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Topoisomerase II Is a Structural Component of Mitotic Chromosome Scaffolds

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ABSTRACT We have obtained a polyclonal antibody that recognizes a major polypeptide component of chicken mitotic chromosome scaffolds. This polypeptide migrates in SDS PAGE with M_r 170,000. Indirect immunofluorescence and subcellular fractionation experiments confirm that it is present in both mitotic chromosomes and interphase nuclei. Two lines of evidence suggest that this protein is DNA topoisomerase II, an abundant nuclear enzyme that controls DNA topological states: anti-scaffold antibody inhibits the strand-passing activity of DNA topoisomerase II; and both anti-scaffold antibody and an independent antibody raised against purified bovine topoisomerase II recognize identical partial proteolysis fragments of the 170,000-mol-wt scaffold protein in immunoblots. Our results suggest that topoisomerase II may be an enzyme that is also a structural protein of interphase nuclei and mitotic chromosomes.

It has been known for many years that the chromatin fiber of both meiotic (1–4) and mitotic (5) chromosomes is folded into loops. In 1977 and 1978, Laemmli and co-workers (6–8) proposed that in mitotic chromosomes these loops are formed as a result of interactions between the nucleohistone fiber and a subgroup of chromosomal nonhistone proteins. A residual structure thought to be enriched in these nonhistone proteins was isolated by extraction of nuclease-digested chromosomes under conditions that solubilized >90% of the chromosomal proteins, including all of the histones (9). Because it retained the size and approximate shape of the mitotic chromosome, this residual structure, was termed the "chromosome scaffold." More recent studies have shown that the scaffold fraction is greatly enriched in two high molecular weight polypeptides, Sc-1 (170,000 mol wt) and Sc-2 (135,000 mol wt; see reference 10).

The existence of a discrete scaffold substructure in intact mitotic chromosomes remains a subject of some debate. The isolated residual scaffold comprises only 3–4% of the chromosome mass (10). Therefore, it is not surprising that direct inspection of whole mounts (11, 12) or thin sections (13) of intact chromosomes does not permit ready visualization of a scaffold substructure, although under certain conditions axial structures can be seen (14). Localization of scaffold components in intact chromosomes has been forced to wait for the availability of anti-scaffold antibodies (15).

Results from a number of laboratories suggest that the chromatin fiber of interphase chromosomes is also topologically constrained in loops (16–22). If correct, this model implies that DNA topoisomerases might be required for the termination of replication. For example, the final stages of SV40 replication involve production of catenated dimers (23, 24). In the absence of nicks, such strucures can only be separated by a type II DNA topoisomerase. This class of enzyme changes the linking number of circular DNA molecules in steps of two, in an ATP-dependent reaction that proceeds via production of protein-linked double strand DNA breaks (recently reviewed in reference 25). If cellular chromosomes are constrained in closed loops, then topoisomerase II might play an essential role in termination of replication and for chromosome segregation. Analysis of conditional lethal topoisomerase II mutants in the unrelated yeasts *Saccharomyces cerevisiae* (26) and *Schizosaccharomyces pombe* (27) confirms that the enzyme is required for chromosome segregation, at least in lower eucaryotes.

We describe experiments performed with an antiserum against a 170,000-mol-wt chromosome scaffold polypeptide from chicken cells. Two independent lines of evidence suggest that this scaffold protein is DNA topoisomerase II. These are inhibition of topoisomerase function by anti-scaffold antibody, and comparison of binding of anti-scaffold and anti-topoisomerase II antibodies to partial peptides derived from

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the scaffold protein 1 (Sc-1)[1] polypeptide. Availability of the anti-topoisomerase antibody permits the first quantitative analysis of the scaffold isolation procedure. Topoisomerase II is recovered at high yield (~70%) in chromosome scaffolds made at either high or low ionic strength. These results have important implications for the control of expression of chromatin domains and for chromosome segregation.

MATERIALS AND METHODS

Cell Culture: MSB-1 cells, the gift of H. Weintraub (Hutchinson Cancer Center, Seattle, WA), were grown in RPMI-1640 (Gibco Laboratories, Grand Island, NY) + 5% Hyclone fetal calf serum (Sterile Systems, Logan, Utah; later replaced with 5% iron-suplemented calf serum from the same supplier). Chicken tumor cells, line 249, originally from the laboratory of M. Wigler (Cold Spring Harbor), were the gift of D. Cleveland (Johns Hopkins School of Medicine). These and primary cultures of chick embryo fibroblasts (28) were grown in Eagle's minimal essential medium with nonessential amino acids (Gibco Laboratories) plus 10% fetal calf serum and 1% chicken serum.

Production of Antibody to Guinea Pig Chicken Scaffold Protein 1: 80 liters of MSB-1 chicken lymphoblastoid cells were grown in spinner culture in batches ranging from 1.5–12 liters. Cells were accumulated in C-metaphase by incubation in the presence of 0.1 μg/ml colcemid for 12–16 h. Chromosomes were isolated by a minor modification of the polyamine method of Lewis and Laemmli (10). We modified the procedure by replacing the 40-ml sucrose gradient with a 16-ml 15–60% gradient over a 10-ml shelf of 80% sucrose. This procedure gives a chromosome recovery of ~25% (Table I). Chromosomes from each preparation were frozen on N_2(l).

All solutions for chromosome and interphase nuclear isolations were prepared fresh from 10× stocks, and the protease inhibitors Trasylol (Mobay Chemical Co., FBA Pharmaceuticals, New York), and phenylmethylsulphonyl fluoride (Sigma Chemical Co., St. Louis, MO) were added to 10 Kallikrein inhibitor units/ml and 0.1 mM, respectively, immediately before use.

Scaffolds were prepared from 95 mg of chromosomes by standard procedures using 2 M NaCl to extract the bulk of the chromosomal protein (9, 14). The scaffolds were resuspended in SDS PAGE sample buffer (10) and electrophoresed in two preparative SDS polyacrylamide gels. The gels were washed with water to remove SDS and stained with 0.1% Coomassie Blue in 1× running buffer minus SDS (L. Gerace, personal communication). Bands to be used as antigen were excised, homogenized in water with a Potter-Elvehjem motor driven homogenizer, and frozen in N_2(l). Guinea pigs were injected with ~100 μg of protein in acrylamide plus complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, MI), given a booster injection of ~50 μg (in incomplete Freund's adjuvant) at 30 d, and bled 14 d later by cardiac punture.

Affinity-eluted antibody was prepared from preparative nitrocellulose blots of SDS polyacrylamide gels, as described previously (29).

