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Detection of Aneuploidy Involving Chromosomes 13, 18, or 21, by Fluorescence In Situ Hybridization (FISH) to Interphase and Metaphase Amniocytes

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Summary

Fluorescence in situ hybridization (FISH) with chromosome-specific probes has been applied to detection of numerical aberrations involving chromosomes 13, 18, and 21 in metaphase and interphase amniocytes. High-complexity, composite probes for chromosomes 13, 18, and 21 were used as hybridization probes for this study. These probes were constructed as chromosome-specific libraries in Bluescribe plasmids and are designated pBS-13, pBS-18, and pBS-21. Elements of these probes bind at numerous sites along the target chromosome and, when detected fluorescently, stain essentially the entire long arm of the target chromosome. The target chromosome number (i.e., the number of chromosomes of the type for which the probe was specific) was correctly determined in 20 of 20 samples in which metaphase spreads were analyzed and in 43 of 43 samples in which interphase nuclei were analyzed; all of these studies were conducted in blind fashion. These results suggest the utility of FISH with composite probes for rapid detection of numerical aberrations in metaphase and interphase amniotic cells.

Introduction

Prenatal diagnosis of fetal genetic disease is routinely accomplished by microscopic analysis of banded metaphase spreads prepared by in vitro culture of cells collected during either amniocentesis (Ferguson-Smith and Yates 1984; Hook and Cross 1987) or chorionic villus sampling (Hook et al. 1988). This technique is accurate and allows detection of a broad range of numerical and structural aberrations. Unfortunately, it is labor intensive, requires a highly trained analyst, and can be applied only to mitotic cells. As a result, prenatal screening for chromosome aberrations is usually performed only for women considered to be at high risk (e.g., for women over 35 years of age or with a low concentration of maternal serum alpha-fetoprotein; Doran et al. 1986; Baird and Sadovnick 1988). Arguably, the most important aberrations found during screening are trisomies for chromosomes 21, 18, and 13 (Lubs and Ruddle 1970; Jacobs 1977; Epstein 1986).

Recent publications on fluorescence in situ hybridization (FISH) with chromosome-specific probes suggests that this technique may facilitate the detection of numerical aberrations in metaphase spreads and in interphase nuclei (Manuelidis 1985; Cremer et al. 1986; Pinkel et al. 1986; Moyzis et al. 1987; Devilee et al. 1988). In this approach, the target nuclei are denatured to produce single-stranded DNA and are hybridized to chemically modified (e.g., by attachment of biotin to the probe DNA), chromosome-specific single-stranded nucleic acid probes under conditions such that the probes bind only to target DNA sequences to which they have high sequence homology. The hybridized probe is then made fluorescent by treatment with an affinity reagent specific for the chemical modification (e.g., fluorescein-labeled avidin). This procedure causes chromosomes of the type

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targeted by the probe to fluoresce intensely in metaphase spreads, so that their number can be determined accurately. In addition, individual chromosomes tend to be tightly localized in interphase nuclei, so that FISH with a chromosome-specific probe produces a fluorescent domain at the location of each target chromosome. When the domains are well separated, aneuploidy can be detected simply by domain counting.

Most aneuploidy analyses using FISH have been accomplished with probes for tandemly repeated DNA sequences usually found near the chromosome centromere (Manuelidis 1985; Pinkel et al. 1986; Moyzis et al. 1987; Devilee et al. 1988). The hybridization signals produced by these probes are intense and well localized and thus are easily counted. However, repeat-sequence probes specific for chromosomes 21 and 13 are not available (although a probe that binds to a tandemly repeated alpha-satellite sequence present on chromosomes 21 and 13 is available; Devilee et al. 1986). More important, the extent of the repeated alpha-satellite sequence on chromosome 21 usually targeted by such probes varies in size among normal individuals and in some is so small that the hybridization signal resulting from FISH with a probe to this region may be missed (Weier and Gray, submitted). This occurrence may lead to missed trisomies involving chromosome 21. An alternative is to employ FISH with composite probes containing elements with DNA sequence homology at multiple sites along the entire target chromosome (Choo et al. 1988; Cremer et al. 1988; Lichter et al. 1988*a*; Pinkel et al. 1988; Fuscoe et al. 1989; Collins et al., submitted) or in subregions thereof (Lichter et al. 1988*b*). FISH with such probes also produces recognizable fluorescent domains in interphase nuclei. However, the intensity of these signals is somewhat lower than that for repeat-sequence probes, and the domain size is larger, so that the probability of domain coalescence is increased. Thus, the accuracy of aneuploidy detection using such probes must be established.

