In Situ Hybridization at the Electron Microscope Level: Hybrid Detection by Autoradiography and Colloidal Gold

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ABSTRACT *In situ* hybridization has become a standard method for localizing DNA or RNA sequences in cytological preparations. We developed two methods to extend this technique to the transmission electron microscope level using mouse satellite DNA hybridization to whole mount metaphase chromosomes as the test system. The first method devised is a direct extension of standard light microscope *in situ* hybridization. Radioactively labeled complementary RNA (cRNA) is hybridized to metaphase chromosomes deposited on electron microscope grids and fixed in 70% ethanol vapor; hybridization sites are detected by autoradiography. Specific and intense labeling of chromosomal centromeric regions is observed even after relatively short exposure times. Interphase nuclei present in some of the metaphase chromosome preparations also show defined patterns of satellite DNA labeling which suggests that satellite-containing regions are associated with each other during interphase. The sensitivity of this method is estimated to be at least as good as that at the light microscope level while the resolution is improved at least threefold. The second method, which circumvents the use of autoradiographic detection, uses biotin-labeled polynucleotide probes. After hybridization of these probes, either DNA or RNA, to fixed chromosomes on grids, hybrids are detected via reaction with an antibody against biotin and secondary antibody adsorbed to the surface of colloidal gold particles (~20 nm in diameter). Gold particles bind specifically both directly over centromeric heterochromatin and along the associated peripheral fibers. Labeling is on average ten times that of background binding. This method is rapid and possesses the potential to allow precise ultrastructural localization of DNA sequences in chromosomes and chromatin.

In situ hybridization has become a classical method for mapping DNA sequences in cytological preparations and it has been used extensively to map repeated genes in polytene and metaphase chromosomes at the light microscope (LM) level (see reference 1 for review). Recent refinements, which enhance the efficiency of hybridization and the sensitivity of hybrid detection, have made it possible to identify and localize specific cellular or viral DNA and RNA sequences even when present in low copy number (2–12). Furthermore, by using high molecular weight radioactive-probe networks (3), or by including dextran sulfate in the hybridization mixture (13, 14), unique sequences have been mapped to metaphase chromosomes after autoradiographic exposure of 5–22 d.

A few attempts have been made to extend *in situ* hybridization to the electron microscope (EM) level. However, most of these involved hybridization to tissue sections, tissue blocks, or whole cells (see reference 15 for review); no general method is available for hybridization to whole mount metaphase chromosomes or chromatin spreads. Such a method would (*a*) allow gene mapping in systems where the metaphase chromosomes are very small (e.g., *Drosophila*, avian microchromosomes, double minute chromosomes), (*b*) provide a technique for fine structure mapping of genes, (*c*) permit investigation of ultrastructural features of chromosome organization, and (*d*) facilitate the analyses of nascent transcripts in chromatin preparations made by the Miller spreading technique (16, 17), thus permitting positive identification of actively transcribing sequences in chromatin and allowing a detailed study of their

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 NOVEMBER 1982 609-618

organization as well as of the structure of the chromatin underlying these regions.

Because of the potential use of a general technique for EM level *in situ* hybridization, we set out to develop the necessary methodology using mouse satellite DNA and whole mount metaphase chromosomes as a simple, well-characterized test system. Since these DNA sequences reside in the centromeric heterochromatin of all mouse chromosomes except for the Y (18, 19), we could rapidly evaluate experiments by examining the centromere region of any metaphase chromosome without the requirement for specific chromosome identification. We first show that it is possible to perform *in situ* hybridization to chromosomes on EM grids using autoradiographic detection. Specific labeling of centromeric heterochromatin is obtained with at least a threefold improvement in resolution over the LM.

To achieve the variety of objectives described above, it is essential to employ a method for detecting hybridized probes with greater speed and resolution than that provided by EM autoradiography. Several groups have attempted to develop sensitive procedures for detecting nonradioactively labeled polynucleotides. Fluorescence detection methods have been described that employ antibodies specific for DNA:RNA hybrids (20, 21) or RNAs that are 3′-end labeled with fluorochromes (22–24). However, fluorescence techniques are unsuitable for EM studies. Manning et al. (25) developed an innovative scanning EM method based on avidin-polymer sphere binding to biotin-coupled nucleic acid probes hybridized to polytene chromosomes. Recently, Langer et al. (26) synthesized nucleotide derivatives directly modified with biotin. Such derivatives are incorporated enzymatically into DNA and RNA molecules that can function efficiently as hybridization probes (26–28). Hybrids are detected in the LM after reaction with a rabbit antibody specific for biotin (RAB) and then either fluorescent or enzymatic markers coupled to a second antibody against rabbit IgG. This approach for preparing and detecting nucleic acids labeled with biotin seemed the one most readily adaptable for EM studies.

Thus, we developed a method for detecting biotin-substituted probes hybridized to chromosomes on grids which employs RAB and secondary antibody adsorbed to colloidal gold particles. This technique provides a simple and rapid method for mapping DNA sequences on chromosomes at the EM level.

MATERIALS AND METHODS

Cell Culture and Chromosome Preparation

Mouse L929 cells (American Type Culture Collection, Rockville, MD) were maintained either in Joklik's modified suspension medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Irvine Scientific, Irvine, CA) or in synthetic medium, Synmed (Centaurus, Anaheim, CA), plus glutamine. Metaphase cells were obtained by selective detachment from semiconfluent cultures after incubation with Colcemid (Gibco Laboratories) at 50–80 ng/ml for 4–12 h. Cells were pelleted by centrifugation at 1,000–2,000 rpm for 10 min at room temperature in a Sorvall GLC-2 centrifuge. Pellets were resuspended in a small volume of growth medium (e.g., cells from a T25 flask were resuspended in 1–1.5 ml).

Cells were lysed by gently mixing an equal volume of cell suspension and 1.0% Nonidet P-40 (NP-40, BRL, Bethesda, MD) (pH 8–9). Alternatively, cells were lysed by 1:150 dilution in 1 mM Tris (pH 7.4); this mode of lysis results in somewhat less condensed chromosomes. Gold EM grids of either 400-mesh (Ted Pella, Inc., Tustin, CA) or 460-mesh hexagonal pattern (Polaron, Doylestown, PA) were coated with 1% parlodion and a thin layer of carbon. Samples of lysed metaphase cells were deposited onto grids by centrifugation through 0.5 M sucrose (pH 8–9), essentially as described by Rattner et al. (29). Grids were rinsed in 0.1% Photoflo (Eastman Kodak, Rochester, NY) and air-dried while held in forceps. Dry grids were stored on filter paper in petri dishes at room temperature and used within 12 wk. All electron microscopy was performed on a Siemens 1A operated at 60 or 80 kV.

Preparation of Mouse Satellite DNA and ^{3}H-cRNA

Mouse satellite DNA was a gift from Dr. Lorraine Lica (University of California, Irvine). Satellite sequences were purified from L929 cells and separated from main band DNA by three cycles of equilibrium centrifugation in cesium chloride plus Hoechst A33258 (Calbiochem-Behring Corp., La Jolla, CA), according to Manuelidis (30). Tritium-labeled RNA complementary to this DNA was prepared using *Escherichia coli* RNA polymerase (the generous gift of Dr. Michael Chamberlin, University of California, Berkeley) and ^{3}H-nucleoside triphosphates (New England Nuclear, Boston, MA), essentially according to Pardue and Gall (31). RNA complementary to *Clostridium perfringens* DNA (Sigma Chemical Co., St. Louis, MO) was synthesized using the same protocol. The specific activity of radio-labeled cRNA was estimated to be 10^6 cpm/μg. Bio-cRNA was prepared according to an identical protocol, except that biotin-labeled UTP (Bio-UTP) (26) was substituted for UTP.

