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TELOMERIC PROBES FOR FISH: TECHNICAL ASPECTS AND CLINICAL APPLICATIONS

by

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LIST OF ABBREVIATIONS

bp	base pairs
BrDU	5-bromodeoxyuridine
CA	congenital anomaly
CHEF	contour clamped electrophoresis
DAPI	diamidinophenylindole
del	deletion
der	derivative chromosome
DS	Down syndrome
FISH	fluorescence in situ hybridization
FITC	fluorescein-isothiocyanate
G-Banding	Giemsa banding
kb	kilobase pairs
Mb	megabase pairs
MR	mental retardation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
SDS	sodium dodecyl sulfate
SINE	simple interspaced repetitive element
SSC	saline sodium citrate
WCPP	whole chromosome painting probe
YAC	yeast artificial chromosome

ABSTRACT

Terminal regions of chromosomes are frequently involved in structural chromosomal abnormalities and are prone to rearrangements. They are also gene rich, and have been shown to be associated with a number of clinical conditions. This study focuses on telomere-specific fluorescence in situ hybridization (FISH) probes. It addresses the technical aspects of their preparation and application, and examines whether FISH using these probes is a valuable tool for detection of aberrations in the terminal regions of chromosomes. A set of telomere specific probes was generated from half yeast artificial chromosome (YAC) and cosmid clones by DNA preparation via Alu-PCR, alkaline lysis, cesium chloride-ethidium bromide centrifugation, or using a "Qiagen" kit. Probe quality and optimal FISH conditions were established by hybridizing half-YACs specific to telomeres 21q, 18q, 10p, and cosmids for telomeres 2q/8p,13q,14q and 20p to normal metaphases. Probes for 10p, 13q, 14q , 18q and 21q yielded clear and specific hybridization signals, present in over 90% of metaphases anlayzed. Interphase analysis using the probes was not accurate. The ability of the telomeric probes to characterize balanced and unbalanced abnromalities was established by hybridization to patients with previously diagnosed chromosomal aberrations. The probes identified and confirmed partial trisomies 21q, 18q, and balanced translocations t(8;14), t(6;14), t(8;18), t(5;10). In three of the cases, partial monosomy 18q, translocation t(10;13), and t(1;10), the probes were able to provide information which was not revealed by banding analysis. This study demonstrates the ability of telomere specific probes to characterize aberrations in the terminal regions of chromosomes, and concludes that FISH using the telomeric probes is a valuable tool for clinical cytogenetics.

RÉSUMÉ

Les régions terminales des chromosomes sont fréquemment impliquées dans les anomalies structurelles des chromosomes et démontrent une tendance à se réarranger. Egalement, ces régions sont riches en génes et sont associées à un certain nombre d'affections cliniques. Cette étude se concentre sur les sondes spécifiques des télomères de chromosomes utilisées pour l'hybridation *in situ* fluorescente (HISF). Elle aborde les aspects techniques de la préparation des sondes et leurs applications, et examine si l'HISF avec ces sondes est un outil valable pour la détection d'anomalies des regions terminales des chromosomes. Un groupe de sondes de demi chromosomes artificiels de levure (half-YAC) et de cosmides spécifiques des télomères de chromosomes ont été préparé par Alu-PCR, lyse alcaline, centrifugation en gradient de chlorure de cesium -bromure d'ethidium, ou en utilisant un kit "Qiagen". La qualité des sondes et les conditions optimales HISF ont été établies par hybridation des chromosomes artificiels de levure spécifiques pour les télomères 10p, 18q, et 21q, et des cosmides pour les telomeres 2q/8p, 13q, 14q, et 20p sur des métaphases provenant de sujets normaux. Les sondes pour les chromosomes 10p, 13q, 14q, 18q, et 21q ont généré des signaux d'hybridation clairs et specifiques presents dans plus de 90% des métaphases analysées. L'analyse en interphase avec les sondes s'est révélée imprécise. La capacité de ces sondes à caractériser des anomalies chromosomiques équilibrées et non-équilibrees a été établie par hybridation sur des préparations chromosomiques de patients ayant eu un diagnostic d'aberration chromosomique dans le passé. Les sondes ont pu identifier et confirmer des trisomies, 18q, et 21q, et des translocations équilibrées: t(8;14), t(6;14), t(8;18), et t(5;10). En trois occasions, dans des cas de monosomie partielle 18q, et de translocation t(10;13) et t(1;10), les sondes ont pu fournir une information que n'avait pas été dévoilee avec les méthodes de marquage cytogénétique. Cette étude démontre l' aptitude des sondes spécifiques des télomères a caractériser les aberrations des régions terminales des chromosomes, et permet de conclure que ces sondes representent des outils valables pour la cytogénétique clinique.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 CHROMOSOME ABERRATIONS

In 1956 Tjio and Leven reported the correct number of chromosomes in a human somatic cell. By the end of 1959 numerical chromosomal abnormalities were already linked to three different clinical disorders. Ford discovered the 45,X association with Turner syndrome. Lejeune discovered trisomy 21 in patients with Down syndrome. Jacob associated Klinefelter syndrome with a 47,XXY karyotype. Today more than 600 different chromosome disorders are known (Connor and Ferguson-Smith, 1991).

Chromosome abnormalities are classified as numerical or structural. Numerical aberrations include aneuploidy and polyploidy. Common aneuploidies seen in newborns are trisomies of chromosomes 13,18 and 21. Also frequent are karyotypes with an abnormal number of sex chromosomes, 47,XXY, 47,XYY, 47,XXX and 45,X. Aneuploidies usually arise during meiosis or mitosis from non-disjunction of chromosomes in anaphase. Of those originating in germ cells, autosomal aneuploidies are predominantly maternal, whereas sex chromosomal aneuploidies mostly originate from paternal non-disjunction (cited in: Van Hummelen et al., 1996). Meiotic non-disjunction occurs at an increased frequency with increasing maternal age.

In polyploidy, the other type of numerical chromosomal abnormality, the extra set of chromosomes usually results either from dispermy or from failure of the maturation divisions of either the egg or the sperm. 69,XXY is the most common polyploidy.

Structural aberrations include reciprocal, Robertsonian and insertions, deletions, ring chromosomes, duplications, inversions, isochromosomes, and centric fragments. The aberrations result from chromosome breakage, followed by incorrect repair. The outcome of this mechanism can be a balanced karyotype, with no excess or lack or chromosomal

material, or an unbalanced karyotype, where modification of DNA took place resulting in partial monosomy or trisomy.

An excess or a lack or chromosomal material is usually associated with pathology. Chromosomal anomalies which cause the most severe effects result in spontaneous abortions. Over fifty percent of live born infants with chromosomal abnormalities show congenital anomalies, or manifest mental retardation, behavioral abnormalities, malformations or other phenotypic changes later in life (reviewed in: Hook 1982; Robinson and Linden, 1993). In general, autosomal abnormalities tend to be more severe than sex chromosomal abnormalities, and deletions more severe than duplications (Connor and Ferguson-Smith, 1991). Sometimes an abnormal phenotype can also be present with an apparently balanced rearrangement.

The estimated frequency of chromosomal anomalies among all conceptions is 7.5% (Connor and Ferguson-Smith, 1991). However this number is only an estimate. The majority of chromosomal aberrations is detected following prenatal diagnosis, upon fetal death, presentation of low birth weight, or the appearance of certain other clinical manifestations. Therefore, chromosomal aberrations associated with a milder phenotype, those present in germ cells, blastocysts, or those resulting in early spontaneous abortions may be under-reported. Furthermore, there may be many cases where the chromosomal aberrations are too small to be detected.

1.2 DETECTION OF CHROMOSOMAL ABERRATIONS

In 1968 Casperson demonstrated that quincarine mustard staining of human chromosomes produced a distinct sequence of bands for each chromosome. Since the

1970s, conventional staining techniques have enabled cytogeneticists to identify all human chromosomes and to diagnose numerical and gross structural chromosomal abnormalities.

G-band analysis following Giemsa staining, has become the primary means of diagnosis of chromosomal aberrations. High resolution G-banding, which involves the use of prophase or prometaphase chromosomes, can yield 800-1200 bands, and allows for detection of duplications or deletions spanning 2-3 Mb in size.

However, this classical technique has limitations. It does not permit detection of the more subtle or "cryptic" aberrations, or of anomalies in G-band negative areas such as the terminal regions of chromosomes. Furthermore, in translocations, it is not possible to establish cytogenetically whether the telomere has remained on the original chromosome (insertion), has been lost or is present in a derivative translocation chromosome.

A technique, which since late 1980s has been used more commonly in cytogenetic laboratories for cases difficult to diagnose by banding analysis, is fluorescence in situ hybridization (FISH). By allowing the visualization of specific nucleic acid sequences on chromosomes, FISH allows for detection of numerical and structural chromosomal abnormalities at a molecular level. With FISH, target sequences as small as 1 kb can be routinely detected, and localization of probes containing as little 250 bp has been reported (Richard et al., 1994).

1.3 FLUORESCENCE IN SITU HYBRIDIZATION

1.3.1 Principles of FISH technique

In FISH: (1) probe containing target sequences of interest is labeled with non-isotopic reporter molecules (2) the labeled probe is denatured (3) sequences in the probe hybridize to the complementary sequences in denatured chromosomes (4) the hybridized

probe is linked to various fluorochrome dyes, and (5) using U.V. microscopy the site of hybridization is visualized as a fluorescent signal in metaphase or interphase cells (Fig. 1).

The most common way to label probes is to incorporate enzymatically nucleotides tagged with biotin or digoxigenin into the probe sequences by nick translation (Rigby et al., 1977). In this technique DNase I enzyme randomly nicks double or single stranded DNA to form mono and oligonucleotides, producing a free 3' hydroxyl and a free 5' phosphate group at each nick site. DNA polymerase I then removes one or more bases at the 5' side of the nick and incorporates unlabeled and biotin or digoxigenin labeled nucleotides in 5' to 3' direction complementary to the template strand.

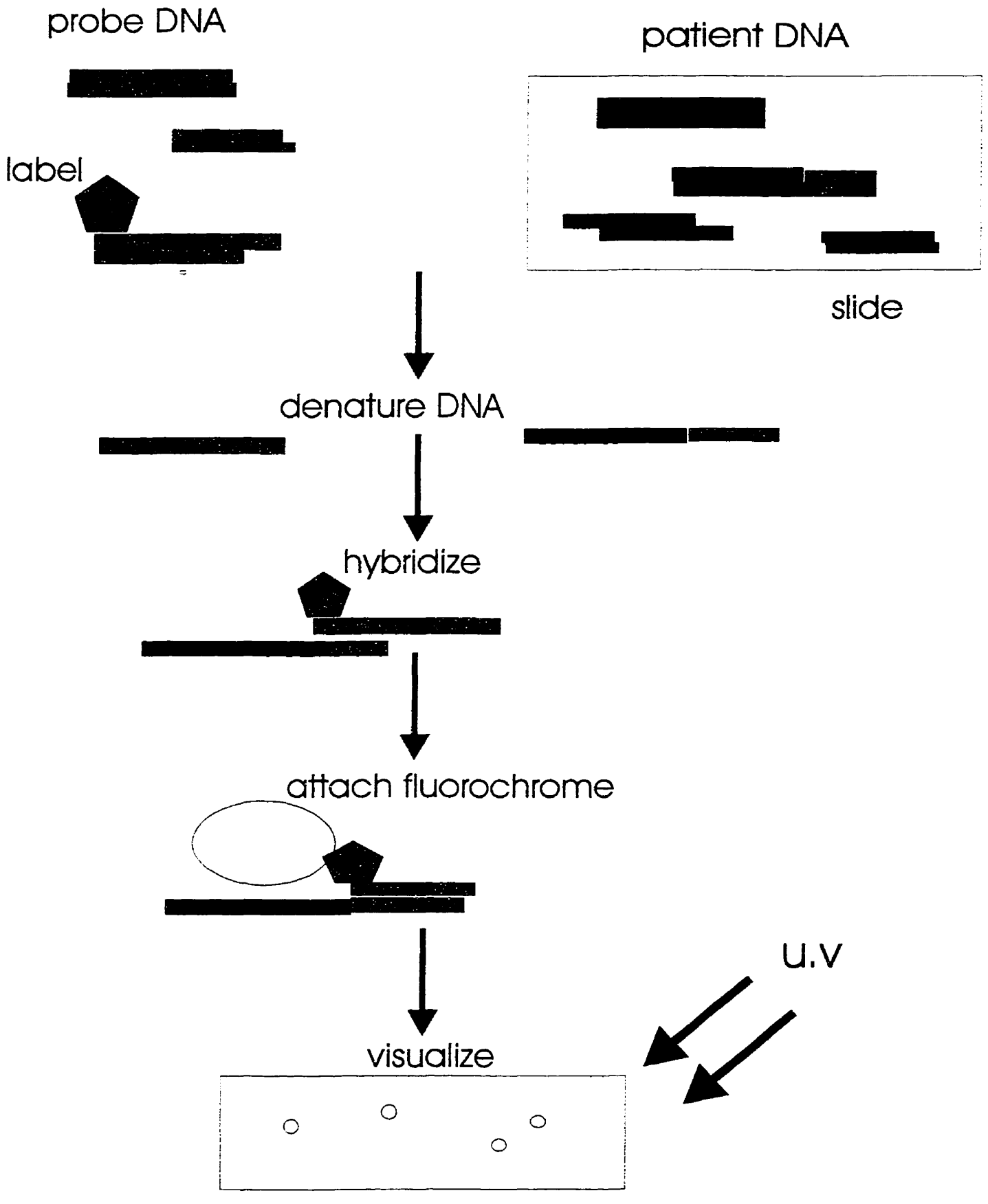
When probes are labeled by nick translation with biotin or digoxigenin, a detection step is necessary following hybridization, to attach detection molecules (usually antibodies) conjugated to fluorescent dyes to the labeled probe. Alternatively, instead of biotin or digoxigenin tagged nucletides, fluorescently labeled nucleotides are incorporated. When this is used, the detection step is eliminated and the probe can be visualized immediately.

Analysis of hybridized sequences consists of examining the number and/or the spatial relation of the fluorescent hybridization signals in metaphase chromosomes or interphase nuclei. A duplication is indicated by the presence of an extra fluorescent signal on the target, or another chromosome. The absence of a signalis interpreted, with proper controls, as a deletion. In cases of deletion, to differentiate between inefficient hybridization and the absence of target sequences, it is important to compare the frequency of cells carrying the deletion in the patient, to the frequency of unlabeled sites following hybridization of probe to normal cells.

Inversions are visualized as relocation of target signal relative to the centromere. Translocations are visualized as (1) separation of a signal generated from a probe

Fig1. Fluorescence in situ hybridization. Probe containing target sequences of interest is labeled with non-isotopic reporter molecules. The labeled probe and patient DNA (chromosomes) are denatured. Sequences in the probe hybridize to the complementary sequences in denatured chromosomes. The hybridized probe is linked to various fluorochrome dyes, and using U.V. microscopy the site of hybridization is visualized as a fluorescent signal in metaphase or interphase cells.

FISH


probe DNA
patient DNA
label
slide
denature DNA
hybridize
attach fluorochrome
u.v
visualize

covering the whole breakpoint area (2) separation of two signals from probes flanking the breakpoint or (3) juxtaposition of signals from two different chromosomal areas that were connected by a translocation event.

To assist analysis, it is possible to visualize the hybridized probe simultaneously with the chromosomal banding pattern by using chromosome preparations from thymidide synchronized and BrDU treated cultures (Lemieux et al., 1992). Alternatively, distinct chromosomal reverse (R) -banding can be achieved by co-hybridization with probes consisting of interspaced repeat sequences such as Alu repeats (Baldini et al., 1991)

1.3.2 Probes for FISH

1.3.2.1 Types of Probes

A variety of probe types are used for FISH (reviewed in:Yung, 1996). Particularly useful for identification of chromosomes and for assessment of numerical anomalies, are centromeric probes. The probes contain highly repetitive alphoid - DNA sequences which hybridize to centromeric or pericentromeric regions of specific chromosomes. They require a short hybridization time, generate strong compact signals, and are applicable to both metaphase and interphase FISH.

Another class of probes used for FISH is the whole chromosome painting probes (WCPP). These probes are composed of a mixture of sequences originating from the entire length of a single chromosome. Because a certain percentage of sequences in WCPP is also common to chromosomes other than the target chromosomes and has to be suppressed, these probes are pre-hybridized with unlabeled total genomic DNA (reviewed by: Trask, 1991). Hybridization with a WCPP probe results in "painting" or highlighting of the target chromosome. The probes are useful in the identification of small marker

chromosomes and for assessing structural rearrangements between different chromosomes.

Unique-sequence FISH probes contain locus-specific DNA segments. They can localize specific genes, and are very valuable for identification of cryptic aberrations undetectable by standard cytogenetic methods.

Most recently, clones specific to each of the human telomeres have also been isolated and characterized. These clones contain unique sequences located within the terminal 100-300kb of chromosomes (Ning et al., 1996). They have great potential for the detection of structural abnormalities in the terminal regions of chromosomes, and for studying of structural rearrangements which involve the telomeres. These probes will be discussed in more detail in section 1.7.

1.2.2 Cloning Vectors

Probes for FISH can be obtained from commercial companies which produce probes by amplifying selected DNA by PCR in presence of reporter or fluorescent labels. (Buckle and Kearney, 1994) Almost all human and centromeric probes are now commercially available. Region specific probes can also be synthesized for any part of the human genome by microdissection, followed by direct enzymatic amplification and labeling (Meltzer et al., 1992).

Unique sequence probes are usually generated in research laboratories, from clones of various vectors obtained from chromosome specific libraries. Probes containing up to 5 kb can be generated from plasmids, up to 50 kb from cosmids (Landegent et al., 1987; Lichter et al., 1990). There is also a number of large -insert libraries. P1 libraries contain clones that carry segments of 100 kb P1-derived clones, PACs, can accommodate inserts of 75-100 kb (Sternberg et al., 1992). Bacterial artificial chromosome clones or

BACs include fragments averaging 90 - 120 kb (Shizuya et al., 1992). Finally, clones containing the largest inserts, over 1 Mb in size, can be obtained from chromosome specific yeast artificial chromosome or YAC (Burke et al., 1987) libraries. Probes generated from YACs can consist of sequences spanning extended genomic regions, and can include unaltered complete genes.

1.2.3 Preparation of Vector Derived DNA for FISH

Plasmids, cosmids, P-1, PACs and BACs

Vector derived DNA may be prepared by standard alkaline lysis procedures. For this purpose, bacteria are suspended in an iso-osmotic sucrose solution. The cell wall and outer membrane are broken down by treatment with a solution containing SDS and NaOH. NaOH creates alkaline conditions which denatures chromosome and vector DNA. The SDS is a detergent which denatures cellular proteins and forms complexes with the bacterial chromosomes, proteins, and membranes. Potassium acetate is used to neutralize the lysate, and the high salt concentration causes denatured proteins, chromosomal, DNA, cellular debris and SDS to aggregate and precipitate from the lysate which retains the shorter vector DNA. Lithium chloride is used to precipitate the RNA in the lysate, and RNase to digest it. A combination of phenol,chloroform, isoamyl alcohol is used to extract further the DNA from the cell lysate. Phenol denatures proteins and removes solutes. Chloroform enhances action of phenol and stabilizes the phenol/aqueous phase. Iosamyl alcohol enhances the separation of organic and aqueous phases. Sodium acetate and ethanol precipitation is then used to concentrate DNA and remove residual organic solvents. DNA can be then labeled by nick - translation, used for PCR amplification, or further purified.

One of the most common purification methods used, which yields high quality DNA, free of most contaminants is cesium chloride-ethidium bromide (CsCl-EtBr) equilibrium centrifugation. In this technique, ethidium bromide binds to DNA by intercalating between bases, the double helix unwinds, and the inserted human DNA becomes linearized and separated from the closed circular vector DNA. The differential binding of the ethidium bromide dye to linear and closed circular vector DNA molecules leads to a difference in densities between the two types of molecules and to the separation of vector and linear DNA in the CsCl gradient. The RNA and protein also become separated.

DNA can also be purified using resin columns found in various commercial isolation and purification kits. RNA and cellular protein go through the column by gravity flow whereas salt and pH conditions cause vector DNA to bind to the resin. RNA and proteins are then eluted from the resin, and all contaminants removed from the column. Finally nucleic acids are eluted, and the DNA is concentrated and precipitated.

YACs

DNA from YAC clones can be prepared in solution or in agarose blocks by a modification of the alkaline lysis method. Total isolated yeast DNA can then be labeled by nick translation. However, because YAC DNA represents only between 8 and 15% of the total yeast DNA, many labs choose to isolate the YAC from the total yeast DNA and prepare FISH probes which contain only the human insert.

Nelson et al. (1989) reported on Alu - PCR as a method for rapid isolation of human-specific sequences from complex DNA sources. Alu is the most common member of the short interspaced repeated sequence (SINE) class, and consists of 300bp repeat sequences in length present every 4-5 kb in the human genome. However, because the

sequences are absent in yeast, when primers anchored in the Alu sequences are used in the PCR reaction, only human specific DNA fragments are amplified.

Lengauer et al. (1991) optimized Alu-PCR for the generation of probes from YACs containing human inserts between 100 and 800 kb in size, and by using this method were able to generate successful probes for FISH. The group designed two primers,. CL1 (5'TCC CAA AGT GCT GGG ATT ACA G'3), and CL2 (5' CTG CAC TCC AGC CTG GG 3'). The primers are located close to the 5' and 3' ends of the 300 bp long Alu block, and were chosen from the most conserved regions of the Alu repeat family. The primers were used in three separate reactions, the first using CL1 primer alone, the second using CL 2 primer alone, and the third using both CL1 and CL2 together, to amplify different regions of the YAC insert, depending on the spacing and orientation of the Alu elements within the insert.

Although the primers were directed outwards from the ends of the Alu sequence, there was still a significant amount of Alu repetitive DNA in the amplification products. To achieve clean and specific FISH signals, the repetitive sequences were supressed prior to hybridization with Cot-1 DNA (Cot-1 DNA is enriched for major classes of human repetitive DNA sequences).

Because Alu sequences are concentrated in the reverse (Giemsa negative) bands (Baldini et al., 1991; Manuelidis and Ward, 1984; Korenberg and Rykowski, 1988; Moyzis et al., 1989), and therefore in the telomeric regions of human chromosomes, this technique is very appropriate for preparation of probes from YACs containing sequences from terminal chromosomal regions.

1.3.3 Applications of FISH

FISH has been a very successful tool in clinical genetics, and reports of cases diagnosed or confirmed by FISH are numerous. The technique has helped to identify marker chromosomes, determine ploidy, and most of all, by allowing the detection of subtle chromosomal aberrations, has solved many diagnostic problems. Commercial FISH kits are available for diagnosis of common micro-deletion syndromes (Buckle and Kearney, 1994) and for sex-determination of pre -implantation embryos for couples at risk for an X-linked disease (Delhanty et al., 1993; Griffin et al., 1993).

FISH has allowed for chromosome abnormalities to be better understood, and has reduced the number of unresolved karyotypes. Because a majority of malignant tumors have an abnormal chromosome number and structure (cataloged in Mitelman, 1994), FISH has also been a very valuable tool for the study of cancer.

