Chapter 36

Gene Mapping to Chromosomes by Hybridization *In Situ*

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1. Introduction

Since the technique of hybridizing labeled nucleic acid sequences to cytological preparations (hybridization *in situ*) was first described in 1969 by Pardue and Gall (*1*), it has undergone considerable refinement. In those early days, the technique was only capable of detecting and locating highly repeated target sequences such as satellite DNAs (2) or the genes for ribosomal DNA (3).

The introduction of methods that combined positive identification of every chromosome in each cell with a quantitative analysis of the distribution of autoradiographic grains (4) opened the way to more sensitive uses of the technique and ultimately to the present situation, in which hybridization *in situ* is routinely used to map the precise chromosomal location of DNA sequences as little as 1000 base pairs (one kilobase, 1 kb) in length.

The technique described here can be broken down into six stages:

1. Chromosome preparation. Chromosomes are prepared from cell cultures, which may be of permanent cell lines (fibroblasts, lymphoblastoid cells, and so on), or primary cultures. The cell type most frequently

used for mapping human DNA sequences is the phytohemagglutinin (PHA) stimulated peripheral blood lymphocyte. The chromosome preparations are spread onto clean glass slides. Since it is essential to be able to identify each chromosome, some form of banding procedure is needed. The method I use, which produces bands on the chromosomes after hybridization *in situ*, requires specific treatment during culture and preparation. The method used must produce a high mitotic index with well-spread metaphase plates and chromosomes of adequate length for good band resolution. It is obviously important that the method minimizes any loss of DNA from the chromosomes, while ensuring that the DNA is readily accessible to the probe.

2. Labeling the probe. The label of choice is tritium, because this provides the best combination of sensitivity and resolution. Alternative labels are discussed in the notes. The maximum specific radioactivity that can be obtained without compromising the integrity of the probe is the ideal. The method of labeling may be either nick translation or random oligonucleotide primed synthesis.
3. Hybridization. This comprises two steps: denaturing the chromosomal and probe DNA, and annealing them together. The denaturing steps must minimize loss or degradation of the DNA while maximizing the separation of the DNA duplex, yet still retain chromosome morphology. The annealing conditions are selected to reduce the chance of producing a poorly matched hybrid duplex, yet obtain the greatest possible amount of specific hybridization. In practice, the hybridization conditions are standard, and the posthybridization wash conditions are varied to increase or reduce the stringency of the reaction as required.
4. Autoradiography. This involves coating the slides with sensitive emulsion, exposing them for a suitable length of time, and developing the autoradiograph.
5. Staining–banding chromosomes after hybridization.
6. Analysis–of grain distribution.

2. Materials

2.1. Chromosome Preparation from Human Peripheral Blood

1. Medium: RPMI 1640 (store at 4°C).
2. Phytohemagglutinin (PHA): dissolved in 5 mL sterile H_2O (store at 4°C).

3. Fetal calf serum (store at –20°C).
4. 5-Bromo-2'-deoxyuridine (BUdR): 10 mg/mL in sterile water, filtered through a sterile 0.2-μm millipore filter (store at 4°C).
5. Tris-EDTA (TE): 10m*M* Tris-HCl pH 7.5; 1m*M* EDTA, (autoclaved).
6. Thymidine: 10^{-3}*M* in TE, filtered through sterile millipore filter (store at 4°C).
7. Colcemid: 10 μg/mL (store at 4°C).
8. Methanol, absolute.
9. Glacial acetic acid.
10. 0.075*M* KCl: 0.56 g/100 mL H_2O.