Nick Translation of Mouse Satellite DNA

Nick-translated DNA probes were prepared essentially according to Rigby et al. (32), except that most reactions were run with biotin-substituted deoxy-UTP (Bio-dUTP). 1 μg of DNA was labeled by *E. coli* DNA polymerase I in the presence of 25 μM each dATP, dCTP, and Bio-dUTP, plus 10 μCi ^{3}H-dATP (14.2 Ci/mmol), after DNase I nicking. Control reactions were carried out in parallel with dTTP instead of Bio-dUTP. Reactions were terminated by the addition of EDTA and the DNA was purified by Sephadex G-50 chromatography. Samples were made 0.1 M sodium acetate (pH 5), tRNA was added (150 μg/ml final concentration), and the DNA was ethanol precipitated.

One DNA sample was labeled using a BRL nick translation kit according to the supplier's instructions with the substitution of Bio-dUTP for dTTP. This reaction was terminated with EDTA and the DNA was phenol-extracted. Most of the resulting phenol phase was discarded and the remainder of the sample was dialyzed against 0.01 M Tris (pH 8), 0.1 mM EDTA. DNA containing biotin nucleotide is referred to as Bio-DNA or Bio-sat DNA (for biotin-labeled satellite DNA). DNA labeled in parallel control reactions with dTTP is referred to as T-DNA.

Colloidal Gold Preparation and Labeling

Gold colloids were prepared as described by Geoghegan and Ackerman (33). 500 ml of aqueous 0.01% wt/vol gold chloride ($HAuCl_4 \cdot 3H_2O$; Fisher Scientific, Pittsburgh, PA) was reduced with 1% wt/vol sodium citrate using 13 ml or 20 ml. The resulting average particle sizes, 20 and 18.5 nm, respectively, were determined relative to 85-nm latex size markers. The protein concentration required for stable labeling of gold colloids was determined by the salt flocculation assay (33) with gold sols adjusted to pH 7.5–8.5. For preparation of stock antibody-labeled gold particles, 10 ml of gold colloid was adjusted to the appropriate pH and mixed with the appropriate amount of secondary antibody (10–20 μg protein/ml colloidal gold, as determined above). After 10 min, polyethylene glycol 20M (PEG 20M, Union Carbide Corp., San Diego, CA) was added to a final concentration of 0.5 mg/ml and unbound antibody was removed by centrifugation at 53,000 *g* for 1.5 h at 4°C in polycarbonate centrifuge tubes in a Beckman J21B centrifuge (Beckman Instruments, Spinco Div., Palo Alto, CA). The resulting pellet was usually very soft, although small dark clumps were occasionally observed. The supernatant, containing unbound antibody, was removed carefully and the pellet resuspended in 10 ml of buffer B (0.15 M NaCl, 0.05 M Tris [pH 7.0], 1 mM $MnCl_2$, 0.5 mg/ml PEG 20M). A second pellet was obtained by centrifugation as before and it was also resuspended in buffer B. Aggregates were removed by low speed centrifugation (400–700 *g*) for 10–20 min. Antibody-labeled colloids were stored at 4°C. These stocks usually remained active and free of precipitates or aggregates for many weeks. Preparations which turned dark or developed precipitates were discarded.

LM In Situ Hybridization and C-banding

Metaphase squashes were prepared from Colcemid-arrested L929 cells using 45% acetic acid fixation; these squashes were hybridized to ^{3}H-cRNA against mouse satellite DNA according to Pardue and Gall (31) with the addition of the 2 × SSC incubation (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate [pH 7.0]) described by Bonner and Pardue (34). Approximately 50,000 cpm was applied to each slide. For C-banding, chromosomes were treated as for *in situ* hybridization with the elimination of the RNase treatments, and incubated in 6 × SSC without nucleic acid probe. After this mock hybridization, slides were stained with

Giemsa's (Harleco Azure B, Scientific Products, McGaw Park, IL) in 0.01 M phosphate buffer (pH 7.2). Slides were analyzed using an Olympus BHA microscope and photographed on Kodak High Contrast Copy film.

EM In Situ Hybridization With Autoradiographic Hybrid Detection

Whole mount chromosome preparations on gold grids were fixed in 70% ethanol vapor for 4–15 h at room temperature and then air-dried. Ribonuclease (RNase) treatment to remove endogenous RNA was usually omitted since its inclusion did not alter the results obtained. Chromosomes were denatured for 2 min at room temperature in 2 × SSC adjusted to pH 12 with NaOH. Specimens were then dehydrated in three 5-min rinses each of 70% and 95% ethanol, followed by air-drying. For convenience during denaturation and ethanol dehydration steps, grids were carried in single grid holders (Ernest Fullam, Schnectady, NY). Use of these holders makes it possible to manipulate many grids simultaneously. For best results, grids were removed from holders and held in forceps for the drying steps.

For hybridization, grids were placed in a petri dish in individual drops (10–50 μl) of 6 × SSC or 6 × TNS (1 × TNS = 0.15 M NaCl, 0.01 M Tris [pH 6.8]) containing 30,000–50,000 cpm/10 μl drop of ^{3}H-cRNA. The petri dish was floated on hybridization buffer in a larger sealed plastic dish. Hybridization was carried out for 4–24 h at 60–65°C. After hybridization, grids were immediately rinsed in a large volume of 2 × SSC and then given a series of 2 × SSC rinses followed by treatment with RNase A (Worthington Biochemical Corp., Freehold, NJ) (20 μg/ml for 30 min at room temperature). The grids were then given additional 2 × SSC rinses, dehydrated with 70 and 95% ethanol, and air-dried. Grids were coated by the loop method (35) with Ilford L-4 emulsion (Polysciences Inc., Warrington, PA) diluted with four parts of water. After coating, grids were secured to microscope slides with tape and stored in light-tight boxes with desiccant at 4°C. Before developing, grids were warmed to room temperature for at least 30 min to prevent wrinkles in the emulsion. Autoradiographs were developed in Kodak Microdol X for 3–5 min at 18–24°C, rinsed in water, fixed in 15% thiosulfate or Kodak Rapid Fixer for 5 min at the same temperature, and dried out of 0.1% Photoflo. For best results, solutions were fresh and Millipore-filtered before use. As a control, ^{3}H-cRNA made against *C. perfringens* DNA, which has about the same base composition as mouse satellite DNA, was hybridized to whole mount chromosomes and EM autoradiography was performed as described.

