Chromosomal In Situ Suppression Hybridization of Immunologically Classified Mitotic Cells In Hematologic Malignancies

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Chromosomal in situ suppression (CISS) hybridization was performed with library DNA from sorted human chromosomes 8, 9, 15, 17, 21, and 22 on immunologically stained bone marrow cells of four patients with a hematologic neoplasm, including two patients with myelodysplastic syndrome and trisomy 8, one patient with promyelocytic leukemia bearing the translocation t(15;17)(q22;q11–12), and one patient with chronic myeloid leukemia and the translocation t(9;22)(q34;q11). In all patients, the results of conventional karyotype analysis could be confirmed by one- or two-color CISS hybridization using the appropriate chromosome-specific libraries. Our results show that CISS hybridization can detect both numerical and structural chromosome changes in immunologically classified cells with high specificity and reliability. The fact that chromosome spreads of very poor quality can now be included in such analyses is a decisive advantage of this approach. In addition, the suitability of this approach for interphase cytogenetics is discussed. *Genes Chrom Cancer 4:135–140 (1992).*

INTRODUCTION

The MAC (morphology, antibody, chromosomes) method is a direct technique that enables karyotypic analysis of morphologically and immunologically classified mitotic cells (Teerenhovi et al., 1984; Knuutila and Keinänen, 1985). The technique has been used mainly in studies of proliferation of normal and neoplastic hematopoietic cell lineages and in studies of cell lineage involvement in hematologic malignancies (Knuutila and Teerenhovi, 1989; Larramendy and Knuutila, 1990). The central problem has been to obtain sufficient chromosome spreading at metaphase and chromosome banding of good quality in cells that are still useful for immunophenotyping. To improve the yield of the MAC technique, we have combined it with in situ hybridization of chromosome-specific repetitive probes (Wessman and Knuutila, 1988). Using this combination, it became possible for the first time to study numerical aberrations not only in immunologically classified mitotic cells but also directly in interphase cells. While chromosome-specific repetitive DNA probes delineate heterochromatic regions with tandemly arranged repetitive sequences, chromosomal in situ suppression (CISS) hybridization with recombinant DNA libraries from sorted human chromosomes has been applied to "paint" complete individual chromosomes (Cremer et al., 1988; Lichter et al., 1988a; Pinkel et al., 1988). We report here the possibility of using CISS hybridization to detect both numerical and structural chromosome aberrations in immunologically classified mitotic cells.

MATERIALS AND METHODS

Cell Material

Bone marrow specimens were obtained from two patients (1 and 2) with myelodysplastic syndrome, one patient (3) with acute promyelocytic leukemia, and one patient (4) with chronic myeloid leukemia. According to a standard cytogenetic study, the bone marrow karyotypes were 46,XY(14/21, 67%)/47,XY,+8 (7/21, 33%) in patient 1 [described previously by Jotterand Bellomo et al., (1990)]; 46,XX,del(7)(q?) (4/12, 33%)/45,X,−X,del(7)(q?) (2/12, 17%)/49,XX,+5,+6,+8,del(7)(q?) (6/12, 50%) in patient 2; 46,XY,t(15;17)(q22;q11–12) (18/18, 100%) in patient 3; and 46,XY,t(9;22)(q34;q11) (21/21, 100%) in patient 4.

MAC Method

The procedure for MAC preparations has been described in detail elsewhere (Knuutila and Teerenhovi, 1989). Briefly, mononucleated bone marrow cells were

TABLE 1. In Situ Hybridization Signal Frequencies (%) for Chromosome 8- and 21-Specific Library Probes on Preparations Stained by Alkaline Phosphatase Antialkaline Phosphatase (APAAP) Technique With Different Monocolonal Antibodies

Patient No.	Percentage of APAAP Positive Metaphase Cells[a]	Slide	Probe[b]	Percentage of Metaphase Cells with Number of Signals					No. of Cells Studied
				1	2	3	≥4	NE[c]	
1	62 (CD13)	IA	8	3	44	38	0	15	102
		IB		1	49	45	0	5	127
	24 (GPA)	2A		2	50	31	0	17	54
		2B		0	50	34	1	15	151
	3 (CD61)	3A		1	46	37	0	16	164
		3B		3	45	29	1	22	146
	24 (GPA)	4A	21	6	72	0	0	22	50
		4B		3	72	0	0	25	88
2	81 (CD13)	1	8	0	40	16	0	44	48
	21 (GPA)	2		3	46	30	3	18	33
	0 (CD61)	3		2	57	21	3	17	153

