

ORIGINAL INVESTIGATION

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Toward a multicolor chromosome bar code for the entire human karyotype by fluorescence in situ hybridization

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Abstract A colored banding pattern for human chromosomes is described that distinguishes each chromosome in a single fluorescence in situ hybridization with a set of subregional DNA probes. Alu/polymerase chain reaction products of various human/rodent somatic cell hybrids (fragment hybrids) were pooled into two probe sets that were labeled differentially and detected by red and green fluorescence. Chromosome regions hybridized by DNA present in both pools appeared yellow. The result was a multi-color set of 110 distinct signals per haploid chromosome set for the human karyotype. Each individual chromosome showed a unique sequence of signals, a result termed the “chromosome bar code”. The reproducibility of the hybridization pattern in various labeling and hybridization experiments was analyzed by computer densitometry. We have applied the chromosome bar code both in diagnostic cytogenetics and in genome studies. The approach allows the rapid identification of chromosomes and chromosome rearrangements. Although not yet showing the resolution of classical banding patterns, the present experiments demonstrate various applications in which the present multi-color bar code can significantly add to the spectrum of cytogenetic techniques.

Introduction

The introduction of in situ hybridization techniques has added significantly to the description of the human karyotype by defining chromosome regions at the molecular level. Recently, in order to simplify chromosome identification, various strategies have been proposed for adding

color information to the classical gray-scale description of chromosome banding. Whole chromosome painting (Pinkel et al. 1988; Lichter et al. 1988; Cremer et al. 1988) can define the gross DNA content of an entire chromosome. This method has also been performed in a multi-color format by labeling probes with different haplotypes in a simple Boolean fashion (Nederlof et al. 1989, 1990; Ried et al. 1992) or by including ratios of differentially labeled probes (Nederlof et al. 1992). Recent studies have successfully demonstrated that each member of the entire chromosome set can be simultaneously distinguished by using different combinations of fluorescent labels (Schröck et al. 1996; Speicher et al. 1996). An alternative approach has been used to label each chromosome by subregional DNA probes in different colors (Lengauer et al. 1992). This pattern has been named the “chromosome bar code” and has simplified chromosome identification by producing a limited number of bars on each chromosome.

Most karyotype analyses, however, still largely rely on the correct interpretation of classical banding patterns, since the new strategies only allow the identification of whole chromosomes without further differentiation of chromosomal subregions. In a different approach, fluorescence in situ hybridization (FISH) with subregional probes has been applied to generate a multi-color banding pattern on a single chromosome pair, so that intrachromosomal rearrangements can be detected (Ried et al. 1992; Lengauer et al. 1993).

Here, we present a novel approach (outlined in Fig. 1) that combines simple chromosome identification and subregional definition of chromosomes with molecular probes in a single hybridization. As Alu repeats are confined to primate DNA, we have used Alu/polymerase chain reaction (Alu-PCR)-generated probes from highly rearranged human/rodent somatic cell hybrids (fragment hybrids) containing up to 15 human chromosome fragments, each ranging in size between approximately 5 Mb and whole chromosome arms, from a panel of cell lines representing over 300 regions of the human genome (Antonacci et al. 1995).

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Fig. 1 Diagram outlining the source of the probes and their preparation for generating the chromosome bar code

1. Preparation of genomic DNA from 11 chosen *fragment hybrid* cell lines, using Alu-PCR for specific amplification of human DNA

19 53 54 78 85 65 56 69 77 75 79

2. Alu-PCR products were split into two pools for differential labeling with two haptens (Biotin-dUTP and Digoxigenin-dUTP) by nick-translation

19 53 78 56 69 75
54 85 65 77 79

3. Precipitation, hybridization and detection of the multiplex probe with the appropriate antibodies (with Avidin-FITC and Sheep-anti-Digoxigenin-Rhodamine)