We report here the result of aneuploidy analysis, by FISH with whole-chromosome probes, on metaphase spreads and on interphase nuclei of cultured amniocytes. The probes for chromosomes 21, 18, and 13 used in this study were the Bluescribe plasmid libraries designated pBS-21, pBS-18, and pBS-13, respectively (Collins et al., submitted). Interphase and metaphase scoring was accomplished in a blind fashion and was compared with results for the same cultures made by banding analysis.

Material and Methods

Cells

Amniocyte cell lines established from frozen stocks in the Department of Obstetrics, Gynecology and Reproductive Sciences at the University of California, San Francisco, were obtained at the Lawrence Livermore National Laboratory (LLNL) and were cultured at 37°C in T-75 flasks in minimum essential medium-alpha supplemented with 20% FCS. The slides were coded so that karyotype information was not available at LLNL during the scoring process. Preparations of interphase nuclei were made from cultures grown to high density so that most were in the G1 phase of the cell cycle. Cells from these cultures were removed from the T-75 flasks by treatment for 5 min with 0.025% trypsin and were centrifuged and resuspended in 75 mM KCl solution at 37°C for 10 min. These cells were then fixed in four changes of methanol:acetic acid (3:1) and were dropped on ethanol cleaned slides and air-dried. Slides were stored at −20°C in plastic bags filled with nitrogen gas. Slides carrying metaphase spreads were prepared from amniocyte cultures in midexponential growth. These cultures were treated with colcemid (0.1 μg/ml) for ~4 h prior to preparation of metaphase spreads. Mitotic cells were shaken from each flask and were centrifuged and resuspended in 75 mM KCl. They were then fixed and dropped onto slides, as described above.

Probes

The libraries pBS-21, pBS-18, and pBS-13 were used as probes for chromosomes 21, 18, and 13, respectively. These libraries were constructed by subcloning *Hin*dIII-digested inserts from the recombinant DNA libraries LL21NS02, LL18NS01, and LL13NS01, respectively (Van Dilla et al. 1986; ATCC, Rockville, MD) into Bluescribe plasmids (Fusco et al. 1989; Collins et al., submitted). Probe DNA was prepared according to a method described elsewhere (Pinkel et al. 1988). The probes were chemically modified by nick-translation with biotin-11-dUTP or biotin-14-dATP (Bethesda Research Laboratories; Gaithersburg, MD) and were recovered at a concentration of ~20 ng/μl by using Sephadex G-50 spin columns. In some studies, pBS-21 was labeled with acetylaminofluorene (AAF) according to a method described by Landegent et al. (1984). All labeling reactions were adjusted to produce labeled probes whose individual elements were 0.3–1.0 kb in length.