EM In Situ Hybridization Using Biotin-substituted DNA

The following hybridization protocol yielded reproducible and specific chromosomal labeling while preserving chromosome morphology. Several modifications of this procedure have been examined and these will be mentioned where appropriate in the text. Chromosomes on grids are fixed with 70% ethanol vapor, as described above, followed by 0.5% glutaraldehyde. A glutaraldehyde (Ted Pella) solution of 0.5% in 2 × SSC is prepared by diluting an 8% stock from a freshly opened ampoule. Grids in single grid holders are fixed for 20 min at room temperature with gentle stirring, followed by three 2 × SSC rinses (with stirring) for 10 min each. Grids are then incubated at 70°C in 2 × SSC for 30 min (34), followed by digestion with 1 μg/ml of Proteinase K (EM Laboratories, Elmsford, NY) in 0.02 M Tris (pH 7.4), 2 mM $CaCl_2$ at 37°C for 15 min (2). Denaturation of chromosomal DNA was performed as described above for autoradiographic experiments. The hybridization buffer used was modified from that described by Brahic and Haase (2) by the addition of 10% dextran sulfate (Pharmacia Fine Chemicals, Piscataway, NJ) as used by Gerhard et al. (13) and Harper and Saunders (14). The buffer also contained 50% formamide (FA), 10 mM Tris (pH 7.4), 1 mM EDTA, 0.6 M NaCl, 0.02% Ficoll, 100 μg/ml *E. coli* DNA, ~100 μg/ml tRNA, 0.5 mg/ml bovine serum albumin, and probe nucleic acid at ~0.8 μg/ml. FA (MCB Reagents, Norwood, OH, or BRL) was twice recrystallized and then deionized with mixed bed resin Amberlite MB-3 (Sigma Chemical Co.) before use. The hybridization medium was heated in a boiling water bath for 3 min to denature the DNA and then quenched on ice. Samples were hybridized in siliconized Reacti-vials (Kontes Co., Vineland, NJ) in 30–50 μl reactions at 30°C. Best results were obtained when two grids, placed back-to-back, were reacted in one vial. After hybridization, grids were washed in 2 × SSC at 37°C or room temperature at least three times for 10 min each to remove unhybridized probe before antibody reactions.

EM In Situ Hybridization Using Biotin-substituted RNA

The hybridization procedure for Bio-cRNA followed that of Stuart and Porter (36). After 0.1% glutaraldehyde fixation and subsequent rinses, chromosomes on grids were denatured and hybridized in 2 × SSC, 50% FA containing tRNA, and Bio-cRNA (or, for controls, cRNA made with UTP) exactly as described (36). This procedure was used since it is simple and has been reported by these investigators to result in superior morphology and excellent hybridization.

Reactions of Hybridized Samples with Anti-Biotin and Secondary Antibody-Colloidal Gold

RAB was prepared as described by Langer-Safer et al. (28). Purified antibody with carrier BSA (5–10 mg/ml) was stored in PBS (per liter, 10 g NaCl, 0.25 g KCl, 1.44 g Na_2HPO_4, 0.25 g KH_2PO_4) at −20°C. Rhodamine- and fluorescein-goat anti-rabbit IgG and sheep anti-rabbit IgG (Rh-GAR, FITC-GAR, and SAR, respectively), were from Miles Laboratories (Elkhart, IN).

In all experiments, grids were not permitted to dry after the rinse before reaction with antibody. Grids were treated with 2–4 μg/ml RAB diluted in PBS or colloidal gold buffer A (0.15 M NaCl, 0.05 M Tris [pH 7]) for 1–24 h at room temperature or 37°C. They were then washed with the appropriate buffer for at least 3 × 10 min before secondary antibody reaction. Colloidal gold preparations labeled with secondary antibody as described above were pelleted immediately before use to remove unadsorbed protein. 1 ml of antibody-labeled gold stock was centrifuged at 16,500 *g* for 30 min at 5°C: the supernatant was discarded and the pellet was resuspended in 1 ml of buffer B for a 1× solution or diluted as required in buffer B for a less concentrated solution. Grids were incubated in either goat or sheep anti-rabbit immunoglobulin on colloidal gold (IgG-CG) at a 1/10 dilution of the stock for 30 min to 24 h. After incubation, unreacted IgG-CG was removed by at least three 10-min rinses in buffer B. Grids were then rinsed in 0.1% Photoflo and air-dried before examination in the EM.

Two types of control experiments were performed. First, hybridization was carried out in buffer without probe or in buffer containing T-DNA or UTP-cRNA, followed by reaction with primary and secondary antibody as described above. In addition, reaction with primary antibody was omitted from other controls to test the specificity of the signal observed.

Quantification of Gold Labeling

Tracing paper was placed over photographs of labeled chromosomes and an area was circumscribed around the centromeric heterochromatin which included the heavily labeled peripheral region. Equal areas on three different noncentromeric chromosomal regions plus supporting film were marked similarly. The number of gold particles in each region was counted and the amount of labeling on centromeric regions relative to the average of the three background regions was used to calculate the signal to noise ratio of this method.

RESULTS

LM In Situ Hybridization to Mouse L929 Chromosomes

Hybridization of ^{3}H satellite cRNA to mouse L929 metaphase chromosomes was first examined at the LM level. Most cells of this heteroploid line contained 60–70 chromosomes, including several metacentric chromosomes. In the autoradiographs (e.g., Fig. 1 *a*), silver grains are present over the centromeric heterochromatin of almost all of these chromosomes. Additional sites of hybridization can be seen at the telomeres and on interstitial arms of a few of the chromosomes.

One large metacentric marker chromosome (solid arrow) contains multiple regions of hybridization, although the exact number is difficult to discern from the dispersed pattern of silver grains. Heterochromatin staining by the C-banding technique demonstrates three C-band positive heterochromatic constrictions in each arm of the marker chromosome, in addition to the stained centromeric region (Fig. 1 *b*). The homologous staining pattern in both arms of this chromosome suggests that it is an isochromosome.

Further examination of the C-band patterns of all the chromosomes shows that the size of the centromeric heterochromatin block differs among individual chromosomes and that it is not correlated with chromosome arm length; we estimated that these C-band regions range from <10% to ~30% of the chromosome length. The ^{3}H-cRNA hybridization levels, as

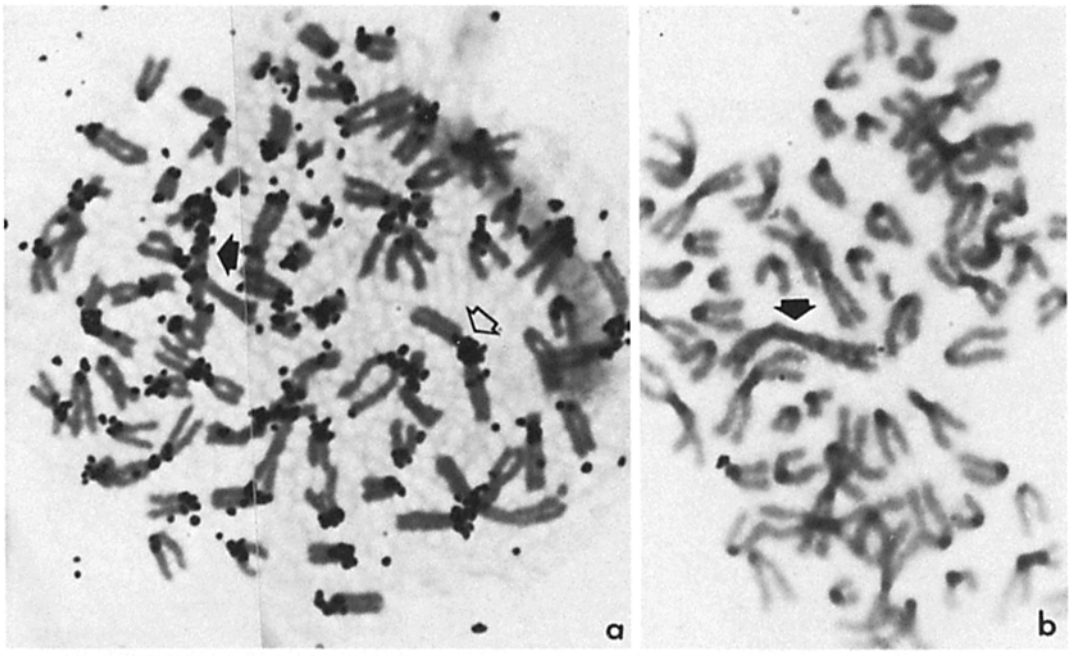

FIGURE 1 Light micrographs of metaphase spreads from mouse L929 cells. (*a*) Autoradiograph of chromosomes after *in situ* hybridization with ^{3}H-cRNA. Centromeric regions of most chromosomes are labeled. The open arrow indicates a chromosome with labeling at two sites and the solid arrow indicates the large metacentric marker chromosome with multiple sites of hybridization. Exposure 11 d. × 3,750. (*b*) C-banded chromosomes to reveal heterochromatin. The size of the heterochromatic block varies among the chromosomes. The metacentric marker chromosome which shows multiple sites of banding is indicated by the arrow. × 3,750.