Production of Antibody to Bovine Topoisomerase II: The first large scale isolation of topoisomerase II from a mammalian source, and preparation of antibody recognizing the purified enzyme are described in detail in reference 30. Important steps in the enzyme purification included the following: extraction of nuclei under conditions in which the chromosome scaffold is solubilized (presence of β-mercaptoethanol [10]); polymin P precipitation of chromatin; ammonium sulphate precipitation; and chromatography on BioRex 70, hydroxylapatite, and blue agarose. This procedure yielded a 1,300-fold purification of the enzyme, which migrated in SDS PAGE as a doublet of M_r 125,000 and 140,000 (30). Both species yielded identical 2-dimensional tryptic maps. A rabbit was injected with a band excised from an SDS gel. Serum from this immunized animal recognizes a single polypeptide of M_r ~ 180,000 in immunoblots of whole cell lysates from calf, human, monkey, hamster (30), and chicken (B. Halligan, unpublished observations).

Gel Electrophoresis: Gel recipes were as previously published (10). 4× sample buffer was 0.125 M trizma base, 0.96 M glycine, 21% glycerol, 2.5% SDS, 0.25% bromphenol blue, and 0.5 M 2-mercaptoethanol. 1× sample buffer was 0.16 M Tris:HCl, pH 8.8, 16% sucrose, 2.2 mM EDTA, 3.3% SDS, 0.01% bromphenol blue, and 22 mM dithiothreitol.

Immunoblotting: The protocol for these experiments has been described previously (29, 31).

Immunofluorescence: Indirect immunofluorescence was performed as previously described (31), except that hepatoma and chick embryo fibroblast cultures were grown on polylysine-coated coverslips. Before processing for immunofluorescence, the coverslips were centrifuged at 1,000 *g* for 2 min to increase adherence of mitotic cells. Subsequent processing was as described previously (31). Rhodamine-conjugated anti-guinea pig IgG was obtained from Cappel Laboratories (Cochranville, PA).

Bookkeeping Experiments: Two methods were used to determine the percentage of total cellular chicken scaffold protein 1 (cSc-1) found in chromosomes and nuclei. Procedure *a* involved spotting solutions containing antigen onto nitrocellulose strips and processing these with iodinated protein A as for normal immunoblots (31). After drying, the strips were sliced up and counted in a gamma counter. In procedure *b*, aliquots of samples were subjected to SDS PAGE as described previously (10). The proteins were then transferred to nitrocellulose paper by electroblotting (32), and processed in the standard way with iodinated protein A. The blots were used to expose preflashed films at −70°C (33). Films were scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, CA), and the amount of material in the cSc-1 band determined by cutting out and weighing the peak. As shown in Table I, procedures *a* and *b* give comparable results.

The procedure used for chromosome isolation was as follows: 8 h before the addition of colcemid to the growing culture, [^{3}H]thymidine was added to 0.12 μCi/ml. The colcemid block was as described above. Next morning, 125 ml of culture was centrifuged and resuspended to swell in 50 ml of RSB buffer (10 mM Tris:HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$). After 5 min at room temperature (during which time the mitotic index was measured), the cells were centrifuged at 800 *g* for 3 min. The swollen cell pellet was vigorously resuspended in 10 ml lysis buffer (15 mM Tris:HCl, pH 7.4, 2 mM K-EDTA, 80 mM KCl, 0.2 mM Spermine, 0.5 mM Spermidine, 0.1% digitonin [10]), and the cells disrupted with 10 strokes of a Dounce homogenizer. 50 μl of homogenate was removed and stored at 4°C for subsequent trichloroacetic acid (TCA) precipitation. Two 0.5-ml aliquots of lysate were removed and processed for electrophoresis as follows: $CaCl_2$ was added to 3 mM and micrococcal nuclease (Worthington Biochemical Corp., Freehold, NJ) to 40 μg/ml. The digestion was allowed to proceed 20 min at 4°C and then terminated in one of two ways. To one aliquot was added ⅓ volume of 4× SDS PAGE sample buffer. This aliquot was then extensively sonicated and boiled, yielding a sample for determination of total antigen. After nuclease digestion, the other aliquot was centrifuged at 16,000 *g* for 10 min and then resuspended in 0.5 ml 1× SDS PAGE sample buffer. This was then sonicated and boiled, yielding the sample for determination of total insoluble antigen. During the processing of the above samples, the rest of the homogenate was processed for chromosome isolation as described by Lewis and Laemmli (10). The final chromosome pellet was resuspended in 0.5 ml of 5 mM Tris:HCl, pH 7.4, 2 mM KCl, 0.25 mM spermidine, 0.1% digitonin (10). The chromosomes were then processed for determination of ^{3}H and total insoluble antigen as described above.

The chromosome yield was calculated as the percent of acid-insoluble ^{3}H recovered, taking into account the mitotic index, since this procedure separates chromosomes from nuclei. The TCA precipitation was performed by standard methods, with nucleic acid being precipitated by 10% TCA in the presence of 1.5% Na-pyrophosphate and 0.1% bovine serum albumin (BSA) as carrier. Precipitates, collected on GF/C (Whatman Chemical Separation Inc., Clifton, NJ) filters were counted in Aquasol-2 after drying. The final yield of cSc-1 was measured by either procedure *a* or *b* as described above. The final yields were normalized by taking into account the chromosome yield calculated above, the relative amounts of original culture loaded per lane (0.15 ml for homogenate, 3.9 ml for chromosomes), and (where applicable) the width of the gel lanes.

The amount of cSc-1 in interphase nuclei was calculated as follows: Cells were grown overnight in the presence of [^{3}H]thymidine as above, but no colcemid was added. Next morning, cells (50-ml culture) were centrifuged at 800 *g* for 3 min and resuspended for hypotonic swelling in RSB buffer (see two paragraphs above) for 5 min at room temperature. They were then centrifuged at 800 *g* for 3 min and resuspended in 4 ml lysis buffer (same as chromosome lysis buffer above, but plus 20% percoll [modified from reference 34]). Cell disruption was accomplished by vigorous Dounce homogenization (20×). An aliquot was taken as above for TCA precipitation, 0.5 ml removed for measurement of antigen, and the rest of the lysate centrifuged at 250 *g* for 5 min. The supernatant was removed by aspiration, and the pellet of nuclei resuspended in 4 ml lysis buffer. After a second centrifugation, the nuclear pellet was resuspended in a volume equal to that of the original homogenate, SDS added to 0.1%, the sample lightly sonicated, an aliquot taken for TCA precipitation, and the remainder processed for detection of antigen by procedure *a* described above.

Enzyme Assays: Mitotic chromosomes were prepared from 500 ml of colcemid-arrested MSB-1 chicken lymphoblastoid cells by gentle differential centrifugation (15). Topoisomerase II extracts were prepared from the chromosomes by the method of Miller et al. (35). Extracts were incubated with

[1] *Abbreviations used in this paper:* CENP-A and CENP-B, human centromere proteins of M_r 17,000 and 80,000, respectively; Sc-1 and cSc-1, human and chicken scaffold protein 1, respectively; TCA, trichloroacetic acid.

