MOLECULAR GENETICS OF CHROMOSOME 21 AND DOWN SYNDROME

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DETECTION OF DOWN SYNDROME BY IN SITU HYBRIDIZATION WITH CHROMOSOME 21 SPECIFIC DNA PROBES

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INTRODUCTION

Down Syndrome is the most common cause of mental retardation in man and afflicts one in every 800-1000 livebirth infants. The majority of Down Syndrome patients (~ 95%) are trisomic for all of chromosome 21 while in about 5% of the cases the trisomy is caused by a Robertsonian translocation (Hamerton, 1981). A Down Syndrome phenotype can also result from a reciprocal translocation involving chromosome 21, although this occurs quite rarely (Hamerton, 1981). Each form of trisomy 21 is routinely diagnosed by conventional cytogenetic banding of metaphase chromosomes, however the detection of translocation chromosomes is often difficult because the terminal segment of chromosome 21 that is translocated can be small. Indeed, cytogenetic studies have shown that only trisomy of subregion 21q22-21qter is required to elicit the Down Syndrome phenotype. An additional diagnostic complication is the occurrence of trisomy 21 mosaicism with a small percentage of trisomic cells. Thus, until the molecular basis of the genetic defect(s) which lead to Down Syndrome are defined more precisely, there is still a need for simple methods, other than conventional karyotyping, for the rapid detection of chromosome 21 abnormalities. In particular, a method that could be directly applied to the analysis of small numbers of amniotic fluid cells, chorionic villi cells, or fetal cells isolated from maternal blood, would have significant clinical utility and circumvent the necessity of cell culture and the preparation of metaphase chromosome spreads.

Several recent publications (Julien et al., 1986; Lichter et al., 1988b; Pinkel et al., 1988) have demonstrated the feasibility of detecting chromosome 21 abnormalities in interphase cell nuclei by in situ hybridization using chromosome 21 specific DNA probes. These studies were predicated on the observation that the DNA of each human chromosome occupied a discrete focal territory within the interphase nucleus (Cremer et al., 1982; Hens et al., 1983). Thus a trisomic karyotype exhibits three discrete foci of hybridization in the majority of nuclei, whereas normal diploid cells show two foci. In this presentation, data on the specificity and efficiency of trisomy 21 detection by in situ hybridization will be presented, the efficacy of different chromosome 21 specific DNA

probes compared, and future diagnostic potential of interphase cytogenetics discussed.

MATERIALS AND METHODS

Cells: Metaphase spreads and interphase nuclei were prepared from lymphocyte cultures of normal (46,XY; 46,XX) individuals and from lymphocytes of Down Syndrome (47, +21) individuals using standard techniques for colcemid treatment, hypotonic swelling, methanol-acetic acid fixation and metaphase chromosome spreading. In some experiments, cell suspensions with methanol-acetic acid fixed cells were washed with 1,6-hexanediol isolation buffer as described by Emmerich et al. (1989). Cells were dropped on slides, air dried, dehydrated through an ethanol series, air dried again, digested with proteinase K, washed with PBS, postfixed with 4% paraformaldehyde in PBS containing 50 mM magnesium chloride, washed with PBS, dehydrated again and air dried. Prior to hybridization, slides were incubated overnight in 50% formamide, 1xSSC, pH 7.0.

DNA Probes: Cosmid clones containing inserts of human chromosome 21 DNA (designated c511, c512, c518, c519, c520, c523, c524, c525, c551 and c552, see Watkins et al., 1985) were obtained from Dr. Paul Watkins (Life Technologies, Gaithersburg, MD) while he was associated with Integrated Genetics, Inc. (Framingham, MA). Clone pHY2.1 (Cooke et al., 1982), containing a Y-chromosome specific repetitive sequence element, was obtained from Howard Cooke. Clone pXBR1 (Yang et al., 1982), containing an X chromosome specific alphoid DNA repeat, was obtained from Barbara Hamkalo. The human chromosome genomic libraries LL21NS02 and LL18N501 were purchased from the American Type Culture collection and DNA prepared as described before (Lichter et al., 1988a). The pool of plasmids containing single copy DNA from band q22.3 of chromosome 21 was described previously (Lichter et al., 1988b).