In addition to its applications in detection of chromosome aberrations, by allowing localization of sequences to chromosomes, FISH also plays an important role in gene mapping (Nadal et al., 1996; reviewed in: Trask, 1991 ; Yung, 1996). It is used in preliminary assignment of clones to a specific chromosomal bands, as well as in ordering of clones along the chromosomal axis. (Lichter et al., 1990; Riethman et al., 1989; reviewed by: Heiskanen et al., 1996; Nadal et al., 1996; Haaf and Ward, 1994). Metaphase FISH allows detection of sequences over 1 Mb apart. Mapping of sequences 200 kb - 1000 kb can be done on mechanically stretched chromosomes. In interphase nuclei, because the chromatin is less condensed than in metaphase chromosomes, FISH can be used to map probes as close as 50 kb apart. Finally, mapping to free chromatin fibers allows resolution of as little as 1 kb (reviewed by: Heiskanen et al., 1996; Heng et al., 1992)

Due to its spatial resolution and sensitivity , FISH also allows for the localization amplified genes in drug-resistant cells (Trask and Hamlin, 1989), and for detection and expression of viruses at a single-cell level (McNeil et al., 1991).

1.4 MULTICOLOR FISH

The potential of detecting chromosomal aberrations by FISH greatly increases with the availability of multicolor detection.

Multicolor FISH enables simultaneous visualization of target signals originating from more than one probe, following a single hybridization experiment. In this technique, multiple probes are labeled by various proportions of reporter molecules. The labeled probes are then tagged with different fluorophore dyes, each generating a different spectral range of emitted light. Probes labeled with a single reporter are visualized as a pure fluorophore , while probes labeled with a number of labels appear as fluorophore mixtures. Using this method, up to seven different probes have been observed at the same time (Ried et al., 1992). With recent development of epifluorescence filter sets and computer software for detection and discrimination of 27 different DNA probes, the number of target sequences visualized following a single hybridization experiment has increased even further (Speicher et al., 1996).

Multicolor FISH has become a very valuable tool in cytogenetics. By allowing incorporation of reference probes (ie. centromeric probes) into hybridization experiments, the technique has been particularly useful in cases where chromosomal identification is not possible, and when clinical samples are limited in number. Two common applications of multicolor FISH with reference probes are sperm analysis (van Hummelen et al., 1996; Martin and Rademaker, 1997) and preimplantation diagnosis (Manor et al., 1996; Munne et al.,1994; Harper et al., 1995).

By allowing simultaneous visualization of multiple chromosomal areas, the technique has facilitated analysis of translocation between chromosomes (Lucas et al., 1993). Finally , multicolor FISH is a very valuable tool for cases where a cryptic aberration is suspected, and where a candidate region has not been identified by clinical information. By utilization of multiple probes together many potential target regions can be screened following a single hybridization experiment.

In addition to its applications in clinical cytogenetics, multicolor FISH has also been very useful for simultaneous gene mapping, and ordering of clones to chromosomes.

1.5 TERMINAL REGIONS OF CHROMOSOMES

1.5.1 Telomeres

Human chromosomes terminate with telomeres. These regions have been extensively studied, and their structure and function are now well understood. Telomere sequence consists of TTAGGG repeated several hundred to several thousand times, and spans 5- 15 kb (Ijdo et al., 1991; Moyzis et al., 1988). This sequence is shared among vertebrates (Meyne et al., 1989), and the length of the repeats is greater in germ line than somatic cells. Studies of telomere length show that it decreases as a function of individual's age, and with *in vitro* cell divisions (Harley et al., 1990).

Telomeres prevent the degradation of DNA at the ends of linear chromosomes, and thus provide chromosomal stability(reviewed in: Blackburn, 1991). Without telomeres, or following telomere disruption, the ends of chromosomes fuse, leading to formation of dicentric, ring, or other unstable chromosomes (Blackburn and Szostak,1994; Park et al., 1992).

In vertebrates, in addition to the ends of chromosomes, telomeric sequences have also been observed interstitially. Rarely, as seen with human chromosome 2, this may be a

fixed characteristic of a certain chromosome pair (Ijdo et al., 1991). More commonly however, interstitial telomere sequences represent constitutional chromosome abnormalities resulting from chromosomal rearrangements (Rossi et al., 1993; Park et al., 1992 ; Boutouil et al., 1996). When observed in an interstitial position, the telomeric sequences appear non functional (Rossi et al., 1993; Park et al., 1992) and can cause chromosomal fragile sites at their location (Boutouil et al., 1996)

Human telomeres are synthesized by an RNA-containing enzyme telomerase. The enzyme was first described in *Tetrahymena* (Greider and Blackburn, 1985). The presence of telomerase in human cells was confirmed by Morin (1989) by identification of its activity in HeLa cell extracts. Telomerase activity occurs mainly in germ cells, and is limited in normal human somatic cells (Kim et al., 1994). These findings, together with evidence of telomerase activity being widespread among various tumors (Kim et al., 1994), associate telomerase with senescence and immortalization, and suggest that it plays a role in the life span of cells.

There is evidence that telomerase is responsible for healing of broken chromosomes. Addition of telomeric repeats into the ends of broken chromosomes that lacked pre-existing telomeric DNA was reported in *Tetrahymena* (Yu et al., 1991). TTAGGGn repeats can lead to telomere formation when re-introduced into mammalian cells (Farr et al., 1991). Addition of telomeric repeats by human telomerase in the alpha-globin region of human chromosome 16 was reported by Morin et al. (1991).

Different telomere binding proteins have been isolated. They alter telomeric chromatin structure and are involved in regulation of telomere length, packaging of telomeric DNA, and attachment of chromosomes to the nuclear matrix (Blackburn et al., 1991; Price, 1992; Zakian et al., 1989).

Studies suggest that telomeric chromatin structure can influence the expression and replication of proximal genes. Heritable and reversible silencing of genes placed near the telomeres occurs in *Saccharomyces* and fission yeast (Aparicio et al., 1991;.reviewed in: Price, 1992; Karpen, 1994). Similar position effects, where gene activity is inhibited by juxtaposition of heterochromatin on euchromatin, is seen in *Drosophila* (reviewed in: Greider, 1992.; Karpen, 1994). Recent work in yeast by Ferguson et al., (1992), suggests that location relative to the telomere also determines replication timing of genes. The extent to which human telomeres influence gene expression or timing of replication of adjacent genomic regions is not known.

1.5.2 Subtelomeric Regions

Proximal to the telomeric region, are subtelomeric regions. These are the sites of telomere- associated repeats (Kipling and Cooke, 1992; reviewed in: Tyler-Smith and Willard, 1993). Also in the subtelomeric region, within 100-300 kb from the end of most chromosomes, is unique sequence DNA.

The telomere-associated repeats often present as short tandem repeats, varying from 29 to 61 bp in length, and some are very G + C rich (Kipling and Cooke, 1992). At some telomeres the repeats are present in variable copy numbers, and at some they are absent (Kipling and Cooke, 1992).

Brown et al. (1990) studied the structure and polymorphism of human telomere-associated DNA and found several interesting properties. Some of the telomere-associated repeats appeared to be unique to a telomere. Most, however, were shared among several chromosomes. Furthermore, by observing that certain repeats could be found on different chromosome ends in different individuals, Brown et al. (1990) showed polymorphism.

A more detailed analysis of the subtelomeric region was performed by Wilkie et al. (1990). The group studied the alpha globin locus which lies in telomeric region of the short arm of chromosome 16 and demonstrated the existence of three different alleles, located at a distance of 170 kb, 350 kb, or 430 kb from the end of chromosome 16p. The two most common alleles, 170 kb and 350 kb, consisted of subtelomeric repeats which were not homologous in sequences to each other, but which were homologous to repeats in other chromosomes. Therefore an individual could have a non homologous chromosome pair,and one of the chromosomes could share subtelomeric sequences with a different chromosome. Homology of the 60 kb telomeric region of 4p to telomeric regions on 13p, 15p, 21p and 22p, has been shown by Youngman et al. (1992).

Studies show that the human subtelomeric regions have a higher than average recombination rates and a higher ratio of male to female recombination than the mean rate for the genome (Bernardi , 1989; Rouyer et al., 1990; Benger et al., 1991). Recently a study of the subtelomeric region of chromosome 14q, showed that the recombination in this region was higher in females than males, indicating that 14q differs from other human subtelomeric regions (Wintle et al., 1996).

Finally, by localizing the richest G+C fraction human DNA (Saccone et al., 1992), and the highest concentration of CpG islands (Wintle et al., 1996) to the telomeric bands (T bands) of chromosomes, it has been demonstrated that terminal chromosomal regions have the highest gene concentration in the genome. Subtelomeric regions of chromosomes may have an average of one gene every 23.4 kb (Fields et al., 1993). Therefore, any disruptions in these regions of chromosomes can affect genes of clinical relevance

1.6 TERMINAL REGIONS AND CHROMOSOME ABNORMALITIES

1.6.1 Terminal Regions and Structural Chromosomal Aberrations

Terminal regions of chromosomes are involved in many structural chromosomal aberrations. In the prenatal diagnosis laboratory at the Montreal Children's Hospital, they are implicated in over 95% of cases with structural anomalies (unpublished data). Exchange of telomeric material between two non homologous chromosomes is seen in balanced reciprocal translocations. Exchange of material between the telomeric regions of the short and long arm of the same chromosome occurs in pericentric inversions. Subtelomeric regions are affected when a loss or duplication takes place in terminal chromosomal segments. Terminal sequences become translocated to interstitial regions, or breakpoint junctions of abnormal chromosomes (Park et al., 1992; Rossi et al., 1993; Boutouil et al., 1996; Park et al. 1992; Rossi et al., 1993).

The higher rate of recombination in the subtelomeric regions could account for the high frequency of involvement of the telomeric regions in structural chromosomal abnormalities. There is also speculation, that this is a result of mispairing of chromosomes due to homology in the subtelomeric regions among non-homologs (Brown et al., 1990; Speed, 1988).

1.6.2 Terminal Chromosomal Aberrations and Clinical Relevance

Many terminal chromosomal abnormalities have been associated with various phenotypic abnormalities. The majority of the initial cases were detected using molecular methods. Lamb et al.(1989) used polymorphic markers to investigate the alpha-globin locus in a patient with hemoglobin H disease, dysmorphic features, and mental retardation, but an apparently normal karyotype. The group demonstrated the presence

of a deletion of one globin locus resulting from inheritance of a cryptic unbalanced translocation from the mother. Overhauser et al. (1989) used restriction fragment length polymorphism (RFLP) analysis to study a family in which several individuals had features of cri-du-chat syndrome, but no abnormalities of 5p detected by G-band analysis. RFLP analysis demonstrated monosomy in the terminal region of the short arm of chromosome 5 in three individuals. Biesecker et al. (1995) used subtelomeric microsatellite markers to screen for unbalanced translocations in children with multiple congenital anomalies of unknown cause. The group detected a segmental aneusomy involving the distal region of 22q in a child with severe multiple congenital anomaly syndrome. Flint et al. (1995) used informative variable number tandem repeats (VNTR) probes mapping to 28 terminal regions of chromosomes to study 99 patients with mental retardation. The study identified three cases of partial monosomy. Two of the deletions, 13q, and 22q were products of unbalanced translocations. The third monosomy, again involving 22q, was a result of a *de novo* terminal or interstitial deletion..

Although useful for diagnosis in certain cases, using polymorphic markers as a method of screening for genetic abnormalities, has disadvantages. Analysis requires samples from both parents. Markers have to be informative for the individual family. Furthermore, although the technique was successful in identifying deletions, duplications, as well as parental disomy, DNA polymorphism studies cannot detect balanced translocations or inversions.

FISH is a more efficient way to study both, visible and cryptic, balanced and unbalanced chromosome anomalies. As a number of telomere specific probes became available for FISH, the reports of cases of aberrations involving the terminal regions of various chromosomes increased.

FISH with telomeric probes was used when karyotype analysis was not consistent with clinical features. Mewar et al.(1994), used a probe specific to telomere of chromosome 1q and reported on an infant who presented with a trisomy 18 syndrome-like phenotype with a duplication/deletion in the terminal region of 1q. Similarly, FISH with a 2q telomeric probe was used to confirm a de novo terminal deletion involving the most distal sub-band of telomere 2q in a patient with a mild phenotype (Lin et al., 1992).

Alther et al.(1991) used telomeric 4p probes to investigate the origin of a 4p deletion detected by using highly informative DNA markers from distal 4p in a child with clinical features of Wolf-Hirschhorn syndrome. FISH with probes from distal 4p showed that the mother was a carrier of a balanced translocation between distal 4p and distal 19p.

Telomeric probes for the short arm of chromosome 8, and long arms of chromosomes 8, and 10 were used with FISH to demonstrate loss of the telomeres on the reciprocal chromosome in three unbalanced translocations involving chromosome 15 in the Prader-Willi and Angelman syndromes (Jauch et al., 1995).

James et al. (1996) used FISH with probe specific to 10q to investigate a patient with developmental delay and what cytogenetically appeared to be a duplication 10q. The studies led to unexpected findings and showed a deletion of 10q telomere and translocation of 19q material.

Kuwano et al. (1991) used probe for telomere 17 p to investigate the potential of FISH for detection of chromosomal abnormalities in patients with Miller-Dieker syndrome. The study showed that FISH using telomeric probes was a rapid and highly efficient method for the detection of deletions and cryptic translocations.

A supernumerary ring chromosome 20 was studied in a patient with mild dysmorphic features and developmental delay. FISH with a probe specific to 20p, distal to

the target site of the all -telomere probe narrowed down the breakpoint region in the ring chromosome (van Langen et al., 1996)

Wong et al. (1997) used FISH with a probe specific to the long arm of chromosome 22 to characterize further a 130 kb microdeletion in the subtelomeric region of 22q in a child with mental retardation. FISH analysis revealed that the deletion breakpoint was healed by the addition of telomeric sequences.

1.7 A COMPLETE SET OF TELOMERE SPECIFIC PROBES

Evidence that the terminal regions of chromosomes (a) contain a high concentration of genes; (b) are involved in many chromosomal aberrations(c) are associated with clinical conditions and (d) cannot be studied accurately by classical cytogenetics, created a demand for FISH probes specific to each human telomere.

In 1996, groups from the National Institutes of Health and the Institute of Molecular Medicine reported on the isolation and characterization of a complete set of FISH probes representing each human telomere (Ning et al., 1996).

1.7.1 Half- YACs

The material for the telomere specific probes was originally derived from Half - YACs. Half-YACs were constructed by ligation of human DNA fragments 50 to 300 kb in size into *Saccharomyces cerevisiae* using a yeast-artificial-chromosome (YAC) vector. This technique was especially designed by Riethman et al. (1989) for capturing terminal ends of human chromosomes.

The half-YAC vector (Fig. 2a) consists of a yeast replication origin ARS1, a yeast centromere sequence CEN, and the *Tetrahymena* telomere-repeat sequence Tr TEL, all

assembled in a plasmid. The vector also contains a selectable marker (URA3) for positive selection of transformants in ura3 hosts, as well as an amp gene for ampicillin resistance (for selection in bacterial cells), and ori (bacterial origin of replication) regions of pBR322 for growth and amplification of the plasmid.. Following linearization of the half-YAC, human DNA fragment containing terminal regions of chromosomes are ligated into the vector. Funtional yeast telomeres are formed by the healing of a TrTEL terminus, and addition of yeast terminal repeats to the human telomere. Following transfection into yeast cells, the half-YACs behave as normal linear yeast chromosomes, and replicate each time the cell goes through mitosis. (Reithman et al., 1989).

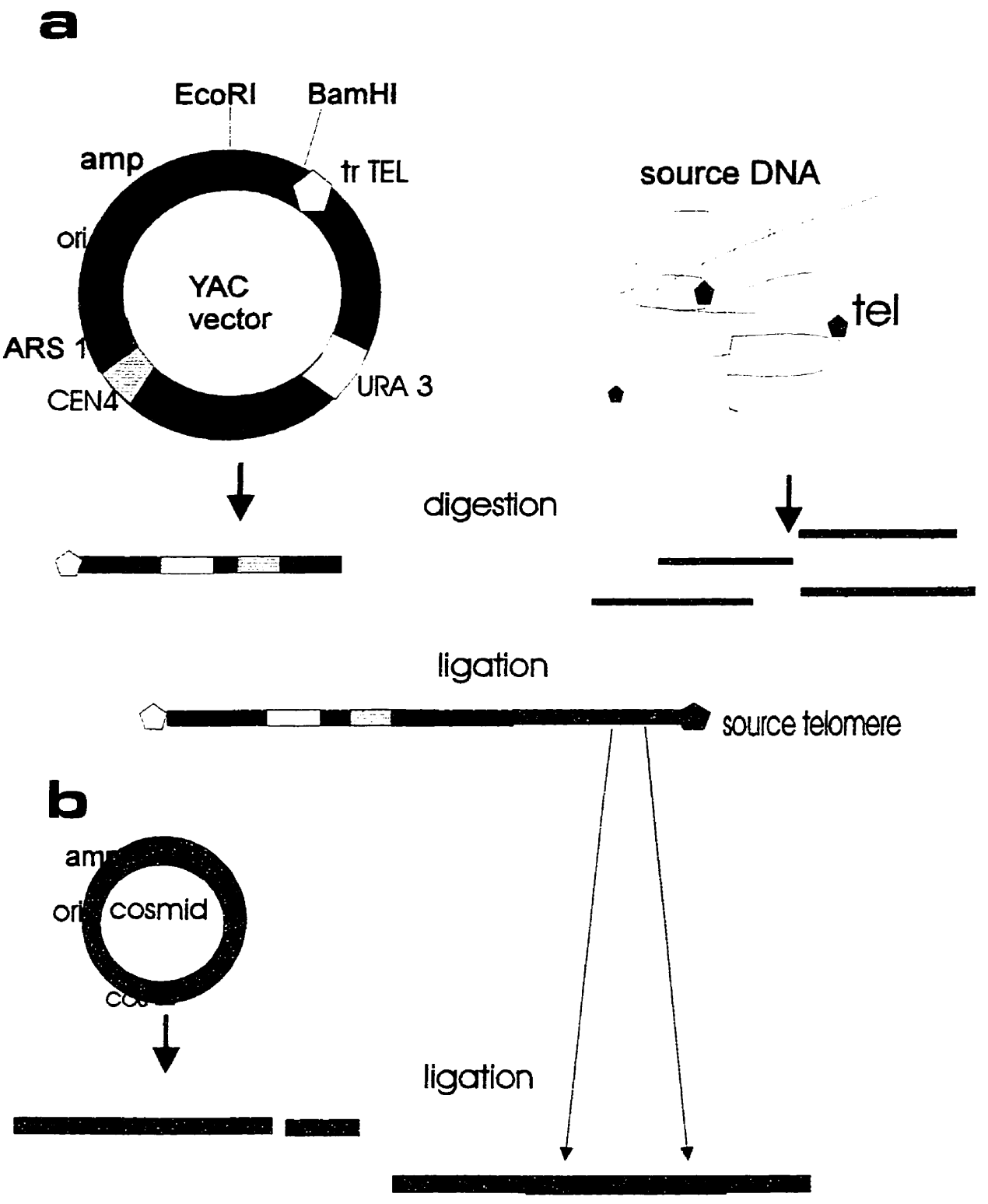
1.7.2 P 1s, PACs and cosmids

The human DNA fragments ligated into the half -YACs contained long stretches of subtelomeric repetitive sequences. In some cases, these sequences were telomere specific. In other cases, where half-YACs contained sequences common to more than one chromosome, a number of methods was used to isolate sequences which were chromosome unique.

The first method of isolating unique sequences from half-YACs involved using the vector -insert junction fragments representing the most proximal sequences of the cloned regions, to screen chromosome specific vector libraries or total human genomic P1 or PAC libraries and to isolate those clones which were telomere specific (Ning et al., 1996) The second method involved cloning of half -YACs into cosmids (Fig 2b), isolating individual clones and testing their specificity by FISH. Another method consisted of genomic walking, starting from a subtelomeric sequence. For telomeres not isolated successfully by these methods, the most distal STS marker on the current integrated map of the chromosome was used to isolate PAC clones. Again location and specificity were

tested by FISH (Ning et al., 1996). Finally the sequences in vectors were localized with FISH , those with telomere specific clones were tested on 5-10 unrelated individuals to

Fig.2. (a) Telomeric YAC cloning system. The vector contains the yeast replication origin ARS, yeast centromere sequence CEN4, the *Tetrahymena* telomere-repeat sequence TR TEL, selectable marker URA3, restriction sites EcoRI and BamHI, as well as origin of replication (ori) and a drug resistance marker (amp) regions of pBR322. Digestion of the vector with EcoRI and BamHI yields a linear fragment which is ligated to eukaryotic source DNA fragments. Functional yeast telomeres are formed by the healing of a TrTEL terminus, and the addition of yeast terminal repeats to the source telomere. (b)Fragments from half-YACs sub-cloned into cosmids. Cosmids carry a cos site required for packaging DNA into bacteriophage particles, an ori region and an amp region.


a
EcoRI
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ARS 1
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URA 3
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exclude polymorphism (Ning et al., 1996). All probes, including those generated directly from the half-YACs map to the terminal 100-300 kb region of each human chromosome arm (Ning et al., 1996).

1.7.3 Potential of Telomere Specific FISH Probes

The availability of FISH probes specific to each of the human telomeres has great potential for clinical applications. Because use of these probes with FISH provides a 10 fold improvement in the detection sensitivity of deletions, as compared to high-resolution banding (Ning et al., 1996), the probes should be suitable for diagnosis of previously undetectable subtelomeric abnormalities. The potential of the probe specific to telomere 1q to detect cryptic aberrations has already been demonstrated during the testing process of the newly isolated telomeric probes. The 1q cosmid probe, upon hybridization to a male patient with a normal phenotype and an apparently normal karyotype, showed an unexpected deletion. This probe was then applied to study the proband's family, and allowed the detection of a cryptic 1q;11p rearrangement in balanced and unbalanced states in multiple members of the family.

The telomeric probes should be suitable for studying translocations involving the terminal regions of chromosomes, and the effects that such rearrangements have on genes proximal to the site of translocation. If the telomeric probes are efficient at interphase FISH, they may be used for analysis of parrafin embedded tissues sections, tumor cells, as well as for non-invasive screening of fetal cells and pre-implantation embryos. Furthermore, telomeric probes should facilitate the search for genes located in their target regions and assist in establishing genotype-phenotype correlation in patients.

1.8 AIMS OF THE RESEARCH PROJECT

The objective of this project was to evaluate laboratory- generated telomere specific half-YAC and cosmid probes for use in FISH and to demonstrate their value for clinical cytogenetic analysis. More specifically the aims were as follows:

1. To generate a set of half-YAC and cosmid probes using various DNA preparation methods
2. To find optimal FISH conditions for each of the probes by application to normal chromosomes
3. To use the probes to study clinical cases of balanced and unbalanced aberrations
4. To evaluate the application of these probes as a diagnostic tool for clinical cytogenetics.

CHAPTER 2

MATERIALS AND METHODS

2.1 OVERVIEW

Yeast strains containing half-YACs specific to the telomeric region of 22 different chromosome arms, and 5 cosmids subclones from the YACs, were kindly contributed by Dr. Reithman from the Wistar Institute, Philadelphia. The half-YAC and cosmid clones, and their corresponding telomeres are listed in the appendix.

The sequence of methods used in this study is outlined in Figure 3. The DNA from the yeast strains was prepared in agarose blocks, the presence of YAC was checked by pulsed field gel electrophoresis and Southern Blot hybridization, and human specific sequences were amplified from the yeast DNA by Alu- PCR. Cosmid DNA was prepared in solution by the alkaline lysis method, and purified by ethidium bromide centrifugation or using a resin column from a Qiagen Kit. Purified half-YAC and cosmid DNA was then labeled by nick translation. To test the probes, FISH was first performed on normal human chromosomes. Multiple hybridization experiments were performed to define the optimal FISH conditions for each probe. Finally, the usefulness of probes for clinical application was determined by hybridizing the probes to patients with various chromosomal abnormalities.

