Localization of a unique gene by direct hybridization *in situ*

(human α -globin/ ^{125}I -labeled cDNA plasmid/metaphase chromosomes/probe network formation/dextran sulfate)

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ABSTRACT Previous determinations of the chromosomal location of unique genes have required that the chromosomes of interest be fractionated, either by species-specific chromosome loss from interspecies hybrids, or by physical fractionation procedures. We have developed a general technique for the localization of a unique gene, which requires no prior chromosome fractionation. The technique involves the use of a labeled hybrid cDNA plasmid for direct hybridization *in situ* to metaphase cells from the organism under investigation. As a model system for development of this technique, we have employed a human α -globin cDNA plasmid (JW101) to localize the corresponding gene cluster. To obtain a sufficiently large autoradiographic signal, we have both labeled this plasmid with ^{125}I to a high specific activity (10^9 dpm/ μg) and taken advantage of the ability of a double-stranded probe to form networks. To obtain sufficient hybridization specificity, various experimental procedures were used, the most important of which was the choice from among a range of probe concentrations of the highest that does not yield excessive background hybridization. We have shown that, with an autoradiographic exposure time of only 12 days, use of this technique correctly localized the human α -globin gene cluster to chromosome 16. This technique should be generally applicable to the localization of any gene for which a corresponding cDNA hybrid plasmid is available.

An understanding of the mechanisms involved in the expression and regulation of any eukaryotic genes will ultimately require a determination of the location of its genetic elements within the genome of the corresponding organism. A first step in this determination is the localization of the gene to a particular chromosome and, if possible, to a particular subchromosomal region.

Early gene localization techniques involved the use of either pedigree analysis or somatic cell genetics. More recently, hybridization *in situ* has been employed to localize reiterated genes in various organisms (see ref. 1 and references therein) and some unique genes in species containing polytene chromosomes (2). By contrast, the use of molecular hybridization procedures to localize unique genes in organisms without polytene chromosomes has depended on prior fractionation of the chromosomes under investigation. This has been achieved either by constructing interspecies (human-mouse) cell hybrids that preferentially lose human chromosomes (3, 4) or by physical fractionation of chromosomes (5, 6). A more direct *in situ* hybridization technique, in which preformed networks tailed with 5-bromodeoxyuridine are used to detect hybrids between chromosomes and a polyadenylated RNA probe, has been employed successfully to localize an integrated retrovirus on chicken chromosomes (7). However, the extension of this method to localize other genes would require the use of probe RNA preparations that are as highly purified as retroviral RNA.

For most mRNA species, this is a goal that would be quite difficult to achieve.

We report here the development of a method for employing a hybrid plasmid containing a cDNA sequence to determine by hybridization *in situ* the chromosomal location of a unique gene. To demonstrate that this method yields the correct result, we have used it to localize the gene that codes for human α -globin, whose chromosomal location has previously been determined through the use of somatic cell hybrids plus molecular hybridization (8, 9).

EXPERIMENTAL PROCEDURES

Precoating of Slides. Slides are incubated for a minimum of 5 hr in $10\times$ Denhardt's solution ($1\times$ is 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone) (10) at 60°C to reduce binding of the probe to the glass slide (11). The slides are then washed profusely in double-distilled water and air dried; they can be stored for several months after preparation.

Preparation of Chromosomes. Penta X (69,XXXXX), a human lymphoblastoid cell line, was kindly supplied by M. Siniscalco of this institute. Metaphase chromosomes are prepared as described (12). The slides are either used for hybridization *in situ* within a day or stored with desiccant at -70°C .