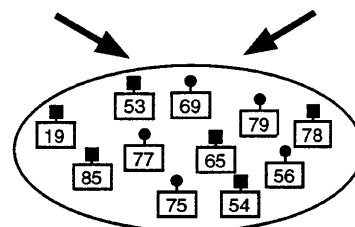
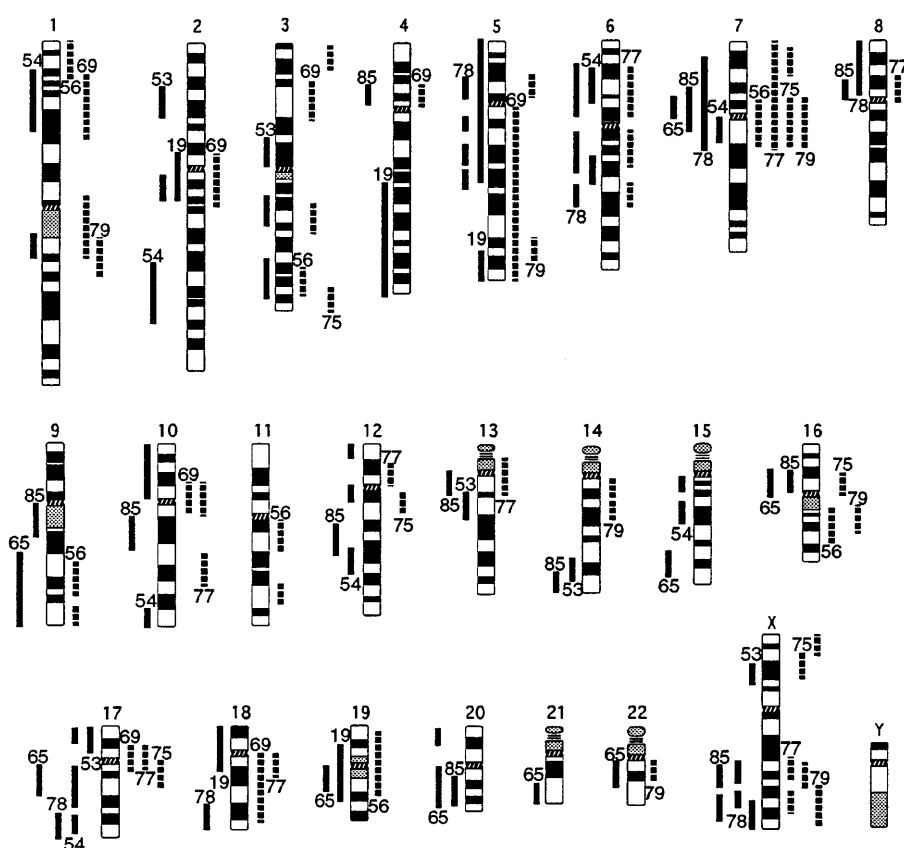


Fig. 2 Summary of the theoretically expected signal composition on human chromosomes, according to Antonacci et al. (1995). Pool 1 was biotin-labeled, is represented by *black bars*, and consists of Alu-PCR products derived from cell lines H-A3 [53], H-B3 [54], H-D3 [65], HY 92F4 [19], Lia 21L [85], and Lia 10L [78]. Pool 2 was digoxigenin-labeled, is represented by *hatched bars*, and is composed of Alu-PCR products from H-B5 [56], H-F4 [69], Lia 4L [75], Lia 7L [77], and Lia 11L [79]



Materials and methods

Cell samples, tissue culture, and chromosome preparation

Metaphase spreads were prepared from normal male blood lymphocytes. Established cell lines were also used: lymphoblastoid cell lines of a normal male and clinical cases (Table 1), a chimpanzee (*Pan troglodytes*; EB(JC)176, ECACC no. 89072704), a gorilla (*Gorilla gorilla*; EB(JC), ECACC no. 89072703), an orangutan of the Bornean subspecies (*Pongo pygmaeus*; EB(JC)185, ECACC no. 89072705), a silver leaf monkey (*Presbytis cristata*), fibroblast cell lines established from a human/hamster somatic hybrid cell line (HY B11T9 [39], Antonacci et al. 1995), pigtailed macaque (*Macaca nemestrina*), and African Green monkey (*Cercopithecus aethiops*). Old World monkey cell lines were as de-

scribed previously (Bigoni et al. 1996; P. Finelli et al. in preparation). Lymphocyte culture followed standard procedures. Metaphase chromosome spreads for in situ hybridization were prepared according to standard protocols.

