Localization of Topoisomerase II in Mitotic Chromosomes

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ABSTRACT In the preceding article we described a polyclonal antibody that recognizes cSc-1, a major polypeptide component of the chicken mitotic chromosome scaffold. This polypeptide was shown to be chicken topoisomerase II. In the experiments described in the present article we use indirect immunofluorescence and immunoelectron microscopy to examine the distribution of topoisomerase II within intact chromosomes. We also describe a simple experimental protocol that differentiates antigens that are interspersed along the chromatin fiber from those that occupy restricted domains within the chromosome. These experiments indicate that the distribution of the enzyme appears to be independent of the bulk chromatin. Our data suggest that topoisomerase II is bound to the bases of the radial loop domains of mitotic chromosomes.

There is currently a substantial body of evidence that suggests that the chromatin fiber in both meiotic (1–4) and mitotic (5–9) chromosomes is folded into radially projecting loops. Little is known, however, about the identity of the structural components that are responsible for tethering these loops along the chromatid axis. Experiments from Laemmli's laboratory (10) suggest that the axial components are part of a set of nonhistone chromosomal proteins that has been termed the "chromosome scaffold."

The study of the arrangement of scaffold components in intact chromosomes has been greatly hampered by the lack of reagents capable of recognizing specific scaffold proteins in situ. Since the structure appears to be a diffuse fibrous network (9), it is difficult to visualize in unextracted chromosomes where it comprises only 3–4% of the total mass (11). Therefore scaffolds have not yet been directly visualized in intact chromosomes (12–14).

The first experiments to achieve some success in detection of a "core" within intact chromosomes used a cytological silver staining procedure (15) that had been previously shown to stain the synaptonemal complex in meiotic prophase chromosomes (16–18). While it was possible to show that scaffold proteins were apparently responsible for the staining (19), the individual polypeptides involved could not be identified. This method also had the drawback that conditions for the silver staining are harsh and might disrupt the chromosome structure.

In the preceding paper (20) we characterized an antibody that recognizes a 170,000-mol-wt antigen found in chicken mitotic chromosomes and chromosome scaffolds. This antigen resembles in electrophoretic mobility a major human chromosome scaffold polypeptide—Sc-1 (11), and we have therefore termed it cSc-1[1] (chicken Sc-1 [20]). Subsequent investigation revealed that cSc-1 was the abundant nuclear enzyme topoisomerase II. Here we present results of a number of different experiments aimed at localizing topoisomerase II within intact chromosomes. Our results indicate that the distribution of the protein appears to be distinct from that of the bulk chromatin. We suggest that topoisomerase II is found at the bases of the radial chromatin loops in mitotic chromosomes.

MATERIALS AND METHODS

General Procedures: Conditions for cell culture, antibody production, and indirect immunofluorescence have been described in the preceding paper (20).

Immunoelectron Microscopy: Chromosomes were isolated by a modification of the procedure of Lewis and Laemmli (11). 40 ml of MSB-1 stock growing in suspension in RPMI-1640 plus 5% calf serum supplemented with iron were poured into a T-75 flask and colcemid was added to 0.1 μg/ml. After 16 h, the cells were centrifuged at 800 *g* for 3 min. The cells were swollen for 5 min at room temp in RSB buffer (see reference 20) before centrifugation and subsequent lysis. After dounce homogenization in 10 ml lysis buffer (20), the lysate was centrifuged for 5 min at 4°C (1,500 *g* for 1 min, then slowed to 250 *g* for the remaining time). The top 90% of the supernatant was removed and brought to a final volume of 40 ml with lysis buffer. After centrifugation for 25 min at 250 *g*, the pellet was gently resuspended in 250–400 μl lysis buffer using a cut pipet tip.

Chromosomes were swollen (9) and centrifuged (1,500 *g* for 15 min at 4°C) onto carbon-coated, alcian blue–treated (21) 400-mesh gold grids. After a rinse in TEEN (1 mM triethanolamine:HCl, pH 8.5 0.2 mM EDTA, 25 mM NaCl),

[1] *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; KB, 10 mM Tris:HCl, pH 7.7, 0.15 M NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin; cSc-1, chicken scaffold protein 1; TEEN, 1 mM triethanolamine:HCl, pH 8.5, 0.2 mM EDTA, 25 mM NaCl.

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grids were fixed for 5 min in 3% paraformaldehyde in TEEN, rinsed 2 min in TEEN, incubated 10 min in 50% calf serum in TEEN, and rinsed again 2 min in TEEN (all at 4°C). The grids were dipped in KB (10 mM Tris-HCl, pH 7.7, 0.15 M NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin), and transferred to 20-μl drops of KB containing first antibody for 60 min at 37°C, followed by rinsing in KB, three times 5 min at 4°C. Subsequent antibody incubations (with biotinylated anti-guinea pig IgG or biotinylated anti-human IgG; both 1:222 in KB; followed by affinity-purified rabbit antibiotin 1:50 in KB) were for 30 min at 37°C and grids were rinsed as for first antibody. Biotinylated antibodies were from Vector Laboratories, Inc. (Burlingame, CA), and rabbit antibiotin was a gift from D. Ward (Yale University).

Colloidal gold (20 nm) was prepared by the procedure of Frens (22) and conjugated to goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) by standard procedures (23, 24). Gold was diluted to an A_{520} of 0.386 in 1% BSA buffer (20 mM Tris:HCl, pH 8.0, 0.15 M NaCl, 1% BSA, 0.01% Na-azide). Grids were incubated in the colloidal gold solution for 60 min at 37°C, rinsed 5 min in KB (at 4°C), and then three times 5 min in TEEN + 0.1% Triton X-100 and 0.1% BSA. They were finally dipped in TEEN and TEEN + 0.4% photoflo, blotted dry, and allowed to stand for ≥60 min. The grids were then stained with phosphotungstic acid and rotary-shadowed with Pt-Pd (9). Electron microscopy was performed on a Zeiss EM 10A at an accelerating voltage of 80 kV.

Treatment of Chromosomes with Antibodies in Solution: Chromosomes were isolated as described in the preceding section and swollen by dilution 40-fold into TEEN buffer. Antibodies were added to a dilution of 1:100 and incubated at 37°C for 30 min. After this incubation, chromosomes were centrifuged onto electron microscope grids through a solution of 0.1 M sucrose in TEEN. The grids were then rinsed, fixed with glutaraldehyde, dried, stained with phosphotungstic acid, and rotary-shadowed as described previously (9).

