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Metaphase and Interphase Cytogenetics with Alu-PCR-amplified Yeast Artificial Chromosome Clones Containing the *BCR* Gene and the Protooncogenes c-*raf-1*, c-*fms*, and c-*erbB-2*[1]

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ABSTRACT

A human yeast artificial chromosome (YAC) library was screened by polymerase chain reaction with oligonucleotide primers defined for DNA sequences of the *BCR* gene and the protooncogenes c-*raf-1*, c-*fms*, and c-*erbB-2*. Alu-PCR-generated human DNA sequences were obtained from the respective YAC clones and used for fluorescence *in situ* hybridization experiments under suppression conditions. After chromosomal *in situ* suppression hybridization to GTG-banded human prometaphase chromosomes, seven of nine initially isolated YAC clones yielded strong signals exclusively in the chromosome bands containing the respective genes. Two clones yielded additional signals on other chromosomes and were excluded from further tests. The band-specific YACs were successfully applied to visualize specific structural chromosome aberrations in peripheral blood cells from patients with myelodysplasia exhibiting del(5)(q13q34), chronic myeloid leukemia and acute lymphocytic leukemia with t(9;22)(q34;q11), acute promyelocytic leukemia (M3) with t(15;17)(q22;q21), and in a cell line established from a proband with the constitutional translocation t(3;8)(p14.2;q24). In addition to the analysis of metaphase spreads, we demonstrate the particular usefulness of these YAC clones in combination with whole chromosome painting to analyze specific chromosome aberrations directly in the interphase nucleus.

INTRODUCTION

Specific chromosomal anomalies play a role in initiation and progression of neoplastic diseases. The unequivocal diagnosis of such aberrations using chromosome banding techniques, however, often suffers from a lack of mitotic tumor cells. In addition, the quality of chromosome spreads is often not sufficient for detailed cytogenetic analyses of the affected chromosomes, and it remains doubtful whether the results of such analyses are representative for the whole tumor cell population. Recent improvements of fluorescence *in situ* hybridization have provided a tool to visualize chromosome aberrations of interest in tumor cells at all stages of the cell cycle. Libraries from sorted human chromosomes have been used for CISS[3] hybridization to "paint" whole chromosomes in tumor cells (1–3), while individual DNA clones have been applied to delineate breakpoint regions with high resolution (4–8). YAC clones (9), which span several hundred kilobases within a chromosome region of interest, provide an ideal tool for such studies (10–13). Recently, Alu-PCR (14) has been applied as a rapid means to amplify human DNA sequences from YAC clones with complexities which generally yield strong signals both on metaphase spreads and in interphase nuclei (15). General use of such clones in tumor cytogenetics, however, is still limited by a lack of appropriate YACs.

In this study we have screened a YAC library for clones containing the *BCR* gene and the human protooncogenes c-*raf-1*, c-*fms*, and c-*erbB-2*. Nine clones were isolated which contained the expected sequences from the respective genes. After CISS hybridization seven of these clones showed signals exclusively within the expected bands of GTG-banded prometaphase chromosomes. We demonstrate that these clones can be used with high reliability for the detection of structural chromosome aberrations in patients with various hematological diseases and constitutive chromosome translocations, respectively, at all stages of the cell cycle. Double color CISS hybridization of YAC clones in combination with whole chromosome painting libraries was used to facilitate the mapping of clones with respect to the localization of the breakpoint in individual tumors.

MATERIALS AND METHODS

Human Cells. Metaphase spreads and interphase nuclei were prepared from phytohemagglutinin-stimulated normal male and female human blood lymphocytes, from Epstein-Barr virus-transformed lymphocytes of a proband carrying the translocation t(3;8)(p14.2;q24), and from nonstimulated lymphocytes of patients with various hematological diseases, including CML and ALL with t(9;22)(q34;q11), acute promyelocytic leukemia (M3) with t(15;17)(q22;q21), and myelodysplasia with del(5)(q13q34). Metaphase chromosome spreads were prepared using standard techniques of Colcemid treatment, hypotonic treatment, and methanol/acetic acid fixation. Conventional chromosome analyses were performed in GTG-banded metaphase spreads. For high resolution mapping, prometaphase chromosomes were obtained by the methotrexate synchronization technique described by Yunis (16). Preparations were stored in 70% ethanol at 4°C until use. Prometaphase chromosome spreads were GTG banded, photographed with a black and white film (AgfaOrtho), and postfixed as described by Klever *et al.* (17) prior to use in CISS hybridization experiments.