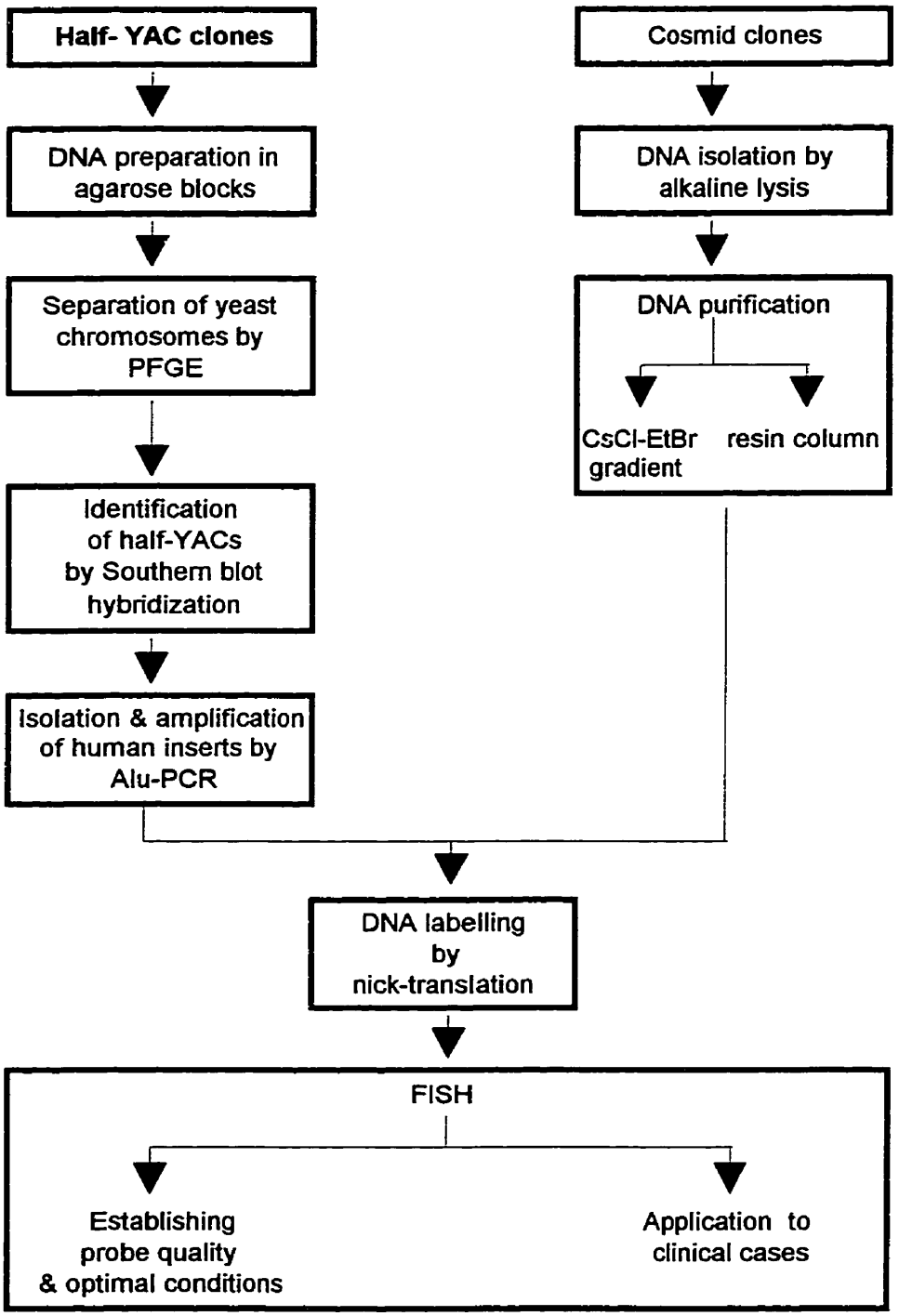
2.2 PROBE PREPARATION

2.2.1 Preparation of total yeast DNA

Colonies of *Saccharomyces cerevisiae* were grown in YPD media (see appendix) at 30^0C in a rotating incubator. Total DNA from YAC bearing yeast was prepared in agarose blocks by a modification of the method in the Current Protocols of Human Genetics (Dracopoli et al., 1996, section 5.7). Briefly, 5ml colonies were pelleted by centrifugation. Cells were washed in TE (see appendix) and spheroplasts

Fig. 3 Overview of methods used in the study. Sequence of steps from the generation of half- YAC and cosmid probes to their application in fluorescence in situ hybridization (FISH).

METHODS


Half- YAC clones
DNA preparation in agarose blocks
Separation of yeast chromosomes by PFGE
Identification of half-YACs by Southern blot hybridization
Isolation & amplification of human inserts by Alu-PCR
Cosmid clones
DNA isolation by alkaline lysis
DNA purification
CsCl-EtBr gradient
resin column
DNA labelling by nick-translation
FISH
Establishing probe quality & optimal conditions
Application to clinical cases

obtained by resuspending cells in 2.5 µl of Lyticase (50 U/µl). Cells were mixed with 2% low melt agarose in 0.125M EDTA, loaded into block molds and allowed to harden. The blocks were incubated overnight at 37 ^{0}C in 10 ml of LET(see appendix) plus 20 µl of ß- mercaptoethanol . Spheroplasts were then lysed by transferring cells into NDS (see appendix) containing N-lauroylsarcosine (1%). Preparations were deprotenized by incubation with Proteinase K (1 mg/ml) at 50 ^{0}C, overnight. The blocks were dialized against TE buffer and stored in the buffer at 4 ^{0}C until use.

2.2.2 Separation of Yeast Chromosomes by PFGE

To verify that the yeast colonies were stable, and contained the half- YACs, entire yeast chromosomes were separated from the half-YACs by pulsed field gel electrophoresis (PFGE).

A contour clamped homogenous electric field (CHEF) electrophoresis type of PFGE was used with 0.5X TBE buffer (see Dracopoli et al., 1996; p.A.2D.9), and 1% agarose gel. The blocks were sealed with 1% low melt agarose. Running conditions included: pulse of 40 s (for 20hrs); voltage of 6 mv/cm; current > 130; temperature of 14 ^{0}C; angle of 120^0. Products were visualized by staining the gel with EtBr and exposing to U.V light.

2.2.3 Identification of YACs by Southern Blot Hybridization

Because YACs can co-migrate with one of the smaller yeast chromosomes, and cannot always be distinguished on the gel following PFGE, YACs were identified using Southern blot hybridization with a YAC specific probe.

Following CHEF, the separated chromosomes were transferred from the gel to a nylon membrane and hybridized with a radioalabelled probe specific for the right arm of

pBR322 (which had been ligated into the half-YAC during construction of the vector) according to standard Southern blot hybridization procedures.

2.2.4 Isolation of Human Sequences by Alu -PCR

Agarose blocks containing total yeast DNA were melted at 65 ^{0}C, for 10 min, and 200 µl of melted preparation was digested using ß Agarase I (1 Unit) at 40 ^{0}C, for 2 hours. DNA was precipitated with 1/10 vol of (3M) sodium acetate and isopropanol, washed with 70% ethanol, dried and resuspended in TE.

Human sequences were amplified from total yeast DNA by Alu-PCR. The method used was a modification of a protocol described by Lengauer et al (1992) who optimized Alu-PCR for the generation of probes from yeast strains containing YACs with human inserts between 100 and 800 kb in size. One hundred ng of yeast genomic DNA was amplified in three separate reactions, the first using primer CL1 (5'TCC CAA AGT GCT GGG ATT ACA G'3), the second using primer CL2 (5' CTG CAC TCC AGC CTG GG 3') and the third using both CL1 and CL2. The three separate reactions amplify different regions of the YAC insert depending on the spacing and orientation of the Alu elements within the insert (Dracopoli et al., 1995).

Two sets of PCR conditions were used. The first included denaturation at 94 ^{0}C for 3 min, 30 cycles of PCR with denaturation at 94 ^{0}C, for 1 min, annealing at 45 ^{0}C for 30 s, and extension at 72 ^{0}C for 2 min, and a final extension at 72 ^{0}C for 10 min. The other conditions included the same parameters as above, but annealing temperature of 37^{0}C instead of 45 ^{0}C.

After visualization of the amplification products on an agarose gel, the products from the three reactions were purified with phenol, ethanol precipitated and polished in exact accordance with the Dracopoli et al., (1996; section 5.9). To ensure that the FISH

probe generated from the Alu-PCR products was highly representative of the DNA sequence, for each YAC, the DNA from the three reactions was ligated using T4 DNA ligase, prior to probe labeling (Dracopoli et al., 1996; section 5.9). Products were quantified on a 1.5% agarose gel.

2.2.5 Preparation of Cosmid DNA

Cosmid colonies were grown at 37 ^{0}C in LB broth (see appendix) supplemented with ampicillin (100 µg/ml). Minipreps of the cosmids were first performed by alkaline lysis according to protocol in Molecular Cloning Laboratory Manual (Sambrook et al., 1989; p.1.25). After precipitation and drying, the pellet was dissolved in TE containing 2 2 µg/ml of RNase. The optical density of the purified DNA was measured at 260 nm using a UV/VIS spectrophotometer. Once the concentration of the purified DNA was known, 1 µg of DNA was digested with 5 units of *Eco*R I for 2 hrs at 37 ^{0}C and products visualized on a 1% agarose gel.

After verification of the presence of inserts by agarose gel electrophoresis, the cosmids were prepared either by alkaline lysis large scale preparation alone, alkaline lysis followed by cesium chloride-ethidium bromide equilibrium centrifugation, or using a Qiagen kit.

2.2.6 Isolation of Cosmid DNA by Alkaline Lysis

Five hundred milliliters of cosmid containing bacterial cultures (LB plus ampicillin) were pelleted by centrifugation at 3000 rpm for 10 min. Cosmids were released from bacterial cells by lysis according to the protocol in Molecular Cloning Laboratory Manual (Sambrook et al., 1989; p. 1.38).

Following isopropanol precipitation, 5 ml of TE 10:10, was added to pellets for cosmids specific to telomere 13q, 20p. 2q/8p. Solutions were thouroughly mixed. Five ml of 8 M cold LiCl was added . Solutions were thoroughly mixed and incubated on ice for 5 min. Solutions were centrifuged at 10 000 rpm for 10 min. Supernatant was transferred to a new oak ridge tubes . One volume of isopropanol was added to each tube, solutions mixed, incubated on ice for 10 min and centrifuged for 10 min at 10 000 rpm. 500 µl of TE 10:10 was added to the pellets, solutions mixed and transferred to microfuge tubes. Following incubation in RNase 50µl (4µg/ul) for 30 min at 37 ^{0}C, the DNA was extracted using phenol-chloroform and precipitated using sodium acetate and ethanol. Pellet was resuspended in TE. DNA concentration was measured, 1 µg of DNA digested with 5 units of *Eco*R I for 2 hrs at 37 ^{0}C and products visualized on a 1% agarose gel.

2.2.7 Purification of DNA from Cosmid by CsCl-EtBr Gradient

Following the isopropanol precipitation step of alkaline lysis, 70% ethanol wash and resuspension in TE, DNA specific for telomere 14q was purified by cesium chloride-ethidium bromide gradient. Equilibrium centrifugation was done according to Sambrook et al. (1989; p. 1.42), using a Ti65 rotor and centrifuging at 65 000 rpm for 24 hrs at 20 ^{0}C. Ethidium bromide was removed from the purified DNA by extraction with 1-butanol saturated with water, followed by a phenol/chloroform extraction and ethanol precipitation. The pellet was resuspended in TE buffer. Following optical density measurements, 1 µg of purified DNA was digested with 5 units of *Eco*R I for 2 hrs at 37 ^{0}C and products visualized on a 1% agarose gel.

2.2.8 Isolation and Purification of DNA from Cosmids by Qiagen Kit

Cosmids specific for telomeres 20p and 2q/8p were prepared using a Qiagen isolation and purification kit. The kit protocol is based on a modified alkaline lysis procedure followed by separation of vector DNA by flow through resin.

Two hundred and fifty milliliters of cosmid containing overnight bacterial culture (LB supplemented with ampicillin) was pelleted by centrifugation at 7000 rpm at 4^0C for 10 min. The supernatant was discarded the pellet was treated exactly according to instructions for a midi purification provided with the kit. Following ethanol wash the purified DNA pellet was resuspended in TE, digested with *Eco*R I and products visualized on a 1% agarose gel.

2.3 LABELING OF PROBES BY NICK-TRANSLATION

One microgram of DNA from half-YAC clones yielding the best PCR products, yRM2050 (18q), yRM2208 (21q), yRM2189 (10p), yRM 2053 (8q) and yRM2195 (Xq/Yq), as well as cosmids subcloned from yRM2006 (14q), yRM2067 (13q), yRM 2003 (2q/8p) and yRM2005 (20p) were labeled with biotin-16-dUTP or digoxigenin 11-dUTP using a nick translation kit (Boehringer Mannheim). The translation was carried out according to the manufacturer's instructions, using a 20 µl reaction volume, and a 90 min incubation with the enzyme mixture (DNA-Polymerase I and DNase I) at 15 ^{0}C . After 90 min incubation, for the initial nick translation of each of the probes, 5 µl of reaction mixture was run on a gel to ensure that the DNA molecules were cut to the optimal size for FISH (100- 500 bp).

Enzymes were inactivated by adding 1/10 volume 0.2M EDTA and 1/10 volume of 5% SDS. Reaction volume was adjusted to 100 µl with distilled water, and unincorporated nucleotides were separated from the labeled probe by using a sephadex G50 spin column. The flow-through was collected. DNA was precipitated using 1/10 volume 3M sodium acetate, 1/10 volume salmon sperm DNA (10 mg/ml), and 2.5 volume of 100% ethanol , placed at -80 ^{0}C for 20 min and centrifuged for 30 min. The pellet was washed with 70% ethanol, dried and resuspended in hybridization buffer containing 50% formamide, 2 X SSC(see appendix), 10% (wt/vol) dextran sulfate to give a final concentration of 25ng/ µl. The probes were stored at -20 ^{0}C.

2.4 CHROMOSOME PREPARATIONS

Harvested lymphocyte pellets remaining from previous high resolution banding analysis and stored at -20^{0}C, were washed once with a 3:1 methanol fixative, centrifuged and resuspended. Fibroblasts and amniocytes cells were thawed from -80^{0} C, placed back in culture, and prepared by technologists according to standard cytogenetic methods. Cells were spread on clean, wet slides using high humidity. Slides were aged overnight at room temperature and frozen at -20 ^{0}C until used for FISH.

2.5 OPTIMIZATION OF FISH CONDITIONS

To establish probe quality and optimal FISH conditions for each probe FISH was first performed on normal metaphases using various probe amounts (50- 300 ng) combined with various amounts of competitor Cot 1 (GIBCO-BRL) (10-60 µg)or total genomic placental DNA (15-88 µg). Different blocking times during detection, as well as post-wash stringencies were also used.

For each probe, 5 random metaphase cells were visually assessed for (a)the presence of a signal on four target chromatids, for (b)the presence of random signals, doublets, or singlets on areas other than target telomeres (cross-hybridization), and for (c) random fluorescent spots in region surrounding the metaphase (background). The effectiveness of a particular "condition" was concluded based on a minimum of two separate FISH experiments, with a minimum of two chromosome preparations per experiment, per probe. "Condition" was viewed optimal when the preparation showed a bright hybridization signal present on four target chromatids, when there was no cross hybridization to chromosomal regions other than the target telomeres, and when background was low (only a few fluorescent spots present in metaphase regions) and did not not interfere with metaphase analysis. When target signal was not bright, or not present on four chromatids the experiment was repeated with an increase in probe amount, decrease in competitor concentration, or decrease in post-wash stringency. When cross-hybridization was apparent, competitor DNA concentration was increased. When background was high, time in blocking solution during detection, as well as stringency of the post-wash were increased.

Preparations considered "optimal" were used to evaluate hybridization efficiency of the probes. For each probe, the number of fluorescently highlighted chromatids was scored in a minimum of 20 metaphase cells. The number of large clear signals, and their appearance (singlet, doublet, or clump) was also counted in a minimum of 60 interphase nuclei.

2.5.1 Chromosome Pretreatment

Prior to hybridization, slides with chromosome preparations were treated with RNase for 1 hr at 37 ^{0}C, rinsed in 2 X SSC and dehydrated in 50%,70%, and 100%

ethanol series. Alternatively the slides were pretreated with 2XSSC for 30 min at 37 ^{0}C and dehydrated.

2.5.2 Chromosome and Probe Denaturation and Suppression

Chromosome preparations were denatured in 70% formamide/2 xSSC at 70 ^{0}C , for 2 min. After denaturation, the slides were immediately transferred to cold 2xSSC for 2 min, and dehydrated in a cold ethanol series 50%, 70% and 100%, 2 min each. Probes were combined with competitor DNA, denatured in a boiling water bath for 10 min and allowed to preanneal for 30-90 min at 37 ^{0}C. Following this the probes were applied to denatured chromosome preparations and slides covered with a plastic coverslip.

2.5.3 Hybridization and Post Hybridization Wash

Hybridization was performed overnight (17-25 hrs) in a humidified chamber at 37^{0}C. The following day slides were washed in 50% formamide, 2 X SSC for periods of 5-8 min, followed by two washes in 2 X SSC , 2-5 min all at 37 ^{o}C, to 41 ^{0}C.

2.5.4 Detection and Couterstaining

Hybridization signals were detected using rabbit anti- biotin (Sigma), biotinylated goat anti-rabbit (GIBCO BRL), and streptavidin FITC (GIBCO- BRL) antibodies for biotin labelled probes and with anti- digoxigenin (Boehringer Mannheim), goat anti-rabbit, rabbit anti-goat FITC (SIGMA) for digoxigenin labeled probes. Incubations were carried out at 37 ^{0}C for intervals ranging from 30 - 60 min. Prior to each antibody incubation the slides were washed in PBS (see appendix) (5 min) and blocked in PBT solution (see appendix) for 5-10 min. Chromosomes were counterstained with propidium iodide (SIGMA; 2 μg/ul) and slides mounted with antifade solution (see appendix).

with propidium iodide (SIGMA; 2 μg/ul) and slides mounted with antifade solution (see appendix).

2.5.5 Microscopy and Image Analysis

Hybridization signals from FITC and rhodamine, as well as DAPI and PI counterstains were visualized using a Zeiss epi-fluorescence microscope equipped with appropriate filters (DAPI: Zeiss #5; FITC: Zeiss # 10; FITC/PI: Chroma #61001; FITC/PI/DAPI: Chroma #51005) and a charged - coupled device (CCD) camera. Images were captured using a Cytovision system software (Applied Imaging) and printed using a Mitsubishi color printer.

2.6 TWO COLOR FISH

Two color FISH experiments were carried out using a biotin labelled telomeric probe combined with a digoxigenin labelled centromeric ONCOR probe. For this purpose, 0.7 μl of ONCOR probe was first combined with 15μl of hybridization buffer and denatured at 80 ^{0}C for 5 min. Ten microliters of this mixture was then combined with the preannealed telomeric probe, and as for the one color FISH experiments above, the probe mixture was then applied to denatured chromosome preparations, allowed to hybridize and washed following hybridization. The detection involved using both a biotin and a digoxigenin antibody system. Rabbit anti- biotin was applied first. The second incubation was with mouse anti - digoxigenin. Following this, both biotinylated goat anti-rabbit antibodies and anti- mouse digoxigenin (Boehringer Mannheim) were applied together. The final incubation was done once again using a combination of two different detection reagents, sheep anti- digoxigenin rhodamine (Boehringer Mannheim) and streptavidin

FITC. The chromosomes were counterstained with DAPI (Boehringer Mannheim; 1mg/ml), and slides mounted in an antifade solution.

2.7 FISH EXPERIMENTS USING CLINICAL CASES

Following the establishment of optimal probe and competitor DNA concentration on control slides, FISH was performed on lymphocytes, fibroblasts or amniocytes from previously diagnosed clinical cases, and on an ovarian tumor cell line. 200 ng of half - YAC probes yRM2208(21q), yRM2050 (18q), and yRM2189(10p), each combined with 30 -50 μg of Cot-1 DNA to analyze trisomy 21q, trisomy 18q, monosomy 18q, and balanced translocations t(8q;18q) and (5q;10p). Probe specific to 10p was also used to study a complex translocation in the cell line of the ovarian tumor. One hundred to 125 ng of cosmid probes yRM2006(14q)and yRM2067 (13q), each combined with 30-45 μg of genomic placental DNA was used to study balanced translocations t(6p;14q), t(8;14q), and an unbalanced translocation t(10q;13q).

Post -wash conditions used consisted of two washes of 50% formamide/ 2XSSC, 7 min each, and two washes in 2XSSC for 4 min each. Prior to application of antibodies, slides were placed in PBT for 10 min (5min x 2). During detection 30 min was used for each antibody incubation.

CHAPTER 3

RESULTS

3.1 SEPARATED YEAST CHROMOSOMES

Entire yeast chromosomes were separated by CHEF. Figure 4 shows yeast chromosomes from three colonies of each of the 22 yeast clones, separated into discrete bands. Sometimes a distinct band representing the migrated YAC may be visualized, however the half-YACs in this study co-migrated with some of the smaller yeast chromosomes and could not be distinguished.

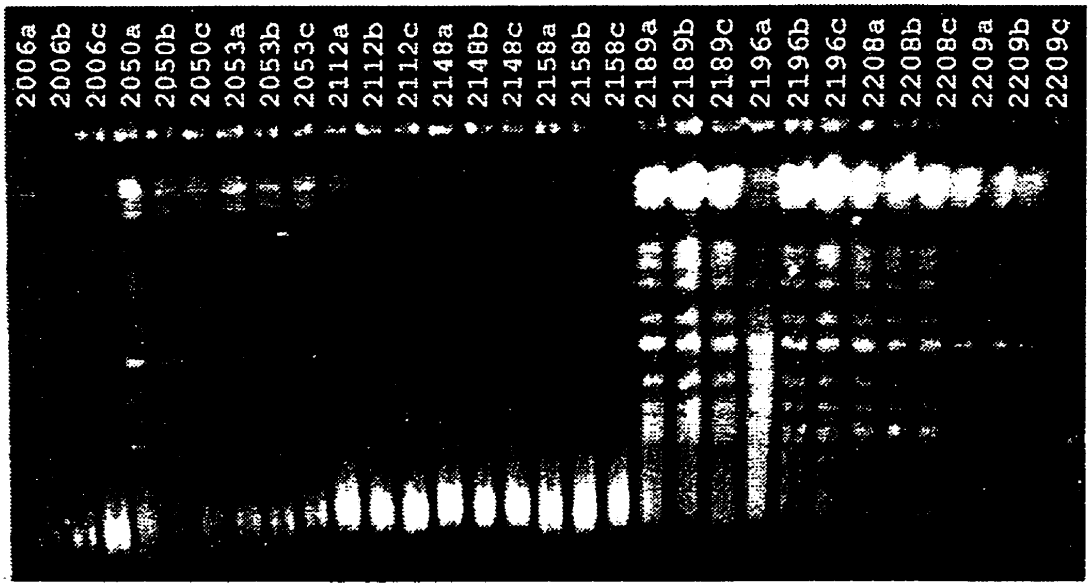
3.2 IDENTIFICATION OF HALF-YACS

Southern Blot hybridization of the separated chromosomes using radiolabelled probe specific for the right arm of pBR322 resulted in hybridization signal locating the half-YAC of each clone (Fig.5). Using the yeast chromosomes as a reference marker, the size of most the half-YACs was estimated to be between 200 - 450 bp as expected. For all but one clone (yRM2052, specific to telomere 2p), the probe hybridized to the same migration distance for all three colonies indicating that they contained half-YACs which were stable. One of the yRM2052 (2p) colonies showed a positive band larger in size than the other two colonies, suggesting that recombination involving the insert had taken place. This colony was eliminated from further steps.

