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Rapid detection of human chromosome 21 aberrations by *in situ* hybridization

(Down syndrome/chromosome translocations/prenatal diagnosis/interphase cytogenetics)

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ABSTRACT **Plasmid clones containing up to 94 kilobases of single-copy DNA from band q22.3 of chromosome 21 and a complete pool of insert DNA from a chromosome 21 recombinant library have been used to rapidly detect numerical and structural aberrations of chromosome 21 by *in situ* hybridization in both metaphase and interphase cells. A trisomic karyotype, diagnostic of Down syndrome, is readily detected in nonmitotic cells because the majority of their nuclei exhibit three discrete foci of hybridization, in contrast to normal diploid cells, which show two foci. Chromosomal translocations involving chromosome 21 sequences were also detected with these probes, and the intranuclear location of 21q22.3 DNA sequences in "normal" human brain neurons was established with the plasmid DNA probe set. These results suggest that chromosome 21-specific probes may have utility in clinical diagnostics, especially by facilitating the direct analysis of interphase cells.**

The smallest human autosome, chromosome 21, has been highly relevant to clinical cytogeneticists because trisomy 21 is the primary cause of Down syndrome (1). The recent mapping of the locus for familial Alzheimer disease (2) and the gene for the amyloid β protein (3, 4) to 21q11.2→21q21 has focused additional attention on chromosome 21. Interestingly, Down syndrome patients and familial Alzheimer patients both develop clinical dementias and have similar brain pathology with plaques rich in the amyloid β protein (5, 6). Cytogenetic studies have indicated that only trisomy of subregion 21q22→21qter is required to elicit the Down syndrome phenotype. There is, however, some disagreement as to which part of this subregion is responsible for the complex pathological effects (reviewed in ref. 7).

The majority of Down syndrome patients (≈95%) show three chromosomes 21, and in about 5% of the cases, the trisomy is caused by a Robertsonian translocation (8). Both forms of trisomy are routinely diagnosed by conventional banding techniques Additionally, a small portion (<<1%) of Down syndrome is caused by reciprocal translocation (8). This is difficult to diagnose because the translocated terminal segment of chromosome 21 can be very small, and it is detectable only by high-resolution banding. Another diagnostic complication is the occurrence of trisomy 21 mosaicism. It has been reported that the incidence of chromosome 21 mosaicism is between 1% and 2%, although the actual frequency may be higher (8).

Recent studies (9–15) have shown that the DNA of each chromosome occupies a discrete focal territory within an interphase nucleus. These observations indicate that chromosomal aberrations can be detected directly in nonmitotic cells by *in situ* hybridization using chromosome-specific probes. Indeed, a successful diagnosis of trisomy 18 has been reported by using amniotic fluid cells hybridized with a repetitive sequence probe that, under high stringency conditions, is specific for the centromeric region of chromosome 18 (16). A similar diagnosis of trisomy 21 also has been reported (17) with probe DNA that was derived from flow-sorted chromosome 21 but was not further defined.

DNA probe sets that specifically label the terminal band 21q22.3 or decorate the entire chromosome 21 are reported here, and the application of these probes to the detection of numerical and structural aberrations of chromosome 21 in both metaphase and interphase cells is described.

MATERIALS AND METHODS

DNA Probes. All plasmids contain inserts of human chromosome 21 that were mapped to 21q22.3 (18–22). All inserts were either known (20, 23–25) or verified by Southern blot analysis to be single-copy DNA; the plasmids other than pS2 are subclones derived from a λ phage library (24) or a cosmid library (25). The plasmids are listed in Table 1 with the Human Gene Mapping Workshop symbols (26) and the approximate insert fragment length.

The human chromosome 21 genomic library LL21NS02 was obtained from the American Type Culture Collection and amplified on agar plates as recommended. Phage DNA was prepared and digested with *Hin*dIII, and the DNA inserts were separated from the vector arms by preparative gel electrophoresis in 0.6% agarose. DNA was isolated from gel slices by electroelution; purified by Elutip-d chromatography (Schleicher & Schuell); extracted with phenol/chloroform, 1:1 (vol/vol); and precipitated with ethanol (14).

