The EMBO Journal vol.7 no.9 pp.2801 - 2805, 1988

Sensitive and high resolution *in situ* hybridization to human chromosomes using biotin labelled probes: assignment of the human thymocyte CD1 antigen genes to chromosome 1

Donna G.Albertson[1,3], Rita Fishpool[1], Paul Sherrington[2], Elisabeth Nacheva[2,4] and Cesar Milstein[1]

[1]Medical Research Council, Laboratory of Molecular Biology, MRC Centre, Hills Road, Cambridge, CB2 2QH and [2]Department of Haematological Medicine, University of Cambridge Medical School, Hills Road, Cambridge CB2 2QH, UK

[3]Present address: Medical Research Council, Molecular Genetics Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, UK

[4]Present address: Oncology Research Institute, Sofia 1156, Bulgaria

Communicated by C.Milstein

A method for *in situ* hybridization originally developed for mapping genes in the nematode, *Caenorhabditis elegans* has been adapted for high resolution cytological mapping of genes in the human. The probe DNAs are labelled by incorporation of biotin dUTP and the site of hybridization detected by immunofluorescence. For the accurate assignment of the hybridization signal to chromosome bands, visualized by staining with Hoechst 33258, a heterologous ribosomal DNA probe is also included in the hybridization reaction. These rDNA signals are used as fiducial markers when aligning the two fluorescent images. We demonstrate the method by assignment of the human thymocyte CD1 antigen genes to human chromosome 1q22-23.

Key words: biotin dUTP/CD1 thymocyte antigen genes/non-isotopic *in situ* hybridization

Introduction

Almost 20 years ago the first demonstration of the localization of genes by *in situ* hybridization was reported (Gall and Pardue, 1969). Since then the method has been refined and has been used routinely to map genes on both normal and rearranged chromosomes. Hybridization to cells or tissue sections has also become an invaluable tool for studying the expression of genes and for demonstrating viral infection. Current research on the human genome, for example, highlights the requirement for long range mapping methods for the analysis of large genes and gene families. Presently, gel electrophoretic techniques can be used to demonstrate linkage betewen two genes within an interval of 10^6-10^7 bases. Beyond this distance *in situ* hybridization is still the only method available for an overall view of the distribution of a cloned sequence in a large genome. Thus the current requirement for *in situ* hybridization methods is that they should be sufficiently sensitive to detect single copy genes, they should be rapid and the resolution should approach that of gel electrophoresis. The use of non-isotopically labelled probes for *in situ* hybridization should be able to meet these requirements, but to date this has not been demonstrated convincingly in mammalian systems. Recently non-isotopic methods for *in situ* hybridization were compared to conventional autoradiographic techniques and it was found that although the non-isotopic method gave higher resolution, autoradiography still provided greater sensitivity (Mitchell *et al.*, 1986). In contrast, a sensitive non-isotopic method has been reported that can detect hybridization of a 1 kb genomic sequence (Garson *et al.*, 1987). However, the method destroyed chromosome bands so it was necessary to score and photograph metaphases before hybridization was carried out. Thus, this method, although sensitive, lacked the resolution of fluorescent techniques, and therefore is not sufficiently rapid and accurate to bridge the gap between molecular biology and gene mapping. We report here the application of mapping methods, originally developed for use in localizing genes on the chromosomes of the nematode, *Caenorhabditis elegans* (Albertson, 1984, 1985) to the mapping of the CD1 gene family on human chromosomes. The method, used successfully to map single copy genes in *C.elegans* (Albertson, 1985) employs biotin labelled probes. The site of hybridization, detected by immunofluorescence, is accurately positioned on a fluorescent chromosome band using fiducial markers incorporated into the hybridization reaction so that a high resolution, sensitive and rapid *in situ* hybridization technique is now available for use in mammalian systems.

