosomes 14 and 22. There was a concomitant loss of one chromosome 14 and one chromosome 22, resulting in an almost balanced karyotype: 45,XX,-14,-22,dic(14;22)(p12;?p11). The mother's karyotype did not include the marker chromosome found in amniotic fluid cells. There was no report of abnormality in the newborn child.

Original cytogenetic analysis

When the patient's samples were initially analyzed during prenatal diagnosis in 1991, G-banding, C-banding, Q-banding, R-banding, NOR staining, and DAPI staining were performed by laboratory personnel according to established Kaiser Permanente Cytogenetics Laboratory protocols.

Slide preparation

In our laboratory, patient amniocyte samples were cultured for 8-12 days according to laboratory protocols, collected for harvest or slide preparation, and the excess cell suspensions were stored in a 3:1 methanol:acetic acid fixative at -15° F. Test tubes containing the suspended cells were centrifuged for 5 minutes at 1000 rpm and the supernatant was removed. The cells were resuspended dropwise in fixative until a slightly cloudy mixture was achieved. The concentration of cells in the fixative solution was therefore subjective. After one or two drops of

the cell suspension were dropped from a pasteur pipette on the middle of a slide, several drops of fixative were allowed to flow down the length of the slide. If done properly, the fixative should remove membranes and other cellular material, leaving chromosomes discretely clustered into metaphase chromosome spread or interphase nuclei. Slides were evaluated by phase contrast microscopy under low power. If metaphase spreads were sparse, the cell suspension was centrifuged again and the supernatant was removed. Less fixative was added to produce a more concentrated cell suspension, and new slides were made. If metaphase spreads and interphase nuclei were not discretely separated from one another, more fixative was added in a dropwise fashion to the cell suspension and new slides were made.

Giemsa staining

G-banding was performed before FISH in many experiments in order to verify the location of the marker chromosome in the metaphases studied. Slides were immersed in a trypsin-EDTA solution and rinsed in fetal bovine serum and distilled water. The slides were then covered with Wright's stain and allowed to stand for 1 minute 15 seconds. After rinsing off excess stain, coverslips were affixed and the slides were photographed.

Sequential method

After G-banding, slides were soaked in xylene for less than five minutes to remove the coverslip and mounting medium. The slides were rinsed in tap water, dried, and placed in 3:1 methanol:acetic acid for 1

minute. After successively dehydrating the slides in 70%, 80%, 90%, and 100% ethanol for 1 minute each time, the appropriate hybridization protocol was used.

Fluorescent in situ hybridization

Complete protocols for these experiments may be found in Appendices I and II. Details of the repetitive sequence and painting probes used may be found in Table I. Repetitive sequence probes are composed of chromosome-specific subsets of the alphoid, beta, or satellite families of sequences. These repetitive sequences are arranged in long arrays of tandemly repeated monomers which are localized to certain regions of human chromosomes. For example, alphoid sequences are based on a 169-171 bp monomer and are found exclusively at all human centromeres (Willard and Waye, 1987). Sequence divergence and arrangement of the monomers within arrays account for chromosome specificity among these repetitive sequences, which is particularly characteristic of alphoid DNA.

Biotinylated alphoid DNA, beta DNA, and satellite probes were obtained from Oncor, Inc. (Gaithersburg, Maryland), and were used according to the manufacturer's instructions. Slides were treated with 2XSSC, pH 7, at 37° C for 1 hour, followed by successive dehydration in 70%, 80%, and 95% ethanol for 2 minutes each. The initial treatment with 2XSSC is intended to preserve chromatin architecture during the subsequent dehydration process, but anecdotal evidence from other researchers suggest that this step may not be necessary. Also, our original

TABLE I. Specifications of Oncor supplied FISH probes

probe	locus or loci detected	DNA concentration in probe	concentration or amount of probe in hybridization mixture	hybridization conditions	post wash conditions
all centromere	mixture of alphoid sequences located at all human centromeres	0.5 mg/1050 ml Hybrisol VII	31.5 ml undiluted probe mixture per slide	50% formamide/ 2XSSC, 37° C	50% formamide/ 2XSSC, 37° C
chromosome 14/22	D14Z1/D22Z1, alphoid DNA at centromeres of chromosomes 14 and 22	10 ng/ml	1.5 ml/30 ml Hybrisol VI	65 % formamide/ 2XSSC, 37° C	65 % formamide/ 2XSSC, 43° C
chromosome 15	D15Z1, short repeats derived from AATGG in satellite DNA found in pericentromeric heterochromatin of chromosome 15	10 ng/ml	1.5 ml/30 ml Hybrisol VI	65 % formamide/ 2XSSC, 37° C	65 % formamide/ 2XSSC, 43° C
chromosome 1/5/19	D1Z7/D5Z2/D19Z3, alphoid DNA at centromeres of chromosomes 1, 5, and 19	10 ng/ml	1.5 ml/30 ml Hybrisol VI	65 % formamide/ 2XSSC, 37° C	65 % formamide/ 2XSSC, 43° C

TABLE I (continued)

chromosome 2	D2Z1, alphoid DNA at centromere of chromosome 2	10 ng/ml	1.5 ml/30 ml Hybrisol VI	65 % formamide/ 2XSSC, 37° C	65 % formamide/ 2XSSC, 43° C
chromosome 18	D18Z1, alphoid DNA at centromere of chromosome 18	10 ng/ml	1.5 ml/30 ml Hybrisol VI	65 % formamide/ 2XSSC, 37° C	65 % formamide/ 2XSSC, 43° C
beta	beta DNA in pericentromeric heterochromatin of all acrocentric chromosomes	10 ng/ml	1.5 ml/30 ml Hybrisol VI	65 % formamide/ 2XSSC, 37° C	65 % formamide/ 2XSSC, 43° C
14 paint	hybridizes to single-copy sequences spanning the length of chromosome 14	concentration varies in each lot; premixed with blocking DNA in Hybrisol VII	10 ml undiluted probe mixture/ slide	50% formamide/ 2XSSC, 37° C	50% formamide/ 2XSSC, 43° C
22 paint	hybridizes to single-copy sequences spanning the length of chromosome 22	concentration varies in each lot; premixed with blocking DNA	10 ml undiluted probe mixture/	50% formamide/ 2XSSC, 37° C	50% formamide/ 2XSSC, 43° C

Locus designation: D = DNA, first number = chromosome, Z = repetitive DNA, second number = order in which locus was discovered on this chromosome

Hybrisol contains reagents which enhance hybridization between probe and target DNAs; Hybrisol VI contains 65% formamide/2XSSC; Hybrisol VII contains 50% formamide/2XSSC

protocol called for RNase to be added to the 2XSSC solution, but we have found that omitting the enzyme did not affect our results.

Chromosomes were denatured in 70% formamide in 2XSSC, pH 7, for 2 minutes at 70° C, then immediately placed in ice-cold 70% ethanol. The slides were rinsed successively in 80%, 90%, and 100% ethanol for 2 minutes each.

A hybridization mixture was prepared containing 1.5 ml of the biotin-labeled probe in 30 ml of a 65% formamide solution. The probe was denatured by heating the hybridization mixture in a 70° C water bath, and then chilled quickly on ice. The denatured hybridization mixture was micropipetted onto the slides, and the slides were incubated overnight at 37° C in a humidified chamber.

Following hybridization, the slides were immersed in a post-wash solution containing 65% formamide for 20 minutes at 43° C. The biotinylated probes were detected by applying 60 ml fluorescein-labeled avidin to the slides. This procedure allows the resulting fluorescent signal to be amplified, if necessary, by the subsequent addition of 60 ml of biotinylated anti-avidin antibody followed by 60 ml fluoresceinated avidin. We found that for our purposes such amplification was generally necessary, and we made a single round of amplification a standard part of our protocol. The chromosomes were counterstained for visualization under the fluorescent microscope by adding 19 ml of a propidium iodide antifade solution to each slide. A glass coverslip was placed directly on the slide, which was then ready to be viewed under the microscope.

Chromosome painting

Slide preparation, dehydration and denaturation were performed as described above for FISH with alphoid DNA probes. Painting probe mixes were then aliquoted (20-30 ml per slide) and denatured at 70° C for 10 minutes. The probes and blocking DNA were allowed to preanneal for 2.5 hours, and then the aliquots were applied to the slides. The slides were incubated overnight at 37° C in a humidified chamber.

After hybridization, slides were immersed in a post wash solution containing 50% formamide for 15 minutes at 43° C. Slides were then rinsed in 0.1 X SSC for 15 minutes at 60° C. Detection and counterstaining were performed exactly as described for alphoid DNA probes.

RESULTS

Tables II and III summarize non-hybridization and fluorescent in situ hybridization results for the marker chromosomes found in the four patients studied.

Patient SD: The marker chromosome from this patient appeared to be smaller than a G-group chromosome, and a single centromeric region was evident (Figure 8). Silver staining of the nucleolus organizer regions labeled both ends of the marker (data not shown). Therefore the marker chromosome appeared to be bisatellited, indicating that the marker is derived from an acrocentric chromosome. The marker was negative for the DA/DAPI stain, suggesting the marker was not derived from chromosome 15 (Figure 8E).

The marker was further analyzed by fluorescent in situ hybridization using alphoid DNA probes for acrocentric chromosomes. The marker was negative for a probe specific for the centromeres of chromosome 13 and chromosome 21 (Figure 8G), but positive for the chromosome 14/22 probe. The 14/22 probe produced five fluorescent signals in metaphase spreads from this patient, one signal at the centromere of each normal chromosome 14 and chromosome 22 homolog, and one signal at the approximate center of the marker chromosome (Figure 8H).

TABLE II. Summary of non-hybridization staining results

				staining results		
patient	karyotype	inheritance	percentage of cells containing marker	C-banding	DA/DAPI	Ag-NOR
SD	47,XX,+mar	paternal	100	ND	-	++
NH	46,XX/47,XX,+mar	de novo	89	+	ND	+
CL	47,XX,+mar	paternal	100	+	-	ND
MP	46,XX/47,XX,+mar	de novo	57*	+	-	-

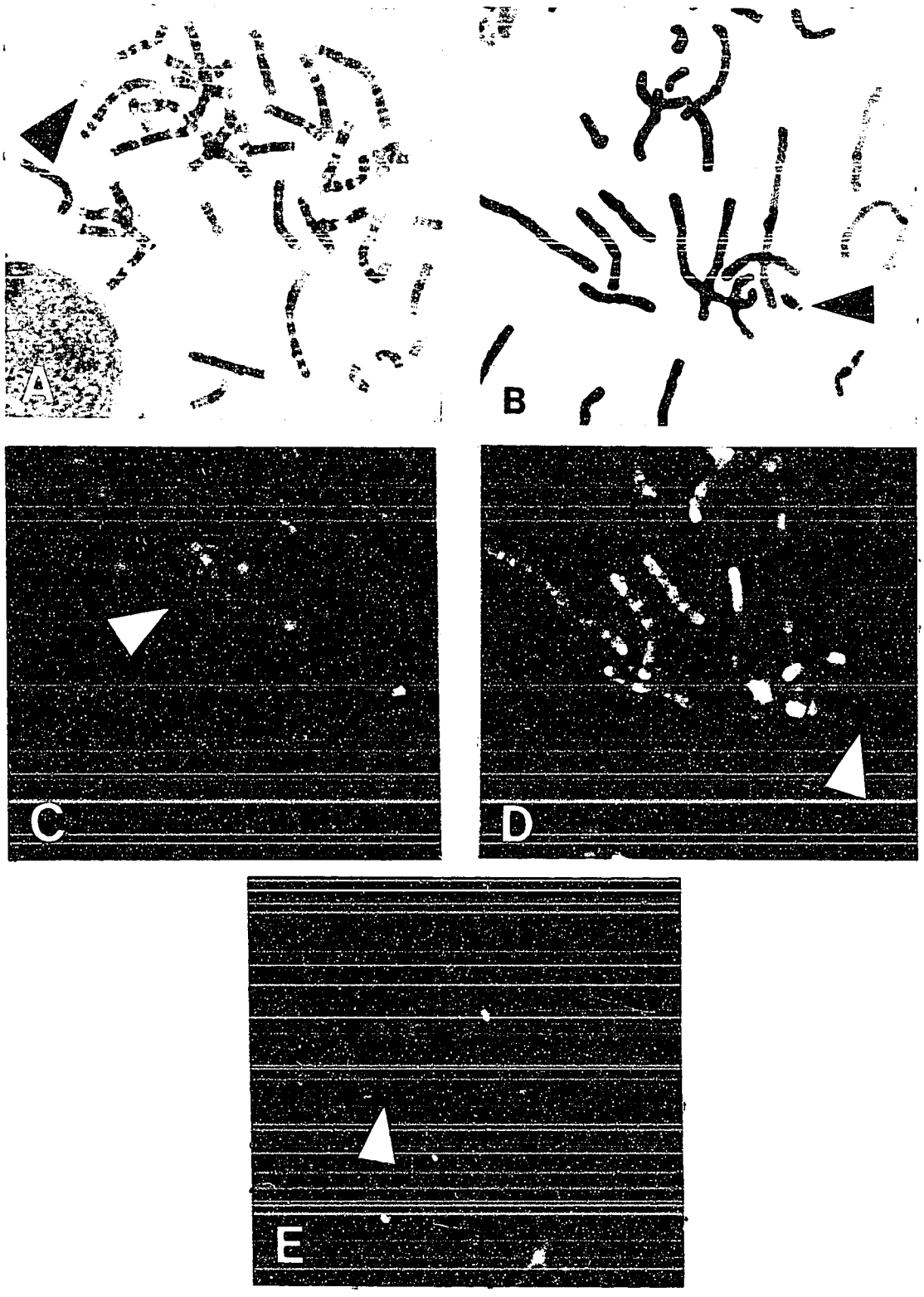
TABLE III. Summary of fluorescent in situ hybridization results

patient	generic centromere	beta satellite	15	13/21	14/22	1/5/19	2	18	14 paint	22 paint
SD	ND	ND	ND	-	+	ND	ND	ND	-	-
NH	+	++	-	-	+	ND	ND	ND	-	-
CL	+	++	-	+	-	ND	ND	ND	-	-
MP	+	-	-	-	-	?	?	?	-	-

Symbols: +, one positive signal; ++, two positive signals; -, negative result; ND, not determined, or stain or probe not used with cells from this patient.