Probe DNA was labeled by nick translation using biotin-11-dUTP, 2,4-dinitrophenol (DNP)-11-dUTP or digoxigenin-11-dUTP as one of the nucleotide substrates (Lichter et al., 1990).

In situ Hybridization: Hybridization reactions using either the chromosome 21 library DNA or individual cosmid DNAs as the probe were carried out under conditions which suppress hybridization signals from repetitive sequence elements in the probe as described (Lichter et al., 1988b). Hybridization of the pool of plasmid DNAs was done under non-suppression conditions (Cremer et al., 1988a). All reactions were incubated at 37°C overnight; posthybridization washes, detection of hybridization signals using fluorochrome-conjugated avidin or antibodies, and microscopic evaluation of the specimens were as described previously (Lichter et al., 1990).

RESULTS

Our initial studies on the detection of numerical and structural aberrations of chromosome 21 in metaphase and interphase cells (Lichter et al., 1988b) used as probes either a complex set of DNA inserts from a chromosome 21 library or a pool of plasmids containing up to 94 kilobase pairs of unique sequence DNA from 21q22.3. A trisomic

karyotype could be detected readily in interphase cells using either probe set since the majority of the countable nuclei (50-65%) showed three distinct foci of hybridization. In contrast, less than 0.2% of nuclei in lymphocytes with a disomic karyotype showed three nuclear signals; interestingly, the corresponding number in chorionic villi cells is higher but still less than 5% (probably due to incomplete hybridization to tetraploid cells, which seem to occur in higher frequencies in chorionic villi cells). In general, the plasmid probe set was superior for the analysis of interphase cells for a number of reasons. First, the specificity of labeling with the chromosome 21 library DNA was less than optimal because of minor hybridization signals generated from sites at or near the centromeric regions of other acrocentric chromosomes, most notably chromosome 13, which could not be suppressed efficiently. Second, the plasmid probe set gave smaller and more focal signals which improved spatial resolution and facilitated more rapid quantitation. Third, the plasmid probe set, because it labeled only the telomeric band of chromosome 21, avoided a potential problem of a pericentromere probe, i.e., sensitivity to interindividual pericentromeric heteromorphisms. The plasmid probe set was not, however, totally satisfactory. Because the unique sequence DNA subset was derived from multiple cosmid clones that spanned several hundred kilobases of band q22.3, in some nuclei the foci of hybridization was actually a cluster of multiple microsignals, possibly reflecting cell to cell differences in chromatin condensation, stage of cell cycle, or levels of transcriptional activity in this chromosomal region. Furthermore, although a higher fraction of the cell population gave the predicted number of nuclear signals with the plasmid pool than with the library pool as a probe (65-75% of countable nuclei versus 50-60%), additional improvements in the efficiency of target sequence detection was desirable. The observation that a significant portion of normal diploid cells gave only one chromosome 21 signal in interphase nuclei (25-30%) or no signal at all (~ 5%) suggested that subtle variations in sample fixation, cell permeabilization, the genetic complexity, chromosomal location or the physical size of the probe, might markedly influence hybridization efficiency. We have, therefore, begun to test some of these parameters in a systematic fashion.

As shown in Figure 1, it is possible to use single cosmid clones from chromosome 21 to detect a trisomic karyotype in interphase cells using Chromosomal In Situ Suppression (CISS) hybridization conditions. Panels A and B show the hybridization signals seen on a metaphase spread and an interphase nuclei from a normal diploid individual while panels C, D and E show the corresponding metaphase and interphase signals from cells of a Down Syndrome individual. The ability to visualize single cosmid clones allowed us to evaluate multiple defined probes from different regions of chromosome 21 in a quantitative fashion and to determine how target detection efficiency is influenced by probe complexity or chromosomal position while keeping other experimental parameters, e.g., fixation conditions, constant.

