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EDITED BY
KIYOSHI OKUDA
Department of Laboratory Medicine,
Osaka City University Medical School,
Japan

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Contents

Human chromosome specific probes for the analysis of genetic and neoplastic disease

D.C. Ward, P. Lichter, T. Cremer, J. Borden and L. Manuelidis

Department of Human Genetics and Section of Neuropathology, Yale University School of Medicine, New Haven, CT 06510

Abstract - A method of in situ hybridization for visualizing individual human chromosomes from pter to qter has been developed and applied to the detection of structural aberrations in both metaphase and interphase cells. Numerical changes, deletions and chromosomal translocations were rapidly delineated in oligodendroglioma and glioblastoma cell lines which possess very complex and highly aneuploid karyotypes. A trisomic chromosome 21 karyotype, diagnostic of Down syndrome, was also readily detected using either a complete pool of insert DNA from a chromosome 21 recombinant DNA library or plasmid clones containing up to 94 kilobases of single copy DNA from band q22.3 of chromosome 21. Such in situ hybridization strategies provide new approaches for the prenatal diagnosis of genetic diseases and the definition of abnormal chromosomes in tumor cell populations.

INTRODUCTION

Chromosome banding techniques have facilitated the identification of specific human chromosomes and presently provide the major basis upon which chromosomal aberrations are diagnosed. The interpretation of chromosome banding patterns, however, requires skilled personnel and is often technically difficult, especially with respect to detecting minor structural changes and when analyzing complex karyotypes, such as those of highly aneuploid tumor cells (ref. 1). An additional complexity is that readable metaphase chromosome spreads are sometimes very difficult or impossible to prepare from certain cell types or tissues. Alternative methods for identifying chromosomal aberrations could augment current cytogenetic analyses, particularly if applicable to both mitotic and interphase cell populations.

Over the past few years a considerable body of evidence has been obtained which indicates that the DNA of individual chromosomes occupies focal territories, or spatially cohesive domains, within mammalian interphase nuclei (refs. 2-6). These observations indicate that chromosome-specific probe sets could be used to detect numerical or structural aberrations of chromosomal domains in non-mitotic cells. Indeed, recent in situ hybridization studies have demonstrated the prenatal diagnosis of trisomy-18 with interphase cells (ref. 7) using chromosome-specific repetitive DNAs as probes. Since all chromosome-specific repetitive DNAs reported to date are localized to discrete subregions of each chromosome, this class of DNA probes would be unsuitable for analyses of many types of chromosomal aberrations, e.g., translocations and deletions. However, the ability to detect uniquely the entire spectrum of sequences comprising a specific chromosome could make these analyses possible. Such a method is described below.

DECORATION OF INDIVIDUAL HUMAN CHROMOSOMES

DNA inserts from genomic DNA libraries derived from flow sorted human chromosomes (commercially available from the American Type Culture Collection) were purified and labeled with biotin by nick translation. This pool of DNA fragments was preannealed with a titrated amount of total human genomic DNA for a short time prior to hybridization with cellular or chromosomal preparations to suppress the cross-hybridization of repetitive sequences to non-targeted chromosomes. This method is referred to as "chromosomal in situ suppression (CISS)" hybridization. Optimized standard conditions for the preannealing step were: 5-30 µg/ml of labeled probe DNA and 100-200 µg/ml of human competitor DNA combined with salmon DNA such that the total DNA concentration was 1.0 mg/ml. The DNA mixture, in a conventional 50% formamide hybridization cocktail, was denatured and partially preannealed at 37°C for 10-20 minutes before addition to the specimen. Hybridization was then allowed