Preparation of ^{125}I -Labeled dCTP. ^{125}I -Labeled dCTP (specific activity 300–1000 Ci/mmol; $1\text{ Ci} = 3.7 \times 10^{10}$ becquerels) is prepared by converting 5-mercuro-dCTP in a thallium-catalyzed reaction using carrier-free Na^{125}I (D. Ward and W. Prensky, personal communication). A standard reaction mixture consists of the following: 10 mCi of Na^{125}I (IMS300, Amersham) neutralized by the addition of a sufficient amount of H_2SO_4 , 0.1 mM NaOAc (pH 4.2), 0.8 mM thallic nitrate, and 0.2 mM Hg-dCTP (P-L Biochemicals). The mixture is incubated at 60°C for 5–10 min, the reaction is terminated by the addition of 50 vol of cold 5 mM triethylammonium carbonate ($\text{Et}_3\text{NHCO}_3\text{H}$) (pH 9.5), and the products are applied to a 0.25-ml DEAE-cellulose column equilibrated in 5 mM $\text{Et}_3\text{NHCO}_3\text{H}$. The column is washed extensively with 0.1 M $\text{Et}_3\text{NHCO}_3\text{H}$ to elute unreacted NaI , and ^{125}I -labeled dCTP is then eluted by the addition of a minimal volume of 0.6 M $\text{Et}_3\text{NHCO}_3\text{H}$. The ^{125}I -labeled dCTP is further purified by passage over a Bio-Gel P2 column (Bio-Rad) in 5 mM $\text{Et}_3\text{NHCO}_3\text{H}$.

Preparation of ^{125}I -Labeled Plasmid DNA. Human α -globin cDNA plasmid JW101, originally constructed by Wilson *et al.* (13), was generously provided by A. Bank of Columbia University. For labeling, the nick-translation protocol of Nordstrom

Abbreviation: NaCl/Cit , 0.15 M NaCl /0.015 M trisodium citrate (standard saline citrate).

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et al. (14) is followed exactly as described, employing ^{125}I -labeled dCTP at a final concentration of $5.0\ \mu\text{M}$ and a reaction volume of $10\ \mu\text{l}$. The reaction volume is then brought up to $0.5\ \text{ml}$ with $10\ \text{mM}$ Tris-HCl (pH 7.6)/ $1\ \text{mM}$ EDTA, and the mixture is extracted with an equal volume of phenol/chloroform (1:1 vol/vol). The phases are separated by centrifugation, and the aqueous phase is passed over a Sephadex G-50 column (Pharmacia). The excluded peak is dialyzed against $1\ \text{liter}$ of $1\ \text{M}$ NaCl/ $10\ \text{mM}$ Tris-HCl (pH 7.6)/ $1.0\ \text{mM}$ EDTA overnight. After addition of $1.0\ \text{mg}$ each of poly(A) and poly(U) (P-L Biochemicals), the nucleic acids are precipitated by adding $2.5\ \text{vol}$ of 95% (vol/vol) ethanol and incubating for $1\text{--}2\ \text{hr}$ at -20°C . The precipitate is then collected by centrifugation ($16,000 \times g$, $30\ \text{min}$), dried, and dissolved at a final concentration of $50\ \text{ng/ml}$ in hybridization buffer: 50% (vol/vol) formamide (Kodak spectral grade), $2\times$ NaCl/Cit (pH 7.0), $1\times$ Denhardt's solution, alkali-sheared salmon sperm DNA at $200\ \mu\text{g/ml}$, $0.1\ \text{mM}$ KI, $5\ \text{mM}$ NaPO₄, and 10% dextran sulfate ($1\times$ NaCl/Cit is $0.15\ \text{M}$ NaCl/ $0.015\ \text{M}$ trisodium citrate). Using ^{125}I -labeled dCTP of specific activity $1000\ \text{Ci/mmol}$, we produce an ^{125}I -labeled plasmid with specific activity of about $10^9\ \text{dpm}/\mu\text{g}$.

Hybridization *in Situ*. Endogenous RNA is eliminated by ribonuclease digestion with RNase A (type IIIA, Sigma) at $100\ \mu\text{g/ml}$ in $2\times$ NaCl/Cit at room temperature for $1\ \text{hr}$. The RNase reaction is stopped by carefully washing the slides with several washes of $2\times$ NaCl/Cit. The immobilized chromosomal DNA is then denatured by incubation at 70°C for $2\ \text{min}$ in 70% (vol/vol) formamide, $2\times$ NaCl/Cit, followed by dehydration through an ethanol series and air drying. The probe DNA is denatured by incubation at 70°C for $10\text{--}15\ \text{min}$. Probe solution ($20\ \mu\text{l}$) is placed on each slide and covered with a coverslip (Corning no. 2). The slides are then placed in a hybridization buffer-saturated environment and incubated at 42°C overnight.