Ten cosmid clones from different loci on chromosome 21 were hybridized individually to lymphocytes from a normal individual after standard methanol-acidic acid fixation. For each cosmid, the number of nuclei giving 0,1,2,3 or 4 signals was determined by evaluating 200 nuclei. The signal distribution observed with each clone is given in Table 1. The percentage of cells giving the expected number of 2 signals per nucleus was found to be

Figure 1: In situ hybridization with cosmid c512 . Metaphase spread (A) and nucleus (B) of acetic acid fixed lymphocytes from an individual with a normal karyotype (46). Metaphase spread (C) and nuclei (C-E) of methanol/acetic acid fixed and 1,6-hexanediol postfixed lymphocytes from a Down Syndrome patient (47, +21). The biotin labeled probe was detected with avidin-FITC (indicated by arrows); chromosomes and nuclei were counterstained with propidium iodide. Digitized images were generated as described by Lichter et al. (1990). In some cases digital image processing was applied for better signal illustration (see panel E).

signal / probe	0	1	2	3	4
c511	2	4	90	4	0
c512	1	5	91	2	1
c518	1	4	89	4	2
c519	2	4	90	3	1
c520	0	2	90	5	3
c523	1	3	91	3	2
c524	1	6	85	6	2
c525	0	5	89	6	1
c551	1	3	93	3	0
c552	2	5	83	7	3

Table 1: Comparison of the hybridization efficiencies of ten different cosmid probes from chromosome 21 to interphase nuclei. Cosmid designations are given in the first column. In each experiment, the number of signals were counted in 200 nuclei and expressed as the percentage of cells exhibiting 0,1,2,3 or 4 signals per nucleus (columns 2-6, respectively).

between 83 and 93%. This is significantly higher than that observed with either the plasmid pool or the library pool probe sets. Since this study revealed a big decrease in cells exhibiting one signal/nucleus versus an increase of cells with two signals/nucleus, the data can be explained by a considerable improvement of hybridization/detection efficiencies. The fraction of cells exhibiting three signals/nucleus varied from 2-7%; this range is higher than that seen in our earlier study, and most likely reflects the increased detection efficiency. A similar quantitative analysis of Down Syndrome cells, but with fewer clones, showed a corresponding increase in target detection efficiency. Whereas prior studies with either plasmid or library probe sets gave 55-65% of countable cells with three nuclear signals, cosmid probes increased this level to ~ 75% of cell (see data with cosmid 512 in Figure 2).

The lack of a statistically significant difference in detection efficiencies between any of the ten cosmid clones examined suggests that the chromosomal origin of the cloned DNA is not a major factor in this particular issue. Another noteworthy observation is that the number of cells which were classified as uncountable was relatively low with cosmid probes and in only one instance was as high as 20% (clone 512). This is in sharp contrast

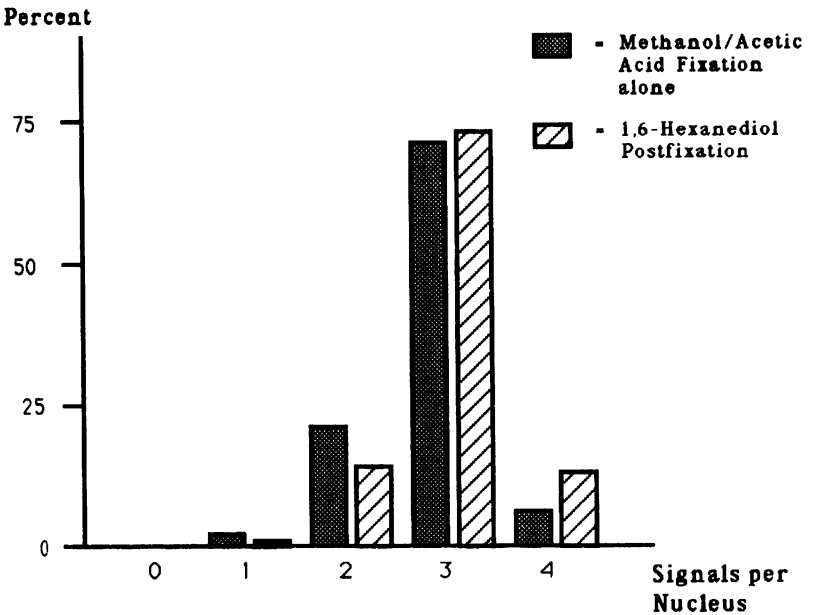

Figure 2: Quantitative analysis of hybridization signals in interphase cells from a Down Syndrome patient (47, +21). Cells were either fixed with methanol/acetic acid alone or with methanol/aceta acid followed by 1,6-hexanediol postfixation treatments. In each experriment, signals were counted in 200 nuclei following CISS hybridization Within the group of countable nuclei (see text) the percentages of nuclei with 0,1,2,3 or 4 signals were compared.

to the chromosome 21 library probe set where one in three cells was considered uncountable. In general, uncountable cells exhibit more than one signal, but due to the frequent close juxtaposition of chromosome 21 around one nucleolus, the actual signal number cannot be determined. The more focal signal of cosmids permits higher resolution thereby facilitating quantitation. Thus, by limiting the total sequence complexity to 40 kb or less it appears that two practical improvements can be achieved ; 1) a reduction in the number of uninformative cells and 2) an increase in the number of cells yielding the predicted number of nuclear signals.