judged by the density of silver grains (Fig. 1 *a*), appear to correspond to the size of the C-banded regions on those chromosomes recognizable by morphology (Fig. 1 *b*). Thus, it is clear that the ^{3}H-cRNA hybridized at the appropriate chromosomal loci and that it could be used for our EM studies.

EM In Situ Hybridization Using Radiolabeled Probes

Chromosomes prepared by standard acid fixation protocols preserve little ultrastructural detail when viewed in the EM (not shown). The modified Miller spreading procedure for whole mount metaphase chromosomes described by Rattner et al. (29) provides specimens with excellent chromosome morphology, including distinct centromeric heterochromatin, attached kinetochores, and higher order nucleosomal chromatin fibers. Chromosomes were deposited onto grids according to this procedure and specimens fixed in 70% ethanol vapor. Reasonably good chromosome morphology was maintained even after all the steps of *in situ* hybridization, and the amount of material lost from grids during the various steps required was reduced.

Chromosomes were observed to occur singly and in groups; chromosomes in groups were always associated at their centromeric regions, possibly reflecting the affinity of these regions for each other in vivo. It should be noted that none of the preparations shown here are stained or shadowed and, as a result, there is less ultrastructural detail than would be seen with these treatments. In these studies we did not use contrast enhancing treatments since relevant loci are visible due to the inherent electron density of the sample.

^{3}H-satellite cRNA was hybridized to chromosomes on EM grids as described in Materials and Methods. EM autoradiographs from typical hybridizations are shown in Fig. 2 *a–c*. In all cases specific labeling was observed directly over the centromeric regions of chromosomes, a result which was expected both from the LM experiments and from the extensive literature demonstrating that mouse satellite DNA is localized predominantly in centromeric heterochromatin (18, 19, 37). As shown in Fig. 2 *a–c*, the labeling typically covers the entire centromeric region. After very long exposures (~1 y, Fig. 2 *c*), centromeric regions are saturated with silver grains that obscure the underlying chromosomal details. Shorter exposures provide results very much like that derived from LM hybridization: silver grain density corresponds to the size of the heterochromatic region of each chromosome.

It has been shown that sequences which hybridize to mouse satellite DNA are present at other noncentromeric sites in the mouse genome (38–40). We also observed chromosomes with labeling at additional noncentromeric sites (e.g. Fig. 2 *b*). In addition to centromeric labeling, the metacentric marker chromosome shown in Fig. 2 *b* was labeled at sites corresponding to the heterochromatic constrictions, consistent with the LM results indicating the presence of sequences homologous to satellite DNA in these regions.

As one test for hybridization specificity, chromosomes were hybridized with ^{3}H-cRNA made from *C. perfringens* DNA. This DNA was chosen for its high (A+T) content of 73.5%, similar to that of mouse satellite. Autoradiographic exposures of up to 1 yr showed no specific chromosomal labeling (data not shown).

Although the specimens were prepared from metaphase-arrested cells, there are some interphase nuclei in these preparations. These nuclei were also labeled following hybridization. A labeled nucleus is shown in Fig. 2 *d*. Silver grains appear in clumps and large clusters, some of which are over nucleolus-associated heterochromatin. In some nuclei (Fig. 2 *d*), the grain clusters are very discrete, while in other nuclei the clusters are large and dispersed, filling most of the nuclear area. These different patterns may represent nuclei at different stages of the cell cycle.

EM In Situ Hybridization Using Biotin-labeled Nucleic Acids and Colloidal Gold

Mouse satellite DNA, biotin-labeled by nick-translation, or as Bio-cRNA, was hybridized to chromosome preparations on grids as described above. After hybridization and rinses, hy-

brids were detected by an antibody sandwich technique using colloidal gold as the electron opaque tag.

Figs. 3 and 4 show examples of chromosomes hybridized with nick-translated Bio-DNA and treated with the appropriate series of antibody reactions. Centromeric regions are readily distinguished and are labeled with the highly electron-dense gold particles. Gold particles are seen either evenly distributed over the centromeric heterochromatin itself with additional labeling of peripheral fibers (Fig. 3 *a*) or as a halo around the periphery of the centromeric heterochromatin (Figs. 3 *b* and 4). The pattern observed was always consistent among all the grids of a given experiment. We believe that the peripheral labeling