DNA probes

Alu-PCR products from fragment hybrids (Antonacci et al. 1995) were pooled to give two probes. Pool 1 consisted of PCR products derived from cell lines H-A3 [53], H-B3 [54], H-D3 [65], HY 92F4 [19], Lia 21L [85], and Lia 10L [78]. Pool 2 comprised PCR products from H-B5 [56], H-F4 [69], Lia 4L [75], Lia 7L [77], and Lia 11L [79]. A detailed description of the human chromosome fragment content of each pool is given in Fig. 2. Yeast artificial

chromosome (YAC) 961f12 was obtained from the Centre d'Etude du Polymorphisme Humain (CEPH) Mega YAC library and maps to chromosome 3p14–21 (Bray-Ward et al. 1996).

Alu-PCR amplification, probe labeling, and hybridization

Alu-PCR was performed as described (Lengauer et al. 1992) for the specific amplification of human DNA retained in the hybrid cell lines. The Alu-PCR products were labeled by nick translation according to a standard protocol; those products chosen for pool 1 were labeled with biotin-dUTP, those for pool 2 with digoxigenin-

dUTP. For in situ hybridization, we pooled 200 ng labeled Alu-PCR product from each cell line, 10 μ g Cot-1 DNA, and 50 μ g salmon testis DNA. The DNA was precipitated and resuspended in 15 μ l hybridization mixture yielding a final concentration of 50% deionized formamide, 10% dextran sulfate, and 1 \times SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0). Slides were denatured in 70% formamide/2 \times SSC at 68°C for 1 min. The probe was denatured at 80°C for 5 min and applied to the dehydrated slides, which were then sealed with a coverslip. The probe mix was hybridized overnight. Post-hybridization washes included 2 \times 5 min in 50% formamide/1 \times SSC at 45°C and 2 \times 5 min in 2 \times SSC at 45°C. Reporter molecules were detected with avidin/fluorescein

Fig. 3 **a** A human metaphase after hybridization and detection of the multiplex probe. The pattern consists of *red and green bars* for fragments represented only in one probe pool, *yellow bars* for fragments present in equal amount in both pools, and bars of defined *mixed colors* derived from fragments overrepresented in the other pool. Chromosomal regions not present in either pool show the *blue* (DAPI) counterstain. **b** Karyotype of the metaphase shown in **a**. **c** The mean *red, green, and blue* fluorescence profiles of all human chromosomes were established on the basis of computer densitometric analysis of 20 metaphases and transferred to a colored idiogram