[a]Antibodies: anti-CD13 (MY7) for detection of granulocytic lineage; anti-GPA (antiglycophorin A) for erythroid lineage, and anti-CD61 (Y2/51) for megakaryocytic lineage.
[b]The probes were labeled with biotin and detected by avidin-FITC.
[c]NE, not evaluable.

isolated by one-step density-gradient Ficoll-Paque and cultured for 1–5 days. For preparation of the slides, 10^5 cells were suspended in a mixture of 1 ml of RPMI-1640 medium supplemented with 20% fetal calf serum and 1 ml of hypotonic solution. The hypotonic solution contained 50 mmol/liter glycerol, 5 mmol/liter KCl, 10 mmol/liter NaCl, 0.8 mmol/liter $MgCl_2$, and 10 mmol/liter sucrose (pH 7.0). After 5 min of incubation, the slides were prepared by means of a cytocentrifuge. After air drying overnight, the cells were classified by alkaline phosphatase-antialkaline phosphatase (APAAP) technique. For recognition of granulocytic, erythroid, and megakaryocytic lineages, the monoclonal antibodies MY7 (anti-CD13; Coulter Clone, Coulter Immunology, Hialeah, FL), anti-glycophorin A (anti-GPA; Edwards, 1980), and Y2/51 (anti-CD61; Erber et al., 1987), respectively, were used. Antibody J5 (anti-CD10; Coulter) was used against common ALL antigen. Giemsa staining was used for morphologic cell classification. Photographic documentation was made on Kodak Ektachrome (50 ASA) film.

CISS Hybridization

For CISS hybridization, the MAC preparations were destained in methanol/acetic acid (3:1) for 1 hr and air dried. To remove the cytoplasm, the preparations were treated with pepsin (0.1 mg/ml in 0.01 N HCl) in 37°C for 4–10 min followed by washing in distilled water or phosphate-buffered saline (PBS) and dehydration in an ethanol series. A postfixation step with 1% formalde-

hyde in PBS with 50 mmol/l $MgCl_2$ for 10 min was included in some of the experiments (Hopman et al., 1989), followed by additional washing in PBS and dehydration in an ethanol series. In all but one patient (2) fresh MAC slides (2 days to 2 weeks) were used for hybridizations. The slides of patient 2 had been stained approximately 2 months before CISS hybridization. The hybridizations were carried out with bacteriophage DNA libraries from sorted human chromosomes 8, 15, 17, 21, and 22 obtained from the American Type Culture Collection (LL08NS02, LL15NS01, LL17NS02, LA21NS01, LL22NS01, respectively). The plasmid library pBS9 for human chromosome 9 was kindly provided by J. Gray (Livermore, CA). Probes were labeled by nick translation using biotin-11-dUTP (Sigma, St. Louis, MO) or digoxigenin-11-dUTP (Boehringer Mannheim), and CISS hybridization was carried out as described in detail elsewhere (Lichter et al., 1988a). The biotinylated probes were detected with avidin conjugated with fluorescein isothiocyanate (FITC), 7-amino-4-methylcoumarin-3-acetic acid (AMCA), or tetramethylrhodamine isothiocyanate (TRITC) (Vector Laboratories, Burlingame, CA). Signals were amplified as described by Pinkel et al. (1986). Digoxigenin-labeled probes were detected by indirect immunofluorescence using a mouse monoclonal antibody against digoxigenin (Boehringer Mannheim) and an antimouse IgG FITC-conjugated antibody made in sheep (Sigma). Cells were counterstained with 1 μg/ml propidium iodide and/or 0.2 μg/ml 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI) and mounted in

TABLE 2. Frequency of Metaphase Cells With t(15;17) Detected by CISS Hybridization With Chromosome 15- and 17-Specific Library Probes on APAAP-Stained Slides of Patient 3 With Promyelocytic Leukemia

Patient No.	Percentage of APAAP Positive Metaphase Cells[a]	Slide	Probe[b]	t(15;17) (%)	NE[c] (%)	No. of Cells Studied
3	0 (GPA)	IA	15	19	81	135
		IB		30	70	10
		IC	17	52	48	21
		ID		54	46	41

[a]Antibody: anti-GPA, anti-glycophorin A for detection of erythroid lineage.
[b]The probes were labeled with biotin and detected by avidin-FITC.
[c]NE, not evaluable.

fluorescence antifading buffer (Johnson and Nogueira Araujo, 1981). Cells were evaluated with a Leitz Laborlux D (single hybridizations), a Zeiss Photomicroscope III, and a Zeiss Axiophot (double hybridizations) equipped with epifluorescence. Photographs were taken on Kodak Ektachrome 400 ASA and on Agfachrome 1000 ASA color slide films.