In Situ Hybridization

Single-color hybridization.—Hybridization was accomplished by using a modification of the procedure described by Pinkel et al. (1988). The slide-mounted cells were treated with pepsin (20 μg/ml in 0.01 N HCl) at 37°C for 10 min and then were dehydrated by immersion in three ethanol solutions in which the successive ethanol concentrations were 70%, 85%, and 100%. The target DNA in the cells was denatured by immersion in 70% formamide, 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7) for 2 min at 70°C and was dehydrated in an ethanol series according to the method described above. The hybridization mixture (10 μl total volume consisting of 50% formamide, 2 × SSC, 10% dextran sulfate, 0.5 μg herring sperm DNA, 1–5 μg proteinase K–treated human placental DNA [both herring sperm DNA and human placental DNA were sonicated to the ~200–600-bp range], and ~40 ng biotinylated probe DNA) was denatured at 70°C for 5 min and incubated at 37°C for 1 h. This mixture was applied to the slides containing the target cells. The hybridization reaction was sealed under a coverslip and incubated at 37°C for 2–3 d. After hybridization, the slides were washed in three changes of 50% formamide and 2 × SSC, pH 7, and twice in PN buffer (a mixture of 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, and 0.1% Nonidet P-40, pH 8) at 45°C for 5 min each. The slides were then treated with alternating layers of fluoresceinated-avidin and biotinylated goat anti-avidin, both at 5 μg/ml in PNM buffer (PN buffer, 5% nonfat dry milk, and 0.02% sodium azide; centrifuged to remove solids) for 20 min each at room temperature until two layers of avidin were applied. After each incubation in avidin or anti-avidin (Vector Laboratories, Burlingame, CA), the slides were washed twice for 3 min in PN buffer. Prior to microscopic analysis, the slides were treated with an antifade solution (Johnson and Arajo 1981) containing 2 μg/ml propidium iodide.

Dual color hybridization.—In some studies, AAF-labeled pBS-21 DNA and biotinylated pBS-18 DNA were used for FISH. The hybridization mixture and prehybridization condition were as described for single-color hybridization, except that the 10-μl hybridization mixture contained both biotin- and AAF-labeled probes. Posthybridization washes were the same as for single-color hybridization. The slides were treated with PNM solution at room temperature for 10 min prior to immunochemical detection of the biotin- and AAF-labeled probes. The PNM-treated slides were incubated, sequentially, with a mixture of mouse anti-AAF and avidin-Texas red (2 μg/ml; Vector Laboratories), a mixture of goat anti-mouse FITC (1:50 dilution; Cal Tag) and biotinylated goat anti-avidin (5 μg/ml; Vector Laboratories), and avidin–Texas red (2 μg/ml; Vector Laboratories) for 60 min each at room temperature in the dark. After each reaction, the slides were washed three times in PN buffer. The FITC signal was amplified, when necessary, by reaction with rabbit anti-FITC (1:100 dilution; Dakopatts), followed by reaction with goat anti-rabbit FITC (1:300 dilution; Sigma). Antibodies used were diluted in 1 × Dulbecco's PBS, 0.05% Tween 20, and 2% normal goat serum. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) in antifade solution.

Hybridization domain scoring.—Fluorescence microscopy with appropriate filters was accomplished according to a method described by Pinkel et al. (1986, 1988), for analysis of hybridization domain size and number. Two domain-scoring procedures were evaluated. Strategy A was designed to minimize the probability of erroneously scoring cells as trisomic. In this strategy, only those cells showing domains of similar size were scored. Strategy B was designed to increase the probability of detecting trisomies and partial trisomies. In this strategy, cells showing unequally sized domains were included in the analysis. In both scoring strategies, cells either (*a*) showing very weak hybridization or highly irregular hybridization domains or (*b*) touching other cells were ignored.

Results

Hybridization Analysis

FISH with pBS-21 resulted in specific, intense staining of the chromosomes 21 in metaphase spreads. Figure 1*A*, for example, shows FISH to a metaphase spread trisomic for chromosome 21. The three chromosomes 21 are clearly visible. Distinct fluorescent domains were visible in interphase nuclei after single-color FISH with the biotinylated chromosome-specific probe. Karyotypically normal interphase cells typically showed one or two domains per nucleus when hybridized to one of these probes for autosomal chromosomes. However, trisomic cells showed one, two, or three domains per nucleus. Figure 1*B* shows FISH with pBS-21 to interphase nuclei prepared from amniocyte cultures determined by banding analysis to have an extra chromosome 21. Three domains are clearly visible in several nuclei in this preparation. Distinct