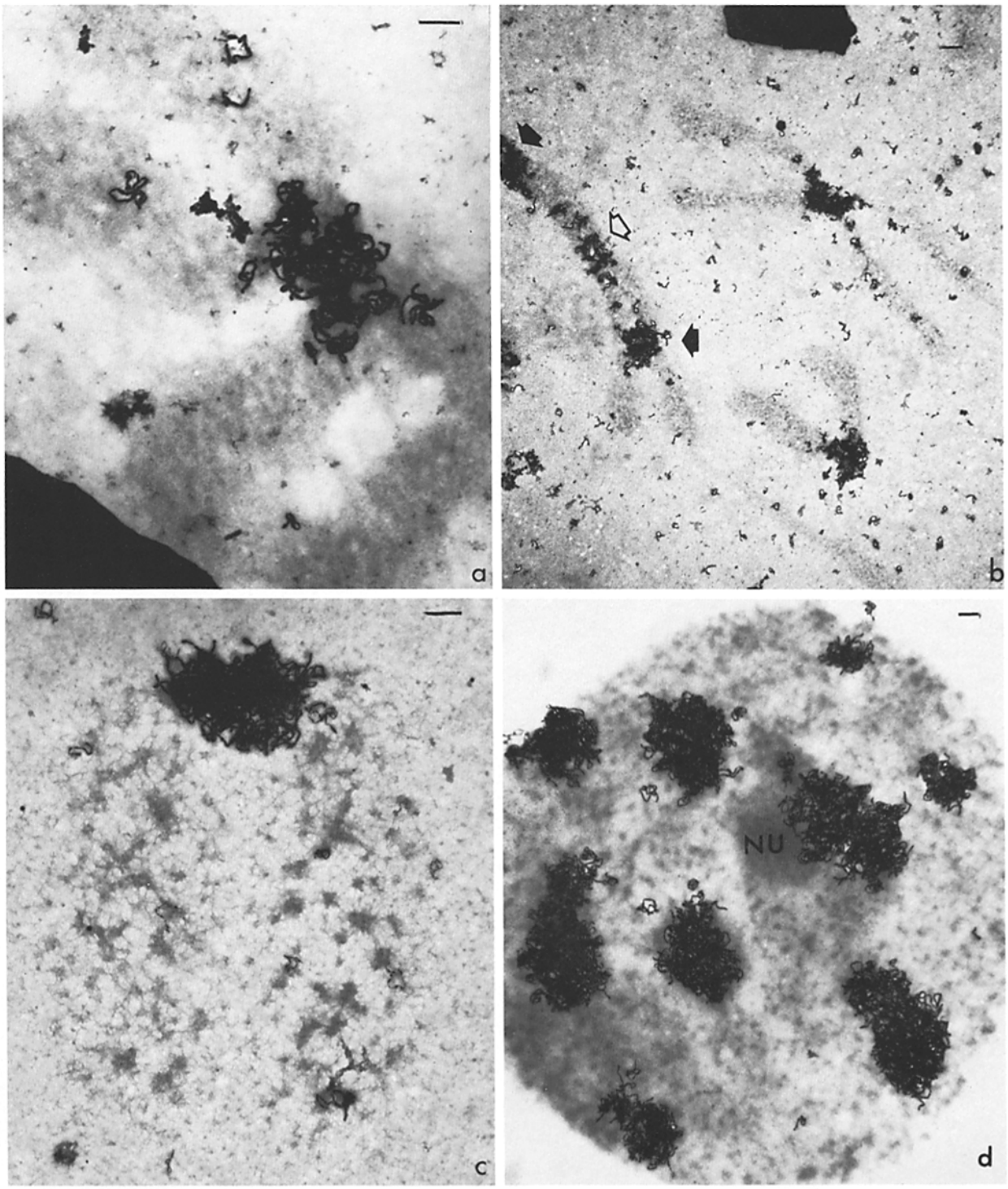

FIGURE 2 Electron microscope autoradiographs of mouse L929 chromosomes (*a–c*) and a nucleus (*d*) after *in situ* hybridization on grids with ^{3}H-cRNA against mouse satellite DNA. Centromeric heterochromatin in (*a–c*) is labeled with silver grains. Part of the marker chromosome is seen in (*b*) but the remainder is obscured by the grid bar. Two labeled sites show tightly clustered grains (solid arrows), whereas grains are dispersed in the central site (open arrow). In *d* the interphase nucleus exhibits discrete clusters of grain tracks; one or more labeled sites are usually associated with the nucleolus (*NU*). Exposure times were 13 d for *a* and *b*; 1 yr for *c*; 11 mo for *d*. Bars, 1.0 μm. *a*, × 6,500. *b*, × 3,000. *c*, × 8,000. *d*, × 5,000.

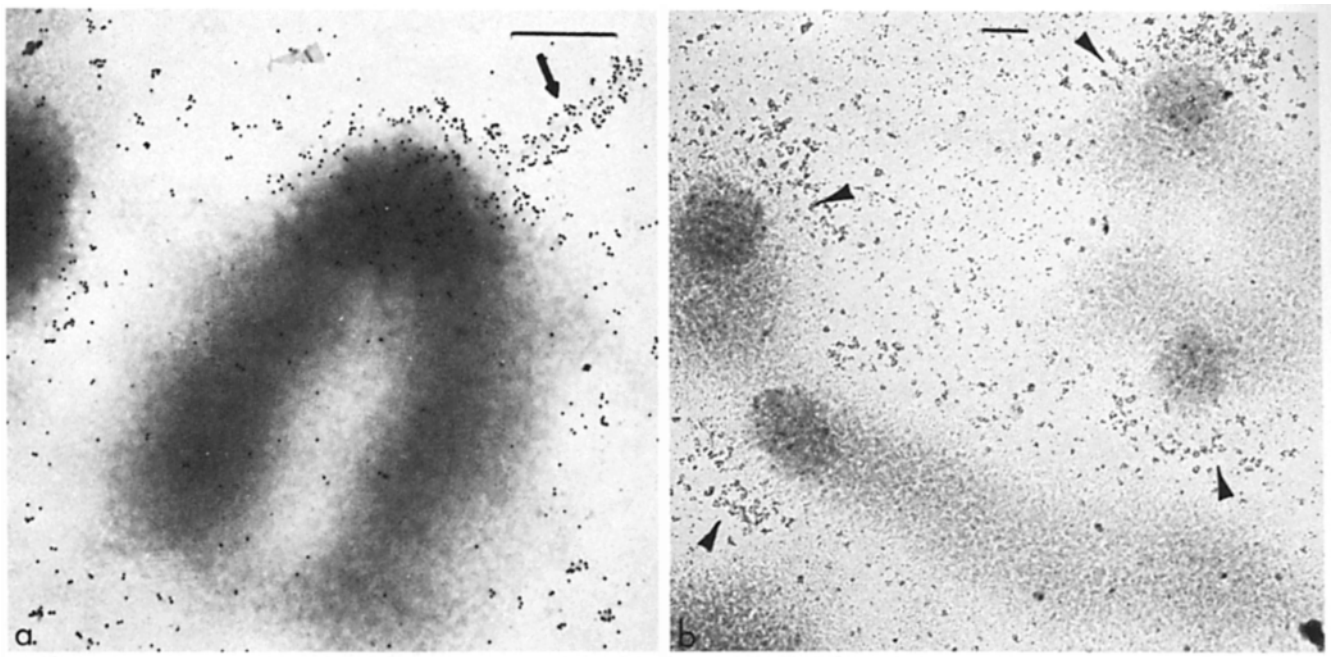

FIGURE 3 Electron micrographs of mouse L929 metaphase chromosomes hybridized with Bio-sat DNA followed by two step Ab-CG detection. Gold particles cover centromeric regions but are present at a higher density over peripheral chromatin fibers. The arrow in *a* indicates a prominent chromosomal fiber extending from the chromosome. In *b*, several chromosomes show centromeric labeling which is primarily peripheral (arrowheads). Reaction in *a* was with FITC-GAR-CG for 1 h. Reaction in *b* was with Rh-GAR-CG for 24 h. Bars, 1.0 μm. *a*, × 14,000. *b*, × 6,500.