RESULTS

Table 1 shows the results of CISS hybridization with chromosome 8 and 21 library DNA probes on mitotic bone marrow cells on APAAP-stained preparations of patients 1 and 2 with myelodysplastic syndrome as well as the proportions of APAAP positive cells with different antibodies. In both patients, the majority of cells were positive for MY7 antibody and thus belonged to the granulocytic lineage. The number of hybridization signals was scored in all mitotic cells detectable on the cytospin preparations. In both patients, most of the mitotic cells were evaluable in several slides with chromosomes 8- and 21-specific library probes (ranges 75–95% for patient 1 and 56–83% for patient 2). Trisomy 8 had been detected in both patients in a portion of the cells when analyzed by standard karyotype analysis (33% in patient 1 and 50% in patient 2). The proportions of mitotic cells with three clear separate chromosome 8 hybridization signals were 29–45% in patient 1 (Fig. 1) and 16–30% in patient 2 (Table 1). The percentages of mitotic cells with trisomy 8 obtained after CISS hybridization appear to be more reliable than those obtained with standard karyoype analysis, since many more metaphases could be analyzed for chromosome 8 copy numbers.

Translocations t(15;17)(q22;q11–12) and t(9;22)(q34; q11) were present in all cells of patients 3 and 4, respectively, as assessed by conventional karyotyping. All mitotic cells on the APAAP-stained preparations were evaluated by CISS hybridization for the presence of t(15;17) in patient 3. The detection of

t(15;17) was possible in 19–30% and 52–54% of the mitotic cells using probes for chromosomes 15 and 17, respectively (Fig. 2; Table 2). In patient 4, 100 mitotic cells on each APAAP-stained slide were evaluated after CISS hybridization with chromosome 22-specific DNA probes. Translocation t(9;22) could be detected reliably in 17–24% of the mitotic cells (Table 3). In two-color hybridization experiments with DNA probes for chromosomes 9 and 22 (patient 4), the der(9)t(9;22) could be unequivocally demonstrated in 60–100% of immunologically stained metaphase cells (Fig. 3; Table 3). In most metaphase cells, the Philadelphia chromosome could also be distinguished from the normal chromosome 22 by painting with the chromosome 22 library probes because of the clear difference in size. The translocated chromosome 9 material in the Philadelphia chromosome, however, was too small to be detected with the chromosome 9-specific library probes (Fig. 3).

In patient 1, chromosome 8 and 21 signals were also analyzed from 200 consecutive interphase cells. Only a minority of interphase cells could be scored (range 33–49%). Trisomy 8 could be detected in 4–11% of the nuclei (Fig. 1). The percentage of interphase nuclei with three chromosome 8 domains is considered to be an underestimate due to merged signals resulting from overlapping chromosome domains or incomplete hybridizations. Such cases would suggest one or two instead of three separate signals. In patient 2, the slides used for CISS hybridization were older than those from patient 1, resulting in a lower hybridization efficiency, which was noted in particular in interphase cells. In patients 3 and 4, interphase cells were not suitable for the analysis of the translocations. Especially when probes for the acrocentric chromosomes 15 and 22 were used, cross hybridization events with the short arms of other nucleolar organizing region (NOR)-bearing chromosomes interfered with a reliable interpretation of interphase nuclear signals.
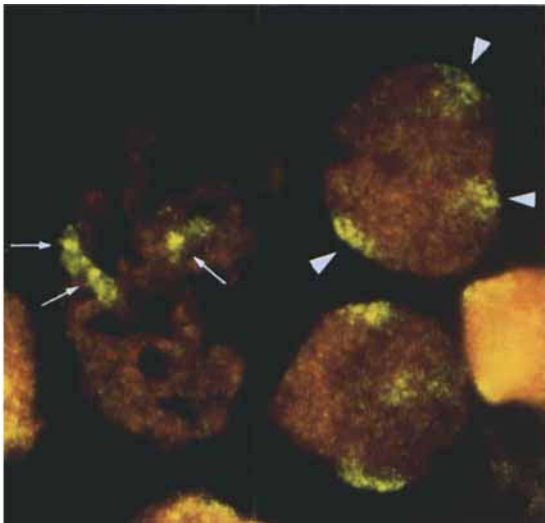
Figure 2. **a:** Translocation t(15;17)(q22;q11–12) in patient 3 with promyelocytic leukemia shown by CISS hybridization with biotinylated chromosome 17-specific library probes detected by avidin-FITC in an APAAP-negative metaphase (antibody anti-GPA against erythroid cells). The arrow points to the der(17)t(15;17)(q22;q11–12) and the arrowhead at the der(15)t(15;17)(q22;q11–12). **b:** Same metaphase spread with DAPI counterstaining.