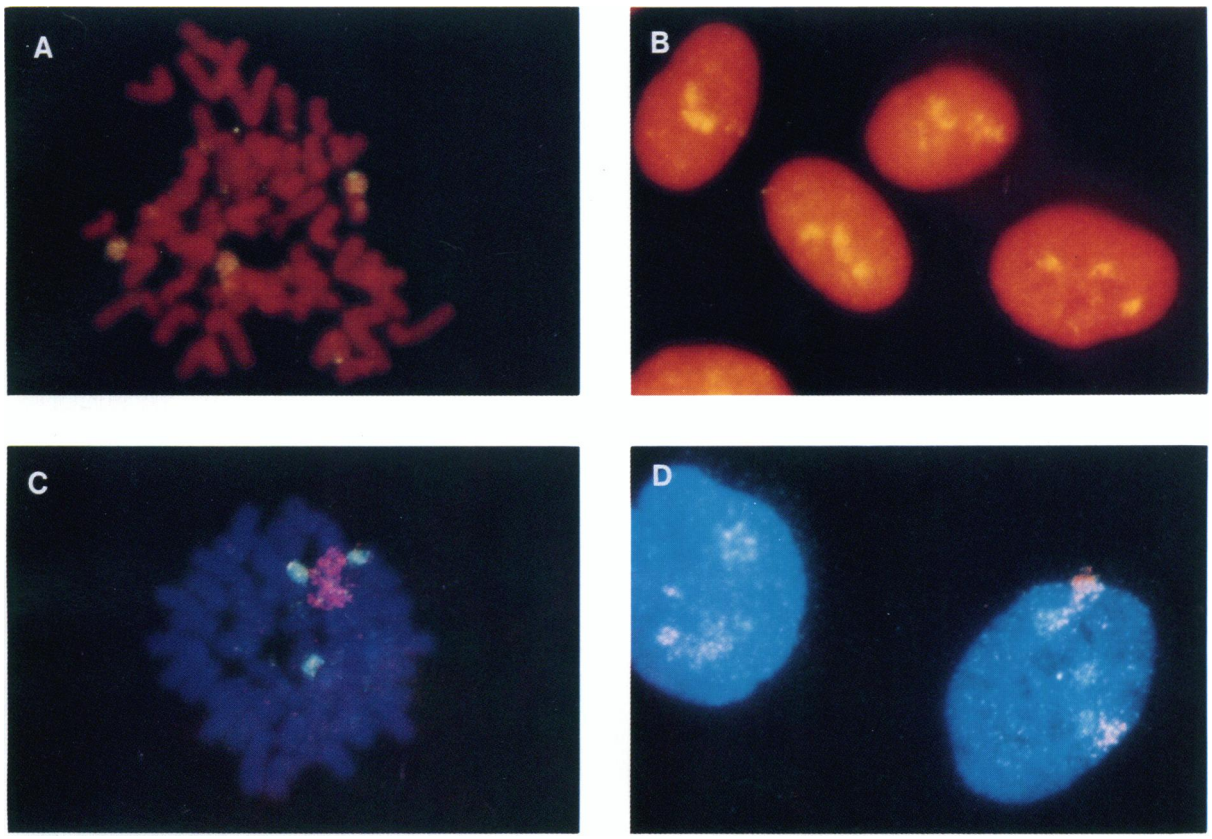

Figure 1 Photomicrographs showing FISH to interphase and metaphase preparations of cultured amniocytes. *A*, FISH with pBS-21 to metaphase spread for trisomy 21 amniocyte culture. *B*, FISH with pBS-21 to interphase nuclei prepared from same amniocyte sample. Hybridization of pBS-21 in panels *A* and *B* was detected by using FITC-labeled avidin, and propidium iodide was used as a counterstain so that the hybridization signals appear yellow and the remaining DNA appears red. The photomicrographs shown in panels *A* and *B* were taken by using a Zeiss Universal, epi-illumination fluorescent microscope equipped with an excitation filter passing ~485 nm and with an emission filter transmitting light of wavelengths >520 nm. This filter arrangement allows simultaneous observation of fluorescence from both fluorescein and propidium iodide. The film was Kodak Ektachrome 400. *C*, Two-color FISH with AAF-labeled pBS-21 and biotinylated pBS-18 to metaphase spread from amniocyte sample that carried two copies of chromosome 18 and three copies of chromosome 21. pBS-21 was detected by using FITC-labeled anti-AAF, and pBS-18 was detected by using Texas red–labeled avidin. DAPI was used as a counterstain. Thus, the pBS-21 domains appear green, the pBS-18 domains appear red, and the remaining DNA appears blue. *D*, FISH with AAF-labeled pBS-21 and biotinylated pBS-18, as described for panel *C*, to interphase nuclei from same amniocyte sample. The photomicrographs in panels *C* and *D* are double exposures. The first exposure was taken by using a dual band filter set for fluorescein and Texas red (Omega Optics, Brattleboro, VT), with excitation bands centered around 495 nm and 580 nm and with emission bands centered around 525 and 635 nm. The second exposure was taken by using both an excitation filter passing 365 nm and an emission filter passing fluorescence at wavelengths >425 nm for DAPI. The film was Kodak Ektachrome 400.

domains also were visible in interphase amniocytes after two-color FISH with biotinylated and AAF-labeled probes. Figure 1*C* and *D*, for example, shows two-color hybridization with the probe pairs pBS-21 and pBS-18, to metaphase spreads and interphase nuclei from amniocytes carrying two copies of chromosome 18 and three copies of chromosome 21. Hybridization of pBS-21 was detected by using fluorescein, and pBS-18 was detected by using Texas red, so that the domains for chromosome 21 appear green and those for chromosome 18 appear red.

Blind Studies

Two studies were conducted to evaluate the utility that FISH with chromosome-specific probes has for aneuploidy analysis. All samples were coded and scored in blind fashion to insure that the scoring results were not biased by prior knowledge of the karyotype.

