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Original investigations

Chromosomal in situ suppression hybridization of human gonosomes and autosomes and its use in clinical cytogenetics*

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Summary. DNA libraries from sorted human gonosomes were used selectively to stain the X and Y chromosomes in normal and aberrant cultured human cells by chromosomal in situ suppression (CISS−) hybridization. The entire X chromosome was stained in metaphase spreads. Interphase chromosome domains of both the active and inactive X were clearly delineated. CISS-hybridization of the Y chromosome resulted in the specific decoration of the euchromatic part (Ypter-q11), whereas the heterochromatic part (Yq12) remained unlabeled. The stained part of the Y chromosome formed a compact domain in interphase nuclei. This approach was applied to amniotic fluid cells containing a ring chromosome of unknown origin (47,XY; +r). The ring chromosome was not stained by library probes from the gonosomes, thereby suggesting its autosomal origin. The sensitivity of CISS-hybridization was demonstrated by the detection of small translocations and fragments in human lymphocyte metaphase spreads after irradiation with ^{60}Co-gamma-rays. Lymphocyte cultures from two XX-males were investigated by CISS-hybridization with Y-library probes. In both cases, metaphase spreads demonstrated a translocation of Yp-material to the short arm of an X chromosome. The translocated Y-material could also be demonstrated directly in interphase nuclei. CISS-hybridization of autosomes 7 and 13 was used for prenatal diagnosis in a case with a known balanced translocation t(7;13) in the father. The same translocation was observed in amniotic fluid cells from the fetus. Specific staining of the chromosomes involved in such translocations will be particularly important, in the future, in cases that cannot be solved reliably by conventional chromosome banding alone.

* Dedicated to Professor Friedrich Vogel on the occasion of his 65th birthday

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Introduction

Chromosomal in situ suppression (CISS−) hybridization has made it possible to stain individual human chromosomes in metaphase spreads and in interphase nuclei. For this purpose, chemically labeled DNA inserts from a library established from sorted human chromosomes can be used as a complex probe and can be visualized using fluorochromes or colorimetric detection procedures (Lichter et al. 1988a; Pinkel et al. 1988). Recent applications of this new approach include the rapid detection of numerical and structural aberrations of chromosome 21 (Lichter et al. 1988b; Pinkel et al. 1988), the analysis of specific chromosome changes in metaphase and interphase tumor cells (Cremer et al. 1988a) and the detection of radiation-induced chromosome aberrations (Cremer et al. 1990). In this study, we demonstrate further the usefulness of CISS-hybridization in clinical cytogenetics. We show that this approach can be used to detect aberrations of the human gonosomes and to characterize reciprocal autosomal translocations.

Materials and methods

Cell material

Metaphase spreads from amniotic fluid cells and from phytohemagglutinin (PHA)-stimulated human lymphocytes were prepared using standard procedures (Schwarzacher and Wolf 1970). Human lymphocytes were obtained from healthy male and female donors (46,XY and 46,XX) and from two phenotypically male babies. Prenatal diagnosis routinely performed by amniocentesis because of the age of the mother had revealed a 46,XX karyotype in both cases. After birth, the karyotype was confirmed in lymphocytes. There was no indication of any mosaicism with cells containing a Y chromosome or a Y-derived chromosomal fragment. In one case, prometaphase analysis suggested that the terminal region of Xp

might be slightly extended in one of the two X chromosomes (data not shown).

Cultured human lymphocytes from a healthy male donor (46,XY) were irradiated with 8 Gy of ^{60}Co-gamma-rays (1.17 and 1.33 MeV) 1 h after stimulation with PHA (Cremer et al. 1990). After an additional 71 h culturing, chromosome preparation was performed.