Human Cells. Metaphase spreads and interphase nuclei were prepared from (*i*) lymphocyte cultures of normal (46, XY) individuals, (*ii*) lymphocytes of Down syndrome (47,+21) individuals, (*iii*) chorionic villi samples cultured for prenatal diagnosis (*ii* and *iii* were provided by T. Yang-Feng, Yale University Cytogenetics Laboratory), and (*iv*) cultures of TC620, an oligodendroglioma-derived pseudotriploid cell line (28). Standard techniques of colcemid treatment, hypotonic treatment, and methanol/acetic acid fixation were used. Biopsy material from the cortical region of a "normal" human brain (46, XX) was fixed, sectioned, and permeabilized as described (29).

***In Situ* Hybridization.** Various combinations of plasmid DNA, labeled with biotin-11-dUTP by nick-translation (30), were used for hybridization at concentrations ranging from 2 to 15 μg/ml depending on the pool size. For example, 15 μg/ml was used when the probe mixture contained 94 kilobases (kb) of insert DNA; the probe concentration was

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Abbreviations: CISS, chromosomal *in situ* suppression; CV, chorionic villi; DAPI, 4′,6-diamidino-2-phenylindole.

Table 1. Plasmids with inserts from chromosome 21

Gene symbol	Plasmid	Insert length, kb	Gene symbol	Plasmid	Insert length, kb
BCEI	pS2 (23)	0.6	*D21S56*	pPW520-10R	4.6*†
D21S3	pPW231F	0.8*†		pPW520-11R	1.8*†
	pPW231G	0.7*†	*D21S57*	pPW523-10B	6.5*
D21S23	pPW244D	1.0		pPW523-1H	7.0*
D21S53	pPW512-6B	3.0*		pPW523-5R	2.2*†
	pPW512-8B	3.8		pPW523-10R	3.8*†
	pPW512-1H	2.9*†		pPW523-19R	2.5*
	pPW512-16P	2.7*	*D21S64*	pPW551-8P	1.9*
	pPW512-18P	1.6*		pPW551-12P	4.2*
	pPW512-4R	4.7	*D21S71*	pPW519-10P	0.8
	pPW512-12R	2.0*		pPW519-11P	3.0
D21S55	pPW518-4H	1.6*		pPW519-1R	6.0*
	pPW518-10P	2.9*		pPW519-8R	2.9*†
	pPW518-5R	5.2*†		pPW519-9R	1.7*
D21S56	pPW520-5B	5.0		pPW519-14R	4.0*†
	pPW520-6B	1.0*		pPW519-22R	1.8*

Preparation of plasmid DNA was according to standard protocols (27). Various probe sets were obtained by pooling plasmids (equal molar amounts), resulting in DNA probe complexities of 94 kb (all plasmids listed), 75 kb (plasmids labeled with an asterisk), or 29 kb (plasmids labeled with a dagger).

decreased in proportion to the sequence complexity of the probe mixture. The size of the probe DNA was adjusted to a length of 150–250 nucleotides empirically by varying the DNase concentration in the nick-translation reaction. The hybridization cocktail also contained 50% formamide, 0.30 M NaCl, 0.03 M sodium citrate (pH 7), 10% (wt/vol) dextran sulfate, and on occasion 0.5 mg of sonicated salmon sperm DNA per ml. Simultaneous denaturation of probe and target DNA was carried out at 75°C for 6 min (metaphase spreads) or 94°C for 11 min (tissue slices). Hybridization reactions were incubated at 37°C overnight.

Delineation of individual chromosomes with DNA probes derived from sorted human chromosomes was done by a method termed chromosomal *in situ* suppression (CISS) hybridization as described (14). Briefly, biotinylated chromosome 21 library DNA inserts (5 μg/ml), DNase-digested human genomic DNA (200 μg/ml), and salmon sperm DNA (800 μg/ml) were combined in the hybridization solution, heat-denatured, and partially prehybridized for 10–30 min at 37°C before application to a separately denatured specimen.

Posthybridization washes, detection of hybridized probe by using either alkaline phosphate-conjugated avidin or fluorescein isothiocyanate-conjugated avidin, and photographic conditions were as described (14). When probe sets containing 29 kb or less of target sequence were used, the fluorescein isothiocyanate detection was generally enhanced by one cycle of signal amplification (31).