DNA Library Probes. Bacteriophage libraries from sorted human chromosomes were obtained from the ATCC (chromosome 3, LA03NS02; chromosome 5, LA05NS01) (18). The plasmid library from sorted human chromosomes 17 was a generous gift from Dr. Joe Gray, University of California, San Francisco (19). Bacteriophage libraries were amplified in liquid culture using *Escherichia coli* LE 392 as the bacterial host. DNA from bacteriophage and plasmid libraries was purified as described by Maniatis *et al.* (20).

PCR Screening of Human YAC Clone Library. Oligonucleotide primers defined for the amplification of DNA sequences of the protooncogenes c-*raf-1* (21), c-*fms* (22), and c-*erbB-2* (23) and for an intron-exon junction region of the *BCR* gene (24) were used for PCR

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[3] The abbreviations used are: CISS, chromosomal *in situ* suppression; YAC, yeast artificial chromosome; CML, chronic myeloid leukemia; ALL, acute lymphatic leukemia; PCR, polymerase chain reaction; PFGE, pulsed field gel electrophoresis; ATCC, American Type Culture Collection; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; APL, acute promyelocytic leukemia.

screening of the Washington University human YAC library as described previously (25).

c-*raf-1*

RAF1: 5′ AGA GGT GAT CCG AAT GCA GGA 3′
RAF2: 5′ TCA ATG GAA GAC AGG ATC TGA 3′
Predicted length of amplification product, 611 base pairs

c-*fms*

FMS1: 5′ TTC TGC TGA GGA GTT GAC GAC 3′
FMS2: 5′ CTT GGT GTG GCC AGC CAA TGC 3′
Predicted length of amplification product, 690 base pairs

c-*erbB-2*

ERB1: 5′ GAC ACC TAC GGC AGA GAA CCC 3′
ERB2: 5′ GTA CAA AGC CTG GAT ACT GAC 3′
Predicted length of amplification product, 510 base pairs

BCR gene

BCR1: 5′ GAC ACT GGC TTA CCT TGT GC 3′
BCR2: 5′ GAC GAT GAC ATT CAG AAA CC 3′
Predicted length of amplification product, 183 base pairs

PCR of human genomic DNA with these primer pairs showed specific amplification products of the predicted length. Clones identified in the appropriate filters by colony hybridization were grown and confirmed by colony PCR (26).

Molecular Characterization of YAC Clones. Methods for the growth of YAC-containing yeast strains, the preparation of DNA for conventional and PFGE, and Southern analysis of DNA have been described previously (9, 27). PCR products to be used as probes were purified using polyacrylamide gel electrophoresis as described previously (25). DNA probes for RAF-1, BCR, FMS, and ERBB2 were obtained from the ATCC (Accession nos. 41050, 59120, 41017, and 57584, respectively). The DNA probes were labeled radioactively (^{32}P) using the random-hexamer priming method of Feinberg and Vogelstein (28).

Alu-PCR Amplification of YAC Clones, CISS Hybridization, and Probe Detection. For CISS hybridization human sequences were generated by Alu-PCR amplification from the YAC clones as described in detail elsewhere (15). Briefly, agarose plugs prepared for pulsed field gel electrophoresis containing individual YAC clones were equilibrated in PCR buffer [10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin]. After careful equilibration, plugs were melted for 5 min at 70°C, and an aliquot equivalent to approximately 100 ng of genomic YAC clone DNA was added to the Alu-PCR assay using the primer pair as follows: CL1: 5′ TCC CAA AGT GCT GGG ATT ACA G 3′; CL2: 5′ CTG CAC TCC AGC CTG GG 3′ (15). Each primer was used at a concentration of 0.25 μM in a total volume of 100 μl of PCR buffer containing 250 μM of each of the four nucleotides and 2.5 units of *Taq* polymerase (Perkin Elmer/Cetus). After initial denaturation at 96°C for 3 min, 30 cycles of PCR were carried out with denaturation at 96°C for 1 min, annealing at 37°C for 30 s, and extension at 72°C for 6 min. Ten-μl aliquots of amplified DNA sequences were fractionated by gel electrophoresis. Alu-PCR amplification products were labeled with biotin-11-dUTP using standard nick translation procedures (29). DNA library probes (see above) were nick translated with digoxigenin-11-dUTP.

