Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6639–6643, September 1990
Genetics

Simultaneous localization of cosmids and chromosome R-banding by fluorescence microscopy: Application to regional mapping of human chromosome 11

(*in situ* hybridization/biotin labeling)

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Communicated by Jean Dausset, June 21, 1990

ABSTRACT **A technique for nonradioactive *in situ* hybridization on human metaphase chromosomes has been developed to localize human cosmid clones. The simple procedure using two fluorescent dyes (fluorescein and propidium iodide) allows the simultaneous identification of chromosomal R-bands and hybridization signal in a single screening of the slides. This technique has been used for rapid correlation of the genetic and physical map of chromosome 11q13–qter in the region of genes responsible for ataxia-telangiectasia and tuberous sclerosis.**

A rapid increase in the number of polymorphic DNA markers has led to the development of primary genetic linkage maps with markers placed at 10- to 20-centimorgan (cM) intervals on all chromosomes (for review, see ref. 1). In principle, the chromosome assignment of the genes responsible for Mendelian diseases can be obtained from primary linkage maps if a sufficient number of informative families can be obtained for linkage studies. After the initial localization of a gene has been obtained, high-resolution linkage and physical maps of the region can be used as tools for isolation and characterization of a gene. A combination of physical and linkage data is important for such studies, since the relationships between physical and linkage distance are not uniform throughout the genome, and recombination frequencies may vary by sex.

Methods for rapid localization and ordering of a large number of markers are needed to supplement linkage methods in the construction of high-resolution chromosome maps. Techniques of *in situ* hybridization on metaphase chromosomes provide the most direct way to establish high-resolution physical maps. Advances in labeling of nonradioactive probes and in methods for amplification and detection of hybridization signals have made possible the localization of unique DNA segments 1–2 kilobases long on metaphase chromosomes (2–4). Nonspecific signals due to the presence of repeated sequences (*Alu*, *Kpn*, etc.) in cloned DNA segments may be suppressed when an excess of total human DNA is present as competitor (5). Thus, nonradioactive *in situ* hybridization techniques can now be exploited for mapping a large spectrum of molecular clones of various sizes, including DNA segments inserted in cosmids and yeast artificial chromosomes (6–8). Lichter *et al.* (7) have demonstrated the effectiveness of these approaches to obtain high-resolution chromosome maps.

Several techniques have been applied to obtain high-precision localizations, with confocal microscopy without chromosome banding (7) or with conventional microscopy and banding techniques (2, 9, 10). Giemsa staining after hybridization necessitates double screening of the slides because of the extinction of fluorescent signal. Other methods compatible with simultaneous detection of banding and the hybridization signal have been used. However, these methods result in band patterns that are slightly different from those obtained by conventional techniques (9).

In this paper, we describe a fluorescent technique for high-resolution R-banding, compatible with *in situ* hybridization of DNA sequences cloned in cosmids and revealed by fluorescence microscopy. Chromosome localization of cosmid clones can be obtained by direct screening of slides. This technique has been applied to obtain precise localization on chromosome 11q for 13 polymorphic cosmid clones and one cloned gene (*ETS1*). Seven of the cosmids had been characterized by linkage in a large panel of reference families (11), allowing a direct comparison of the physical and genetic maps of chromosome 11q.

MATERIALS AND METHODS

Chromosome 11q Markers. Clones containing human inserts were selected as described (11) from a cosmid library constructed from a somatic hybrid cell line CF52-46/5 (12) containing 11q13–11qter::16p11–16qter translocation as its only human component. Cosmids were screened for polymorphisms and mapped to chromosome 11 or 16 by linkage, somatic cell hybrids, or *in situ* techniques. A primary map of 31 markers, including 13 loci detected by these cosmids, was constructed from genotype data obtained on 59 reference families [including the 40 families from the Centre d'Etude du Polymorphisme Humain (CEPH) panel] (11). Five of the cosmids isolated from this library that are included in the genetic map were chosen for study by *in situ* hybridization: CJ52.4 (D11S388), CJ52.5 (D11S386), CJ52.208 (D11S351), CJ52.12 (D11S385), and CJ52.15 (D11S383). HBI18 (D11S147), also contained in the linkage map, was included in the *in situ* studies (gift from Y. Nakamura and R. White, Salt Lake City). One of the other cosmids (CJ52.92) from this library that we studied has also been mapped by linkage in reference families to chromosome 11q; the six other cosmids (CJ52.3, CJ52.24, CJ52.34, CJ52.114, CJ52.20, and CJ204) detect polymorphisms but have not yet been studied by linkage. In addition, *in situ* mapping was obtained for *ETS1* (pHE5.4; gift of D. Stehelin, Lille).