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antisera (all used at a dilution of 1:50) for 1 h at 4°C with gentle agitation. Immune complexes were adsorbed by addition of fixed *Staphylococcus aureus* to ~0.6% (wt/vol). This suspension was incubated for 1 h at 4°C with agitation, and the immune complexes subsequently removed by centrifugation at 16,000 *g* for 5 min. Assay conditions for topoisomerase I (relaxation of supercoiled pBR322 DNA [36]) and topoisomerase II (unknotting of phage P4 DNA [37]) were as previously described, except that the incubation was done at 37°C for 2 h.

RESULTS

Production of Antibodies to a Polypeptide from Chicken Chromosome Scaffolds

Fig. 1 shows the protein composition of mitotic chromosomes and chromosome scaffolds isolated from colcemid-blocked chicken cells (line MSB-1). Chromosomes (Fig. 1 *b*) were isolated by the polyamine method of Lewis and Laemmli (10). Nonhistone scaffolds isolated from these chromosomes after digestion with micrococcal nuclease and subsequent extraction with 2 M NaCl (9) are shown in Fig. 1 *c*. Chromosomal proteins solubilized by the extraction are shown in Fig. 1 *d*. The protein composition of chicken chromosome scaffolds is similar to that of human scaffolds, as described by Lewis and Laemmli (10). Scaffolds of identical protein composition were obtained if a low ionic strength polyanion extraction procedure (9, 14) was used in place of 2 M NaCl (data not shown).

A guinea pig immunized with a high molecular weight polypeptide from chicken chromosome scaffolds (indicated by arrows in Fig. 1) produced antibodies that bind to a single band (M_r 170,000) on nitrocellulose blots of an SDS PAGE of whole cells (Fig. 2 *b*). This is the antigen we refer to as cSc-1. These antibodies bind to cSc-1 and a number of smaller peptides present in isolated chicken chromosomes (Fig. 2 *c*). Preimmune serum did not bind to any chromosomal proteins (Fig. 2 *d*). The smaller peptides appear when cells are homogenized in the presence of detergent, and co-purify with chromosomes. Antibody fractions that had been affinity-eluted from either cSc-1 or from the smaller peptides gave identical patterns when used to reprobe blots of total chromosomal proteins (Fig. 2, *e* and *f*). This, together with the observation of a single antigenic species in boiled whole cell extracts (Fig. 2 *b*), suggests that the smaller peptides are proteolytic fragments derived from cSc-1.

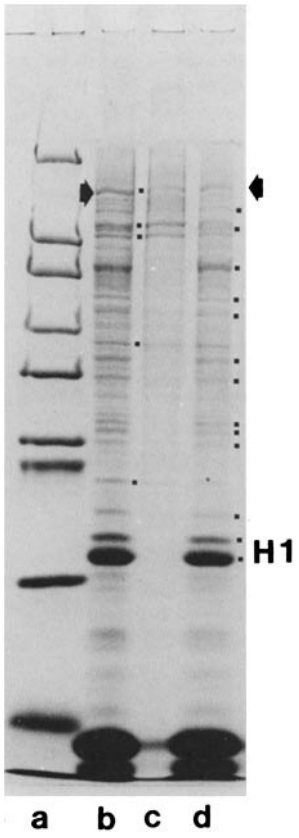

FIGURE 1 SDS PAGE of chicken chromosomes and nonhistone scaffolds isolated from them. Lane *a*, marker proteins myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (95 kD), bovine serum albumin (68 kD), catalase (60 kD), actin (43 kD), aldolase (40 kD), carbonic anhydrase (29 kD), and beta-lactoglobulin (18.4 kD). Lane *b*, isolated chromosomes. Lane *c*, chromosome scaffolds isolated after 2 M NaCl treatment. Lane *d*, proteins extracted from chromosomes by the 2 M NaCl treatment. The proteins were electrophoresed in a 10% polyacrylamide gel (10) and visualized by staining with Coomassie Blue. The profile of scaffolds isolated after extraction with dextran sulfate and heparin (9) was identical to that in lane *c*; however, patterns of the proteins extracted by the polyanion procedure were difficult to obtain as the polyanions interfere with the mobility of the proteins in the gel lane. Several prominent scaffold proteins are indicated by dots between lanes *b* and *c*. Major extracted proteins are shown by dots next to lane *d*. The position of cSc-1 is indicated by arrows.

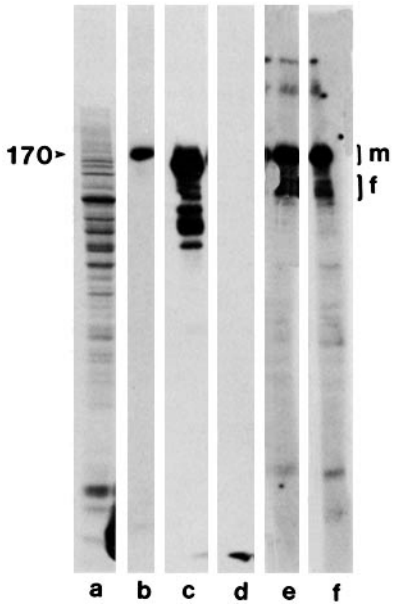

FIGURE 2 Immunoblots of cellular and chromosomal proteins probed with anti-cSc-1. Bound antibody was detected by autoradiography after reaction with ^{125}I-protein A. Lane *a*, mitotic cells centrifuged and resuspended in boiling SDS PAGE sample buffer; Coomassie Blue stain. Lane *b*, parallel gel lane probed with anti-cSc-1. In all subsequent lanes the antigen mixture came from isolated mitotic chromosomes and the antibodies used to probe the blots were as follows: (lane *c*) whole serum anti-cSc-1; (lane *d*) preimmune serum; (lane *e*) anti-cSc-1 affinity eluted from the cSc-1 band of a preparative immunoblot (indicated to the right of lane *f* and marked *m*); and (lane *f*) anti-fragments affinity eluted from the indicated region (marked *f*) of a preparative immunoblot. Whole serum was used at a dilution of 1:500. Affinity-eluted antibody fractions were used at a dilution of 1:20. For a Coomassie Blue-stained view of the antigen mixtures in lanes *c–f* see Fig. 1 *b*.

Evidence That the Scaffold Protein cSc-1 Is Topoisomerase II

Both cSc-1 and topoisomerase II isolated from a number of species, migrate in SDS PAGE with M_r 170,000 (30, 35). The following experiments suggest that cSc-1 is, in fact, topoisomerase II.