All quantitative analyses of interphase signals were carried out by using slides from several independent experiments, with more than 100 nuclei being analyzed per slide. Comparison of signals in normal and trisomic samples was done in a blind-study fashion.

RESULTS

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 ≈94 kb; this probe set resulted in a clearly visible labeling of the terminal region of both chromatids of the chromosome 21 homologs (see Fig. 1*B*). 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 (see Fig. 1*C*), 25–30% showed one signal, and less than 5% showed no signal (for discussion of signal distribution in interphase nuclei, see refs. 13-15). Nuclei with three signals were found only rarely (<0.2%) and may reflect incomplete hybridization to a few tetraploid cells in the sample. Similar results were obtained with probe sets containing 29 or 75 kb of DNA. With probe sets containing fewer than 20 kb of insert DNA, there were increased numbers of cells with less than two signals. Thus, these probe sets were deemed unsuitable for diagnostic purposes. However, such probes still yielded specific signals on the majority of chromosomes 21, even with a 6-kb single-copy DNA (see Fig. 1*A*), especially when signal amplification was used.

The usefulness of chromosome library DNA CISS hybridization (14) for detecting chromosome 21 was also evaluated. 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 (normal karyotype not shown; see Fig. 1*E*). Suppression with additional DNA including the plasmid L1.26, which detects a repetitive DNA located predominantly at the centromeric region of chromosomes 13 and 21 (32), did not efficiently suppress the minor non-21 chromosomal signals. Quantitative evaluation of interphase nuclei signals again showed a negligible portion of nuclei with three signals; however, a significant increase in nuclei with less than two signals was observed (50–60% with two signals, 35–45% with one signal, and 5–10% without a signal). The numerical differences observed with the two different probes can be explained in part by the number of nuclei (up to one of three) that were excluded from the latter analysis because they exhibited larger and more diffuse signals, most likely from more than one chromosome that could not be resolved unambiguously as two separate chromosome domains in a two-dimensional representation. The minor cross-hybridizing sites noted above presented a second experimental complication but did not adversely influence data interpretation.

The optimal (94 kb) plasmid pool as well as CISS hybridization with chromosome 21 library inserts were tested further by using cells containing chromosome 21 aberrations. Both probe sets permitted a fast and unambiguous diagnosis of trisomy 21 in all metaphase spreads from Down syndrome lymphocyte cultures (see examples in Fig. 1 *D* and *E*). Furthermore, the quantitative distribution of hybridization signals in interphase nuclei of the same preparation, analyzed as described above, was similar with either type of probe [<5% of cells with no signal, 5–15% with one signal, 25–35% with two signals, and 55–65% with three signals (Fig. 1 *F–J*)]. Although the library DNA inserts gave up to 15% of four-signal nuclei (compare Fig. 1 *F* and *G*), most likely because of the minor binding sites on other chromosomes, the plasmid pool revealed only a negligible percentage of nuclei (<0.2%) with four signals. These results indicate that trisomy 21 can be detected in a diagnostically meaningful way with small populations of nonmitotic cells.

Embryonic chorionic villi (CV) cells were also investigated with the 94-kb plasmid probe set in a case where the father had a reciprocal t(4;21) translocation. Hybridization to metaphase spreads of the CV cells showed that the translocated chromosome (4pter→4q33::21q11.2→21qter) was indeed inherited by the fetus (see Fig. 1 *L* and *M*). The signals in the interphase cell nuclei (see Fig. 1*K*) of the CV cells had a distribution that paralleled that of cells with a normal karyotype (see above), indicating a balanced representation of

