High-resolution Mapping of Satellite DNA Using Biotin-labeled DNA Probes

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ABSTRACT We have developed a novel method for high resolution mapping of specific DNA sequences after *in situ* hybridization. DNA probes, labeled with biotin-nucleotides in conventional nick-translation reactions, are hybridized to cytological preparations and detected with affinity-purified rabbit antibiotin antibodies followed by antibodies to rabbit IgG that are conjugated to fluorescent or enzymatic reagents. Using peroxidase labeled anti-rabbit IgG, we are able to detect and localize specific sequences at both the light and electron microscopic levels. Initial studies were done with repeated DNA sequences previously mapped by light microscope autoradiography to assess the fidelity and resolution of this method. An analysis using biotin-labeled mouse satellite DNA is presented here.

In situ hybridization, a procedure for the localization of specific polynucleotide sequences, was introduced in 1969 (1, 2). It has been used subsequently by numerous investigators to examine the intracellular or chromosomal location of specific DNAs or RNAs in many different species (3). Recently, the method had been refined to permit the direct localization of single copy genes on mammalian metaphase chromosomes (4–6), using autoradiographic exposures of 5–22 d. These procedures are invaluable for a first assessment of chromosome organization and an understanding of how different sequences are arranged, i.e., whether they occupy one or several sites in the genome. Since interphase chromosomes appear to be highly ordered in the nucleus (7, 8), possibly with specific arrangements or patterns, a simple method for analyzing the organization of specific DNA sequences with high resolution in both interphase and metaphase chromosomes would be of considerable use. We developed an immunological method for localizing polynucleotide sequences after *in situ* hybridization that exploits the interaction between biotin-labeled nucleotides and antibiotin antibodies. This technique has been used to map genes on *Drosophila* polytene chromosomes with a spatial resolution and signal to noise ratios superior to those routinely achieved using autoradiography (9).

Here we report the first application of this methodology to the localization of DNA sequences in mammalian cells and a simple procedure for extending the analysis to the level of the electron microscope. We tested the specificity and resolution of this method by hybridizing mouse satellite DNA to conventional acid-fixed chromosome and nuclear preparations. These studies indicate that our detection method can unambiguously and reproducibly delineate the location of specific sequences in such samples with a resolution greater than that previously achieved by either light or electron microscope (EM) autoradiography.

MATERIALS AND METHODS

Mouse glioblastoma cells TC 509 (10) and mouse A9 cells (11) were treated with colcemid for 40 min and then spread using conventional acetic acid-methanol fixation methods. Slides were treated with 100 μg/ml of RNase in 2 × SSC for 1 h at 37°C, washed in 2 × SSC (2 × 10 min), dehydrated in graded ethanols (70, 80, 90, and 100%), and air-dried.

Chromosome spreads were denatured immediately before hybridization. The slides were incubated in 70% formamide (freshly deionized and recrystallized) in 2 × SSC (standard saline-citrate buffer) at 70°C for 2–4 min (5), dehydrated in 70% ethanol through 100% ethanol for 2–5 min each, and air-dried. Formamide was preferred over other denaturing agents, such as NaOH or acetic acid, as these latter treatments were found to alter chromosome structure more drastically and/or to give less reproducible hybridization results.

Mouse satellite DNA was isolated as described (12) and was >98% pure as determined by restriction enzyme analysis and by unambiguous sequencing (13). Biotin-labeled satellite DNA probes were prepared using Bio-dUTP (14) in a nick-translation mixture (50 μl) containing 0.5 μg of DNA, 10–12 U of DNA polymerase I, 0.5 mM deoxy-ATP (dATP), dGTP, dCTP, and standard nick-translation buffers (15). The reaction mixture was then incubated at 14°C for 90 min. In most experiments ^{3}H-dCTP (19 Ci/mol, New England Nuclear) was used to monitor the extent of nucleotide incorporation into TCA precipitable counts. Reactions containing Bio-dUTP as a substrate gave the same levels of incorporation as reactions containing TTP. The reactions were terminated by the addition of EDTA to a final concentration of 10 mM, the reaction mixture was heated to 60°C for 10 min, and unincorporated nucleotides were separated from DNA on

Sephadex G-50 using H_2O or 10 mM Tris-Cl, pH 7.5, as eluates. Stock solutions of biotin-labeled DNA probes were stored frozen at −20°C.