First, both guinea pig anti-cSc-1 and rabbit anti-topoisomerase II (prepared against enzyme purified to homogeneity from calf thymus [30]) bind to identical ladders of polypeptides on immunoblots of chicken chromosomes and scaffolds (Fig. 3, *b* and *c*). The major immunoreactive polypeptides that migrate ahead of cSc-1 have apparent M_r 126,000, 117,000, 106,000, 98,000, 91,000, and 81,000. These are presumed to be proteolytic fragments of cSc-1 (preceding

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section). On overexposed autoradiographs at least nine additional bands (corresponding to smaller fragments) are recognized by both sera. Neither preimmune serum (Fig. 3*a*) nor guinea pig sera raised against other high molecular weight chromosome scaffold components bind to any of these bands (W. C. Earnshaw, unpublished observations). We therefore conclude that both antisera recognize specific epitopes that are present on intact cSc-1 and on various fragments derived from it.

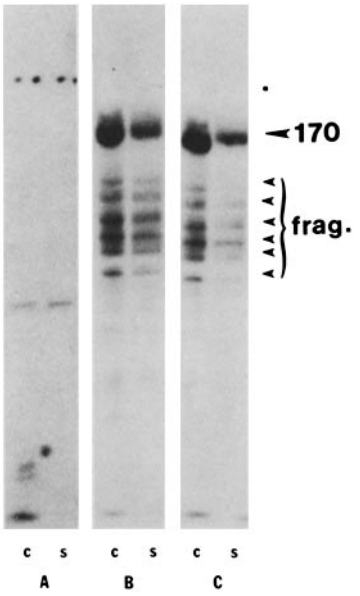

FIGURE 3 Topoisomerase II is found in chicken chromosome scaffolds. Chromosomes and scaffolds were electrophoresed in parallel lanes of an SDS polyacrylamide gel, transferred to nitrocellulose, and probed with the following sera: (*a*) guinea pig preimmune serum; (*b*) guinea pig anti-cSc-1; (*c*) rabbit anti-topoisomerase II. Bound antibody was detected by incubation of the blot with ^{125}I-protein A, followed by autoradiography. The figure shows immunoblots of isolated chromosomes (*c*) and chromosome scaffolds (*s*) prepared by extraction of nuclease-digested chromosomes with a 2 M NaCl lysis mix. The positions of the fragments are indicated by arrowheads to the right of c. The core histones migrate with the dye front in this 7.5% acrylamide gel. *frag.*, fragments.

The results of Fig. 3*c* indicate that topoisomerase II is a component of chicken mitotic chromosomes and chromosome scaffolds. Similar results have been obtained for HeLa chromosomes and scaffolds using a similar antibody (W. C. Earnshaw, B. Halligan, and L. F. Liu, unpublished observations). Comparison of Fig. 3, *b* and *c*, suggests strongly that the scaffold antigen cSc-1 is topoisomerase II. While our data do not establish unambiguously that topoisomerase II is the only 170,000-mol-wt antigen present in scaffolds, this is the most likely interpretation of the results.

This interpretation of our results is supported by fractionation of the proteins of isolated chromosomes by hydroxylapatite chromatography in the presence of SDS (data not shown). This method has been shown to effectively resolve high molecular weight structural proteins (38). Column fractions were analysed by SDS PAGE and immunoblotting with guinea pig anti-cSc-1 and rabbit anti-topoisomerase II. Both antibodies gave identical patterns, with 170,000-mol-wt polypeptide eluting at 0.52 M $NaPO_4$, 0.1% SDS, and the fragments eluting somewhat earlier (0.46 M $NaPO_4$, 0.1% SDS). Most polypeptides elute from hydroxylapatite by ~0.4 M $NaPO_4$ (39), and this late elution of topoisomerase II, which was noted previously (30), was interpreted as an indication that the polypeptide may be rich in hydrophobic residues.

The identity of cSc-1 as topoisomerase II has been supported by showing that both topoisomerases I and II can be extracted in active form from chicken chromosomes (Fig. 4) and that antibodies to cSc-1 can absorb the topoisomerase II activity from the extracts (Fig. 4, *left*; compare lanes *E* through *H* with lanes *A* through *D*, respectively). Note that topoisomerase II is extracted from chromosomes under conditions in which the chromosome scaffold has been shown to fall apart (presence of 2-mercaptoethanol [10]). Preimmune serum had no effect on the activity of the enzyme (Figure 4*A*; compare lanes *I* through *L* with lanes *A* through *D*, respectively), and neither preimmune serum nor anti-cSc-1 had any effect on the activity of topoisomerase I in these extracts (Fig. 4, *right*). Identical results were obtained if the rabbit anti-topoisomerase II antiserum was substituted for anti-cSc-1 (data not shown).

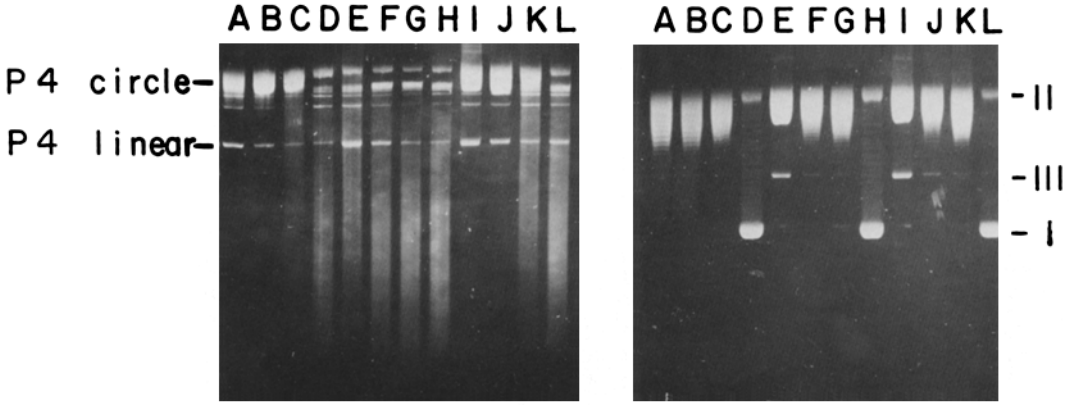

FIGURE 4 Effect of various antisera on the activity of topoisomerases I and II in extracts prepared from isolated mitotic chromosomes. Extracts were exposed to (lanes *A–D*) no antibody, (lanes *E–H*) anti-cSc-1, and (lanes *I–L*) preimmune serum. An assay for topoisomerase II is shown on the left. The results of a parallel assay for topoisomerase I are shown on the right. The amount of extract in each reaction mixture (20 μl volume) is as follows: 1 μl for samples in lanes *A*, *E*, and *I*; 0.25 μl for samples in lanes *B*, *F*, and *J*; 0.0625 μl for samples in lanes *C*, *G*, and *K*; and 0.0156 μl for samples in lanes *D*, *H*, and *L*. The smear in the left panel represents the heterogeneously knotted P4 DNA used to assay topoisomerase II (37). The migration of form I (supercoiled), form II (relaxed), and form III (linear) pBR322 DNA used in the assay for topoisomerase I (36) is indicated to the right of the right-hand panel.