Somatic Cell Hybrids. Additional physical mapping data was obtained by Southern blot hybridization of somatic cell hybrids containing parts of chromosome 11 and other human chromosomes in a rodent background. The two cell lines used for this purpose were derived from (*i*) a constitutional t(11;22)(q23;q11) and (*ii*) a neuroepithelioma tumor with t(11;22)(q24;q11) (13).

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Abbreviation: FITC, fluorescein isothiocyanate.

Metaphase Chromosome Preparation. Phytohemagglutinin-stimulated blood lymphocytes of healthy males were cultured for 72 hr. For synchronization, methotrexate (10 μM) was added for 17 hr, and 5-bromodeoxyuridine (0.1 mM) was then introduced for 6 hr. Colcemid (1 μg/ml) was added for the last 15 min before harvesting [hypotonic solution, 0.075 M KCl; fixative, methanol/glacial acetic acid, 3:1 (vol/vol)]. After chromosome spreading, the slides were kept in darkness at −20°C until use.

Probe Preparation. Cosmid probes were labeled by nick-translation with bio-11-dUTP (Sigma) according to the BRL protocol, purified over a Sephadex G-50 column, and precipitated. The pellet was dissolved in TE (10 mM Tris·HCl/1 mM EDTA, pH 8) and stored at −20°C. The insert of probe pHE5.4, homologous to *ETS1*, without the plasmid was labeled by enzymatic incorporation of dUTP biotinylated at position 11 by the random-priming technique (14).

Prehybridization of Cosmid Clones. Nick-translated probes and competitor DNA [human DNA (Sigma), sonicated to 300–500 base pairs] were dissolved at, respectively, 1 μg/ml and 200 μg/ml in hybridization mixture [50% (vol/vol) formamide/2× SSC, pH 7/10% (wt/vol) dextran sulfate/sonicated salmon sperm DNA (1 mg/ml)]. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.) After denaturation at 70°C for 5 min, DNA was preannealed at 37°C for 3 hr, according to Kievits *et al.* (15).

***In Situ* Hybridization.** Slides kept at −20°C were rinsed three times in isotonic phosphate-buffered saline at room temperature and then dehydrated in an ethanol series of increasing concentrations (70, 80, 90, and 100%). Slides were treated for 1 hr at 37°C with RNase (RNase A at 100 μg/ml in 2× SSC), rinsed for three 5-min periods in 2× SSC (pH 7) at room temperature, and then dehydrated as above. Just prior to hybridization, chromosome preparations were denatured in 70% (vol/vol) formamide/2× SSC (pH 7) for 2 min at 70°C, and then dehydrated at 4°C. The slides were treated with proteinase K (10 μg/100 ml in 20 mM Tris·HCl, pH 7.4/2