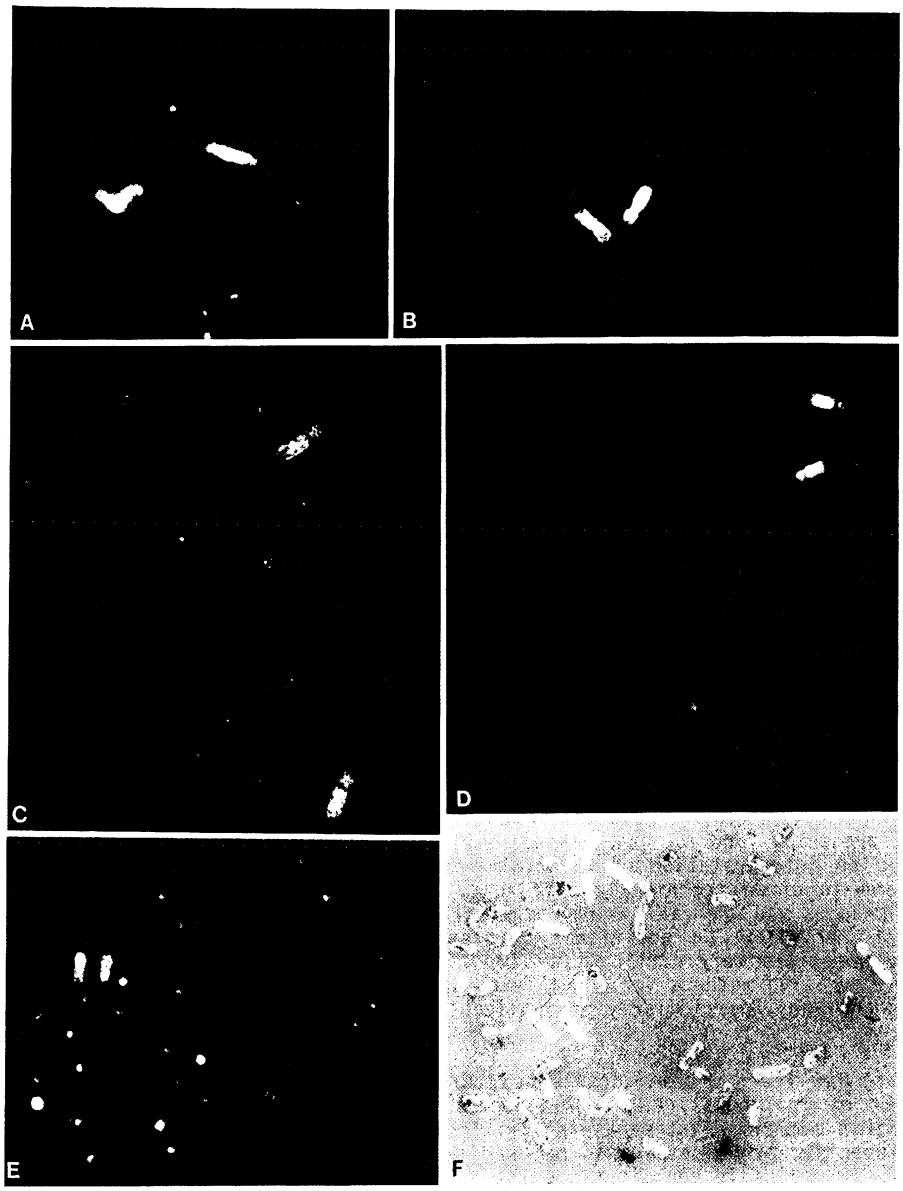

Fig. 1 A-F. Decoration of (A) chromosome 1, (B) chromosome 7, (C) chromosome 4, (D) chromosome 18, (E) chromosome 13, (F) chromosome 20 in normal human lymphocytes. Only the chromosome 13 insert DNA pool shows significant cross-hybridization to other chromosomes after the pre-hybridization suppression step. Detection was with fluorescein isothiocyanate-conjugated avidin (A-E) or with avidin-alkaline phosphatase using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as the enzyme substrate mixture (F). The signal of chromosome 1 (A) was amplified by the sandwich technique of Pinkel et al. (ref. 6)

to proceed for 8-14 hours. Following post-hybridization washes, the sites of hybridization were detected using fluorescent or enzyme labeled avidin. DNA from recombinant libraries for chromosomes 1,4,7,8,9,12,13,14,16,17,18,20,21,22 and X were assessed for their ability to decorate specifically their cognate chromosome and most libraries proved to be highly specific. Representative examples of such in situ hybridization results are shown in Figure 1. Note that some library DNA pools gave highly specific labeling of their cognate chromosomes (e.g., panel 1 B and C) while others (e.g., panel 1E) showed various extents of non-suppressible cross-hybridization. Table 1 lists the tested libraries with scores according to their labeling specificity. All scores are positive because the chromosome of interest was always decorated. The highest score (4+) is used when no significant cross-