The human CD1 thymocyte antigens (Thy, gp45, 12) are

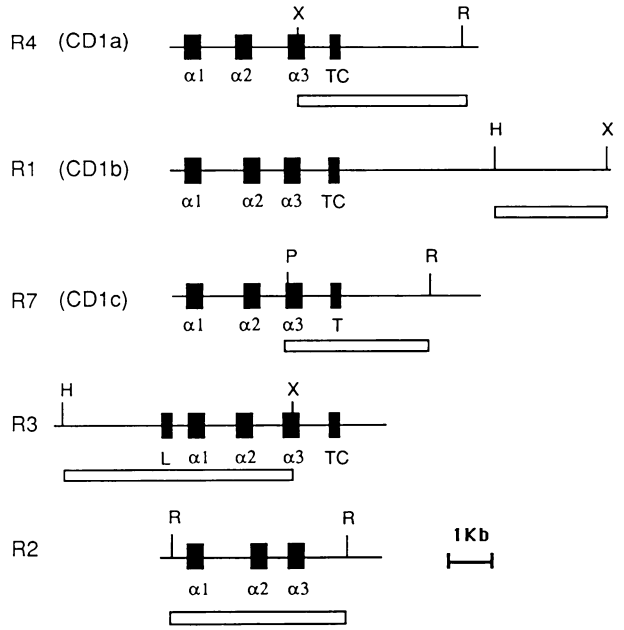

Fig. 1. CD1 gene probes used for *in situ* hybridization. Portions of the CD1 genomic clones reported previously (Martin *et al.*, 1986) were subcloned into pUC9 or pUC18. The single copy probe used for each gene is indicated by the open box. Filled boxes denote the locations of the CD1 exons. The leader (L), transmembrane (T), α1 and α2 domains show 50-70% nucleotide sequence conservation between genes. The α3 domain is most highly conserved and in paired comparisons shows at least 77% [R3 versus R4 (CD1a)] and as much as 94% [R4 (CD1a) versus R7 (CD1c)] identity at the nucleotide level. Restriction sites are X, *Xba*I; R, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

