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Mapping small DNA sequences by fluorescence *in situ* hybridization directly on banded metaphase chromosomes

(biotinylated probes/fluorescent detection of small probes/bromodeoxyuridine synchronization/simultaneous visualization of chromosome bands and signal/chromosome 11 mapping)

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ABSTRACT **A procedure for mapping small DNA probes directly on banded human chromosomes by fluorescence *in situ* hybridization has been developed. This procedure allows for the simultaneous visualization of banded chromosomes and hybridization signal without overlaying two separate photographic images. This method is simple and rapid, requires only a typical fluorescence microscope, has proven successful with DNA probes as small as 1 kilobase, is applicable for larger probes, and will greatly facilitate mapping the vast number of probes being generated to study genetic disease and define the human genome. Human metaphase chromosomes were prepared from phytohemagglutinin-stimulated lymphocyte cultures synchronized with bromodeoxyuridine and thymidine. Probes were labeled with biotin–dUTP, and the hybridization signal was amplified by immunofluorescence. Chromosomes were stained with both propidium iodide and 4′,6-diamidino-2-phenylindole (DAPI), producing R- and Q-banding patterns, respectively, allowing unambiguous chromosome and band identification while simultaneously visualizing the hybridization signal. Thirteen unique DNA segments have been localized to the long arm of chromosome 11 by using this technique, and localization of 10 additional probes by using radioactive *in situ* hybridization provides a comparison between the two procedures. These DNA segments have been mapped to all long-arm bands on chromosome 11 and in regions associated with neoplasias and inherited disorders.**

The current effort toward mapping the human genome requires a rapid, simple, and direct technique to identify the chromosomal location of the very large number of small single-copy DNA segments being generated. Previously, the technique of radioactive *in situ* hybridization on metaphase chromosomes was the most direct procedure for visually mapping genes or other DNA segments onto a chromosome band (1–3). Disadvantages of radioactive hybridization include a prolonged development time for the autoradiographic procedure, probe instability over time, the necessity for statistical analysis of a large number of metaphases, and imprecision caused by the different focal planes of silver grains and the chromosomes. However, the principal advantage is that unique sequences can be localized directly on banded metaphase chromosomes without further manipulations and photographic overlays. The development of nonradioactive fluorescence *in situ* hybridization procedures has overcome many of these disadvantages, but certain technical aspects have restricted this procedure from universal applicability.

Fluorescence *in situ* hybridization with repetitive human probes has allowed rapid identification of human chromosomes in somatic cell hybrids (4, 5). Chromosome-specific centromeric and telomeric probes have been used to detect numerical and structural chromosome aberrations (6, 7). Hybridization with pools of probes from chromosome-specific libraries has identified individual human chromosomes in metaphase and interphase cells (8). Hybridization signals of cosmid clone probes, with suppression of repetitive sequences, have been detected by using a confocal microscope with laser excitation (9). However, identification of hybridizing chromosomes depended on cohybridization with chromosome-specific probes, and the probe location was determined by its relative distance from an arbitrarily chosen reference point, rather than assigned directly to a chromosomal band.

Recent advances have been made in mapping single-copy genes and DNA segments by nonradioactive *in situ* hybridization. The high level of resolution and sensitivity achieved using fluorescence *in situ* hybridization was demonstrated when single-copy sequences as small as 5 kilobases (kb) were detected in metaphase chromosomes (10). However, the hybridization signals were not visualized directly on banded chromosomes and required subsequent staining [with the DNA fluorochrome 4′,6-diamidino-2-phenylindole (DAPI)] and alignment of the images to identify the chromosome and position of the hybridizing signal. In a similar study that used small DNA segments, subsequent chromosome staining, and overlaying of images for chromosome identification, complex digital-imaging microscopy was used for optimal sensitivity and resolution (11). In another study using standard fluorescence microscopy, hybridization signals of biotin-labeled single-copy genes were visualized by immunofluorescence, but banding quality sufficient to identify individual chromosomes was not achieved (12).

The data presented here demonstrate significant improvements leading to a rapid procedure that is sensitive enough to permit direct visualization of discrete fluorescent signals on R-banded chromosomes enabling detection of probes as small as 1 kb. Unique DNA segments were mapped to the long arm of chromosome 11. Several of these probes were mapped to regions associated with disease.

