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Topoisomerase II Forms Multimers In Vitro: Effects of Metals, β-Glycerophosphate, and Phosphorylation of Its C-Terminal Domain

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We present a novel assay for the study of protein-protein interactions involving DNA topoisomerase II. Under various conditions of incubation we observe that topoisomerase II forms complexes at least tetrameric in size, which can be sedimented by centrifugation through glycerol. The multimers are enzymatically active and can be visualized by electron microscopy. Dephosphorylation of topoisomerase II inhibits its multimerization, which can be restored at least partially by rephosphorylation of multiple sites within its 200 C-terminal amino acids by casein kinase II. Truncation of topoisomerase II just upstream of the major phosphoacceptor sites reduces its aggregation, rendering the truncated enzyme insensitive to either kinase treatments or phosphatase treatments. This is consistent with a model in which interactions involving the phosphorylated C-terminal domain of topoisomerase II aid either in chromosome segregation or in chromosome condensation.

Eukaryotic DNA topoisomerase II is a nuclear enzyme that relaxes supercoiled DNA and decatenates interlocked circles of DNA in a reaction that involves the introduction of double-strand breaks, strand passage, and religation (for a review see reference 42). Topoisomerase II has also been identified as a major component of histone-depleted metaphase chromosomes (12, 16) and as a factor required for chromosome condensation in *Xenopus* extracts in vitro (4, 19). Genetic evidence in support of this role for topoisomerase II was provided by analysis of a cold-sensitive *top2* mutant in fission yeast cells: in the presence of a mutation in β-tubulin it could be shown that proper condensation of mitotic chromosomes requires a functional *top2*$^+$ allele (39).

The molecular mechanisms that control or catalyze chromosome condensation are unknown. In higher eukaryotic cells, histone H1 probably folds the nucleosomal fiber into a compact 300-Å (30-nm) fiber, but this level of folding is insufficient to account for the degree of compaction observed in mitotic chromosomes (reviewed in reference 14). In early attempts to identify other chromosomal proteins involved in chromatin folding, highly purified mitotic chromosomes were subjected to various extraction procedures (5, 16, 24, 30). A subpopulation of nonhistone chromosomal proteins were recovered after the extraction of histones, which maintained the genomic DNA in a partially folded, torsionally constrained state (5, 24). The single most abundant protein in this subfraction, called the chromosomal scaffold, was identified as DNA topoisomerase II (12, 16). Roughly 60% of the topoisomerase II that copurified with mitotic chromosomes from HeLa cells was recovered in the scaffold fraction (16). Consistent with this, different populations of topoisomerase II have recently been distinguished in *Drosophila* embryos on the basis of the distribution of injected, rhodamine-labelled enzyme (37). Roughly 75% of the topoisomerase II in prophase nucleus remained associated with condensed chromosomes, and of this fraction half remained chromosome associated at late telophase, suggesting the presence of three populations of topoisomerase II, perhaps each with a different function, at least in fly embryos (37).

One of the basic tenets of the radial loop model of chromosome organization is that nonhistone proteins play a role in the maintenance of looped domains (5, 15, 28, 30). Since not only protein-DNA interactions but protein-protein interactions would thus be important for the compaction of DNA in mitotic chromosomes, it is important to ask whether these interactions exist, whether they involve topoisomerase II, and how they might be regulated through the cell cycle. The chromosomal scaffold is thought to be a good source for isolating proteins involved in long-range chromosomal organization. However, because it is the result of a subfractionation technique, the presence of a protein in this fraction does not address the question of whether the protein plays a structural role in vivo. Immunostaining of topoisomerase II in condensed, intact mitotic chromosomes shows a general staining much like the DNA stain 4′,6-diamidino-2-phenylindole (DAPI) (13b, 37), while in extracted human chromosomes antitopoisomerase II stains a series of foci extending the length of the chromatid, located centrally in comparison with the surrounding halo of DNA (12, 13, 16). Similar staining was observed in Indian muntjac chromosomes with the MPM-2 antibody, which in mitotic chromosomes specifically recognizes a phosphorylated form of topoisomerase IIα (38).