TABLE 1. Relative quality of specific chromosome labeling in situ using preannealed biotinylated library DNA

Chromosome No.	Library Used (ATTC Designation)	Relative Specificity of In Situ Hybridization Signal[a]
1	LA01NS01	3+
4	LL04NS01	4+
7	LA07NS01	4+
8	LL08NS02	4+
9	LL09NS01	3+ (-h)[b]
12	LA12NS01	4+
13	LA13NS03	1+
14	LL14NS01	2+
16	LA16NL02	4+
17	LL17NS02	4+
18	LL18NS01	4+
20	LL20NS01	4+
21	LL21NS02	3+
22	LA22NS03	3+[c]
X	LA0XNL01	4+
X	LA0XNS01	4+

[a]See text for score definition; [b](-h): using this library as probe, the centromeric heterochromatin region of chromosome 9 was not labeled; [c]under standard preannealing conditions the chromosome 22 library gave a score of 1+; a value of 3+ was achieved only with a human competitor DNA concentration of $\geq$ 700 µg/ml (total DNA concentration 1.0 mg/ml).

hybridization to other chromosomes was observed and the scores decrease (3+ to 1+) with an increasing amount of cross-hybridizing sequences. All attempts to reduce the additional signals on other chromosomes by varying experimental conditions failed except in experiments with chromosome 22; in this case higher concentrations of human competitor DNA (700 µg/ml) resulted in a significant improvement of signal specificity. Additional details of the CISS hybridization protocol are described by Lichter et al. (ref. 8).

DETECTION OF HUMAN CHROMOSOME ABERRATIONS

Various combinations of cloned DNA fragments from human chromosome 21, previously localized to the 21q22.3 band, were tested for their ability to specifically label the cognate chromosomal region in lymphocyte metaphase spreads and interphase nuclei after *in situ* hybridization. The maximal amount of unique-sequence DNA in the probe set was $\sim$ 94 kb; this probe set, labeled with biotin, resulted in a clearly visible labeling of the terminal region of both chromatids of the chromosome 21 homologs in normal diploid metaphase spreads or interphase nuclei (data not shown). These signals were seen unambiguously and without exception in all metaphase spreads, even in spreads of poor quality or from prophase cells (not shown). In normal interphase cells, the majority (65-75%) of nuclei exhibited two signals, 25-30% showed one signal, and less than 5% showed no signal (for discussion of signal distribution in interphase nuclei, see refs. 8 and 9). Nuclei with three signals were found only rarely (< 0.2%) and may reflect incomplete hybridization to a few tetraploid cells in the sample. The 94 kb pool of 21q22.3 DNA permitted a fast and unambiguous diagnosis of trisomy 21 in all metaphase spreads from Down syndrome lymphocyte cultures (see Fig. 2A) as well as in interphase nuclei (Fig. 2 C and D). Furthermore, the