MATERIALS AND METHODS

Metaphase Chromosome Preparations. Metaphase chromosome spreads were prepared from 5-bromodeoxyuridine-synchronized lymphocyte cultures as described by Zabel *et al.* (2) with modifications. Six milliliters of heparinized blood was cultured in 54 ml of RPMI 1640 medium with phytohemagglutinin/20% fetal calf serum for 72 hr at 37°C. 5-

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Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

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Bromodeoxyuridine (0.18 mg/ml, Sigma) was added for 16 hr; cells were washed with medium once, and then incubated in 60 ml of RPMI 1640 medium containing thymidine (2.5 μg/ml, Sigma) for 6 hr. Cells were harvested by using standard procedures, and metaphase chromosomes were prepared. Each 6 ml of culture typically yields 80–120 slides with a high mitotic index and metaphase chromosomes extended to a length appropriate for banding at the 400–550

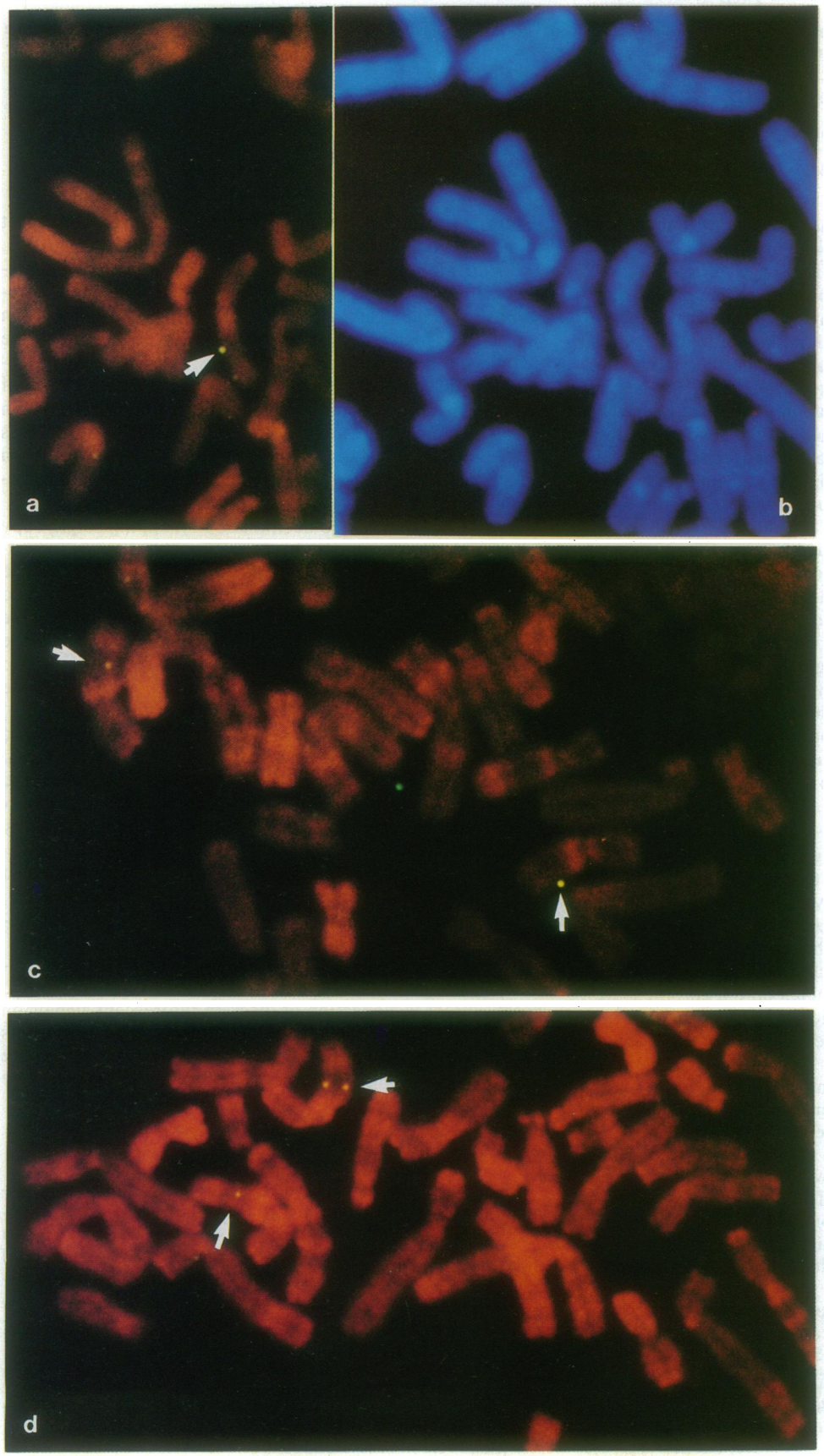

FIG. 1. Localization of D11S268 by fluorescence *in situ* hybridization. The chromosomes were stained simultaneously with propidium iodide and DAPI. Arrows indicate hybridization signals at 11q14.3–22.1. (*A*) Partial metaphase with R-bands, observed with filter combination B-2A. The yellow-green hybridization signal is seen on one of the chromosomes 11. (*B*) The same metaphase demonstrating Q-bands, observed with filter combination UV-2A. (*C*) R-banded partial metaphase, showing hybridization signals on both chromosomes 11. (*D*) R-banded partial metaphase with hybridization signals on both chromosomes 11, with one chromosome showing signal on both chromatids. The chromosomes were photographed with Kodak Ektachrome 400. (Magnification ×2000.)