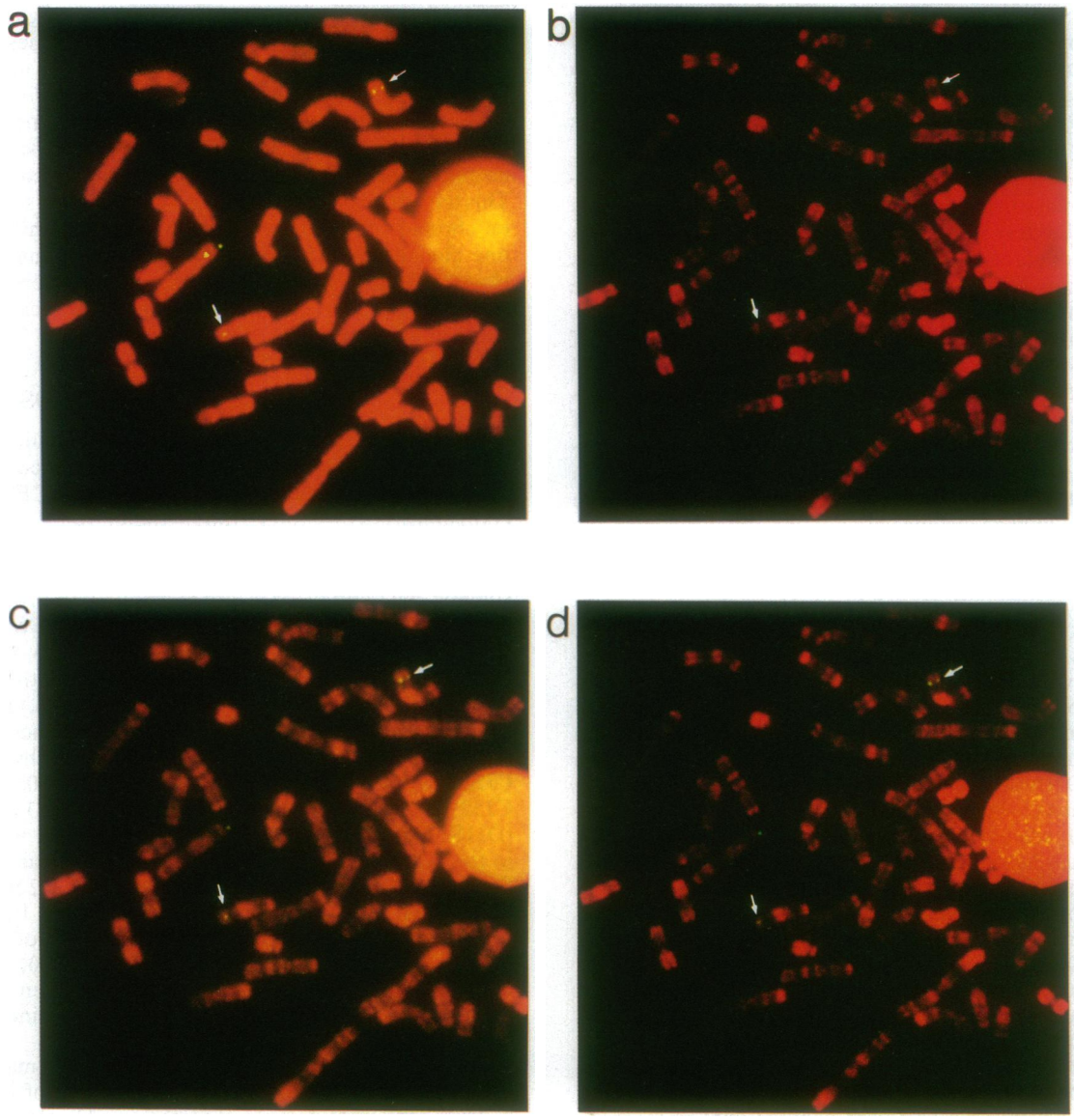

FIG. 1. Chromosomal localization of cosmid CJ52.208 by *in situ* hybridization. (*a*) Competitive *in situ* hybridization of the biotinylated cosmid and FITC staining after one amplification (two layers of avidin-FITC) of the signal. Chromosomes were counterstained with propidium iodide (filter combination I3 Leitz). (*b*) Pattern of R-bands in the same metaphase observed after hybridization and detection of the probe (filter combination N2.1). (*c*) Simultaneous visualization of R-bands and hybridization signal after a second round of amplification (filter combination I3). (*d*) Double exposure showing the fluorescent signal (filter combination L3) and R-bands (filter combination N2.1) on the same microphotograph. (×1550.)

mM $CaCl_2$) at 37°C for 7–8 min and dehydrated. The hybridization mixture (50 μl) was placed on the slide, covered with a coverslip, and sealed with rubber cement. After overnight incubation in a humid chamber at 37°C, the slides were washed at 45°C for three 3-min periods in 50% formamide/2× SSC, pH 7, for five 2-min periods in 2× SSC (pH 7), and once for 1 min in 1× BN (0.1 M sodium bicarbonate/0.05% Nonidet P-40, pH 8).