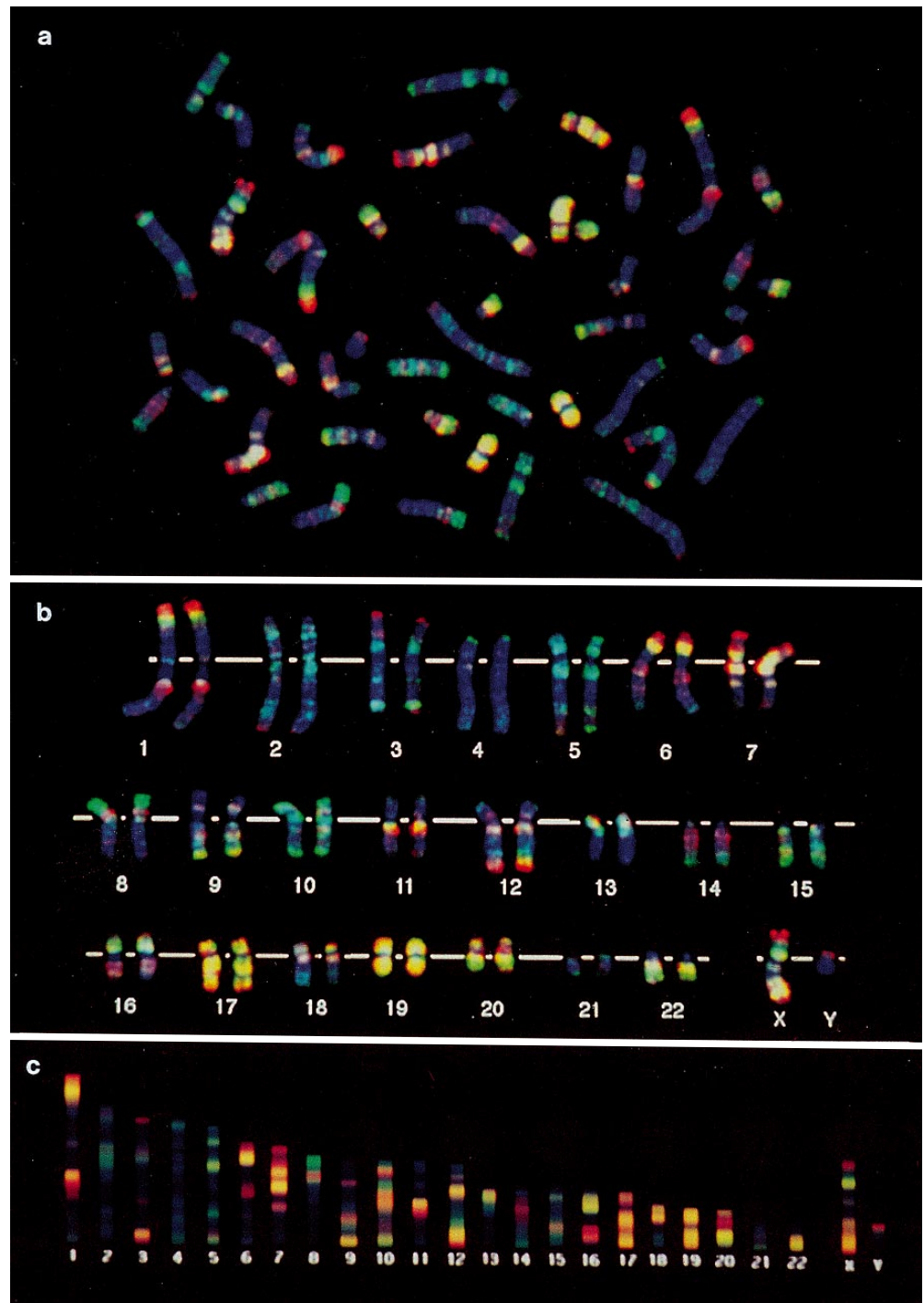
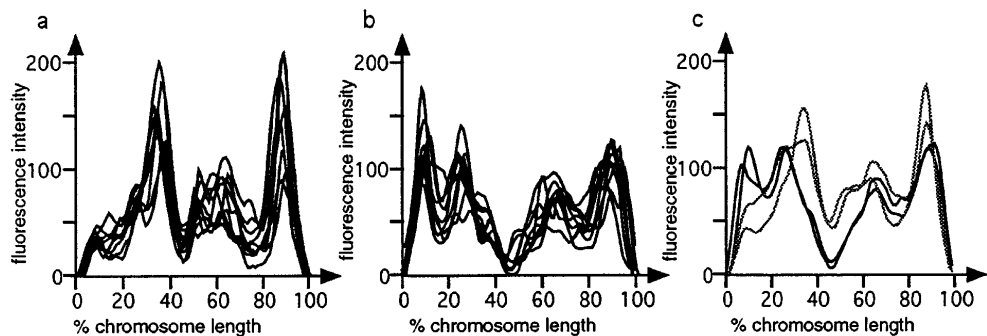


Fig. 4 Computer densitometric analysis of the observed signal composition on human chromosome 3. The green (a) and red (b) fluorescence profiles observed for 15 chromosomes 3 in one experiment are shown. c A comparison of mean red (solid lines) and green (dotted lines) fluorescence profiles observed on each of the 15 chromosomes 3 derived from two independent Alu-PCR labeling and hybridization experiments



isothiocyanate (avidin-FITC) and a sheep anti-digoxigenin antibody coupled to rhodamine. In the experiment mapping both a YAC and the color bar code probe chromosomes, pool 1 was labeled with digoxigenin-dUTP and detected with a sheep anti-digoxigenin antibody coupled to FITC, whereas pool 2 was labeled with Cy3-dUTP. The YAC was labeled with biotin-dUTP and detected with avidin-Cy5.

Microscopy and image analysis

Metaphases were analyzed with a cooled charge-coupled device camera (Photometrics CH250/A equipped with a KAF1400 chip) attached to the microscope. Camera control and digital image acquisition was as described by Ried et al. (1992). Merging of chromosome images were performed by using the software Adobe Photoshop 3.0. Computer densitometric analysis of the fluorescence profiles was performed with modified IP Lab/Smart Capture extensions, kindly provided by Digital Scientific, Cambridge, UK.