3.3 ALU-PCR

Figure 6 shows distinct bands representing specific Alu-PCR products for half-YACs amplified at annealing temperature of 45 ^{0}C, (which yielded more products than when 37^{0}C was used). The PCR products from the CL1 reaction were larger in size than those of CL2, or CL1 +CL2. Fewer bands were obtained from the two individual reactions than

Fig. 4 An ethidium bromide stained agarose gel (1%) following PFGE (CHEF), showing the separation of *Sacchromyces cerevisiae* chromosomes for 2 to 3 colonies (a,b,c), from 22 different clones. Each clone is representative of a half-YAC specific for a different human telomere. Size in kilobase pairs (kb), and the corresponding yeast chromosomes are indicated. The half-YACs can not be distinguished from the migrated yeast chromosomes.


2006a
2006b
2006c
2050a
2050b
2050c
2053a
2053b
2053c
2112a
2112b
2112c
2148a
2148b
2148c
2158a
2158b
2158c
2189a
2189b
2189c
2196a
2196b
2196c
2208a
2208b
2208c
2209a
2209b
2209c
945kb XVI
915kb XIII
815kb II
785kb XIV
745kb X
680kb XI
610kb V
555kb VIII
450kb IX
375kb III
295kb VI
225kb I

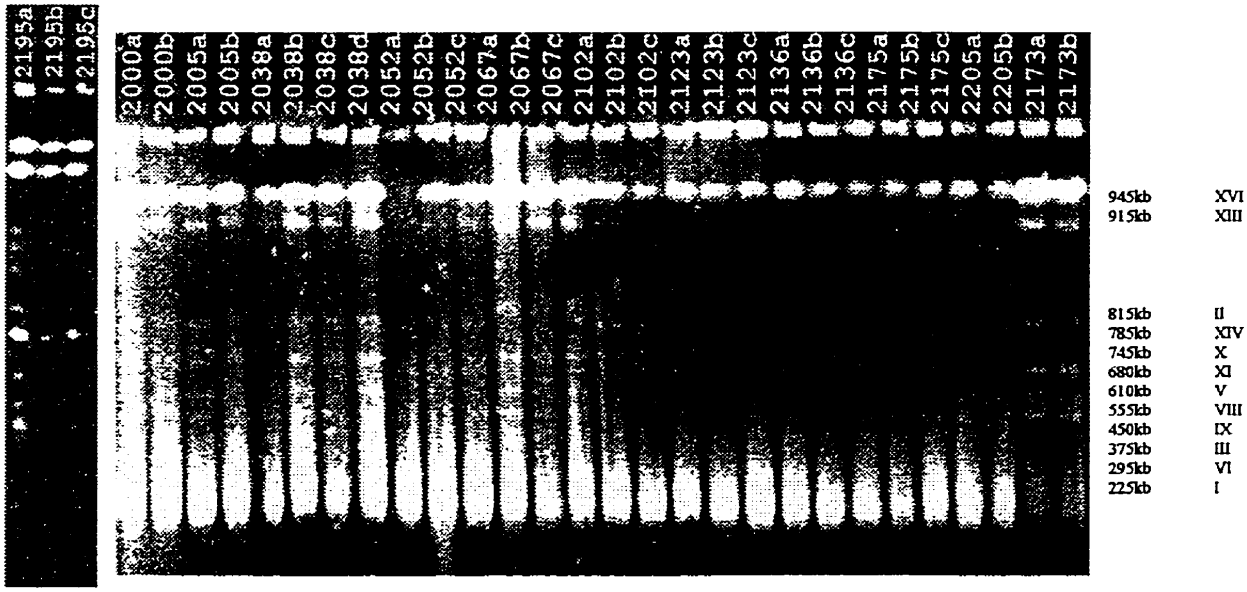
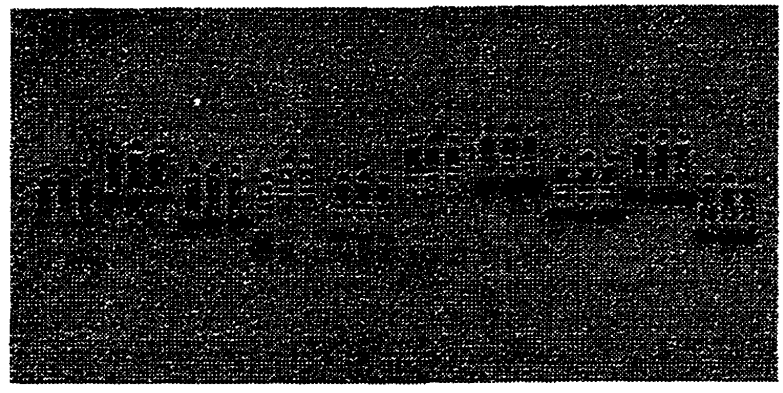
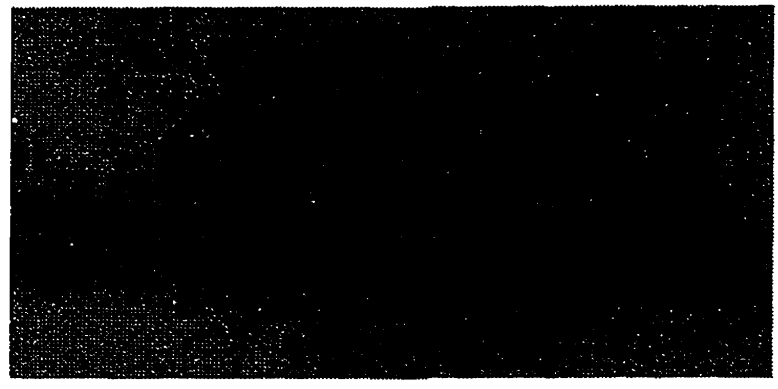

2195a
2195b
2195c
2000a
2000b
2005a
2005b
2038a
2038b
2038c
2038d
2052a
2052b
2052c
2067a
2067b
2067c
2102a
2102b
2102c
2123a
2123b
2123c
2136a
2136b
2136c
2175a
2175b
2175c
2205a
2205b
2173a
2173b
945kb XVI
915kb XIII
815kb II
785kb XIV
745kb X
680kb XI
610kb V
555kb VIII
450kb IX
375kb III
295kb VI
225kb I

Fig.5 An autoradiogram showing hybridization of half-YACs from 22 yeast clones (2 to 3 colonies each) to ^{32}P-labeled pBR322 probe. The half-YACs range in size from 200-450 kb. Note, half-YAC yRM 2112 (2q) in one of the three colonies differs in size from the half-YACs in the other two colonies.

450kb
350 kb
225 kb



450 kb
225kb



291 kb

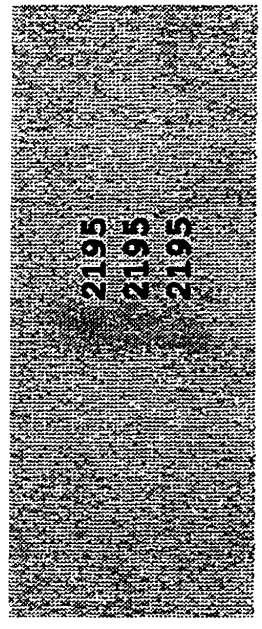
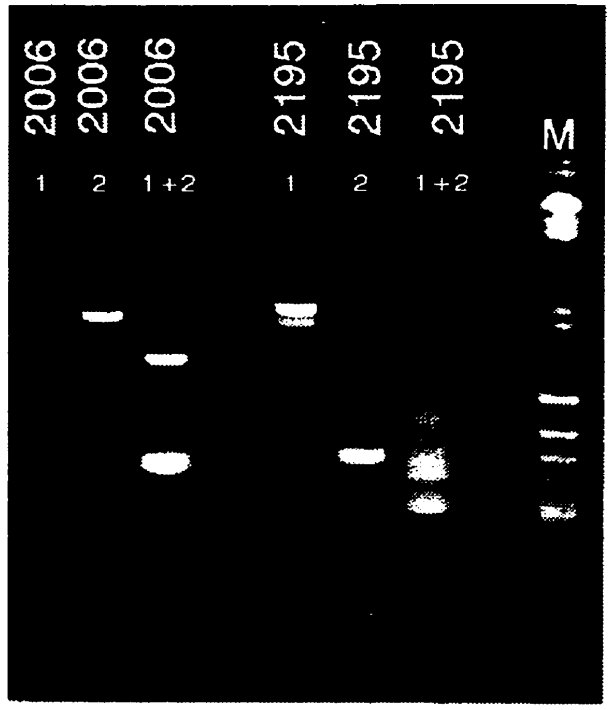
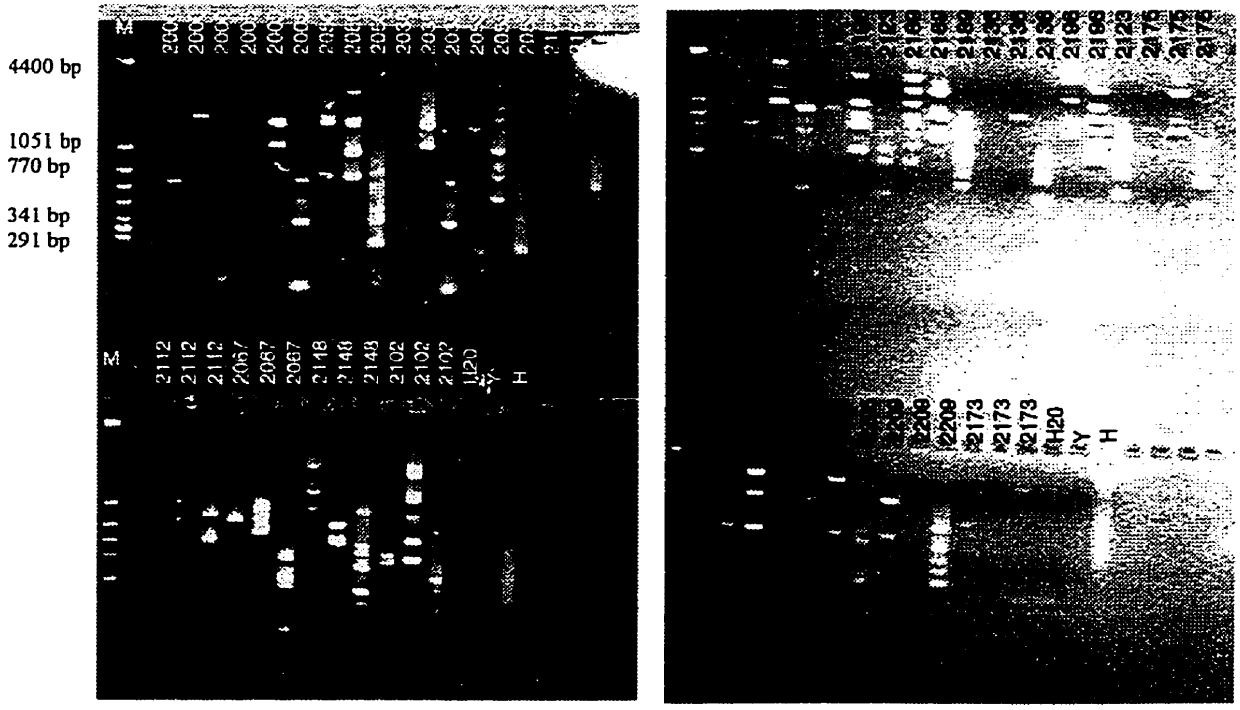


Fig.6 Agarose gel (1.5%) showing Alu- PCR products from half-YACs following amplification with three different primer sets CL 1, CL2, and CL1+CL2. Positive bands of variable size and number indicate differences among products from the three reactions. **M**: marker- Lambda DNA- Hind III digest plus 0 X-174-RF DNA Hinc II digest. **H_20**: no templete DNA, **Y**: total yeast DNA as templete, **H**: total human DNA as templete.


2006
2006
2006
2195
2195
2195
M
1
2
1+2
1
2
1+2
4400 bp
2300 bp
1051 bp
770 bp
341 bp
291 bp


4400 bp
1051 bp
770 bp
341 bp
291 bp
M
M
H

the combined CL1+CL2, however, the individual reactions included products not well represented in the combined reaction. CL1+CL2 contained some of the product present in individual reactions in addition to several new products formed as a result of having both primers present.

Figure 7 shows Alu-PCR products after combining the DNA from the three separate reactions (CL1 + CL2 + [CL1+CL2]). YACs yRM 2050 (18q), 2053 (8q), 2189 (10p), 2208 (21q) and 2195 (Xq/Yq) showed the highest number of bands which suggested that they would be most successful as FISH probes.

3.4 ISOLATED AND PURIFIED COSMID DNA

Table 1 shows the maximum yield of purified cosmid DNA following preparation by three different DNA isolation and purification methods. The highest yield of DNA was obtained for cosmid 2006 prepared by cesium chloride- ethidium bromide equilibrium cenrifugation. For cosmids prepared by both techniques, alkaline lyisis and Qiagen kit, reproducibly higher yields of DNA were obtained following alkaline lysis than after isolation and purification using the Qiagen kit.

Figure 8 shows a 1% gel electrophoresis products of EcoRI 1 digest of purified DNA from cosmids yRM 2006 (14q), yRM 2069 (13q), yRM 2005 (20p), and yRM 2003 (2q/8p). Multiple bands ranging from 2 - 10kb representing digested human insert, and 7 kb plasmid band, was observed for all but one cosmid (yRM 2000). The clone yRM2000, due to unsuccessful growth in selective media (amp +) had been grown without ampicillin. Extractions and purifications of the clone were repeated a number of times, but no insert bands were observed.

Fig.7 Agarose gel (1.5%) showing DNA from 22 different half-YACs after ligation of products from the three separate reactions (CL 1 + CL 2 + [CL1+CL2]). **M**: marker-Lambda DNA- Hind III digest plus 0 X-174-RF DNA Hinc II digest. **H_20**: no templete DNA, **Y**: total yeast DNA as templete, **H**: total human DNA as templete. Note, half-YACs for telomeres 18q, 8q, 21q, and Xq/Yq yielded the highest number of bands

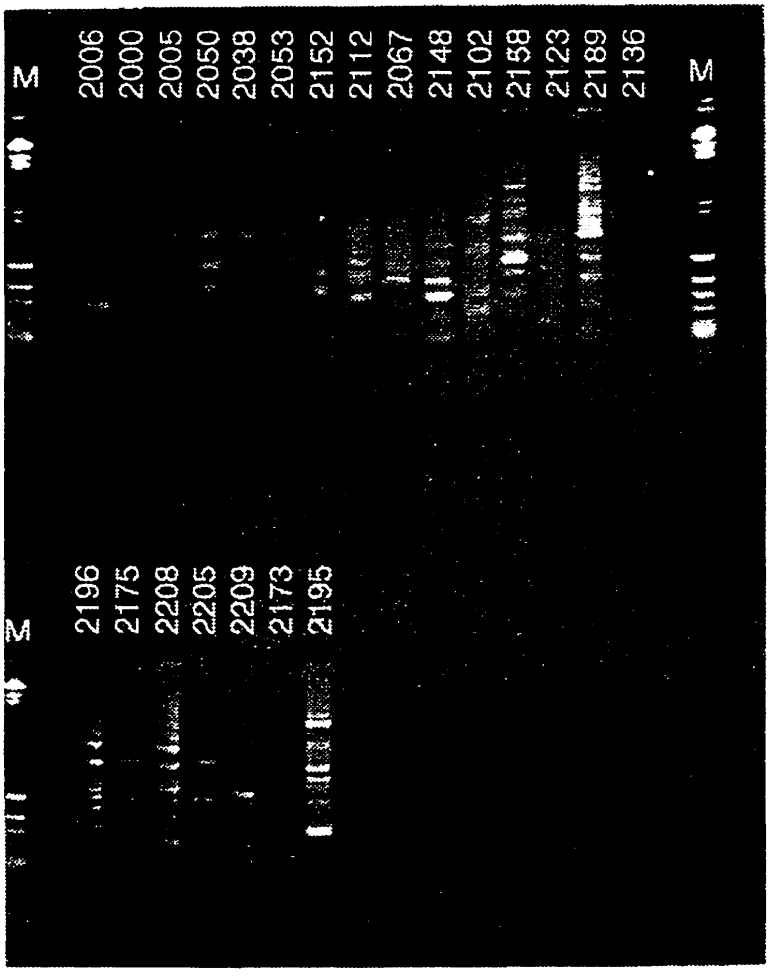
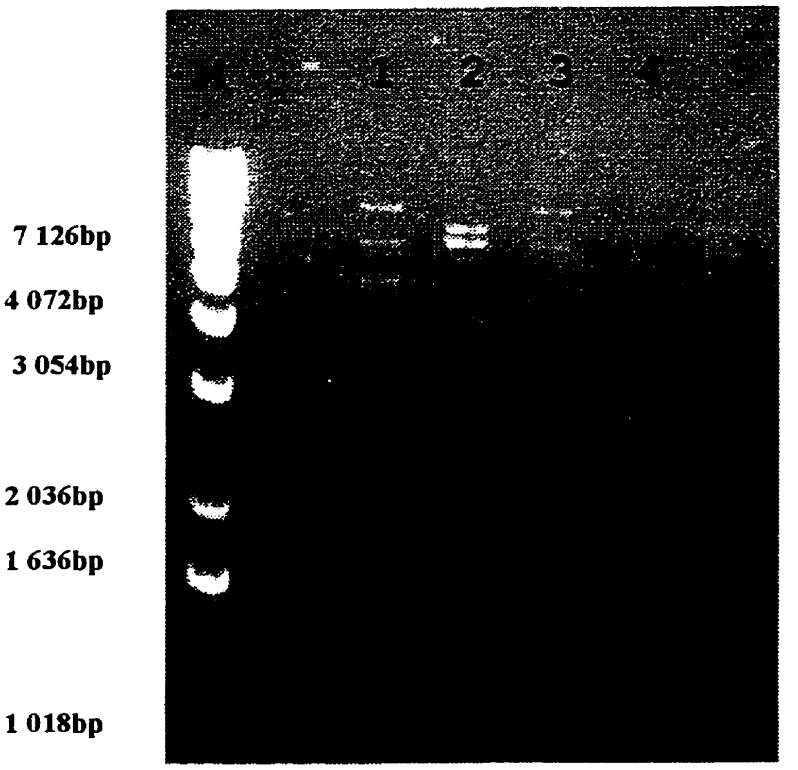

M
2196
2175
2208
2205
2209
2173
2195
M
2006
2000
2005
2050
2038
2053
2152
2112
2067
2148
2102
2158
2123
2189
2136
M
4400 bp
2300 bp
1051 bp
770 bp
341 bp
291 bp

Table 1. Maximum yields of purified cosmid DNA prepared by three different methods

Cosmid	DNA Preparation Method	Maximum Yield
yRM 2006 (14q)	CsCl-EtBr centrifugation	1720 μg
yRM 2067 (13q)	Alkaline Lysis	1600 μg
	Qiagen Kit	17 μg
yRM 2005 (20p)	Alkaline Lysis	1026 μg
	Qiagen Kit	9 μg
yRM 2003 (2q/8p)	Alkaline Lysis	1229 μg
	Qiagen Kit	19 μg
yRM 2000 (7q)	Alkaline Lysis	8 μg

Fig.8 Agarose gel (1%) showing products of EcoRI digest of purified cosmid DNA. From left to right: **M**: marker- Hinf I 1kb DNA ladder; Lane **1**: yRM 2003 (2q/8p); lane **2**: yRM 2006 (14q); lane **3**: yRM 2069 (13q), lane **4**: yRM 2000 amp$^-$ (7q); lane **5**: yRM 2005 (20p). Note consistent plasmid band at approximately 7 kb, and absence of product in lane 4.


M
-
1
2
3
4
7 126bp
4 072bp
3 054bp
2 036bp
1 636bp
1 018bp

3.5 LABELED PROBE DNA

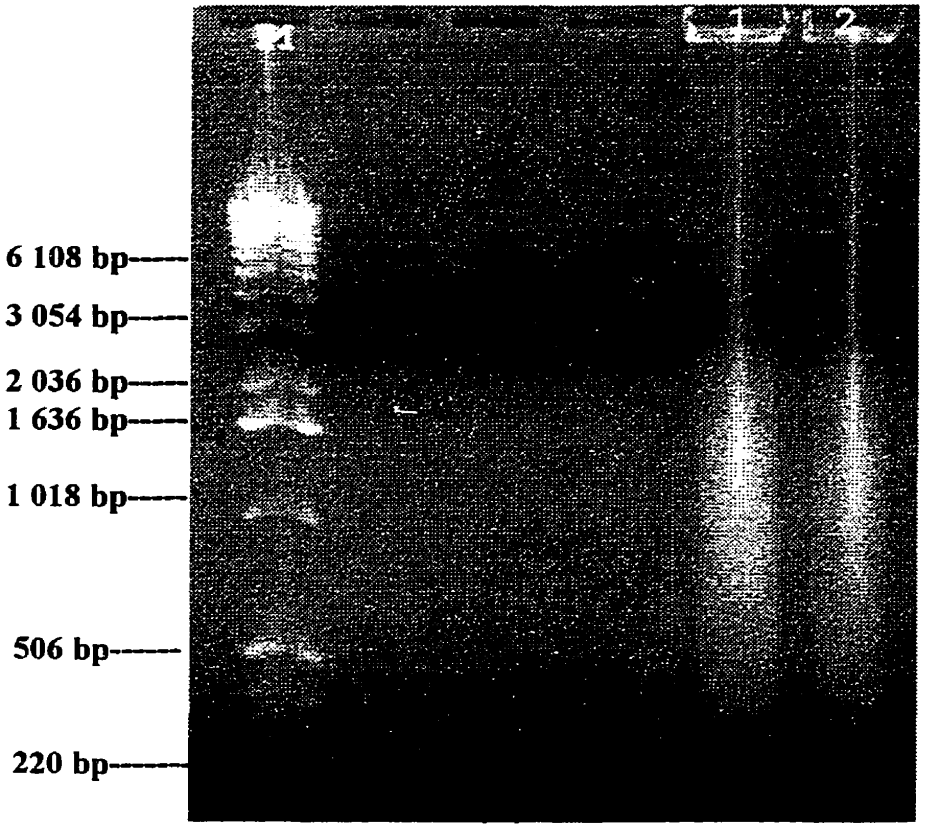
DNA from four half-YACs that yielded the best Alu-PCR products were chosen and labeled by Nick Translation. Fragments ranged in size from 100-4000 bp. Smaller fragments could not be obtained despite longer incubation periods, and increasing enzyme mixture concentrations. Nick translation of cosmid DNA also yielded fragments in the same size range.

Variability in size of fragments, was observed among different probes as well as for the same probe DNA labeled in different reactions. Figure 9 shows DNA fragments from half-YACs yRM 2208 (21q) and yRM 2189 (10p), as seen on a 0.8% agarose gel after a 90 min incubation with enzyme mixture at 15 ^{0}C. The fragments ranged from 200 to 3000bp.