Biotin-labeled mouse satellite DNA (~0.6 μg/ml, 3 × 10^6 cpm/μg) was denatured in the presence of sonicated herring sperm carrier DNA (250 μg/ml) for 4 min at 92–94°C, quenched on ice, and ice-cold 20 × SSC was added to give a final concentration of 4 × SSC. Approximately 35 μl of the probe solution was added to each of the dry slides, the solution was overlaid with a 24-mm^2 cover slip, and the slides were incubated at 60°C in a moist chamber for 4 h to complete the *in situ* hybridization. After removal of the cover slips, the slides were washed in 2 × SSC at 60°C (20 min) and then washed at room temperature in 2 × SSC for 10 min followed by three changes of PBS for 5 min each. Slides were drained (but not allowed to dry) and then incubated overnight at 37°C with 35 μl of affinity-purified rabbit antibiotin antibody (2.5 μg/ml) containing 10 mg/ml of bovine serum albumin carrier. They were then washed in PBS 3 × 5 min, drained, and incubated for 4 h at 37°C with a 1:40 dilution of affinity purified goat anti-rabbit IgG conjugated to horseradish peroxidase, made as described (16) (a gift of J. Madri, Yale University). After the slides were washed, insoluble peroxidase products were developed using a freshly prepared solution of 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) at a concentration of 0.5 mg/ml in 0.05 M Tris-Cl, pH 7.6, containing 0.01% H_2O_2. Development was generally allowed to proceed for up to 40 min at room temperature. In some cases strips cut from an autoclave bag (VWR Scientific) were used to overlay solutions on slides; this flexible covering allowed complete removal of trapped bubbles, and scratching or distortion of the chromosome surface was avoided.

For light microscopy, slides were lightly counterstained with Giemsa stain, dehydrated, and mounted in Permount. For electron microscopy, unstained slides were first examined by light microscopy using an overlay of 90% glycerol in 0.1 M Tris-Cl, pH 8.0 (see Fig. 1*b*); they were then washed extensively in PBS, dehydrated, air-dried, and coated by dipping the sides in with 0.7% parlodion in amyl acetate. After air-drying slides, 100-mesh copper grids were placed over chromosome spreads. The chromosomes, film and copper grids were etched off the glass surface by slowly immersing the slide at an angle of ~20° on to a solution of 0.5% hydrofluoric acid, as originally suggested by Lubit et al. (17). The film-coated grids were washed 2 × 2 min in H_2O, picked up on Saran Wrap, and air-dried. Osmium tetroxide treatment was found not to be necessary for electron microscope detection of the peroxidase precipitates in our preparations.

In some experiments, for comparison of resolution and detection levels, FITC-labeled goat anti-rabbit antibody (Miles Laboratories, Elkhart, IN) was used (at a dilution of 1:1,000) instead of the peroxidase-labeled second antibody. The results in these experiments were comparable, however photography of the less intense and less stable fluorescent signals required long film exposure. In addition, the fluorescence detection method often gave less resolution of general chromosome structure.