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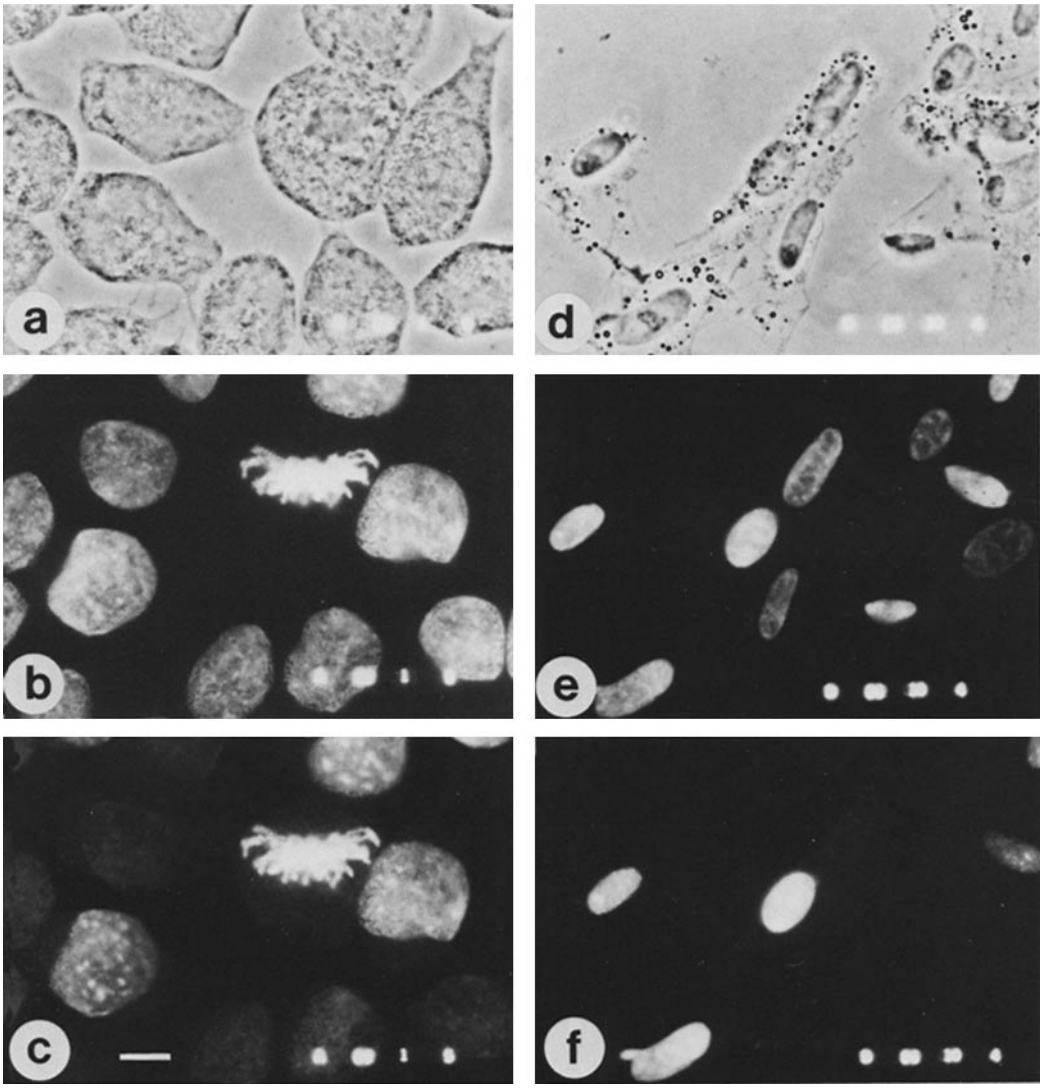

FIGURE 5 Indirect immunofluorescence using whole serum and affinity-eluted anti-cSc-1. The cells used for *a–c* were line 249, a transformed chicken cell line. *d–f* show a parallel experiment performed using an untransformed primary culture of chick embryo fibroblasts. *a* and *d* are phase-contrast images. *b* and *e* show staining of the DNA using DAPI, a nonintercalating dye that is brightly fluorescent when bound to DNA (58). *c* and *f* present the localization of topoisomerase II in these same cells using anti-cSc-1 (*c*, whole serum; *f*, affinity-eluted anti-cSc-1) and rhodamine-conjugated anti-guinea pig second antibody. Bar, 10 μm.

Therefore, we conclude that anti-cSc-1 recognizes functional topoisomerase II.

Localization of Topoisomerase II in Mitotic and Interphase Cells: Immunofluorescence Results

Topoisomerase II was localized in detergent extracted cells by indirect immunofluorescence using anti-cSc-1 (Fig. 5). The enzyme was found in nuclei and mitotic chromosomes of both transformed cell lines (Fig. 5, *a–c*) and untransformed primary cultures (Fig. 5, *d–f*). Little or no antigen was detected in the cytoplasm. Similar results were obtained with cells fixed with organic solvents. All mitotic chromosomes were found to label intensely (see reference 15 for details), but the staining of interphase nuclei varied considerably. Many nuclei exhibited bright punctate fluorescence, while others stained only weakly.

Using MSB-1 cells separated into G_1, S, and G_2 + M populations by a fluorescence-activated cell sorter, we found that virtually all cells that fail to stain with anti-cSc-1 have a G_1 DNA content. Negative cells represent the following percentages of the cells in each cell cycle phase: G_1, 14% (71/494); S, 2% (13/488); and G_2 + M, 0% (0/297). Labeled cells from different cell cycle phases were indistinguishable with regard to the distribution of fluorescent antibody binding.

We have considered two possible explanations for these results. The antigen may be masked (or degraded) during a brief portion of G_1. Alternatively, cells that are no longer cycling (and that would have a G_1 DNA content) may no longer contain topoisomerase II.

Independent confirmation that topoisomerase II is found in mitotic chromosomes is presented in Fig. 6. This figure shows chromosomes from chicken mitotic cells that were gently disrupted by centrifugation onto glass coverslips (for methods, see reference 15). The coverslips were then stained with preimmune serum (Fig. 6*b*) or rabbit anti-topoisomerase II (Fig. 6*d*). Fig. 6, *a* and *c*, show the distribution of chromosomal DNA (stained with 4′,6-diamidino-2-phenylindole dihydrochloride) in the corresponding fields.