Probe Detection and R Banding. The hybridization signal was revealed by avidin-conjugated fluorescein isothiocyanate (FITC; 5 μg/ml; Vector Laboratories) and amplified once with additional layers of biotinylated goat anti-avidin (5 μg/ml; Vector Laboratories) and avidin-FITC, as described by Pinkel *et al.* (16). R-bands were obtained by a modification of the technique of Camargo and Cervenka (17, 18) that omits Giemsa staining. Slides were rinsed in distilled water, stained with Hoechst 33258 (0.1 μg/ml for 15 min), and rinsed. They were then UV-irradiated (365 nm) for 20 min and immersed in Earle's solution (pH 6.5) at 87°C for 2 min. After UV exposure, a second round of signal amplification was necessary for the detection of the hybridized probe on the banded chromosome. The chromosomes were counterstained with propidium iodide (1 μg/ml) in an antifade solution (19).

The slides were screened with a Leitz fluorescence microscope (Aristoplan). The hybridized probes appeared as yellow-green spots on red chromosomes with the filter combination I3 (BP 450-490/LP 515). For visualization of the fluorescent R-bands, a selective filter (combination N2.1: BP 515-560/LP 580) that permits visualization at wavelengths >580 nm was used. With the filter combination L3 (BP 450-490/BP 525/20), the red-emission ranges were blocked, and only the fluorescence of FITC was seen. For screening, the filter combination I3 was used. Microphotographs were taken with a film Kodak Ektachrome 400 after a double exposure of the FITC signal (filter combination L3) and fluorescence R-banding (filter combination N2).

RESULTS

***In Situ* Hybridization.** Under the prehybridization and hybridization protocols detailed above, a specific signal appearing as symmetrical spots on both chromatids of at least one of the two homologues of chromosome 11 was obtained in >90% of the metaphases with all the cosmids used as probes, after a single amplification (Fig. 1*a*), and a specific signal was observed on both homologues of chromosome 11 in 70–80% of the metaphases analyzed. The high hybridization efficiency is similar to that obtained by others (5, 7) using a different approach.

R-bands were prepared after hybridization and detection of specific signals (Fig. 1*b*). Experiments were undertaken to determine the most effective sequence of steps. When R-bands were prepared (without staining) prior to hybridization or prepared between hybridization and detection, the efficiency of the hybridization and the intensity of the signal decreased, possibly due to DNA loss by photolysis, and the background was increased. With the chosen protocol (i.e., preparation of R-bands after hybridization and detection of specific signals), UV irradiation resulted in fading of the fluorescence of the signal, which could be overcome by a second cycle of amplification without significant increase of the background. Under these conditions, simultaneous observation of hybridization signal and chromosome banding was possible either with the filter combination I3 (Fig. 1*c*) or with a double-exposure photograph (Fig. 1*d*) using two combinations of selective filters: FITC fluorescence with Leitz filter L3 (BP 450-490/BP 525/20) and propidium iodide with Leitz filter N2.1. The latter provides better resolution of banding.

The 13 cosmids we studied were all localized on chromosome 11q. The accuracy of the localization on subbands is in part related to the degree of elongation of chromosomes, and a small dispersion was noted with every probe, except for cosmid CJ52.15 (Table 1). Counting a sufficient number of spots, however, allowed us to choose the most probable localization (Fig. 2). In addition, a genomic probe was used to localize the gene *ETS1* by fluorescence. From the results presented in Table 1, it can be seen that the localization of *ETS1* was found at the borderline between bands 11q24 and 11q25, thus more distal than usually described.