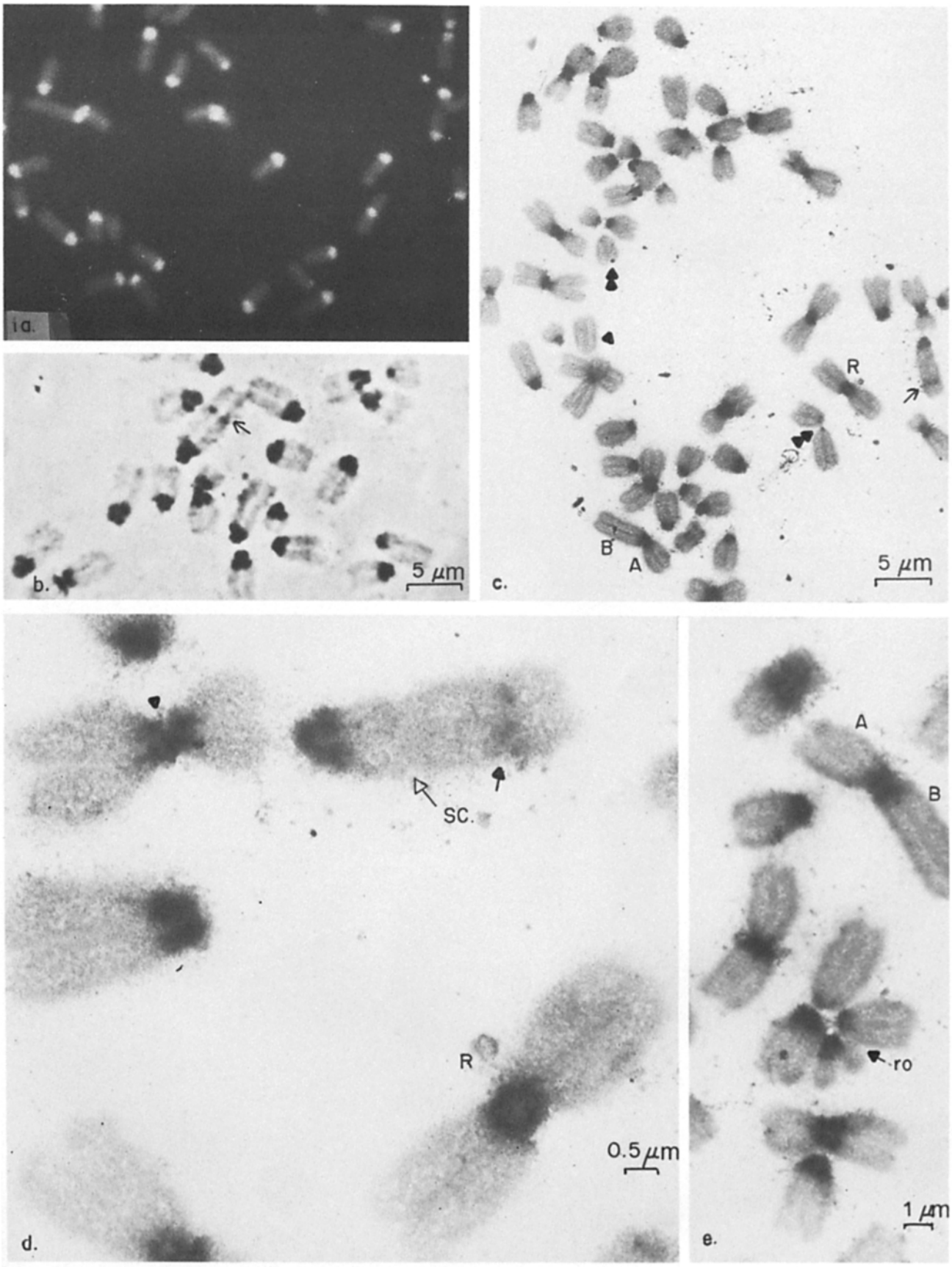
The sequential addition of all three reagents (Bio-DNA, rabbit antibiotin antibody, and peroxidase-labeled goat anti-rabbit IgG) was essential to generate a positive hybridization signal. No staining was observed after hybridization with Bio-DNA if the primary incubation with antibiotin antibody was omitted or if it was substituted by an incubation with nonimmune rabbit serum. Similarly, no signal was observed after hybridization of nonbiotinized probes, using either antibiotin or control antibodies.

RESULTS

By light microscopy both FITC-labeled and peroxidase-labeled antibodies showed specific localization of satellite DNA to centromeric heterochromatin (Fig. 1 *a* and *b*). Metaphase chromosomes and nuclei showed well delineated labeling with very low background and little scatter of signal. In peroxidase-labeled preparations centrometric satellite DNA showed well defined extension into the initial segment of each sister chromatid (Fig. 1 *b*). In both cell lines, A9 as well as TC 509, ~10% of the spreads also exhibited a long acrocentric chromosome that was labeled distally on the arms; this was more easily photographed and with higher resolution in peroxidase-labeled preparations (Fig. 1 *b*) as compared to experiments using FITC-labeled antibodies. When sister chromatids were separated, such labeled "intercalary" regions appeared as discrete bands or punctate regions (Fig. 1 *b*). Intercalary regions hybridizing to mouse satellite DNA were less intensely stained than those in the centromeric heterochromatin. The centrometric staining also appeared as larger, more tightly associated clumps of precipitate.