3.6 CHROMOSOME PREPARATIONS

The morphology, spreading, mitotic index and amount of cytoplasm varied with different cell types. Lymphocytes, which had been synchronized, especially freshly harvested lymphocytes yielded preparations with a high mitotic index, good spreading, no cytoplasm and nice morphology. The quality of preparations decreased when slides were prepared from previously harvested cells, with the oldest pellets giving the poorest results. Fibroblasts, and especially amniocytes, yielded preparations with a much lower mitotic index, and chromosomes which were very hard to spread. The worst preparations were obtained from amniocyte and fibroblast cells which had been frozen at -80^{0}C, and had already been thawed and placed back in culture a number of times prior to the present time.

Fig. 9 Example of an agarose gel (1%) showing DNA fragments after nick translation. **M**: marker- Hinf I 1kb DNA ladder, lane **1** half-YAC yRM 2208 (21q), lane **2** half-YAC yRM 2189 (10p). Bands are in the size range of 200-3000 bp.


M
1
2
6 108 bp
3 054 bp
2 036 bp
1 636 bp
1 018 bp
506 bp
220 bp

3.7 HALF-YACS AND COSMID PROBES HYBRIDIZED TO NORMAL CHROMOSOMES

Hybridization signals were generated after FISH with four cosmids, specific for telomere 13q, 14q, and 20p, and 2q/8p. Hybridization signals were also obtained with three out of the 5 half-YACs which had been selected based on quality of PCR product, half-YACs specific to telomeres 10p, 18q, and 21q. Half-YACs for 8q and Xq/Yq, even after multiple hybridization experiments, involving various conditions, did not produce telomere specific signals.

3.7.1 Optimal FISH Conditions

For each of the cosmids and half-YACs, multiple hybridization experiments with varying probe and competitor amount, as well as different post hybridization parameters yielded "optimal" FISH conditions which resulted in a bright target specific signal, a high efficiency of hybridization, and minimal background.

Although adequate suppression was achieved using total genomic DNA for cosmid probes, Cot- 1 was a better competitor for Alu-PCR generated half-YAC probes, eliminating unwanted hybridization without compromising the intensity of the signal. The best results were obtained using 200 ng of the half-YAC probes combined with 40-50 μg of Cot-1 DNA, and 125 ng of cosmid probes combined with 20-40 μg of genomic placental DNA, all pre-annealed at 37 ^{0}C for 60 min. Minimum background (only a few fluorescent spots in regions close to metaphase cells, which did not interfere with metaphase analysis), was achieved using post wash conditions of 50% formamide, 2 X SSC (2 times, 7 min each) followed by two washes in 2 X SSC , 4 min each, all at 39^{0}C , and a PBT block of 2X 5 min prior to each antibody incubation.

No differences were noted in results between slides pretreated with 2XSSC instead of RNase, when rinces in PBS were shortened from 5 to 2 min, or when incubations with antibodies were shortened from 1 hr to 30 min each.

3.7.2 Hybridization to Metaphase Chromosomes

For all probes at "optimal" conditions the background was low; only a few fluorescence spots within metaphase regions were present, and they did not interfere with metaphase analysis. In almost all cells assessed, there was no cross hybridization to chromosomal regions other than the target telomeres; no doublets were present on regions other than target telomeres, and presence of any singlets was inconsistent among cells.

Table 2 shows the efficiency of hybridization for the six probes, as determined by hybridization to normal chromosomes under "optimal" conditions. All three PCR-generated half-YAC probes produced large and intense signals present on both target telomeres, in over 90% of metaphases analyzed. Signal quality and hybridization efficiency among the cosmids was varied. A higher quality signal (brighter, clearer) was obtained from hybridization to probe specific to 14q prepared by the CsCl gradient, compared to that from the other two cosmids prepared by Alkaline Lysis or using the Qiagen Kit. Despite repeated experiments, cosmid yRM2003 (2q/8p) did not yield a high level of hybridization efficiency, or consistent specific signal.

On most homologs, especially for the half-YAC probes, the signal was present on both chromatids (doublets). Usually the signal consisted of one spot per chromatid.

Table 2. Signal quality and hybridization efficiency of probes following hybridization to normal metaphase chromosomes at optimum conditions.

Probe	Method of Preparation	Signal Quality	Hybridization Efficiency [a]	Hybridization Efficiency[b]
YAC yRM2189 (10p)	Alu-PCR	large, intense	100% (n=58)	90% (n=116)
YAC yRM2050 (18q)	Alu-PCR	large, intense	100% (n=50)	99% (n=100)
YAC yRM2208 (21q)	Alu-PCR	large, intense	98% (n=60)	90% (n=120)
cos yRM2006 (14q)	CsCl-EtBr gradient	large, intense	98% (n=60)	95% (n=120)
cos yRM2067 (13q)	Alkaline Lysis	fair, intense	94% (n=64)	78% (n=108)
cos yRM2005 (20p)	Qiagen Kit	small, weak	75% (n=40)	63% (n =60)
cos yRM200 (2q/8p)	Qiagen Kit	small, weak	[c]46% (n=40)	[c]32% (n=60)

[a] hybridization to both of the target telomeres

[b] hybridization to four of the target chromatids ("a doublet" on each telomere)

[c]hybridization to two 2q and two 8p telomeres

However, sometimes signals consisting of two fluorescent spots per chromatid, or a fluorescent clump covering the whole terminal region were noted. The different types of metaphase signals observed are shown in Figure 10.

3.7.3 Hybridization to Interphase Nuclei

Target sequences were also visualized in interphase nuclei. The majority of nuclei contained signals consisting of two spots closely spaced together, or doublets (Fig. 11a). Other cells contained singlets. Certain cells contained both a doublet and a singlet. In some of the interphase cells, three co-localized spots, or triplets were observed (Fig. 11b). This was especially frequent for probe 14q. Other times, FISH, especially with probe 18q, generated interphase signals with a clump- like appearance, larger and more diffuse than those seen as doublets (Fig. 11c). Unlike in metaphase cells, even with optimal suppression and post wash conditions, background was apparent in interphase nuclei, making interphase analysis more difficult. This problem varied from probe to probe, with 10p generating highest cross hybridization or the greatest percentage of "undetermined" number of signals per nuclei (Table 3).

Fig.10 Fluorescence in situ hybridization of half-YAC and cosmid probes to normal metaphase chromosomes. **(A)** cosmid probe yRM 2006 hybridized to telomeres 14q **(B)** half- YAC probe yRM 2189 hybridized to telomeres 10p. Note variety in signals **a:** one fluorescent spot per chromatid, **b:** two fluorescent spots per chromatid, **c:** fluorescent clump covering entire telomeric region. Chromosomes are R-banded.

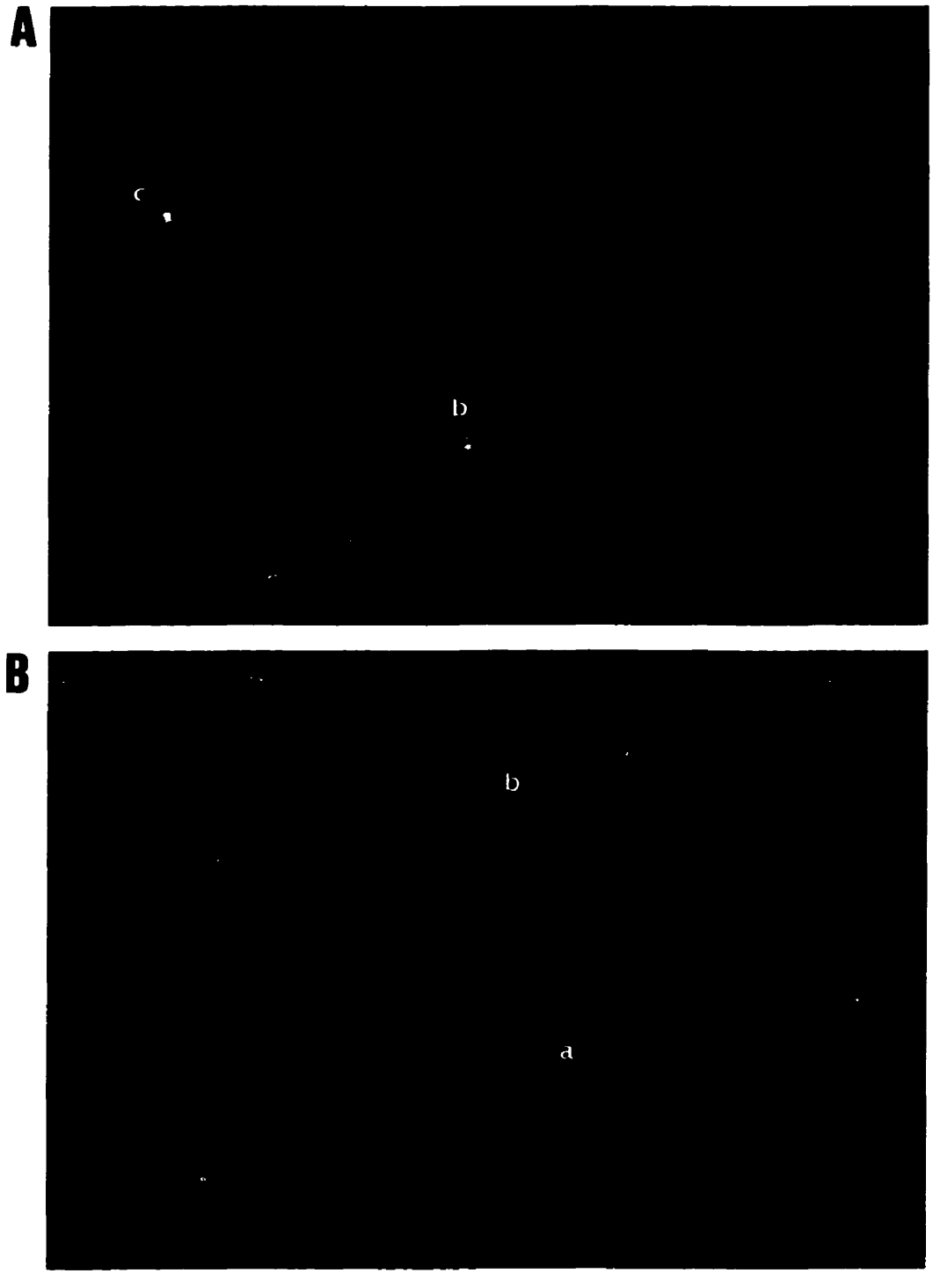

A
c
b
B
b
a

Fig. 11 FISH to interphase nuclei. Hybridization signals can appear as **(a)** doublets - two spots closely spaced together, **(b)** three co-localized spots or triplets, or **(c)** clumps.

a

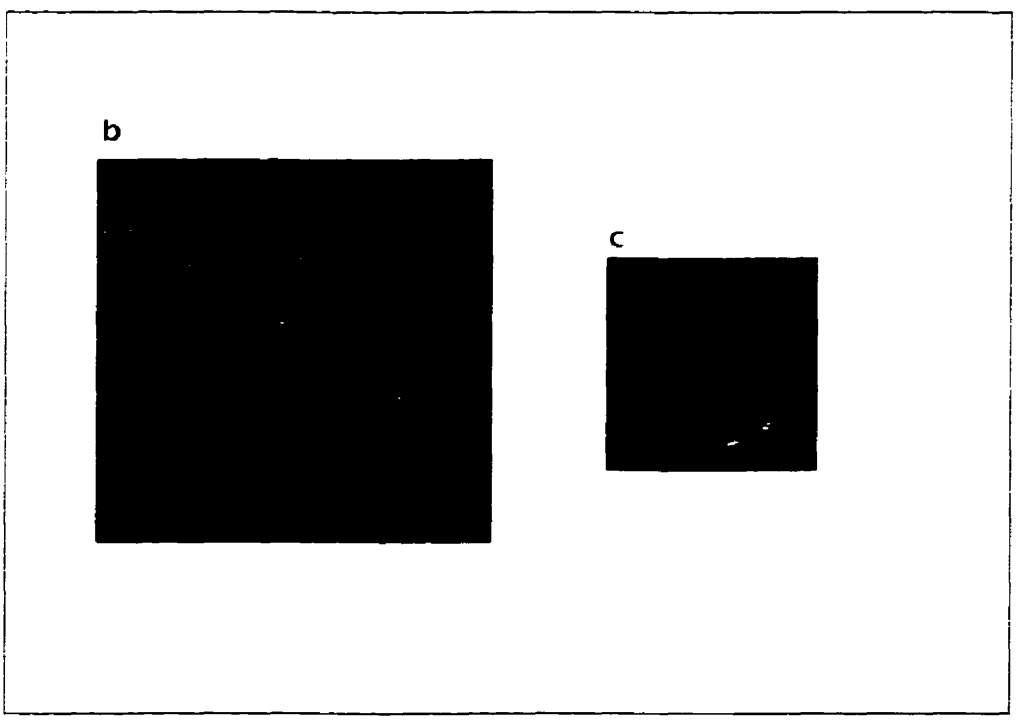


Table 3. Percentage of interphase nuclei with a determined number of signals following hybridization to normal cells at optimum conditions.

Probe	YAC yRM2189 (10p)	YAC yRM2050 (18q)	YAC yRM2208 (21q)	cos yRM2006 (14q)	cos yRM2067 (13q)	cos yRM2005 (20p)	cos yRM 2003 (2q/8p)
% cells	48 (N=106)	62 (N=113)	76 (N=110)	74 (N=108)	57 (N=97)	ND	ND

ND not scored due to low hybridization efficiency, and high background.

3.8 APPLICATION OF PROBES TO CLINICAL CASES

Following the establishment of optimal FISH conditions, probes for subtelomeric region of chromosomes 10p, 13q, 14q, 18q, and 21q were applied to study balanced and unbalanced chromosomal aberrations of 9 different patients and one ovarian tumor cell line (Table 4).

Although observation of identical labeling of sister chromatids in just one metaphase was sufficient to reveal the abnormality and to demonstrate that the probe was successful in its detection, to ensure consistency of the observed results, over 10 metaphase cells in cases of translocations, and over 20 cells in cases of trisomies and monosomy were analyzed.

FISH was affected by the quality of the chromosome preparations. Differences in the intensity of the signal and hybridization efficiency were noted between different patients as well as between slides from the same individual run in the same FISH experiment (which had therefore all been subjected to the same conditions). Chromosome identification following hybridization was not possible for all cases. This was due to either insufficient quality of chromosome banding, or its complete absence when chromosome preparations did not originate from BrDU treated cultures. In these instances the chromosomes involved in the rearrangement were identified using centromeric ONCOR probes.

3.8.1 Unbalanced Aberrations

Case 1

FISH using half-YAC yRM2050 specific to telomere 18q, was done on lymphocytes of a patient referred for diagnosis due to tracheal esophageal fistula, and

Table 4. Clinical cases studied by FISH using telomere specific half-YAC and cosmid probes.

Case	Cell type	Reason for referral	G-Banding result	Expected Result	Probes used in FISH	FISH result (subtelomeric region)
1	lymphocytes	CA MR	46,XX, rec,dup, inv(18)(p13;q11) de novo duplication or translocation between homologs,	partial trisomy 18q	YAC 18q	signals on three telomeres (trisomy)
2	lymphocytes	CA MR	46,XY, der(21;21)(q10;q10) de novo Robertsonian translocation	trisomy 21q	YAC 21q	signals on three telomeres (trisomy)
3	amniocytes	advanced maternal age	46,XX, der(21;21)(q10;q10) de novo Robertsonian translocation	trisomy 21q	YAC 21q	signals on three telomeres (trisomy)
4	lymphocytes	CA	46,XX,del(18)(q21.3) de novo interstitial or terminal deletion	partial monosmy 18q	- YAC 18q - centr 18	signal on 1 telomere (monosomy; terminal deletion or a semi cryptic unbalanced translocation)
5	lymphocytes	CC	46,XX,der(10)t(10;13) (q25;q13) de novo derivative from a reciprocal translocation	partial monosomy 10q, partial trisomy 13q	- Cos 13q	signals on two telomeres (disomy; insertion ?)
6	lymphocytes	familial study	47,XY,+der(14)t(8;14) (q24.1;q22) mat derivative from a reciprocal translocation	balanced disomy	- Cos14q - centr 14/22	signals on 2 telomeres (disomy) reciprocal translocation
7	lymphocytes	DD	46,XX,t(6;14) (p11.2;q11.2) reciprocal translocation	balanced disomy	- Cos 14q - centr 6	signals on two telomeres(disomy) reciprocal translocation
8	lymphocytes	CA DD	46,XY,t(8;18) (q22;q11.2) mat reciprocal translocation	balanced disomy	YAC 18q	signals on two telomeres (disomy) reciprocal translocation
9	amniocytes	advanced maternal age	46,XX,t(5;10)(q11.2;15) de novo reciprocal translocation	balanced disomy	- YAC 10p - centr 10	signals on two telomeres (disomy) reciprocal translocation

10	ovarian tumor cell line (OV90)	serous papillary adeno-carcinoma	46,XX, hsr (3)(p11),der 9(9;17)(q10;q10),add(13)(p 11)	possible complex rearrangement	-YAC 10p	signals on two telomeres (disomy) translocation

CA: congenital abnormalities

DD: developmental delay

MR: mental retardation

mental retardation (MR). High resolution investigation at the age of one month had revealed a 46, XX, rec,dup,inv(18)(p13;q11) karyotype. The parents' karyotypes were normal. Chromosome painting using chromosome 18 painting probe had confirmed partial trisomy18. FISH using chromosome 18 telomeric probe produced signal on three telomeres, normal 18q and the two telomeres of the recombinant chromosome in the 95% of metaphases analyzed (Fig. 12A), indicating trisomy for subtelomeric region of 18q. Due to the presence of background signals interphase analysis was difficult, however three signals, two of which were oriented closer together were observed in 65% of nuclei (Fig.12B). This observation is compatible with the recombinant chromosome, from a pericentric inversion or a translocation plus a duplication between the two 18 homologs.

Case 2

Half-YAC yRM2208 (21q), was applied to study lymphocytes of a 20 year old patient with Down syndrome and a carrier of a Robertsonian translocation (21q;21q) as shown by GTG banding. The mother's karyotype was normal. FISH with the telomeric probe specific to 21q produced signal on the normal 21q, and on both telomeres of the derivative 21 in all of metaphases analyzed (Fig.13A). In 34% of interphase cells three large and clear signals, distinguishable from the smaller background signals were present . By demonstrating trisomy for the subtelomeric region of 21q, FISH confirmed previous GTG -banding based diagnosis.

Case 3

Half-YAC for telomere 21q was also used to study amniocytes. The mother was referred for prenatal diagnosis due to advanced maternal age. Amniocentesis had revealed a 46,XX, der21, (21;21) karyotype compatible with the clinical diagnosis of Down

Fig.12 FISH with 18q telomeric probe (yRM 2050) to chromosomes of patient in case 1. **(A)** Hybridization to metaphase chromosomes showing signal on three telomeres, on the normal 18q (yellow arrow), and on the two telomeres of the recombinant chromosome (white arrow). **(B)** Interphase nuclei showing three hybridization signals. The two signals closer together (white arrow) likely represent hybridization to the recombinant chromosome, whereas the signal further from the previous two (yellow arrow) represents hybridization to the normal chromosome 18. **(C)** G-banded 18 and recombinant (rec) 18.

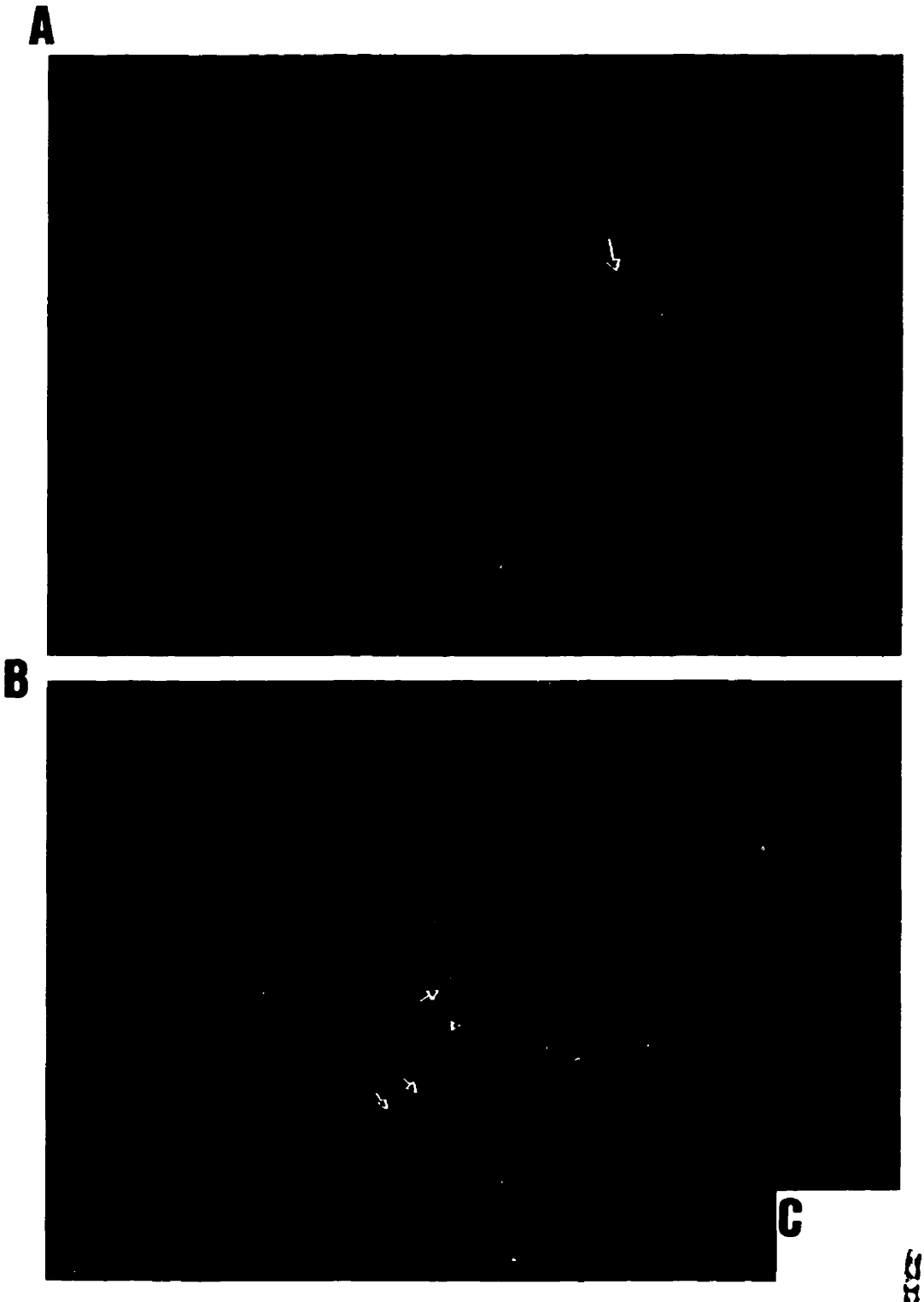
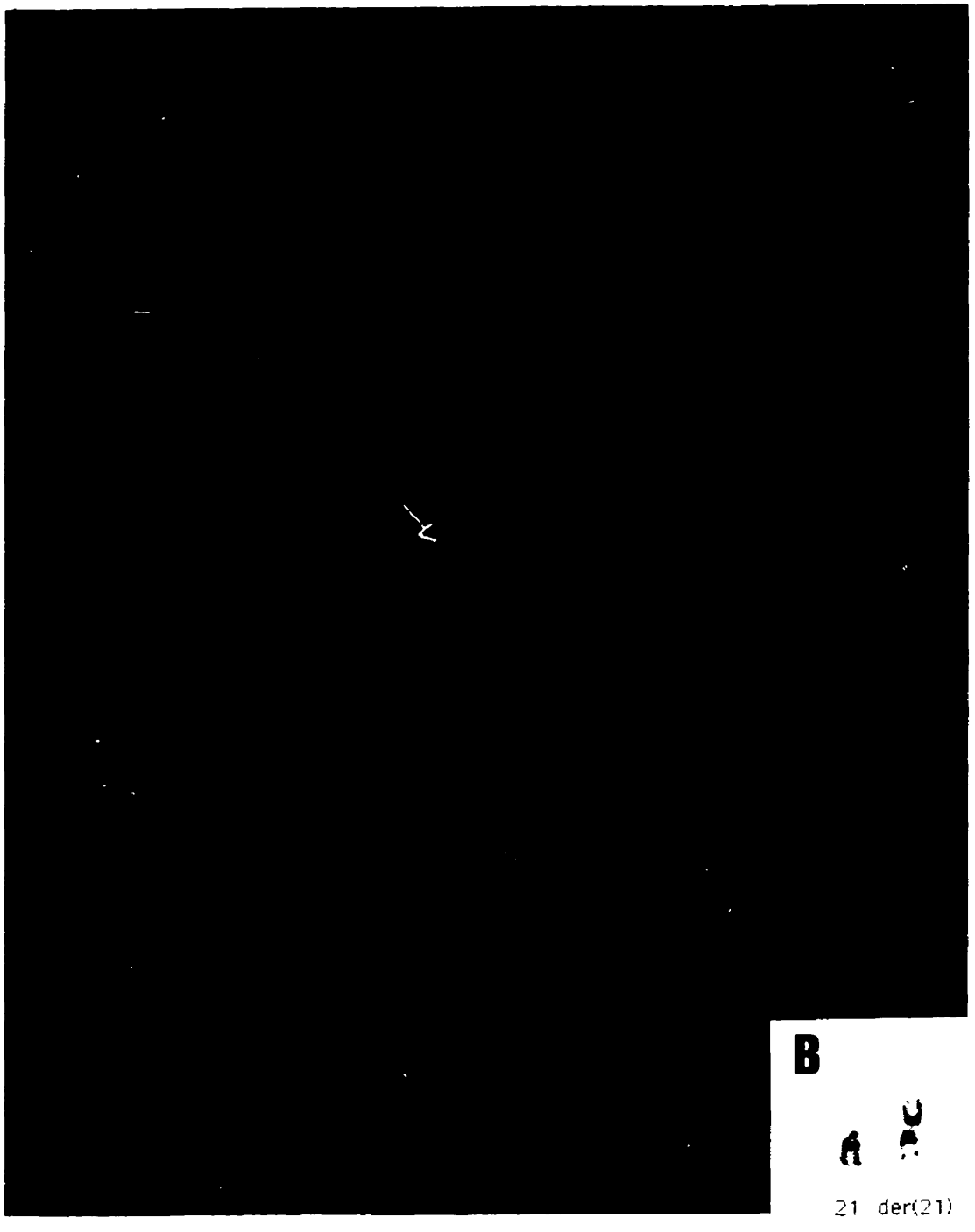