Chromosome preparations were pretreated with RNase A and pepsin, postfixed for 10 min in 3% paraformaldehyde, and stored in 70% ethanol overnight (8). CISS hybridization and detection of YAC probes with FITC conjugated to avidin were carried out as described previously (30) with the following modifications. For hybridization, 100 to 150 ng of the Alu-PCR-amplified YAC DNA were used as a probe after preannealing with various amounts (30 to 100 μg) of an unlabeled Cot1 DNA fraction (BRL/Life Technologies; Catalogue No. 5279SA). The signals were amplified once (31). In two-color CISS hybridization experiments with biotinylated YAC probes and digoxigenin-labeled library probes, the latter were detected by indirect immunofluorescence using mouse antidigoxin (Sigma) and goat anti-mouse Ig-TRITC (Sigma). After FITC signal detection, cells were counterstained with 1 μg/ml of DAPI and 0.2 μg/ml of propidium iodide. In two-color CISS hybridization experiments DAPI was used as the only counterstain. Cells were mounted in fluorescence antifading buffer (1 mg of *p*-phenylendiamine in 1 ml of glycerine buffer, pH 8.0) and evaluated with a Zeiss Photomicroscope III or a Zeiss Axiophot equipped with a double band pass filter (Zeiss). Microphotographs were taken with AGFACHROM 1000 RS or Kodak Ektachrome 400 color slide films.

RESULTS

Isolation and Molecular Characterization of YAC Clones for the *BCR* Gene and the Protooncogenes c-*raf-1*, c-*fms*, and c-*erbB-2*

Nine YAC clones containing the *BCR* gene or the protooncogenes c-*raf-1*, c-*fms*, or c-*erbB-2*, respectively, were isolated by PCR screening of the Washington YAC library established from human genomic DNA. Each of the nine isolated YAC strains was checked for the presence of expected gene-specific sequences by Southern blot analysis of digested DNA samples. The amplified PCR products and previously characterized oncogene sequences from the cloned region [obtained from ATCC (see "Materials and Methods")] were used as DNA hybridization probes for these analyses. Each isolated YAC carried the expected sequences (data not shown), although two clones which contained c-*erbB-2* sequences (A153G3 and B217G7) were later found to contain additional DNA from chromosomal regions apparently unrelated to the c-*erbB-2* gene (see the CISS hybridization experiments described below). PFGE of three of the nine clones (A230A7, D107B4, and A168H4) showed minor YAC bands which were related to the major YAC band of the respective clone as indicated by Southern blot hybridization with the respective oncogene probes. The seven oncogene-bearing YAC clones which apparently contain DNA only from the expected human genomic regions are listed in Table 1.

Alu-PCR amplification from an aliquot of melted agarose plugs containing 100 ng of YAC clone DNA yielded numerous bands of 0.1 to at least 4 kilobases in length for each clone (Fig. 1). The size and intensity of individual bands were found to be specific and reproducible for each YAC clone. YAC clones which were isolated for the same genomic site all showed some bands with apparently identical position and intensity, while other bands were distinctly different. These results confirm that PCR screening of the YAC library yielded different YAC clones for the *BCR* gene and the protooncogenes c-*raf-1* and c-*erbB-2*.

High Resolution Mapping of Alu-PCR-amplified YAC Clones

Alu-PCR-amplified probes obtained for each of the nine isolated YAC clones were used for CISS hybridization on

Table 1 *YAC clones with DNA only from expected human genomic regions*

YAC clone	Gene	Size (kilobases)
B99E1	*BCR*	115
D107F9	*BCR*	215
D73A7	c-*fms*	205
D122A5	c-*erbB-2*	500
A230A7	c-*erbB-2*	115 (135)[a]
A168H4	c-*raf-1*	550 (420)
D107B4	c-*raf-1*	240 (210)

[a] Numbers in parentheses, secondary YAC band of the indicated size, related to the primary YAC band (both contain the expected sequences), also present in the YAC-bearing yeast strain.

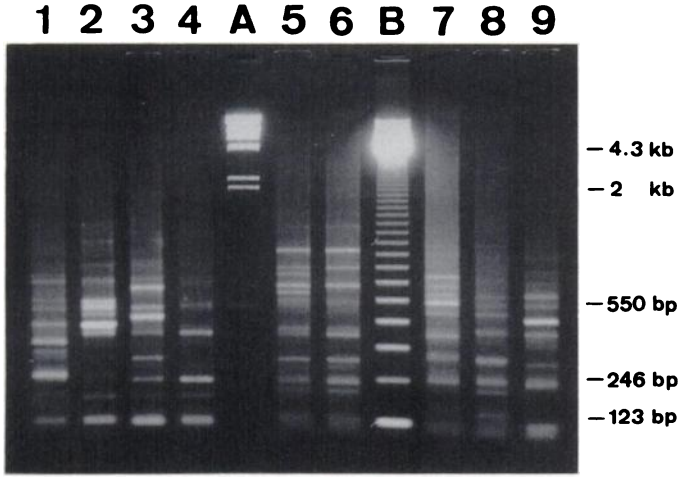

Fig. 1. Agarose gel (1.2%) of Alu-PCR-amplified YAC clone DNA. *Lanes 1* to *4*, c-*erbB-2* YAC clones A153G3, B217G7, D122A5, and A230A7; *Lanes 5* and *6*, *BCR* YAC clones D107F9 and B99E11; *Lanes 7* and *8*, c-*raf-1* YAC clones A168H4 and D107B4; *Lane 9*, c-*fms* YAC clone D73A7; marker *Lane A*, λ/*Hind*III; marker *Lane B*, 123-base pair ladder.