Figure 1. Trisomy 8 in patient 1 with myelodysplastic syndrome after CISS hybridization with biotinylated chromosome 8-specific library probes detected with avidin-FITC on APAAP-stained preparation (antibody anti-CD13 for detection of granulocytic cells). Arrows point to the centromeric regions of chromosomes 8 in a metaphase plate, and arrowheads show three separate chromosome 8 domains in an interphase nucleus.
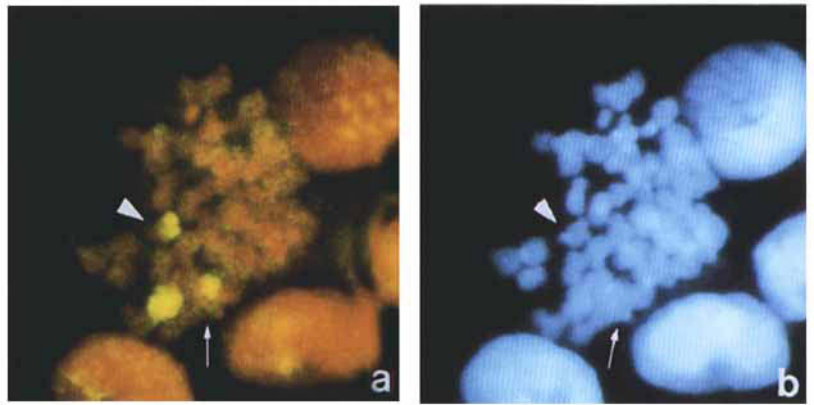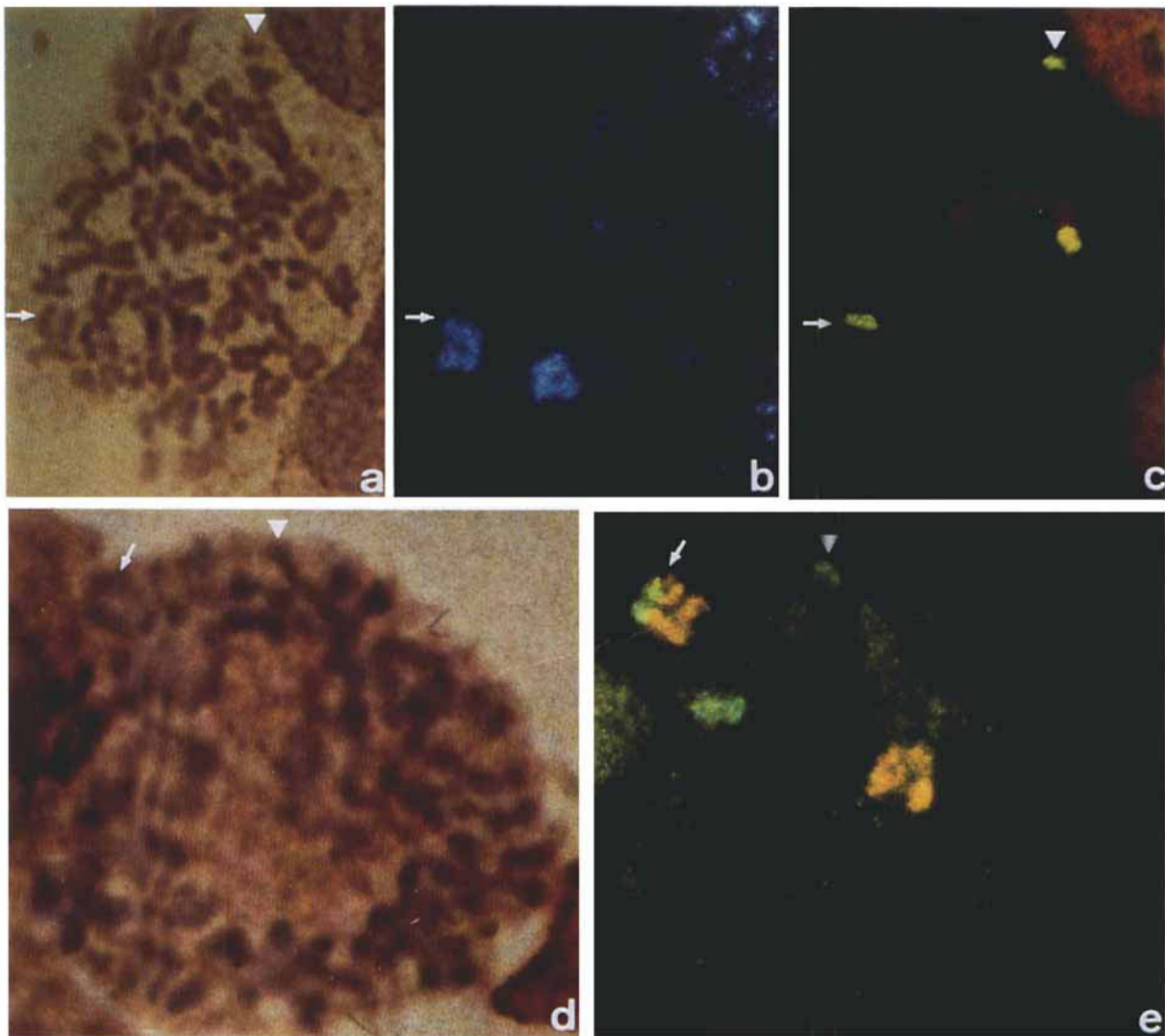