Another experimental variable examined was cell fixation conditions. Cosmid clone 512 was hybridized to nuclei of Down Syndrome cells after fixation with either methanol-acetic acid alone or additional treatments with 1,6-hexanediol buffer, proteinase K, etc., as outlined in Materials and Methods. The fraction of cells exhibiting 0,1,2,3 or 4 signals per nucleus was quantitated as before by analyzing 200 nuclei from each experiment. The

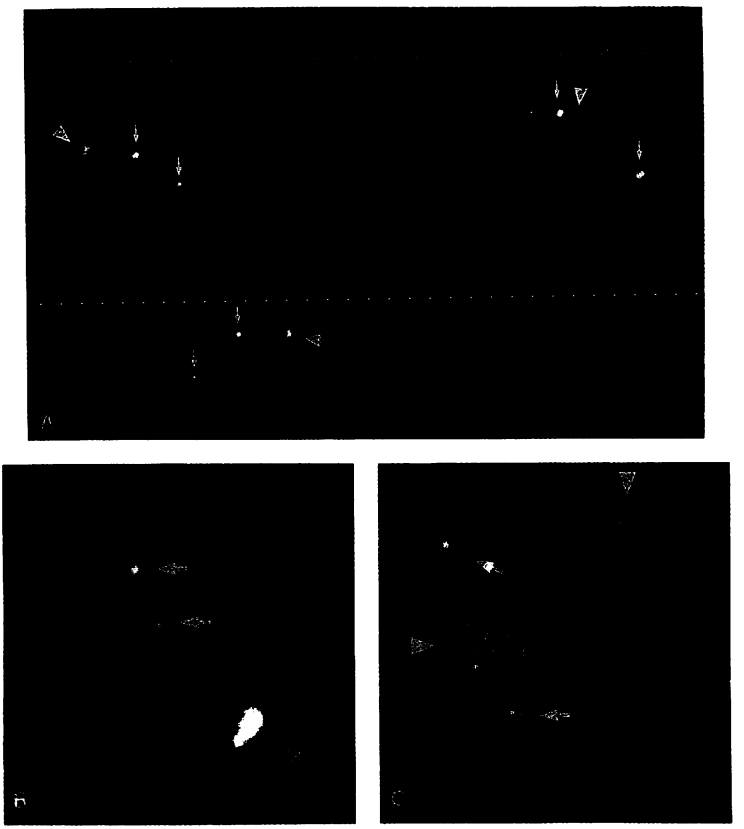

Figure 3: Simultaneous detection of two chromosome specific probes in interphase nuclei by in situ hybridization. A) Chromosome 21 and Y chromosome: three lymphocyte nuclei from a male individual after CISS hybridization with biotin labeled probe c519 (detected via FITC, signals indicated by arrows) and cohybridized with DNP labeled probe pHY21, containing a Y chromosome specific repetitive DNA (detected by indirect immunofluorescence with rhodamine, signals indicated by arrowheads). B) Chromosome 21 and X chromosome: the experiment was as in A but digoxigenin labeled c519 (FITC detection, signals indicated by arrows) and biotin labeled X chromosome specific probe pXBR1 (detected via avidin-texas red, signal indicated by arrowhead) were used. C) Double CISS hybridization with digoxigenin labeled c519 (arrows) and biotin labeled DNA from chromosome 18 library LL18NS01 (arrowheads, detection as in B). Note the doublet signals with the cosmid probe and the size of the chromosome 18 domains both of which indicate that this is a G2 phase nucleus. A to C are digitized images where the part of the image generated by rhodamine or texas red, respectively, is enhanced to show the outline of the nuclei.

numerical distribution of nuclear signals is shown in Figure 2. Although both fixation methods yield comparable numerical data, the intensity of the hybridization signal obtained after the additional treatments was always appreciably stronger than that observed after methanol-acetic acid fixation (data not shown), thus making aneuploidy analysis faster and easier.