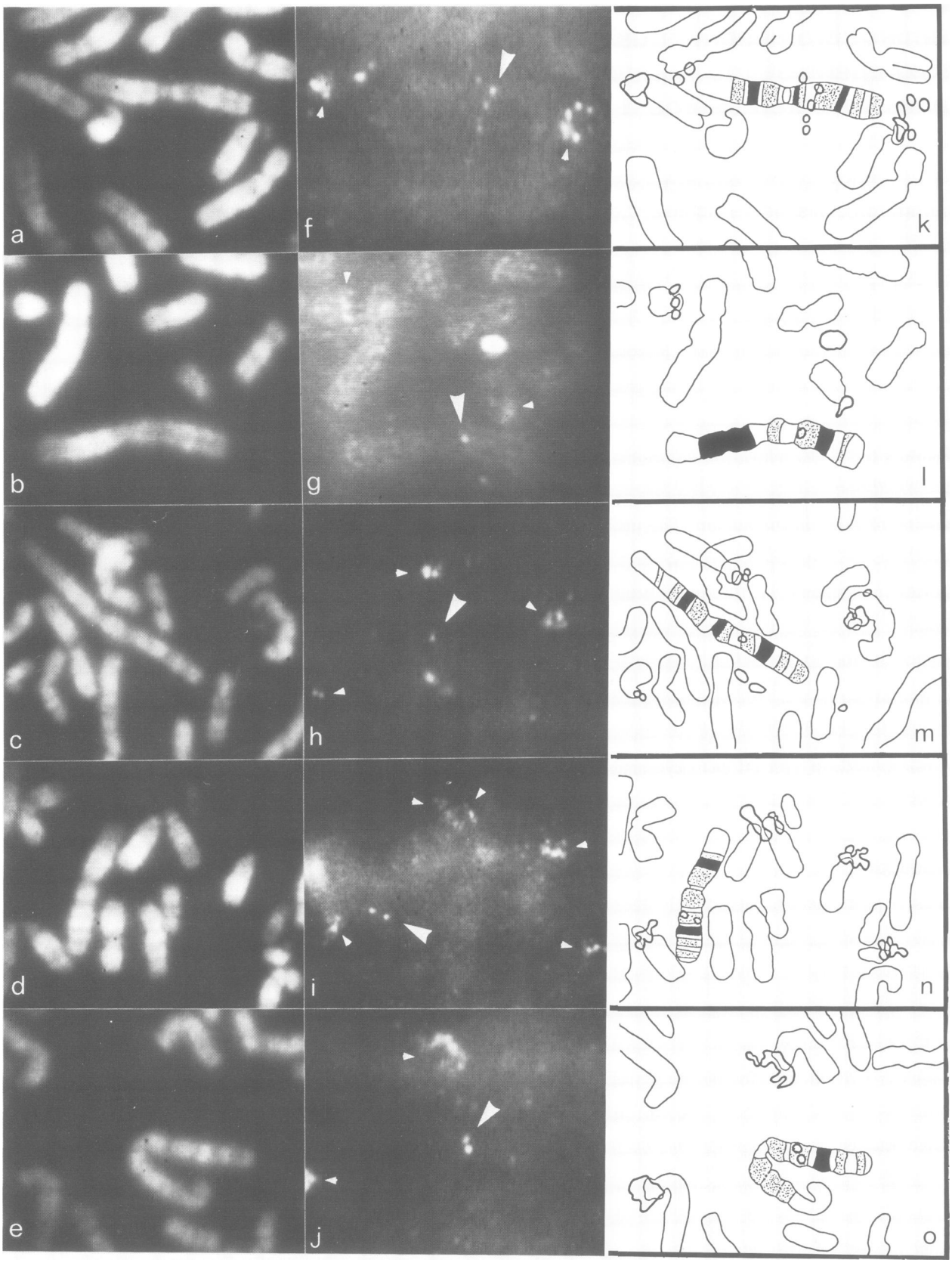

Fig. 2. *In situ* hybridization of the CD1 gene probes to human chromosomes. A representative spread is shown for each CD1 gene, R4 (**a,f,k**), R1 (**b,g,l**), R7 (**c,h,m**), R3 (**d,i,n**) and R2 (**e,j,o**). (**a–e**) Hoeschst 33258 stained chromosomes. (**f–j**) Texas red fluorescent image of the fields shown by the adjacent Hoechst 33258 image. The hybridization signal from the CD1 probe is indicated by the large arrow. The smaller arrows indicate the hybridization of the ribosomal probe to the ends of the acrocentric chromosomes. (**k–o**) Composite drawings showing the Texas red image overlying the Hoechst 33258 images were made by placing a clear acetate sheet over the photograph of the Texas red fluorescence, tracing the fluorescent signals and then aligning the sheet over the photograph of the Hoechst 33258 fluorescence.

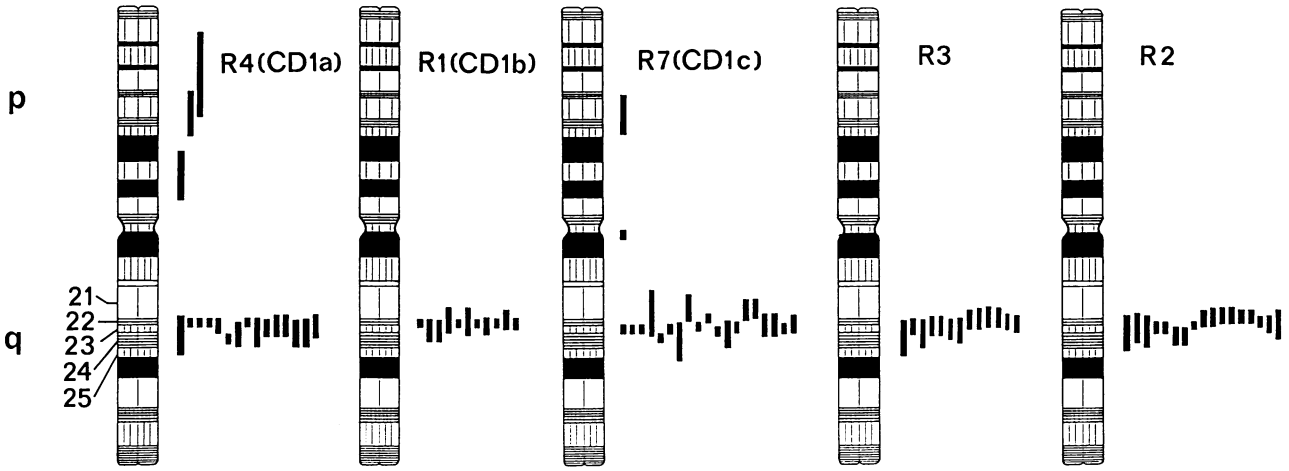

Fig. 3. Position of hybridization signals from CD1 probes on chromosome 1. The hybridization signals from the CD1 probes were recorded by a bar that spans the chromosome bands covered by the fluorescent signal.

expressed on the surface of immature thymocytes, where they are associated with β2-microglobulin (Bernard *et al.*, 1984). A family of five genes has been identified (Calabi and Milstein, 1986; Martin *et al.*, 1986) and the corresponding protein products of three of these have been identified (Martin *et al.*, 1986). The CD1 proteins are evolutionarily related to the MHC class I and class II proteins and have a highly conserved α3 domain that binds β2-microglobulin (Martin *et al.*, 1986). However, these genes do not appear to map in the MHC, as preliminary experiments have mapped four of these genes to chromosome 1 (Calabi *et al.*, 1987).