normal human metaphase and prometaphase spreads (46, XY, approximately 800 to 1000 band level) (Fig. 2). Prometaphase chromosome spreads were GTG banded prior to CISS hybridization. For each YAC clone, at least 50 metaphase spreads and 25 prometaphase spreads were evaluated, and the localization of the signals was compared with the localizations of the respective genes reported elsewhere (32–34). Fig. 2, *E* to *L*, shows examples of the high resolution mapping of site-specific YAC clones. The two *BCR* YACs mapped to 22q11, the two c-*raf-1* YACs mapped to 3p25, the c-*fms* YAC mapped to 5q33, and two of the c-*erbB-2* clones (D122A5 and A230A7) mapped exclusively to 17q12–21.1 (see Fig. 2, *A* to *D*, for examples). However, two of the four YAC clones obtained for the c-*erbB-2* protooncogene (A153G3 and B217G7) showed additional band-specific signals on other autosomes (data not shown). These clones were excluded from further experiments.

Application of Alu-PCR-amplified YAC Clones in the Analysis of Structural Chromosome Aberrations

Analysis of Metaphase Spreads. The Alu-PCR-amplified YAC clones were used to delineate specific deletions and translocations (Fig. 3). In each case a minimum of 50 tumor metaphase spreads was evaluated. Even metaphase spreads with very poor spreading could be easily evaluated.

Fig. 3*A* shows a metaphase spread from a patient with 5q− syndrome after two-color CISS hybridization with a human chromosome 5 genomic DNA library and the biotinylated c-*fms* YAC probe D73A7. The hybridization signal of the c-*fms* YAC clone is visible only on the normal chromosome 5. In the other chromosome 5 the signal is lost because of an interstitial deletion involving the c-*fms* protooncogene (32).

Fig. 3*D* shows a metaphase spread with t(9;22)(q34;q11) from a patient with CML after CISS hybridization with the *BCR* YAC clone D107F9. The normal chromosome 22, the Philadelphia (Ph^1) chromosome, and the der(9) are clearly labeled. These results were obtained with both *BCR* YAC clones D107F9 and B99E1 in three CML patients and in one patient with ALL carrying the Ph^1 chromosome (data not shown). These results demonstrate that both clones span the breakpoint cluster region on chromosome 22 involved in these patients. Notably, the relative signal intensities observed on the der(9) differed for the two clones; *i.e.*, the signals obtained with B99E1 were considerably weaker than with D107F9.

Fig. 3*F* shows a metaphase spread from a patient with APL carrying the translocation t(15;17)(q22;q21). Two-color CISS hybridization was performed with a human chromosome 17 genomic library and the c-*erbB-2* YAC clone A230A7. The hybridization signal of the YAC clone is visible on the normal chromosome 17 and the translocation chromosome (17pter→17q21::15q22→15qter). The same result was obtained with the c-*erbB-2* clone D122A5 (data not shown). These findings confirm directly that the breakpoint in APL is distal to the c-*erbB-2* sequences contained in these clones (35).

Fig. 3*G* shows a metaphase spread from an individual carrying the constitutional translocation t(3;8)(p14.2;q24). Two-color CISS hybridization was performed with a chromosome 3 genomic library and the c-*raf-1* YAC clone A168H4. The hybridization signal of the YAC clone is visible on the normal chromosome 3 and the translocation chromosome (8pter→8q24::3p14.2→3pter). As expected, the breakpoint in 3p is proximal to the c-*raf-1* sequences contained in these clones.