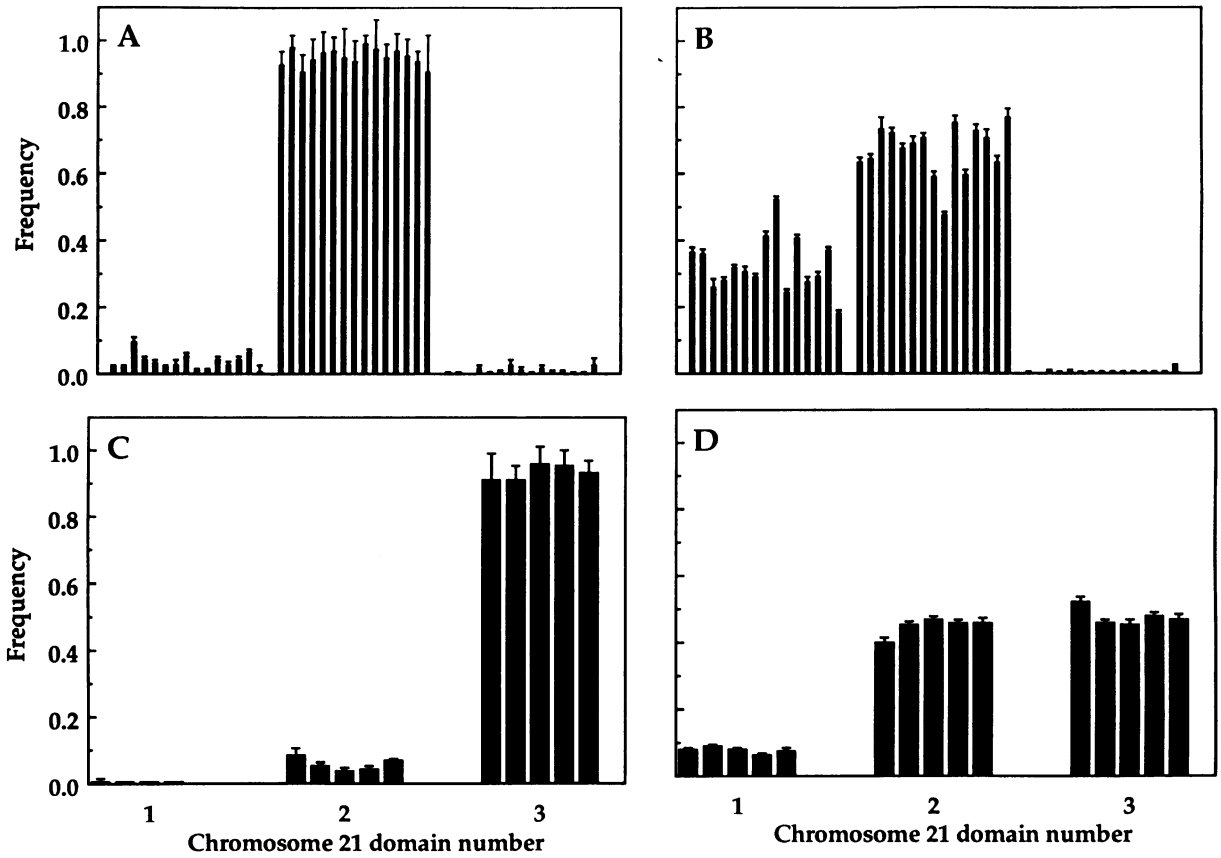

Figure 2 Results of hybridization with pBS-21 to metaphase and interphase amniocyte preparations. *A* and *C*, Frequencies of metaphase spreads showing hybridization to one, two, or three small chromosomes in metaphase spreads. *A*, Results for normal spreads. *C*, Results for samples trisomic for chromosome 21. *B* and *D*, Frequencies of interphase nuclei showing one, two, or three hybridization domains. *B*, Results for normal samples. *D*, Results for samples trisomic for chromosome 21. Each vertical bar represents the results for one sample in all panels. The bars above each frequency measurement show the counting uncertainty for that measurement (square root of the number of cells showing that number of domains, divided by the total number of cells scored).

All scoring was completed before the code was broken.

Study 1.—The goal of this study was to determine the utility that hybridization with pBS-21 has for detection of aneuploidy involving chromosome 21. Both metaphase and interphase slides were prepared from 20 different amniocyte cultures and were hybridized with biotinylated pBS-21. The number of metaphase spreads scored for each sample was 75–1,100, while the number of interphase nuclei scored was 500–3,600. All slides (metaphase and interphase) were coded separately and independently, so that the interphase analyses were not influenced by the results of the metaphase analyses. Furthermore, the interphase preparations were made so that metaphase spreads were not present. The results are summarized in figure 2.

The frequency of metaphase spreads showing hybridization, with pBS-21, to two small chromosomes was 90%–99% for the normal samples, while the frequency of spreads showing hybridization with pBS-21 to three chromosomes was 91%–96% for the trisomic samples. The frequency of normal spreads showing hybridization to three small chromosomes was <1.3%.

In study 1, the interphase nuclei were scored by strategy A. The frequency of normal interphase nuclei showing two domains was 47%–75%, and the frequency showing three domains was 0.1%–2%. The frequency of trisomic interphase nuclei showing three domains was 45%–52%, while the frequency of these nuclei showing two domains was 40%–47%.