* initial finding; after several passages in culture, mosaicism for cells containing the marker chromosome had decreased to 8%.

Figure 8. Results of cytogenetic analysis of the marker chromosome found in amniotic fluid samples from patient SD. The position of the marker in each photograph is indicated by an arrow. Staining results are shown for (A) G-banding; (B) C-banding; (C) Q-banding; (D) R-banding; (E) DA/DAPI staining. Fluorescent in situ results are shown for the (F) chromosome 13/21 and (G) chromosome 14/22 probes. Sequential G-banding and FISH results are shown for the (H) chromosome 14 paint and (I) chromosome 22 paint probes.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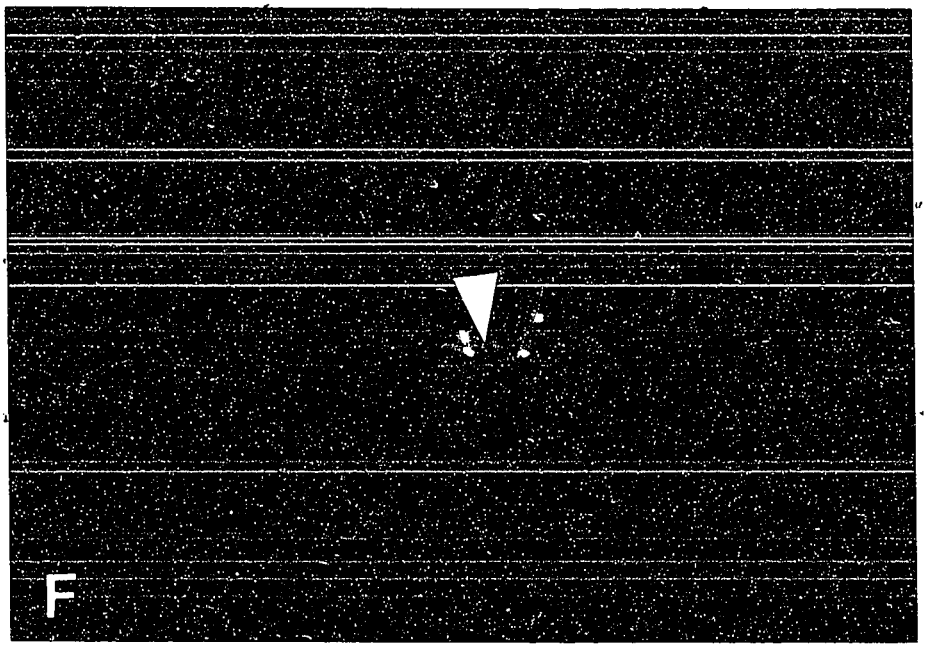
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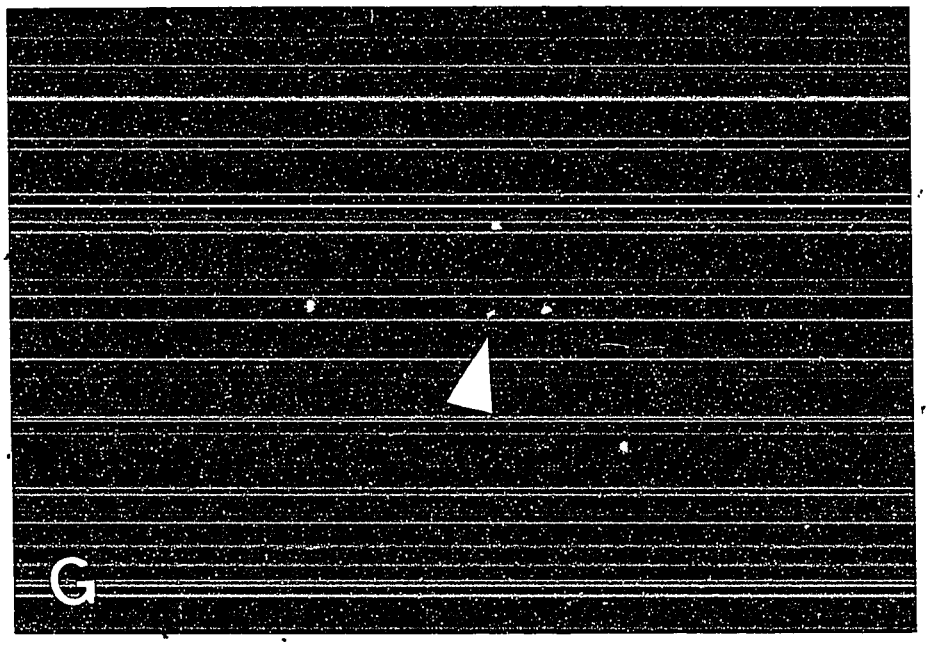
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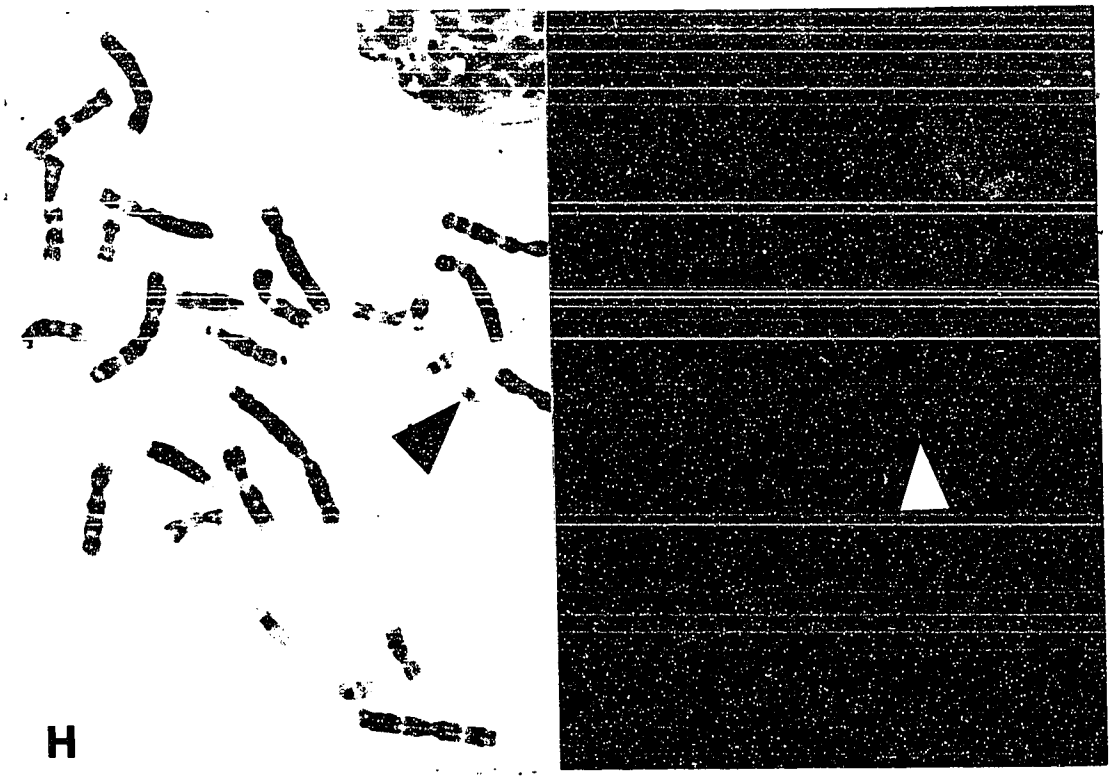

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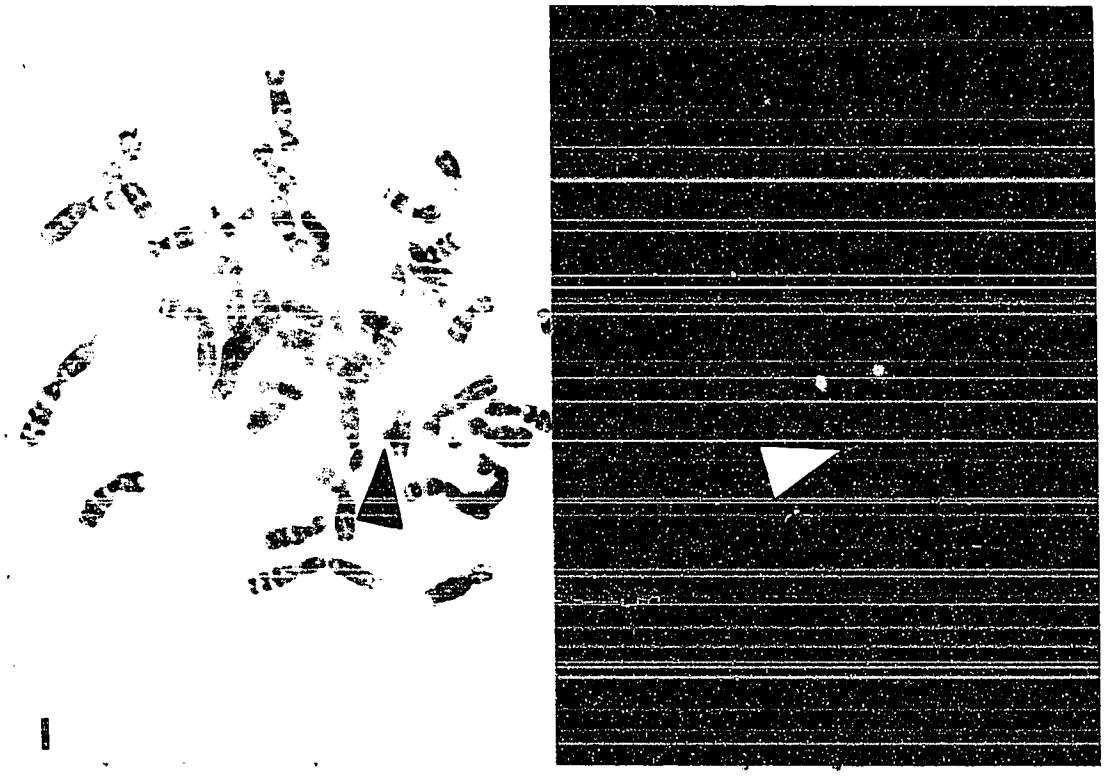

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The results from non-hybridization and FISH methods therefore suggest that the marker chromosome in this case was an isochromosome derived from the short arm (including stalks and satellites) and the centromere of either chromosome 14 or chromosome 22. To confirm that unique or low-repetitive sequences derived from the long arm of one of these chromosomes was not present, chromosome painting was performed using probe mixtures from chromosome 14 or chromosome 22. These probe mixtures contain single copy or low copy number DNA sequences, which are contained primarily, if not exclusively, on the long arms in acrocentic chromosomes. While the experiment using the chromosome 14 probe mixture was characterized by extensive cross-hybridization, the marker chromosome was not intensely decorated with fluorescent signals as were the two normal chromosome 14 homologs (Figure 8I), and was interpreted as being negative for this probe mixture. The marker chromosome was also determined to be negative for the chromosome 22 paint probe, which almost exclusively labeled the two normal 22 homologs (Figure 8J). These results support the conclusion that the marker chromosome from this patient was a monocentric isochromosome containing only short arm and centromere material from either chromosome 14 or chromosome 22.

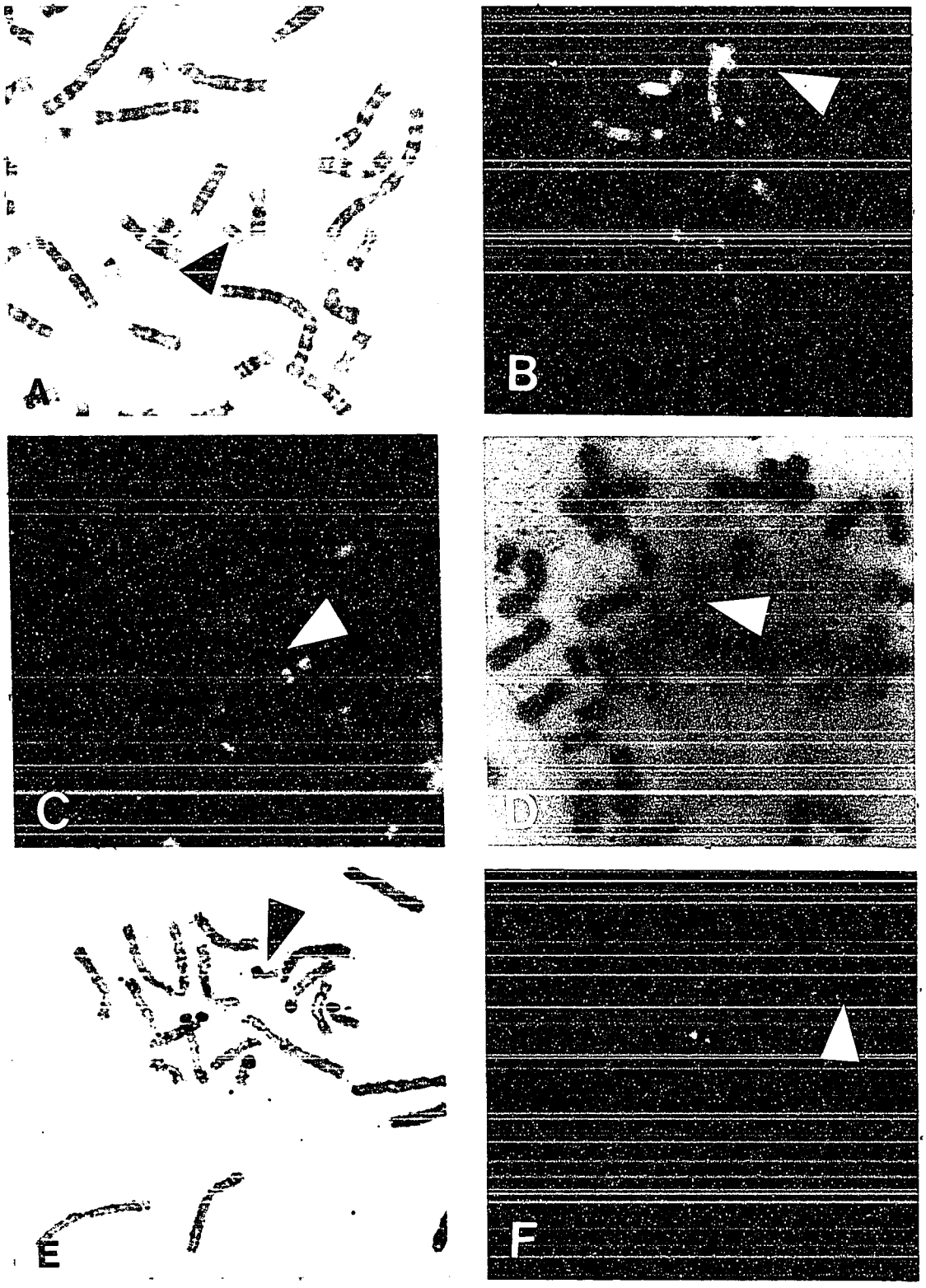
Patient NH: The marker chromosome in this case was slightly smaller than a G-group chromosome, and appeared to have satellites on

each end by G-banding (Figure 9A). Silver NOR staining labeled only one satellite, but FISH using a beta DNA probe labeled both ends of the marker (Figure 9G), suggesting that the marker was bisatellited. C-banding indicated the presence of one centrally located centromeric region (Figure 9D). FISH with an alphoid probe generic for all human chromosomes also produced a single broad centromeric signal (Figure 9H).