2.2. Labeling Probe DNA by Random Oligonucleotide Primed Synthesis

1. Deoxy(1',2',2,8–^{3}H) adenosine 5'-triphosphate (50–100 mCi/mmol) (dATP) (store at –20°C).
2. Deoxy(1',2',5–^{3}H) cytidine 5'-triphosphate (50–85 mCi/mmol) (dCTP) (store at –20°C).
3. (*Methyl*,1',2'-^{3}H) thymidine 5'-triphosphate (90–130 mCi/mmol) (dTTP) (store at –20°C).
4. 2'-deoxyguanosine 5'-triphosphate (dGTP): 0.1 m*M* in TE (store at –20°C).
5. Mixed sequence hexadeoxynucleotides: 1 mg/mL in TE (store at –20°C).
6. DNA Polymerase 1: large fragment, Klenow enzyme (store at –20°C).
7. 10 x concentration salt solution: 0.5*M* Tris-HCl pH 7.8; 50 m*M* $MgCl_2$; 10 m*M* β-mercaptoethanol; 500 μg/mL bovine serum albumin (BSA) (store at –20°C).
8. TNE column buffer: 0.2*M* NaCl; 0.01*M* Tris-HCl pH8.0; 1.0 m*M* EDTA (store at room temperature (RT).
9. Water-saturated, distilled phenol (store at RT).
10. Chloroform: octan-2-ol (24:1) (store at RT).
11. Sonicated salmon sperm DNA: 10 mg/mL in TE (store at 4°C).
12. 3*M* ammonium acetate (store at 4°C).
13. Scintillation fluid made as follows: 2(4'-t-Butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole (Butyl-PBD), 8 g, dissolved in 1000 mL of toluene, to which is added 600 mL 2-ethoxyethanol (Scintillation grade).
14. Nick column (Pharmacia).

2.3. Hybridization

1. 20 x SSC: 3*M* NaCl; 0.3*M* tri-sodium citrate.

2. Ribonuclease A (10 mg/mL in 2 x SSC, boiled 5 min to inactivate DNAse) (store at 4°C).
3. Formamide (Analar).
4. Dextran sulfate.
5. 10 x SSCB: 1.5*M* NaCl; 0.15*M* tri-sodium citrate; 0.25*M* Tris-HCl pH 7.4; 0.5m*M* EDTA (store at 4°C).
6. *E. coli* tRNA: 4 mg/mL in TE (store at 4°C).
7. Rubber solution (e.g., Pang Super Solution, Fritz Hesselbein Chemischefabrik, Nurderstedt).

2.4. Autoradiography

1. Dark room with safelight (Kodak S902 filter), water bath at 45°C, light-tight cabinet.
2. Ilford L4 Nuclear Emulsion (Kodak NTB2 emulsion can be substituted) (store in dark at 4°C).
3. Dipping vessel (flat Pyrex tubing, 30 x 7 mm inside measurements, 80 mm long, sealed and rounded at one end).
4. Light-tight slide containers.
5. Kodak D19 developer.
6. Kodafix.

2.5. Staining–Post-hybridization Banding and Analysis

1. Bisbenzimide H33258 fluorochrome (H33258): 500 µg/mL in H_2O (store in dark at 4°C).
2. Giemsa stain: 2% in Gurr's buffer pH 6.8, diluted fresh on each occasion.
3. 2 x SSC: 0.3*M* NaCl; 0.03*M* tri-sodium citrate (store at RT).
4. D.P.X. mountant.
5. Printed chromosome idograms for grain distribution analysis (*see* Fig. 1).

3. Methods

3.1. Chromosome Preparation from Human Peripheral Blood Lymphocytes

All solutions used prior to harvest *must* be sterile.

1. Medium: To a sterile medical flat or other closed glass vessel containing 200 mL sterile RPMI 1640 medium, add 2mL PHA (dissolved in

sterile distilled water as directed on container) and 30 mL sterile fetal calf serum. For culturing, this is then dispensed with a sterile pipet in 9-mL aliquots into sterile glass vessels of 20–25 mL capacity.