RESULTS

Location of Topoisomerase II in Intact Chromosomes

We have used a gentle centrifugation procedure to lyse mitotic cells in situ for light microscopy. This enables us to obtain preparations in which all chromosomes from a given cell lie in a single plane of focus, while avoiding the rigors of acid fixation and drying that are necessary for standard cytological spreads. For this procedure, cells are maintained in a buffer that favors chromosome compaction before centrifugation (i.e., containing 5 mM $MgCl_2$), and all steps subsequent to centrifugation are performed at physiological ionic strength in a phosphate-buffered saline buffer. The chromosomes should therefore incur minimal structural damage and should retain the full protein complement present in vivo. In general, chromosomes seen on these slides resembled those seen by staining intact cells (see Fig. 5 of reference 20 [preceding paper]). Occasionally, however, it was possible to find regions where the chromosomes had become slightly expanded during centrifugation. These regions proved to be informative when examined in detail.

We studied the distribution of topoisomerase II in these expanded chromosomes by double-label fluorescence microscopy. Fig. 1 *a* shows the appearance of centrifuged mitotic cells following the immunofluorescence procedure using phase-contrast optics. In Fig. 1 *b*, the DNA is visualized by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) fluorescence (25). Fig. 1 *c* shows the same field observed by rhodamine fluorescence after staining with preimmune serum. Preimmune serum contains no antibody species that bind to native chromosome antigens.

Fig. 1, *d* and *f*, presents fluorescence images of centrifuged mitotic cells stained with DAPI. On this area of the coverslip, the chromosomes were expanded to the extent that the paired chromatid morphology was obscured and (in the lower portion of Fig. 1 *d*) the structures were unrecognizable as chromosomes. Nonetheless, when the location of topoisomerase II is displayed for these same chromosomes using the anti-cSc-1 antiserum (Fig. 1, *e* and *g*), the paired chromatid axes are easily observed. The arrows in Fig. 1, *d–g*, provide assistance in aligning the two images obtained using DAPI and specific antibody. Immunoelectron microscope results (see below) indicate that this apparent difference is not simply due to differing photographic exposure levels for the DAPI and rhodamine images. Our interpretation of these results is that in these chromosomes the distribution of the enzyme differs from that of the bulk chromatin. Topoisomerase II appears to be concentrated towards the center of the expanded chromatids.

This conclusion is strongly supported by examination of double exposures of cells stained with DAPI and anti-cSc-1. Fig. 2 presents a pair of images of a particularly informative mitotic cell. This cell was evidently exposed to shear forces during centrifugation (causing a portion of the chromatin to stream around the periphery giving the appearance of a continuous boundary). The chromatin, visualized with DAPI, is found in radially projecting fibers (Fig. 2 *a*). The axes of individual chromosomes are not apparent. However, when the location of topoisomerase II is revealed with anti-cSc-1 (Fig. 2 *b*) the chromosome axes are readily distinguished. The antigen is found in chromosome-shaped structures with radially projecting chromatin fibers. No evidence is obtained for the presence of antigen in the peripheral (loop) chromatin, though small amounts would not be observed by this technique. It is important to note that this sample was subjected only to hypotonic swelling and gentle centrifugation. No extractions or other perturbations of the chromatin organization were performed. To our knowledge, this is the first time that radial chromatin loops have been observed in mitotic chromosomes by light microscopy.

High-Resolution Localization of Topiosomerase II in Swollen Mitotic Chromosomes

We next sought to localize topoisomerase II in mitotic chromosomes at higher resolution by immunoelectron microscopy. To permit optimal accessibility of the antibody to the antigens, we performed the experiments using chromosomes swollen in hypotonic buffer. These chromosomes readily return to the condensed state when exposed to divalent cations (9, 26). The results of one such experiment are presented in Fig. 3. The chromosomes seen in this figure were subjected to a four-step immunocytochemical procedure, culminating in attachment of antibody bound to colloidal gold. The structural alterations observed in Fig. 3, *b–d*, are therefore due both to the presence of protein aggregates that have been positively stained and rotary-shadowed, and to the presence of the colloidal gold marker.

Swollen chromosomes reacted with preimmune serum were found to remain as undifferentiated puddles of chromatin whose only discernable structural landmark was the centromeric region (see arrow in Fig. 3 *a*). All chromosomes on the grid had this appearance, which was indistinguishable from untreated swollen chromosomes (not shown). A striking morphological change was observed in 96% (198/206) of chromosomes that were exposed in parallel to anti-cSc-1. Discrete clumps of aggregated material appeared along the chromatid axes (Fig. 3, *c* and *d*). These clumps were ~120–200 nm across, and showed differing degrees of aggregation in different