Amniotic fluid cells were obtained by diagnostic amniocentesis in the second trimester of pregnancy and cultured as described by Rooney and Czepulkowski (1986). In one case, the indication for amniocentesis was a known balanced reciprocal translocation (7;13) in the father. This was detected by a routine analysis of the parents' lymphocytes because of two previous miscarriages. The analysis of metaphase spreads of the amniotic fluid cells showed the same balanced reciprocal translocation as the father. In another case, kindly provided by Professor H.-D. Rott (Institute of Human Genetics, University of Erlangen-Nürnberg, FRG), amniocentesis was performed on a 36-year-old woman, who had suffered three spontaneous abortions for unknown reasons. A ring chromosome was observed in 7 out of 8 amniotic fluid cell clones (47,XY, +r (?)). Cytogenetic analysis revealed that this ring consisted of euchromatin and contained a centromere. After induced abortion, at the parents' request, the presence of the ring chromosome was confirmed both in placental and several fetal tissues. Cytogenetic analyses showed the karyotype of the mother to be normal (46,XX), but the presence of a ring chromosome in 3 out of 50 lymphocyte metaphase spreads from the father (H.-D. Rott, unpublished results).

Storage of slides

Chromosome preparations were stored in 70% ethanol at 4°C up to 2 months until CISS-hybridization was performed. In fresh preparations, preservation of chromosome morphology may be improved by heating the air-dried slides at 60°C–65°C for 3 h prior to the denaturation step. Prolonged storage of air-dried slides at room temperature (> 2 weeks) may impair the efficiency of in situ hybridization. The following protocol can be recommended, if storage is intended for long periods. Air-dried slides are washed in PBS (3×, 3 min each) to remove traces of acetic acid, put through an ethanol series (70%, 90%, 100%, 3 min each), air dried again and stored in sealed boxed (in the presence of silica gel to avoid moisture) at −70°C. In this way, we have kept slides for more than 1 year with no noticeable loss of hybridization efficiency.

DNA libraries and labeling procedure

Phage DNA libraries from sorted human chromosomes were obtaines from the American type culture collection: LA07NS01 (Chromosome 7), LL13NS02 (Chromosome 13), LA0XNL01 (Chromosome X) and LL0YNS01 (Chromosome Y). Amplification of these phage libraries in liquid culture (using *E. coli* LE392 as the bacterial host), purification of the phage and extraction of phage DNA were carried out as described by Maniatis et al. (1982). Phage DNA containing the human library inserts was labeled with biotin-11 dUTP by nick-translation (Langer et al. 1981).

CISS-hybridization and probe detection

Denaturation of chromosomal DNA, CISS-hybridization with biotin-labeled total library DNA and detection of hybridized probes using fluorescein isothiocyanate (FITC)-labeled avidin was performed as described by Lichter et al. (1988a). For fluorescence microscopy, all preparations were counterstained with 0.2 μg/ml 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) and 1 μg/ml propidium iodide and mounted in fluorescence antifading buffer (1 mg p-phenylendiamine in 1 ml glycerine buffer, pH 8.0). Cells were evaluated using a Zeiss Photomicroscpe III equipped with epifluorescence and photographs were taken on Agfachrome 1000 RS color slide film.

Results

CISS-hybridization of human gonosomes

Figures 1a–f shows metaphase spreads after CISS-hybridization of the human gonosomes with the respective chromosome-specific libraries. Staining of the Y chromosome was restricted to the euchromatic part (Ypter-q11); the heterochromatic part of the long arm (Yq12) remained unstained (Fig. 1c). In contrast, the entire X chromosome was delineated (Fig. 1a, b). As expected, library probes both from sorted Y and X chromosomes showed cross hybridization to evolutionarily conserved regions present on both human gonosomes (Bickmore and Cooke 1987). Sequences contained in the Y-library stained the pseudoautosomal region on the X chromosome, and a discrete band (Xq13) on the long arm of the X chromosome (Fig. 1c, f). Similarly, sequences in the X-library delineated the pseudoautosomal region and also, to some extent, the short arm of the Y chromosome (Fig. 1a). Implications of this finding for chromosome evolution will be discussed elsewhere (J. Wienberg et al. 1990).