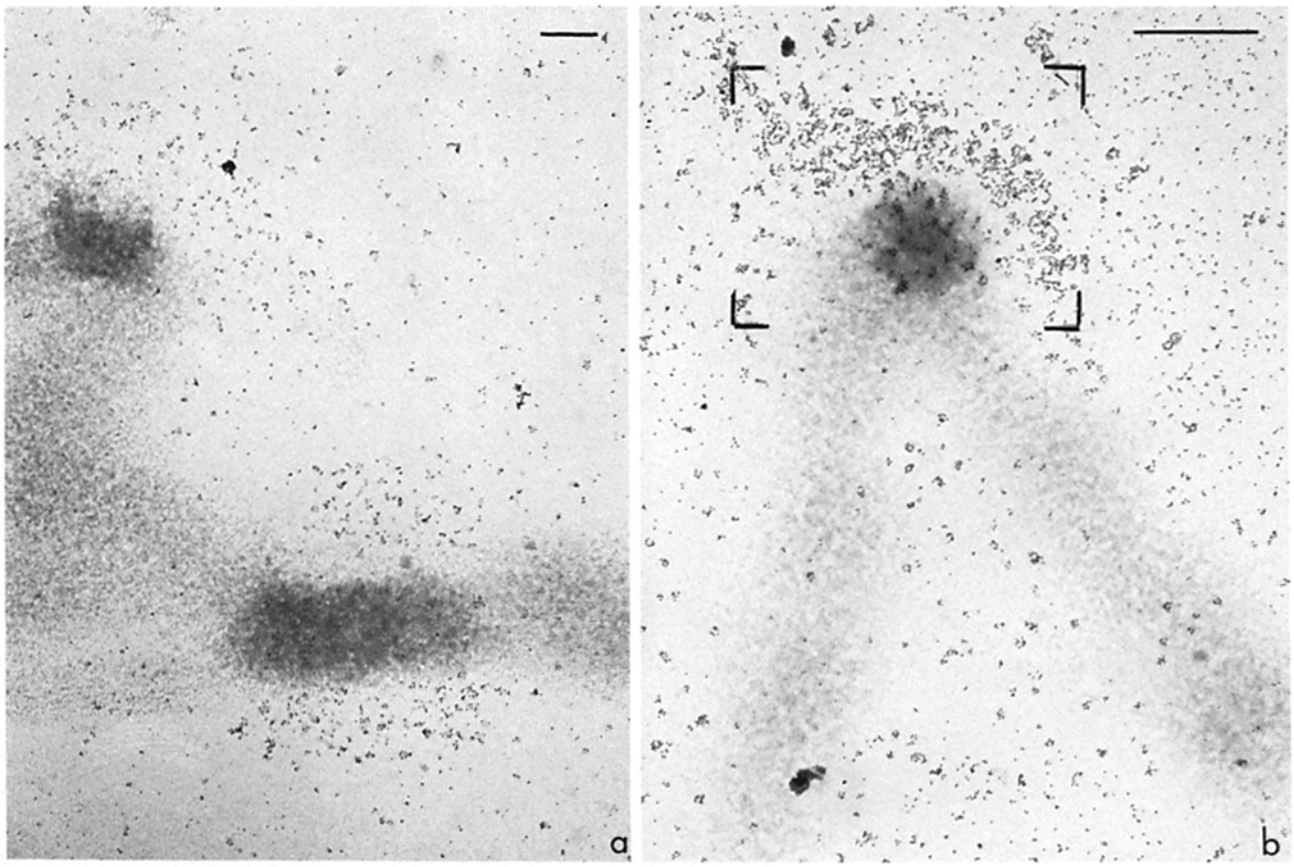

FIGURE 4 Time-dependent labeling of secondary antibody on colloidal gold. (*a*) Incubation with secondary antibody-CG was for 1 h. (*b*) Incubation was for 24 h. The circumscribed area was selected for gold particle quantification of this chromosome. Bars, 1.0 μm. *a*, × 8,000. *b*, 12,000.

represents hybridization to DNA within chromatin fibers which are somewhat stretched or unfolded from the highly condensed centromeric heterochromatin. This is seen clearly in Fig. 3 *a* where gold labeling is intense along a fiber which extends some distance from the centromere region. Although we do not fully understand the source of these different patterns some possibilities will be discussed below.

We have been comparing different protocols in the prehybridization, hybridization, and antibody labeling steps in an attempt to determine an optimal set of conditions. Although these experiments are still in progress, some data are available. The two micrographs in Fig. 4 represent a comparison of reaction of chromosomes hybridized and labeled with RAB in the same experiment and then labeled with a secondary antibody-gold conjugate (Rh-GAR-CC) for either 1 h (Fig. 4 *a*) or 24 h (Fig. 4 *b*). Similar results were also obtained with SAR-CG. It is obvious that the longer labeling time increases the intensity of specific labeling as well as the level of background labeling. Nonetheless, in both cases chromosome arms routinely absorb less gold than comparable areas of the support film. In contrast to these results, no difference in the intensity of the signal was observed when the incubation time with the primary antibody was varied from 1 h to 24 h. This differential behavior may be a reflection of colloidal gold particle size that may sterically inhibit efficient delivery of the detector-antibody conjugate to the target sequences. In all the experiments described above, control chromosomes hybridized with T-DNA were carried through all the same steps and these chromosomes never showed specific centromeric heterochromatin labeling (data not shown, but see Fig. 5 *b*).

In addition to the experiments with nick-translated DNA, we carried out hybridizations using Bio-UTP-containing cRNA. The cRNA synthesis reaction in the presence of Bio-UTP is only ~10% as efficient as a parallel reaction with UTP. The cRNA/chromosome hybridizations followed the procedure described by Stuart and Porter (36) with hybrid detection as described for the Bio-DNA probes. Labeling is observed prominently over the centromere region (Fig. 5 *a*), with little peripheral fiber labeling. Also, the level of labeling is routinely less intense than that observed with the nick-translated probe (compare with Figs. 3 and 4). This is probably due to the inefficient labeling in the presence of Bio-UTP.

Fig. 5 *b* represents one of a series of control experiments in which chromosomes were hybridized with UTP-containing cRNA, followed by reactions with primary antibody and secondary antibody on colloidal gold. Neither centromeric nor chromosomal labeling is seen, arguing that the signal observed (Fig. 5 *a*), even though it is low in these experiments, is due to the specific interaction between antibiotin and biotin-containing hybrids and does not result from nonspecific adsorption of antibody or antibody-gold to the centromeric region. As noted above, similar results are seen when the probe was T-DNA.

We have attempted to quantify the specificity of gold labeling in these experiments using the procedure described in Materials and Methods. In general, there is usually a higher level of labeling with DNA:DNA compared to cRNA:DNA hybridization. The centromere region/background labeling ratio varies from 3.7 to 37, with a mean of 9.4, based on