To examine the hybridization data with greater resolving power, we adopted the hydrofluoric acid etching procedure (17) for lifting specimens from glass slides onto EM grids. When such preparations were examined by electron microscopy the extent and configuration of satellite sequences in chromosomes and nuclei were more strikingly resolved. The intercalary labeled regions in chromosomes from A9 cells (Fig. 1 *c* and *d*) and from TC509 cells (Fig. 2 *c* and *d*) were easily detected and were distinctly different from the larger centromeric arrays. Although at least one particular site of satellite DNA on chromosome arms in these two different tissue culture cell lines was reproducibly detected, we also were able to note some other more minor sites of hybridization of satellite DNA on chromosome arms. These appeared as small precipitates of peroxidase products along other regions of the chromosome arms (Fig. 2 *e*). Although precipitates near the centromere regions could reflect artifactual spread of the very heavy centromeric label, scattered clusters of peroxidase precipitates were also observed in more telomeric regions in some spreads, and these latter precipitates were less likely to represent labeling artifacts. It is thus possible that, besides the one prominent intercalary array observed reproducibly in each cell line, other more minor sites of satellite DNA reside on some chromosome arms. Since both the A9 and TC509 cell lines are tumorigenic and have been maintained in culture for many years, they may have undergone extensive chromosomal rearrangements. Nor-

FIGURE 1 (*a*) Mouse satellite DNA detected with FITC-labeled antibody following hybridization. Centromeres of TC 509 chromosomes show bright (yellow-green) fluorescent labeling. The general outline of the chromosome is revealed (in red) by counterstaining with ethidium bromide. (*b*) TC 509 chromosomes showing satellite DNA detected after hybridization with peroxidase-labeled antibody. The chromosome preparation is unstained and is viewed mounted in buffered glycerol. Arrow shows intercalary labeling on both separated sister chromatids of one chromosome. Many of the centromeres have a tightly associated cap of label which, after a small constriction, extends into the initial segment of each sister chromatid. There are differences in the extent of satellite on different chromosomes, e.g., labeled centromeres in lower left are smaller than those of the other chromosomes. (*c*) Low power electron micrograph of A9 chromosomes following hybridization with satellite DNA. Several centromeres in this spread showed only very faint centromeric labeling (double arrowheads), and one chromosome showed no detectable label (single arrowhead). An intercalary band is seen on one chromosome (arrow) and a chromosome interpreted as a Robertsonian fusion is noted (*R*). (*d*) Higher power view of a portion of *c*. Intercalary band on both sister chromatids (arrow) is less dense than centromeres. This chromosome was identified by its secondary constriction (arrow) and by the configuration of its centromere which was roughtly C-shaped. The chromosome labeled *R* in *c* is shown at higher magnification; the labeled region is circular and without interruption. In contrast, a higher power view of two centromeres that are closely associated but not fused at the centromere is seen in the upper left. These latter centromeres are shorter than others in the field. Arrowhead points to one centromere where the most dense peroxidase product appears to form a twisted, fiberlike structure of ~2,000 Å. (*e*) A spread of A9 chromosomes that shows close association of centromeres of two apparently biarmed chromosomes (*A* and *B*) which are similar to *A* and *B* noted in *c*. Other centromeres were seen associated in a rosettelike pattern, an example of which is seen in the cluster designated *ro*, and could give rise to interphase satellite aggregates.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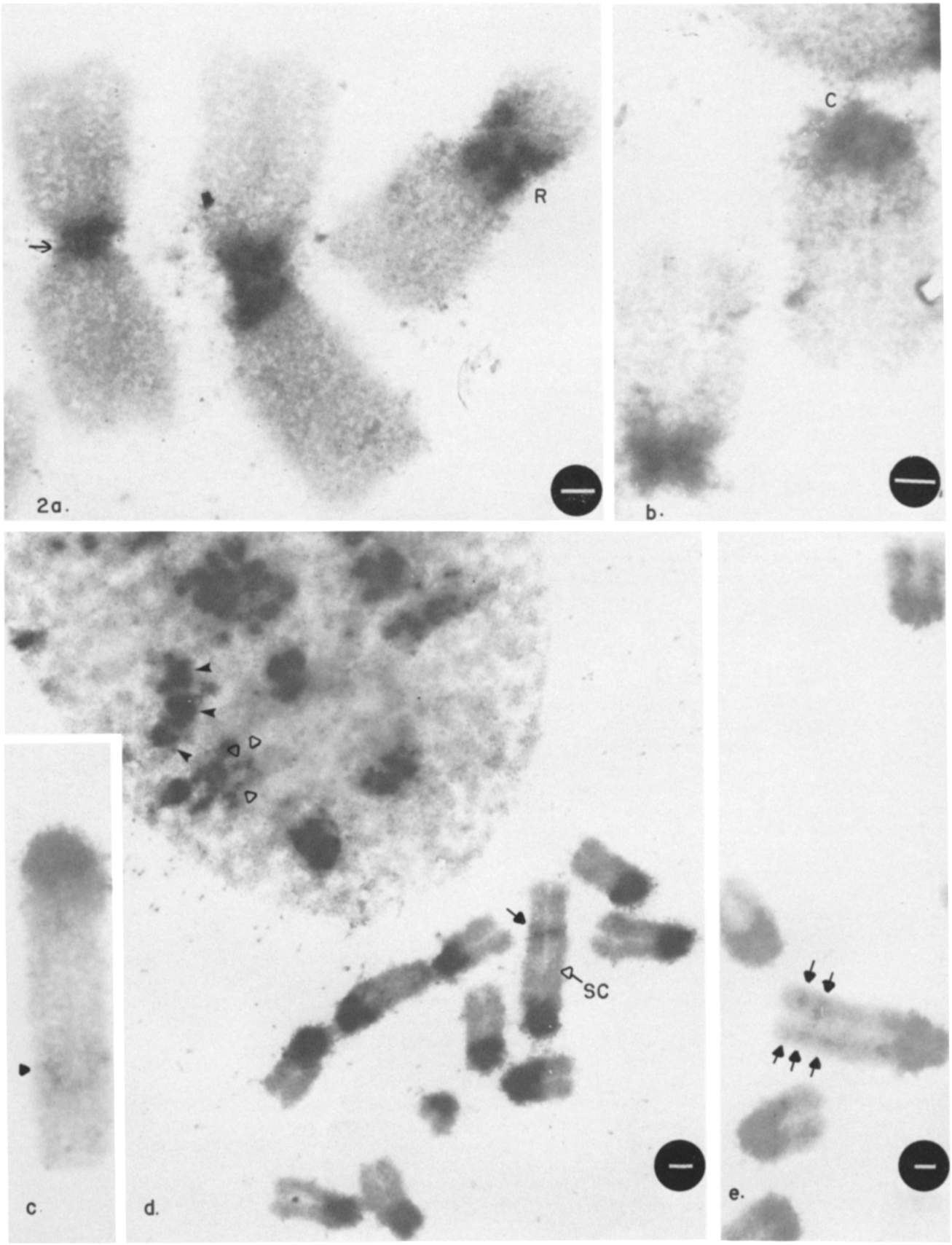
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mal mouse cells could lack the prominent intercalary band of satellite sequence observed in these cell lines, and experiments to test this possibility are in progress.