2. Culture: Blood is obtained by venipuncture with a sterile syringe and needle and placed in a vial containing lithium heparin as an anticoagulant. Of this fresh whole blood 1 mL is added to 9 mL of medium, gently mixed, and placed in a water bath or incubator at 37°C for 72 h. It will be found convenient to do this at about 3 PM on Friday. After 72 h (3 PM Monday) 200 µl of BUdR solution is added, gently mixed, and incubation is continued for a further 18 h (*see* Note 1).
3. At 8 AM the following day (Tuesday), transfer the cultures to sterile capped centrifuge tubes and spin them in a bench centrifuge at 1800 rpm for 5 min.
4. Gently decant the supernatant, without disturbing the pellet, and gently resuspend the cultures in RPMI 1640 (without PHA or serum).
5. Repeat the centrifugation and resuspension, and then centrifuge for a third time. On this occasion, the cultures are resuspended in complete medium and transferred to fresh, sterile glass culture vessels.
6. To each, add 100 µL of sterile $10^{-3}M$ thymidine. Incubate the cultures for a further 5 h 15 min at 37°C, at which time add 100 µL colcemid solution to each culture and continue incubation for a final 40 min.
7. Transfer the cultures to fresh, sterile capped centrifuge tubes, and spin at 1800 rpm for 5 min.
8. Gently remove the supernatant by pipet, and add 5 mL freshly made hypotonic (0.075*M*) KCl solution at RT drop by drop while mixing on a vortex mixer.
9. Leave the cultures at RT for 10 min, and then centrifuge again.
10. Carefully remove the supernatant and add (drop by drop on a vortex mixer as before) 3 mL of a freshly made mix of methanol: glacial acetic acid (3:1).
11. Top the tube up to the 10 mL level with fix, and mix well. Leave in this first fix for up to 30 min, then spin again, decant the supernatant and add a further 10 mL of 3:1 fix, mixing well.
12. Centrifuge again, decant the supernatant, and add 10 mL of fix.
13. At this stage, a test slide can be made by dropping one drop of the resuspended chromosome preparation onto a clean glass slide and examining it under phase contrast. If phase optics are not available, an approximation can be made by removing the condenser from a standard microscope. This will let the observer see how many cells are in metaphase, how well the chromosomes are spread, and how much cy-

toplasm is still attached to the chromosome spreads. It is worth staining one slide (2 min in 2% giemsa) to check that nuclear envelopes are removed to permit access of probes to chromosomes. This cannot be readily determined under phase contrast.

14. A further two or three changes of fix will be necessary in most cases. After the final change, resuspend the pellet in 1 mL of fix.
15. Double-frosted glass slides should be used. The slides are cleaned prior to use by soaking in absolute ethanol to which 1% HCl has been added, and polishing with fine muslin. (The same treatment is used for coverslips. These will be termed clean slides and coverslips.)
16. One drop of cell suspension is dropped into the center of a clean slide. The height from which it is dropped, and whether the slide should be warm or cold, wet, dry, or damp (from being breathed on) will vary from day to day according to the local climatic conditions. Each worker will find the best treatment for the local conditions by trial.
17. The slides should be dried at room temperature and then stored in light-tight containers in desiccators under vacuum. Under these conditions, a batch of slides should remain useful for at least 2 mo, and may remain so for much longer.

3.2. *Labeling Probe DNA by Random Priming* (See *Note 2*)

1. A minimum of 0.3 µg of linear DNA is needed, and the maximum to be labeled at any one time is 1 µg.
2. In a sterile plastic microcentrifuge tube, place 0.5 nmol of each of ^{3}H-labeled dATP, dCTP, and dTTP. Dry down by blowing a gentle stream of air into the tube (just sufficient to ruffle the surface of the fluid) from a glass Pasteur pipet connected to an aquarium aeration pump. This procedure takes about 3 h, and may conveniently be done overnight.
3. Mix the probe DNA, 2 µLof hexanucleotide solution and sterile, distilled H_2O to a total volume of 16 µL in a second sterile 1.5 mL plastic microcentrifuge tube. Spin briefly to collect, pierce a fine hole in the lid with a heated syringe needle, and place in a boiling water bath for 3 min. Remove from the bath, cool to room temperature, and spin again to collect.
4. Transfer the contents of the second tube to that in which the labeled nucleotides have been dried. Add 2 µL of 10 x salt solution and 1 µL of unlabeled dGTP. Mix well (ensuring that all the dried nucleotides

are redissolved), and add 1 µL of Klenow enzyme. Mix gently and incubate in a water bath at 37°C for 90 min to 2 h.