Analysis of Interphase Nuclei. Alu-PCR-generated probes from YAC clones yielded specific signals in interphase nuclei. The intensity of these signals varied with different clones, but was generally comparable to signals obtained from chromosome-specific alphoid probes. For example, Fig. 4 shows the result of CISS hybridization of the c-*raf-1* YAC probe A168H4 to normal lymphocyte nuclei. Table 2 shows the results of interphase cytogenetics performed with the seven band-specific YAC clones both in normal lymphocytes and in cells with the specific structural aberrations described above. These data were fully consistent with the conclusions obtained by the analysis of metaphase spreads. Interphase nuclei carrying the t(3;8)(p14.2;q24) translocation showed two signals after CISS hybridization with the c-*raf-1* YAC clone A168H4. Additional painting of chromosome 3 showed large and small domains both carrying a c-*raf-1* YAC-specific signal, while a third medium-sized domain did not (Fig. 3*I*). CISS hybridization of the c-*fms* YAC clone D73A7 to lymphocyte interphase nuclei of three patients with 5q− syndrome showed one distinct green signal only in most nuclei (Table 2), which was located within one of the two red-painted chromosome 5 domains (compare Fig. 3, *B* and *C*). Interestingly, Patient 1 showed only 2 of 200 interphase nuclei with a normal painting pattern; *i.e.*, two chromosome 5 domains both carrying the c-*fms* YAC clone signal, while 24% and 35% normal nuclei, respectively, were observed in Patients 2 and 3. For comparison, Patient 1 showed only metaphase spreads carrying the del(5q) ($n = 34$), while Patient 2 and Patient 3 showed 9% ($n = 46$) and 34% ($n = 21$) normal metaphase spreads. The differences between the percentages of normal and aberrant metaphase spreads and interphase nuclei in Patient 2 suggest that metaphase cell populations are not necessarily representative of the heterogeneity of normal and tumor cells present in the interphase cell population. CISS hybridization of the *BCR* YAC clone D107F9 to tumor interphase nuclei of a patient with CML showed that most nuclei contained three signals, as expected from a clone overlapping the breakpoint region in 22q11 (Fig. 3*E*).

DISCUSSION

In this study we have screened a human YAC library for YAC clones containing the *BCR* gene and the protooncogenes c-*raf-1*, c-*fms*, and c-*erbB-2*. Seven YAC clones contained sequences from these genes and showed signals exclusively within the bands to which the respective genes have been previously mapped. The localization of the two c-*erbB-2* clones could be