Since silver staining and FISH indicated that the marker contained satellites and thus was derived from an acrocentric chromosome, FISH with alphoid acrocentric probes was used to further examine the marker. The marker chromosome was positive for the chromosome 14/22 probe (Figure 9I), which produced one strong signal on the marker, but negative for the chromosome 15 and chromosome 13/21 probes (Figures 9J and 9K).

Although the combination of non-hybridization and in situ hybridization techniques indicated that the marker chromosome from this patient contained a single centromere, the appearance of the fluorescent signal produced by the chromosome 14/22 suggested the possibility that this marker was dicentric, with centromeric regions too close together to be resolved by C-banding or FISH on metaphase chromosomes. Koch et al. (1993) describe a marker chromosome that produced a single hybridization signal with an alphoid probe specific for chromosome 18 that was more intense than the signals on the normal chromosome 18 homologs. Inspection of interphase nuclei hybridized with the same probe

Figure 9. Results of cytogenetic analysis of the marker chromosome found in amniotic fluid samples from patient NH. The position of the marker in each photograph is indicated by an arrow. Staining results are shown for (A) G-banding; (B) Q-banding; (C) R-banding; (D) C-banding; (E) silver NOR staining; (F) DA/DAPI staining. Fluorescent in situ hybridization results are shown for the following probes: (G) beta DNA; (H) generic centromere; (I) chromosome 15; (J) chromosome 13/21; (K) chromosome 14/22. Several additional examples of hybridizations with the chromosome 14/22 probe are shown: (L) an interphase nucleus shows the variability of fluorescent signals in chromosomes at this stage of mitosis; (M) a metaphase showing that the probe signal overwhelms the propidium iodide signal at an exposure time of 20 seconds; (N) an apparently doublet signal in one metaphase; (O) a metaphase with extended chromosomes showed a single marker signal. Sequential G-banding and FISH results are shown for the (P) chromosome 14 and (Q) chromosome 22 paint probes. These paint probes did not produce marker signals of comparable intensity to those of the normal homologs.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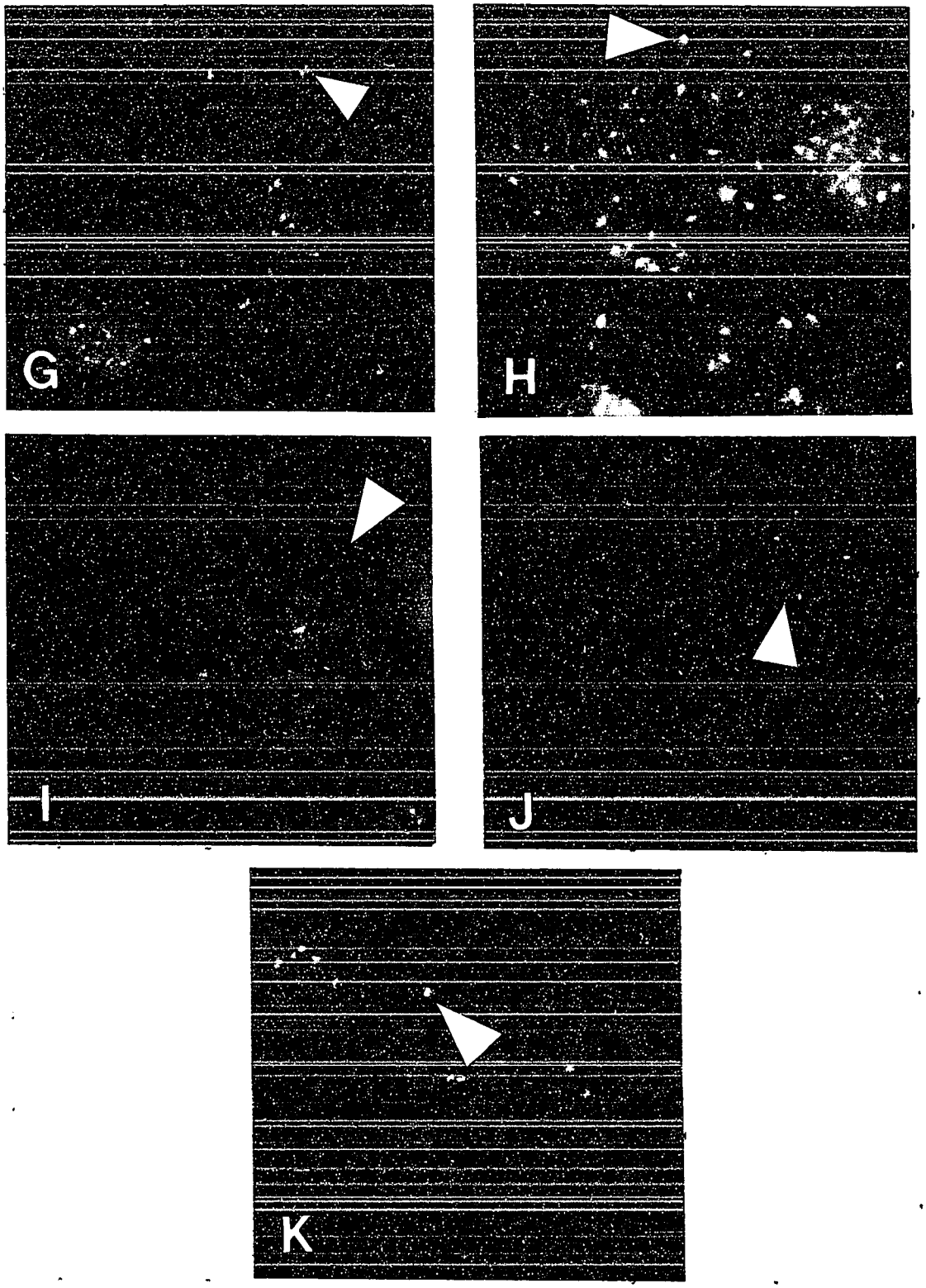
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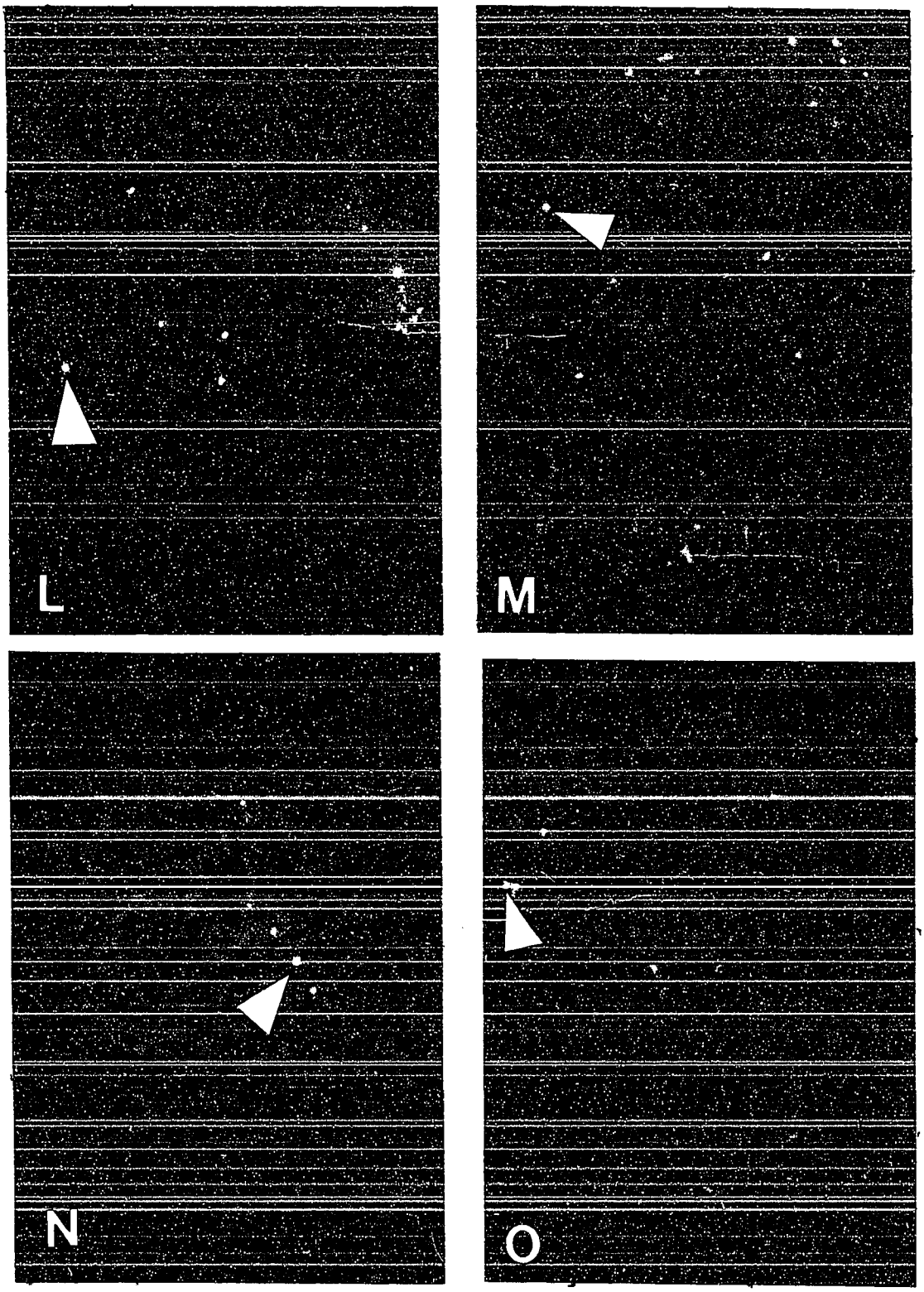
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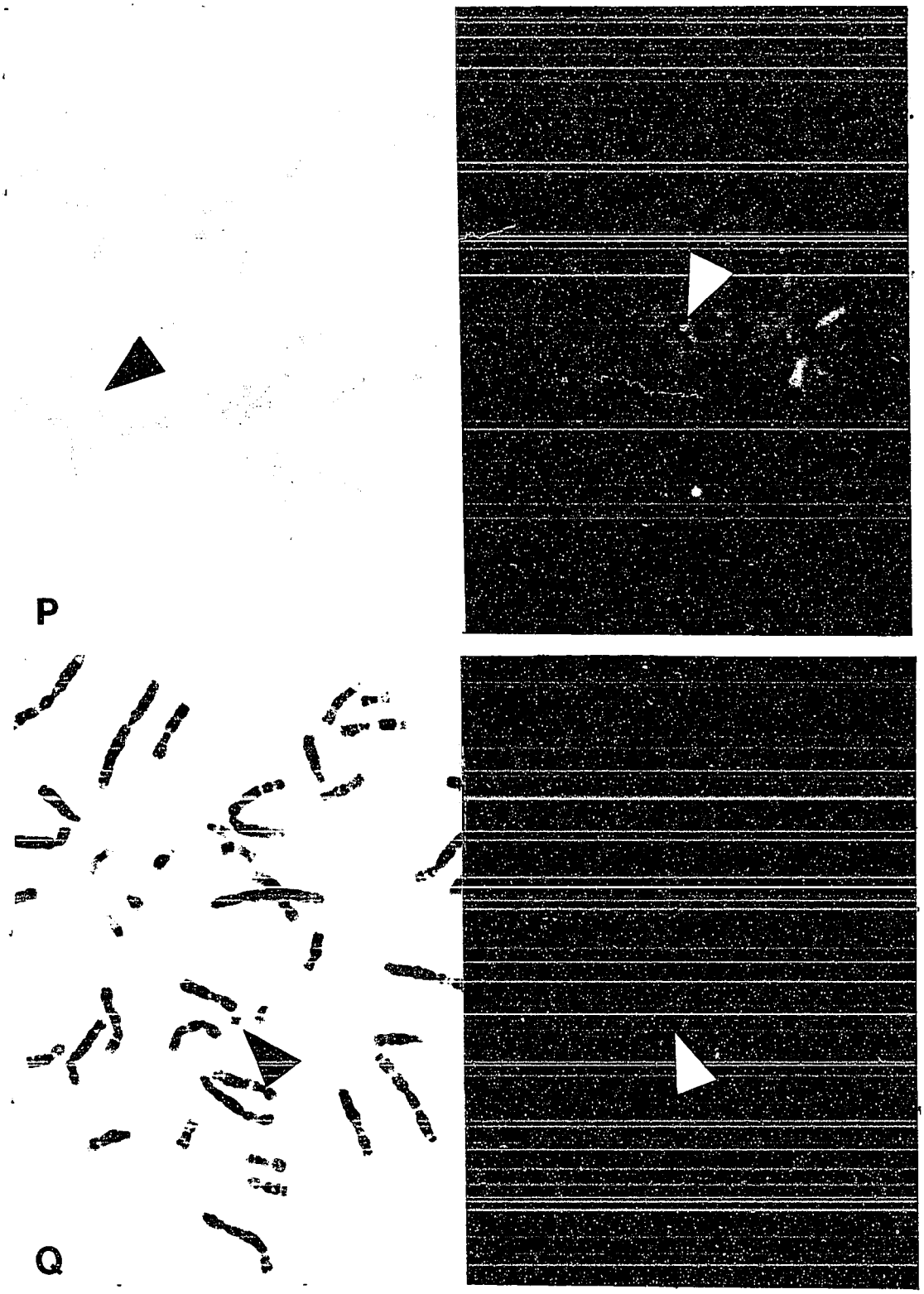
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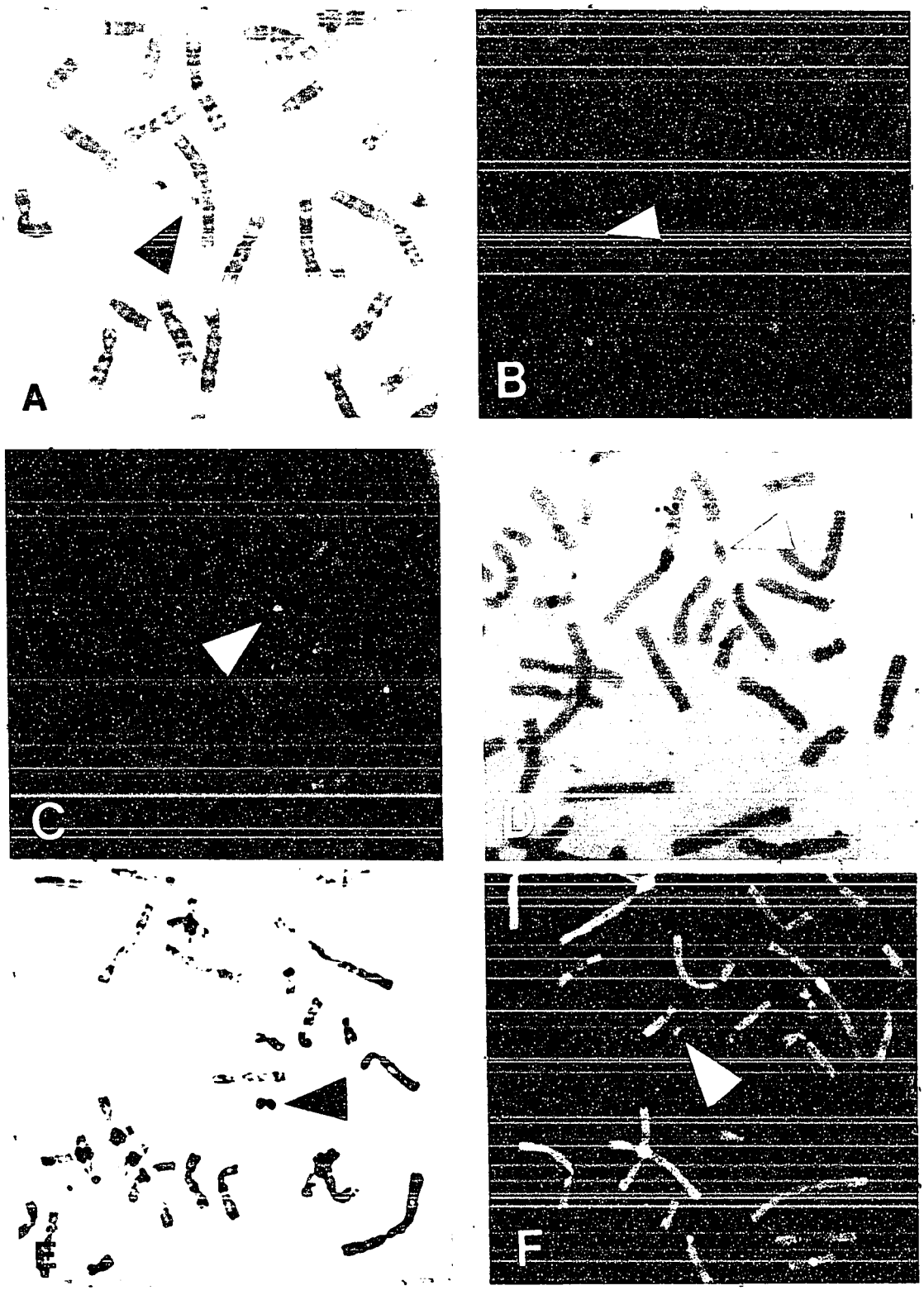
found four signals including a doublet signal, suggesting that the marker chromosome was dicentric. Unfortunately, in the FISH experiments reported here, the number of hybridization signals for the chromosome 14/22 probe in interphase nuclei was highly variable (cf. Figures 9I and 9L). Furthermore, these signals were quite variable in shape, making interpretation of signal number much more difficult. In metaphase spreads, the marker chromosome signal was often stronger than the signals on the normal chromosome 14 and chromosome 22 homologs, and at longer photographic exposure times (› 20 seconds), the 14/22 probe fluorescence overwhelms the propidium signal on the marker (Figure 9M). In one metaphase, the marker chromosome appeared to carry a doublet signal (Figure 9N), but less condensed metaphase chromosomes did not have signals that had a different appearance from those seen on more compact chromosomes (Figure 9O). A doublet signal would be expected to be better separated on such extended chromosomes.