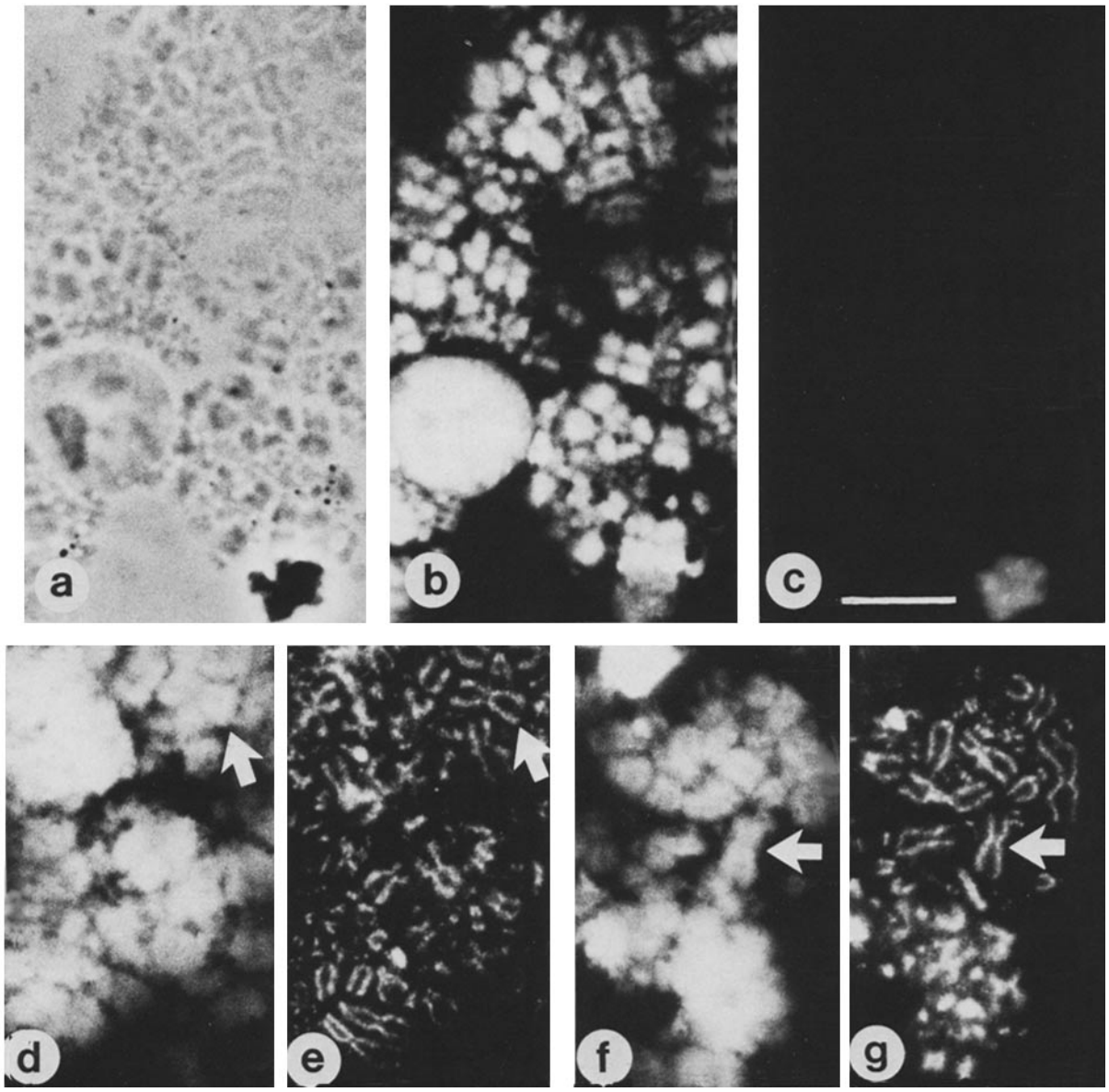

FIGURE 1 Indirect immunofluorescence of MSB-1 mitotic cells broken open by centrifugation onto glass coverslips. The first antibody used was (*a–c*) preimmune whole serum (1:500), and (*d–g*) anti-cSc-1 whole serum (1:500). The panels are visualized by (*a*) phase contrast, (*b, d, f*) fluorescence of DAPI bound to the DNA, and (*c, e, g*) fluorescence of rhodamine-conjugated anti-guinea pig IgG. *a–c*, *d* and *e*, and *f* and *g* show parallel exposures of the same field. Arrows in *d–g* indicate two chromosomes visualized by both DAPI staining and binding of anti-topoisomerase II. Bar, 10 μm.

chromosomes (compare Fig. 3, *c* and *d*). The clumps may result from aggregation of the chromatin through cross-linking of topoisomerase subunits by the antibodies. Alternatively, they may simply be large aggregates of immunoglobulin.

We were concerned that the axial density increase observed in Fig. 3, *c* and *d*, might be a nonspecific effect that would result from binding of any antibody to chromosomal antigens. Such an effect might be similar to the chromatin precipitation induced by divalent cations (9). We therefore exposed chromosomes on parallel grids to a human autoimmune serum containing a high titer of anti-DNA antibodies (from a patient with systemic lupus erythematosus). This serum caused an overall alteration of the chromosome structure consistent with either random local aggregation of the chromatin or with deposition of large quantities of antibody (Fig. 3*b*). No preferential effect was seen along the chromosome axes. All chromosomes on the grid had this appearance.

To exclude the possibility that the different appearance of the chromosomes in Fig. 3, *b* and *c*, was due to the trivial effect of binding vastly different quantities of antibody, we examined the selected regions of the chromosomes indicated in Fig. 3 at higher magnification. This permits visualization of the colloidal gold used to detect bound antibody. Fig. 4 shows that similar amounts of bound antibody were detected when chromosomes had been exposed to either anti-cSc-1 or anti-DNA. In addition, experiments like that of Fig. 3 were performed with anti-topoisomerase II over the dilution range 1:125–1:1,000 and anti-DNA over the range 1:50–1:1,000. At all concentrations, the effects of treatment of chromosomes with the two sera remained readily distinguishable.

Treatment of Chromosomes in Solution with Anti-Topoisomerase II: Experimental

Together the results obtained by indirect immunofluorescence and electron microscopy suggest that topoisomerase II