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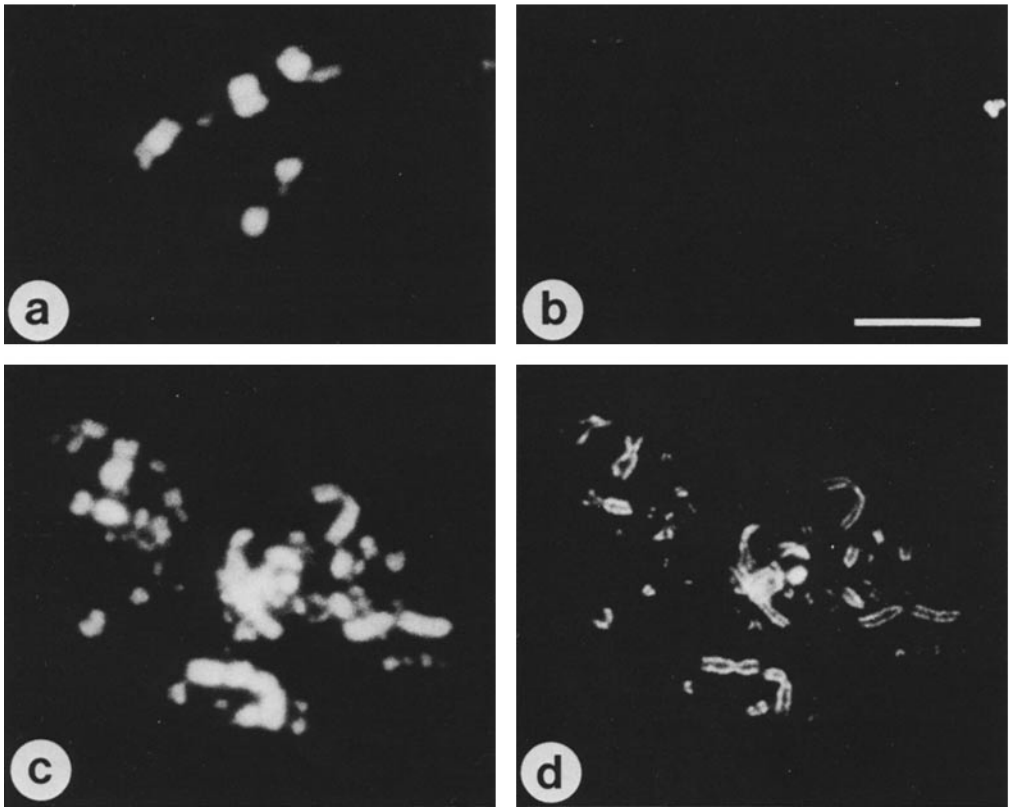

FIGURE 6 Identification of topoisomerase II in mitotic chromosomes. Chicken mitotic cells were hypotonically swollen and centrifuged onto coverslips (15). *a* and *c* show the distribution of DNA (stained with 4',6-diamidino-2-phenylindole dihydrochloride) in these chromosomes. *b* and *d* show the immunofluorescence obtained with (*b*) rabbit preimmune serum and (*d*) anti-topoisomerase II from this animal. Bar, 10 μm.

TABLE I
Data

Fraction	Recovery of structure	Normalized recovery of topoisomerase II*		N‖	Procedure
		Total‡	Sedimentable§		
Interphase nuclei	65 ± 11%	91 ± 15%	ND	20	*a*
Chromosomes	16 ± 8%	56 ± 21%	ND	4	*a*
Chromosomes	28 ± 9%	64 ± 21%	87 ± 35%	20	*b*

ND, not determined.
* Expressed as 100 × (recovery of topoisomerase II)/(recovery of structure).
‡ Calculated as 100 × ($tII_{chrom,nuc}/tII_{tot}$), where $tII_{chrom,nuc}$ is the amount of topoisomerase antigen in chromosomes (tII_{chrom}) or nuclei (tII_{nuc}) isolated from a known culture volume and tII_{tot} is the amount of topoisomerase II in an equivalent volume of the crude whole-cell homogenate from which the structures had been isolated.
§ Calculated as 100 × ($tII_{chrom,nuc}/tII_{pel}$), where tII_{pel} is the amount of topoisomerase antigen that could be pelleted from an equivalent volume of whole-cell homogenate by centrifugation at 16,000 *g* for 10 min.
‖ Number of independent determinations.

Localization of Topoisomerase II in Mitotic and Interphase Cells: Cell Fractionation Experiments

Quantitative cell fractionation and immunoassays (Table I) support the conclusions of immunofluorescence experiments. Interphase nuclei, isolated by a simple differential centrifugation procedure in the presence of detergent, contain 91% of the total immunoreactive topoisomerase II present in the cells. Therefore, neither immunofluorescence nor this cell fractionation experiment provide evidence for the existence of a pool of cytoplasmic topoisomerase II in interphase cells.

Similarly, the bulk of topoisomerase II from mitotic cells is found in isolated chromosomes. When a total cell homogenate from mitotic cells was centrifuged at 16,000 *g* for 10 min (to pellet all chromosomes and nuclei), ~70% of the topoisomerase II (the sedimentable antigen) was recovered in the pellet. When chromosomes were isolated from similar homogenates, 87% of the sedimentable antigen co-purified with the chromosomes through sucrose and Percoll gradients. This corresponds to ~60% of the total cellular pool of topoisomerase II.

Not all topoisomerase II from mitotic cells co-purifies with chromosomes. About 30% of the antigen is apparently soluble in total cell homogenates. We have considered three likely explanations for this result. First, it is possible that the soluble antigen is proteolysed. However SDS PAGE/immunoblotting

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appears to rule this out, since the ratio of 170,000-mol-wt polypeptide to smaller fragments is essentially identical for both soluble and sedimentable antigen. Alternatively, there may be two pools of enzyme in mitotic cells. While it is clear that most enzyme is associated with mitotic chromosomes, the remainder may either be soluble (and completely extracted from cells by our immunofluorescence procedure), or it may be reversibly bound to chromosomes. Finally, it is possible that the soluble antigen is derived from chromosomes damaged by the homogenization, or from microchromosomes which may not sediment under the centrifugation conditions used.