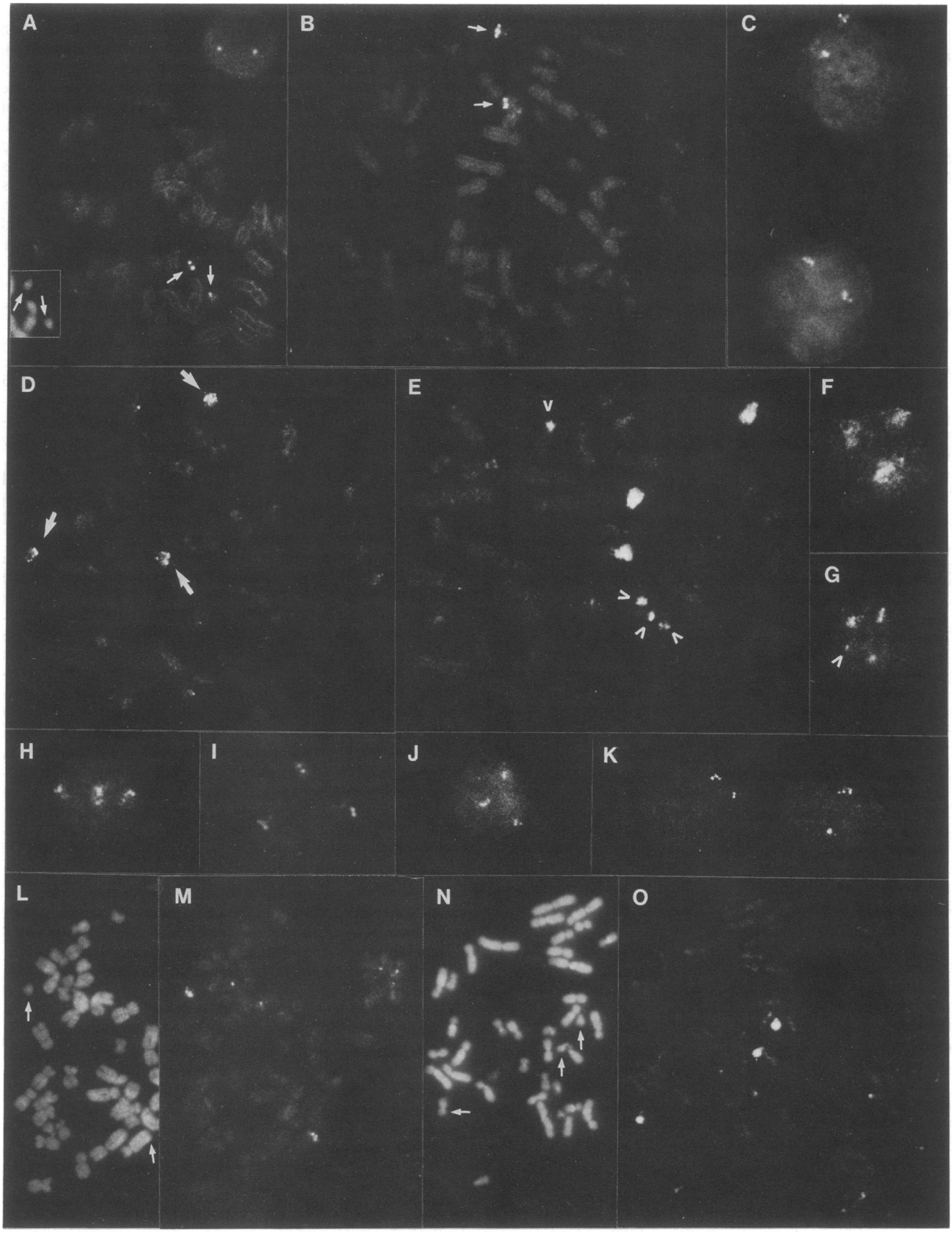

FIG. 1. Specific labeling of human chromosome 21 by *in situ* hybridization with biotinylated DNA probe sets. (*A*) Plasmid pPW519-1R (6-kb insert) hybridized to a normal lymphocyte metaphase spread. Signals are located on the termini of 21q [see 4',6-diamidino-2-phenylindole (DAPI)-stained chromosomes in *Inset*] as verified by DAPI banding (not shown). (*B* and *C*) Normal human lymphocyte metaphase (*B*) and nuclei (*C*) after hybridization with the 94-kb plasmid pool probe set. The terminal band 21q22.3 is specifically labeled. (*D* and *E*) Signals on trisomy 21 (47,+21) lymphocyte metaphase spreads after hybridization with the 94-kb probe set (*D*) or chromosome 21 library DNA inserts with the CISS hybridization (14) protocol (*E*). Three chromosomes 21 are entirely delineated by the library inserts; additional minor signals (see text) are indicated by arrowheads (also in *G*). (*F–J*) Labeling of trisomy 21 lymphocyte nuclei by the library inserts (*F* and *G*; compare with *E*) and

21q22.3 and excluding Down syndrome as a possible diagnosis. A small increase of nuclei with three and four signals (both <5%) over that of normal lymphocytes was also observed, probably reflecting a higher portion of tetraploid cells in such CV samples.