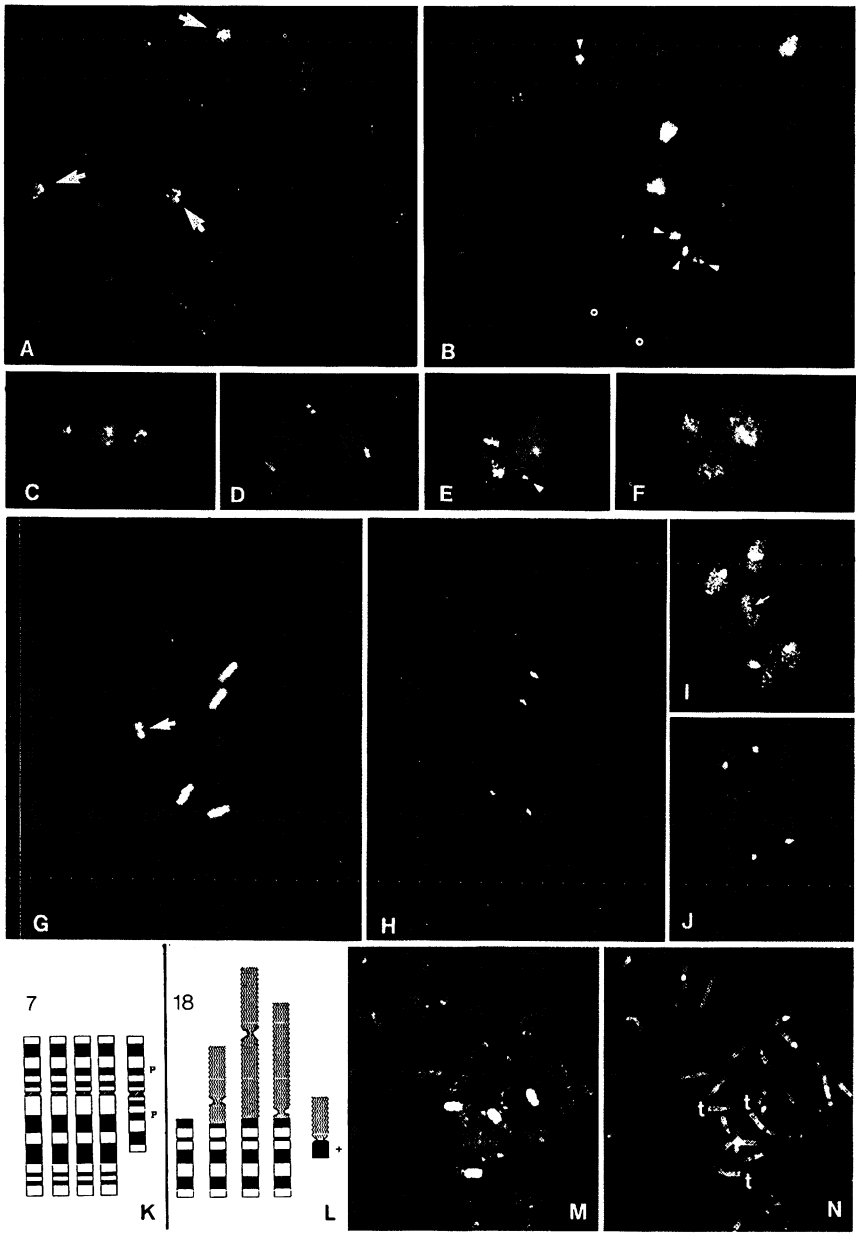

Fig. 2. Specific labeling of human chromosomes and chromosomal subregions by in situ hybridization. Signals on trisomy 21 (47, +21) lymphocyte metaphase spreads (A) after hybridization with the 94 kb probe set specifically labeling 21q22.3 and (B) after hybridization with chromosome 21 library DNA inserts using the CISS hybridization (ref. 8) protocol. Three chromosomes 21 are entirely delineated by the library inserts; additional minor signals (see text) are indicated by small arrowheads (also in E). (C-F) Labeling of trisomy 21 lymphocyte nuclei by the 94 kb probe set (C and D) and the library inserts (E and F). (G-J) Double hybridization of chromosome 7 library DNA inserts and a chromosome 7 specific alphoid repeat to the glioma cell line TC593. (G) Chromosome 7 inserts detect five entirely decorated metaphase chromosomes. Four of them are complete chromosomes 7, the fifth is an iso(7p) (see arrow). (H) The same field as G showing the chromosome 7 alphoid signals on only four decorated chromosomes; no signal is detected on the iso(7p). (I) Interphase nucleus of a TC593 cell showing five domains delineated by chromosome 7 inserts (the arrow represents the iso(7p) marker in interphase). Four of these are labeled by 7 alphoid probes (J). (K and L) Summary chromosome idiogram of complete and aberrant chromosomes detected by CISS-hybridization of chromosome library inserts 7 (K) and 18 (L) in TC593. G-bands (black) with breakpoints suggested

by data; grey regions are from other chromosomes that constitute part of the marker translocation chromosomes. A small translocation of chromosome 18 material in ~ 20% of TC593 metaphase spreads (+) could not be further identified. (M and N) TC593 hybridized to chromosome 18 inserts. Four decorated 18 chromosomes are shown (M) and three of them (DAPI stain in N) clearly translocated (denoted by t). All probes (with one exception) were biotinylated and detected after hybridization by FITC-conjugated avidin. The chromosome 7 alphoid repeat probe (H and J) was AAF modified and after hybridization detected via indirect immunofluorescence with rhodamine-conjugated antibodies.