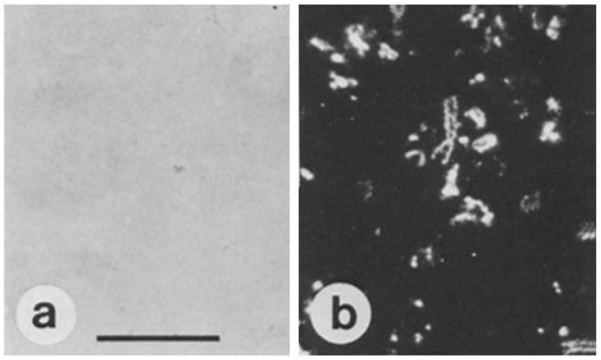

FIGURE 7 Chromosome scaffolds reacted with affinity-eluted anti-topoisomerase II (undiluted). The views shown are: (*a*) phase contrast; (*b*) indirect immunofluorescence using rhodamine-coupled anti-guinea pig IgG. Scaffolds were produced by extraction of nuclease-digested chromosomes with dextran sulfate/heparin (14). After the extraction, aliquots were sedimented onto coverslips through a cushion of 0.1 M sucrose in extraction buffer. The remainder of the material was analyzed by SDS PAGE to confirm that the extraction had proceeded normally. A parallel control experiment with preimmune serum is not shown since both phase-contrast and fluorescence microscopy lacked any visible structures. The paired and single dots are scaffolds of chicken microchromosomes. Note that of the scaffolds from larger chromosomes, only those that impact the coverslip en face will present a classical paired chromatid appearance. Bar, 10 μm.

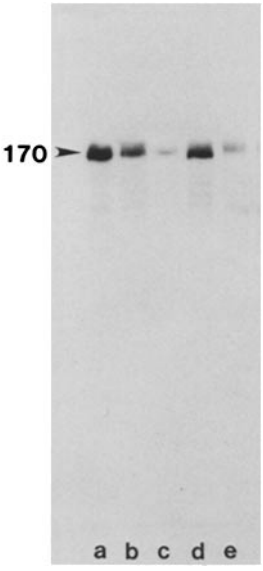

FIGURE 8 Immunoblotting of proteins of isolated chromosomes, chromosome scaffolds, and extracted fractions using anti-topoisomerase II. In all cases the samples were prepared as for those in Fig. 1, electrophoresed in a 7.5% SDS polyacrylamide gel, and immunoblotted using whole serum diluted 1:500. The samples electrophoresed in the lanes were (*a*) chromosomes, (*b*) scaffolds isolated after extraction of nuclease digested chromosomes with dextran sulfate/heparin, (*c*) proteins released by the polyanion extraction, (*d*) scaffolds isolated after extraction of nuclease digested chromosomes with 2 M NaCl, and (*e*) proteins released by the 2 M NaCl extraction. The position of cSc-1 is indicated.

Recovery of Topoisomerase II in Isolated Chromosome Scaffolds with High Yield

Indirect immunofluorescence confirms that topoisomerase II is found in structures with the morphology expected for chicken mitotic chromosome scaffolds (Fig. 7). Even though isolated scaffolds are not visible under phase contrast (Fig. 7*a*), they are easily visualized using the specific antibody (Fig. 7*b*). In addition to a number of chromosome-shaped structures, Fig. 7*b* also contains numerous single and paired fluorescent dots. These are the scaffolds of microchromosomes (minute chromosomes that are a natural component of the chicken karyotype and are present in about 60 copies per cell [40]).

At least 70% of the chromosomal topoisomerase II is recovered in scaffold fractions prepared by extracting isolated chromosomes at either low or high ionic strength. In the experiment shown in Fig. 8, equal starting volumes of isolated chromosomes (Fig. 8*a*), scaffolds (Fig. 8, *b* and *d*) and proteins solubilized during the extraction (Fig. 8, *c* and *e*) were electrophoresed and analysed by immunoblotting. The recovery of topoisomerase II in scaffolds prepared using the 2 M NaCl extraction (Fig. 8, *b* and *c*) was virtually identical to that found using the low salt dextran sulphate:heparin extraction (Fig. 8, *d* and *e*). In four independent experiments, the average values obtained were the following: NaCl scaffolds, 72 ± 10% recovery; and polyanion scaffolds, 72 ± 14% recovery. The 72% figure is a minimum value for the fraction of topoisomerase II associated with scaffolds, since some fragmentation of scaffolds occurs during the extraction procedure (W. C. Earnshaw and U. K. Laemmli, unpublished observations). In addition, scaffolds of microchromosomes may be too small to sediment under the conditions we have used.

DISCUSSION

Until recently, the literature on eucaryotic topoisomerase II has dealt largely with in vitro studies, and little is known about the function of the enzyme in living cells (reviewed in reference 25). The bacterial enzyme (DNA gyrase) has been implicated in replication, transcription, repair, and recombination (reviewed in references 41, 42) and it has been assumed that the eucaryotic enzyme is required for similar functions. Recent isolation of conditional lethal topoisomerase II mutants in yeast confirms that the enzyme is essential for cell growth (26, 27). Furthermore, at least in synchronized cells, the phenotypic block appears to affect chromosome segregation. Involvement of topoisomerase II in segregation had been predicted earlier (23, 24), based on detailed analysis of the terminal stages of SV40 replication.

Analysis of the yeast mutants suggests that topoisomerase II has a physiological role in regulating events at the chromosomal level. Our observation that the enzyme is a structural component of the mitotic chromosome scaffold suggests that specific ultrastructural interactions might contribute significantly to regulation of the activity of topoisomerase II in vivo.

Topoisomerase II Is a Structural Component of Chicken Chromosome Scaffolds

Published data on the composition of mitotic chromosome scaffolds indicate that the structures are composed of a well defined subset of chromosomal proteins (10, 14). The basis

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of the co-fractionation of these components during scaffold preparation is currently unknown, although one likely explanation is that the proteins exhibit some association within intact chromosomes. Whether or not these proteins are assembled into a discrete scaffold substructure remains the subject of ongoing investigation.

Because chromosome scaffolds contain the most insoluble proteins found associated with mitotic chromosomes, it is necessary to demonstrate that specific proteins that appear in scaffolds are bona fide chromosomal components and not insoluble contaminants of the scaffold fraction. Four independent arguments suggest that the association of topoisomerase II with mitotic chromosomes and scaffolds results from high affinity interactions rather than random precipitation or adventitious binding.

(*a*) Enzyme assays, immunofluorescence, and cell fractionation all indicate that topoisomerase II is an integral component of mitotic chromosomes. We find no evidence for a substantial cytoplasmic pool of the enzyme in interphase cells. It has been suggested that certain scaffold components might be insoluble fragments of cytoskeleton that contaminate isolated chromosome fractions (10). Clearly, this objection does not apply to topoisomerase II.

As expected, the enzyme occurs in interphase nuclei. Surprisingly, however, it is greatly reduced or absent from some cells. These cells were shown to have a G_1 DNA content, and may no longer be cycling. That topoisomerase II is found only in cycling cells is supported by the observation that enzyme activity is difficult to detect in normal rat liver (43; Halligan and Liu, unpublished), but is readily detected in regenerating liver (43).

(*b*) Results of high resolution localization of topoisomerase II in intact chromosomes suggest that the enzyme is located at the base of the radial chromatin loops (15). Both immunofluorescence and immunoelectron microscopy indicate that the enzyme occupies a restricted region around the axis of swollen chromosomes. In addition, structural alterations resulting from treatment of swollen chromosomes in solution with anti-topoisomerase II support the conclusion that the enzyme is not found in the radial chromatin loops.