While additional experimental variables are still being explored, these two seemingly minor alterations have enhanced both the qualitative and quantitative aspects of intranuclear trisomy 21 detection. Other chromosomes may be included in this intranuclear analysis as internal controls and for added diagnostic value. Although non-isotopic reporter/fluorophore detector systems for the visualization of three probes simultaneously have already been reported (Nederlof et al., 1989; our own unpublished data), our current digital imaging hardware, based upon the BioRad Lasersharp MRC-500 confocal laser-scanning microscope, is presently capable of analyzing only two fluorophores (i.e., probes) at the same time. As seen in Figure 3, simultaneous analysis of two chromosomes is not problematic. Panel A shows an example of normal diploid lymphocyte nuclei (46,XY) that have been cohybridized with a chromosome 21 cosmid probe (seen in yellow) and a Y chromosome specific repeat (seen in red). Panel B shows another sample of the same individual's cells cohybridized with a chromosome 21-specific cosmid (yellow) and an X chromosome specific repeat (red). While both results indicate that the cells are male and diploid for chromosome 21, a simultaneous triple hybridization (for chromosomes 21, X and Y) would be necessary to rule out the possibility of an X, O phenotype for the cell in Panel B. Another example of a double label hybridization experiment is illustrated in Panel C which shows a chromosome 21 cosmid (yellow) cohybridized with a chromosome 18 library probe set (red) under CISS hybridization conditions. Instrumentation developments currently in progress, the installation of a tunable-dye laser on the confocal microscope and the assembly of an epifluorescent microscope equipped with a cooled CCD digital imaging camera, should in the near future permit the analysis of multiple chromosomes simultaneously.

DISCUSSION

The results presented here demonstrate that the quantitation of trisomy 21 in interphase cells improves significantly when single cosmid clones are used as probes. This is of considerable interest, since the preparation of one particular probe is much less time consuming than preparing a set of probes which has to be pooled. Although the overall fluorescent signal is less than seen with either the chromosome 21 library or the band q22.3 probe set, the smaller foci of hybridization are better spatially resolved in a larger fraction of the cells and thus are easier to count. Indeed, between 80 and 90% of all cells generally yield clear-cut quantitative information. The highly focal nature of the intranuclear signal should also make it amenable to simple automated image analysis.

The application of such probes, either composite or single, in a clinical situations could provide an unequivocal diagnosis with a relatively small sample size. A clinical trial to evaluate the effectiveness of chromosome 21 probes for the prenatal diagnosis of Down Syndrome using amniotic fluid and chorionic villi samples is in progress (K. Klinger,

Integrated Genetics, Framingham, MA; personal communication). Thus, the general utility of a hybridization based-interphase cell assay for the diagnosis of Down Syndrome should be known in the near future.

Chromosomes 13, 18, 21, X and Y account for most of the chromosome abnormalities identified during prenatal karyotype screening for age-related maternal risk. Technically, it may become feasible to quantitatively assess the aneuploidy of these five chromosomes in a single assay using interphase cells. However, since the clinical consequences of numerical gonosomal aberrations can be comparatively mild, detection of the sex chromosomes might be excluded from such an assay. Nevertheless, it may be beneficial for parents, physicians and genetic counselors to know that a child will be afflicted with syndromes, such as Turner's or Klinefelter's, to provide psychological guidance, emotional support and opportunities for possible treatment at an early fetal stage.

Considerable effort has been made over the past decade to develop efficient methods to isolate fetal trophoblast cells from maternal blood, with varying degrees of success. The recent report (Bianchi et al., 1989) that fetal reticulocytes can be isolated in reasonable numbers from the maternal circulation opens another avenue to fetal cells for diagnostic purposes. Success in either of these endeavors when coupled with polymerase chain reaction or interphase cytogenetic techniques, could provide a universal, low-fetal risk, diagnostic modality. As faster and easier diagnostic tests are developed, they could be applied to an increasing percentage of all pregnancies and performed by a broader spectrum of physicians. In this future context, we feel it is important to emphasize that such procedures must always be coupled to high quality, non-directive, genetic counseling.

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