Results

The multiplex probe produces a set of about 110 distinct colored bars unique for each human autosome and for the sex chromosomes. The pattern observed consists of red and green bars for fragments represented only in one probe pool, yellow bars for fragments present in equal amount in both pools, and bars of defined mixed colours derived from fragments overrepresented in the other pool. A human metaphase after detection of the probes with red and green fluorescence is shown in Fig. 3a. Chromosomal regions not present in either pool show the blue (4, 6 di-amidino-2-phenylindole; DAPI) counterstain. The probe set chosen covers approximately 40% of the human genome. The same metaphase with chromosomes karyotyped is given in Fig. 3b. Karyotyping followed the system based on classical banding as provided by the contrast-enhanced DAPI pattern (not shown).

The reproducibility of this artificially generated pattern was verified by computer densitometry and analysis of the fluorescence intensity profiles. Analysis of each of the 10 metaphases derived from two independent Alu-PCR labeling and hybridization experiments showed no obvious differences in the observed fluorescence profiles. As an example, the individual and the mean density profiles of chromosome 3 are shown in Fig. 4.

On the basis of these data, the means of red, green, and blue fluorescence profiles of all 24 different human chro-

somes based on 20 metaphases were established and transferred to a colored idiogram (Fig. 3c). Both the mean hybridization profiles (not shown) and the colored idiogram show a unique pattern for each chromosome. Intensities and location of hybridization signals in most chromosome subregions agreed with the expected values based on mapping positions described in detail above (Fig. 2; Antonacci et al. 1995).

The potential of the "multi-color bar code" established from the present probe pools as a screening method for chromosomal aberrations was explored by analyzing clinical samples. The bar code was also used in various applications in chromosome mapping and comparative genome analysis.

Diagnostic applications in clinical cytogenetics

We analyzed 10 clinical cases with aneuploidies, balanced translocations, inversions, and duplications, where conventional banding methods or FISH with chromosome painting probes had been previously performed. The hybridization of the bar code probe was performed without

Fig. 5 a The bar code probe hybridized to a metaphase of a clinical case in which G-banding analysis revealed additional material on chromosome 13qter. *Arrows* The normal and the derived chromosomes 13 (enlarged in the *box*). Bar coding excluded chromosome 13 as the origin of the chromosomal material and narrowed down the possible sites of origin to chromosome bands 1p33–35, 9q34, 16p, 17, 19, 20q, or 22q. b Chromosome bar coding as a means of chromosome identification to map simultaneously a DNA probe (YAC clone 961f12) labeled with a third hapten. c The same metaphase, false colored, with counterstain and the YAC hybridization only. d The single human chromosome present in a human/hamster somatic cell hybrid (HY B11T9) was identified (*arrow*) by the bar code probe on metaphase chromosomes from the somatic hybrid cell line. *Inset* This chromosome is a translocation product of human chromosome arms 9q and Xp (*left to right* the human chromosome 9, the human chromosome retained in the hybrid cell line, and the human X chromosome). e A typical gorilla metaphase after FISH with the bar code probe. f Homologous human (*left*) and gorilla (*right*) chromosomes showing rearrangements. *Arrowheads* Positions of centromeres. g Human chromosome 3 homologs were studied in the great apes (*PTR Pan troglodytes*, *GGO Gorilla gorilla*, *PPY Pongo pygmaeus*) and Old World monkeys (*PCR Presbytis cristata*, *MNE Macaca nemestrina*, *CAE Cercopithecus aethiops*)