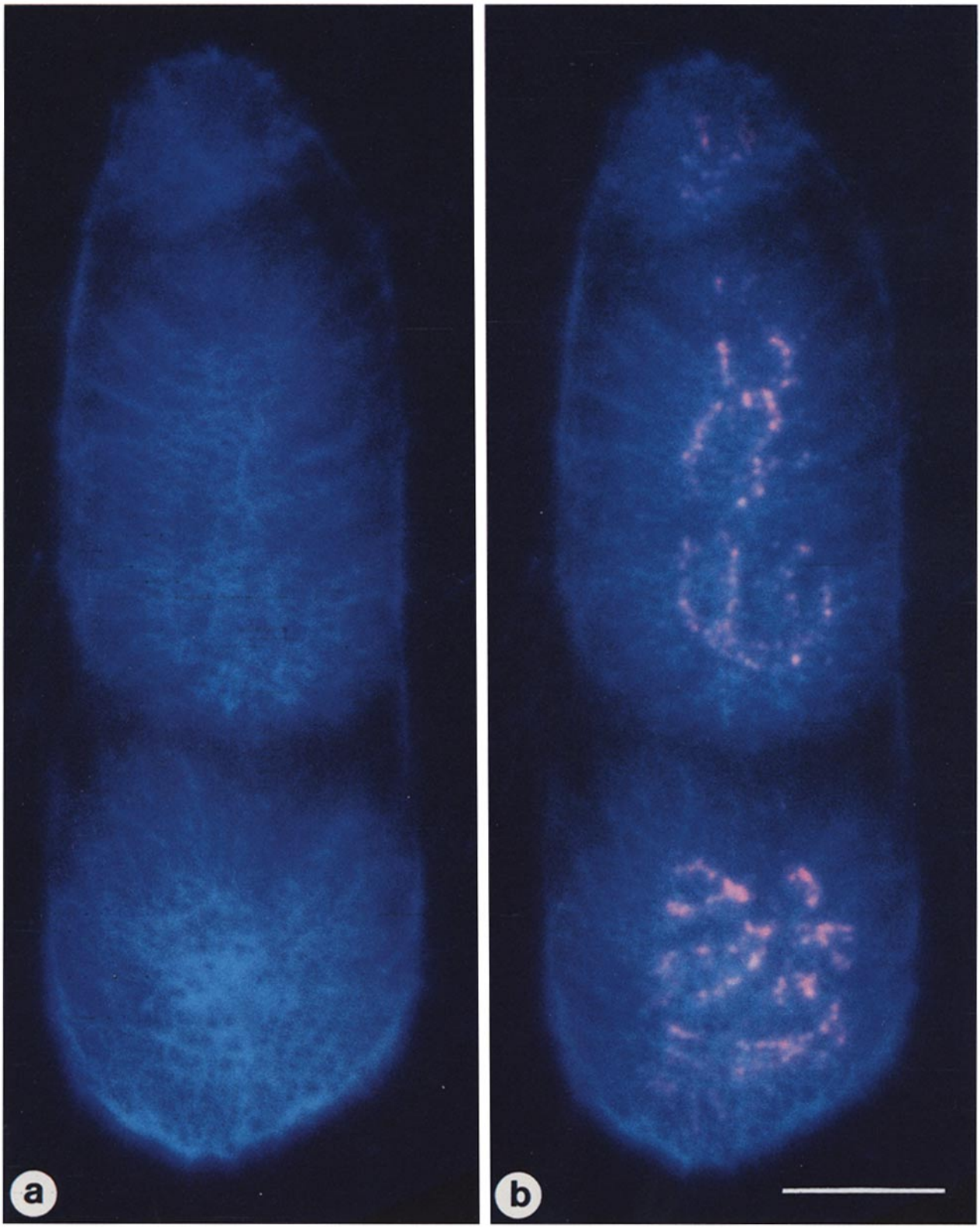

FIGURE 2 Double exposure showing the relative distribution of DNA and scaffold antigen within unextracted mitotic chromosomes. (*a*) DAPI staining of the DNA, present as chromatin. (*b*) Superimposition of DAPI staining with immunolocalization of topoisomerase II, using anti-cSc-1. Note the radial distribution of chromatin fibers and the absence of distinct chromatid axes in *a*. Bar, 1 μm.

is located near the axes in swollen chromosomes. Such a result is consistent with structural models in which the enzyme is located at the base of chromatin loops that radiate outward from the chromosome axes in swollen chromosomes (7, 9). It was possible to test this hypothesis by using the following experimental protocol: Isolated chromosomes were swollen by resuspension in low ionic strength buffer. To this suspension was then added either preimmune serum or specific antibody. After an incubation to allow interaction of the antibody with its antigen, the chromosomes were sedimented

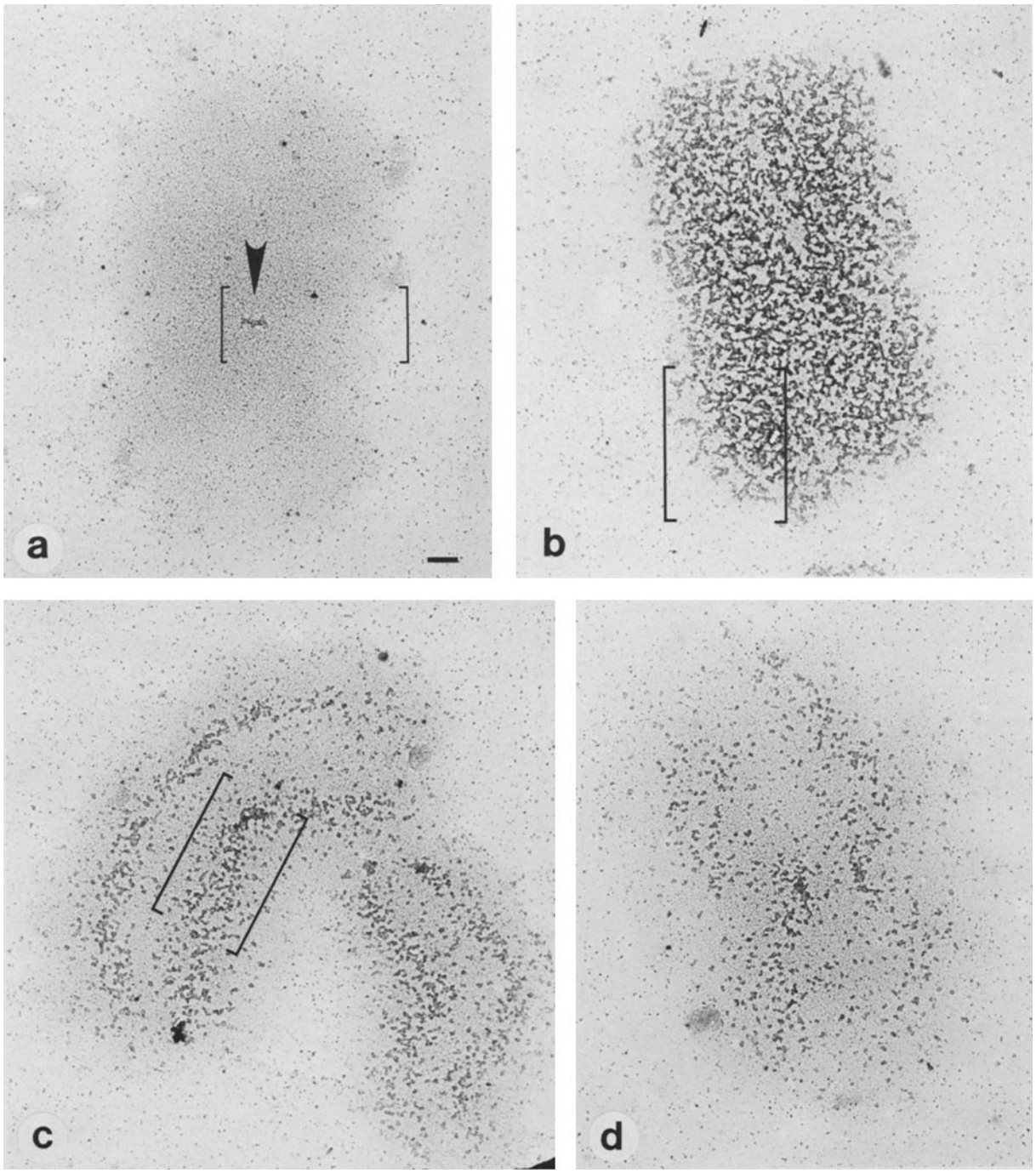

FIGURE 3 Immunoelectron microscopy of isolated MSB-1 chromosomes treated on the grid with various antibodies. The first antibody used in each case was: (*a*) guinea pig preimmune serum (1:50); (*b*) autoimmune serum from a patient with high levels of anti-DNA antibodies (1:200); (*c* and *d*) anti-cSc-1, whole serum (1:250). After treatment with first antibody, the samples were treated with biotinylated goat anti-human or anti-guinea pig IgG, affinity purified rabbit anti-biotin, and goat anti-rabbit IgG coupled to colloidal gold. In all cases the chromosomes were stained and shadowed with metal as described in Materials and Methods. The chromosomes shown are typical of the entire population and are not highly selected views. In a, condensed centromeric chromatin is indicated by an arrowhead. The approximate areas displayed at higher magnification in Fig. 4 are indicated by brackets. Bar, 1 μm.

onto electron microscope grids through a buffer that greatly favors chromosome swelling. No secondary antibodies were used.