Chromosome painting was used to determine whether the marker chromosome contained q arm euchromatin, which could have been present if the marker were indeed dicentric. Painting with the chromosome 14 probe was performed under the same conditions as reported above for cells from patient SD, and the same amount of cross-hybridization was evident on slides from patient NH. The marker chromosome from patient NH also did not exhibit the intensity of hybridization as was evident on the two normal

chromosome 14 homologs, and was interpreted as being negative for the chromosome 14 paint probe (Figure 9P). This marker was also negative for the chromosome 22 paint probe (Figure 9Q). Thus no evidence could be found that the marker chromosome was dicentric, and it was interpreted as being a monocentric isochromosome derived from the short arm and centromere of either chromosome 14 or chromosome 22.

Patient CL: The marker chromosome in this case contained a single centrally located C-band (Figure 10D). A single centromeric region was also observed with the generic FISH centromeric probe (Figure 10G). The marker was positive at both ends for the beta DNA FISH probe, indicating that it was bisatellited (Figure 10H). Because the marker was negative for the DA/DAPI stain (Figure 10F), it appeared to have originated from an acrocentric chromosome other than chromosome 15. Accordingly, the marker was negative for the chromosome 15 alphoid probe (Figure 10I). The marker was negative for the 14/22 probe as well (Figure 10J), but was weakly positive for the 13/21 probe (Figure 10K). Although the 13/21 probe signal on the marker chromosome was smaller and often much less intense than signals on the normal chromosome 13 and chromosome 21 homologs, it appeared consistently in metaphases from this patient. On the basis of this evidence, the marker chromosome was identified as a monocentric

Figure 10. Results of cytogenetic analysis of the marker chromosome found in amniotic fluid samples from patient CL. The position of the marker in each photograph is indicated by an arrow. Staining results are shown for (A) G-banding; (B) Q-banding; (C) R-banding; (D) C-banding; (E) silver NOR staining; (F) DA/DAPI staining. Fluorescent in situ hybridization results are shown for the (G) generic centromere probe. FISH using both beta DNA and generic centromere probes (H) produced a single broad centromeric signal on the marker flanked by two beta DNA signals. FISH results are also shown for the following probes: (I) chromosome 15; (J) chromosome 14/22; (K) chromosome 13/21.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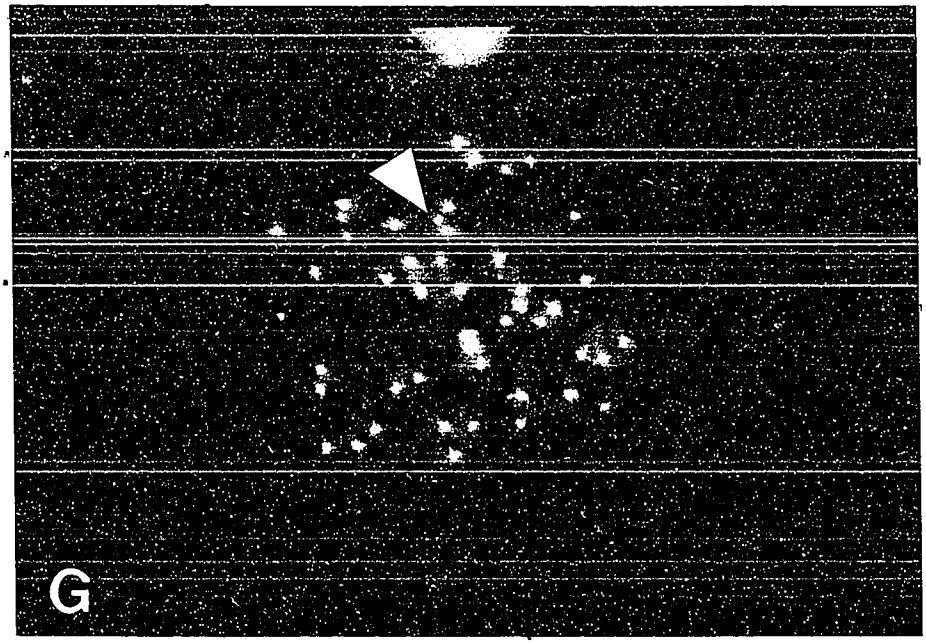
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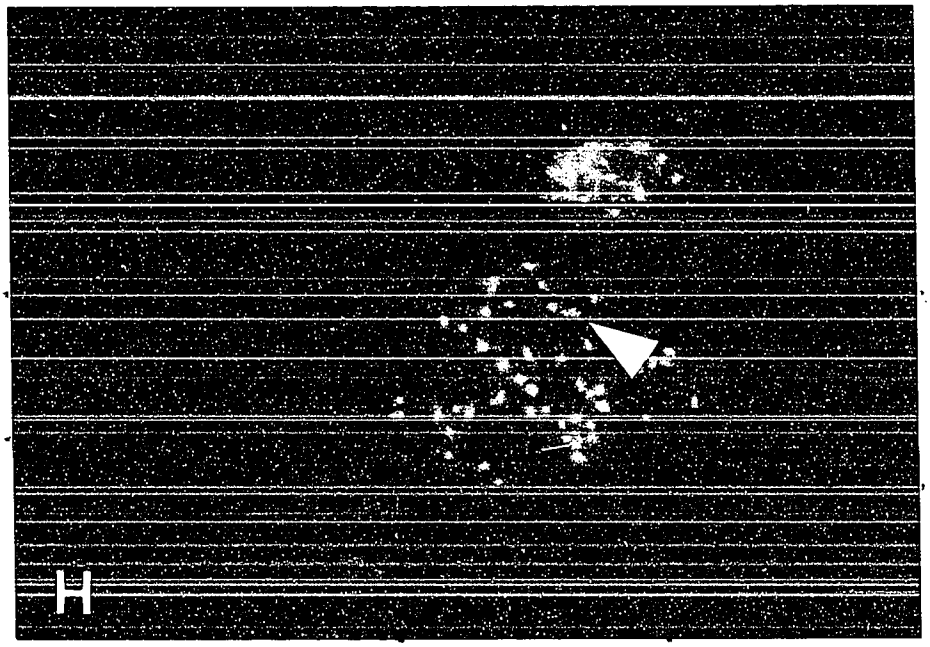
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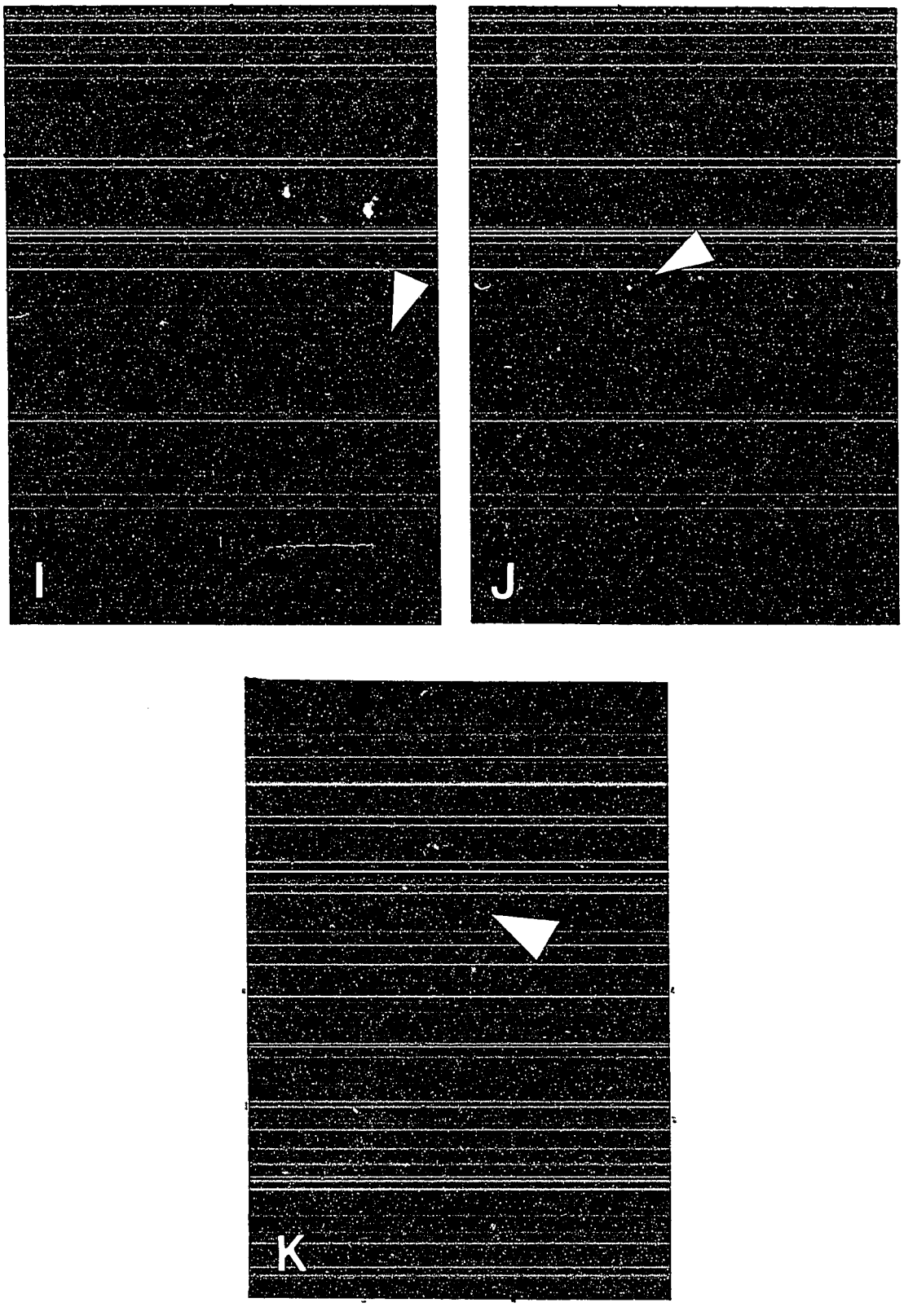
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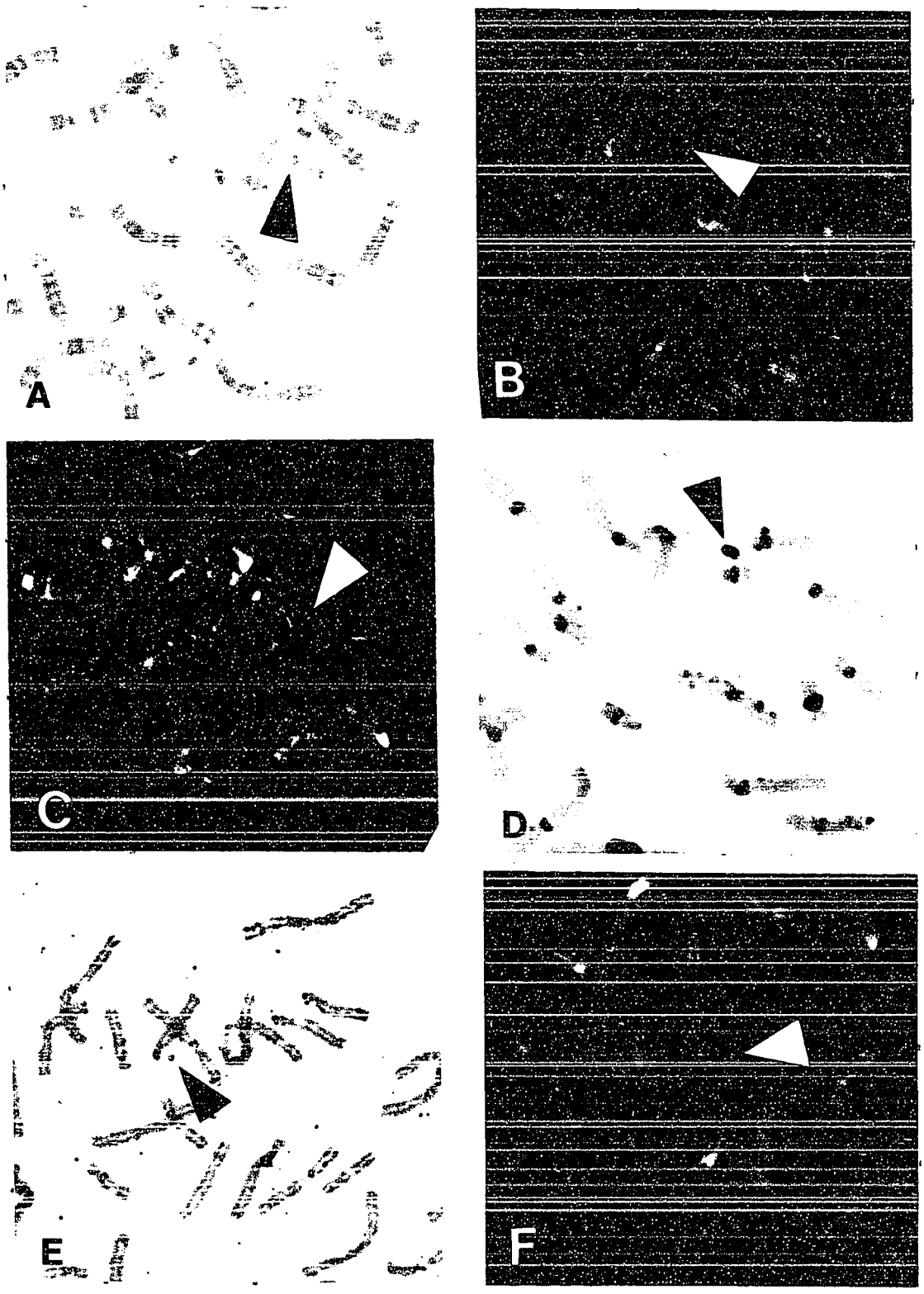
isochromosome derived from the short arm and centromere of either chromosome 13 or chromosome 21.