At the end of the reaction, the coverslips are removed by soaking the slides in a large container filled with $2\times$ NaCl/Cit until they slide off gently. The slides are then washed in four changes of $2\times$ NaCl/Cit at room temperature followed by two 15-min washes in $2\times$ NaCl/Cit at $65\text{--}68^\circ\text{C}$. The slides are equilibrated to room temperature in $2\times$ NaCl/Cit, followed by four 1-hr washes in $0.1\times$ NaCl/Cit. A final wash is done overnight at 4°C in $4\ \text{liters}$ of $0.1\times$ NaCl/Cit. The slides are then dehydrated and air dried prior to autoradiography.

Autoradiography. The slides are coated with a $1:1$ (wt:vol) aqueous dilution of NTB (Kodak) liquid photographic emulsion, dried, and stored at 4°C in light-tight slide boxes with desiccant. The slides are developed by using a standard protocol (15).

Staining and Photography. The developed slides are stained with Giemsa stain as described (16), washed in tap water, and air dried. Metaphase cells are located and photographed by using a Zeiss photomicroscope with Ilford pan F film. The film is developed in Perceptol (Ilford).

RESULTS AND DISCUSSION

Two technical difficulties have hindered previous attempts to localize single-copy genes by hybridization *in situ*. One problem has been the preparation of sufficiently pure probes. Thus, it has been observed that the presence in the probe of even trace levels of a nucleic acid species that is encoded by repeated sequences in the genome can yield nonspecific signals that are larger than the signal from the specific hybrid under investigation (17). The other technical difficulty has been the inability to prepare probes of sufficiently high specific activity to obtain an interpretable signal in a reasonable amount of time.

The general problem of pure probe preparation has been virtually eliminated by the availability of hybrid cDNA plasmids. To overcome the problem of low probe specific activity,

we have employed the technique of Ward and Prenskey to synthesize ^{125}I -labeled dCTP with a specific activity of $300\text{--}1000\ \text{Ci/mmol}$. In addition, it has been shown that recombinant plasmids can be used to amplify the signal from a given hybrid through the formation of double-stranded networks. Wahl *et al.* (18) have discussed the formation of such networks when randomly sheared double-stranded DNA probes are employed, and they have shown that the rate of hybridization is increased by addition of dextran sulfate to the hybridization buffer. In collaboration with L. Lederman we have recently shown that the rate of probe hybridization to cytological preparations is also increased $20\text{--}30\text{-fold}$ when dextran sulfate is present in the hybridization buffer (unpublished data).

In the present studies, we have adapted the principle of probe network formation to the localization of a unique gene by hybridization *in situ*. The technique employed is illustrated schematically in Fig. 1. A recombinant plasmid that has been labeled with ^{125}I to a high specific activity by nick-translation is randomly sheared and denatured, and then is annealed on a cytological preparation. In the two extreme cases shown, either networks of probe molecules can form in solution and then hybridize to a homologous sequence in chromosomal DNA (pathway I), or networks can be built onto a free single-stranded tail of a probe molecule that has hybridized to a homologous sequence in chromosomal DNA (pathway II). The hybridization

FIG. 1. Model for the formation of probe networks during hybridization *in situ*. Probe strands that were originally in a double-stranded molecule can, after random shearing and denaturation, hybridize to each other and form networks. When the annealing is carried out in the presence of denatured chromosomal DNA, the probe networks can form and hybridize to homologous chromosomal sites. This may occur either by formation of probe networks in solution, followed by hybridization to chromosomal DNA (pathway I) or by formation of a probe network on the single-stranded tail of a probe molecule which has hybridized to chromosomal DNA (pathway II). The actual mechanism probably involves a combination of both pathways.

steps that actually occur most likely involve a combination of both pathways.