In typical low power EM views, the level of staining on most chromosome arms was low and several of the centromeric regions were only weakly stained (Fig. 1 *c*). Although it is possible that the hybridization protocol does not detect every satellite array in every spread, it is also possible that there are chromosomes in these cultured cell lines that have little, or no, satellite DNA sequences in their centromeric heterochromatin. This latter interpretation is supported by both Hoechst 33258 staining and by *in situ* hybridization studies using ^{3}H-autoradiography, which also fail to label all centromeres in the chromosomes of the TC509 cell line (18). Although only one chromosome in normal cells (the Y chromosome) is known to lack centromeric satellite DNA, we believe that the light staining of some centromeric regions is an intrinsic characteristic of the cells analyzed and not a limitation of the detection method employed.

We observed three different patterns of satellite DNA hybridization in the acrocentric chromosomes. The most common pattern showed a band of stained material across the top of each chromosome, with the stained region extending for a variable length into each sister chromatid (Fig. 1 *d*). In some cases the most acrocentric region appeared capped by a small stained ball or fiberlike region of ~2,000-Å diameter (Fig. 1 *d*), giving the centromere a horizontally banded, or C-shaped appearance. In other chromosomes, each of the satellite-stained regions showed separate parallel arrays that did not appear to connect at their acrocentric extremity (Fig. 2 *b*). In some of these latter forms a constriction was noted just before entry into the sister chromatid. Finally, in some centromeres the satellite region appeared as a circular or looped structure similar to that seen in some centromeres with Robertsonian fusion (Fig. 2 *b*). Some apparently biarmed chromosomes showed interruptions in the satellite DNA label, whereas others showed a continuous, uninterrupted, satellite array. The former could represent a fusion variation or, alternatively, a close association of the centromeric heterochromatin of two separate chromosomes. Although different centromeric configurations could reflect subtle differences in centromere morphology on different chromosomes, additional studies will be necessary to establish the significance of these centromeric patterns.