Correlation with Genetic and Other Physical Mapping Data. Seven of the cosmids chosen for study by *in situ* hybridization are contained in the linkage map of chromosome 11q (11). They provide wide coverage of chromosome 11q (Fig. 2) and include the most distal and proximal markers obtained from cosmids isolated from the CF52-46/5 cell line. As shown in Fig. 2, the *in situ* hybridization results confirmed the order of the cosmids as established from multilocus linkage analysis, including two markers (HBI18 and CJ52.12) at ≈2% recombination distance in the genetic map. The placement of these markers and others derived from *in situ* hybridization is also consistent with those obtained from somatic cell hybrids.

DISCUSSION

Nonradioactive *in situ* hybridization provides a rapid method for obtaining localization and gene order for cosmid and yeast artificial chromosome clones in high-resolution maps of hu-

Table 1. Regional localization of cosmid clones and *ETS1* on chromosome 11 by *in situ* hybridization

Cosmid clone or gene	Chromosomal band(s)	No. of spots per chromosome examined	Chromosomal localization
CJ52.92	q13.5	12/88 (14)	
	q13.5–q14.1	49/88 (56)	q13.5–q14.1
	q14.1–q14.2	27/88 (30)	
CJ52.4	q14.3	41/90 (46)	q14.3
	q14.3–q21	28/90 (31)	
	q21	21/90 (23)	
CJ52.20	q14.3	13/87 (15)	
	q21	52/87 (60)	q21
	q22.1	22/87 (25)	
CJ52.24	q22.1	58/82 (71)	q22.1
	q22.2–q22.3	24/82 (29)	
CJ52.5	q22.1	21/93 (23)	
	q22.2	59/93 (63)	q22.2
	q23.1	13/93 (14)	
CJ52.3	q22.3	34/70 (49)	q22.3–q23.1
	q23.1	36/70 (51)	
CJ52.208	q23.1–q23.2	47/82 (57)	q23.1–q23.2
	q23.3	35/82 (43)	
CJ52.114	q23.1	20/79 (25)	
	q23.2	54/79 (68)	q23.2
	q23.3	5/79 (6)	
CJ52.12	q23.1–q23.2	14/66 (21)	
	q23.3	52/66 (79)	q23.3
HBI18	q23.3	10/93 (11)	
	q23.3–q24	68/93 (73)	q23.3–q24
	q24	5/93 (16)	
CJ52.204	q23.3	11/82 (13)	
	q23.3–q24	65/82 (79)	q23.3–q24
	q24	6/82 (7)	
ETS1	q24	13/44 (30)	
	q24–q25	31/44 (70)	q24–q25
CJ52.34	q24–q25	48/63 (76)	q24–q25
	q25	15/63 (24)	
CJ52.15	q25	54/54 (100)	q25

Numbers in parentheses are percentage of counts.