This experiment was designed to examine structural alterations caused by antibody-induced aggregation of antigens in intact unfixed chromosomes. The consequences expected to result from two different distributions of antigen are diagrammed in Fig. 5. A uniform distribution of antigen (Fig. 5 *a*) would be expected to result in condensation of the entire chromosome if the antigen were aggregated by antibody. The condensation would be due, at least in part, to a collapse of the radial chromatin loops. If the antigen were localized in

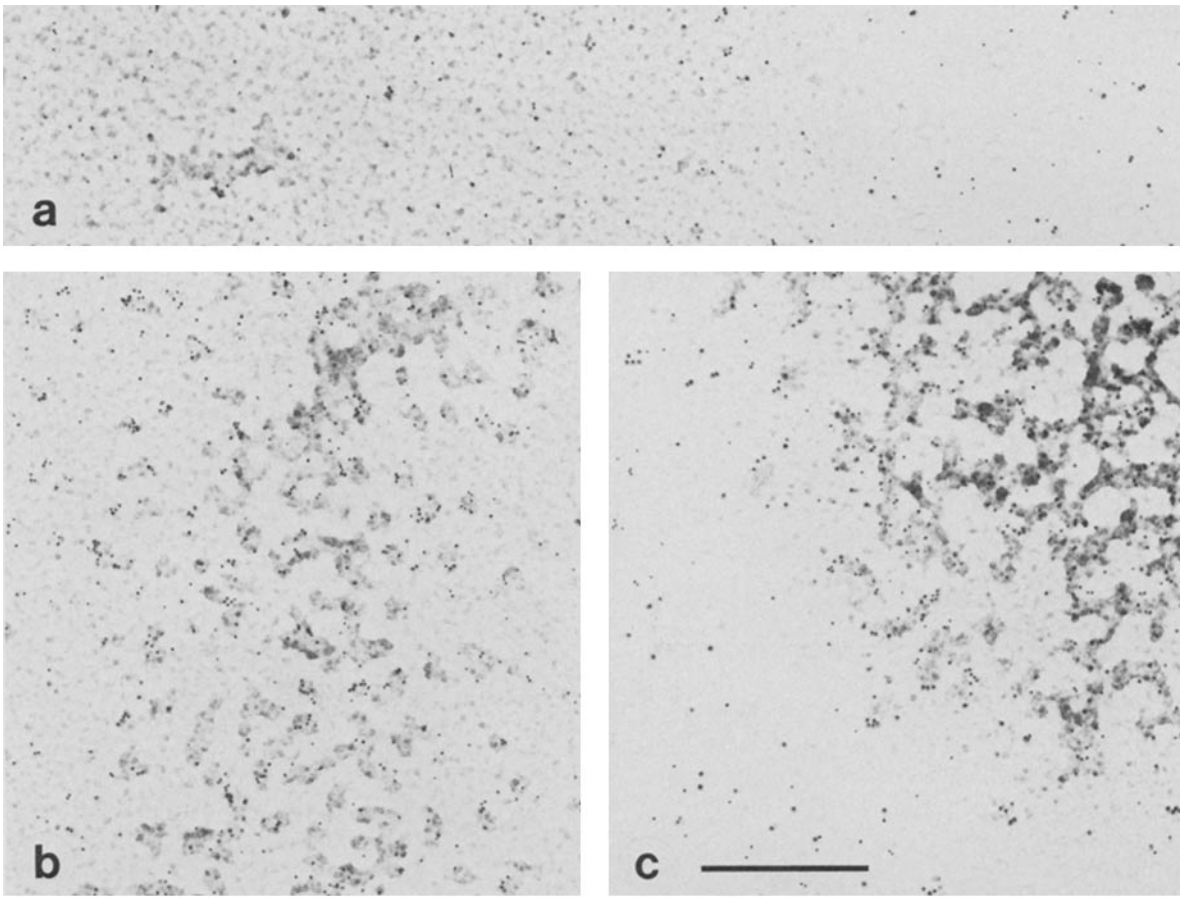

FIGURE 4 Selected areas of the images of Fig. 3 a–c printed at higher magnification to permit detection of bound antibody. In all cases the samples were processed as described in Fig. 3 and in Materials and Methods. The first antibody used in each case was: (a) preimmune serum; (b) anti-cSc-1; and (c) human autoimmune serum containing anti-DNA antibodies. The bound antibody is revealed by the presence of colloidal gold particles. Bar, 1 μm.

the axial region of swollen chromosomes and was not associated with the radial loops, exposure to antibody should result in aggregation of the axial region but leave the radial loops extended (Fig. 5*b*). The results of one such experiment are shown in Fig. 6, in which microchromosomes (which resemble in size the double minutes found in some tumor cell lines, and of which there are approximately 60 per diploid chicken cell [27]) have been marked with the letter "*m*."

Chromosomes exposed to preimmune serum in solution resembled untreated swollen chromosomes (Fig. 6, *a* and *b*). Except for the presence of condensed centromeric regions (arrowhead, Fig. 6*a*), these chromosomes did not appear to contain any regions of aggregated chromatin. In four repetitions of this type of experiment, all chromosomes exposed to a given antiserum had the same appearance when examined in the electron microscope.

Chromosomes exposed to anti-DNA antibodies in solution became highly condensed and did not reexpand even when exposed to solution conditions that favor swelling (Fig. 6*d*). This suggests that DNA throughout the chromosome became cross-linked by anti-DNA antibodies, and that this cross-linking was responsible for the chromosome condensation.