A
B
C

Fig. 13 (A) FISH of telomere 21q probe (yRM2208) to metaphase chromosomes of case 2. Hybridization signals are observed on the telomere of the long arm of normal chromosome 21 (yellow arrow), and on both telomeres of the derivative 21 (white arrow). **(B)** G-banded chromosome 21 and derivative (der) 21.


A
B
21 der(21)

syndrome. Similarly to the previous case, hybridization of the 21q telomeric probe produced three signals in metaphase cells, one on normal 21q, the other two signals on both telomeres of the abnormal 21 (Fig.14A,a). This was observed in 91% (n=11) of the metaphases analyzed. Three distinct signals were evident in 74% (n=34) of the interphase nuclei (Fig. 14A,b). These FISH results are in agreement with the previous findings from GTG banded analysis .

Case 4

To demonstrate the potential of telomere specific probes for studying deletions a half-YAC for 18q was hybridized to lymphocytes of a patient with a partial monosomy 18q. Monosomy could have resulted either from an interstitial or terminal deletion. One signal, on the long arm of the normal chromosome 18 was present in 95% of metaphase cells analyzed. Due to difficulty in identifying the abnormal 18, two color FISH using the 18q probe combined with a chromosome 18 centromeric probe was used. This experiment identified both of the chromosomes 18 and demonstrated the absence of 18q probe specific sequences in the deleted 18 (Fig.15A,a). Figure 15A,b. shows interphase nuclei with one clear distinct telomere 18q hybridization signal as seen in 79% of interphase cells that contained a determined number of signals. These results are compatible with a terminal deletion which could have resulted from a deletion and telomere reconstruction, or from a semi-cryptic balanced translocation of 18q to another chromosome in the parental germ cell with inheritance of an unbalanced derivative in the patient. Unfortunately, no samples were available from the parents to confirm or disprove the second hypothesis.

Fig. 14. FISH of telomere 21q (yRM 2208) to amniocytes of case 3.

(A) Metaphase chromosomes with hybridization signal on the telomere of the normal chromosome 21 (yellow arrow), and on both telomeres of the derivative 21(white arrows). **(B)** Interphase cell showing hybridization to the normal (yellow arrow) and to the derivative (white arrow) chromosome 21. **(C)** G-banded normal chromosome 21 and derivative 21.


A

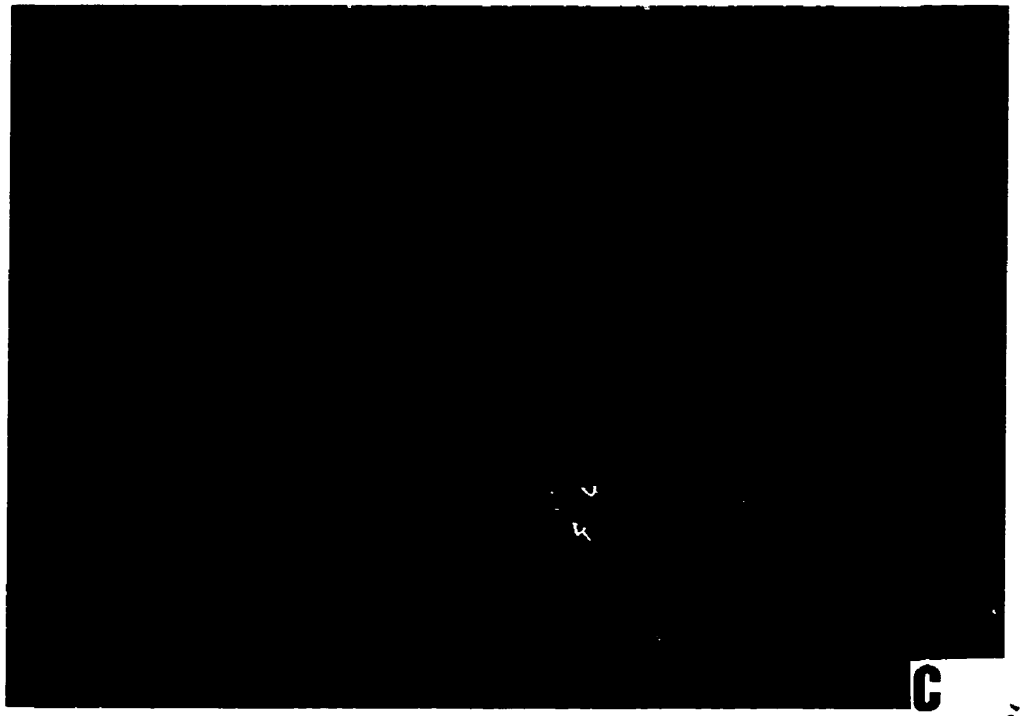
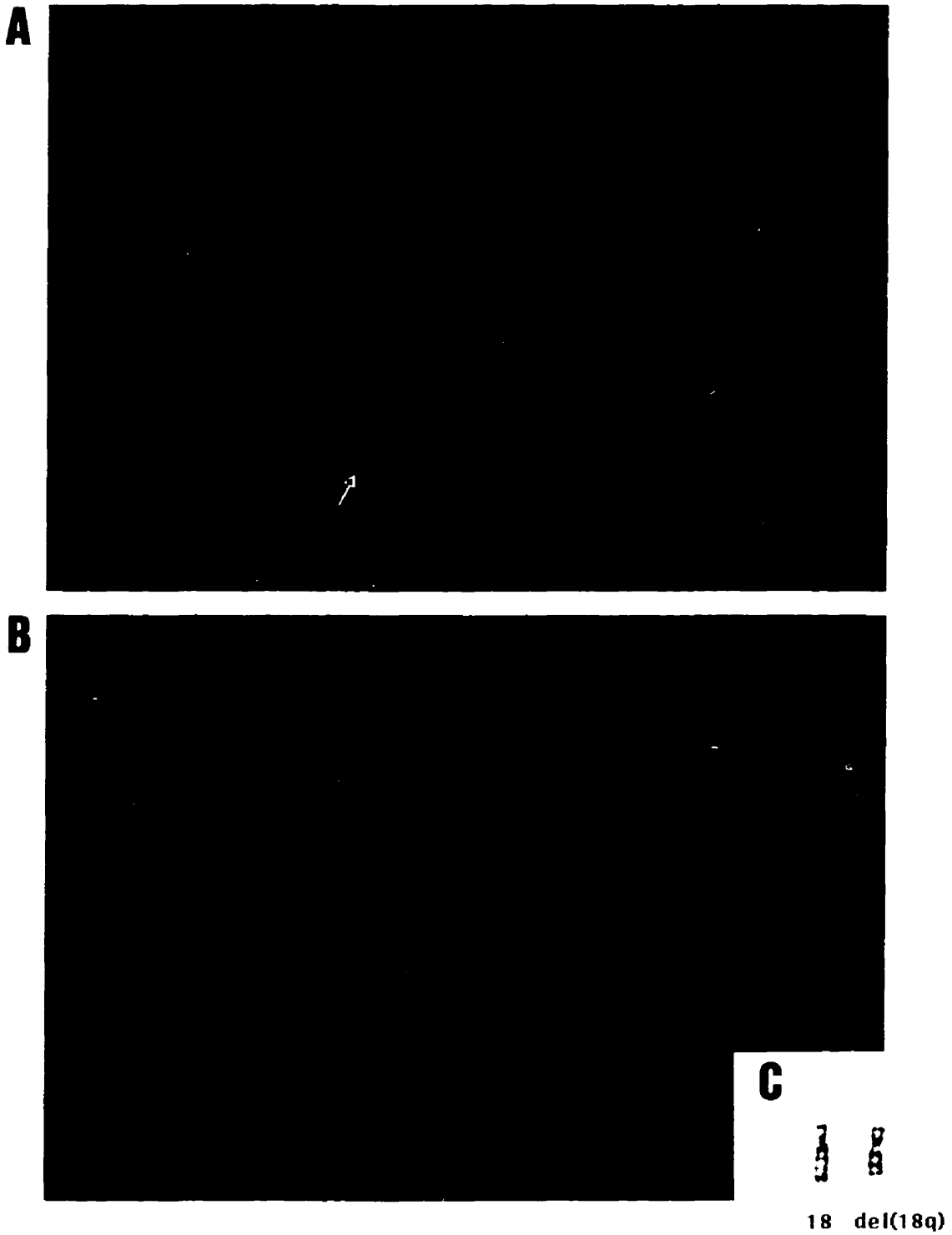

B
C

Fig. 15. FISH of telomere 18q probe in case 4. ONCOR probe specific to the centromeric region of chromosome 18 was also hybridized to help identify chromosomes 18. Green (FITC) represents hybridization with the 18q telomeric probe, red (rhodamine) represents hybridization with the centromeric probe **(A)** Metaphase cell showing hybridization of 18q probe to only one of the 18 homologs (yellow arrow). White arrow shows deletion of the 18q probe sequences from the other chromosome 18. **(B)** Interphase nuclei with two chromosome 18 centromeric signals (red) , but only one chromosome 18q telomeric hybridization signal (green). **(C)** G-banding pattern of normal and deleted chromosomes 18.


A
B
C
18 del(18q)

Case 5

A telomere 13q cosmid probe was used to study cells from a one year old female with a 46,XX, der(10)t(10;13)(q25;q13) karyotype as revealed by high resolution banding analysis. Both parents' karyotypes were normal. Although the karyotype suggested partial trisomy 13q, and the patient had dysmorphic features, the clinical picture did not correspond to the phenotype of trisomy 13 . FISH resulted in two 13q specific hybridization signals in metaphase cells, localized to the distal portion of the two 13 homologs (Fig. 16A). The fact that a third 13q signal is not present on the distal portion of the derivative 10 suggests that the aberration originating from a parental germ cell was not a result of a reciprocal translocation, but an insertion, comprised of two breaks in the long arm of chromosome 13q, and one or two breaks in chromosome 10q. This finding suggests three possibilities for the origin of the abnormality (Fig 17): (a) two breaks in chromosomes 13 and 10, with insertion of chromosome 13 into chromosome 10; (b) two breaks in chromosome 13 and one break in chromosome 10, with translocation of chromosome 13q to chromosome 10, a deletion of the 13q subtelomeric region, and reconstitution of the telomere; or (c) two breaks in chromosome 13, insertion of the 13q fragment located above the subtelomeric region of 13q into chromosome 10, and a translocation of a telomere of another chromosome below the inserted 13q. The finding of 10q subtelomeric sequences in the derivative 10, distal to the translocated region of 13q, would confirm possibility "a".

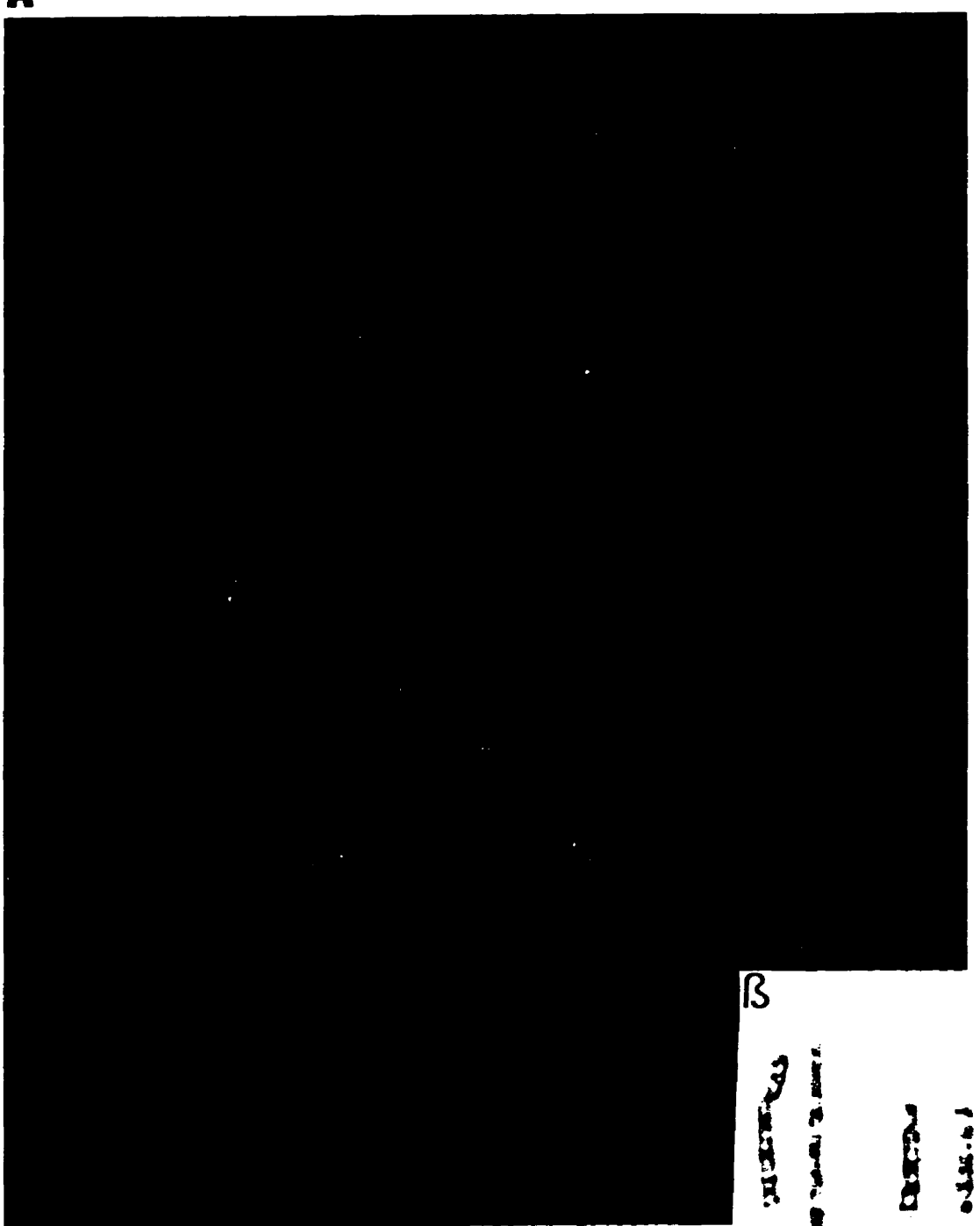
3.8.2 Balanced translocations

Case 6

Cosmid probe yRM2006 (14q), was applied to the study of a familial translocation involving chromosome 8 and 14. The father's karyotype was normal whereas the mother

Fig. 16. (A) FISH of cosmid probe yRM 2067 (13q) to chromosomes of patient in case 5. Arrows show hybridization to the distal portion of chromosome 13 homologs, indicating that the patient is balanced for the telomeric region of the q arm of chromosome 13. More detailed information on this aberration is provided in Fig. 15. **(B)** G-banded chromosomes 10, derivative (der)10, and 13.

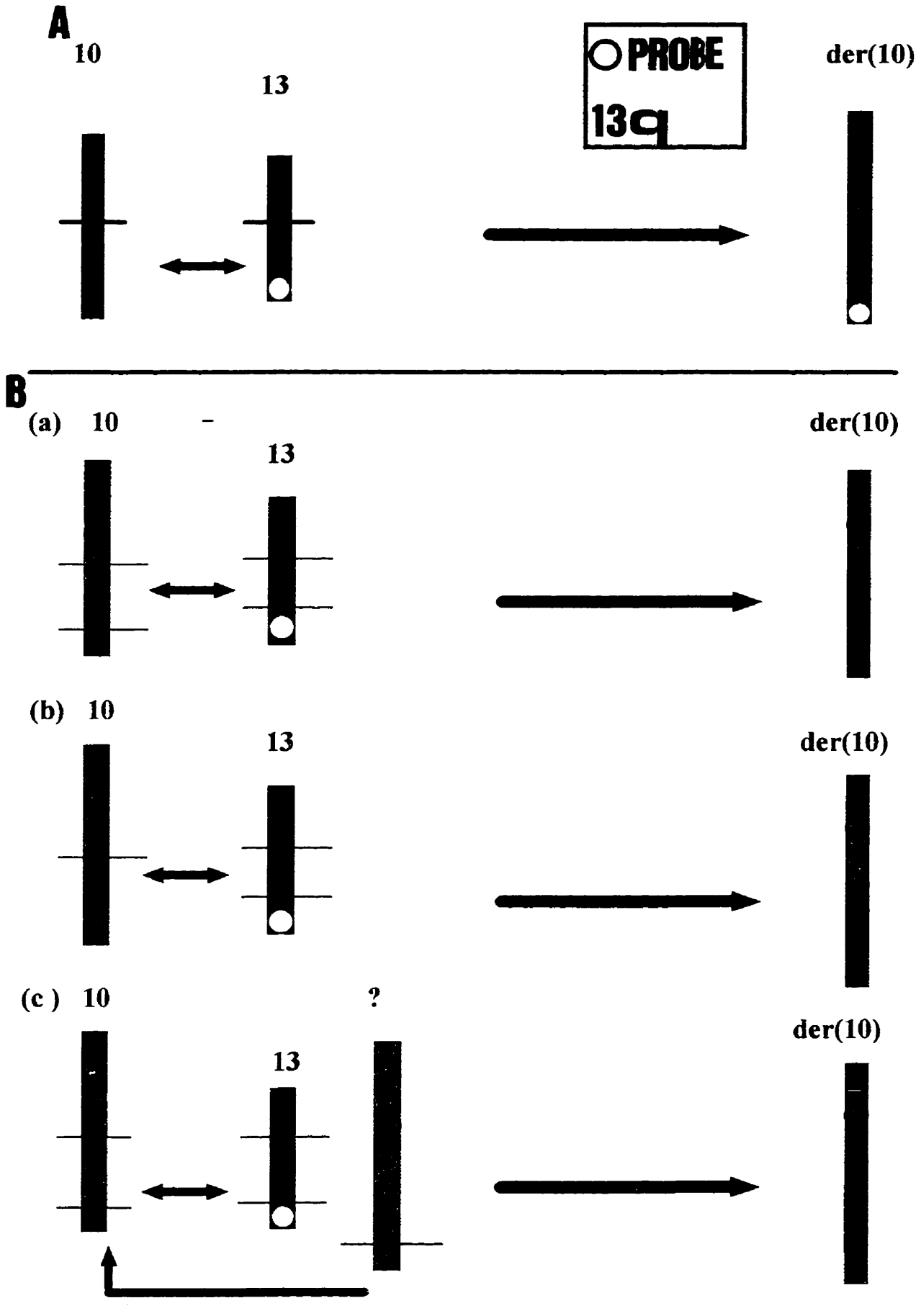
A



B

10 der(10) 13 13

Fig. 17. Origin of the aberration in patient in case 5. **(.)** Based on G-band analysis the aberration was thought to result from a reciprocal translocation in the parent. The patient was expected to be monosomic for the telomeric region of 10 q, and trisomic for the telomeric region of 13q. **(B)** FISH results show that the patient is disomic for the telomeric region of 13q, suggesting that the inherited aberration was a result of (a) two breaks in chromosomes 13 and 10, with insertion of chromosome 13 into chromosome 10; (b) two breaks in chromosome 13 and one break in chromosome 10, with translocation of chromosome 13q to chromosome 10, a deletion of the 13q subtelomeric region, and reconstitution of the telomere.; or (c) two breaks in chromosome 13 and one in chromosome 10, with an insertion of the 13q fragment located above the subtelomeric region of 13q into chromosome 10, and a translocation of a telomere of another chromosome below the inserted 13q


A
10
13
PROBE
13q
der(10)
B
(a) 10
13
der(10)
(b) 10
13
der(10)
(c) 10
?
13
der(10)

carried a reciprocal balanced translocation, 46,XX,t(8;14)(q24.1;q22) discovered as a result of amniocentesis findings from her first pregnancy (the same translocation as the mother's). Amniocentesis results from her second pregnancy had shown a 47,XY,+der(14) t(8;14)(q24.1;q22) karyotype. At birth, the child had a cleft palate and multiple physical anomalies. High resolution banding of lymphocytes at age 7 days showed the same karyotype as the amniocentesis. Cosmid probe for telomere14q was hybridized to the child's lymphocytes. Two signals, on telomeres 14q were observed in the majority of metaphases analyzed (Fig. 18A,a) Two color FISH using the 14q probe in conjunction with a centromeric probe mixture consisting of sequences specific to centromeres 14 and 22 confirmed that the translocated derivative did not include subtelomeric sequences from 14q (Fig.18A,b) and that the child was balanced for the terminal region of the long arm of chromosome 14.