Figure 3.

TABLE 3. Frequency of Metaphase Cells With t(9;22) Detected by Single and Double CISS Hybridization on APAAP-Stained Slides of Patient 4 With Chronic Myeloid Leukemia

Patient No.	Percentage of APAAP Positive Metaphase Cells[a]	Slide	Probe/Detection System	t(9;22) (%)	NE[b] (%)	No. of Cells Studied
4	99%(CD13)	IA	22/biotin FITC	17	83	100
		IB		24	76	100
	99%(CD13)	2	9/biotinTRITC 22/digoxigeninFITC	100	0	50
	0.5%(CD61)	3	9/digoxigeninFITC 22/biotinTRITC	66	34	68
	0.5%(GPA)	4	9/biotinAMCA 22/digoxigeninFITC	100	0	50
	0%(CD10)	5	9/digoxigeninFITC 22/biotinAMCA	60	40	66

[a]Antibodies: anti-CD13 (MY7) for detection of granulocytic lineage; anti-CD61 (Y2/51) for detection of megakaryocytic lineage; anti-GPA, antiglycophorin A for detection of erythroid lineage; anti-CD10 (J5) against common ALL antigen.
[b]NE, not evaluable.

DISCUSSION

While the detection of balanced translocations in G-banded leukemic MAC metaphase cells is extremely difficult, the results obtained in the present study, using chromosome 8, 9, 15, 17, 21, and 22 recombinant DNA libraries as probes for CISS hybridization, show that both numerical and structural chromosome aberrations can be detected in immunologically classified mitotic cells with high specificity and reliability. This is true even for cases when the chromosomes are very poorly spread or extremely condensed. Thus long colcemid treatment periods can be used to arrest a maximum number of mitotic cells.

In this study, t(15;17) was clearly detectable in approximately 53% of metaphases on APAAP-stained slides when chromosome 17-specific library probes were used. The percentage was smaller (25%) with chromosome 15-specific library DNA, which—as with chromosome 21 library DNA—gives cross-hybridization signals to the short arm of acrocentric human chromosomes. Translocation t(9;22) was detectable in

approximately 20% of the MAC metaphases when chromosome 22-specific library probes only were used. After two-color CISS hybridization with chromosome 9- and 22-specific library probes, 60–100% of the metaphases could be analyzed. While these differences may in part indicate the variability of hybridization experiments independently performed in two laboratories, two-color in situ hybridization experiments are generally superior. They provide complementary information for both chromosomes involved in a translocation and thus increase the reliability with which a diagnosis can be obtained even in metaphase spreads of very poor quality.