band stage. Slides are stored in a desiccator for at least 2 weeks before hybridization and are baked at 56°C overnight before use. Both of these drying steps are required for good banding. Slides stored in a desiccator with Drierite (W. A. Hammond Drierite, Xenia, OH) are stable for at least 4 months.

Biotinylation of Probes. DNA probes were isolated from the Livermore Laboratory chromosome 11 library (identification code LL11NS01) (13). The library was originally constructed in the phage vector Charon 21A and recloned into pUC13; 59 clones were assigned to the long arm of chromosome 11, including the centromeric area, on a panel of somatic cell hybrids, without subregional band localizations (14). Most probes were 1.5–2.5 kb in length and in this study were labeled without linearization or purification of insert.

DNA probes were labeled with biotinylated 11-dUTP (0.02 mM, Enzo Biochemicals) and Klenow fragment (15). Unincorporated nucleotides were separated by chromatography (Sephadex G-50, Pharmacia). The recovered probe was ethanol precipitated, vacuum-dried for 10 min, and dissolved in hybridization mix.

***In Situ* Hybridization.** *In situ* hybridization was carried out by treating metaphase chromosomes on slides with RNase (100 μg/ml, Sigma) in 2× standard saline citrate (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) for 1 hr at 37°C, washing twice in 2× SSC, and dehydrating in 70% (vol/vol) and then 90% (vol/vol) ethanol. Chromosomes were denatured in 70% (vol/vol) formamide/2× SSC at 70°C for 3 min, quickly dehydrated with three rinses of cold ethanol (70%, 70%, 95%, at −20°C), and air dried. Chromosomal proteins were digested with proteinase K (0.06 μg/ml in 20 mM Tris/2 mM $CaCl_2$, pH 7.5) at 37°C for 8 min and dehydrated. The biotinylated probe was added to the hybridization mixture (see below), denatured at 70°C for 3 min, and quick-chilled on ice for 5 min. The probe was hybridized to metaphase chromosome slides (100 μl of hybridization mix with 25 ng of biotinylated DNA probe per slide) at 37°C overnight in a humid chamber sealed with Parafilm. Hybridization mix was 50% (vol/vol) formamide/2× SSC/10% (wt/vol) dextran sulfate/40 mM sodium phosphate/0.1% SDS/1× Denhardt's solution (Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and sonicated salmon sperm DNA at 100 μg/ml.

Detection by Immunofluorescence. Immunofluorescence was achieved by using the following procedures. After hybridization, slides were washed twice, 10 min each in 50% (vol/vol) formamide/2× SSC and twice for 10 min each in 2× SSC at 45°C. Slides were placed in 1× BN buffer (0.1 M sodium bicarbonate/0.5% Nonidet P-40, pH 8.0) for 10 min at room temperature. One hundred microliters of fluorescein avidin DCS (Vector Laboratories) (5 μg/ml in 1× 100 μl BN/5% (wt/vol) nonfat dry milk/0.02% NaN_3) was placed on each slide. Slides were covered with coverslips and incubated at 37°C in a humid chamber for 1 hr, rinsed briefly twice with 1× BN and then in 1 liter of 1× BN at 45°C for 10 min. Slides were treated with biotinylated anti-avidin D (Vector Laboratories) (12 μg/ml in 1× BN with 5% (vol/vol) goat serum and 0.02% NaN_3) at 37°C for 30 min. After the washing, another layer of fluorescein avidin DCS was added for amplification. Slides were washed and mounted in antifade solution (16) containing both DAPI (0.8 μg/ml) and propidium iodide (0.4 μg/ml) and observed with a Nikon fluorescence microscope.