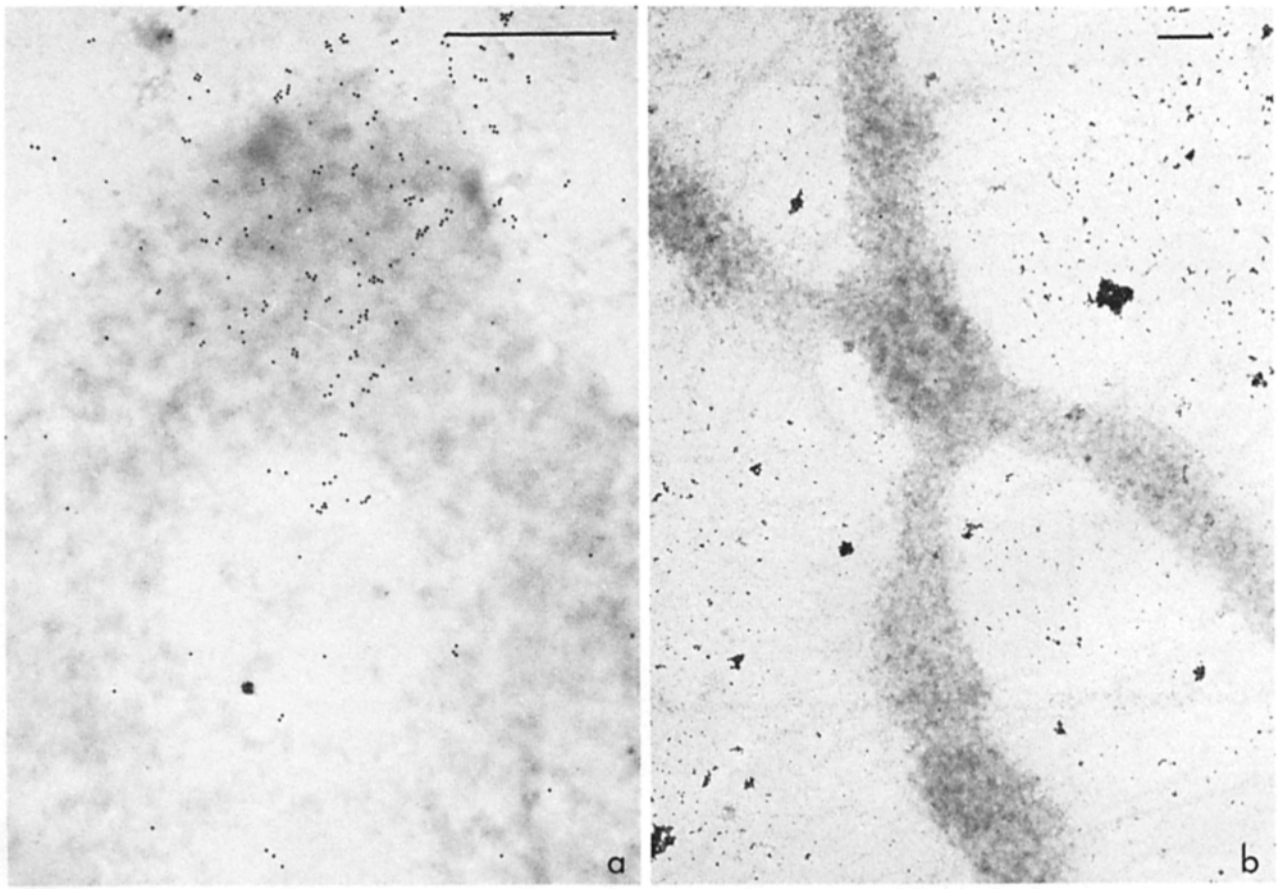

FIGURE 5 Comparison of hybridization with Bio-cRNA against mouse satellite (*a*) and U-cRNA (*b*). The centromeric region is labeled with gold only when biotinated probe is used. Hybridization was according to Stuart and Porter (36). Bars, 1.0 μm. *a*, × 23,000. *b*, × 7,500.

quantifying 36 chromosomes. This is to be compared with a typical control chromosome which showed a ratio of 0.9. Thus the technique described affords a substantial signal to noise ratio which may be improved in several ways as discussed below.

DISCUSSION

We have described procedures to extend in situ hybridization to whole mount metaphase chromosomes prepared by a minor modification of the Miller spreading technique with detection in the transmission electron microscope. Mouse satellite DNA was chosen as a test system for the development of EM level *in situ* hybridization because the centromeric localization of this sequence is well-documented (18, 19, 39–41), the DNA is readily obtained in pure form, and, since satellite DNA represents a large portion of the mouse genome, detection times should be minimal. The presence of satellite DNA on almost every chromosome also simplifies EM analysis since specific chromosome localization and identification is unnecessary. In addition, we felt that the increased resolution afforded by the EM could add to our understanding of the spatial arrangement of satellite DNA. Two different systems of hybrid detection were developed for localizing mouse satellite DNA in chromosomes and interphase nuclei from the heteroploid mouse L929 cell line.

Autoradiographic Detection of EM In Situ Hybrids

To compare our results directly to the published LM *in situ* hybridization results, we first developed an autoradiographic detection method. The chromosome preparation and hybridization methods used are simple and essentially follow published procedures for whole mount chromosome preparation (29) and for *in situ* hybridization to specimens on slides (2, 13, 14, 31). The fixation step is of critical importance in preserving morphology, maintaining adherance of chromosomes to the grid, and obtaining a strong hybridization signal. Because of the deleterious effects of acetic acid treatment on the ultrastructure of chromosomes, experiments were performed with fixation in ethanol vapor.

The data presented on autoradiographic detection of mouse satellite DNA of metaphase chromosomes in EM preparations provide some insight into satellite DNA organization in mouse L929 cells. For most chromosomes, satellite sequences appear to be distributed throughout centromeric heterochromatin in proportion to the amount of heterochromatic material. Because the satellite in these cells represents only 6% of the genome, whereas the estimated proportion of the genome which is centromeric is an average of 20%, determined by measurements of centromeric heterochromatin vs. chromosome lengths (42), this observation suggests that satellite DNA sequences are not organized as a single block, but are interspersed with nonsatellite DNA. Our cell line possesses a metacentric marker chromosome labeled at several constricted sites in addition to the main centromeric region. This pattern of labeling was seen both in LM and EM hybridization experiments. Interstitial or noncentromeric satellite sequences associated with constrictions have been observed in many cell lines (41, 43–51).

Certain chromosomes exhibit associations at metaphase such that centromere regions are closely associated and chromosome arms point away from centromere clusters. These clusters of centromeric regions may coalesce to form chromocenters which are visible in interphase nuclei. Determination of the exact number of chromocenters is somewhat subjective; however, the number of grain clusters we observed was much lower than the number of chromosomes (or centromeres) in these cells. Thus, satellite DNA-containing sequences appear to be associated in interphase nuclei (see also reference 52). In the nuclei examined in detail, chromocenters appear to be randomly distributed except for asosciation with the nucleolus. Note, however, that Colcemid treatment has been reported to interfere with normal nuclear positioning of chromatin regions (53). If one avoids use of this drug, *in situ* hybridization with EM level autoradiography should provide a promising method for mapping sequences within the interphase nucleus.

On the basis of the theoretical and experimental estimations of autoradiographic resolution as discussed by Jacob (54), we estimate the resolution in our autoradiographic experiments using tritiated probes to be 0.1–0.3 μm, compared to the reported resolution of $\sim$1 μm for LM autoradiography. Therefore, the adaptation of EM autoradiography to *in situ* hybridization has achieved at least a threefold increase in resolution. With this resolution, DNA sequences specifically associated with the kinetochore potentially could be resolved from DNA in other regions of centromeric heterochromatin.

An accurate estimate of the overall sensitivity or efficiency of the EM method requires a large number of measurements, most of which we can only estimate. These include the specific activity of the probe, the number of satellite DNA repeats per chromosome, the efficiency of hybridization, and the efficiency of autoradiographic detection. Because of the number of uncertainties in these factors, it is probably of more use to compare the grain densities in the light and electron micrographs (Figs. 1 *a* and 2 *a* and *b*). The same cRNA preparation was used for both hybridizations, hybridization conditions were very similar, and the exposure times were approximately the same (11 d for LM and 13 d for EM). The resultant grain densities are of the same order of magnitude, arguing that the sensitivity of the autoradiographic EM *in situ* hybridization procedure is at least that obtained at the LM level. The thinness of the emulsion layer used in EM ARG results in lower autoradiographic sensitivity. Thus, the similar EM and LM grain densities observed suggest that another factor(s) is responsible for the greater efficiency in the EM procedure: e.g., hybridization may be more efficient; loss of chromosomal DNA may be reduced; or self-absorption might be decreased in these specimens. We are pursuing a more precise estimate of sensitivity using probes to sequences which occur in lower copy numbers in the genome.

Although the autoradiographic procedure is adequate for certain problems, we found it difficult to reproducibly coat grids with a uniform monolayer of emulsion, despite our attempts to control darkroom temperature and humidity (35, 55, 56). We believe that the problem of variability in emulsion deposition is reflected by some variability in the level of labeling in a single experiment. The variability typically was confined to individual grids and, in the extreme cases, neither specific nor background silver grains were apparent.