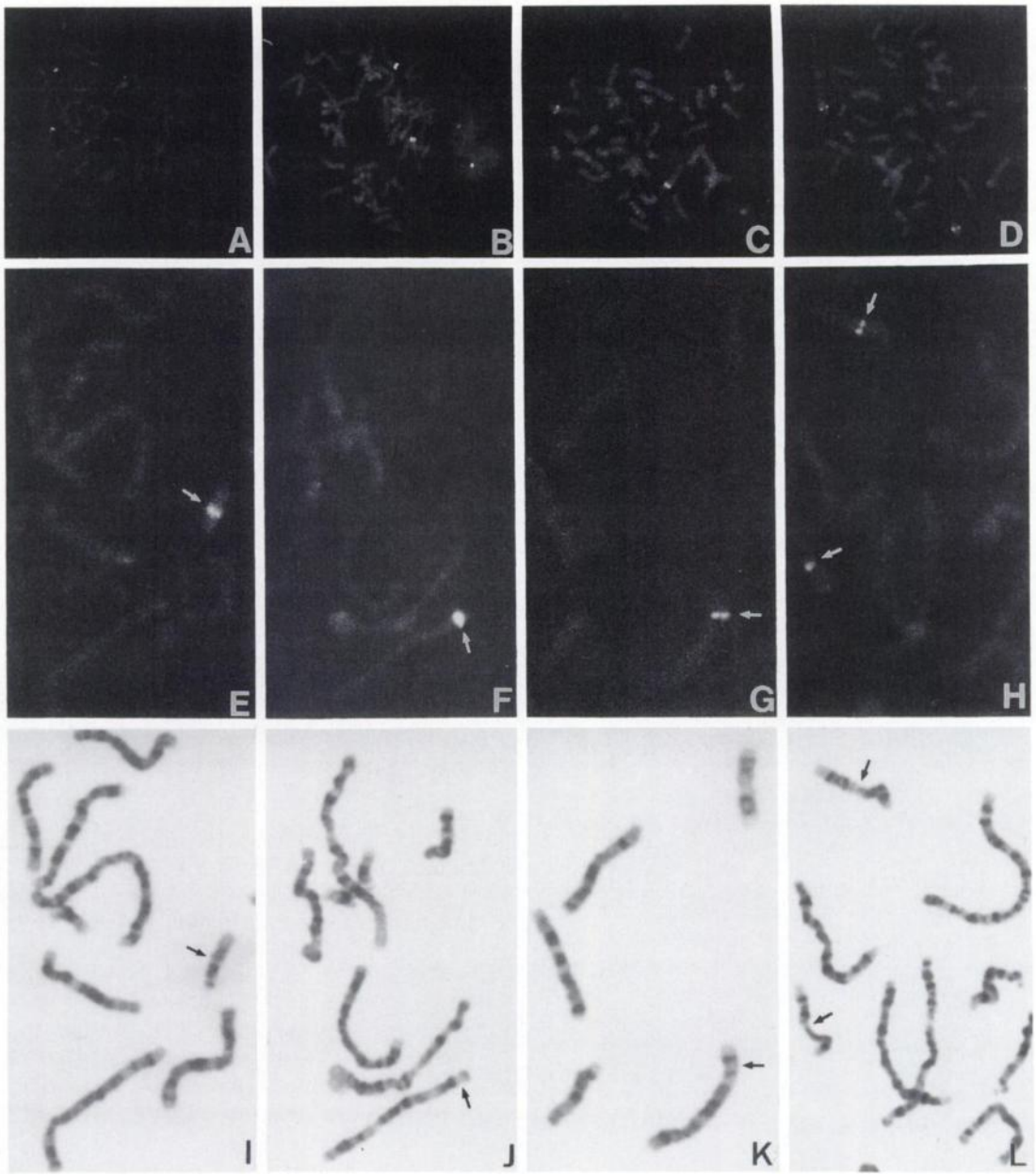

Fig. 2. Whole metaphase spreads after CISS hybridization of Alu-PCR-amplified probes from the *BCR* YAC D107F9 (*A*), the c-*raf-1* YAC A168H4 (*B*), the c-*fms* YAC D73A7 (*C*), and the c-*erbB-2* YAC D122A5 (*D*). Biotinylated probes were detected with avidin-FITC. Chromosomes were counterstained with propidium iodide. *E* to *L*, high-resolution mapping of Alu-PCR-amplified YAC clones. Prometaphase chromosomes were GTG banded (Fig. 3, *I* to *L*) and used for CISS hybridization after destaining. Biotin-labeled probes were detected with avidin-FITC. Chromosomes were counterstained with propidium iodide (Fig. 3, *E* to *H*). *E* and *I*, partial prometaphase with chromosome 22 indicating the localization of the *BCR* YAC clone D107F9 on 22q11 (*arrow*). *F* and *J*, partial prometaphase with chromosome 3 indicating the localization of the c-*raf-1* YAC clone A168H4 on 3p25 (*arrow*). *G* and *K*, partial prometaphase with chromosome 5 indicating the localization of the c-*fms* YAC clone D73A7 on 5q33 (*arrow*). *H* and *L*, partial prometaphase with two chromosomes 17 indicating the localization of the c-*erbB-2* YAC clone A230A7 on 17q12–21.1 (*arrows*).

narrowed down to 17q12–q21.1. The signals obtained with Alu-PCR-amplified YAC clones in metaphase spreads and interphase nuclei are clearly superior to signals which can generally be obtained with individual bacteriophage or cosmid clones. These strong signals, combined with a low background, make these probes ideal for tumor interphase cytogenetics and allow analyses using conventional fluorescence microscopy.

Recently, it has been demonstrated that interphase cytogenetics can also be applied to paraffin-embedded sections from solid tumors (36–38). We are presently exploiting the possibilities of using the c-*raf-1* YAC clones to diagnose deletions of 3p in several solid tumors, such as renal cell carcinomas (39) and small cell carcinomas of the lung (40). In addition, the c-*erbB-2* YACs may provide a useful tool to study the possible amplification of this region in patients with breast cancer (41, 42).

As demonstrated for the two *BCR* YAC clones in the case of CML and ALL cells with t(9;22)(q34;q11), small populations of interphase tumor cells carrying a specific translocation can be easily distinguished from normal interphase cell populations with YAC clones spanning the breakpoint region of interest. Similarly, tumor cell populations carrying a specific deletion can be discriminated from normal cells as exemplified for the c-*fms* YAC clone in several cases with the 5q− syndrome. The ability to diagnose specific chromosome aberrations at the single cell level makes interphase cytogenetics particularly useful to study heterogeneous cell populations. The unequivocal classification of individual nuclei, however, may be complicated