Case 7

FISH with 14q specific probe was used to study a *de novo* translocation (46,XX, t(6;14)(p11.2;q11.2)) in lymphocytes of a four year old female with developmental delay. FISH resulted in two 14q telomere specific signals in the majority of metaphases analyzed. Two color FISH using the 14q telomeric probe and a chromosome 6 centromeric probe demonstrated that one of the signals originated on the telomere of chromosome 14, whereas the other localized to the distal portion of the chromosome 6 derivative (Fig.19A) These results are consistent with a balanced reciprocal translocation (6;14).

Fig. 18. FISH of cosmid probe yRM 2006 (14q) to chromosomes of patient in case 6. **(A)** Metaphase cell showing hybridization of 14q probe to the telomeric regions of two chromosomes 14 **(B)** Co- hybridization with ONCOR probe (red) specific to centromeres of chromosomes 14 and 22 identifies the two normal chromosomes 14, and the derivative 14 (yellow arrows), and confirms the absence of 14q subtelomeric sequences (green) on the derivative chromosome. **(C)** G-banded chromosomes 8, 14, and derivative (der) 14.

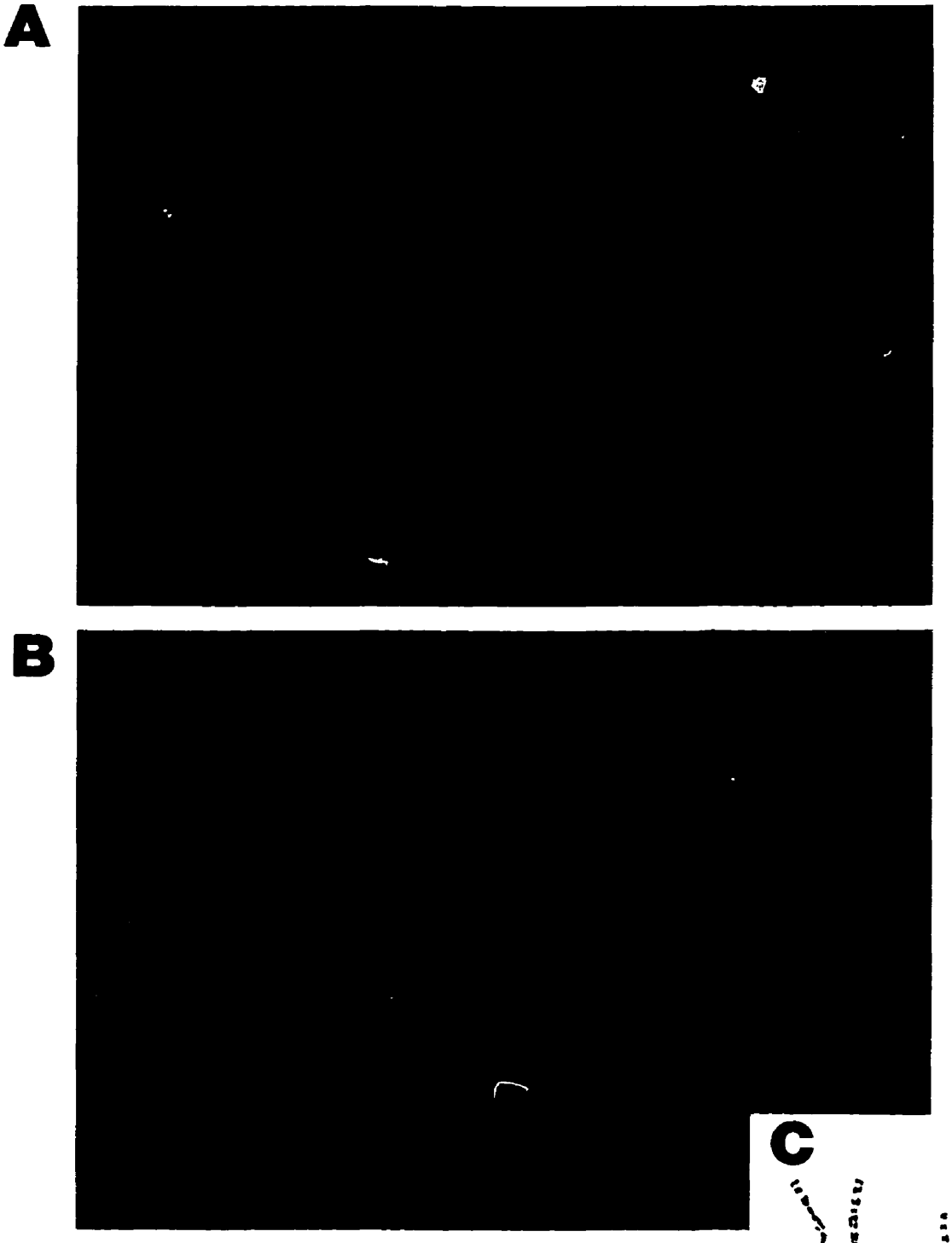
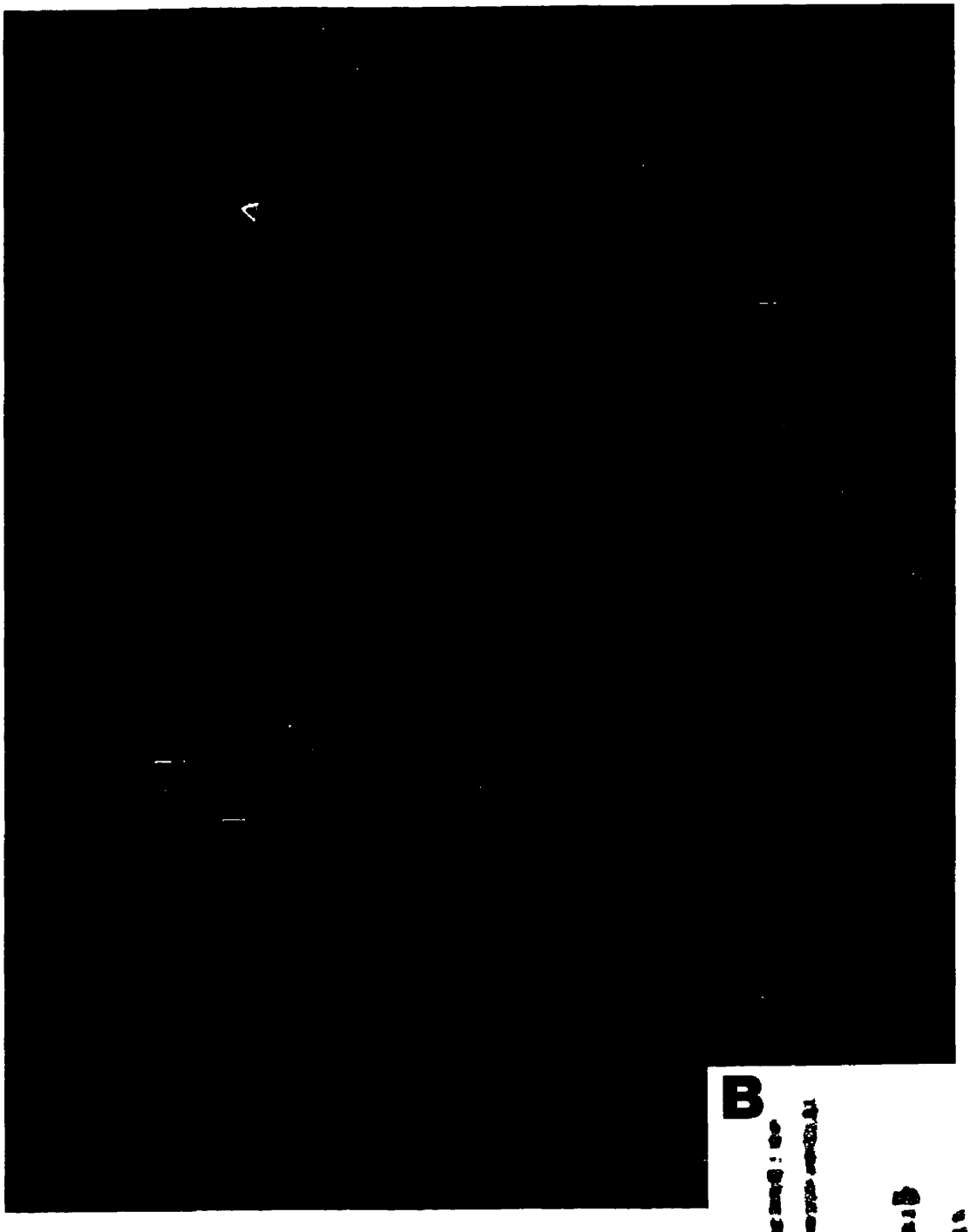

A
B
C
8
8
14
14
der(14)

Fig. 19. (A) FISH with 14q cosmid probe in conjuction with ONCOR probe specific to centromeric region of chromosome 6 , to chromosomes of case 7. The centromeric probe (red) identifed chromosome 6 (a), and the chromosome 6 derivative (b). Probe specific to telomere 14q (green) hybridized to normal chromosome 14 (yellow arrow), and the telomere of derivative 6 (white arrow). **(B)** G-banding pattern of the chromosomes involved in the aberration.


A
B
6 der(6) 14 der(14)

Case 8

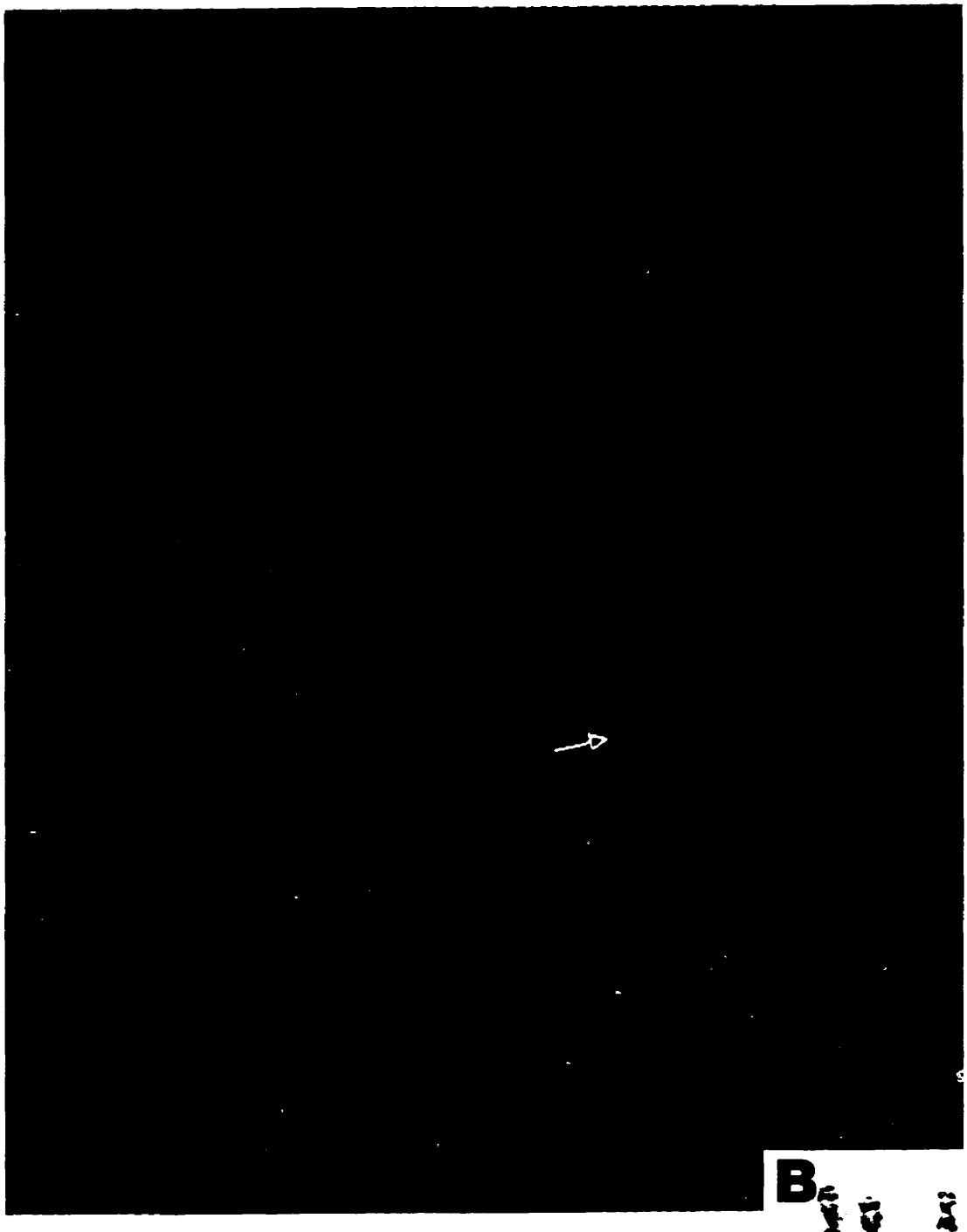
Half-YAC for telomere 18q was applied to the study of another familial translocation involving chromosomes 8 and 18. The proband was referred for chromosome analysis at age 11 due to fragile X like clinical manifestations, including cranio facial dysmorphism and developmental delay. High resolution banding revealed a 46,XY,t(8;18)(q22;q11.2) karyotype. FISH using half-YAC 18q probe produced two signals in majority of metaphase cells analyzed, one on the telomeric region of 18q, and one on the distal portion of derivative 8 (Fig. 20A). The mother's G- banded karyotype had also shown the same translocation, however FISH was not done due to unavailability of another maternal sample. These results confirm a balanced reciprocal translocation between the long arms of chromosomes 8 and 18.

Case 9

Half-YAC probe yRM2189 (10p) was used to study a *de novo* translocation which had been revealed by amniocentesis done for advanced maternal age. The results had shown a 46,XX,t(5;10)(q11.2;p15) karyotype in all cells analyzed. The translocation appeared balanced, however the presence of a submicroscopic deletion or duplication could not be ruled out. FISH using 10p specific probe was done to screen for a duplication or deletion of the distal region of the short arm of chromosome 10. Two color FISH using the 10p telomeric probe in conjunction with a 10 centromeric probe showed two 10p sequence specific signals, one on a normal 10, and the other on the derivative 5 (Fig.21A), thus ruling out a submicroscopic deletion or duplication of the distal region of the short arm of chromosome 10.

Fig. 20. (A) FISH of half-YAC 2050 (18q) to chromosomes of case 8. Metaphase shows hybridization signal on the telomeric region of 18q (white arrow) and on the distal portion of derivative 8 (yellow arrow). **(B)** G-banded chromosomes involved in the aberration.

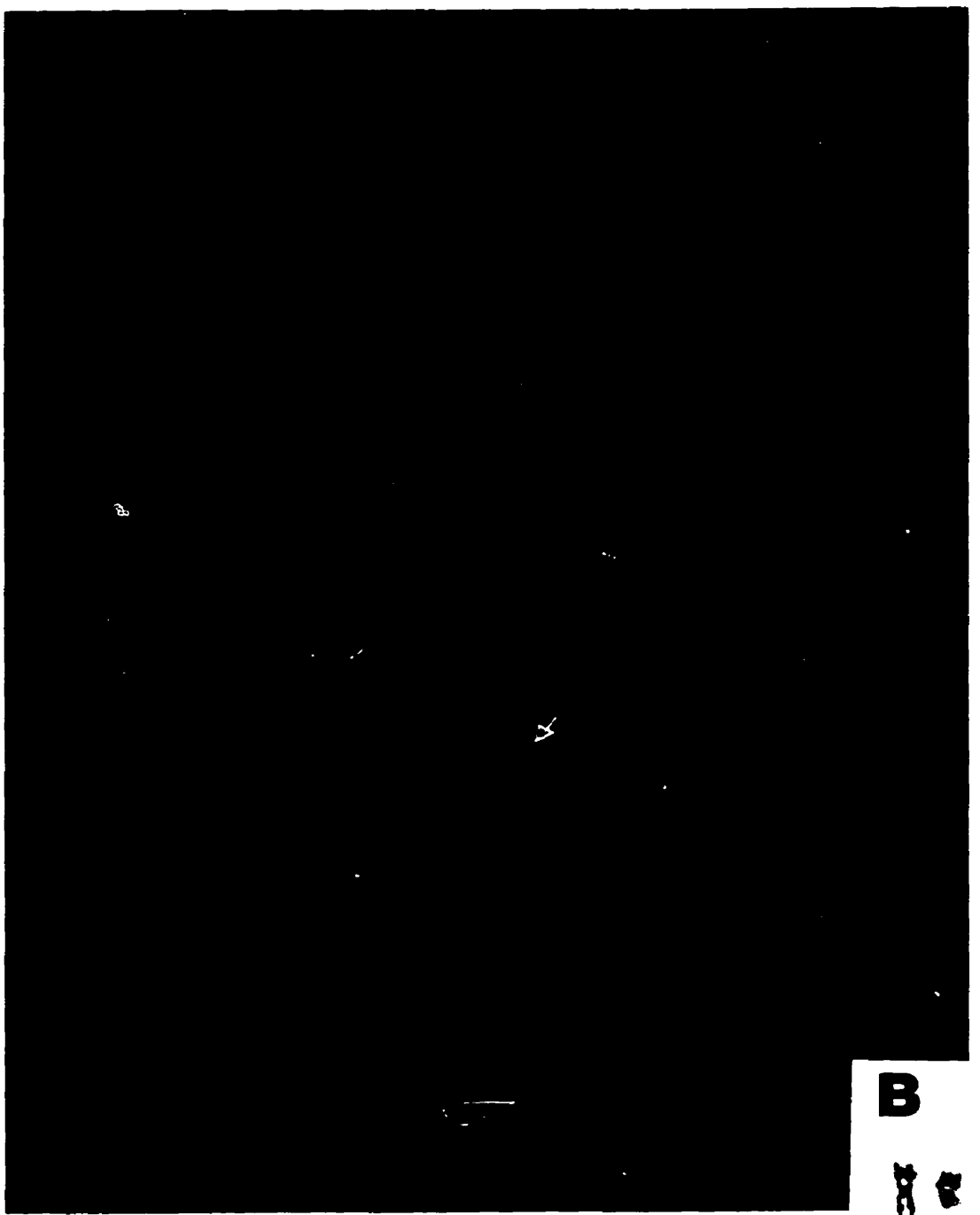
A



B

8 der(8) 18 der(18)

Fig. 21. (A) FISH of half-YAC telomeric 10p probe, in conjunction with ONCOR probe specific to the centromeric region of chromosome 10, to amniocytes of patient in case 9. Centromeric probe (red) identified chromosome 10 (a) and derivative 10 (b). Telomeric 10 p probe (green) hybridized to the telomere of the normal 10p (c), and to the telomere of the derivative 5 (d). **(B)** G-banded chromosomes involved in the aberration.



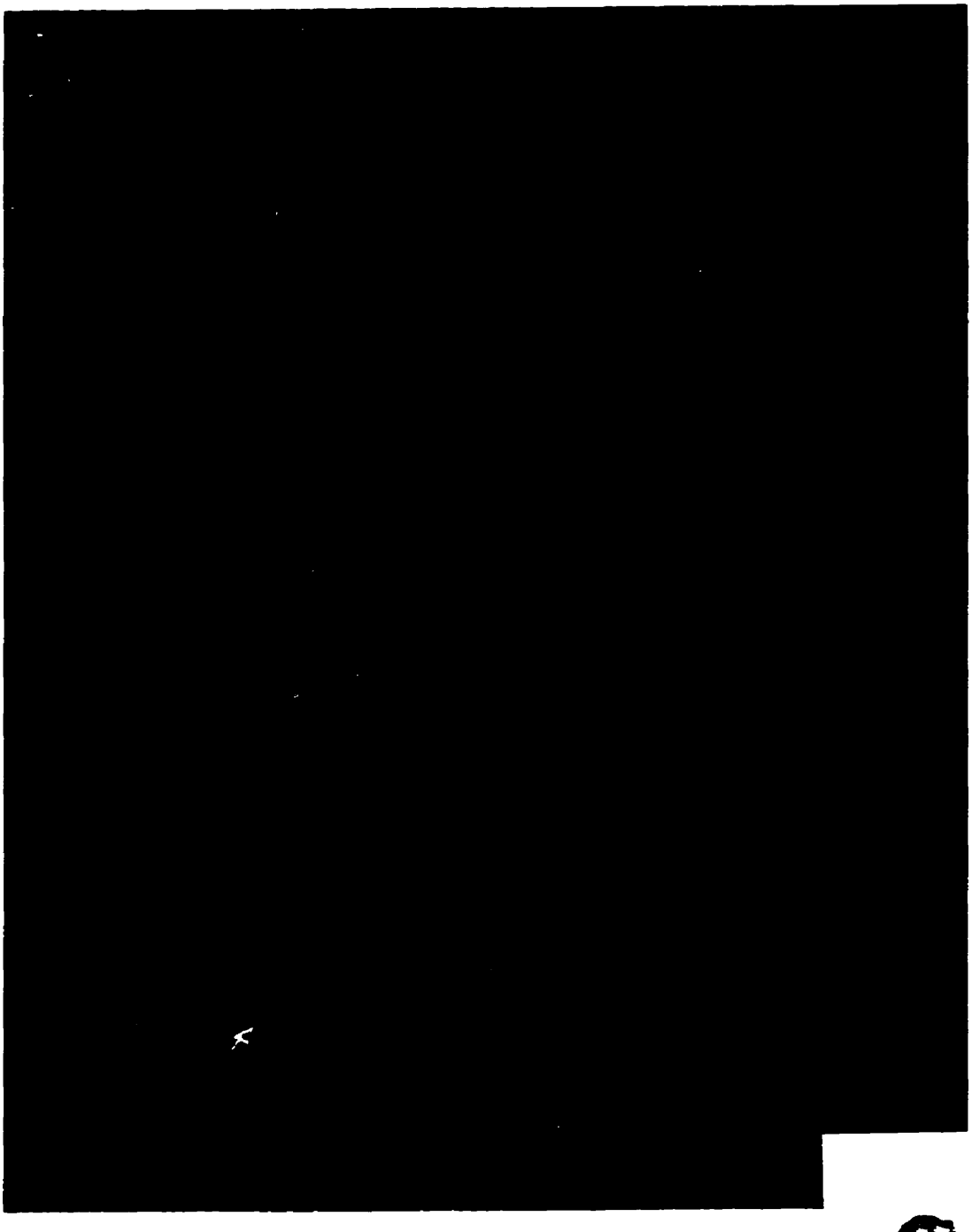
5 der (5) 10 der (10)

Case 10

FISH using a probe specific to telomere 10p was used to study a complex translocation in an ovarian tumor cell line (OV90). The cell line originated from ascites of a 64 year old patient with serous papillary adenocarcinoma, stage III, grade III and was contributed to our laboratory for FISH studies by Dr. Provencher and Dr. Mes-Masson from the Notre -Dame Hospital. G-banding showed a female karyotype which included a homogenously stained region (HSR) replacing chromosome 3p, a complex translocation involving chromosome 9 and 17 with a loss of the derivative (9)(p10;p10), and an add(13)(p11). This case was first studied with chromosome painting probes and centromeric probes. A translocation between chromosome 10 and 17 was detected. The 10p half-YAC probe was applied to determine whether the translocation was reciprocal or insertional. Hybridization of the 10p probe resulted in two signals, one on the telomere of 10p, the other on the p arm of a group A chromosome (Fig.22A). G-banding of the hybridized chromosome preparation indicated that the 10p probe sequences were translocated to the distal portion of the short arm of chromosome 1.