Patient MP: When this patient was referred for amniocentesis in 1991, the marker chromosome in this case was reported to be present in 57% of cells in the sample. However, at the time of the experiments reported here, the marker was found in only about 8% of the patient's amniocytes. The marker was extremely small, causing poor resolution of bands. Q- and R-bands were difficult to interpret with respect to recognizable chromosomal banding patterns (Figures 11 B and 11C). The marker appeared to have two C-band positive regions (Figure 11D), but FISH with the generic centromere alphoid probe indicated only a single centromeric region. Further attempts to characterize the marker chromosome by non-hybridization methods were also unsuccessful: Silver NOR staining and DA/DAPI staining were both negative (Figures 11E and 11F).

Silver staining indicated that the marker chromosome did not bear satellites; this result was confirmed by FISH using the beta DNA probe, which was also negative (Figure 11H). The marker was also negative for the 15 (data not shown), 13/21, and 14/22 probes (Figures 11I and J), confirming that it was not derived from an acrocentric chromosome. Alphoid probes for several other chromosomes were used in an attempt to determine the origin of the marker. Inconclusive results were found with a probe for an alphoid domain shared by chromosomes 1,5, and 19, as well as with

Figure 11. Results of cytogenetic analysis of the marker chromosome found in amniotic fluid samples from patient MP. The position of the marker in each photograph is indicated by an arrow. Staining results are shown for (A) G-banding; (B) Q-banding; (C) R-banding; (D) C-banding; (E) silver NOR staining; (F) DA/DAPI staining. Fluorescent in situ results are shown for the following probes: (G) generic centromere; (H) beta DNA; (I) chromosome 13/21; (J) chromosome 14/22; (K) chromosome 1/5/19; (L) chromosomes 2 and 18.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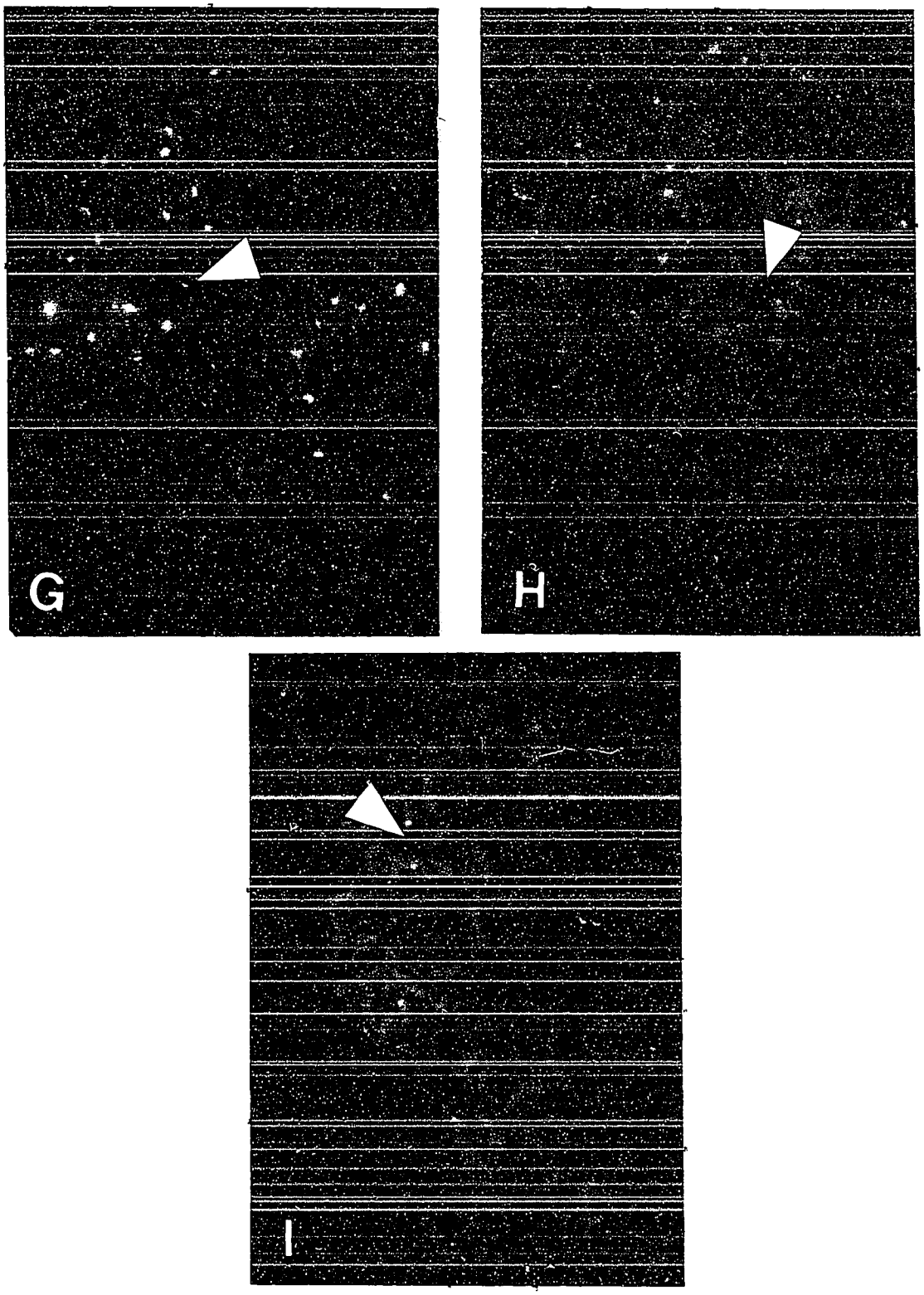
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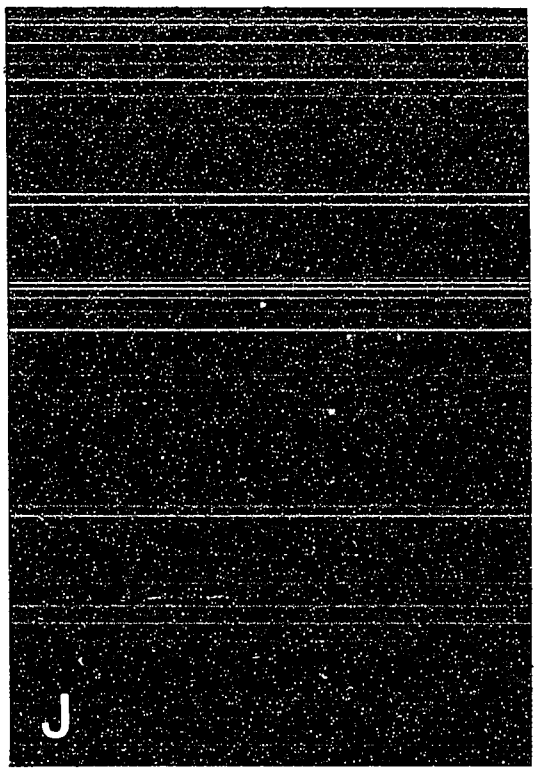
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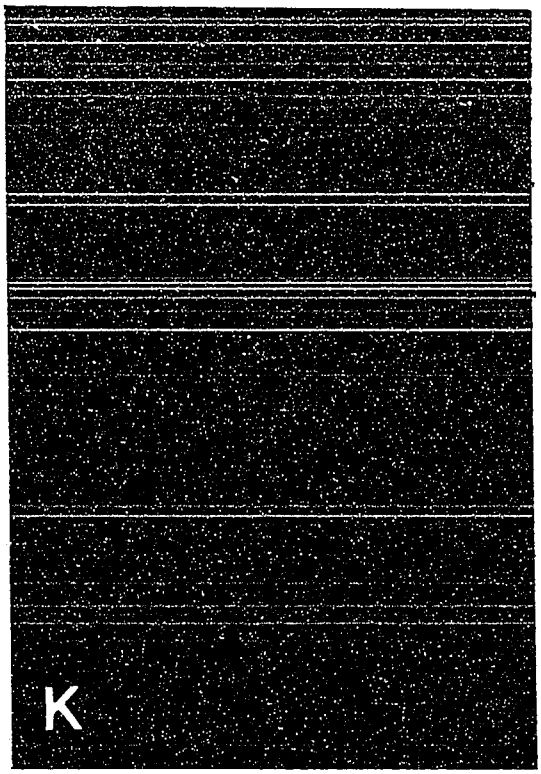
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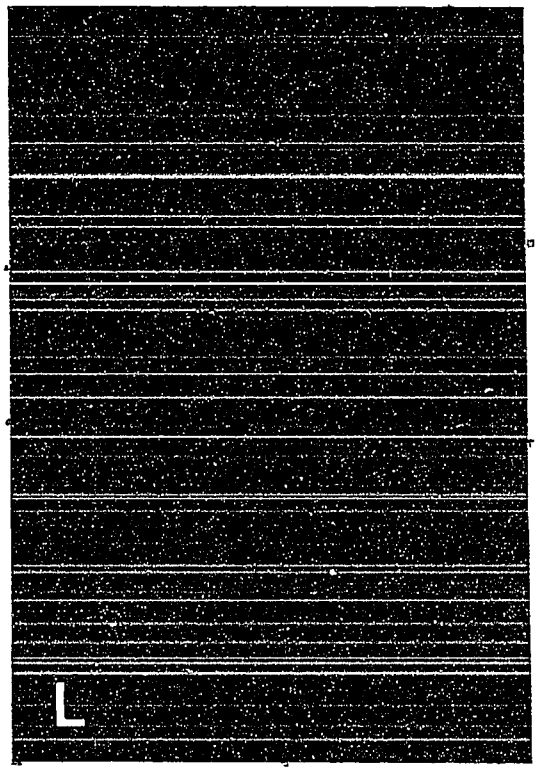
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probes for chromosome 2 and chromosome 18 (Figures 11K and L). Strong fluorescent signals generally appeared only on the normal chromosome 1 homologs with the chromosome 1/5/19 probe, and on the chromosome 18 homologs when the chromosome 2 and chromosome 18 probes were used together. Signals from these probes on the normal homologs were weak or nonexistent in amniocyte metaphases from this patient. Analysis of this marker chromosome was discontinued because the low level of mosaicism for the marker prevented accurate assessment of negative results. Due to its small size, the marker was difficult to identify in metaphase spreads even under optimal conditions, and the fact that it was present in so few cells complicated interpretation of the absence of extra fluorescent signals.