We have employed the human α -globin gene as a model system to determine whether this technique can be employed to localize a unique gene. This gene is known from previous studies to be located on chromosome 16 (8, 9), a metacentric chromosome of the E group that can be identified in most metaphase spreads without significant ambiguity. We have employed as probe hybrid plasmid JW101, which contains about 800 bases [including poly(dA)-poly(dT) tails] of human α -globin cDNA in the vector pMB9 (13).

Choice of Probe Concentration. In preliminary studies with rat growth hormone and prolactin cDNA plasmids, we found that there was a tremendous increase in the background when plasmid concentrations of 100 ng/ml or greater were used for a 12- to 16-hr hybridization reaction. We believe that this was probably due to the formation of very large probe networks, which interacted with the substrate in a manner such that, short of denaturing the networks, they could not be removed from the slides by our posthybridization treatments. To circumvent

this problem we normally hybridize samples on a series of slides with several concentrations of the probe and analyze those at the highest probe concentration that does not yield an excessive background. In the experiment reported here, the probe concentrations used were 50, 20, and 10 ng/ml, and the hybridization time was 16 hr. The slides with the probe at 20 ng/ml were chosen for further analysis.

Although we cannot directly determine whether the above conditions are sufficient to saturate all available gene sites, the extent of the reaction can be estimated as follows. For the slides on which probe had been hybridized at 20 ng/ml, the final product of probe concentration and time, C_0t , was $0.003 \text{ mol} \cdot \text{sec} \cdot \text{liter}^{-1}$. Because the hybridization was done in the presence of 10% dextran sulfate, this would be equivalent to an actual C_0t on the order of $0.06\text{--}0.09 \text{ mol} \cdot \text{sec} \cdot \text{liter}^{-1}$. The complexity of our probe, JW101, is about 6300 nucleotides, which is comparable to mammalian rRNA. We estimate that a C_0t of $0.06\text{--}0.09 \text{ mol} \cdot \text{sec} \cdot \text{liter}^{-1}$ would be sufficient to saturate about 50–70% of the homologous gene sequences if the probe were single-stranded, because ribosomal RNA hybridization to cytological preparations has a $C_0t_{1/2}$ of about $0.05 \text{ mol} \cdot \text{sec} \cdot \text{liter}^{-1}$ (15). However, because the probe is double-stranded, the formation of hybrids in solution would lower the concentration of hybridizable sequences, thus affecting the kinetics of hybridizing to the chromosomal DNA. We therefore estimate that no more than 50% of the α -globin genes are saturated.

Localization of the α -Globin Gene. A typical metaphase chromosome set after hybridization and autoradiography is shown in Fig. 2. In this particular cell, silver grains are observed over two of the three chromosomes 16. Some background silver grains can also be seen. However, because about 25% of all silver grains detected over chromosomes are associated with chromosome 16, it is quite clear that the signal-to-noise ratio is more than sufficient for detection of specific hybrids by this technique.

FIG. 2. Hybridization of ^{125}I -labeled α -globin cDNA plasmid to human metaphase chromosomes. (Upper) Giemsa-stained Penta X metaphase cell hybridized for 16 hr with 20 ng/ml of plasmid JW101 DNA that had been labeled with ^{125}I to a specific activity of $10^9 \text{ dpm}/\mu\text{g}$. The exposure time of the autoradiogram was 12 days. (Lower) Karyotype of the same cell. Silver grains are seen over two chromosomes 16, and also over other chromosomes. However, in almost all metaphase spreads examined, only chromosome 16 was found to be consistently labeled (see Fig. 3 and Table 1). It should be noted that in the metaphase spread shown here, the C band at the centromere of chromosome 16 was not clearly stained. However, in many other metaphase spreads hybridized by employing the procedures described, this C band was clearly stained, assisting in the identification of chromosome 16.