The copy number of chromosomes 8 and 21 was detectable in over 90% of APAAP-stained metaphases from two patients with myelodysplastic syndrome. In that the frequency of hybridization signals for chromosome 8 in patient 1 was similar on slides where most of the cells were positive after APAAP staining (anti-CD13; Table 1) and on slides where most of the cells were negative (anti-CD61), the APAAP reaction itself does not appear to affect the hybridization efficiency.

Figure 3. The t(9;22)(q34;q11) detected in two immunologically stained metaphases of patient 4 with chronic myeloid leukemia after two-color CISS hybridization with library probes from sorted chromosomes 9 and 22. a: Negative APAAP staining with antiglycophorin A antibody against erythroid lineages. Chromosomes are counterstained with Giemsa. The arrow points at the der(9)t(9;22) and the arrowhead at the Philadelphia chromosome, der(22)t(9;22) (compare b and c). b: Same metaphase spread after CISS hybridization with biotinylated chromosome 9 library probes detected with avidin-AMCA shows the bluepainted normal chromosome 9 (right) and the der(9)t(9;22) (left). The arrow indicates the translocation breakpoint at 9q34. c: The same metaphase spread after CISS hybridization with digoxigenin-labeled chromosome 22 library probes detected with FITC-conjugated antibodies shows a green painted normal chromosome 22 (middle, right), the Philadelphia chromosome (arrowhead), and the chromosome 22-derived part of the translocation chromosome der(9)t(9;22) (arrow). d: Another metaphase after positive APAAP staining with antibody anti-CD13 specific for granulocytic lineages. Note the poor chromosome spreading. The arrow indicates the position of the translocation chromosome der(9)t(9;22) and the arrowhead the position of the Philadelphia chromosome (compare e). e: The same metaphase spread after two-color CISS hybridization with biotin-labeled chromosome 9 library probes detected with avidin-TRITC (red) and with digoxigenin-labeled chromosome 22 library probes visualized with FITC-conjugated antibodies (green). Arrow and arrowhead indicate the translocation breakpoints in the der(9)t(9;22) and in the Philadelphia chromosome, respectively. In addition, the normal green-painted chromosome 22 and the red-painted chromosome 9 are clearly detectable.

The copy number of chromosome 21 was also evaluable in a high percentage (75%) of mitotic cells. Even though CISS hybridization with a human chromosome 21-specific recombinant DNA library probe results in cross hybridization to the short arm of other acrocentric human chromosomes (Lichter et al., 1988b), these cross-hybridization signals do not interfere with the counting of painted chromosomes 21 in mitotic cells. The latter chromosomes are painted completely along the whole long arm in contrast to the case with other acrocentric chromosomes, in which the long arm remains unpainted.

In contrast to mitotic cells, signals obtained with chromosome-specific library probes in interphase nuclei were often of unsatisfactory quality or tended to merge or overlap. Counting of clearly separate chromosome signals can often be improved by the use of DNA probes that delineate a restricted chromosomal segment instead of a complete chromosome domain. Chromosome-specific repetitive centromeric DNA probes useful in this respect have been described for most human chromosomes (Willard and Waye, 1987). For example, we have previously demonstrated that the copy numbers of chromosome 12 can be reliably evaluated in over 90% of immunologically classified interphase cells after in situ hybridization with a chromosome 12-specific repetitive alphoid DNA probe (Perez Losada et al., 1991).

Recently, it has been demonstrated that chromosome translocations and inversions can also be detected in interphase nuclei by in situ hybridization with DNA probes that flank specific breakpoints on both sides (Arnoldus et al., 1990; Dauwerse et al., 1990; our unpublished data). These considerations demonstrate the necessity to construct optimum probe sets for each application. Future applications will not be restricted to tumor cytogenetics but will also include the characterization of abnormal tissues in mosaic trisomy syndromes as well as studies of the effect of clastogenic agents (Cremer et al., 1990; Popp et al., 1990; Larramendy and Knuutila, 1991).

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