Results

The probe used for each CD1 gene (Figure 1) was labelled with biotin dUTP in a nick translation reaction. Included in each labelling reaction was the plasmid pCe7 (Files and Hirsh, 1981) that contains a single 7 kb ribosomal gene repeat from *C.elegans*. The *C.elegans* rDNA hybridizes weakly to the human ribosomal genes, which are located at the 'satellites' of the acrocentric chromosomes (D and G group). By aligning the fluorescent hybridization signals from the ribosomal probe with the ends of the 10 D and G group chromosomes it was possible to assign unambiguously any other fluorescent signals to chromosome bands. Figure 2 shows a representative spread for each of the five CD1 genes. The hybridization signal from the rDNA (small arrows) is diffuse, representing hybridization to the repeated ribosomal genes on the acrocentric chromosomes and is easily distinguished from the smaller, discreet fluorescent dots attributable to the hybridization of the CD1 genes (large arrows). The alignment of the two fluorescent images for each of the genes is shown in Figure 2 (k–o). Frequently, the site of hybridization was distinctly seen as two adjacent fluorescent dots (Figure 2f, i and j). These appear to represent hybridization of the probe to the homologous sequences on each of the two chromatids of the metaphase chromosome. In contrast, very few 'background' signals of comparable intensity are seen in the field. One can be seen in Figure 2 (g and h) and two in Figure 2f. None of these overlay chromosomes. By comparing the other chromosomes shown in Figure 2 to chromosome 1, it can be seen that the hybridization signals on chromosome 1 are distinctive.

The regions of chromosome 1 covered by the hybridization signals have been recorded in Figure 3 for each of the CD1 gene probes. As can be seen in Figure 2 (k, n and o), when both chromatids are labelled the signals do not lie opposite one another across the long axis of the chromosome, but are staggered slightly. Since the paired signals were recorded as a single hybridization event, the displacement of these spots therefore results in the longer bars drawn for some of the data points. All five members of the CD1 gene family map to chromosome 1q22-23. It is unlikely that hybridization of all five genes to this locus is due to cross-hybridization between the genes. The probes cover regions of moderate or negligible homology, except for the α3 exon. The R7 (CD1c) probe which contains the complete α3 is the least homologous to the others. As for the probe R4 (CD1a), it contains only 30 bases of the α3 domain, and the probe for the R1 gene (CD1b) was prepared from genomic DNA that did not contain any of the conserved sequences. The CD1 gene R4 was also mapped to 1q22-23 by *in situ* hybridization using a radioactively labelled probe and autoradiography (data not shown).

Discussion

The genes encoding the human thymocyte CD1 genes have been mapped to chromosome 1q22-23. Human metaphase chromosome preparations vary in the degree of condensation of the chromosomes and hence the number of bands that can be discerned; prophase chromosomes being the longest and displaying as many as 2000 bands over the entire human karyotype (Yunis, 1981). The pattern of bands seen on chromosome 1 in this work corresponded to the mid-metaphase pattern in which ~30 bands can be distinguished on chromosome 1 and over the entire karyotype ~400 (Yunis, 1973). Since the human genome contains 3×10^9 bp, then an average band we have seen would contain 7.5×10^6 bp. Thus, the CD1 gene family is contained within a chromosomal region that spans in the order of 10^7 bp.

The double hybridization signals, probably due to hybridization to each chromatid of the metaphase chromosome do not identify precisely the same chromosome band.