Study 2.—This study involved aneuploidy analysis, in 23 amniocyte cultures, for chromosomes 21, 18,

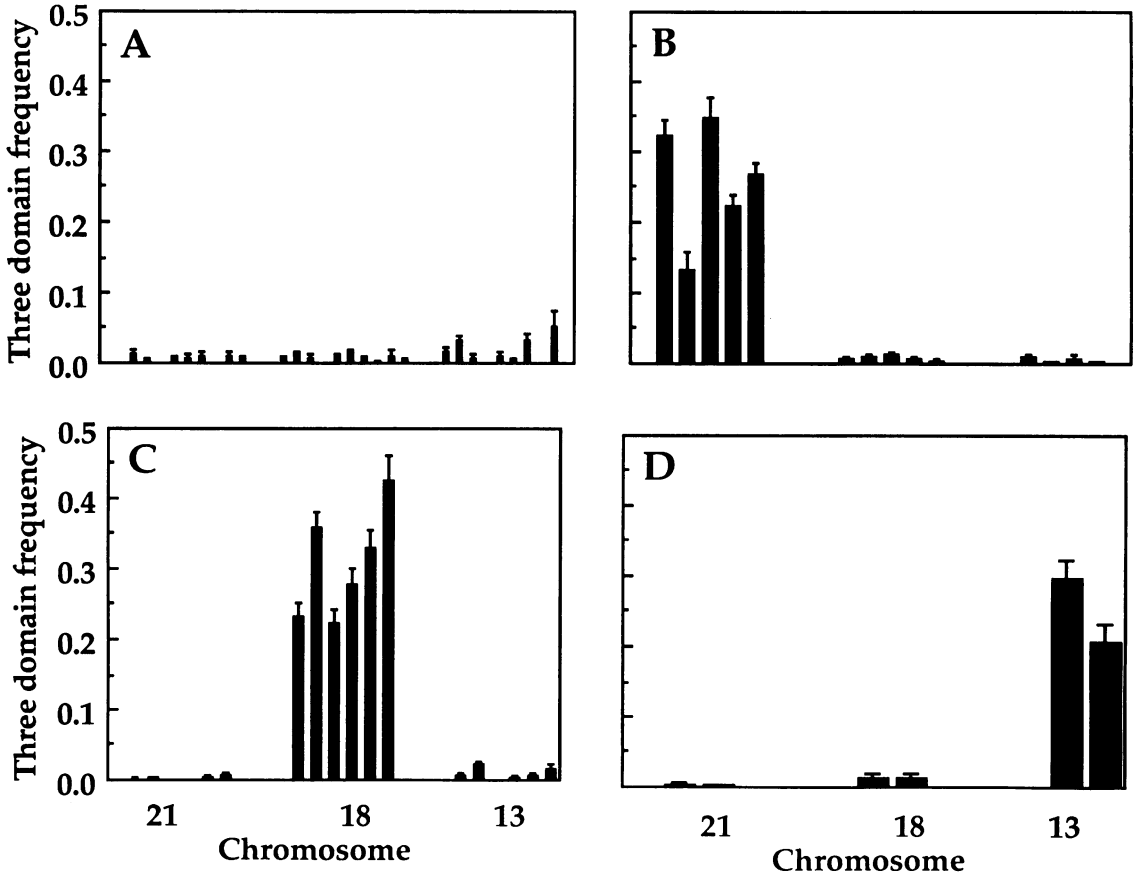

Figure 3 Results of FISH with pBS-21, pBS-18, and pBS-13 to interphase amniocytes. The results for the hybridizations with the three probes are shown separately in all panels. *A*, Frequencies of normal interphase nuclei that showed three domains. *B*, Frequencies of cells trisomic for chromosome 21 that showed three domains. *C*, Frequencies of cells trisomic for chromosome 18 that showed three domains. *D*, Frequencies of cells trisomic for chromosome 13 that showed three domains. Each vertical bar represents one sample in all panels. The bars above each measurement are as described in the legend to fig. 2.