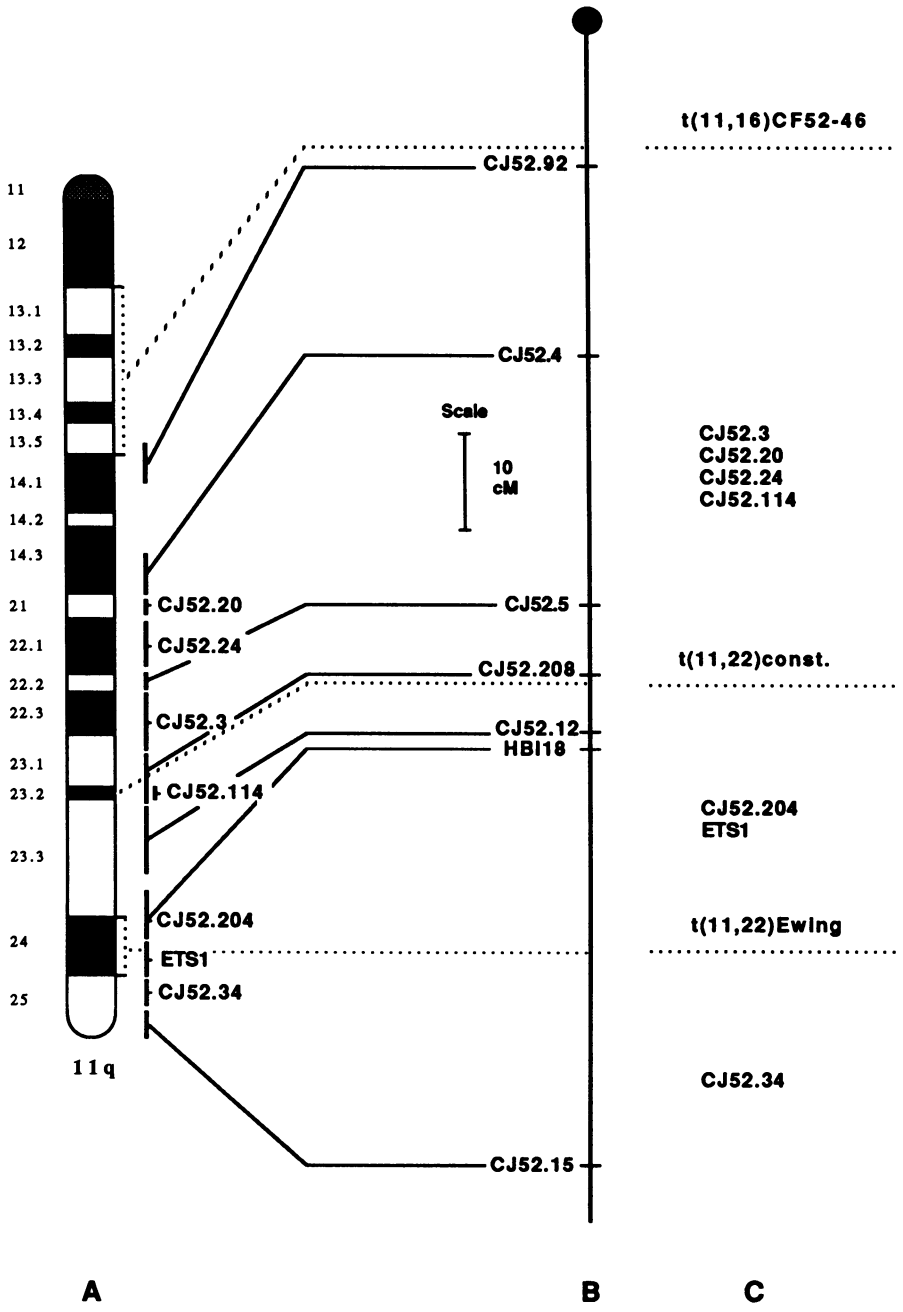

FIG. 2. Comparison of the maps of the long arm of chromosome 11 obtained by three methods. (*A*) Physical map obtained by *in situ* hybridization of biotinylated cosmids and of a probe corresponding to *ETS1*. (*B*) Linkage map. (*C*) Physical map obtained by Southern blot hybridization of human–rodent somatic cell hybrid DNA.

man chromosomes (5, 7, 8). Lichter *et al.* (7) have demonstrated the effectiveness of this approach by the construction of an *in situ* map of 50 markers on chromosome 11.

One limitation of these methods has been the difficulty of obtaining conventional chromosome banding to serve as a reference for mapping studies. Several different techniques have been proposed to resolve this problem. Methods for incorporation of 5-bromodeoxyuridine into chromosomes have been proposed (20–22) and used to study patterns of chromosome replication and their correlation with bands (23–25). Camargo and Cervenka (17, 18) have shown that the introduction of 5-bromodeoxyuridine for 5–6 hr in cell cultures, after methotrexate synchronization, and the use of the fluorescence-plus-Giemsa method (21) resulted in high-resolution R-bands perfectly classifiable in the international nomenclature (26). This technique or a variant (27) has been used to localize precisely DNA sequences with nonradioactive probes (10, 28). The major disadvantage of this method is the necessity of a double screening for hybridization signal detection and for band recognition. On the other hand, the use of fluorescence to reveal *in situ* hybridization signal may be an advantage if the banding technique also uses a fluorochrome at the same time.