5. Meanwhile, prepare a Nick column by washing with three reservoirs full of TNE to replace storage buffer with column buffer, and ensure column bed is properly equilibrated.
6. Add 80 µL of TNE and 50 µL of water-saturated phenol to the incubation mix. Mix well and incubate for a further 10 min at 37°C. Add 50 µL of chloroform: octan-2-ol, mix again, and spin for 2 min to separate the aqueous and phenol phases.
7. With a glass Pasteur pipet, carefully transfer the upper (aqueous) phase to the Nick column being scrupulously careful to avoid picking up any of the lower (phenol) phase. Having allowed the aqueous phase to run into the column bed, add 400 µL TNE to the column and let this run through. Add a further 400 µL of TNE and collect the drops from the column (total volume should be approximately 400 µL) in a clean, sterile plastic microcentrifuge tube.
8. Take a 4-µL aliquot from this, place in a scintillation vial containing 3 mL scintillation fluid, mix well, and count in a liquid scintillation counter. There should be no less than 50,000 cpm (about 100,000 dpm) in this sample. If there are fewer counts than this, incorporation of label may well be insufficient to detect in the hybridization.
9. If the counts are adequate, add 10 µL of sonicated salmon sperm DNA (to act as coprecipitant) and 50 µL of 3*M* ammonium acetate to the microcentrifuge tube, mix well, and add 1 mL of absolute ethanol. Mix again and place the tube at –20°C for at least 1 h.
10. Centrifuge for 5 min, decant the supernatant (into a new tube) and place the sample in a lyophilizer (Freeze-drier) to evaporate the residual alcohol.

3.3. *Hybridization* (See *Note 3*)

1. Make a stock solution of 20% dextran sulfate in formamide. Since this takes a long time to dissolve and seems stable at room temperature for several months, it is worth making a large volume such as 10 mL.
2. Treat the slides with ribonuclease A to remove any endogenous RNA that might otherwise compete for the probe. Make a solution of 100 µg/mL RNAse A in 2 x SSC by adding 1% of the stock solution to the appropriate volume of 2 x SSC. For a normal experiment, using six slides, 50 mL final volume in a Coplin jar is sufficient. Place the jar in a water bath at 37°C and incubate the slides in it for 1 h. Remove the

slides and pass them through 70%, 90%, and 100% ethanol (5 min each). Dry the slides under vacuum.