In nearly every species examined, topoisomerase II is modified during the cell cycle by phosphorylation, with the level at metaphase being significantly higher than in G_1 (for a review see reference 8). Dephosphorylation in vitro significantly lowers the decatenation activity of topoisomerase II (9, 33) while rephosphorylation by casein kinase II (CKII) restores catalytic activity (9, 13a, 33). Moreover, the pattern of phosphotryptic peptides from $^{32}PO_4$-labelled yeast cells shows that CKII is the major kinase modifying topoisomerase II in vivo (7), with the 10 major CKII target sites localized within the C-terminal 200 amino acids (aa) (7, 11a). Surprisingly, it was observed that a C-terminal truncation of either the *Saccharomyces cerevisiae*

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enzyme or the *Schizosaccharomyces pombe* enzyme, which removes the major phosphate acceptor sites, does not eliminate the enzyme's catalytic activity in vitro (10, 36). The truncated enzymes also could support cell division, although decatenation activity in vivo was significantly less efficient without the C-terminal domain (10, 17). These observations raise the questions of what the function of the C-terminal 200 aa of topoisomerase II might be and why the enzyme should be hyperphosphorylated in the G_2 and M phases of the cell cycle. One possibility is that topoisomerase II's phosphorylated C terminus aids in interaction with other proteins involved in chromosome condensation or segregation. While perhaps not an essential function in yeast cells, the activity could be more important in cells with significantly larger chromosomes. Such a role would be consistent with the observation that a phosphorylated epitope of mammalian topoisomerase II is specifically recognized by the MPM-2 antibody in mitotic chromosomes (38).

The aims of the present study were to find a simple assay to study the interaction of topoisomerase II with itself and other nuclear proteins in vitro and to examine the role of topoisomerase II phosphorylation in these interactions. We have devised a sedimentation assay that monitors topoisomerase II multimerization and have examined its sensitivity to phosphorylation, nucleotide binding, DNA, and various buffer components. In addition we show that certain proteins solubilized from an interphase nuclear scaffold interact with purified topoisomerase II, particularly under the conditions used for isolation of nuclear and mitotic chromosomal scaffolds.

MATERIALS AND METHODS

Topoisomerase II purification. Yeast DNA topoisomerase II and a C-terminally truncated form were isolated from the yeast strain GA-24 (9) bearing either YEpTOP2-PGAL1 or a mutated form of this plasmid. Purification was as described in reference 44, except that β-glycerophosphate was omitted from the final phosphocellulose column, as it interferes with the retention of topoisomerase II. The truncation introduces a frameshift at aa 1235 of the yeast enzyme, with termination 8 aa later. This eliminates the major phosphoacceptor sites in tryptic peptides 8, 9, and 10 (see reference 7). For separation of the DNA topoisomerase II from the copurifying CKII, the samples were sedimented in a high-salt glycerol gradient as described in reference 9. Specific activity and characterization of the overexpressed yeast topoisomerase II have been described elsewhere (6, 9).

DNA sedimentation assay. Sedimentation of DNA by topoisomerase II was carried out in buffer B1 (20 mM Tris-HCl [pH 7.6], 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, and 8% glycerol). The DNA used was pBR322 carrying the 1.3-kb *Eco*RI-*Hin*dIII fragment of *Drosophila* DNA containing the histone scaffold attachment region (SAR) (28). The radioactive probe was prepared by digestion with *Eco*RI and *Hin*dIII and filling in with [α-^{32}P]dATP and other cold deoxynucleotides, such that both fragments have roughly equal specific activities. Two hundred nanograms of topoisomerase II was added to 50 μl of the reaction buffer. Active or heat-inactivated (95°C for 10 min in the presence of EDTA) calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) was added to some of the samples as indicated. In others ATP was adjusted to 10 μM, and all samples were incubated at 30°C for 30 min. One to two nanograms of labelled probe and 400 ng of salmon sperm DNA were then added to the reaction mixture. The sample was incubated at 30°C for 30 min and was spun in an Eppendorf Microfuge for 10 min at 4°C. The pellet and supernatant fractions were separated, and the pellets were resuspended in the reaction buffer. Proteinase K and sodium dodecyl sulfate (SDS) were added to the samples and were incubated at 45°C for 60 min. The samples were then phenol extracted and were precipitated with ethanol. The precipitates were dissolved in the DNA sample buffer and were run on a 1% agarose gel in TBE (89 mM borate, 89 mM Tris base, 1 mM EDTA), and the gel was dried and autoradiographed.