The sensitivity of the method allows us to test whether small chromosome fragments, whose origin cannot be determined by conventional staining procedures, are de-

Fig. 1a–n. CISS-hybridization of human chromosomes X (**a, b, d, e, g, h**), Y (**c, f, i, j**), 13 (**k, l**) and 7 (**m, n**). Biotinylated DNA from libraries of sorted human chromosomes was used as probe and detected with avidin-FITC. Cells were counterstained with DAPI (**b, e, l, n**) and propidium iodide (**a, c, d, f, g–j**). **a, b** Metaphase spread and interphase nucleus from amniotic fluid cells with 47,XY; +r. The X chromosome is clearly delineated, whereas the ring chromosome remains unlabeled *(arrows)*. **c** Metaphase spread from 47,XY; +r. The euchromatic part of the Y chromosome is selectively labeled. The heterochromatic region of the Y is stained with DAPI and propidium iodide only. The ring chromosome *(arrow)* remains unstained. Note: *small arrows* indicate two faintly stained regions with cross-hybridization of Y-library sequences on the X chromosome (compare **f**). **d, e** Labeling of the X chromosome in a metaphase spread after irradiation with 8 Gy of ^{60}Co-gamma-rays indicates several translocations and fragments *(arrows)*. **f** Lymphocyte metaphase spread from XX-male (case 1). The two X chromosomes are specifically labeled with the Y-library probe; the *large arrow* indicates translocated Y chromosome material at the terminal region of Xp, *small arrows* point to the pseudoautosomal region of the normal X and the homologous region in Xq13. **g** Single X-chromosome domain in two amniotic fluid cell nuclei from a normal male (46,XY). **h** Two X-chromosome domains in a lymphocyte nucleus of a normal female (46,XX). **i** Single domain of the euchromatic part of the Y chromosome (Ypter-Yq11) in a male cell nucleus (46, XY) from amniotic fluid. **j** Two lymphocyte nuclei from XX-male (case 1) with single hybridization spots representing translocated Y-chromosome material (compare **f**). **k–n** Metaphase spreads from amniotic fluid cells with balanced translocation 46,XY; t(7;13). **k** Labeling of a normal chromosome 13 *(large arrow)* and deleted chromosome 13q- *(small arrow)*. The deleted chromosome 13 material is translocated to a chromosome 7 *(arrowhead)*. Note that the heterochromatic part of the Y chromosome *(triangle)* is also labeled by the chromosome 13 library used in this experiment. **l** Same metaphase as in **k** after DAPI staining. **m** Labeling of a normal chromosome 7 *(large arrow)* and deleted chromosome 7 *(small arrow)*. The deleted chromosome 7 material is translocated to a chromosome 13 *(arrowhead)*. **n** Same metaphase as in **m** after DAPI staining