FIG. 3. Distribution of silver grains over human chromosomes. The average number of silver grains per chromosome determined from karyotypes of 10 randomly selected Penta X metaphase cells is shown. Data for individual chromosomes whose identification was difficult were pooled; the B, F, and G group chromosomes were pooled in their respective groups, and the C and D group chromosomes were pooled by size into, respectively, four and two subgroup categories. In general, excluding chromosome 16, the larger chromosomes were found to be labeled more often than the smaller ones, suggesting that the background is randomly distributed. The average number of silver grains over chromosome 16 is seen to be approximately 15 times larger than the average number over other chromosomes comparable in size (cf. chromosome categories D_I, D_{II}, 17, and 18). Analysis of an additional 50 metaphase spreads has yielded results in agreement with those shown here.

Table 1. Analysis of the silver grain distribution over human metaphase chromosomes

Chromosome category	% of haploid genome*	Number of silver grains			P values of positive deviations†
		Observed	Expected‡	χ^2	
1	8.64	10	15.9	2.19	—
2	8.42	7	15.5	4.66	—
3	7.00	7	12.9	2.70	—
B	13.04	15	24.0	3.38	—
C _I	11.58	25	20.3	1.09	0.3
C _{II}	10.46	12	19.2	2.70	—
C _{III}	9.34	6	17.2	7.29	—
C _{IV}	9.40	16	17.3	0.10	—
D _I	5.54	9	10.2	0.14	—
D _{II}	5.18	2	9.5	5.92	—
16	3.10	47	5.7	299.24	$<<10^{-4}$
17	2.96	3	5.4	1.07	—
18	2.80	2	5.2	1.97	—
F	4.56	17	8.4	8.80	10^{-2} – 10^{-3}
G	3.36	6	6.2	0.01	—

* Values determined by Mayall *et al.* (19).

† The expected number of silver grains for each chromosome category was calculated, assuming a random distribution, by multiplying the total number of silver grains detected (184) by the fraction of the haploid DNA content in each chromosome category.

‡ For each chromosome category where the observed grain number was greater than the expected number, a P value was determined from the χ^2 value for one degree of freedom.

The grain distribution over 10 randomly selected Penta X metaphase cells is shown in Fig. 3, and a χ^2 test for significance of the distribution is given in Table 1. The distribution of silver grains over the chromosomes is clearly not random ($\Sigma\chi^2 = 341.2$, degrees of freedom = 14, $P < 10^{-4}$). The labeling seen over chromosome 16 is highly significant, $P < 10^{-4}$. The remaining silver grains appear to be randomly distributed, except for the F group, which is labeled at a slightly higher frequency than expected. This may have been due to an occasional error in karyotyping, because the F group consists of two pairs of small metacentric chromosomes that in highly condensed metaphase cells can be confused with chromosome 16.

Does Probe Network Formation Contribute to α -Globin Gene Localization? An answer to this question requires estimations of both the average size of the specific hybrids formed and of the average specific hybrid size that would have been expected in the absence of probe network formation. We estimate the former quantity as follows. The mean number of silver grains over chromosome 16 was 1.6 (Fig. 3). However, the distribution of silver grain number over chromosome 16 appeared to be bimodal, with means of about 0.1 and 3.0 grains per chromosome, respectively. Because the former value was close to that observed for other chromosomes of comparable size, we conclude that the former value represents nonspecific labeling of chromosome 16. Therefore, on the basis of a value of 3.0 specific grains per chromosome and a 20% detection efficiency for ^{125}I (20), we calculate that the number of nucleotides in the average specific hybrid was about 1600.‡

We estimate the average specific hybrid size that would have been expected in the absence of probe network formation as follows. The maximal amount of homologous sequence at the α -globin gene cluster when all the gene sites are saturated would be: 800 nucleotides per haploid copy $\times$ 5 genes [one copy