Protein sedimentation assay. Two hundred nanograms of purified yeast topoisomerase II and, when required, 10 μM nucleoside triphosphate (NTP) or analogs, 1.5 μg of bovine serum albumin (BSA), 10 μg of yeast nuclear protein extract (41), or 5 μg of yeast nuclear scaffold protein extract (20), were incubated at 30°C for 30 min in buffer B1 and were then centrifuged in an Eppendorf Microfuge at 10,000 × *g* for 10 min at 4°C. The pellet and supernatant fractions were separated on an SDS–7% polyacrylamide gel. The gel was stained with Coomassie blue and was scanned on a Hirschmann Elscript 400 scanning densitometer. To test the effects of phosphorylation, topoisomerase II was incubated at 30°C for 30 min before centrifugation, either with or without 1 μl (<0.5 U) of purified protein phosphatase I (PP1; a gift of B. Hemmings, Basel, Switzerland) or 0.2 μl (1 U) of CIP. Where required, topoisomerase II was labelled by the copurifying CKII by using [γ-^{32}P]ATP prior to dephosphorylation, which was carried out at 37°C for 2 or 5 min with 1 U of CIP. The dephosphorylation was nearly equivalent to the longer reaction at 30°C. To test the possible effects of contaminating DNA, either 10 or 100 ng of either DNase I or micrococcal nuclease with 0.5 mM $CaCl_2$ was added, and samples were incubated at 30°C for 30 min before centrifugation. Other additions to the above incubation conditions are explained in the figure legends.

Glycerol gradient centrifugation. The topoisomerase II multimers formed as described above were cross-linked by the addition of glutaraldehyde at 0.02% for 20 min at room temperature and then were loaded onto a 4-ml linear gradient of 15 to 40% (vol/vol) glycerol in buffer B1. The gradient was centrifuged for 16 h at 35,000 rpm (120,000 × *g*) in a Kontron TST60 rotor. The fractions were collected starting from the bottom of the gradient and were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Native high-molecular-weight standards (Pharmacia) were centrifuged in similar conditions for standards. The gel was then stained with Coomassie blue and was scanned as described above.

Glycerol gradient purification. Four hundred micrograms of topoisomerase II was loaded onto a 12-ml linear gradient of 15 to 40% (vol/vol) glycerol in buffer B1 containing 400 mM KCl as described in reference 9. The gradient turned for 68 h at 39,000 rpm (200,000 × *g*) in a Kontron TST41 rotor. Topoisomerase II-containing fractions were pooled and used for further analysis. For some experiments, topoisomerase II was treated with 10 U of CIP for 30 min at 30°C in a CIP buffer prior to being loaded onto the glycerol gradient.

Electron microscopy. For microscopy of topoisomerase II with DNA, 600 ng of topoisomerase II was incubated in the presence of 250 μM ATP for 15 min at 30°C with 100 ng of plasmid pSW301 containing the ARS1 sequence cut with *Eco*RI and *Hin*dIII. They were immediately diluted into 100 mM ammonium acetate and 60% glycerol, sprayed on a freshly cleaved mica surface, and shadowed with platinum at a 7° angle, and a carbon replica was made at a 90° angle. Replicas were floated off on a water surface and were observed under a JEOL 100× electron microscope. The same technique was used for samples without DNA. For quantitation of multimers the samples were also diluted into 600 mM ammonium acetate

and 60% glycerol prior to being sprayed, to reduce nonspecific aggregates. For images of negatively stained topoisomerase II, 1% uranyl acetate was used to stain the protein adhered to glow-discharged grids.