Exposure of chromosomes in solution to anti-cSc-1 caused a marked aggregation of material around the chromatid axes (Fig. 6*c*). These antibody-induced changes were observed only in the axial region. This suggests that the topoisomerase II is not interspersed among the nucleosomes on the radial chromatin loops. The structural changes observed along the chromatid axes in Fig. 6*c* indicate that substantial quantities of antibody were bound. Therefore, the differences between Fig. 6, *c* and *d*, are not likely to be due merely to the presence of vastly different amounts of DNA and topoisomerase II (see below).

Quantitative analysis of the results of the above experiment supports the conclusion that topoisomerase II is not found along the radial chromatin loops in swollen chromosomes. If the enzyme were located in the loop region, we would expect that chromosomes treated with anti-cSc-1 would undergo some degree of contraction (see Figs. 5*a* and 6*d*). Measurement of a number of isolated macrochromosomes indicates that exposure to anti-DNA causes a marked decrease in the

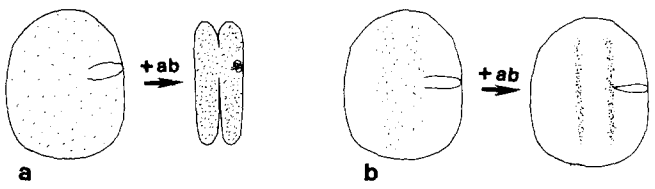

FIGURE 5 The effects of two different distributions of antigen on the final appearance of unfixed chromosomes exposed to antibody (*ab*) in solution.

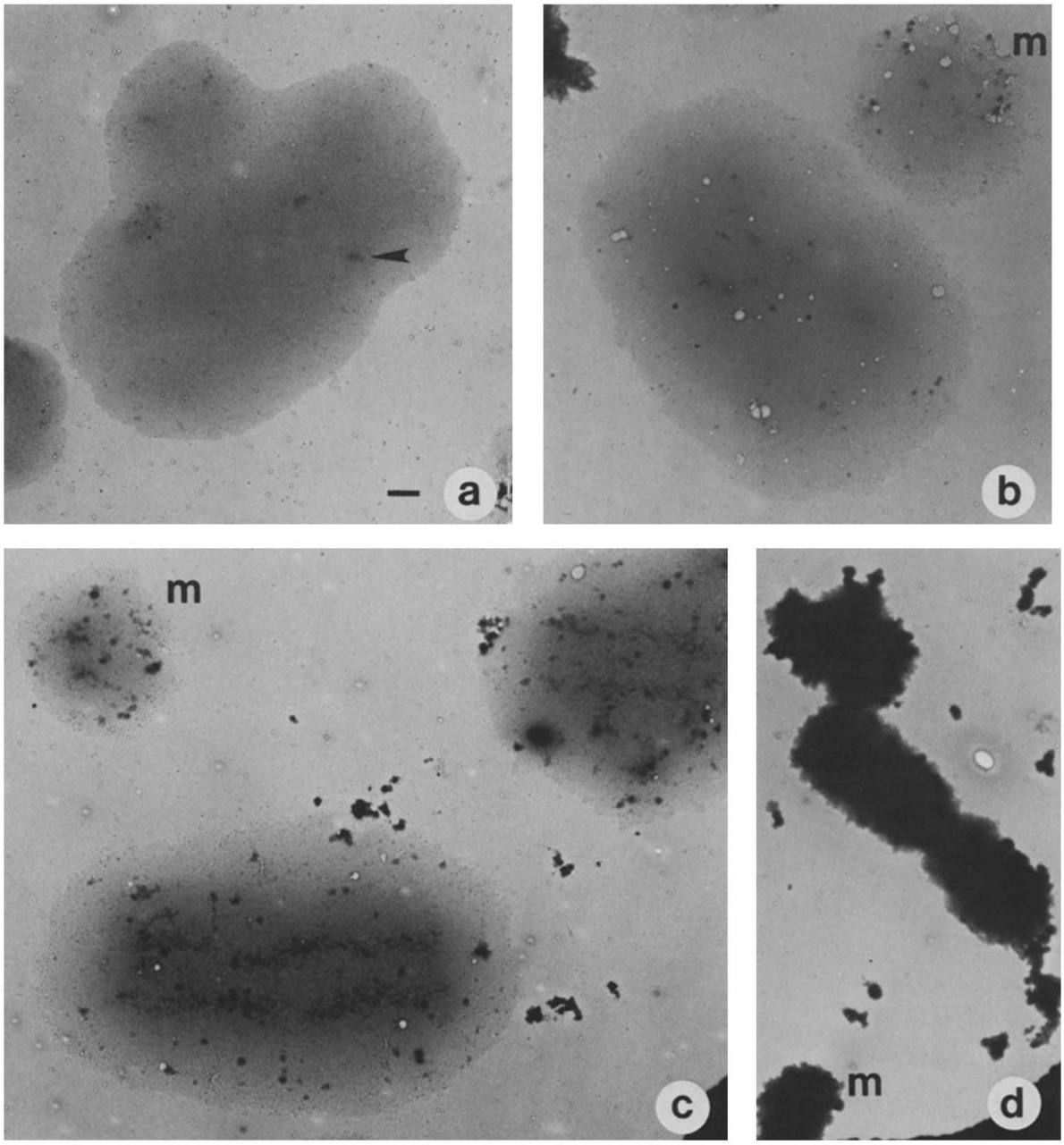

FIGURE 6 The effects of treatment of unfixed chicken mitotic chromosomes with various antisera in solution. The chromosomes were swollen by dilution into a low ionic strength buffer. To this suspension were then added the following antisera: (*a*) none; (*b*) guinea pig preimmune serum; (*c*) anti-cSc-1; (*d*) anti-DNA (lupus patient serum). All sera were added to a final concentration of 1:100. After antibody treatment, chromosomes were sedimented onto electron microscope grids, fixed, stained with PTA, and rotary shadowed. The arrowhead in *a* indicates the condensed centromeric region in a swollen chromosome. *M*, microchromosomes. Bar, 1 μm.

diameter of swollen chromosomes, but that exposure to anti-cSc-1 has no such effect. The results of measurements of the chromosome width across the midpoint of the longer arms are as follows: anti-DNA, 3.8 ± 0.6 μm (25 measured); no antibody exposure, 9.1 ± 1.4 μm (16 measured); preimmune serum, 10.2 ± 1.2 μm (14 measured); and anti-cSc-1, 9.6 ± 1.4 μm (12 measured).