The present technique is based on the spectral characteristics of two fluorochromes (FITC for the visualization of the probe and propidium iodide for chromosome staining; Fig. 1). It allows identification of the bands and location of the signals on the same preparation, by only changing the filters. Excitation and visualization of the two fluorochromes in the same time are made possible by use of the filter combination Leitz I3 (BP 450-490/LP 515). The signal of the probe is yellow-green on red chromosomes. Filter combination N2.1 (BP 515-560/LP 580), which stops FITC emission (between 500 and 550 nm), allows an optimal selective observation of

propidium iodide, which is excited at the maximal wavelength of 534 nm. High-resolution banded chromosomes can be observed with increased fluorescent staining. Importantly, simultaneous observation of chromosomal bands and hybridization signals eliminates the need for a reference system for gene localization, as has been proposed for techniques based on confocal microscopy (7), and permits localization and gene order to be determined with fluorescent microscopy. Moreover, the presence of banding allows an extremely rapid analysis of the mapping results, as chromosomal localization is acquired the day after hybridization.

As an example of our technique, we have applied it to obtain physical data on 13 cosmids localized to chromosome 11q13–q24 and a DNA fragment corresponding to *ETS1*. This region of chromosome 11 is of interest because it contains genes responsible for tuberous sclerosis (29) and ataxia-telangiectasia (30). Chromosome 11q, particularly bands 11q23 and 11q24, is frequently involved in chromosomal rearrangements associated with some types of cancers, including acute leukemia, Ewing sarcoma, and neuroepithelioma (31).

Loci detected by seven cosmids included in this study have been mapped (11) to chromosome 11q by linkage. Comparison of the genetic and physical maps in Fig. 2 shows that identical gene orders were obtained by the two methods. Interestingly, two of the markers (CJ52.12 and HBI18) were linked at a recombination distance of 0.02 (0.01–0.07; confidence interval, 1 lod unit) with a lod score of 24 and yet could be mapped to 11q23.3 and 11q24, respectively, suggesting that this region of chromosome 11 may have a low ratio of recombination to physical distance. Four cosmids that had not been studied by linkage could be accurately ordered with respect to markers in the genetic map. Two other cosmids (CJ52.114 and CJ52.204) mapped closely to CJ52.208 and HBI18, respectively, but the order of these pairs could not be determined with the present data. However, these orders might be resolved by the use of two fluorophores for simultaneous hybridization. At present, the maximal resolution possible with nonradioactive *in situ* methods is unknown, but our results demonstrate accurate mapping is possible on the order of 2 cM in some chromosome regions without the use of confocal microscopy if chromosome banding is used. Three of the previously unlocalized cosmids used in this study (CJ52.114, CJ52.24, and CJ52.3) fall into the region likely to contain genes responsible for ataxia-telangiectasia and tuberous sclerosis. This illustrates the power of the technique to obtain markers for disease studies once a preliminary chromosomal assignment has been made.

Finally, the localization of the protooncogene *ETS1* to 11q24–q25 and its presence in a somatic cell hybrid involving a rearrangement present in neuroepithelioma is consistent with its presence on the rearranged chromosome 11 in Ewing sarcoma with a similar translocation (32). The localization of cosmid CJ52.34 is distal from *ETS1*, and the breakpoint on chromosome 11 is thus localized between the two DNA sequences in the t(11;22)(q24;q12) recurrent translocation. It is likely that further high-resolution *in situ* studies will aid in the identification of the translocation breakpoints involved in these diseases.

We sincerely acknowledge T. Kievits and R. Slim for their help in the competitive hybridization technique and B. Boursin for the photographic work. We thank Y. Nakamura, R. White, and D. Stehelin for the gifts of probes HBI18 and pHE5.4. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, Ministry of Research and Technology (Grant 0878), Association pour le Developpement de la Recherche sur le Cancer, and the Human Frontiers Program. Photography was supported by Wild-Leitz.

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