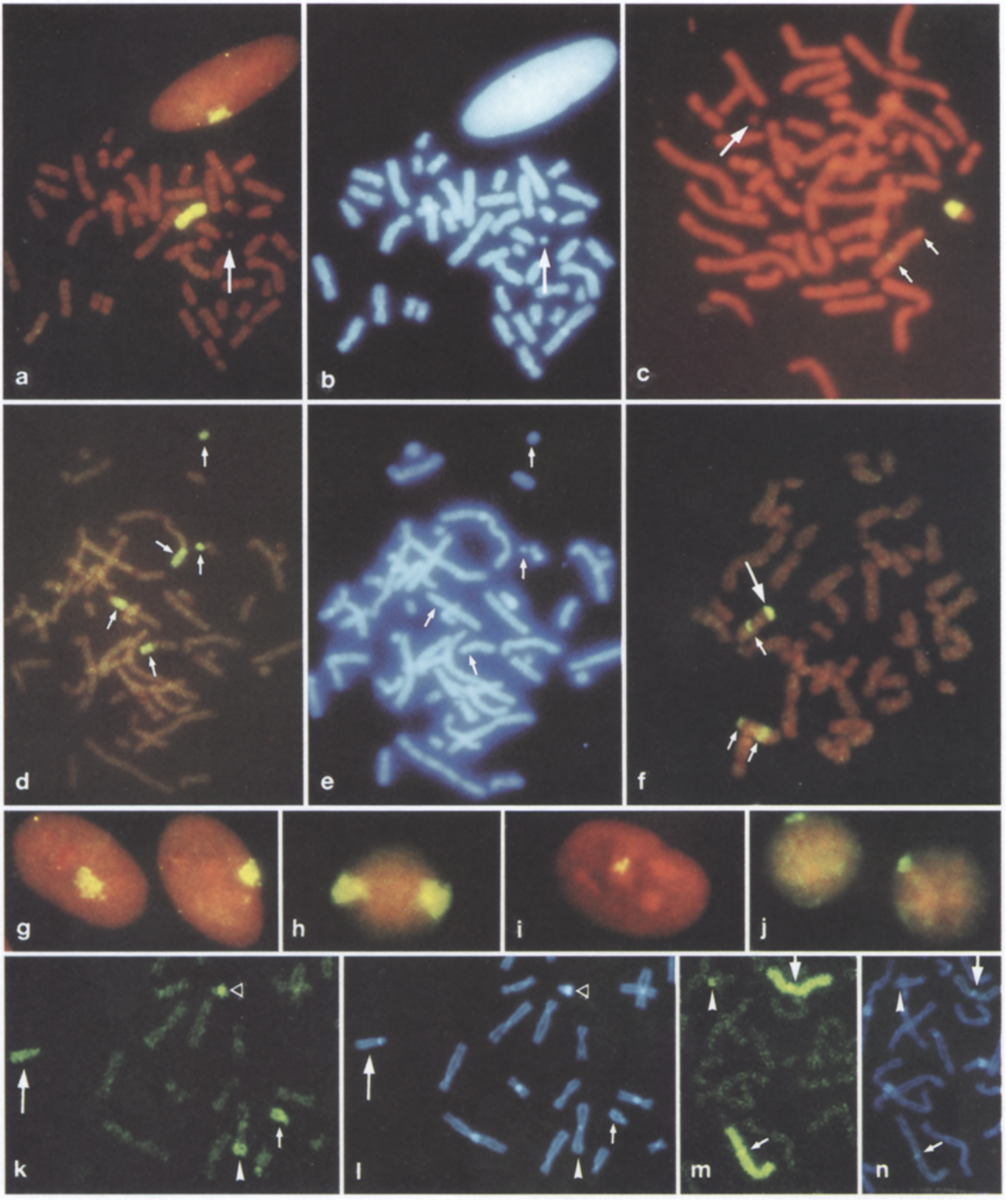
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Table 1. Frequencies of hybridization spots detected in interphase nuclei of PHA-stimulated human lymphocytes and amniotic fluid cells after CISS-hybridization with biotinylated X- and Y-library DNA

Cell type	Karyotype	Library DNA	No. of nuclei	Percentage of nuclei with spot number		
				0	1	2
Lympho-cytes	46,XY	X	200	23	77	–
	46,XY	Y	200	3	97	
	46,XX	X	200	8	–	92
	46,XX	Y	200	100	–	–
	46,XY t(X;Y) case 1	Y	200	13	87	–
	46,XX t(X;Y) case 2	Y	200	11	89	–
Amniotic fluid cells	47,XY; +r	X	200	6	94	–
	47,XY; +r	Y	200	17	83	–

rived from the human gonosomes or from autosomes. Figure 1a–c shows a small unidentified ring chromosome in the DAPI-stained metaphase spread of an amniotic fluid cell. The fact that this ring-chromosome could be stained neither by the Y- nor by the X-library indicates its autosomal origin. Figure 1d, e presents examples of X-chromosome aberrations detected in a lymphocyte metaphase spread from a normal male individual (46,XY) after irradiation of the cells with ^{60}Co-gamma-rays and CISS-hybridization with the X-library. Translocations or fragments comprising a few megabases can be clearly detected in this way.