Treatment of Chromosomes in Solution with Anti-Topoisomerase II: Controls

Three alternative explanations of the conclusions of the previous section are addressed below. These are as follows: (*a*) The difference between treatment of chromosomes with anti-cSc-1 and anti-DNA might be due to vast differences in the amount of antigen present. (*b*) The anti-cSc-1 antibody might recognize only a limited subset of the total topoisomerase II. Other enzyme subtypes might occur in the radial loop chromatin. (*c*) The binding of antibody to topoisomerase II might sterically inactivate the second antigen-binding site, effectively preventing the antibody from freely cross-linking its antigen.

We have addressed the first point by comparing the effect caused by binding of anti-topoisomerase II to chromosomes with that caused by antibodies to topoisomerase I. This enzyme is not found in the chromosome scaffold (B. Halligan,

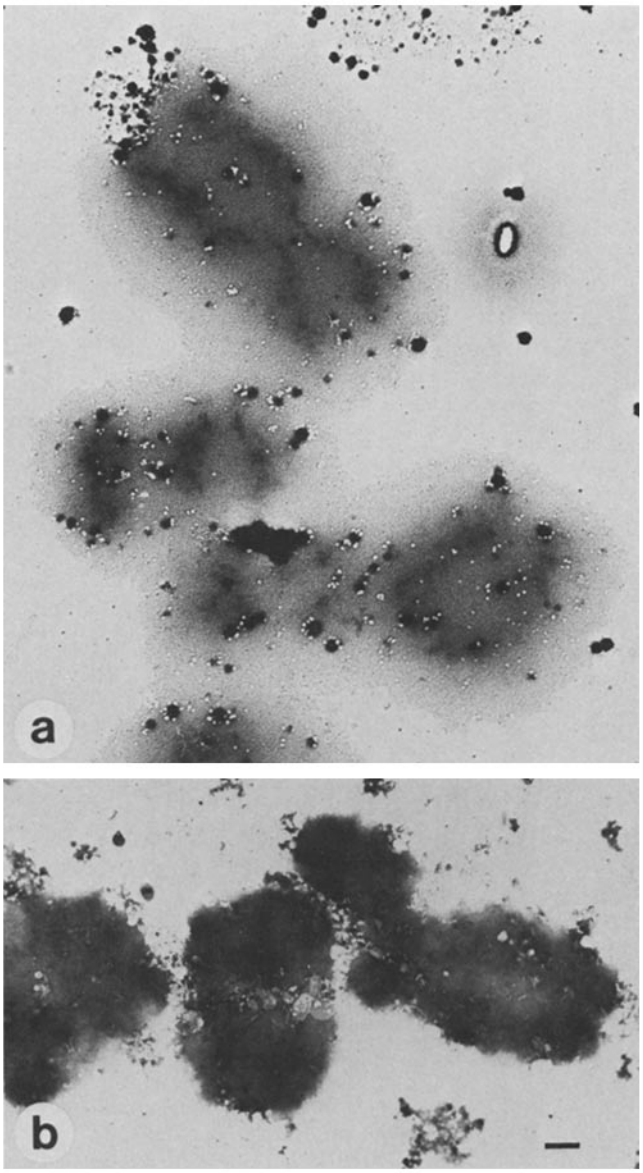

FIGURE 7 Treatment of chromosomes in solution with rabbit antisera that recognize topoisomerases I and II. The samples were processed as for Fig. 6. The antibodies used to treat chromosomes in solution were (*a*) anti-topoisomerase II (1:50) and (*b*) anti-topoisomerase I (1:50). Bar, 1 μm.

W. C. Earnshaw, and L. F. Liu, unpublished observations). Anti-topoisomerase I (the gift of L. Liu, Johns Hopkins University School of Medicine) caused a condensation of unfixed chromosomes resembling that caused by anti-DNA (Fig. 7 *b*). An identical result was obtained with antibodies to HMG-17 (the gift of M. Bustin, National Institutes of Health). When these antibodies were tested over a wide range of dilutions, they were found to cause a gradient of changes involving the whole chromosome. We have not observed any preferential effect along the chromosome axis at any intermediate concentration of antibody.

We have addressed the second explanation above by treating chromosomes in solution with rabbit anti-topoisomerase II (20, 28). This antibody shows a wide interspecies cross-reactivity not exhibited by the anti-cSc-1 antibody discussed above. Nonetheless, when it is used to treat swollen chromosomes in solution, the resultant structural changes are indistinguishable from those caused by anti-cSc-1 (Fig. 7 *a*). This confirms that few, if any, previously undetected topoisomerase II molecules are found in the chromatin of the radial loops.

We have also shown that the difference between the effects caused by anti-topoisomerase I and the two independent anti-topoisomerase II antibodies is unlikely to be due to functional monovalency of the antibodies. Chromosomes were treated in solution with both anti-topoisomerase II sera or with low levels of human serum containing anti-DNA antibodies (an amount insufficient to cause the dramatic condensation seen in Fig. 6). The chromosomes were removed from antibody by gentle sedimentation through a 0.1-M sucrose cushion and then reacted with second antibody (anti-guinea pig, anti-rabbit, or anti-human). They were subsequently processed either for electron microscopy or immunofluorescence (data not shown). Immunofluorescence experiments provided the proof that significant amounts of second antibody were bound.

Treatment with second antibody caused further condensation of chromosomes previously exposed to low levels of anti-DNA. Chromosomes treated with this level of anti-DNA alone measured 9.0 ± 1.5 μm across (32 measured). After treatment with second antibody, this value decreased to 7.0 ± 1.2 μm (34 measured). However treatment with second antibody had no visible effect on chromosomes treated with either guinea pig or rabbit anti-topoisomerase II. Chromosomes treated with anti-cSc-1 alone measured 9.8 ± 1.3 μm across, while after second antibody treatment the corresponding value was 10.1 ± 1.5 μm (21 measured in both cases). These latter values are in excellent agreement with results of an independent experiment described above. The second antibody should have been fully capable of cross-linking the first antibodies, and would have been expected to cause chromosome condensation if the original antigens were uniformly distributed.

Given that both anti-topoisomerase sera are polyclonal, that binding of second antibody had no additional effect, and that immunogold detection of the bound antibody was limited to the axial region (Figs. 3 and 4), we consider it highly unlikely that the effect observed following treatment of swollen chromosomes with anti-topoisomerase II is limited to the axial region as a result of functional monovalency of the antisera.