and 13. Slides carrying interphase nuclei were hybridized separately with pBS-21, pBS-18, and pBS-13. Interphase samples were analyzed by using FISH, and scoring strategy B was employed. The number of interphase nuclei scored for each sample was 98–1,100. Figure 3 shows the frequencies of interphase nuclei showing three hybridization domains after hybridization with pBS-21, pBS-18, and pBS-13. The data are divided so that the three domain frequencies for the normal nuclei are shown in figure 3*A*, while the frequencies of nuclei trisomic for chromosomes 21, 18, and 13 are shown in figures 3*B*, 3*C*, and 3*D*, respectively. The frequency of nuclei carrying two copies of the chromosome targeted by the hybridization probe and showing three domains is <5% in all cases, even though scoring strategy B was employed. The frequencies of nuclei carrying three copies of the chromosome targeted by the hybridization probe and showing three hybridization domains was always above 10% and usually was 25%–35%.

Discussion

We have described the use of FISH with whole-chromosome plasmid-library probes for chromosomes 21, 18, and 13, for detection of trisomies involving these chromosomes in interphase nuclei and in metaphase spreads prepared from cultured amniocytes. Detection of trisomies involving these chromosomes is particularly important for prenatal diagnosis, both because these aberrations have serious medical consequences and because they occur at relatively high frequencies in live-born infants (Lubs and Ruddle 1970; Jacobs 1977; Epstein 1986).

Detection of trisomies involving chromosomes 21, 18, and 13 is particularly easy and rapid in metaphase spreads when FISH with chromosome-specific probes is used. After FISH with pBS-21, pBS-18, and pBS-13, trisomies involving the target chromosomes, spreads can be scored as rapidly as they can be located (typically a few metaphase spreads per minute). In addition, spreads of low quality (e.g., those in which the chromosomes are highly condensed or not well spread) can be scored accurately. Thus, the number of spreads that can be analyzed by using this approach is limited only by the number of spreads that can be conveniently produced. If sufficient spreads are available, the sensitivity of this approach to detection of subpopulations may be limited only by the frequency with which trisomic cells appear in normal populations (Eastmond and Pinkel 1989). Analysis of metaphase spreads also has the advantage that it allows detection of structural aberrations (e.g., partial trisomies) involving the target chromosomes. However, this approach still requires extensive cell culture to produce sufficient metaphase spreads for statistically precise analysis.

Analysis of trisomies in interphase nuclei reduces the time required for cell culture and, when techniques for FISH to uncultured cells are fully developed, may eventually eliminate the need for this entirely. Studies 1 and 2 demonstrate the feasibility of interphase trisomy detection in cultured amniocytes. These studies show that a substantial fraction of cells in each trisomic sample display the expected three domains after FISH, with a composite probe, against the trisomic chromosome type. The frequencies of trisomic cells showing three domains is 10%–>50%. The lowest frequencies were observed in samples in which the hybridization intensity was not high, so that domain definition was difficult in some cells. This was more of a problem in study 2 than in study 1. As a result, the average domain number for the trisomic samples in study 1 was ~50% but was only ~25% in study 2 (even for chromosome 21). This indicates the need, in studies of this type, for high-quality hybridization probes and high-quality hybridization protocols. However, ≤50% of the cells in a trisomic sample will show less than three hybridization domains, even when the hybridization intensity and specificity are high. We attribute this to domain coalescence caused by the juxtaposition of two or more target chromosomes in the interphase nucleus. This problem is worse in uncultured amniocytes (data not shown). However, it can be reduced by using probes with more limited spatial extent (i.e., composed of elements with homology only to a subregion of the target chromosome). Domain counting may be made more accurate by using computer-assisted fluorescence microscopy to measure domain size and total intensity. This approach reduces ambiguities arising from domain coalescence, because merged domains are expected to be larger and more fluorescent. In spite of these limitations, perfect discrimination between trisomic and normal samples was accomplished in our studies by scoring as trisomic all samples in which >10% of the cells showed three domains.

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