each of $\zeta 1$, $\zeta 2$, $\psi\alpha 1$, $\alpha 2$, and $\alpha 1$ (see ref. 21)] $\times$ 2 haploid copies per chromosome $\times$ 2 strands = 16,000 nucleotides. Even if saturation of all available gene sites is achieved, the efficiency of hybridization *in situ* is low and can be affected by a number of variables involved in manipulation of chromosomal preparations (15, 22). By using procedures comparable to those employed in the present studies, efficiencies of 2% and 6.8% have been determined for hybridization of histone mRNA (23) and 5S rRNA (unpublished observation), respectively, to human metaphase chromosomes. Assuming these efficiencies, the maximal number of nucleotides of hybrid on chromosome 16 would be between 320 and 1088 nucleotides. Because we estimate that the hybridization is no more than 50% complete (see above), these values become 160 and 544 nucleotides. The number of nucleotides of hybrid on chromosome 16 is likely to be closer to 160, because: (i) hybridization to the two fetal ζ -globin genes may not be complete, due to sequence divergence (21), and (ii) earlier studies using iodinated β -globin mRNA at the same specific activity yielded a mean of less than one grain per chromosome 11 [the chromosome containing the β -globin gene cluster (3, 24)] in a 50-day exposure (unpublished studies by D. M. Steffensen, P. Szabo, W. Prenskey, and B. Forget), suggesting the hybridization efficiency may be as low as 1–2%.

On the basis of these calculations, the observed signal was about 10 times (1600/160) larger than expected in the absence of probe network formation, strongly suggesting that networks of probe molecules were formed. This conclusion is further strengthened by our preliminary studies with rat growth hormone and prolactin cDNA hybrid plasmids. Our observation in those studies, that the use of probes with lower specific activities ($1\text{--}5 \times 10^8$ dpm/ μg) yielded signals at least as large as those obtained in the present study, suggests that probe network size, and thus ultimately the number of grains detected at a gene site, is very sensitive to the probe size.

CONCLUDING REMARKS

The method for gene localization we have described here has several advantages over previously described techniques for localization of unique genes. It requires only a 10-ml cell culture plus 100 ng of ^{125}I -labeled plasmid DNA and yields results in a couple of weeks. By contrast, previous methods for mapping unique genes, involving prior fractionation of chromosomes, are less direct and more complex, time-consuming, and costly. The use of somatic cell hybrids for gene mapping requires the generation of a panel of hybrid cell clones containing subsets of chromosomes from the species under investigation. Such panels do exist for the human genome (4, 8). However, unless the gene under investigation is linked to a selectable marker, its chromosomal location can only be inferred from statistical analysis, and errors can occur. Physical fractionation of chromosomes by sucrose density gradient ultracentrifugation can be used to localize genes to specific chromosomes (5). However, except with species in which individual chromosomes vary greatly in size, sorting by this technique yields limited resolution of chromosomes. It is now possible to achieve high resolution of chromosomes with a fluorescence-activated cell sorter, but this method requires specialized equipment and large-scale chromosome preparation.

We would like to emphasize two technical aspects of our direct *in situ* hybridization technique. The most serious problem we have encountered is the high background that results from the use of high probe concentrations (>100 ng/ml). We therefore employ a series of probe concentrations less than 100 ng/ml and analyze in detail only those slides on which hybridization took place at the highest probe concentration with a low background. The other technical aspect is the relationship between

‡ [(3 grains) (5 disintegrations/grain) (6×10^{17} nucleotides/ μmol) $\div$ [(12-day exposure) (1.44×10^3 min/day) (10^9 disintegrations/min/ μg) ($330 \mu\text{g}/\mu\text{mol}$)] = 1600 nucleotides.

the number of silver grains obtained at a gene site and the size of the probe molecules. In general, small probe molecules do not form large networks (we have yet to determine the optimal probe size). This is especially true for hybrid plasmids in which tailing with poly(dA) and poly(dT) was employed, because the hybridization conditions used here do not yield stable poly(dA)·poly(dT) hybrids. Therefore, if the poly(dA)·poly(dT) tails are large and the probe size, after nick-translation and denaturation, is small, little vector DNA is covalently linked to hybrids and few probe networks are formed. This problem would not be expected to arise when hybrid plasmids constructed by direct insertion, by using synthetic linkers, or by tailing with poly(dG) and poly(dC) are employed as probes.

In summary, we have described a general method for the localization by direct hybridization *in situ* of any gene for which a cDNA hybrid plasmid is available. Experiments employing a human α -globin cDNA plasmid as probe have shown that use of this method yields the correct chromosomal location of the corresponding α -globin genes.

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