The diagnostic potential of the chromosome 21 probes was further tested by using a glioma tumor cell line, TC620, known to be pseudotriploid with a highly rearranged genome (13, 15, 28). The metaphase spreads revealed two apparently normal chromosomes 21 and one translocation chromosome (see Fig. 1 *N* and *O*). Interestingly, the chromosome 21 DNA on the translocation chromosome labeled by the library probe has a size equivalent to a normal 21q region, thus suggesting a Robertsonian translocation event. However, fine structural aberrations of 21q (i.e., small deletions etc.) cannot be excluded by this analysis. The interphase signals seen with both the plasmid probe set and the library inserts were consistent with trisomy 21q22.3 and trisomy 21, respectively.

Finally, we determined if the 94-kb plasmid probe set could be used to localize chromosome 21 DNA sequences in solid tissues. Fig. 2 shows the nucleus of a cortical neuron from "normal" human brain tissue after *in situ* hybridization. Both chromosomes 21 are clearly labeled by the probe, and they are located near the nucleolus; this nuclear location is consistent with the fact that chromosome 21 contains a ribosomal gene cluster that is usually localized in the nucleolus. This observation suggests that these probes may also prove useful for evaluating the frequency of chromosome 21 mosaicism in specific cell or tissue types. In addition, it should be of interest to see if the various karyotypic changes associated with the Down syndrome phenotype alter the normal nuclear topography of chromosome 21 in neuronal tissue.

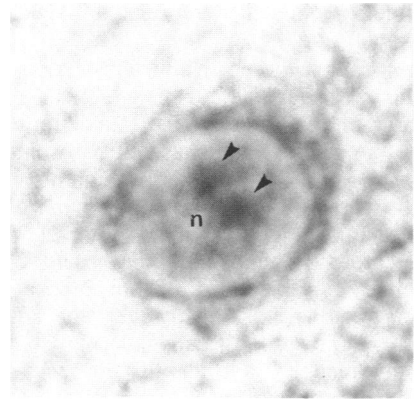

FIG. 2. Visualization of 21q22.3 in a nucleus of a large neuron in human parietal lobe tissue. The biotinylated plasmid pool probe set (94 kb) was detected by the purple-colored precipitate generated by alkaline phosphatase (conjugated to avidin). The arrows indicate the two signals; "n" indicates the center of the nucleolus.

DISCUSSION

We have demonstrated a method to rapidly detect numerical and structural aberrations of chromosome 21 in metaphase and in interphase cells. A trisomic karyotype can be diagnosed easily in interphase cells because the majority of the nuclei (55–65%) exhibit three distinct foci of hybridization. In contrast, less than 0.2% of nuclei in lymphocytes with a disomic karyotype show three nuclear signals; interestingly, the percentage of such nuclei in normal CV cells was higher but still considerably less than 5%. In general, as few as 20–30 cells were sufficient to unambiguously distinguish between disomic and trisomic cell populations. However, in view of the uncertainty of the level of chromosome 21 mosaicism in clinical samples, the number of cells required to make an unambiguous diagnosis will likely be higher. Additional clinical correlations will be required to establish the absolute number. Nevertheless, this analytical approach could allow the diagnosis of Down syndrome without the need to culture cells or to obtain metaphase spreads.