An analysis of nuclei present in the chromosome preparations indicated that many of them had multiple satellite DNA regions which were similar in both size and number to the centromeres seen in adjacent metaphase chromosome spreads (Fig. 3 *a*). Other nuclei exhibited varying degrees of aggregation or clustering of satellite DNA sequences into larger arrays (Fig. 3 *b–g*). Dispersion, rather than aggregation, would be expected by the hypotonic spreading treatments, thus the more aggregated clusters of satellite DNA sequences are unlikely to be hybridization artifacts. Indeed, the results obtained by the antibody detection method are entirely consistent with previous studies (19–23) that have shown intranuclear clustering of centromic heterochromatin and satellite DNA sequences using autoradiographic detection protocols. However, the immunological method employed here offers an improved spatial resolution over that obtained with tritium autoradiography, by circumventing the inherent limitations of decay-particle path length and emulsion thickness.

Some of the satellite DNA regions in these nuclei appear aligned in rows (Fig. 3 *a*); close-packing of these aligned centromeric regions (Figs. 2 *d* and 3 *b*) is likely to be followed by further condensation into the few large, possibly specifically positioned, aggregates. After studying several hundred nuclei from both cell lines, it was apparent that certain clusters of satellite DNA appeared to occupy defined regions of the nucleus relative to heterochromatic dense regions and the nuclear membrane, often suggesting a pattern of fourfold symmetry (e.g., Fig. 3 *d* and *e*). The pattern of centromere arrangement in the interphase nucleus has been shown to differ in different cell types of the mouse and to follow a defined pattern according to the stages of cellular development (20). The different patterns of satellite DNA distribution observered here may reflect dynamic changes in specific chromosome segments during different stages of the cell cycle. The immunological method of hybrid detection, when applied to ultra-thin sections of synchronized cell populations or tissue sections, should provide a rapid and sensitive approach for examining dynamic cell cycle and differentiation parameters in the nucleus.

DISCUSSION

The method presented here allows unambiguous localization of specific DNA sequences in mammalian chromosomes or nuclei with a resolution greater than that previously obtained using conventional autoradiography. We studied mouse satellite DNA as a starting point because this repeated sequence is easy to purify and it is represented in large arrays in the genome. We also used conventional acid-fixed chromosomes because they are routinely employed for many types of identification in cytogenetic analysis. Centromeric labeling also provides a convenient reference point for later studies where several different labeled probes may be used.

It is clear from the data presented here that the immunological method of hybrid detection is suitable for analyzing the location or distribution of nucleotide sequences of high abundance. However, the ultimate sensitivity of the method has yet to be established rigorously, although we have been able to

FIGURE 2 (*a*) A TC 509 chromosome interpreted as a Robertsonian fusion (*R*) since it shows a continuously labeled centromere extending as twisted parallel fibers down into each sister chromatid. Two other chromosome sets in the field are closely associated but do not appear fused since there is a small gap between each centromere; these gaps are much more apparent at higher magnification. Arrow shows labeled fiber of ~500 Å in one of these centromeres. (*b*) Acrocentric TC 509 chromosome at *C* shows a looped configuration with substructure. Chromosome at bottom left shows label in roughly parallel fibers with a small indentation or constriction in the middle. (*c*) TC 509 chromosome with localized light labeling on each sister chromatid arm. (*d*) TC 509 chromosome with intercalary labeling on the arms (arrow). This chromosome has a secondary constriction (*SC*) and is structurally similar to that seen in Fig. 1 *b* and in other spreads of this cell line. Nucleus shows dense clusters of satellite DNA, some of which are similar in size to centromeric label on chromosomes (arrowheads). Other labeled regions appear more extended and these, particularly at higher power, contained a banded appearance (open triangles). (*e*) Small, dense precipitates were sometimes seen distally on chromosome arms (arrows). TC 509 chromosomes are illustrated. Bars, 0.5 μm.