Fig. 22. (A) FISH of 10p telomeric probe to ovarian tumor cell line described in case 10. Metaphase shows hybridization of 10 p probe to telomere 10p (yellow arrow) and to theterminus of the long arm of a group A chromosome (identified as 1q by G-bandedanalysis). **(B)** G-banded chromosome 10, and derivative (der) chromosome 1.

A



CHAPTER 4

DISCUSSION

This study has focused on the recently isolated and characterized telomeric probes. It has addressed the different methods of their preparation, their performance in FISH, as well as their usefulness for clinical cytogenetics.

4.1 PROBE PREPARATION

4.1.1 Preparation of Half-YAC Probes

Because of the substantial role YACs have in gene mapping, many protocols have been developed for preparation of DNA from this vector. In this study, total yeast DNA was prepared in agarose plugs. This protocol was tedious and time consuming, however it was necessary because intact yeast chromosomes were required for PFGE in order to determine whether the yeast colonies represented faithful replicas of the source DNA.

Where clones were known to be stable, alternative methods have been used to prepare YAC DNA for FISH probes (Yurov et al., 1995; Nadal et al., 1996; Lengauer et al., 1993). The DNA can be prepared from lysed spheroplasts in solution. This DNA is not suitable for PFGE, however it can be used as template source for Alu-PCR or labeled directly. The yeast spheroplasts can be obtained by using lytic enzymes such as Lyticase, or Zymolase. Another variation of this technique, is to break the yeast cell wall using glass beads. In addition to preparing yeast DNA in agarose blocks, a number of YACs in this study was also prepared using glass beads. The DNA was used for Alu-PCR, however these PCR products showed significantly fewer bands compared to PCR products from agarose block DNA preparations.

FISH probes may be generated from total yeast DNA prepared in solution by lysis methods. Although this DNA is of sufficient purity and can be labeled, YAC DNA

represents only between 8 and 15% of the yeast DNA. The preferred protocol is to isolate the YAC from the total yeast DNA.

The isolation method used in this study, Alu -PCR, yielded probes which showed a high hybridization efficiency and which generated strong, clear signals. This was in agreement with Alu-PCR results reported by Lengauer et al., (1992), with the exception that the highest yield of amplification products in this study was obtained at annealing temperate of 45^0C and not 37^0C.

The yield of Alu -PCR product is YAC dependent. It is determined by the size of the YAC, and the number, type, frequency, spacing, and relative orientation of Alu elements within it. Experiments showed that Alu sequences are concentrated in the reverse (Giemsa negative) bands of chromosomes (Baldini et al., 1991; Manuelidis and Ward, 1984; Korenberg and Rykowski, 1988; Moyzis et al., 1989). Therefore, clones containing fragments from reverse band rich telomeric regions should yield optimal results (Lengauer et al., 1993).

An alternative method used to isolate human DNA from yeast is to separate the YAC from the yeast chromosomes by PFGE. Following migration, the band representative of the YAC is excised, purified and labeled. However, as seen in this study, YACs may co-migrate with other yeast chromosomes, and therefore may be difficult to isolate. In such cases, preparation can be enriched in YAC DNA by excising the YACs along with a number of yeast chromosomes located in the approximate region of YAC migration. The disadvantage of this method is that in order to purify the DNA from the gel, the gel has to be made of a high grade low melt agarose which is very expensive.

Philippsen et al. (1991) describes isolation and fractionation of yeast DNA following alkaline lysis, by cesium chloride -ethidium bromide equilibrium centrifugation. This method a is modification of the protocol for isolation of plasmid DNA. An attempt

was made in the present study to isolate a number of YACs which did not yield high quality PCR products by cesium chloride gradient. These experiments were unsuccessful, and did not yield DNA. The technique requires further optimization, including centrifugation conditions, and CsCl densities.

4.1.2 Preparation of Cosmid Probes

Alkaline lysis, although tedious, produced cosmid probes which yielded good quality FISH results. Purification by CsCl- EtBr centrifugation was very time consuming (2-3 days depending on the centrifuge), and the procedure involved handling of ethidium bromide which is toxic and use of CsCl which is expensive. However, this technique produced a high yield of DNA. Also, FISH with the probe yRM 2006 (14q), which had been purified using this technique, produced intense specific signals and little background. This may have been due to this method of purification yielding exceptionally clean DNA compared to other isolation and purification techniques (Sambrook et al., 1989). Due to generation of a high quality FISH probe, CsCl-EtBr equilibrium centrifugation is a worth while procedure when large amounts of cosmid DNA are required. This is the reason why equilibrium centrifugation has been the method of choice for preparation of plasmid or cosmid DNA in many laboratories despite the technical disadvantages.

Use of the commercial DNA purification and isolation Qiagen kit was simple, and rapid, requiring 3-4 hours of labor. However, the yield of the midi -prep was much smaller than expected. Perhaps not all DNA has been eluted from the resin, or DNA was lost in the tube following isopropanol precipitation. Furthermore, probes prepared by this method hybridized with lower efficiency, higher background, and weaker signals than did DNA prepared by CsCl EtBr gradient or alkaline lysis. Due to its ease and speed, the

technique might be advantageous in cases where small quantities of many different probes are needed (i.e. for testing of probes by FISH).

4.2 LABELING BY NICK TRANSLATION

The fragment size after labeling, affects the penetration of the probe into the chromosome preparation as well as the non-specific adherence (or background)of the probe to the preparation . Therefore, two labeled preparations of the same probe DNA may not yield identical FISH results (McNeil et al., 1991). Most protocols report that for optimum hybridization conditions, the probe length following incubation with DNA polymerase I and DNase I should be between 100 and 500 bp (Dracopoli et al., 1996; Lichter and Ried., 1994). Verma and Baboo (1995), advise that the optimal length is 500-1000 bp. Fragments which are larger are supposed to produce significant background with little or no detectable hybridization signal (Dracopoli et al., 1996). Although differences in FISH results in this study were noted among DNA labeled in different reactions, good quality hybridization signals and low background were obtained even with probe fragments of 500 bp- 2000 bp in size.

4.3 FISH

4.3.1 Optimal Conditions

A number of factors influenced FISH results. Success of FISH depends critically on the accessibility of the target DNA sequences to the hybridization reagents (probe and antibodies), and on the degree to which nonspecific reagent binding can be suppressed (Pinkel et al., 1986).

Multiple hybridization experiments to normal chromosomes confirmed that adequate suppression, blocking prior to incubation with antibodies, and stringent post

wash conditions are all very significant steps in obtaining good results. With the exception of concentration of probe and competitor DNA, the "optimal" FISH conditions were the same for all the half- YAC and cosmid probes.

The factor which yielded most notable differences in results, was the quality of the target chromosome preparation. It is best to use fresh samples, and when given the choice of cell type to choose synchronized lymphocytes.

Whereas some parameters influenced significantly the quality of results, others had no effect. Many FISH protocols specify pre-treatement in RNase, and longer rinses in PBS and incubations with antibodies than those used in this study. This study shows these parameters are flexible, and observing the specified conditions is not necessary for obtaining good FISH results with the telomeric probes.

4.3.2 Interphase FISH

Multiple studies report on identification of genomic constitution at the level of interphase nuclei. Interphase FISH enables the assessment of the number of hybridization targets in a large number of cells, and is helpful in determining percentage of abnormal karyotypes in cases of mosaicism, and in non-dividing tissues (Novak et al., 1995). The technique has been applied to, and is a very useful method for assessing numerical aberrations in tumors (Anastasi et al., 1992; Arnoldus et al., 1991; Cremer et al., 1988; Hopman et al., 1991; Gray et al., 1992; Xu and Wang., 1994), in amniocytes and chronic villi (Pinkel et al., 1986; Cremer et al ., 1986), early embryos (Ma et al., 1996; Manor et al., 1996; Harper et al., 1994), and sperm (Martin et al., 1990; Martin and Rademaker., 1995; Van Hummelen et al., 1996; Williams et al., 1993). Furthermore, it can be used on archived, paraffin embedded material (Novak et al., 1995). However, most of the studies have used centromeric probes (Anastasi et al., 1992; Bartsch et al., 1991; Pinkel et al.,

1988; Van Hummelen et al., 1996; Ma et al., 1996; Novak et al., 1996) or whole chromosome paints (Pinkel et al., 1988; Lichter et al ., 1988), probes which provide large, clear signals which are easily distinguished from the background.

This study shows that although subtelomeric probes provide high efficiency metaphase analysis, they are not very successful for interphase FISH. The number of signals seen in interphase nuclei does not necessarily correspond to the number of signals in metaphase cells under the same conditions. In addition, due to their small size it is difficult to distinguish the target signals from the background. This result was observed for both the half-YAC and cosmid probes, indicating that the problem was not related to the method of probe generation.

The varied appearance of hybridization signals in interphase nuclei may reflect replication timing of the telomeres. Studies suggest that singlets are representative of non replicated telomeric DNA , whereas doublets represent DNA sequences which had been replicated (Selig et al., 1992). Therefore, interphase nuclei containing both, a doublet and a singlet (Fig. 9A,10c,11B,13B) suggest replication-asynchrony of homologous telomeres.

The variability of interphase FISH results may be related to chromatin packing. The coils and loops in the chromatin may cause portions of DNA strand to be inaccessible to the probe or the detection reagents. When this occurs probe or reagents may bind only to some of the sequences in the probe specific stretch of DNA, resulting in inconsistent numbers of fluorescent, background -like spots, instead of one larger signal. Varied accessibility of probe and reagents to sequences within a stretch of DNA may also explain the appearance of fluorescent doublets, singlets, triplets, or clumps in some of the interphase cells seen in this study. The inconsistent appearance of interphase signals may

also be due to difficulty in visualizing target sequences, which due to the three dimensional organization of chromatin, lie on different planes of focus.

The variability in the appearance of fluorescent hybridization signals in interphase nuclei was a very interesting finding. Although this subject is not within the scope of the present study, future investigations, including confocal microscopy may provide more information on the structure and replication of telomere chromatin.

4.4 VALUE OF PROBES FOR CLINICAL APPLICATIONS

FISH with subtelomeric probes provided valuable information supplementary to G-banded karyotypes. The high efficiency of metaphase analysis with the half-YAC and cosmid probes allowed identification of duplications, and deletions. In cases 2 and 3, a subtelomeric probe specific to 21q confirmed trisomy for terminal regions of the long arm of chromosome 21 in patients with clinical manifestations of Down syndrome (DS) Although most cases of DS result from a supernumerary chromosome 21, a small percentage of cases result from partial trisomy 21q (Delabar et al., 1993). Furthermore, a majority of the phenotypic features is associated with the presence of three copies of region 21q ter (Nadal et al., 1996). Due to alphoid sequences common between chromosomes 13 and 21 (Jorgensen et al., 1987) chromosome 21 centromeric alphoid DNA probes are not very accurate for diagnosis of DS. Construction of new pericentromeric chromosome 21 specific YAC probes has been reported (Yurov et al., 1995) however these probes are still not useful for cases with partial trisomy 21q. YAC 21q subtelomeric probe described in this study, will provide an additional probe to the commercially available 21q cosmid probes, and its use can help identify cases of partial

trisomy 21. Furthermore the probe could help determine the breakpoint in translocations involving telomere 21 q.

In case 4 the subtelomeric 18q YAC probe aided in the understanding of the origin of the aberration. Visualization of the probe by FISH indicated that the deletion in the abnormal 18q was terminal and not interstitial. This suggests that the aberration resulted from a semi-cryptic unbalanced translocation between chromosome 18 and another chromosome.

The telomere specific probes also identified and yielded valuable information about translocations. By hybridizing to two chromosomes per metaphase, the probes confirmed that the translocations seen were balanced (cases 6,7,8,9). In one of the cases, (case 5), FISH yielded results which were unexpected. The G-banded analysis of the patient's chromosomes showed a trisomy 13q. This abnormality was thought to be the result of the malsegregation of a reciprocal translocation t(10;13) in a parental germ cell, and the resulting karyotype in the child to be unbalanced. Hybridization using a subtelomeric probe for 13q however, demonstrated that the patient was balanced for the terminal region of chromosome 13, suggesting another mechanism for the imbalance.

4.5 FUTURE STUDIES

Ledbetter (1992) proposed to use telomere -specific probes as a diagnostic approach for assessing telomere integrity in cases where a particular region has not been identified by clinical information but when a chromosomal abnormality is suspected. To allow simultaneous visualization of the maximum number of telomeres, multicolor FISH with telomere specific probes could be used to screen all the telomeres of patients with unexplained MR or couples with multiple miscarriages. This method could also be used to analyze the integrity of a fetal karyotype. Once common abnormalities are found in certain

conditions, genes in the regions could be isolated, and phenotype-genotype correlations established.

Evidence indicates that there are attachments between telomeres of a chromosome, and between telomeres and the nuclear envelope (Blackburn and Szostak, 1984). How the telomere-telomere interactions and attachments to the nuclear envelope contribute to the three dimensional organization in the nucleus, and their implications in meiotic chromosome behaviour and processes such as gene expression, has been studied in yeast *Drosophila* (reviewed in Price, 1992; Kipling and Cooke, 1992; Karpen, 1994; .82.) , as well as *Tetrahymena thermophilia* (Kirk et al., 1997). However investigations of these aspects in the human have been limited. Studies of X inactivation using a subtelomeric probe for chromosome X, show that the inactive human X chromosome forms a loop via telomere-telomere association near the nuclear envelope, whereas the active X chromatin orientation is linear (Walker et al., 1991). This was seen in both interphase and mitosis, as well as in mouse-human hybrids. Whether the observed telomere configuration is unique to inactive chromosomes and plays a role in silencing transcription, has not yet been established. Probes for the telomeres of other chromosomes, such as the ones developed in this study, could be used to expand further our knowledge of human telomere organization and their role in gene expression.

4.6 CONCLUSION

This study addressed the technical aspects and clinical applications of telomere specific FISH probes. The results generated from preparation of half-YAC and cosmid probes showed that a variety of methods can be used to produce telomeric probes successful for FISH studies. High quality FISH probes can be generated by Alu-PCR of those half-YACs which yield high amounts of amplification products. Good quality

cosmid probes can be prepared by alkaline lysis alone, or in conjunction with DNA purification by CsCl -EtBr centrifugation or by a resin column. Alkaline lysis followed by purification with CsCl-EtBr centrifugation yields the largest amount of probe DNA with best FISH results.

This study showed that the telomeric half-YAC and cosmid probes are efficient for the detection of duplications and deletions at the metaphase stage, and are able to provide information on chromosomal translocations which was not available by the conventional methods. The telomeric probes, however, are not very precise for interphase analysis.

When used on metaphases, telomeric probes would be very valuable tools for clinical diagnosis of abnormalities involving their target telomeric regions. For example, because Down syndrome is the most common autosomal aneuploidy (overall incidence estimated at about 1/600; cited in: Robinson and Linden, 1993), the half-YAC probe 21 q would be very useful in ascertaining cases of partial trisomy 21q. A probe specific for 18q, would also be very useful for detection of another common aneuploidy, trisomy or partial trisomy 18.

In the future, the set of probes used in this study, in conjunction with the remainder of the human telomeric probes, could be used in a telomere-integrity assay to help establish whether cryptic aberrations in terminal regions of chromosomes account for a significant source of human pathology. In addition to clinical applications, the probes could be used to study nuclear organization and gene expression.

SUMMARY

1) Half-YACs and cosmid clones specific to 22 different telomeres were obtained and probes generated using different DNA preparation methods.

2) Alu-PCR was successful for some, but not all half-YAC clones. Alkaline lysis and Qiagen kit generated probe DNA which could be used for FISH. However the largest amount of very pure DNA was obtained following CsCl-EtBr eqilibrium centrifugation.

3) Half-YAC and cosmid probes at optimal FISH conditions, produced large, clear signals with a high level of hybridization efficiency in metaphase cells. However, interphase analysis using these probes was not accurate.

4) Characterization of cases of partial trisomy 21q, 18q, and cases of balanced translocations t(8;14), t(6;14), t(8;18), t(5;10) using these telomeric probes, confirmed previous G-banding based diagnosis. The probes also provided information which conventional methods were not able to reveal, in cases of partial monosomy 18q, translocation t(10;13), and t(1;10).

5) FISH with half-YAC and cosmid telomere specific probes would be a valuable tool for clinical cytogenetics. By supplementing conventional analysis, metaphase FISH with telomeric probes would provide more information on abnormalities, and would reduce the number of unresolved cases.

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APPENDIX I: CLONES

Half-YAC	Telomere
yRM 2123	(1q)
yRM 2052	(2p)
yRM 2112	(2q)
yRM 2175	(3q)
yRM 2038	(4p)
yRM 2173	(4q)
yRM 2158	(6q)
yRM 2000	(7q)
yRM 2205	(8p)
yRM 2053	(8q)
yRM 2189	(10p)
yRM2136	(10q)
yRM2209	(11p)
yRM2196	(12q)
yRM 2006	(14q)
yRM2067	(13q)
yRM2148	(16q)
yRM2102	(18p)
yRM2050	(18q)
yRM2005	(20p)
yRM2208	(21q)
yRM2195	(Xq/Yq)
cosmid yRM2003	(2q/8p)

APPENDIX II : SOLUTIONS

Antifade solution
100 mg p- phenylenediamide dihydrochloride
10 ml PBS
adjust to pH8
90 ml glycerol

Denaturation solution

20 X SSC	4ml
distilled H_20	8ml
deionized formamide	28ml

pH 7

Hybridization buffer
50% formamide, SxSSC
10% Dextran suphate

LB medium

bacto-tryptone	10g
yeast extract	5g
NaCl	10g
H_20	to:1L

LET solution

0.5 M EDTA	500 ml
0.01M Tris	5 ml

pH 7.5

NDS solution

0.5 M EDTA	500 ml
0.01M Tris	5 ml

pH 7

N-lauryl sarcosine	5 g

PBS, pH ~ 7.3

NaCl (137 mM)	8.0 g
KCl (2.7 mM)	0.2 g
$Na_2HPO_4 7H_2O$ (8mM)	2.16g
KH_2PO_4 (1.5 mM)	0.2 g
H_2O	to 1 L

PBT

PBS	100 ml
BSA	1 g
Tween 20	100 µl

Post wash solution 50%

formamide	50ml
20 x SSC	10ml
H_20	40 ml

RNase pre-treatment solution

RNase (4mg/ml)	1ml
2 x SSC	39ml

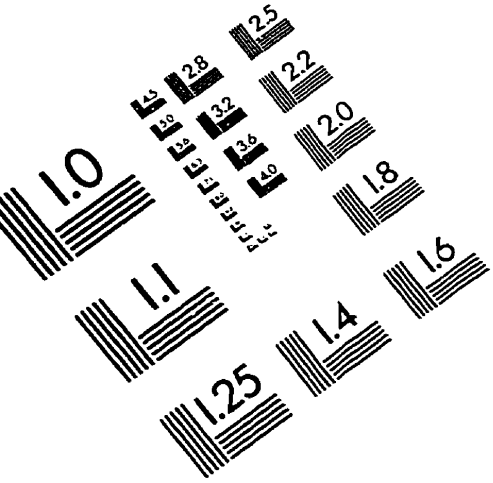
TE buffer

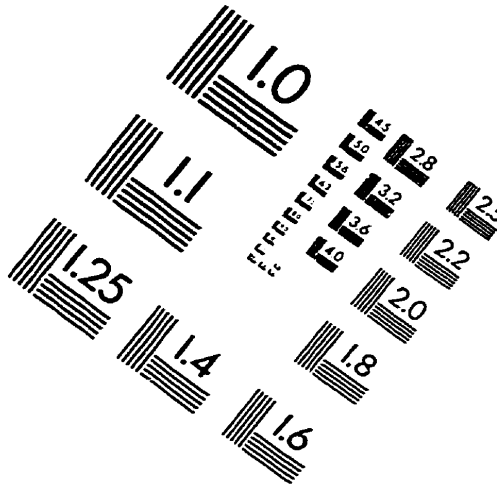
1M Tris Cl	10ml
0.5M EDTA, pH8	2ml
H_2O	to 1 L
pH 7.5	

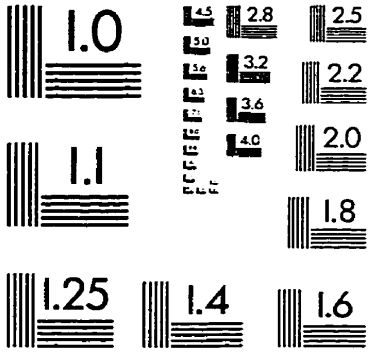
YPD media

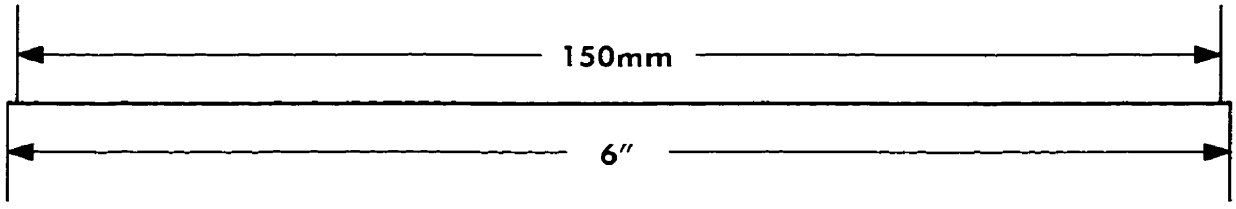
1% yeast extract	10g
2% dextrose	20g
2% bacto-peptone	20g
H_20	to 1 L

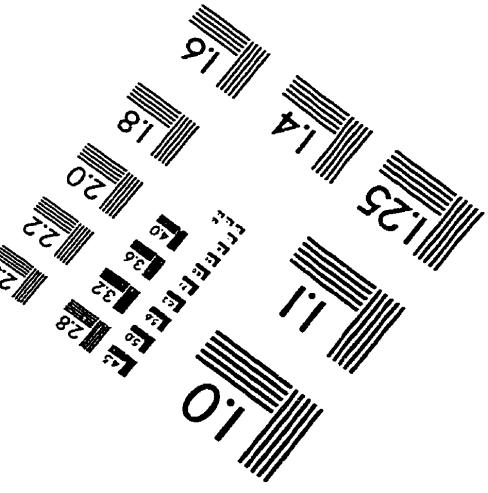
IMAGE EVALUATION TEST TARGET (QA-3)

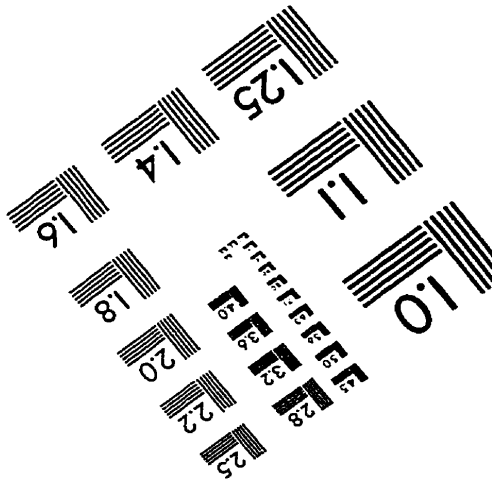












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