3. Denature the chromosomal DNA by placing the slides in a solution of 0.6 x SSC; 70% formamide at 70°C for 2 min, transferring them instantly to 70% ethanol after this time, and passing through the alcohol series as above, before drying under vacuum. At this stage, provided the slides are kept dry, they are stable for at least several hours, if not days.
4. Meanwhile, make up the hybridization mix as follows: For each slide, allow 20 µL of mix, plus an extra 40 µL, i.e., for six slides (6 x 20) + 40 = 160 µL. Of this total, 20% is the labeled probe dissolved in sterile distilled water, 20% is 10 x SSCB, 10% is *E. coli* tRNA solution, and 50% is 20% dextran sulfate in formamide. For the six slides, dissolve the dry, labeled probe in 33 µL sterile distilled water, leave 5 min to allow complete solution, and take 1 µL and count in a scintillation counter (leaving the 32 µL needed for the hybridization mix). Add 32 µL 10 x SSCB, 16 µL tRNA and 80 µL dextran/formamide. This last is very viscous: it is best to cut off the end of a micropipet tip to dispense this material, and use the same tip to mix it with the remainder of the hybridization mix by pipeting up and down. Mix very thoroughly and place in a water bath at 70°C for 5 min. At the end of this time, transfer to an ice bath.
5. Prepare six 20 x 40 mm clean coverslips. Place 20 µL of hybridization mix on each coverslip, and invert a slide over each. Allow the mix to spread, ensuring that there are no air bubbles; when the area under the coverslip is covered, seal with rubber solution to prevent evaporation of hybridization mix.
6. When the rubber solution is dry, transfer the slides to a thin metal vessel floating in a 37°C water bath with a lid, or a closed container in a 37°C incubator. Leave overnight.
7. Meanwhile, prepare the washes. In 1000 mL reagent bottles or flasks, make (a) 50% formamide; 1 x SSC and (b) 1 x SSC. Place in a water bath at 42°C.
8. The following morning, carefully peel the rubber solution away from the coverslips, and remove them from the slides. (They should come away easily if the rubber solution provided a good seal.)
9. Place the slides in a glass stain rack, and place this in a 250-mL capacity stain dish in the 42°C water bath. Add 50% formamide; 1 x SSC, agitate gently, and leave for 5 min.

10. Repeat three times, and then give four washes of 5 min each in 1 x SSC.
11. Transfer to 70% ethanol and pass through the series to absolute alcohol as before. Air dry or dry under vacuum.

3.4. Autoradiography

1. In a darkroom under a safelight, heat the water bath to 45°C. In the water bath place: (a) a 25-mL measuring cylinder containing 5 mL distilled water and (b) a 100-mL beaker full of water, in which the dipping vessel is supported. Place the slides to be dipped face up on a hot plate that is just warm to the touch. (This helps the emulsion to spread thinly and uniformly.)
2. With a pair of sterile plastic forceps, transfer emulsion from the container to the measuring cylinder until the volume in the latter is 10 mL. Allow to stand 5 min to reach melting temperature. Mix very gently, avoiding air bubbles, and pour the diluted emulsion into the dipping vessel. Take two slides, back to back, and dip into the emulsion, so that the frosted area is just clear of the emulsion surface.
3. Remove gently (without scraping the sides of the vessel), separate the slides, and place in a rack to dry. (A standard test-tube rack, with the slides diagonally across the apertures will do.)
4. When all the slides have been dipped, place the rack in a locked, light-tight cabinet, and leave for 2.5–3 h to dry.
5. When dry (still under safelight), transfer the slides to a light-tight box containing a small amount of silica gel separated from the slides by a piece of tissue paper. Seal the box and place at 4°C in a refrigerator free from all sources of radiation.
6. After 6 d, bring the slides to room temperature (this takes about an hour) and, in the dark room, under safelight, remove one slide from the box.
7. Develop for 5 min in Kodak D19, agitating once a minute, stop in water, and fix for 5 min in Kodafix, diluted 1:3 with water, again agitating once a minute.
8. Wash under gently running cold water for 6 min.
9. Allow to dry, and stain in 2% Giemsa in Gurr's buffer for 5 min.
10. Air dry, and examine under a microscope. If there are more than 2 or 3 autoradiographic grains/metaphase, develop the remainder of the slides. If not, leave a further 6 or 7 d, by which time, if the experiment has worked, there should be enough grains.

3.5. Staining—Banding

1. After the slides have been developed and washed, allow them to dry thoroughly—at least overnight—to ensure that the emulsion is hard again.
2. Stain slides for 15 min in H33528 diluted to 50 µg/mL in 2 x SSC. Rinse in 2 x SSC.
3. Mount in 2 x SSC under a 20 x 40 mm coverslip and, in a darkroom, place under UV lamp, set to long wave, at a distance of 10–12 cm, for 30–60 min.
4. Place slides in a Coplin jar containing 2 x SSC for 35–45 min at room temperature.
5. Dry slides, and stain for 15 min in freshly diluted 2% Giemsa. It is probably wise to treat only three or four slides at a time. The chromosomes should show clearly defined G-bands.