Topoisomerase II activity assay. Topoisomerase II activity was detected by ATP-dependent relaxation of supercoiled pBR322 DNA. The reaction mixture contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 30 μg of BSA per ml, 1 mM ATP, and 20 μg of DNA per ml, unless otherwise indicated. Reaction mixtures were incubated for up to 1 h at 30°C after the addition of topoisomerase II and were stopped by the addition of SDS to 1%, EDTA to 10 mM, and proteinase K to 25 μg/ml. After digestion at 56°C, the reaction mixtures were analyzed by 1% agarose gel electrophoresis in Tris-acetate buffer.

RESULTS

Precipitation of AT-rich DNA by topoisomerase II is influenced by phosphorylation. The catalytic activity of topoisomerase II from different species is stimulated by a variety of protein kinases (1, 2, 9, 32–34) including CKII, which copurifies with topoisomerase II when it is isolated from yeast cells (9). One means by which phosphorylation might stimulate topoisomerase II activity is by modulation of its interaction with DNA. Adachi et al. (3) have shown that topoisomerase II binds cooperatively and preferentially to AT-rich DNA fragments (SARs) in vitro and that the topoisomerase II-DNA complex can be sedimented by centrifugation. We have carried out a similar assay with topoisomerase II from *S. cerevisiae* and an AT-rich 1.3-kb fragment found upstream of the *Drosophila* histone H1 gene (28). Purified yeast DNA topoisomerase II was incubated at 30°C with the radiolabelled fragments, the topoisomerase II-DNA complex was recovered by centrifugation in an Eppendorf Microfuge, and the distribution of DNA was analyzed by agarose gel electrophoresis and autoradiography. As previously shown, the 1.3-kb histone SAR sediments efficiently in the presence of topoisomerase II, while vector DNA is less efficiently bound (Fig. 1A, lanes 1; see figure legend for quantitation). The pBR322 vector is less AT rich, yet it does contain short regions with an A + T content over 80%, which like the oligo(dA) or oligo(dT) stretches in SAR fragments may serve to nucleate the binding of topoisomerase II.

To examine the effects of phosphorylation on this topoisomerase II-DNA interaction, we incubated topoisomerase II and its copurifying CKII with ATP, prior to incubation with DNA. No significant effect on the sedimentation of topoisomerase II-DNA complexes was observed (Fig. 1A, lanes 2). We reasoned that this lack of effect on DNA precipitation may reflect a basal level of phosphorylation on the CKII acceptor sites. Indeed, the average molar ratio of phosphate incorporation into this same yeast topoisomerase II, when phosphorylated by CKII in vitro, is approximately 2:1, although two-dimensional phosphopeptide maps show three C-terminal peptides containing from two to four phosphorylated amino acids each (7, 8, 11a). This suggests that yeast topoisomerase II, as we isolate it, has a significant number of stably phosphorylated residues but is still not fully phosphorylated.

To reduce the basal level of phosphorylated residues, we have treated topoisomerase II with CIP. The efficiency of the phosphatase treatment was checked by phosphorylating topoisomerase II with [γ-^{32}P]ATP and its copurifying CKII and subsequently treating with phosphatase (either at 37°C for 2 min or at 30°C for 30 min). During these incubations roughly