The phenomenon of chromosome banding is not understood, but is thought to result from differences in packaging of the DNA that reflects the underlying mosaicism of G+C content along the human genome. The chromosomes that were used for mapping were in transition from prophase to metaphase and hence were undergoing further condensation at the time of fixation. Thus, either due to pre-treatment of the chromosome, aimed at loosening the structure to allow access for the probe DNA, or as a result perhaps of differential condensation of the DNA in the two chromatids, the site of hybridization identifies slightly different locations for this small fraction (0.0001%) of the human genome. These phenomena may always present a limit to the resolution that can be achieved in mapping genes cytogenetically.

As noted previously (Calabi and Milstein, 1986), the human CD1 antigen genes, mapping to a single region of human chromosome 1q, are the only genes known to bind β2-microglobulin that do not map to the short arm of human chromosome 6. Within the CD1 gene family, the nucleotide sequences are highly conserved in the α3 exon, but much less so in other segments (Martin *et al.*, 1987). At the nucleotide level, the homology between CD1 genes and MHC genes has been lost. At the cytogenetic level, the CD1 genes also appear to be tightly clustered, as are the MHC genes. The results are all consistent with a gene family that separated from the MHC very near to the time when the duplications took place, and which expands and contracts during evolution. Recent results show that in the mouse, a pair of highly homologous CD1 genes are also found on a chromosome different from the MHC (A.R.M.Bradbury *et al.*, personal communication).

We have described here a technique for mapping cloned DNAs to a 10 Mb region of the human genome. It has now been used to map more than a dozen human genes on metaphase spreads with both normal and abnormal karyotypes. The fluorescent method has several advantages over bright field techniques (Garson *et al.*, 1987) in that chromosome bands may be obtained without pre-treatments of cell cultures or chromosome spreads, it is not necessary to pre-band and the hybridization signals are distinctive, making it possible to resolve hybridization to individual chromatids. Although the hybridization signals were recorded electronically in this work, it is possible to see the fluorescent signals by eye, suggesting that it would also be possible to record them photographically as has been done for large probes (Lawrence *et al.*, 1988). This reliable and fast method for mapping cloned DNAs by *in situ* hybridization in the human genome should also be applicable to other large genomes such as plants. Current molecular biological techniques for the study of the organization of genes and gene families in a genome are limited to regions of 10^6-10^7 bases that can be analysed by PFGE. We have demonstrated that *in situ* hybridization is at a similar level, and that we are closing the gap between molecular biology and cytogenetics. Further refinements in resolution will be achieved by using DNA probes labelled with different non-isotopic labels, detected by two different fluorochromes that will permit fine structure mapping within chromosome bands. Thus, these cytological mapping methods will, in combination with molecular biological techniques, facilitate the study of the organization of gene families in large genomes and the characterization of rearrangements resulting from such complex chromosomal translocations as seen in neoplasia. For the future, it is worth noting that non-isotopic techniques are suited to use with thick specimens such as tissue biopsies or whole organisms. The fluorescent techniques, when combined with laser scanning confocal light microscopy, provide a sensitive means of assessing the distribution of nucleic acids in three dimensions. It remains to be seen if further developments could lead to automated DNA hybridization-based diagnostic tests for virus infections and chromosomal aberrations such as trisomies.

Materials and methods

Metaphase spreads

Metaphase spreads were prepared from normal cultured lymphocytes that had been exposed to 0.01 μg/ml colcemid for 40 min before harvesting and fixation. Fixed cells were dropped onto cleaned glass slides. Either chilled, wet or dry slides were used to obtain the desired degree of spreading and reduction of cytoplasm retained with the chromosomes. Air dried slides were then fixed a further hour in methanol:acetic acid 3:1, and air dried. For hybridization the slides were rinsed briefly in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7), then incubated in 20 μg/ml ribonuclease in 2 × SSC at 37°C for 1 h. The slides were rinsed by incubating in 2 × SSC at 65°C for 15 min, then dehydrated by passing through two changes of 70 and 90% ethanol and air dried. To denature the chromosomes, slides were incubated in 70% formamide at 70°C for 3.5 min, dehydrated through ethanol and air dried as before.