Figure 1f demonstrates a lymphocyte metaphase spread from a male individual previously karyotyped by conventional banding analysis as 46,XX (data not shown) after CISS-hybridization with the Y-library. One X chromosome displays an abnormally extended fluorescent region at the tip of the short arm, whereas the corresponding region of the other X chromosome shows the smaller fluorescent band representative of the pseudoautosomal region. This result clearly indicates a translocation of Y-chromosomal material containing the male determining gene(s) to one X chromosome.

The X chromosome in male nuclei and both the active and the inactive X chromosome in female nuclei occupied discrete interphase domains (Fig. 1g, h). The sizes of these domains varied in different nuclei. Surprisingly, the differences, if any, between the active and inactive X-domains in each nucleus appeared small in the cell types investigated so far. The euchromatin of the Y chromosome (Ypter-q11) was also visualized as a compact domain in normal male interphase nuclei (Fig. 1i) and the same result was found for the translocated piece of the Y chromosome in interphase nuclei obtained from the XX-male (Fig. 1j). In some nuclei, additional minor signals were seen; these probably resulted from cross-hybridization of Y-library sequences to the X chromosome as described above. These signals were clearly weaker than labeled Y-chromosome material and were not further evaluated.

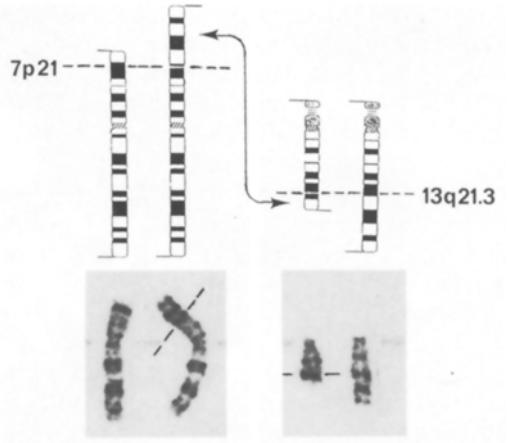

Fig. 2. GTG-banded chomosomes 7 and 13 from the metaphase spread of an amniotic fluid cell with t(7;13). Breakpoints were defined in agreement with additional information from CISS-hybridization experiments (compare Fig. 1 k–n)

Table 1 presents counts of interphase domains specifically stained with either the X- or the Y-library. Most males and female nuclei showed the expected number of X- and Y-domains, respectively. The high percentage of nuclei from the XX-male showing a distinct Y-euchromatin fluorescence clearly contrasts with the total lack of such a signal in normal female nuclei. Interphase signal resulting from cross-hybridization of X- or Y-library probes to the pseudoautosomal region of the gonosomes were very weak and not counted.

CISS-hybridization of human autosomes

Figure 2 shows the GTG-banded normal chromosomes 7 and 13 and the corresponding translocation chromosomes from a metaphase spread of an amniotic fluid cell with 46,XY, t(7qter-7p21::13q21-13qter;13pter-13q21::7p21-7pter). Using CISS-hybridization (Fig. 1k–n), the translocated chromosome material from both chromosomes could easily be distinguished, even in metaphase spreads of low quality with very short or overlapping chromosomes; these were unsuitable for conventional banding analysis. The chromosome 13 library used in the present experiments showed cross-hybridization with the heterochromatic region of the Y chromosome (Fig. 1k, l) (Lichter et al. 1988a). This unexpected hybridization pattern indicates the importance of rigorous testing of complex library probes by CISS-hybridization on normal metaphase cells to avoid misinterpretation in pathological cases.

Discussion

In the following, we will discuss the potential of CISS-hybridization in clinical cytogenetics compared with conventional cytogenetic analyses; we will also consider possible future improvements.