DISCUSSION

The chromosomal origins of accessory marker chromosomes in amniotic fluid samples from three patients were identified with fluorescent in situ hybridization. In each case, a small bisatellited marker was found to be derived from either of a pair of acrocentric chromosomes. The commercially available probes used to identify the centromeres of these markers contained subsets of alphoid DNA sequences shared between chromosomes 14 and 22 or between chromosomes 13 and 21. Thus the markers from patients SD and NH were derived from either chromosome 14 or chromosome 22, and the marker from patient CL was derived from either chromosome 13 or chromosome 21. To date, commercial probes are unavailable that are able to distinguish between chromosomes 14 and 22 or between chromosomes 13 and 21. The appropriate chromosome painting experiments indicated that euchromatin derived from the long arms of these chromosomes was not present in these markers.

It should be noted that each marker chromosome has been identified as being derived from a single chromosome, although the alphoid probes used could not exclude the possibility that the marker was formed from the short arms and centromeric heterochromatin of two different chromosomes. For example, the marker chromosome from patient CL might have been derived from both chromosomes 13 and 21. A bisatellited, monocentric marker such as this would be the expected reciprocal product of a Robertsonian translocation with the breakpoints at the centromeres of two

acrocentric chromosomes. Reciprocal products like these have rarely been reported, although Robertsonian translocations are one of the most common structural chromosome abnormalities observed (Heppell-Parton and Waters, 1991). These reciprocal products have been presumed to be lost during subsequent cell divisions, and would be unlikely to contribute to many cases of marker chromosomes, which appear at amniocentesis with a frequency similar to that of Robertsonian translocations (approximately 1/1000 cases). However, as noted earlier, recent evidence suggests that Robertsonian translocation chromosomes may be generally dicentric, with breakpoints in the proximal short arms of two acrocentric chromosomes (Callen, 1992), and any reciprocal bisatellited products would be acentric fragments. Thus bisatellited isochromosomes may be a kind of Robertsonian translocation chromosome composed of two acrocentric short arms rather than two long arms. It would be expected that bisatellited markers could be formed from two different acrocentric chromosomes, since Robertsonian translocations occur most frequently between chromosomes 13 and 14 (Callen, 1992). However, bisatellited markers have not been observed to be derived from two different acrocentric chromosomes in studies using FISH probes specific for individual chromosomes (Callen, 1992).

The mechanism of formation of bisatellited marker chromosomes is not clear, and may be heterogeneous. Many researchers believe that these markers may result from abnormal exchange either between sister chromatids or between homologous non-sister chromatids from acrocentric chromosomes to form isochromosomes or isodicentric chromosomes (Buckton

et al., 1985; Callen et al., 1992; Romain et al., 1979; Stetten et al., 1981; Rasmussen et al., 1976). Before the advent of molecular cytogenetics, Q-band or DA/DAPI fluorescence and Ag-NOR staining polymorphisms on markers were examined and compared with the corresponding normal chromosomes from the proband's parents in an attempt to determine which mechanism was involved in the formation of a particular marker chromosome (Plattner et al., 1991; Hoo et al., 1986; Schmid et al., 1986; Maraschio et al., 1981). Thus marker chromosomes with two morphologically distinct satellites, and dicentric markers having Q- or DAPI-bands at each end that could be distinguished by differing amounts of fluorescence, were interpreted as being derived from homologs. Although based on subjective interpretation of staining results, the available evidence suggest that most markers appear to have been formed by non-sister chromatid exchange between homologs. The asymmetry of the satellites and the position of the centromere in the marker chromosomes from patients SD, NH, and CL suggest that these markers were also formed from non-sister chromatids of acrocentric chromosomes.

In order to postulate how monocentric and dicentric bisatellited markers may appear in karyotypes in addition to a normal human complement of 46 chromosomes, the formation of these supernumerary markers should be explored in greater detail. Isodicentrics and isochromosomes may be formed by breakage and reunion between chromatids during either mitosis or meiosis (Daniel, 1988). Non-disjunctional events would also be required to replace the homolog involved in the formation of the marker (Daniel, 1988). These events would produce

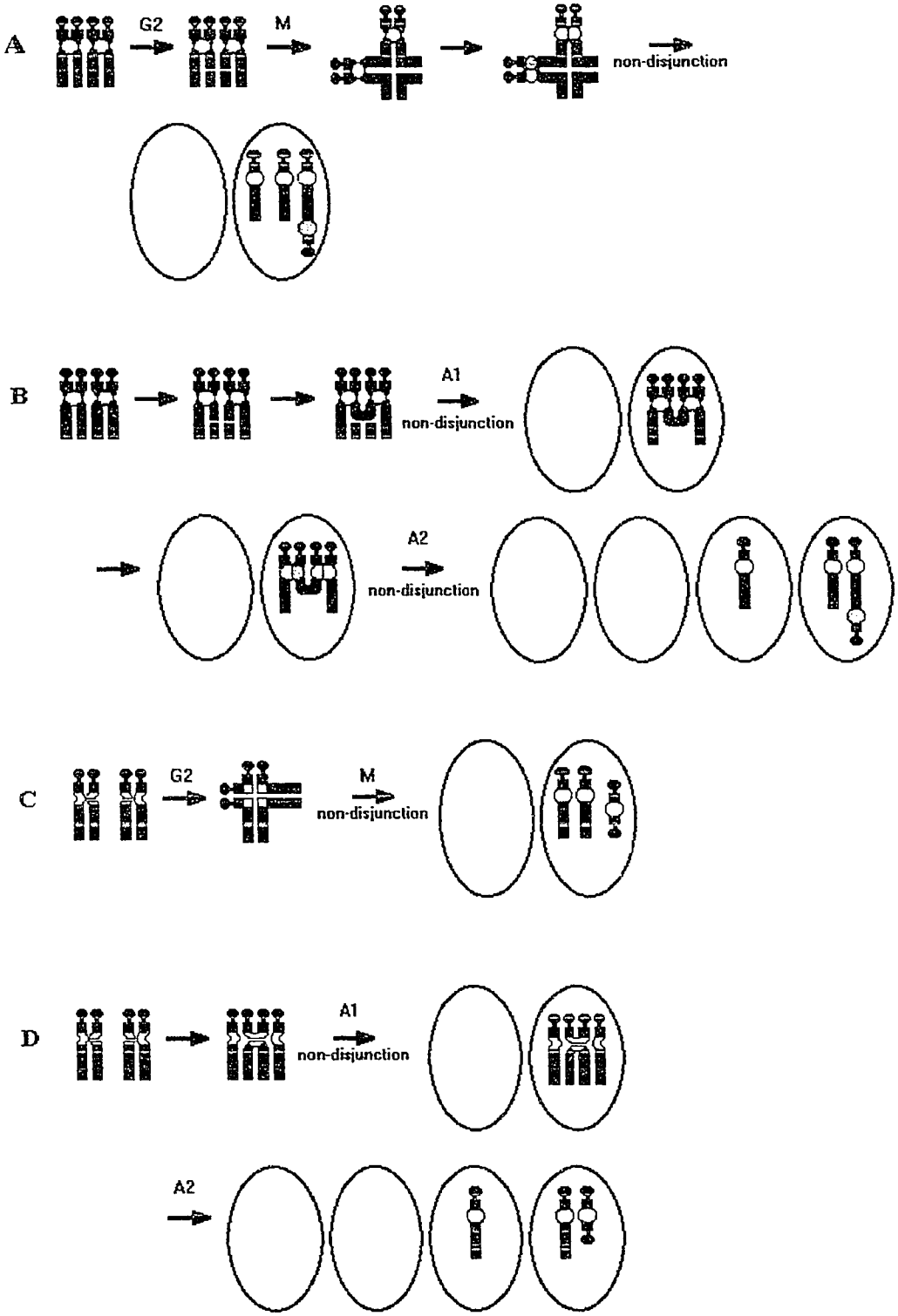
partial tetrasomy for the material duplicated in the marker chromosome. Thus, a karyotype including a monocentric bisatellited marker will contain four copies of the short arm of one of the acrocentric chromosomes.

During mitosis, a U-type exchange between homologous chromatids could produce an isodicentric bisatellited marker from acrocentric chromosomes (Figure 12A). Breakage and reunion between non-sister chromatids would both take place during G2 (Figure 12A). Chromatid exchange would form a quadriradial structure at metaphase (Figure 12A). Non-disjunctional segregation of the dicentric chromatid and one of the normal chromatids, coupled with independent non-disjunction of the remaining normal chromatid, would produce a daughter cell containing the two normal homologs as well as the isodicentric chromosome (Daniel, 1988).

A simpler mechanism to produce partial tetrasomy involving an isodicentric is a U-type exchange between homologous non-sister chromatids during meiosis I (Figure 12B). Because homologous chromosomes ordinarily segregate during anaphase I, non-disjunction is not required to ensure that the dicentric chromatid and one of the normal chromatids segregate together. A non-disjunctional event is required, however, for the remaining normal chromatid to segregate to the same pole during anaphase II (Figure 12B). Supernumerary bisatellited markers which are dicentric may be more likely to be formed in this manner, since this mechanism does not require two non-disjunction events to produce partial tetrasomy after fertilization (Daniel, 1988). This may explain why

Figure 12. Formation of asymmetrical bisatellited isodicentric chromosomes (A, B) and isochromosomes (C, D) by U-type exchanges between acrocentric chromosomes during mitosis or meiosis. Open circles (A, B) or segments of the chromatid (C, D) represent centromeres; gray circles represent inactivated centromeres and are indicated in order to clarify the segregation of chromatids during mitosis or meiosis; ellipses represent daughter cells or gametes; G1, S, G2, and M are the stages of mitosis; AI and AII are meiotic anaphase I and II. Acentric fragments (A, B) are assumed to be lost. (A) Breakage and reunion between non-sister, homologous chromatids during mitosis will produce partial tetrasomy with an isodicentric chromosome if there is non-disjunction between the dicentric chromatid and one of the normal chromatids in the quadriradial structure as well as non-disjunction of the remaining normal chromatid. (B) Breakage and reunion of non-sister homologous chromatids during meiosis I. Both homologs segregate together during anaphase I. Non-disjunction between the dicentric chromatid and one of the normal chromatids during anaphase II would produce a gamete containing a normal homolog and an isodicentric chromosome. Fertilization with a normal gamete will produce partial tetrasomy in the zygote. (C) Breakage and reunion of homologous non-sister chromatids within the centromeric region during mitosis. The non-satellited reciprocal product is assumed to be lost. Non-disjunction of the bisatellited chromatid and the two normal chromatids would produce a

Figure 12 (continued) partially tetrasomic daughter cell. (D) Breakage and reunion of non-sister chromatids during meiosis I. Non-disjunction during anaphase II would produce a gamete containing the bisatellited isochromosome and a normal homolog. The non-satellited reciprocal product is assumed to be lost. Fertilization with a normal gamete would produce partial tetrasomy in the zygote.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staining polymorphisms seem to suggest that most bisatellited marker chromosomes arise from non-sister chromatids.

Because the DNA strands of the chromatids are continuous and separate throughout the length of the centromere, monocentric bisatellited isochromosomes may also be formed by a U-type exchange between homologous non-sister chromatids within the centromeric region (Daniel, 1988). Of course, if these markers are actually dicentric as Robertsonian translocation chromosomes are now believed to be, the breakpoints involved in the formation of these markers would be located in the short arms of the parent chromosomes rather than within the centromeres. However, if recombination between centromeric sequences is possible, then a bisatellited isochromosome may be formed by this type of exchange during mitosis (Figures 12C; Daniel, 1988). Non-disjunction between the bisatellited chromatid and one of the normal chromatids, and independent non-disjunction of the remaining normal chromatid, would produce partial tetrasomy in one of the daughter cells (Figure 12C). Lack of mosaicism with a cell line containing only the reciprocal isochromosome (see Figure 12C) may be due to inviability of cells containing this isochromosome in the absence of a normal homolog. Alternatively, the isochromosome might be lost in anaphase, leaving one daughter cell nullisomic for the appropriate acrocentric chromosome, which would presumably be an inviable condition as well (Figure 12C; Daniel, 1988). Recent research has identified several centromeric sequences associated with proteins involved in forming the kinetochore, the point of attachment between the centromere and the spindle (Willard, 1992). It is conceivable that only one

of the isochromosomes created by centromere misdivision would contain the appropriate sequences to form a functional centromere, leaving the reciprocal product to be lost by anaphase lag.

Homologous chromatid exchange during meiosis I would require only a single non-disjunctional event during anaphase II, involving one of the normal chromatids, to produce a gamete containing an isochromosome and a normal homolog (Figure 12D). Fertilization with a normal zygote would produce partial tetrasomy in the zygote. Thus, isochromosomes as well as isodicentric chromosomes may be more likely to result from meiotic non-sister chromatid exchange.