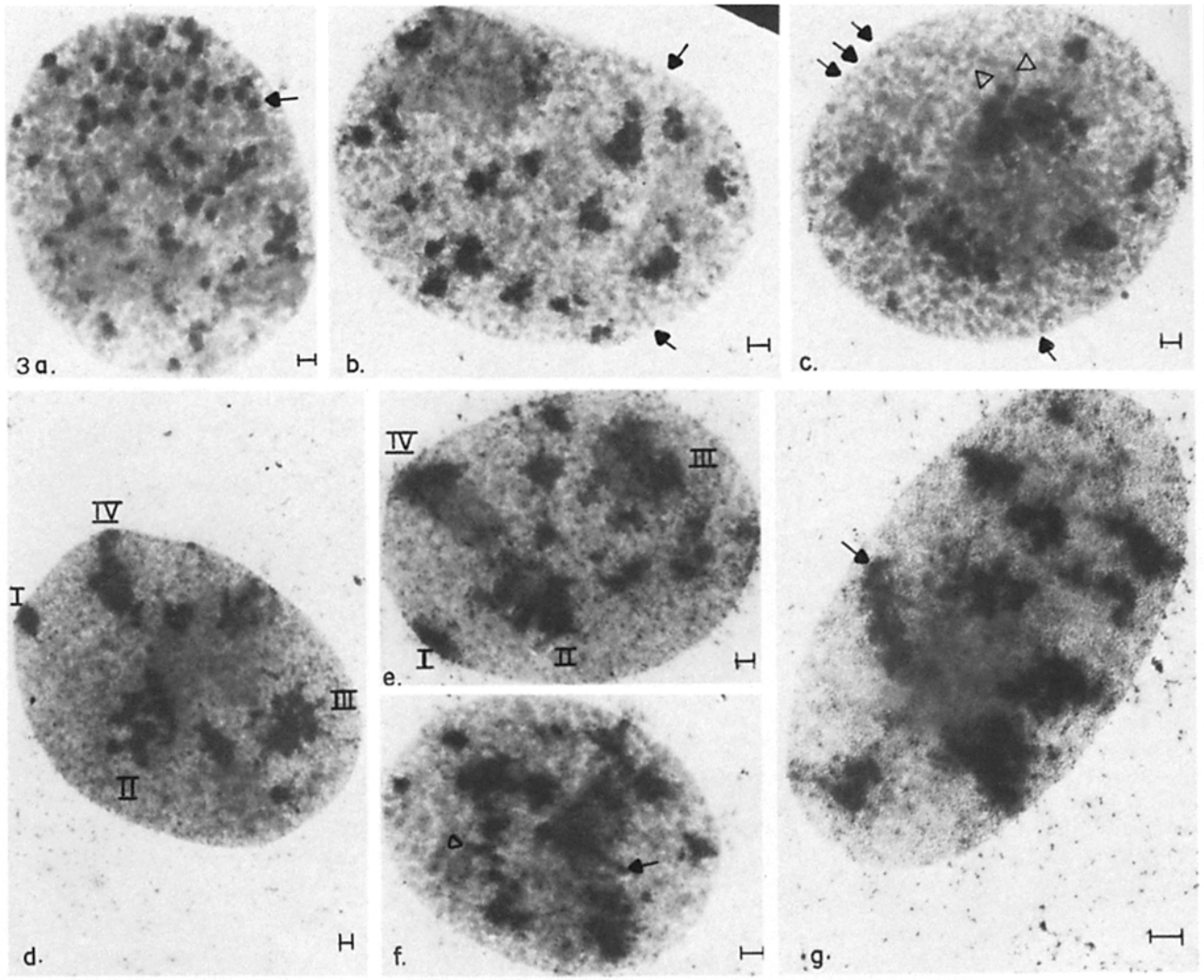

FIGURE 3 Whole nuclei showing various configurations of satellite labeling. (*a*) Shows separated satellite regions in some cases aligned in a row (arrow). There are about 50 separate densely labeled satellite regions in the nucleus; the number of satellite DNA positive loci is approximately the same as the number of chromosomes present in this aneuploid cell line (TC 509). (*b*) Shows aggregation of satellite DNA into larger clumps that are still widely separated in the nucleus. Arrow shows 2,000 Å diameter, unlabeled fiber. (*c*) Interphase nucleus showing clustering of satellite DNA regions into a few large aggregates. Four of these are roughly symmetrically arranged around the central heterochromatin. Arrows show possible ~2,000-Å twisted interphase fibers. Similar fibers can be seen extending from a satellite stained region (triangles). (*d* and *e*) The spatial relationship between clumps of aggregated satellite containing regions did not appear to be entirely random; in many instances the distribution patterns suggest ordered and/or symmetrical arrays. For example, note the relative positions and sizes of the satellite labeled regions I–IV in the nuclei shown in *d* and *e*. (*f*) In addition to larger aggregates of satellite DNA, apparent labeled ~2,000-Å fibers could be seen (arrow). In some cases these satellite positive fibers appeared aligned and in this two-dimensional view appeared as a twisted fiber (triangle). (*g*) Another example of "C" shaped satellite regions aligned with each other (e.g., at arrow). Again larger clumps of satellite DNA as well as more extended regions of staining are seen. Bars represent ~1 μm in each case.

detect chromosomal loci that contain 15–20 kibobases of a unique sequence (L. Manuelidis and D. C. Ward, unpublished results). The sensitivity parameter has not been studied extensively since the reagents used both for tagging the DNA probe and for detecting the probe following hybridization are still prototypes which can be improved upon significantly. For example, we have observed recently that DNA probes containing pyrimidine nucleotides which possess a longer linker arm between the biotin moiety and the pyrimidine ring interact with antibiotin antibodies or avidin with greater affinity than do the biotin-labeled DNA probes used in this study (L. Manuelidis and D. C. Ward, unpublished results). In addition, we have also obtained avidin derivatives or avidin analogs, e.g., streptavidin from *Streptomyces avidini* (24) which do not exhibit nonspecific binding to chromatin or chromosomes. Complexes between avidin and biotinated enzymes (e.g., peroxidase) have been shown to be more sensitive as indicator reagents than antibody-peroxidase conjugates (25). We are currently evaluating a combination of these reagents in an attempt to increase the sensitivity of this hybrid detection method to the level of a single gene copy. By tagging avidin or avidin-biotinated protein complexes with colloidal gold as described in the preceding paper (23) it should be possible to obtain a resolving power and sensitivity even greater than that achieved with peroxidase-labeling methods.