DISCUSSION

Current Status of the Scaffold Model

While other models of mitotic chromosome architecture have been proposed (29, 30), considerable evidence has been obtained supporting a radial loop model (6, 7). Any loop model for chromosome structure requires a mechanism to explain how the loops are fastened together along the chromatid axis, and Laemmli et al. (10) proposed that this loop closure was one function of the mitotic chromosome scaffold. Such a role for the scaffold proteins does not require the existence of a rigid scaffold structure within the chromosome, although self-association among scaffold components provides one simple model for a mechanism of chromosome condensation.

Recent experimental evidence indicates that the mitotic chromosome scaffold is composed of a discrete and reproducible set of nonhistone proteins that remain associated following nuclease digestion and extraction of the bulk of the

proteins from isolated chromosomes (11, 20). The degree to which this association reflects the existence of a discrete scaffold substructure within intact chromosomes remains an open question.

The experiments presented above provide the first high resolution localization of a scaffold protein in intact, unextracted chromosomes. We have found no evidence for the existence of a rigid core-like scaffold, such as has been suggested by silver-staining experiments (15, 19). Rather, our localization of topoisomerase II in swollen chromosomes (Fig. 3*d*) suggests that under some circumstances this scaffold component is localized in numerous separate "islets" that appear to be 120–200 nm across when covered with bound antibody. These may represent areas in which several chromatin loops are clustered either as a result of topoisomerase-topoisomerase interactions or as a result of antibody cross-linking. Such "islets" could be related to the "chromomere loops" or "rosettes" previously proposed by Comings (31) and Okada and Comings (32).

One implication of the above results is that scaffold-scaffold interactions within the mitotic chromosome may be short range rather than global. That is, scaffold components might cause closure of loop domains and local clustering of these domains into annuli or rosettes. The sum of many such local interactions could result in chromosome condensation. This model is distinct from the original view of the scaffold as a robust structure running rod-like along the axis of each sister chromatid. The exact distribution of scaffold molecules within the intact chromosome is likely to be extremely difficult to resolve by direct immunocytochemical examination of chromosomes (even in ultrathin sections), given the complexity and packing density within the structure. Functional experiments, like that of Fig. 6 may yield a more accurate view of the interactions between scaffold proteins and the chromatin of the loop domains. Eventually, it will be necessary to develop antibody-based assays to study the function of individual scaffold proteins in vivo.

Distribution of Topoisomerase II in Intact Chromosomes

Indirect immunofluorescence, immunoelectron microscopy, and treatment of unfixed chromosomes in solution with anti-topoisomerase II all indicate that the distribution of the enzyme differs from that of bulk chromatin in swollen chromosomes. The enzyme does not appear to be randomly interspersed among the nucleosomes along the entire length of the chromatin fiber. Rather, it appears to be localized in the axial region of swollen chromosomes.

While we cannot directly extrapolate from studies of expanded chromosomes to predict the detailed spatial distribution of topoisomerase II in condensed chromosomes in vivo, it is unlikely that the gentle conditions we have employed cause gross rearrangements of the enzyme-binding sites along the chromatin fiber. We have examined chromosomes expanded by two different methods—mechanical forces occurring during centrifugation in the presence of divalent cations, and charge repulsion following removal of divalent cations. The latter is unlikely to disrupt chromosome structure, since chromosomes may be subjected to repeated cycles of swelling and shrinking in solution without undergoing any apparent alteration in their macroscopic structure (26). Furthermore, unfixed SV40 chromatin has been shown to retain the nucleosome-free region around the origin of replication when exposed to low ionic strength during preparation for electron microscopy (33). Both types of expanded chromosomes yield similar results with respect to the relative distribution of topoisomerase II and bulk chromatin.

Our immunolocalization data are consistent with two possible distributions of the enzyme in chromosomes. (*a*) It could occur at the base of each loop, possibly forming the link responsible for topological closure of the loop. (*b*) It could form an independent network that need not follow the path of the chromatin fiber at all. In this case, the scaffold could be thought of as a meshwork permeating the entire chromatid. This second model is unlikely, since it is known that topoisomerase II is bound to the cellular DNA (34). In addition, cross-linking such a meshwork with anti-scaffold antibodies would be expected to cause some condensation of the entire chromosome as a result of simple entanglement of the radial chromatin loops. No such condensation occurs following antibody treatment (Fig. 6).

Our data permit further interpretation of the biochemical results of Chen et al. (34), which suggested that a subset of topoisomerase II–binding sites is widely spaced in interphase chromatin. They showed that lysis (in SDS) of cells exposed to the epipodophyllotoxins VP-16 and VM-26 results in fragmentation of the cellular DNA into linear pieces of ~50–100 kilobase pairs with topoisomerase II covalently attached to the 5′ end. This spacing suggests that there may be a single high-affinity binding site for the enzyme in each loop domain. The immunolocalization results presented above suggest that this binding site occurs at the base of the loop domains in mitotic chromosomes.

The original experiments describing the properties of histone-depleted chromosomes (6, 35) indicated that the scaffold structure retains the ability to constrain the metaphase DNA in a looped form. This implies that at least one component of the scaffold is responsible for topological closure of the loops. It is therefore tempting to speculate that topoisomerase II may be directly involved in topological closure of the loops. It is, however, possible that loop closure is mediated by other scaffold components, which may or may not be associated with the enzyme.

This discussion raises the fundamental question of whether topoisomerase II is an enzyme that is specifically associated with structural components of the scaffold, or whether the protein is, in fact, an integral structural component itself. Enzymatically active structural proteins are well known in other contexts (e.g., dynein and myosin), but to our knowledge, so far unknown in the cell nucleus. It must also be noted that we have no evidence that the topoisomerase of chromosome scaffolds is enzymatically active (although we have shown that active enzyme may be recovered from intact chromosomes [20]). It is possible that the scaffold antigen is an inactive subtype that has retained close structural homology to the active enzyme, but lost its catalytic functions. This question will only be answered following the development of assays suitable for detecting topoisomerase II activity in intact chromosomes and scaffolds.

The role of topoisomerase II in vivo is not known, although it seems likely to be involved in both replication and transcription (36; see also Discussion of preceding paper [20]). It is interesting to note that the non–DNA-binding drugs VP-16 and VM-26 cause a dramatic increase in the frequency of sister chromatid exchange (37). Since the experiments of Chen

et al. (34) suggest that these drugs act directly on topoisomerase II, it is therefore possible that the enzyme plays a central role in sister chromatid exchange. The observation that inappropriate exchange events between nonsister chromatids are relatively rare might be due both to restrictions imposed by DNA sequence homology, and to regulation at the level of chromosome structure through involvement of chromosome scaffold proteins such as topoisomerase II.

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