Hybridization

Probes for hybridization were prepared by labelling 2–3 μg of cloned human DNA and 1 μg of pCe7 in an *in vitro* nick translation reaction incorporating 5-allylaminobiotin-labelled deoxyuridine triphosphate (Langer *et al.*, 1981) in place of thymidine as described previously (Langer-Safer *et al.*, 1982; Albertson, 1984). The plasmid pCe7 contains a single 7 kb repeat from the ribosomal genes of the nematode *C.elegans* (Files and Hirsh, 1981). Hybridization and washing of slides were carried out as described previously except that washing times were reduced to 10 min (Albertson, 1984, 1985). The site of hybridization was detected by incubating slides at 37°C for 1 h with rabbit anti-biotin (Enzo) at a final dilution of 1:500. Slides were rinsed in phosphate buffered saline (PBS; 12 mM NaCl, 16 mM Na_2HPO_4, 8 mM NaH_2PO_4) containing 0.1% Tween 20 and then incubated a further 20 min with Texas red labelled goat anti-rabbit IgG (Jackson Immuno-Research Laboratories Inc.). Slides were rinsed again in PBS containing 0.1% Tween 20, stained for 1.5 min in 1 μg/ml Hoechst 33258 and mounted in 2% propylgallate in glycerol buffered to pH 8.

Each probe was hybridized to metaphase cells prepared from at least two different subjects. Hybridization was seen over 50–70% of the chromosome 1s examined.

Microscopy

Slides were viewed by epifluorescence using a Zeiss planapo 63/1.4 oil objective, and appropriate filter sets for Hoechst 33258 and Texas red fluorescence. A 4× lens was mounted on the camera tube and images were recorded with an RCA ISIT camera with two stages of intensification, time averaged for noise reduction using an Intellect 100 image processing system (Micro Consultants, UK Ltd) and stored digitally on magnetic disk. At the high magnification used, the displacement of the image, as viewed through two different filter sets for the two fluorochromes, is ~1 cm on the video screen. Therefore, to align the hybridization signals with the image of the chromosomes, the hybridization signals (Texas red fluorescence) were drawn on the video screen. The Hoechst image was then aligned such that the hybridization signals from the ribosomal probe lay over the ends of the acrocentric chromosomes that carry the human ribosomal genes. The single copy hybridization signals were then assigned to chromosome bands. Mammalian ribosomal gene probes are not suitable markers for alignment in this system as the hybridization signal is too large, causing a reduction in sensitivity by the automatic gain control on the camera.

Acknowledgements

We would like to thank F.Calabi, L.H.Martin and Chack-Yung Yu for supplying the CD1 gene probes used in this work. D.G.A. was the recipient of a DuPont Fellowship.

References

Albertson,D.G. (1984) *EMBO J.*, **3**, 1227–1234.
Albertson,D.G. (1985) *EMBO J.*, **4**, 2493–2498.
Bernard,A., Boumsell,L. and Hill,C. (1984) In Bernard,A., Boumsell,L., Dausset,J. and Milstein,C. (eds), *Leucocyte Typing*. Springer, Berlin, pp. 9–142.
Calabi,F. and Milstein,C. (1986) *Nature*, **323**, 540–543.
Calabi,F., Schroeder,J., Martin,L.H. and Milstein,C. (1987) In McMichael,A.J. (ed.), *Leucocyte Typing III*. Oxford University Press, Oxford, pp. 72–74.
Gall,J.G. and Pardue,M.L. (1969) *Proc. Natl. Acad. Sci. USA*, **63**, 378–383.
Files,J.G. and Hirsh,D. (1981) *J. Mol. Biol.*, **149**, 223–240.
Garson,J.A., van den Berghe,J.A. and Kemshead,J.T. (1987) *Nucleic Acids Res.*, **15**, 4761–4770.
Langer,P.R., Waldrop,A.A. and Ward,D.C. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6633–6637.
Langer-Safer,P.R., Levin,M. and Ward,D.C. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4381–4385.
Lawrence,J.B., Villnave,C.A. and Singer,R.H. (1988) *Cell*, **52**, 51–61.
Martin,L.H., Calabi,F. and Milstein,C. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9154–9158.
Martin,L.H., Calabi,F., Lefevre,F.-A., Bilsland,C.A.G. and Milstein,C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 9189–9193.
Mitchell,A.R., Ambros,P., Gosden,J.R., Morten,J.E.N., Porteous,D.J. (1986) *Somat. Cell Mol. Genet.*, **12**, 313–324.
Yunis,J.J. (1973) *Cytogenet. Cell Genet.*, **22**, 679–683.
Yunis,J.J. (1981) *Hum. Genet.*, **56**, 293–298.

Received on May 16, 1988