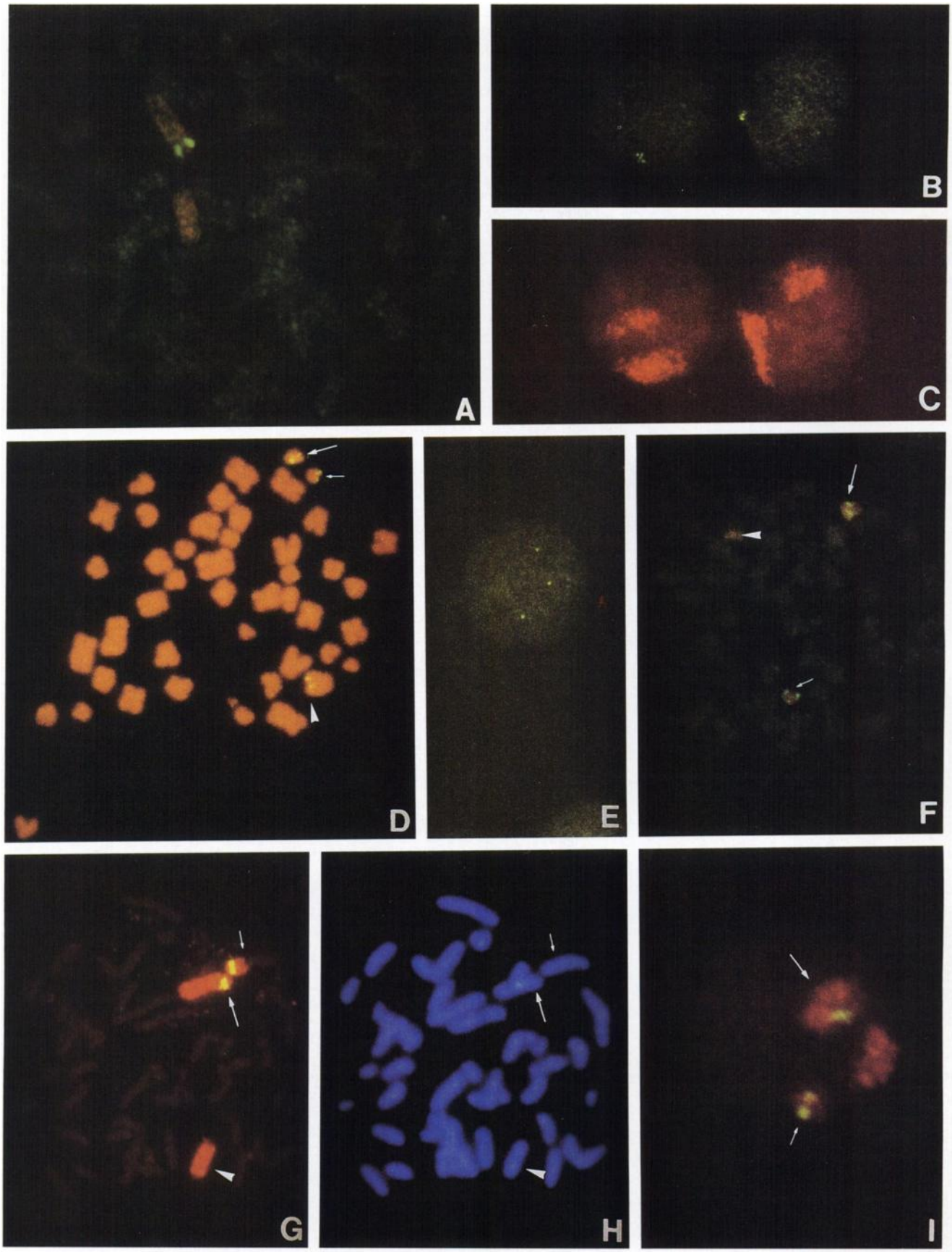

Fig. 3. Metaphase spread (*A*) and interphase nuclei (*B* and *C*) of a patient with 5q− syndrome after two-color CISS hybridization with a biotinylated Alu-PCR-amplified probe of c-*fms* YAC clone D73A7 (detection with avidin-FITC, green) and with a digoxigenin-labeled bacteriophage library from sorted human chromosomes 5 (detection with TRITC-conjugated antibodies, red). In *A*, the normal chromosome 5 shows strong specific labeling with the c-*fms* YAC on both chromatids, while the signal is missing on the deleted chromosome 5. In *B*, two nuclei exhibit one signal of the c-*fms* YAC clone only. In *C*, the same nuclei show two chromosome 5 domains. Note that the c-*fms* signal shown in *B* colocalizes with one domain. Metaphase spread (*D*) and interphase nucleus (*E*) of a patient with CML exhibiting the

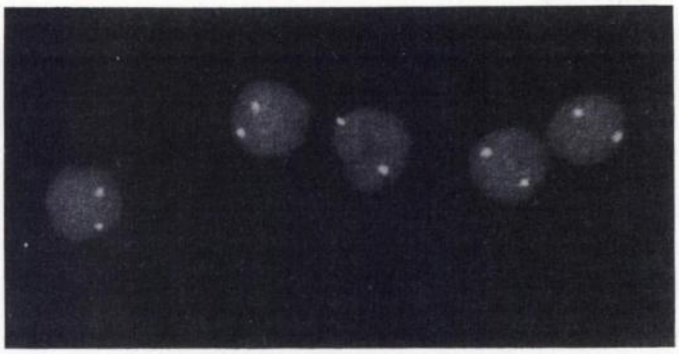

Fig. 4. Five normal lymphocyte nuclei with two signals each obtained after CISS hybridization with the c-*raf-1* YAC A168H4. Alu-PCR-generated, biotinylated probes were detected with avidin-FITC. Nuclei were counterstained with propidium iodide.

Table 2 *Results of interphase cytogenetics performed with the seven band-specific YAC clones*

The number of evaluated nuclei was 200 to 400.