It should be noted that isochromosomes and isodicentrics might also be formed by breakage and reunion between sister chromatids during either mitosis or meiosis (Daniel, 1988). However, this type of chromatid exchange would produce bisatellited markers with a symmetrical appearance. Since the marker chromosomes found in amniotic fluid cells from patients SD, NH, and CL were generally asymmetrical in appearance (and for the sake of simplicity), the possible mechanisms of sister chromatid exchange will not be considered here. It should be further noted that in the formation of isochromosomes or isodicentrics by mitotic chromatid exchange, mosaicism with a normal cell line could result if chromatid exchange takes place after the first postzygotic division (Daniel, 1988). If these abnormal chromosomes are formed during meiosis, mosaicism would have to result from the postzygotic loss of the chromosome in a cell line.

The clinical outcome of prenatal diagnosis in patients SD and CL was normal, but the newborn child of patient NH presented a number of abnormalities. A diverse combination of anomalies might be expected to arise from a chromosomal abnormality rather than from a mutation at a single locus. Supernumerary chromosomal material, such as a marker chromosome, represents a karyotypic imbalance that may involve many different active genes, leading to a complex phenotype. Such an interpretation is difficult to support in the case of the child of patient NH, in which no chromosomal abnormalities other than the marker chromosome were found. The centromeres and short arms of acrocentric chromosomes are thought to be primarily composed of heterochromatin, and acrocentric stalks and satellites display high interchromosomal polymorphism without phenotypic consequence. The genes for the 18S and 28S ribosomal RNA subunits are located at the nucleolus organizer regions on the satellite stalks, and are among very few genes known to be located on acrocentric short arms. Thus a monocentric, bisatellited marker chromosome such as the one found in patient NH would be expected to have minimal phenotypic effect, since current cytogenetic methods suggest that these markers are composed of little active genetic material.

However, a survey of the literature reveals that abnormalities have been found in patients with a monocentric marker chromosome derived from an acrocentric chromosome, but these phenotypes, as well as those in patients with dicentric bisatellited markers, do not closely resemble the clinical picture of the newborn child of patient NH. Furthermore, when comparing phenotypes from different patients, it is important to emphasize

that similar abnormalities may have appeared at different stages of development. Also, it may be generally impossible to state definitively whether a patient's phenotype may be attributed to the presence of a marker chromosome. Even well-characterized syndromes such as the cat-eye syndrome may encompass a variety of symptoms which appear in different combinations in different patients. Many of the cases reported in the literature were studied using non-hybridization techniques, which can at best only identify whether a bisatellited marker might be derived from chromosome 15 or not by DA/DAPI staining. Cheung et al. (1990) report one case of a bisatellited marker chromosome in which the fetus experienced intrauterine growth retardation, but the child appeared normal. Rasmussen et al. (1976) report the case of a 14 year old girl with a dicentric bisatellited marker, slightly larger than a G group chromosome, who was severely mentally retarded. Additional anomalies included autism in early infancy, ataxia in the extremities, and hypotonia of the legs. DA/DAPI staining was not performed. Like the newborn child of patient NH, adult patient K59/79/69 in Buckton et al. (1985), who also had a bisatellited monocentric marker chromosome, was found to have an asymmetrical face. However, additional abnormalities included a flat occiput, bilateral coloboma and cataract, hypertelorism tooth enamel hypoplasia, low hairline, prepubertal genitalia, bilateral extensor plantar reflex. This patient was ascertained through a survey of patients in mental deficiency hospitals. The presence of coloboma in this patient, a symptom of the cat-eye syndrome, suggests that the marker may have been derived from chromosome 22.

Among marker chromosomes reported to have been derived from either chromosome 14 or chromosome 22, several cases have included abnormalities similar to those seen in the child of patient NH. Crolla et al. (1992) report the case of a 3 year old male with a marker chromosome derived from chromosome 14. This patient had hypermobile joints, like the child of patient NH, as well as an undescended testicle, bilateral preauricular sinuses, bifid tongue, wide set and slightly downward sloping eyes, and was referred because of delayed speech. The cat eye syndrome, associated with markers derived from chromosome 22, has a wide ranging clinical picture. Ear abnormalities are frequently reported, primarily skin tags (Hoo et al., 1986; Liehr et al., 1992; Callen et al., 1992; Reiss et al., 1985; Duncan et al., 1986). Rosenfeld et al. (1984) report the presence of a dicentric marker in a child who, like the child of patient NH, had posteriorly rotated ears.

With the exception of the chromosome 15 probe, the commercially available acrocentric probes used in the FISH experiments reported here could only distinguish between two pairs of acrocentric chromosome. The alphoid domains detected by each of these two probes represent sequences on either chromosome 13 and 21 or chromosomes 14 and 22 that share sufficient homology for each chromosome within a pair to hybridize equally well with the same probe even under high stringency. However, researchers have isolated repetitive sequences specific for individual acrocentric chromosomes as well. Charlieu et al. (1993) report the discovery of a clone, YSG1, derived from a partial chromosome 21 library, which is specific for the pericentromeric region of that chromosome. The

probe p22hom48.4 is specific for chromosome 22, and an alphoid repeat fragment isolated by McDermid et al. (1986) selectively hybridizes to the chromosome 22 centromere under conditions of high stringency. Waye et al. (1988) have described a low-copy number or single-copy alphoid sequence localized only to chromosome 14. Finally, Kalitsis et al. (1993) isolated a satellite I DNA clone specific for the chromosome 13 centromere and satellites.

Although the ability to identify the chromosomal origin of marker chromosomes with FISH centromeric probes may allow researchers to evaluate the prenatal risk of individual markers with greater precision, marker heterogeneity underscores the need to better understand the functional significance of the genomic material contained within a particular aberrant chromosome. Especially in the case of small dicentric markers, it would be advantageous to know what genetically active DNA lies near the centromere and may be included in a particular marker. The work of Cooper et al. (1993), which characterized the boundaries of the Y chromosome centromere, exemplifies how this may be done. In order to orient yeast artificial chromosome and cosmid clones of sequences at the edges of the array of alphoid and satellite repetitive DNA forming the Y centromere, digests of these clones were made with enzymes which cut frequently in the genome as a whole but lack sites in most of the repetitive DNA of the Y centromere (Cooper et al., 1993). At one end of the centromere, two clones were found which extend into a region rich in Alu sequences, which may represent typical euchromatin and thus the true edge of the centromere (Cooper et al., 1993). Mapping centromere

boundary regions as well as more distal sequences may provide panels of FISH probes to more accurately identify the extent of interstitial euchromatin found in dicentric marker chromosomes.

Even though the proliferation of probes for use with FISH will undoubtedly aid marker chromosome analysis, it would be simpler to identify abnormal chromosomes directly by comparison with normal chromosomes. Several recent papers have described "reverse" chromosome painting, a technique in which sequences isolated from abnormal genomes or chromosomes are used to paint chromosomes in normal metaphase spreads (Blennow et al., 1992). This may be contrasted with "forward" painting, the technique used in the experiments reported here, in which painting probes derived from normal chromosomes are hybridized to metaphases containing the chromosome under study. One form of reverse painting, comparative genomic hybridization (CGH), compares the relative sequence copy number between normal and tumor genomes, and can detect gains and losses of sequences or chromosomes in tumor DNA (Kallioniemi et al., 1992). Tumor DNA from a particular tissue specimen may be labeled with biotin and normal control genomic DNA labeled with digoxigenin, and then hybridized together to normal metaphases in the presence of unlabeled Cot-1 blocking DNA (Kallioniemi et al., 1992; Joos et al., 1993). The biotin label is detected by the addition of FITC-avidin and digoxigenin is detected by rhodamine anti-digoxigenin. Competitive hybridization between tumor and normal DNA allows changes in the karyotype of tumor cells to be detected by a change in the ratio of fluorescent intensity between the yellow-green FITC signal of the tumor

DNA and the red digoxigenin signal of the normal genomic DNA. Thus, DNA amplification or duplication of a chromosome in the tumor karyotype results in an increased green-to-red ratio (Kallioniemi et al., 1992). Conversely, DNA deletions or loss of a chromosome would result in a decreased green-to-red ratio. Fluorescent signals are quantitatively analyzed with digital image analysis equipment (Kallioniemi et al., 1992; Joos et al., 1993). In the system used by Kallioniemi et al. (1992), a fluorescence intensity profile of a chromosome was produced by summing the pixel values of green and red signals in strips across the width of the chromosome. The values of DA/DAPI counterstain images were used as an intensity reference.

Comparative genomic hybridization has been used successfully to determine the complex karyotypes of several cancer cell lines (Kallioniemi et al., 1992; Joos et al., 1993; Du Manoir et al., 1993), and it was able to verify an amplification at the c-myc locus in tumor DNA. To verify that the amplification occurred at the c-myc locus, a c-myc cosmid probe was hybridized to tumor metaphases and the position of the hybridization signal was compared with the site of amplification (Joos et al., 1993). This technique is particularly applicable to the analysis of tumor genomes, since metaphases for use with in situ hybridization may not be found, particularly in some hematological and many solid tumors, and tumor genomic DNA may be the only material available for study (Joos et al., 1993; Du Manoir et al., 1993).

Other advances in the acquisition of FISH probes may be more applicable to the study of individual marker chromosomes. Repetitive

DNA probes have been generated by the polymerase chain reaction using primers derived from chromosome-specific alphoid DNA monomers. This approach is quicker and easier to perform than producing these probes by cloning (Dunham et al., 1992). Painting probes may also be derived by PCR with microdissected chromosomal material. Banded chromosome regions may be cut out by laser or a fine glass needle and handled with an electronic micromanipulator (Deng et al., 1992; Bohlander et al., 1992). DNA extracted from the isolated chromosomal material is then used as a template for PCR. Flow-sorted marker chromosomes have also been used as a source of PCR-generated probes for reverse painting. Blennow et al. (1992) used this approach to identify a large marker chromosome, derived from chromosomes 5, 7, and X, by both reverse and forward painting. A probe library derived from the marker hybridized to regions of these three chromosomes in normal metaphase spreads (Blennow et al., 1992). The orientation of the material from each chromosome within the marker was then determined by forward painting, in which probe mixtures from chromosomes 5, 6, and X were hybridized to metaphases containing the marker (Blennow et al., 1992).

The increasing sophistication of the FISH technique offers the opportunity to study individual marker chromosomes in great detail. Of course, these new methods are not presently applicable for routine prenatal diagnostic cases. FISH with commercially available repetitive DNA and painting probes, on the other hand, may be performed in two days, with consistent results, and is becoming a commonly used adjunct to prenatal cytogenetic analysis. Furthermore, large surveys based on the

chromosomal identity of the material in individual markers have not yet been performed. In order to contribute to one such effort, the results from the experiments reported here will be sent to D. F. Callen of the Adelaide Children's Hospital, North Adelaide, SA, Australia. Dr. Callen is gathering an international collection of prenatal diagnoses of marker chromosomes identified by FISH in an attempt to understand the correlation between genotype and phenotype in marker cases. Such collections of FISH data may produce a clearer picture of the risk presented by individual markers found prenatally than has been possible from larger surveys based on staining analysis.

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APPENDIX I

FLUORESCENT IN SITU HYBRIDIZATION METHOD FOR ONCOR SUPPLIED CENTROMERIC PROBES

This method is adapted from the ONCOR Chromosome In Situ Protocol

Day 1

1. Prepare metaphase chromosome spreads or interphase nuclei on a glass microscope slide. Do not heat age the slides. For best results use prepared slides within two days. If using older slides (1 week to 1 month) denature slides between 2 and 3 minutes.

RNASE TREATMENT AND DEHYDRATION

2. Prepare 40 ml of 1XRNase solution per 4 test slides.
3. Prewarm the RNase solution to 37 C.
4. Add the slides to the prewarmed RNase and incubate for 1 hour in the water bath.

   Record the temperature and monitor the temperature every 15 minutes.

   Shake slides periodically.
5. Rinse slides 4 times in 40 ml of 2XSSC at pH 7.0, at room temperature for 2 minutes each.

6. Dehydrate slides in a series of ice cold (-20 C) ethanol washes: 70%, 80%, and 95%. Use glass coplin jars for washes, 2 minutes each and gently shake slides.

7. Dry slides quickly under an air jet. Warm slides to 37 C.

DENATURATION

8. Prepare 40 ml of denaturation solution per 3 test slides. Prewarm denaturation solution to a minimum of 70 C. If multiple slides are processed simultaneously, each slide will cause the solution to drop 1 C. Therefore the temperature of the solution must be raised 1 C for each slide to be added to denaturation solution. Time and temperature are very important to maintain chromosome morphology and allow hybridization of the probe.

9. Denature the slides by immersing them in the glass coplin jar for 2 minutes at the appropriate temperature with periodic agitation.

10. Immediately transfer slides to glass coplin jars containing 40 ml ice cold (-20 C) 70% ethanol and rinse for 2 minutes.
    Repeat rinse in cold 80%, 90%, and 100% solutions for 2 minutes each.

11. Dry under an air jet immediately.

12. Prewarm slides to 37 C.

HYBRIDIZATION

13. For each slide to be hybridized with a chromosome-specific satellite DNA probe add the following:

    1.5 ml of biotin-labeled DNA probe

30.0 ml of Hybrisol VI

Make a tube for each slide. do not make a batch preparation.

14. Denature probe by heating the tube in a 70 C water bath for 5 minutes. Quickly chill in a 4 C ice bath.

15. Spin probe in eppendorf tube for 1 minute in nanofuge to collect all probe solution.

16. Place 30 ml of the denatured hybridization mix in a continuous stream along each slide and cover with a glass coverslip.

Be careful to avoid air bubbles under the coverslip.

Seal glass coverslip with the sealant by applying sealant all along the perimeter of the coverslips using a 1cc syringe.

17. Incubate slides at 37 C for 4-`6 hours in a humidified chamber. Construct the humidified chamber using a small Tupperware tray with a lid. Soak several paper towels in water and place at the bottom of the tray. Place two wooden dowels across the towels. Place slides across dowels. Put the lid on top and place chamber in the 37 C incubator.

Day 2

POST WASHING

1. Prewarm post washing solution to appropriate temperature:

chromosome-specific alphoid probe	PW1 at 43 C
all human centromeric probe	PW2 at 37 C
beta DNA probes	PW1 at 37 C

2. Carefully remove the coverslip sealant with forceps. Do not remove the coverslip; it will fall off during the washing steps.