Pools of plasmid DNA from 21q22.3 and a "complete" set of DNA inserts from a chromosome 21 library were compared as probes. In general, the plasmid probe set was superior for interphase diagnosis because smaller and more focal areas were labeled with improved spatial resolution. This probe set, which labels 21q distal to the centromere, also has the particular advantage of relative insensitivity to interindividual pericentromeric heteromorphisms. Furthermore, unlike the library DNA inserts, there were no minor nonspecific hybridization signals. Eventually, removal of the cross-hybridizing sequences from the library DNA [e.g., as by subtractive hybridization (33)] could make chromosomal decoration from pter to qter more attractive for diagnostic purposes. It should also be noted that the 94-kb plasmid probe exhibited several types of hybridization patterns in interphase cells, each exhibiting subtle differences in structural detail (see Fig. 1 *C* and *H–K*). For example, in many G_1-phase cells, each nuclear domain can be resolved as a doublet of closely juxtaposed signals. The variability in the nuclear signal patterns may reflect dynamic changes in this segment of euchromatic DNA that occur at different stages of the cell cycle or during transcriptional activation.

Although we have used here selected plasmid clones containing only unique human DNA sequences, cosmid clones containing repetitive sequences can also be used to specifically label their cognate genomic region in metaphase and interphase cells by applying hybridization protocols like CISS hybridization that suppress the signal contribution of repetitive sequence elements (14, 34, 35). Therefore, single or nested sets of cosmids could be used as diagnostic tools for other genetic diseases in a fashion similar to that reported here. Trisomy of chromosomes 13, 18, 21, X, and Y together account for the vast majority of numerical and/or structural chromosome abnormalities identified during prenatal karyotyping. With the continued development of multiple nonisotopic probe labeling and detection systems (13, 15), it should be possible to visualize three or more chromosomes simultaneously following *in situ* hybridization. Thus, the development of a rapid and automated screening test to detect the major trisomic disorders directly in interphase cells from amniotic fluid or chorionic villi cells is a viable future objective. The analysis of specific human chromosomes by *in situ* hybridization has already been used to complement conventional cytogenetic studies of highly aneuploid tumor

the 94-kb probe set (*H–J*; compare with *D*). (*K–M*) Nuclei (*K*) and metaphase spread (*M*) of chorionic villi cells containing a translocation chromosome but showing a balanced karyotype with regard to 21q22.3, and DAPI-counterstained metaphase (*L*) indicating (lower arrow) the terminal region of 21q, which is translocated to a B-group chromosome independently identified as chromosome 4 by banding analysis (T. Yang-Feng, Yale University, personal communication). (*N* and *O*) Chromosome 21 delineated in a metaphase spread of the oligodendroglioma-derived cell line TC620 by using the library inserts probe (*O*) and DAPI staining (*N*). TC620 exhibits two chromosomes 21 and one translocation chromosome (see *N*, left arrow) whose short arm contains an apparently complete 21q, suggesting a Robertsonian translocation event. Arrows indicate either hybridization signals or the corresponding DAPI-stained chromosome. Detection of the hybridized probes was with fluorescein isothiocyanate-conjugated avidin [the signal of the 6-kb probe (*A*) was amplified (31)]. Similar results were obtained when the detection was mediated by alkaline phosphatase-conjugated avidin (not shown).

lines (15), and the extension to prenatal diagnostic applications seems warranted.

The analysis of karyotypes with translocations of chromosome 21 shows the usefulness of a regional probe set to rapidly identify and characterize even small translocations by unambiguous signals on metaphase chromosomes, thus circumventing an extensive analysis by high-resolution banding. In contrast, the library insert probe is more suitable for defining the relative amount of chromosome 21 DNA that has been translocated. By analyzing interphase nuclei, one can also determine if a balanced or unbalanced number of chromosomal regions exists. However, the detection of a translocated chromosome directly in nuclei would require double-labeling techniques to identify the recipient chromosome to which the chromosome 21 material was translocated. With prior knowledge of the chromosomes in question, such translocation events could be assessed by measuring the juxtaposition of the nuclear signals (9).

Finally, we have shown that probes containing 6 kb of sequence can be localized in both metaphase spreads and interphase cells with high efficiency. This detection sensitivity with nonisotopic reagents is similar to that achieved in other recent reports (36–38). The combination of nonisotopic *in situ* hybridization with DAPI or BrdUrd banding or total chromosome decoration with library DNA probes thus provides a simple and general approach for gene mapping. It also should facilitate the use of small DNA probes to rapidly pinpoint the breakpoints on translocation chromosomes, which could further aid in defining the genomic segment critical for Down syndrome.

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