An accessory small chromosome of unknown origin can occasionally be found de novo when prenatal diagnosis is performed (Sachs et al. 1987). The origin of such marker chromosomes often cannot be deduced from banding patterns. Recently, Crolla and Llerena (1988) have demonstrated the X-chromosomal origin of a ring chromosome using in situ hybridization of biotinylated X and Y centromer-specific probes. Our results indicate that CISS-hybridization with library DNA from sorted human gonosomes can be used to investigate whether the whole marker chromosome is constituted from gonosomal material. Such a distinction can be of great importance for genetic counseling of the parents, since marker chromosomes containing euchromatin from autosomes may have clinical consequences of greater severity (Steinbach et al. 1983).

Application of CISS-hybridization can also be useful in familial cases with known balanced reciprocal translocations of autosomes (Boué and Gallano 1984). Identification of the translocation chromosomes may require optimal banding of long chromosomes in well-spread metaphases or prometaphases. Such cells may not be available for prenatal diagnosis. CISS-hybridization of the two chromosomes in question can provide definitive results, even with chromosome spreads of poor quality.

A desirable new strategy would be one that helped to pinpoint unequivocally the chromosomal origin of genetic material in de novo structural chromosomal rearrangements (Warburton 1984). Such a strategy could be based on CISS-hybridization with combinations of multiple chromosome libraries. At present, we can stain simultaneously up to five chromosomes in one color (S. Popp, unpublished results). Advances in multicolor detection protocols add another possibility that can be exploited in this context (Nederlof et al. 1989). By staining groups of chromosomes selectively, one should rapidly be able to narrow down possible candidates from which the unidentified material originates.

When the origin of a marker chromosome or translocated chromosome material is known, in situ hybridization with probes mapped to chromosomal subregions can be used to characterize further the genetic content and the breakpoints. At present, the sensitivity of nonradioactive in situ hybridization procedures is sufficient to localize single copy sequences of a few kb (Landegent et al. 1985; Lichter et al. 1988b; Viegas-Pequignot et al. 1989). CISS-hybridization makes it possible to achieve the site-specific hybridization of DNA clones, even if these clones contain repetitive sequences distributed throughout the whole genome (Landegent et al. 1987; Lichter et al. 1990). We expect that future in situ hybridization strategies in clinical cytogenetics will be based on combinations of mapped probes such as cosmid- or YAC-clones. The realization of such strategies seems clearly feasible in the near future because of the rapid increase of precisely mapped sequences.

Interphase cytogenetics provides another rapidly developing application of in situ hybridization (Rappold et al. 1984; Burns et al. 1985; Cremer et al. 1986, 1988a, b; Pinkel et al. 1986, 1988; Devilee et al. 1988; Hopman et al. 1988, 1989; Emmerich et al. 1989; Walt et al. 1989). The development of this new approach has become possible after the demonstration that chromosomes occupy distinct territories in the interphase nucleus of somatic mammalian cells (Cremer et al. 1982; Schardin et al. 1985; Manuelidis 1985; Lichter et al. 1988a). Interphase cytogenetics can be used in prenatal diagnosis to decide whether the fetus is balanced for specific chromosomes or chromosomal subregions, even in those cases where metaphase spreads for conventional analysis cannot be obtained (Lichter et al. 1988b).

Our preliminary data suggest a correlation between the DNA content of a specifically stained metaphase chromosome or chromosome fragment and the size of its corresponding interphase domain (Cremer et al. 1988a; Lichter et al. 1988a). For example, in the XX-male described above, the translocated piece of Yp was delineated in most lymphocyte nuclei, but was clearly smaller than the domains of complete Y or X chromosomes. The sensitivity with which chromosome material can be detected in interphase nuclei may vary in different cell preparations. In the present experiments, attempts at staining the translocated chromosome fragment in nuclei from cells of amniotic fluid in the t(7;13) case were not successful, although interphase detection of autosomal fragments of similar size was clearly possible in other cases (unpublished data). We expect that the sensitivity of interphase detection can be improved by methods that will enable better probe penetration to the nuclear hybridization targets (Emmerich et al. 1989; Hopman et al. 1989), and by the use of DNA-probes specific for a particular chromosome band (Lichter et al. 1988b). Under optimized conditions, a few kb of DNA can presently be visualized in the interphase nucleus (Lawrence et al. 1988).

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