YAC clone	Chromosome status	No. of signals in interphase nuclei (%)				
		0	1	2	3	4
c-*raf-1*						
D107B4	Normal diploid	1	1	97	1	0
A168H4	Normal diploid	0	3	97	0	0
A168H4	t(3;8)	0	2	95	2	1
c-*erbB-2*						
D122A5	Normal diploid	1	3	86	10	0
A230A7	Normal diploid	1	8	89	2	0
A230A7	t(15;17)	1	7	91	1	0
c-*fms*						
D73A7	Normal diploid	1	3	93	3	0
D73A7	del(5q); Patient 1	1	98	1	0	0
D73A7	del(5q); Patient 2	0	76	24	0	0
D73A7	del(5q); Patient 3	0	65	35	0	0
BCR						
B99E11	Normal diploid	0	5	93	2	0
D107F9	Normal diploid	1	3	90	6	0
D107F9	t(9;22)	2	6	28	62	2

by incomplete hybridization of a fraction of nuclei resulting in an underestimation of the true number of specific targets present in these cells (false negatives) or by artifactual fluorescent spots in nuclei which are counted as true signals (false positives). In cell populations containing both normal cells and tumor cells, false negatives would simply result in an underestimate of the fraction of tumor cells. A definitive decision, however, of whether or not the cell population would contain tumor cells could still be reached by a sufficiently large sample size. In contrast, false positives could lead to the erroneous diagnosis that a small percentage of tumor cells were still present within a large majority of normal cells. A strategy to discriminate artifactual from true signals could be based on multiple-color *in situ* hybridization experiments (8, 43, 44). For example, the discrimination of background spots from true hybridization signals can be facilitated by simultaneous painting of the target chromosome to which the respective YAC clone maps. In this case, interphase signals are counted only if they are found within the corresponding chromosome domain. Alternatively, false positives could be avoided by applying multiple-color *in situ* hybridization of probes which span the breakpoint of interest and flank it on both sites (8). Obviously, the chance of side-by-side localization of differently colored artifactual spots should be a rare event. Evaluation of such multiple-color *in situ* hybridization experiments will be very much facilitated by recent developments in digital fluorescence microscopy (45). In particular, the intriguing prospects of the automated evaluation of cells with specifically painted chromosome aberrations using digital image analysis could result in a tremendous reduction of the human workload (46). Direct labeling of DNA probes with fluorochromes has recently become possible and will further facilitate the application of multiple-color *in situ* hybridization (8, 43).

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translocation t(9;22)(q34;q11) after CISS hybridization with the biotinylated Alu-PCR-amplified *BCR* YAC D107F9 detected with avidin-FITC. In *D*, note signals on both chromatids of the normal chromosome 22 (*large arrow*), the Ph^1 chromosome (*small arrow*), and the derivative chromosome 9 (9pter→9q34::22q11→22qter) (*arrowhead*). Chromosomes are counterstained with propidium iodide. In *E*, in correspondence with the result obtained in the metaphase spreads, the interphase nucleus clearly exhibits three signals. *F*, metaphase spread with a translocation t(15;17)(q22;q21) obtained from a patient with APL after two-color CISS hybridization with the biotinylated Alu-PCR-amplified c-*erbB-2* YAC A230A7 (green signals) and with a digoxigenin-labeled plasmid library from sorted human chromosomes 17 (red). Signals from the c-*erbB-2* YAC can be observed on both chromatids of the normal chromosome 17 (*large arrow*) and the derivative chromosome 17 (*small arrow*). The *arrowhead* points to the translocated chromosome 17 material on the derivative chromosome 15. Metaphase spread (*G*) and interphase nucleus (*I*) of a proband carrying the constitutional translocation t(3;8)(p14.2;q24) after two-color CISS hybridization with the biotinylated Alu-PCR-amplified c-*raf-1* YAC A168H4 (green signals) and with a digoxigenin-labeled bacteriophage library from sorted human chromosomes 3 (red). Signals of the c-*raf* YAC clone can be observed on both chromatids of the normal chromosome 3 (*large arrow*) and the derivative chromosome 8 together with the translocated chromosome 3 material (*small arrow*). In the derivative chromosome 3, the chromosome 3 material (3p14.2→3qter) is painted red (*arrowhead*). *H*, DAPI picture of the same metaphase as shown in *G*. In *I*, the corresponding interphase nucleus is weakly outlined by background red fluorescence and shows three bright red domains representing the normal chromosome 3 and the two translocation chromosomes with chromosome 3 material. Note that only the large normal chromosome 3 domain (*large arrow*) and the small domain (3p14.2→3pter) contained in the derivative chromosome 8 (*small arrow*) show colocalization with green c-*raf-1* signals.

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