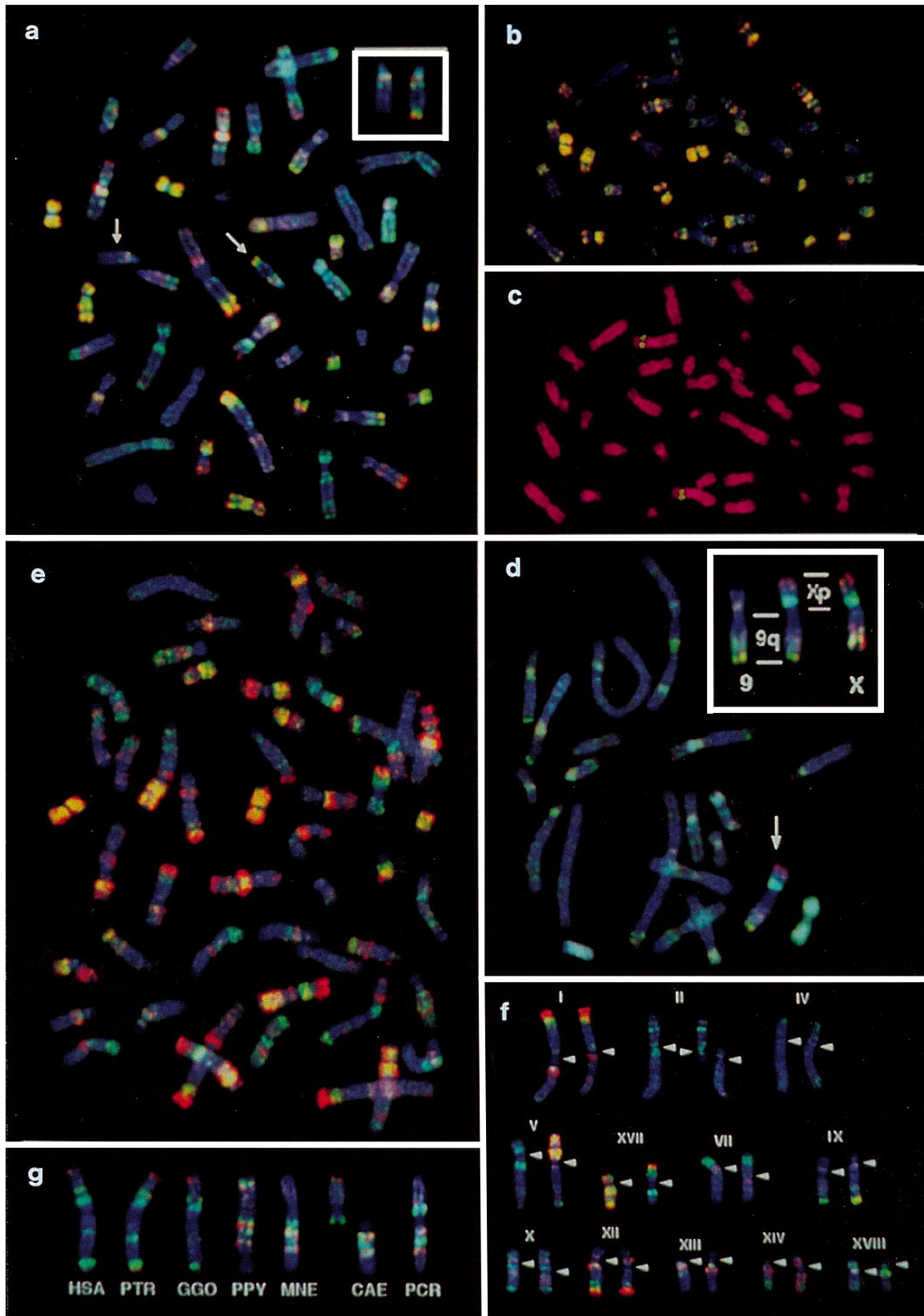


Table 1 Comparison of chromosome banding and bar code results from 10 patients with chromosome abnormalities. For the bar code experiments, band assignments were obtained from DAPI patterns. Bar coding provided more information in patients bo915, WA, and bo769. For bo769, who possessed an unbalanced chromosome aberration, bar coding narrowed down the origin of additional chromosomal bands to 1p33–35, 9q34, 16p, 17, 19, 20q, or

22q. Subsequent painting experiments revealed a translocation t(13;19)(q33;p13), whereas G-banding analysis of a relative with the balanced translocation revealed a der(13)t(13;19)(q34;p13.2). In all other cases, bar coding detected specific rearrangements. However, because of poor differentiation with the present probe pools in some chromosome regions, G-banding gave more information than bar coding in cases bo663, PB, and bo737

Case	Result: G-banding	Result: bar coding
GB	47, XY, +21	47, XY, +21
JM	48, XXXX	48, XXXX
bo 663	46, XY, inv (5)(p13.3;q13.3), add (3)(q26)	46, XY, inv (5)(p14;q13.3)
OJ	46, XX, inv (2)(p23;q13)	46, XX, inv (2)(p23;q13)
bo 706	46, XX, add (12)(q24.2)	46, XX, add (12)(q24.2)
bo 915	46, XY, 2p+	46, XY, add (2)(p12–p15)
WA	46, XX, t(8;18)(?q11.2;?q12.2)	46, XX, t(8;18)(q12;q12.1)
bo 769	46, XY, 13q+	46, XY, t(13;19?) see figure 5a
PB	46, XX, t(6;13)(q25.3;q22)	46, XX, 6qter+
bo 737	46, XY, t(1;22)(p36;p11.2)	46, XY, 1pter+