We initially used EM autoradiographic *in situ* hybridization as an independent method for detecting hybrids while developing the Bio-CG detection method. Although EM ARG is technically demanding, it remains a reliable method for localizing sequences on chromosomes and in interphase nuclei. Furthermore, the ARG method should be useful for simultaneous detection of multiple probes where one is tritium-labeled and a second biotin-labeled. Such an approach would be particularly useful in studies of interphase nuclear organization and changes during the cell cycle. In addition to DNA sequence

mapping, a combination of in vivo RNA labeling and EM ARG of nuclei may be of use in determining the position of the genes coding for newly synthesized RNAs in nuclei. At present this cannot be done with the biotin-substituted nucleotide since it is not incorporated by eukaryotic RNA polymerases.

Nonautoradiographic Detection of In Situ: Hybrids

To provide a rapid and simple method for routine hybrid detection we turned to a nonautoradiographic system. The work by Manning et al. (25) described a promising detection system based on biotin-labeled probes and an appropriate tag coupled to a molecule with a high affinity for biotin. We were unsuccessful in adapting to our system the procedure described by these workers. However, the synthesis of biotin-substituted nucleotides and generation of biotin-specific antibodies (26–28) provided an ideal detection system. We chose colloidal gold as our reporter tag, for several reasons: it is inexpensive and simple to prepare in a variety of sizes; it is extremely electron-opaque; and it does not bind adventitiously to chromatin or chromosomes. Using this system, we showed that one can localize highly repeated mouse satellite sequences to centromeric regions in whole mount metaphase chromosomes. The protocol takes a day or two, depending upon the incubation times with primary and secondary antibodies, and the labeling is centromere region-specific. The data presented represent the first stage in the development of the nonautoradiographic detection method.

In developing this method, we tested many steps in the standard procedure given in the Materials and Methods, examining the effects on chromosome morphology and overall specific labeling. We determined that glutaraldehyde concentrations from 0.1 to 1.0% may be used (we have not tested concentrations out of this range) without alteration in labeling efficiency. In a single test of acetic anhydride treatment (57) as recommended by Tereba et al. (3), we found reduced specific labeling with no difference in background labeling. In the hybrid detection system we have also employed protein A-CG, using protein A from three different sources; in all cases, only slight centromeric labeling was observed. Background binding is relatively low in these experiments, but it might be reduced further by inclusion of a mild detergent in the labeling and rinse steps and/or by incubation with unlabeled heterologous antibody before specific antibody reactions.

With the available data we cannot compare the efficiency of the nonautoradiographic procedure to ARG. The overall efficiency of the new technique may be affected adversely by steric hindrance between reacting components at each step in the detection. First, biotin-nucleotide which is in a hybrid duplex may not be as accessible to reaction with other molecules as it is in solution. Second, gold particles of the size used (~20 nm in diameter) may render adsorbed antibodies less accessible to ligands in the central region of the centromeric heterochromatin. Finally, the net negative charge of the gold particles may cause them to be repelled from chromosomes. This last possibility could explain the very low background staining of chromosome arms in our experiments but may also contribute to inefficient labeling. The observation that antibody-peroxidase conjugates can be used to detect satellite DNA sequences in centromeric regions with high efficiency (52) supports these interpretations.

Although we do not have definitive data on those factors which determine the final level of labeling, modifications can be incorporated into the protocol to reduce possible problems. First, biotin-nucleotides can be synthesized with longer linker arms so that the biotinyl group is further from the immobilized hybrid and, therefore, should be more reactive. Preliminary results with DNA probes containing such derivatives along with 5 nm of gold indicate that significantly better hybridization signals can be obtained (S. Narayanswami and B. A. Hamkalo, unpublished data). De May et al. (58) have, in fact, shown massive labeling of antigens when antibodies are coupled to 5 nm of gold particles. Although these particles may be too small to use as a convenient tag for hybrid detection at low magnification, one can react samples with a mixture of large and small gold particles such that the large particles locate the hybrid region and the small particles provide the accessibility which may be necessary for optimal antigen-antibody complex formation. Finally, although standard commercial avidin preparations stain chromosomes nonspecificially and essentially irreversibly (59; N. J. Hutchison, unpublished data; P. R. Langer-Safer and D. C. Ward, unpublished data), new sources of repurified avidin which do not have this problem are available from Vector Systems (Burlingame, CA). Therefore, work is in progress using avidin on colloidal gold as a hybrid detection system.

The two patterns of hybridization observed with the biotin-CG system merit discussion. Hybridization over the centromere region is the expected result, based on the LM and EM autoradiographic experiments. However, except with cRNA, this pattern of hybridization is not always observed in the nonautoradiographic experiments. Rather, one also sees hybridization to a halo surrounding a centromeric region which itself is only lightly labelled. There may be some relationship between lowered accessibility due to fixation and the degree of labeling over centromeres since this labeling is typically lower than that observed when gold reacts at peripheral regions. Obviously this question also is related to the previous discussion of labeling levels.

An additional explanation for the observed peripheral labeling is based on the fact that all hybridizations, except those using the Stuart and Porter (36) protocol, were performed in the presence of dextran sulfate. This compound increases the efficiency of hybrid detection (13, 14, 60), possibly by forming DNA networks in solution which then hybridize to target sequences and amplify the hybridization signal. Since these networks may be quite large, it is reasonable that they would react preferentially with the chromatin fibers which extend out from the highly condensed centromeric region and, therefore, produce an amplified signal which gives a halo effect. Although dextran sulfate may contribute to this labeling pattern, it is not solely responsible since preferential peripheral labeling is not always observed in its presence. Beyond differences in protocol, differences in the way chromatin fibers are adsorbed to EM grids may contribute to the labeling pattern observed.

Future Applications of the EM In Situ Hybridization Techniques

The autoradiographic hybrid detection method presented is particularly suited to the localization of DNA sequences in interphase nuclei. Using this method it should be possible to identify the positions of specific sequences in different cell types and at different stages of the cell cycle. Such data may be of use in understanding the relationship between nuclear organization and gene expression.

The biotin-antibody-colloidal gold methodology has rather different applications. We do not yet have a measure of the sensitivity of the technique but we estimate the resolution (using 20 nm gold particles and IgGs) to be 15–45 nm or better. This method has numerous advantages over autoradiography, as has already been shown at the LM level by Langer et al. (27, 28). It will be useful for fine resolution of sequences relative to identifiable chromosome structural elements; in addition, it should be applicable to the identification and location of nascent RNA transcripts associated with active transcription units in chromatin spreads. Hybridization to nascent transcripts would allow the examination of chromatin structure around specific genes, the relative activity levels of genes which exist in more than one copy in the genome, and could provide a way of finding chromosomal loci which are being transcribed and are also involved in other functions such as replication. Finally, with appropriate DNA probes, this technology can be used to study processing of specific nascent RNAs concomitant with transcription.

Since first preparing this manuscript, we have made additional observations that simplify the basic technique as described. We have determined that the 70% ethanol fixation step, the 70°C incubation, and the Proteinase K digestion can be eliminated without affecting the results obtained. Using this simplified protocol and long linker biotin-substituted probes, intense labeling directly over the centromere is observed with an increase in average signal to noise to 40:1.

The authors would like to thank the members of their respective laboratories for encouragement and useful discussions. B. A. Hamkalo and D. C. Ward thank Hal Weintraub for introducing us to each other, which began our collaboration.

N. J. Hutchison was supported by National Institutes of Health (NIH) training grant GM 07311; B. A. Hamkalo was recipient of RCDA GM 002333. Our research was also supported by NIH grants GM 23241 to B. A. Hamkalo and GM 20124 and CA 16038 to D. C. Ward.

Received for publication 28 April 1982, and in revised form 9 July 1982.

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