(*c*) The enzyme is found in chromosome scaffolds prepared in two different ways. These involve extraction of chromosomes with 2 M NaCl or with a low ionic strength polyanion mixture. The conductivity of the latter is equivalent to 20 mM NaCl (14). Evidence presented elsewhere indicates that these two methods extract chromosomal proteins by different mechanisms. The polyanion extraction is inhibited by as little as 12 mM added NaCl (14). In addition, the morphology of scaffolds produced by these two procedures is extremely different (14). Despite these differences, the protein compositions of both types of scaffolds are similar (10), and the percent recovery of topoisomerase II is identical for both procedures.

(*d*) Topoisomerase II is recovered in scaffolds with high yield. In fact, 70% of the enzyme present in isolated metaphase chromosomes is recovered in scaffolds. Therefore, isolation of the scaffold is an efficient procedure. Some of the material lost from scaffolds is presumably lost due to fragmentation of the structures during extraction, and as a result, the 70% figure for topoisomerase II is probably an underestimate.

Our interpretation of the above results is that topoisomerase II is a bona fide structural component of mitotic chromosome scaffolds. Counter arguments that the enzyme occurs in scaffolds as a result of adventitious sticking (13, 44) are difficult to reconcile with our observation that the enzyme is recovered with high yield in scaffolds prepared at both low and high ionic strength (points *c* and *d* above).

Comparison with Other Scaffold Proteins

Comparison of the above results with those obtained for other scaffold proteins is difficult, since this report describes the first experimentally raised serum to recognize a major scaffold protein. One unrelated scaffold protein has been characterized in some detail, however, This is CENP-B, an 80,000-mol-wt human centromere protein that is detected by autoimmune sera from certain patients with rheumatic diseases (29, 31). Both electron microscopy (14) and a combination of immunoblotting and immunofluorescence using the autoimmune sera (31) indicate that centromere components are retained in chromosome scaffolds prepared by our methods. Analysis of immunoblots similar to those shown in Fig. 8 indicates that 61 ± 13% of the CENP-B is retained in scaffolds (W. C. Earnshaw, unpublished observations).

The same immunoblots show that CENP-A (a related centromere protein of M_r 17,000 [29]) is completely solubilized during the scaffold extraction procedure (31). CENP-A and CENP-B share antigenic determinants (29). Therefore, structurally related proteins localized in close proximity in the chromosome may differ greatly in their binding to the chromosome scaffold. This is further evidence that the protein composition of the scaffold fraction is determined by specific interactions.

Is Human Sc-1 Topoisomerase II?

When the protein composition of human chromosome scaffolds was analyzed in detail, the structures were found to be significantly enriched in two polypeptide species, Sc-1 (M_r 170,000) and Sc-2 (M_r 135,000 [10, 14]). We have now shown that a chicken scaffold protein of M_r 170,000 is topoisomerase II. However, recent evidence suggests that human Sc-1 is composed of at least three co-migrating polypeptides.

(*a*) Both chicken and human (W. C. Earnshaw, B. Halligan, M. M. Heck, and L. F. Liu, unpublished observations) chromosome scaffolds contain topoisomerase II as demonstrated by immunoblotting and immunofluorescence.

(*b*) A second polypeptide of M_r 170,000 in humans is antigenically related to a chicken chromosomal antigen of M_r 135,000 (M. M. Heck and W. C. Earnshaw, manuscript in preparation). This antigen is not topoisomerase II.

(*c*) A third human chromosomal polypeptide of M_r 170,000, recognized by a human autoimmune serum (W. C. Earnshaw and N. F. Rothfield, unpublished observations) is antigenically related to a chicken chromosome protein of M_r 160,000. This species is also apparently not topoisomerase II, and is not recognized by our anti-cSc-1 serum.

These results suggest that human Sc-1 is composed of a mixture of polypeptides. Therefore any functional analysis of this species that relies solely on one-dimensional SDS PAGE must be interpreted with caution.

Implications for the Organization of Interphase Nuclei

While loop models provide a convenient framework for understanding the structure of mitotic chromosomes and interphase nuclei, most interest on chromatin domains centers on interphase, where the genome is physiologically active,

and where the structural organization is much less well characterized. Data consistent with the existence of chromatin loop domains in interphase cells has been obtained using several different techniques (16–22). These domains are similar in size to the radial loops observed directly in swollen and dehistonized mitotic chromosomes (7, 14, 45).

Our data suggest that the cell might use an enzymatic mechanism to control the topology of these loop domains. Previous workers have pointed out correlations between the size of loops in interphase chromatin and the size of both replicons and regions of intermediate DNase I sensitivity flanking actively transcribed genes (20, 46–48). The importance of loop structures in transcription has long been known for the case of lampbrush chromosomes (49, 50). More recently, it was proposed that promoters might be activated or repressed through alterations in superhelicity (51, 52). Superhelicity might be controlled by alterations in the secondary structure of the DNA (53, 54), transient dissociation of nucleosomes from control regions (55), or by the action of an ATP-requiring eucaryotic DNA gyrase (as suggested by recent drug studies [56]). Although isolated eucaryotic type II topoisomerases lack gyrase activity (25), the logical candidate for the putative gyrase in vivo remains topoisomerase II.

One role for topoisomerase II in vivo has been shown to be resolution of catenated loops that result from replication of closed circular DNA molecules. The enzyme is required for chromosome segregation in procaryotes (*Escherichia coli* [57]), viruses (SV40 [23, 24]), and lower eucaryotes (*S. cerevisiae* [26]; *S. pombe* [27]).

The radial loop model of chromosome structure proposed that each chromosome consists of loops that are topologically fixed due to binding of scaffold proteins (6, 7, 10, 45). One efficient way of regulating the segregation of these domains after replication would be to make the enzyme required for decatenation (topoisomerase II) part of the framework responsible for establishment and maintenance of the loop structure. Therefore, topoisomerase II would be expected to be a component of the insoluble "nuclear matrix" fraction prepared from interphase cells. That this is indeed the case has been demonstrated by the experiments of Berrios et al. (59) using antibody recognizing the *Drosophila* enzyme, and also by experiments from this laboratory using the rabbit anti-topoisomerase II antibody discussed above and nuclei prepared from *Drosophila* and rat (Halligan, B., D. Small, B. Vogelstein, T.-S. Hsieh, and L. F. Liu, manuscript submitted for publication).

If topoisomerase II were part of the structural framework responsible for the loop organization of the chromatin of mitotic chromosomes, the enzyme would be expected to be concentrated at the base of the radial chromatin loops. Our data concerning the distribution of topoisomerase II in chicken mitotic chromosomes (15) are consistent with this model.

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