3.6. Analysis (see Note 4)

1. When completely dry, dip the slides in xylene for 5 min before covering with a 20 x 40 mm coverslip mounted in D.P.X. When they are completely dry, examine under microscope.
2. A suitable form of idiogram is shown in Fig. 1. It is important that the resolution implied by the bands on the idiogram is equivalent to that seen down the microscope!
3. Identify those chromosomes on which grains can be seen, and mark the grains in the correct location on the idiogram. Only grains in contact with a chromosome should be scored. In this way, the total distribution of grains is accumulated, and it is possible to distinguish specific signals from the background.
4. The number of cells needed to obtain a significant result varies from probe to probe. In general, the highest proportion of grains seen on the specific chromosome is about 20%, i.e., only one grain in five is actually found on the correct chromosome, and of these between 60 and 90% may be located at the correct band. It is therefore rarely possible to get a significant result without scoring at least 20 cells, and it may be necessary to score many more—perhaps as many as 80 or 100. It should be possible to resolve the location of a single copy sequence to within a major band, and in most cases to within one or two minor bands.

4. Notes

1. Chromosome preparation: It is important to remember that the BUdR treatment makes the chromosomal DNA sensitive to light, and there-

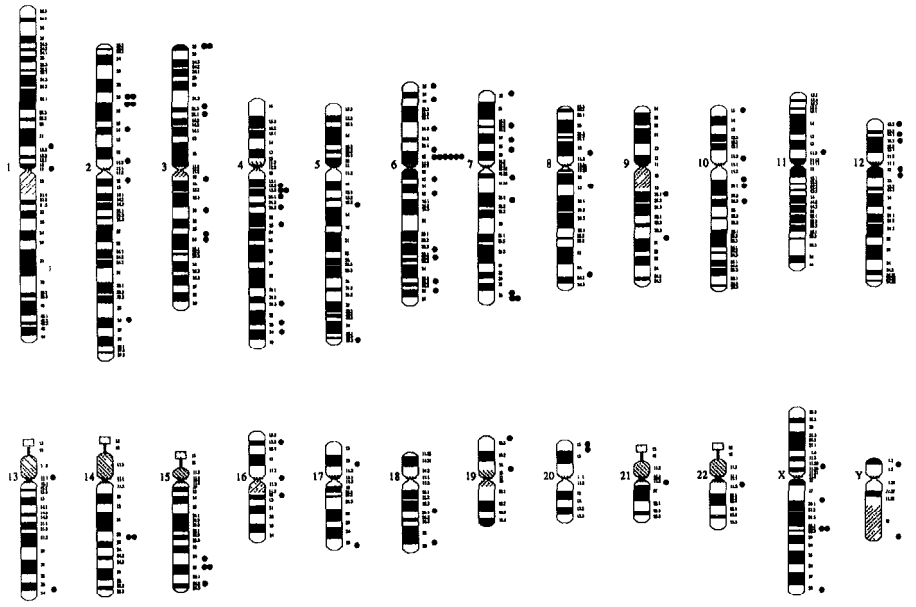

Fig. 1. Hybridization *in situ* of λ4c11 to human metaphase chromosomes. λ4c11 is a cloned genomic probe containing 5.3 kb of human DNA. It was labeled with tritium by random priming and hybridized as described in the text. The autoradiographic exposure was for 7 d. Forty-nine cells were analyzed, with a total of 100 grains on the chromosomes. Of this total, 17 (17%) were on chromosome 6, and of these, 7 grains (41%) were on band 6p12.

fore to minimize exposure to light, the slides should be stored in light-tight containers. Storage of the slides in dessicators under vacuum seems to slow down the aging process which eventually reduces their hybridization efficiency.