prior knowledge of the chromosomal aberrations. In all cases, chromosome anomalies were readily detected. Most of the results were consistent with previous G-banding analysis. In three cases, the bar code probe provided a more complete diagnosis of chromosomal aberrations that was then confirmed by chromosome painting analysis. A case in which G-banding analysis revealed additional material on chromosome 13qter was interpreted as a duplication (Fig. 5a). Bar coding excluded chromosome 13 as the origin and narrowed down possible sites of origin to chromosome bands 1p33–35, 9q34, 16p, 17, 19, 20q, or 22q. Subsequent painting experiments revealed a translocation t(13;19). This was confirmed by G-banding analysis of a relative with the balanced translocation (Table 1).

DNA probe mapping

We used the multi-color bar code to map simultaneously a further DNA probe (YAC clone 961f12) labeled with a third hapten and visualized in the near-infrared. Because of higher non-specific background caused by Cy3-dUTP-labeled DNA probes, the colored pattern was slightly different from the original labeling in previous experiments. However, as before, each chromosome exhibited a unique and highly specific bar code (Fig. 5b). The hybridization signal of the YAC was readily identified and assigned to chromosome 3 (Fig. 5c). Fractional length measurement ranged between $Fl_{\text{pter}_{\min}} = 28$ and $Fl_{\text{pter}_{\max}} = 32$ corresponding to chromosome band 3p14–p21, in agreement with the previous published assignment of this clone (Bray-Ward et al. 1996).

Somatic cell hybrid genetics

The human chromosome content in a human/hamster somatic cell hybrid (HY B11T9) was analyzed by using the

bar code probe on metaphase chromosomes from the somatic hybrid cell line (Fig. 5d). The bar code probe identified one chromosome of human origin. Further analysis of the colored “bars” allowed us to identify this chromosome as a translocation of the short arm of the human X chromosome and the long arm of chromosome 9 to a single translocation product. This interpretation was consistent with the previous description of this cell line (Antonacci et al. 1995).

Comparative cytogenetics

The human bar code probe allowed us readily to identify all gorilla (*Gorilla gorilla*) homologous chromosomes. This was not trivial, since various gorilla chromosomes exhibit chromosome rearrangements when compared with the human karyotype. A typical gorilla metaphase after in situ hybridization is shown in Fig. 5e. The rearrangements that distinguish human and gorilla chromosomes are highlighted in Fig. 5f. All pericentric and paracentric inversions on gorilla chromosomes homologous to human chromosomes proposed by classical banding techniques (Dutrillaux 1979; Yunis and Prakash 1982) were confirmed by the bar code probe with the exception of inv(VII)(q21.1;q22.2). This region was poorly differentiated with the bar code probe. The two chromosomes homologous to human chromosome 2p and 2q, and the reciprocal translocation between chromosomes homologous to human chromosomes 5 and 17 (Stanyon et al. 1992) could also be verified by the bar code probe, as could the size difference of the chromosome 1q12 heterochromatic block. In addition to previously known rearrangements, an as yet undescribed inversion was observed (inv(XIII)(q12;q13)).

The evolution of changes in human chromosome 3 homologs was studied in the great apes (*Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*; Fig. 5g) and Old World monkeys (*Presbytis cristata*, *Macaca nemestrina*, *Cerco-*

pithecus aethiops; Fig. 5g). The bar code hybridization pattern on the human and African great ape homologs appear to be identical. However, the orangutan shows a pattern resembling the hybridization on Old World monkeys. The hybridization pattern also suggests that the homologs of the macaque and the African green monkey differ only by a fission, and the homologs of the macaque and the langur by a pericentric inversion.

Discussion

Chromosome identification is important in various experimental applications but is often difficult following in situ hybridization experiments. Enhanced DAPI staining of chromosomes provides a clear G-banding pattern. Alternatively, probes are co-hybridized with Alu-sequences (Baldini and Ward, 1991; Matera and Ward 1992), or chromosomes can be stained with classical banding techniques before or after in situ hybridization (Arnold et al. 1992). However, these classical banding patterns only provide gray-value differences for chromosome identification. The color information provided by the present probe set simplifies chromosome identification, so that it can be performed even by untrained personnel or can be automated with high performance. Idiograms presenting the rough values for the mean hybridization sites (Fig. 3c) can assist in rapid chromosome identification.