RESULTS

Thirteen small chromosome 11 DNA segments have been mapped (Fig. 1). The probes were labeled with biotinylated 11-dUTP by using the random primer method described. *In situ* hybridization was according to Zabel *et al.* (2) and Fan

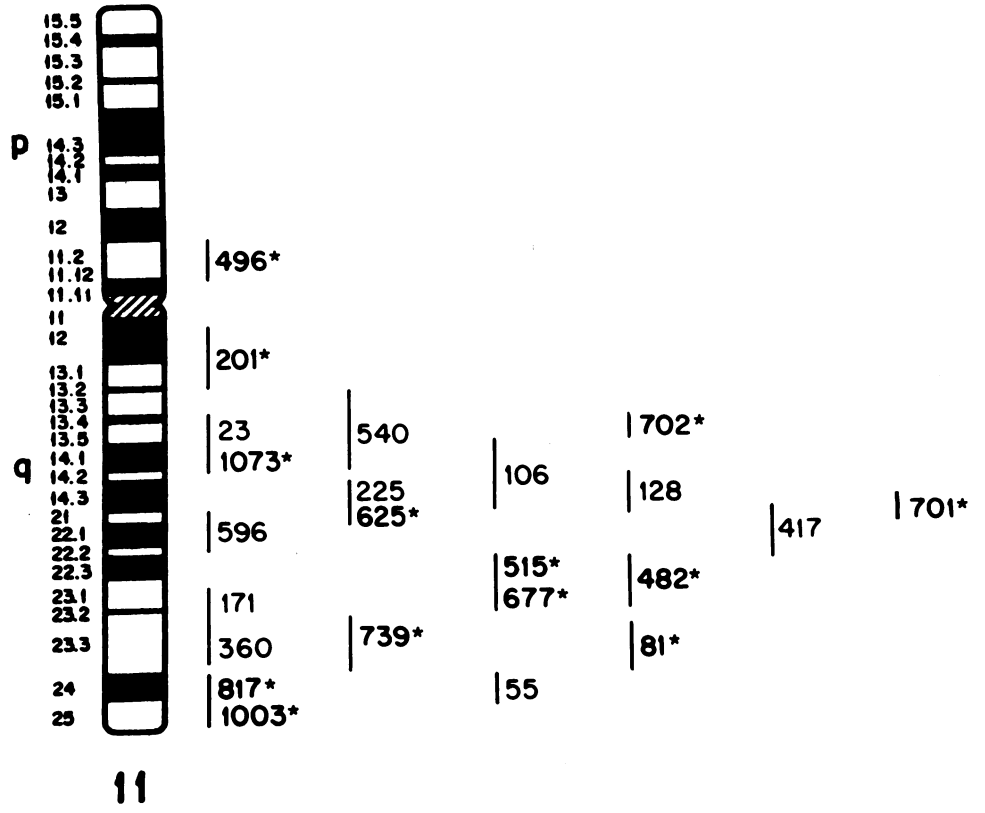

FIG. 2. The assignment of 23 chromosome 11 DNA segments by *in situ* hybridization with either ^3H- or biotin-labeled probes. Biotin-labeled probes are indicated by stars. Vertical bars represent the spread of grains over the region. Conversion to Human Gene Mapping Workshop 9 symbols is found in Tables 1 and 2.

Table 1. Assignment of unique DNA sequences on chromosome 11 by immunofluorescence

HGM symbol D11S-	Marker name D11RP-	DNA size, kb	Metaphases counted, no.	Fluorescent signals on chromosomes, no.	Signals at specific bands, %	Metaphases with signals at specific bands, %	Localization
214	201	1.5	26	44	84	69	11q12–13.1
219	1003	2.5	23	40	90	86	11q24–25
221	1073	1.5	42	31	81	42	11q13.4–14.1
232	496	2.5	32	22	100	65	11p11.2
241	482	2.0	40	32	88	48	11q22.3–23.1
242	739	1.0	30	35	86	60	11q23.3
255	81	3.2	20	22	82	60	11q23.3
262	515	2.0	30	37	81	87	11q22.3–23.2
266	625	1.5	30	41	68	80	11q14.3–21
267	677	2.2	30	37	84	70	11q22.3–23.2
268	701	1.5	29	31	74	69	11q14.3–22.1
269	702	1.6	22	20	70	58	11q13.4–13.5
273	817	3.0	15	40	83	100	11q24–25
				Mean	82	70	

HGM, Human Gene Mapping Workshop.

et al. (3). Immunofluorescence amplification of the hybridization signal was modified (see above) from Cherif *et al.* (12), and chromosomes were stained simultaneously with propidium iodide and DAPI. Slides were screened and photographed by using a Nikon fluorescence microscope. To simultaneously detect fluorescence hybridization signals on banded chromosomes, the fluorochrome dye must produce a characteristic banding pattern in a color distinct from that of the fluorescein-labeled probe in the same excitation range. Counterstaining with propidium iodide facilitates the visualization of whole chromosomes without interfering with the yellow-green hybridization signal, but such counter-staining does not help identify the chromosomes. By use of a bromodeoxyuridine block synchronization technique (see above) of lymphocytes and combined propidium iodide and DAPI staining, the chromosomes can be banded by both dyes.