quantitative distribution of hybridization signals in interphase nuclei of the same preparation, analyzed as described above, showed 55-65% of cells with three signals, 25-35% with two signals, 5-15% with one signal, and < 5% with no signal. Similar results, both qualitative and quantitative, were obtained when the chromosome 21 library was used with CISS hybridization for the detection of chromosome 21 abnormalities. Chromosome 21 was specifically and entirely decorated in normal lymphocyte metaphase spreads, although some additional minor binding sites were seen at or near the centromeric region of other acrocentric chromosomes, especially chromosome 13. Examples of the results obtained on trisomic metaphase spreads and interphase nuclei are shown in Fig. 2B and Fig. 2 E-F, respectively. Arrowheads in these panels denote the non-specific binding sites. The pool of unique DNA sequences from 21q22.3 was superior for interphase diagnosis since there are no additional signals and the signals are more focal with a better spatial resolution. Additional details on the application of these probes to the diagnosis of Down syndrome, including the detection of translocations involving chromosome 21q terminal sequences, are described by Lichter et al. (ref. 9).

Genetic changes are central to the initiation and progression of neoplasias. Some changes, such as point and insertional mutations, are submicroscopic. Other changes are quite large, and can be detected grossly in chromosome analyses. Non-random chromosomal changes, especially in hematopoietic malignancies, have been well established in recent years, and often mirror events at the molecular level. Such non-random changes may occur at an early stage in tumorigenesis and can be characteristic or even unique for specific tumor types. We have also demonstrated that it is possible to rapidly screen both mitotic and interphase tumor cells for complex numerical and structural aberrations of individual chromosomes during an analysis of oligodendroglioma and glioblastoma cell lines using several chromosome libraries under CISS hybridization conditions. Despite the fact that these lines were propagated in long term culture, they displayed several cytogenetic features common to many glioma cells. Two examples from this study, using chromosome 7 and 18 library probes and the glioma cell line TC593, are shown in Fig. 2, panels G-N. Four apparently normal chromosomes 7 and one smaller metacentric chromosome with 7 sequences (see arrow) are detected in metaphase (panel G) and interphase (panel I) cells. The comparison with the simultaneously labeled chromosome 7 alphoid repeat DNA in both metaphase (panel H) and interphase (panel J), demonstrates the lack of the alphoid sequences in the small decorated chromosome. The latter chromosome was identified as iso(7p) by DAPI staining (not shown). A more complex picture is seen with chromosome 18 DNA. Three translocation chromosomes involving chromosome 18 material were typically detected in addition to an apparently normal chromosome 18 (compare fig. 2M and N). In a minor proportion of metaphases there was a small additional translocation observed (not shown). The exact chromosomal region from which this translocated 18 material derived could not be resolved by DAPI staining. The chromosomal representation of chromosome 7 and 18 in the TC593 cell line is schematically shown in Fig. 1K and L, respectively. These data show that numerical as well as structural chromosomal aberrations can be rapidly detected by the CISS hybridization procedure.

Digitized images were used to quantitatively measure decorated areas in metaphase preparation and interphase cells where chromosomal domains were well resolved. Quantitative evaluation of chromosome equivalents indicated highly concordant numbers for interphase versus metaphase (not shown). We analyzed the chromosomal dosage in both glioma lines relative to their status of (pseudo)ploidy. Accordingly, an increase or a decrease of the chromosome copy number of 4 in the pseudotetraploid line TC593 (or 3 in the pseudotriploid line TC620) was considered an overrepresentation or underrepresenatation, respectively. In spite of the fact that both glioma lines have been passaged in vitro for many years, an underrepresentation of chromosome 22 and an overrepresentation of chromosome 7 (specifically 7p) were observed. These observations agree with previous studies on gliomas. In addition, sequences of chromosome 4 were also found to be underrepresented, especially in TC593. These analyses indicate the power of these methods for pinpointing chromosome segments that are altered in specific types of tumors. Additional details of the karyotypic analysis of these glioma cells are presented by Cremer et al. (ref. 10). In summary, our results demonstrate that chromosome specific DNA probes can be applied successfully to tumor cell cytogenetics and prenatal diagnosis.

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