A two hapten hybridization was chosen for the present experiments for simplicity and reliability. Visualization of the probes in red and green together with the blue counterstain allowed the merging of the fluorescent signals in a simple RGB format. Most commercial image-processing computer software and software recently developed specifically for FISH applications can perform this task.

Computer densitometry of a set of metaphases hybridized with the bar code probe revealed consistent results, not only within, but also between different experiments. As is evident from Figs. 2 and 3, not all chromosome segments identified in the original analysis of the single hybrid cell lines appear in the hybridization pattern of the two pools of the bar code probe. For example, the green signal observed on chromosome 4p16 is not represented in the map obtained from the previous analysis of the single hybrid cell lines, whereas the 5q fragment from cell line H-F4, which was expected to yield a red signal, was lost. This is because of the variable copy number of some chromosome fragments in somatic cell hybrids and the loss of others. A more general use of this bar code probe would therefore require a more stable source from a DNA library established for each probe pool, which can be recovered repeatedly. Cloning of flow-sorted chromosomes (Collins et al. 1991) and microdissection fragments (Trautman et al. 1991), and repeated DNA preparations from these libraries has shown that the probes can be kept sufficiently complex to provide a permanent resource for FISH experiments.

Bar coding allows simplified karyotyping for diagnostic applications in clinical cytogenetics. The origin of

chromosome rearrangements and aneuploidies has been readily established in this study. The present experiments also provide a highly efficient protocol for large-scale chromosome mapping of DNA probes on the human karyotype. The hybridization signal of the YAC to be mapped was analyzed in the near-infrared by a Cy5-coupled reporter molecule. This allowed the probe to be analyzed in a different color "channel" than the bar code display. The distinct pattern of the bar code on each chromosome suggests that this approach can be used to identify isolated chromosomes. An example is the analysis of somatic cell hybrids containing a few human chromosomes or chromosome fragments. Striking evidence for the reliability of the technique has been demonstrated in the identification of human chromosome homology in primate metaphase preparations (Fig. 3e–g). Subregional mapping is laborious, and classical banding has often proved to be misleading in comparative cytogenetics. Our experiments show that a bar code using human probes on non-human chromosomes should provide useful additional pattern information. Even under conditions of several chromosome rearrangements, we were able to identify all gorilla homologs. Moreover, Old World monkey chromosomes displayed a specific bar code pattern that allowed intrachromosomal rearrangements to be defined. Most chromosome rearrangements known from previous banding studies were evident from the hybridization pattern. In addition, the analysis revealed a rearrangement not yet reported for the gorilla and provided a more complete overview of the evolution of the human chromosome 3 homologs in the great apes and Old World monkeys. The multi-color bar code can be used to guide further fine mapping studies in comparative cytogenetics, whereas specific subregional probes, such as YACs or microdissection paints flanking or spanning the proposed breakpoints, should verify these results.

The chromosome bar code complements, but cannot replace, classical chromosome banding at this stage. Our experiments, however, demonstrate the power of introducing color information into chromosome identification and into molecular definition by coloring chromosome subregions. As previously suggested (Nederlof et al. 1989, 1990, 1992; Lengauer et al. 1993; Ried et al. 1992), in situ hybridization of defined DNA probes should allow the design of color banding appropriate to a particular application. This makes banding patterns with molecular probes entirely different from any classical chromosome banding patterns, which are, in general, either G-banding or R-banding or variations thereof. The resolution of the bar code pattern can be increased in the future by adding other bars and combining other probe sets. This is especially true for chromosomes 2q, 4, 7q, and 8q, which to date show a poorly differentiated pattern with the probe pools currently used. Resolution can be increased further by adding YACs or band-specific microdissection probes, especially telomere-specific probes for the efficient detection of cryptic translocations (NIH and IMMC Collaboration 1996) This should allow various other multi-color bar codes far beyond the classical G-band and R-band spectrum.

Recent developments in multicolor FISH indicate that color information can be increased to seven or even more distinct subdivisions of the spectrum (Schröck et al. 1996, Speicher et al. 1996). These approaches should significantly increase the color differentiation shown in our present experiments, since the origin of the "bars" can be defined more precisely. However, although our method does not yet yield the resolution of classical banding patterns, our experiments demonstrate various applications where the present multi-color bar code can add significantly to cytogenetic methods.

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