Other methods of banding can be used that do not require the BUdR treatment. These include methods of banding the chromosomes before hybridization, either by Lipsol-Giemsa (*5*) or Trypsin-Giemsa (*6*). These have several disadvantages: First, selected cells must be photographed to record the bands, since these are lost during the hybridization process. The photographed cells are relocated after autoradiography, and the labeled chromosomes identified by reference to the photograph. Only a proportion of the selected cells prove suitable for analysis, which means some labor and time has been wasted. Secondly, the staining treatment results in some loss of DNA from the chromosomes. Should the lost sequences include that of interest, the efficiency of hybridization will be reduced. A method of banding after hybridization using Wright's stain on cells synchronized with methotrexate has been described (*7*), but in my hands, this does not give the

consistency or quality of bands produced by the method I have described. Some methods for producing elongated chromosomes have depended on the incorporation of ethidium bromide into the DNA. This seems to reduce the efficiency of hybridization, probably because the dye is intercalated into the DNA, and the use of such methods is not encouraged.

2. Labeling the probe: In order to obtain the highest specific activity with ^{3}H, three labeled nucleotides are used. Since the incorporation of dGTP is less efficient, this base is not normally labeled. Of the two possible methods of labeling DNA, nick translation (*8*) and random oligonucleotide primed synthesis (*9*), the latter usually gives higher specific activity, but in some circumstances (*see below*), nick translation may be preferable.

   Random priming requires the substrate probe to be linearized, by cutting with a restriction endonuclease with a single recognition site. This method gives longer stretches of intact labeled DNA than nick translation. Most experiments with *in situ* hybridization are attempts to map the location of a sequence that exists in a single copy at one site in the haploid genome. However, if the probe is derived from a cloned genomic DNA sequence it may, in addition to the single copy sequence of interest, include sequences that are repeated and dispersed throughout the genome. Even if the probe is derived from a cDNA, it may still include sequences that are repeated elsewhere in the genome. In either of these cases, it may be difficult or impossible to distinguish the site of interest from the other labeled sites and background. In this case, hybridization of the repeated elements can be reduced or even eliminated by preannealing the probe with an excess of unlabeled sheared total DNA of the same species (*10*). This process (known as "stripping") requires that the fragments of labeled probe containing the repeats can form duplexes with the unlabeled total DNA (and thereby be prevented from taking part in the hybridization reaction) without compromising the unique sequence of interest. For this purpose, the lower mol wt product of nick translation may be preferable.

   Alternative radioisotopes are ^{125}I and ^{35}S. These have the advantage over ^{3}H that higher specific activities can be obtained using only one labeled nucleotide. However, because of the greater energy of emission from these isotopes, the resolution is poorer and background higher. Nevertheless, some groups have success, particularly with ^{125}I (*11*). Nonradioactive systems, most of which are based on the use of biotin incorporation into the probe, and its detection by immunologi-

cal methods, hold hope for the future (*12*). Despite encouraging reports (*13*), however, they are not yet sufficiently sensitive for routine use.

3. Hybridization: All glass pipets and containers, micropipet tips, microcentrifuge tubes, and solutions must be sterilized by autoclaving or filtration to reduce the risk of contamination with DNase. The number of slides suggested for an average experiment (six) is not an arbitrary figure. It provides one or two slides for flat staining (unbanded) to check the progress of the autoradiographic exposure, plus four slides for banding and full analysis. This should give an adequate number of analyzable metaphase chromosomes to obtain a result (*see above*).
4. Analysis: It is possible to carry out analysis of grain distribution by photographing suitably banded and labeled cells, identifying the labeled chromosomes on the photograph, and transferring the accumulated data to an idiogram. This has the advantage of providing a permanent photographic record of the analyzed cells, but the disadvantage that the autoradiographic grains are frequently in a different focal plane from that of the chromosomes. In half-tone photographs, these are not always readily distinguished from stain debris. The method that I use, however, stores the permanent data in the form of the slides themselves, with microscope vernier references to identify the analyzed cells.

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