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.
were lysed by 1:150 dilution in 1 mM Tris (pH 7.4); this mode of lysis results in metaphase cells were deposited onto grids by centrifugation through 0.5 M sucrose. Recently, Langer et al. (26) synthesized nucleotide derivatives directly modified with biotin. Such derivatives can function efficiently as hybridization probes (26-28).

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. 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 with at least a threefold improvement in resolution over the LM.

To perform in situ hybridization to chromosomes on EM grids using autoradiographic detection. Specific labeling of centromeric heterochromatin is obtained with the LM. The achievement 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.

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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 32P-labeled mouse satellite DNA according to Pardue and Dug (31) plus labeled RNA complementary to this DNA was prepared using *E. coli* DNA polymerase II in the presence of 25 nM each dATP, dCTP, and Bio-dUTP, plus 10 nM 32P-dATP and dGTP. RNA was hybridized to this DNA (Sigma Chemical Co., St. Louis, MO) that had been labeled with fluorescein labeled UTP (Bio-UTP) (26) was substituted for UTP.

Preparation of Mouse Satellite DNA and 32P-labeled DNA

Mouse satellite DNA was a gift from Dr. Lorraine Lica (University of California, Irvine). Satellite sequences were purified from L929 cells and separated from the main band DNA by three cycles of equilibrium centrifugation in cesium chloride plus Hoechst 33258 (Calbiochem-Behring Corp., La Jolla, CA), according to Manuell (30). Tritium-labeled RNA complementary to this DNA was prepared using *E. coli* DNA polymerase II in the presence of 25 nM each dATP, dCTP, and Bio-dUTP, plus 10 nM 32P-dATP and dGTP. RNA was hybridized to this DNA (Sigma Chemical Co., St. Louis, MO) that had been labeled with fluorescein labeled UTP (Bio-UTP) (26) was substituted for UTP.
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 2 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, Schenectady, 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 of 3H-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 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-developed in Kodak Mierodol X for 3-5 min at 18-24°C, rinsed in water, fixed, and then dehydrated in three 5-min rinses each of 70% and 95% ethanol, and air-dried. Kodak Rapid Fixer was used at 1:1 dilution of the stock for 30 min at 37°C. They were then washed with the appropriate buffer for at least 3× 10-min rinses before antibody reaction. Kodak 3M silver intensification kit was used (total exposure time). The 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

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. 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 1% NaCl, 0.05 M Tris (pH 7.1) for 24 h at room temperature or 37°C. They were then washed with the appropriate buffer for at least 3× 10-min rinses in buffer B. Grids were then rinsed in 0.1% Photoflo and air-dried before examination in the EM.

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 Antibiotin 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 (pH 7.4, 0.15 M NaCl, 0.025 M KCl, 1.44 g Na2HPO4, 0.25 g KH2PO4) at −20°C. Rhodamine- and fluorescein-antibiotin IgG and sheep anti-rabbit IgG (Rh-GAR, FITC-GAR, and AR, 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 (0.05 M NaCl, 0.05 M Tris [pH 7.1] 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.

Hybridization of L929 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 autoradiograms (e.g., Fig. 1a), 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. 1b). 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 3H-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. X 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, X 3,750.

judged by the density of silver grains (Fig. 1a), appear to correspond to the size of the C-banded regions on those chromosomes recognizable by morphology (Fig. 1b). 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. 2a–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. 2a–c, the labeling typically covers the entire centromeric region. After very long exposures (~1 yr, Fig. 2c), 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. 2b). In addition to centromeric labeling, the metacentric marker chromosome shown in Fig. 2b 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 labelled following hybridization. A labeled nucleus is shown in Fig. 2d. Silver grains appear in clumps and large clusters, some of which are over nucleolus-associated heterochromatin. In some nuclei (Fig. 2d), 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-
bridges 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 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, X 14,000. b, X 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, X 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 SARC-G. 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/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 adherence 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 association 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 (34), we estimate the resolution in our autoradiographic experiments using tritiated probes to be 0.1–0.3 μm, compared to the reported resolution of ~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. 1a and 2a 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. Hamaklo, 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 nonspecifically 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-collodial 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(195,177),(980,243)