3. Immerse slides in appropriate post washing solution at the appropriate temperature.

   Frequently shake slides and incubate for 20 minutes.

4. Remove slides from post wash solution, blot off excess liquid from slide by placing edge of slide along a paper towel.

5. Quickly place slides into 40 ml of 2XSSC and gently shake slides for 4 minutes at 37 C. Repeat once.

6. Remove slides, blot off excess fluid and place slides in 40 ml 1XPBD at room temperature and proceed immediately to detection.

   Do not allow slides to dry beyond this step.

   If necessary, slides can be stored at 4 C in 1XPBD for up to 2 weeks.

DETECTION

7. Remove slides from 1XPBD and blot excess fluid from the edge.

8. Apply 60 ml of blocking reagent 1 to each slide.

   Place parafilm coverslips over the solution.

   Incubate for 5 minutes at room temperature.

9. Carefully peel back coverslip with forceps, tilt slide and allow fluid to drain.

10. Apply 60 ml of fluorescein-labeled avidin to each slide and replace plastic coverslip.

    Incubate 20 minutes in a humidified chamber at 37 C.

11. Wash slides 3 times in 40 ml of fresh 1XPBD at room temperature for 2 minutes each wash.

AMPLIFICATION

12. Apply 60 ml of blocking reagent 2 to each slide.
    Add fresh parafilm coverslip.
    Incubate for 5 minutes at room temperature.
13 Carefully peel back coverslip with forceps, tilt slide and allow fluid to drain.
14. Apply 60 ml of biotinylated anti-avidin antibody to each slide and replace parafilm coverslip.
    Incubate for 20 minutes at 37 C in a humidified chamber.
15. Wash slides 3 times in 40 ml of fresh 1XPBD at room temperature for 2 minutes each wash.
16. Apply 60 ml of blocking reagent 1 to each slide and replace plastic coverslip.
    Incubate for 5 minutes at room temperature.
17. Peel back coverslip and allow fluid to drain.
18. Apply 60 ml of fluorescein-labeled avidin to each slide and replace parafilm coverslip.
    Incubate 20 minutes at 37 C in humidified chamber.
19. Wash slides 3 times in 40 ml fresh 1XPBD at room temperature for 2 minutes each wash.

CHROMOSOME STAINING

20. Stain chromosome by adding 19 ml of a 1v:1v solution of propidium iodide/antifade:antifade solution to each slide.

21. Cover with a parafilm coverslip and view under the microscope.

APPENDIX II

FLUORESCENT IN SITU HYBRIDIZATION METHOD FOR ONCOR SUPPLIED PAINT PROBES

Day 1

1. Prepare metaphase chromosome spreads or interphase nuclei on a glass microscope slide.

   Do not heat age the slides. For best results use prepared slides within two days. If using older slides (1 week to 1 month) denature slides between 2 and 3 minutes.

2. Follow sequential GTW to FISH procedure unless otherwise specified.

SEQUENTIAL METHOD

3. Immerse slides in solution containing 30 ml of trypsin-EDTA and 10 ml of distilled water for 15 seconds.
4. Immediately rinse slides in two changes of a 40 ml solution containing fetal bovine serum and distilled water, and then a 40 ml solution of distilled water.
5. Cover slides with 5 ml of Wright's stain and let stand for 1 minute, 15 seconds. Rinse slides with tap water.
6. Apply mounting medium to glass coverslip and place coverslip on slide.
7. Photograph G-banded metaphases according to established cytogenetics laboratory protocols.

8. Soak slides in xylene minimum amount of time to remove coverslip. Put slide in fresh xylene (5 minutes or less) to clean off all mounting medium. Rinse in tap water and dry.

9. Rinse Wright's stain from slides by immersing slides sequentially in the following solutions for 1 minute each:

    30 ml of methanol/10 ml acetic acid

    40 ml of 70% ethanol

    40 ml of 80% ethanol

    40 ml of 90% ethanol

    40 ml of 100% ethanol

10. Dry under air jet.

RNASE TREATMENT AND DEHYDRATION

11. Prepare 40 ml of 1XRNase solution per 4 test slides.

12. Prewarm the RNase solution to 37 C in a water bath.

13. Add the slides to the prewarmed RNase and incubate for 1 hour.
    Record and monitor the temperature every 20 minutes.
    Shake slides periodically.

14. Rinse slides 4 times in 40 ml of 2XSSC (pH 7.0), at room temperature for 2 minutes each time.

15. Dehydrate slides in a series of ice cold (-20 C) ethanol washes: 70%, 80%, and 95%. Use glass coplin jars for washes, 2 minutes each and gently shake slides.

16. Dry slides quickly under an air jet. Warm slides to 37C.

DENATURATION

17. Prepare 40 ml of denaturation solution per 3 test slides. Prewarm denaturation solution to a minimum of 70 C. If multiple slides are processed simultaneously, each slide will cause the solution to drop 1 C. Therefore the temperature of the solution must be raised 1 C for each slide to be added to the denaturation solution.

    Time and temperature are very important to maintain chromosome morphology and allow hybridization of the probe.

18. Denature the slides be immersing in the glass coplin jar for 2 minutes at the appropriate temperature with periodic agitation.

19. Immediately transfer slides to glass coplin jars containing 40 ml ice cold (-20 C) 70% ethanol and rinse for 2 minutes.

    Repeat rinse in cold 80%, 90%, and 100% solutions for 2 minutes each time.

20. Dry under an air jet immediately.

21. Prewarm slides to 37 C.

HYBRIDIZATION

22. Incubate the stock painting probe for 5 minutes at 37 C.

23. Aliquot 20-30 ml into a small eppendorf tube for each slide. Denature probe at 70 C for 10 minutes. Spin for 2-3 seconds in a vortex.

24. Place in a 37 C water bath and preanneal for 2.5 hours. Spin for 2 or 3 seconds to collect contents.

25. Place 20-30 ml of the paint probe in a continuous stream along each slide and cover with a glass coverslip.

    Be careful to avoid capturing air bubbles under the coverslip.

    Seal glass coverslip with sealant by applying sealant all along the perimeter of the coverslip using a 1cc syringe.

26. Incubate slides at 37 C for 4-16 hours in a humidified chamber.

Day 2

POST WASHING

1. Prewarm post wash solution 2 to 43 C.
2. Carefully remove the coverslip sealant with forceps.

   Do not remove the coverslip, it will fall off during the washing steps.
3. Place slides in the prewarmed post wash solution 2 at 43 C with constant shaking for 15 minutes.
4. Remove slides from post wash solution, blot off excess liquid from slides by placing edge of each slide along a paper towel.
5. Quickly place slides in 40 ml of 0.1XSSC and gently shake slides for 15 minutes at 60 C.
6. Remove slides and blot off excess fluid and place slides in 40 ml 1XPBD at room temperature for 5-10 minutes, allowing the slide to cool to room temperature before going on to detection.

   Do not allow slides to dry beyond this step.

   If necessary, slides can be stored at 4 C in 1XPBD for up to 2 weeks.

DETECTION

7. Remove slides from 1XPBD and blot excess fluid from the edge. Do not allow the slides to dry.
8. Apply 60 ml of blocking reagent 1 to each slide. Place parafilm coverslip over the solution. Incubate for 5 minutes at room temperature.
9. Carefully peel back coverslip with forceps, tilt slide, and allow fluid to drain.
10. Apply 60 ml of fluoroscein-labeled avidin to each slide and replace plastic coverslip. Incubate 20 minutes in a humidified chamber at 37 C.
11. Wash slides 3 times in 40 ml of fresh 1XPBD at room temperature for 2 minutes each wash.

AMPLIFICATION

12. Apply 60 ml of blocking reagent 2 to each slide. Add fresh parafilm coverslip. Incubate for 5 minutes at room temperature.
13. Carefully peel back coverslip with forceps, tilt slide, and allow fluid to drain.
14. Apply 60ml of anti-avidin antibody to each slide and replace parafilm coverslip. Incubate for 20 minutes at 37 C in a humidified chamber.
15. Wash slides 3 times in 40 ml of fresh 1XPBD at room temperature for 2 minutes each wash.
16. Apply 60 ml of blocking reagent 1 to each slide and replace parafilm coverslip. Incubate 5 minutes at room temperature.

17. Peel back coverslip and allow fluid to drain.

18. Apply 60 ml of fluorescein-labeled avidin to each slide and replace parafilm coverslip. Incubate 20 minutes at 37 C in humidified chamber.

19. Wash slides 3 times in 40 ml of fresh 1XPBD at room temperature for 2 minutes each wash.

CHROMOSOME STAINING

20. Stain chromosome by adding 19 ml of a 1v:1v solution of propidium iodide/antifade solution to each slide.

21. Cover with a glass coverslip and view under the microscope.

APPENDIX III

PREPARATION OF WORKING REAGENTS

Adapted from Oncor Chromosome In Situ Protocol

1. Prepare 2XSSC by adding:

    50 ml of 20XSSC (supplied by Oncor)

    + 450 ml of distilled water

    500 ml Total

    Adjust pH to 7.0 using HCl. Solution may be prepared and stored as 2XSSC at room temperature for up to 1 year.

2. Prepare 1XRNase solution by adding:

    40 ml of 1000XRNase (supplied by Oncor)

    + 40 ml of 2XSSC

    40.04 ml Total

    Prepare fresh.

3. Prepare Denaturation Solution (70% formamide) by adding:

    4 ml of 20XSSC

    8 ml of distilled water

    + 28 ml of formamide

    40 ml Total

    Prepare fresh. Heat to 70 C in a glass coplin jar by immersing in a 70 C water bath.

4. 10XPBD settles into two phases during shipping. In order to prepare 1XPBD the entire volume of 10XPBD must be diluted. To prepare 1XPBD add :

390 ml	10XPBD
+3510 ml	distilled water
3900 ml	Total

Store 1XPBD at 4 C for up to 1 year.

5. Prepare Post Washing Solution 1 by adding:

4 ml	20XSSC
10 ml	distilled water
26 ml	formamide
40 ml	Total

6. Prepare Post Washing Solution 2 by adding:

4 ml	20XSSC
16 ml	distilled water
20 ml	formamide
40 ml	Total

APPENDIX IV

FLUORESCENCE MICROSCOPY OF ONCOR REPETITIVE DNA, SINGLE-COPY, AND PAINTING PROBES

Adapted from Zeiss Axiophot Photomicroscope Operating Instructions

VIEWING

1. Turn on microscope lamp power supply at least 15 minutes before using microscope.
2. Place slide on specimen stage so that it is held in place against the specimen holder by the spring clip.
3. Use the 10X objective to locate metaphases. Pull out light block slider, located on the side of the microscope housing near the right camera port, to the second stop. It is important to keep the slider closed when not viewing or photographing slides in order to prevent fading of fluorescent signals. When propidium iodide is used as the conter-stain, set filter slider so that the slide is viewed through the Texas Red filter. Chromosomes and interphase nuclei will be colored red only.
4. Scan slide for suitable metaphases for observation at higher power. To move the stage, use the coaxial controls suspended from the stage. Suitable metaphases should have chromosomes which are spread out with few overlapping chromosomes. Focus with coarse and fine adjustments.
5. Switch to higher power with the 63X oil immersion lens. Turn the nosepiece so that no objective is positioned above the slide. Place a small drop of immersion

oil on the coverglass below where the lens would be positioned. Turn the 63X objective from the side through the oil drop, passing back and forth through the locked position of the objective untel a bubble-free layer of oil is formed between the lens and the cover slip. When the specimen is in focus with the 10X objective, the 63X objective should be at the proper height to come into contact with the oil drop when locked into position above the slide. Focus with the fine adjustment.

6. Move filter slider so that slide is observed through the FITC filter. Focus once again. Evaluate the metaphase under observation for the quality of its fluorescent signals. These signals should be bright, well defined, and limited to the chromosome structure being probed (e.g., the centromere or the locus of a single-copy probe). Cross-hybridization should be minimal, whether cross-hybridization to incorrect chromosomes or chromosome regions or extrachromosomal background hybridization.

PHOTOGRAPHY

7. Turn on camera control panel.
8. Set film speed (ASA) to 800 on the side of the camera by pressing the button on top of the camera and adjusting the switch on the side of the camera. The adjusted ASA will automatically appear on the control panel.
9. To load film, detach the camera from the microscope by pressing the button on top and pulling off the camera. Move the lock on the bottom of the camera in the direction of the arrow. The back can then be removed. Load the cartridge, insert the film leader into the slot in the take-up spool so that the sprocket teeth catch

the perforations in the leader. Tighten the film by turning the take-up spool outward. Replace the back of the camera and attach the camera to the microscope.

10. Make sure that the camera selector on the control panel indicates the side of the microscope to which the camera has been reattached (35L or 35R). If the appropriate camera selector button has been pushed, the film leader will automatically advance.

11. Set exposure time manually by pressing the button labeled MAN, entering the time in seconds on the keypad, and pressing ENTER. An exposure time of 10-15 seconds has been found to be ideal for Oncor supplied repetitive DNA or painting probes, but a range of times should be examined in order to determine the best compromise between the brightness and the clarity of probe and propidium iodide fluorescent signals.

12. In order to position the metaphase under observation properly within the camera's field, pull the beam splitter out to the first stop and press the FRAME button. Luminous frame line reticles will be displayed in the field. Lower the intensity of these lines by holding down the FRAME button. Bring the reticles into focus with the fine focus knob. Position the metaphase within the frame lines, and then turn off the reticle display.

13. Pull beam splitter out to the second stop, so that all light reflected from the slide goes to the camera. Press START to open the shutter and take an exposure. The exposure time will count down to zero on the control panel. It is important to make sure that the microscope is not moved during the exposure.

14. Note the X, Y coordinates of the metaphase on a count sheet, referring to the numbered gradations engraved at the back and right side of the stage. Also note

the exposure number, slide number, and patient's name on the count sheet. The number of exposures taken on a roll of film is displayed on the control panel under the label COUNT.

15. Push beam splitter all the way in, return to the 63X objective, and locate the next metaphase to be photographed.

16. After photography is completed, rewind the film by sliding the switch labeled R on the back of the camera to the right. Detach the camera from the microscope, remove the back of the camera, and take out the roll of film.