Propidium iodide and fluorescein were viewed through filter combination B-2A with an excitation wavelength of 450–490 nm (Fig. 1 *A*, *C*, and *D*). Fluorescein-detected hybridization signals appear yellow-green on a background of red chromosomes with an R-banding pattern. Observing the same field of view, with filter combination UV-2A and an excitation wavelength of 330–380 nm, the chromosomes appear blue, with a characteristic Q-banding pattern confirming chromosome identification (Fig. 1*B*). The combination of dual staining with a simple change of filters allows simultaneous identification of chromosomes according to the banding pattern and visualization of hybridization signal on the same metaphase image, allowing unambiguous assignment and linear ordering of the 13 probes to specific chromosome 11 bands (Figs. 1 and 2, and Table 1).

Ten different probes were labeled with [^{3}H]dATP, [^{3}H]TTP, and [^{3}H]dCTP and hybridized to the prepared metaphase chromosomes; the distribution of grains on the whole-chromosome complement, including chromosome 11, was analyzed as described (2, 3) (Table 2). Comparison of radioactive and fluorescent hybridization procedures shows that the fluorescent technique is more efficient. Radiolabeled probes require that 100 cells be scored for grain distribution, with hybridization signals detected in only 20% (on average) of the cells counted. Fluorescent hybridization signals are detected in 70% of the cells (on average) observed, against much lower background than the radiolabeled probes, as indicated by the percentage of signals at specific bands (82% for immunofluorescence method, 12% for radioactive labeling, respectively) (see Tables 1 and 2). Thus, fewer slides are needed for each probe analysis, and the need for statistical analysis of the data is eliminated. Both members of a chromatid pair and both pairs of chromosomes frequently show distinct hybridization signals (Fig. 1 *C* and *D*). The delay in time from hybridization to data collection is also reduced from 2 weeks in the radioactive procedure to 2 days in the fluorescent procedure.

DISCUSSION

We have developed a rapid, efficient method for mapping small, single-copy DNA probes directly to chromosome bands using fluorescence *in situ* hybridization. Simultaneous visualization of banded chromosomes and the hybridization signal allows unambiguous assignment of a probe to a specific

Table 2. Localization of DNA segments by radioactive *in situ* labeling

HGM symbol D11S-	Marker name D11RP-	DNA size, kb	Metaphases counted, no.	Grains on chromosomes, no.	Grains at specific bands, %	Metaphases with grains at specific bands, %	Localization
239	171	2.5	100	219	8	14	11q23
240	360	1.5	100	264	15	26	11q23
253	23	1.0	100	225	13	17	11q13.4–14.1
254	55	1.5	100	171	9	15	11q24
256	106	1.5	100	229	8	25	11q14
257	128	2.0	100	219	8	12	11q14.3
259	225	1.5	100	216	12	22	11q14.3–21
261	417	1.5	110	263	7	18	11q22.1–22.2
264	540	1.5	100	179	19	25	11q13.3–14.1
265	596	2.5	100	193	17	21	11q21–22.2
				Mean	12	20	

HGM, Human Gene Mapping Workshop.

band, without the need to overlay two separate photographic images. The procedure can be used directly for single-copy probes as small as 1 kb and requires no previous knowledge of probe location. A standard fluorescence microscope was used without laser excitation, complex computer analysis of the data, image amplification, or overlay of two separate photographic images. The procedure has been successful with a larger cosmid probe, together with competitive suppression of repetitive sequences (data not shown), and should be equally successful with λ clones or yeast artificial chromosome sequences.

In this study we have localized 23 anonymous DNA segments to specific chromosome 11 bands by using this modified technique of fluorescent hybridization and conventional radiolabeled *in situ* hybridization (Fig. 2). A number of these markers have been assigned to regions associated with disease or growth control genes. Four probes map to 11q13, which is involved in translocations frequently seen in neoplasias of the hematopoietic system, such as acute myeloid leukemia, B-cell chronic leukemia, multiple myeloma/plasma cell leukemia, B-cell prolymphocytic leukemia, and malignant lymphoma (17). Six probes were assigned to 11q23, which is reported as a consistent breakpoint in rearrangements in acute leukemias, Ewing sarcoma, and ataxia-telangiectasia (18–20). These probes may prove useful in investigating the genetic alterations in these diseases. This study will contribute to expediently and accurately mapping markers to specific chromosome bands by the simultaneous visualization of fluorescence signals on banded metaphase human chromosomes.

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