The development of highly sensitive protocols for *in situ*

hybridization is likely to be of importance not only in localizing genes and their transcripts but also in obtaining a three-dimensional view of morphologically preserved preparations that may well give us more insight into the dynamics and arrangement of specified sequences in different cell types. In differentiated cells of the brain, for example, the position and orientations of nucleolus organizer regions appear to be quite specific in different cell types (26). Indeed, study of mouse brain tissue sections, fixed in paraformaldehyde and hybridized with biotin-labeled satellite DNA, show aggregates of satellite in these G1 nuclei. The distribution of satellite sequences in glial cells and neurons is distinctly different and reproducible in each cell type (L. Manuelidis and D. C. Ward, manuscript in preparation), as observed earlier by Hsu et al. (20) using strains specific for heterochromatin.

Our study also shows different nuclear patterns of centromeric heterochromatin (satellite) distributions which are likely to reflect different functional states in interphase (e.g., cell cycle changes). Dynamic changes are still compatible with the concept that specific chromosomes occupy defined three dimensional positions relative to each other (26–30). Although classical cytological studies have indicated that there may be a single polar collection of centromeres in some interphase nuclei (27–29) with telomeres oriented at the opposite side of the nucleus, we observe more than one collection of centromeres in mouse nuclei, and these centromeric aggregates are not oriented in a single polar fashion.

Using variations of the method presented here, it should be possible to directly examine the definition of chromosome substructure and higher order folding with reference to specific sequences. Analysis of the orderly arrangement of defined chromosome segments within nuclei during cell division and differentiation is also possible. Such studies are in progress.

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Note Added in Proof: Biotin nucleotides containing linker arms with 11 or 16 atoms have been used successfully in hybridization experiments and can be detected in a single step using complexes of avidin and biotinated horseradish peroxidase. This reduces the time required for probe detection to 1–2 h, and omits antibody reagents.

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