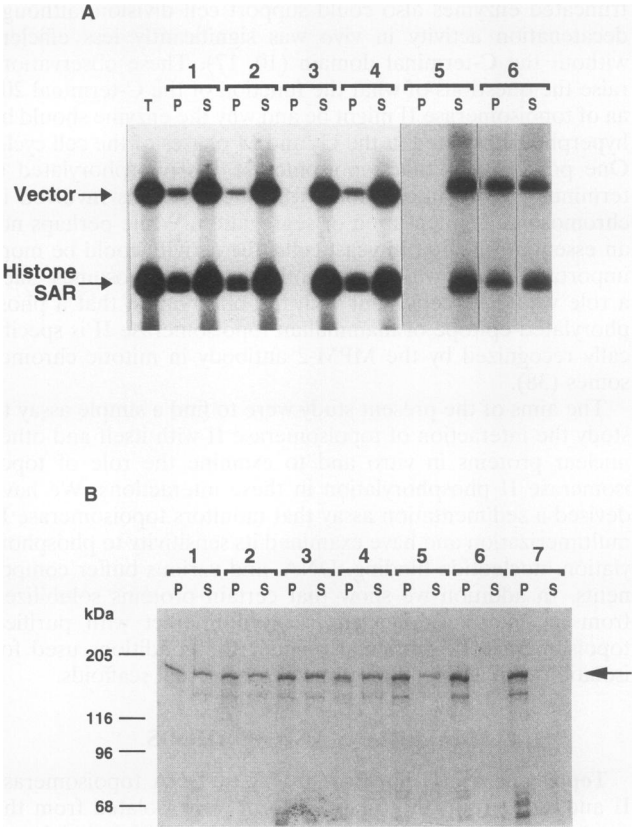

FIG. 1. (A) Precipitation of a *Drosophila* SAR fragment with topoisomerase II. Topoisomerase II was preincubated at 30°C for 30 min alone (lane 1) or with 1 U of active CIP (lanes 3 and 5), 1 U of heat-inactivated CIP (lane 6), 0.5 μg of BSA (lane 4), or CKII in the presence of 10 μM ATP (lane 2) as described in Materials and Methods. The histone spacer SAR sequence and its vector were end labelled and added to all samples with competitor poly(dA-dT) DNA (lanes 1 to 6). After 30 min at 30°C the complexes were sedimented in a Microfuge and the pellets and supernatants were analyzed by gel electrophoresis and autoradiography. Preincubation of topoisomerase II with ATP has been shown to lead to phosphorylation by the copurifying kinase. Note that lanes 5 and 6 come from one experiment and lanes 1 to 4 from another experiment. T, total probe; P, pellet fraction; S, supernatant fraction. Quantitation of precipitated versus supernatant DNA demonstrated that the addition of ATP increased the SAR:vector ratio in pellets approximately twofold in the experiments shown in lanes 1 and 2, while the absolute amounts of SAR in the pellet varied from 28 to 23% of the input SAR fragment in these two samples. (B) Topoisomerase II forms readily sedimented aggregates with or without DNA fragments. Two hundred nanograms of purified topoisomerase II was incubated in B1 buffer in the presence of DNA at 30°C for 30 min and was then centrifuged at 12,000 rpm (10,000 × *g*) at 4°C for 10 min in an Eppendorf Microfuge. The supernatant and pellet were collected, diluted with the SDS sample buffer, and separated on SDS-PAGE (23). P, pellet fraction; S, supernatant fraction. Lanes: 1, no DNA added; 2 to 4, incubation carried out in the presence of 10, 100, and 500 ng of oligo(dT-dA)$_{14}$, respectively; 5 to 7, incubation carried out in the presence of 10, 100, and 500 ng of sheared polymers of d(A-T), respectively. Arrow indicates the positions of intact topoisomerase II. Bars indicate positions of high-molecular-weight standards (Sigma).

90% of the in vitro-incorporated ^{32}P could be removed (11a). The phosphatase-treated topoisomerase II was incubated with the radiolabelled fragments and could not provoke the sedimentation of either the 1.3-kb SAR or the vector DNA (Fig. 1A, lanes 3 and 5). This loss of sedimenting DNA does not reflect nonspecific effects of added protein or of the CIP buffer, since the addition of an equal volume of heat-inactivated CIP, or of an equivalent amount of BSA, had no effect (Fig. 1A, lanes 6 and 4, respectively). These results suggest that dephosphorylated topoisomerase II either has a lower affinity for DNA or interacts poorly with itself, reducing the sedimentation coefficient of the topoisomerase II-DNA complex.

Topoisomerase II is able to form rapidly sedimenting multimers without DNA. Most protein-DNA complexes do not sediment at the 10,000 × *g* attained in a Microfuge. Therefore, we reasoned that the SAR-topoisomerase II sedimentation may also reflect protein-protein interactions, which help form large complexes. To test this we asked whether topoisomerase II would multimerize in the absence of DNA. Purified yeast topoisomerase II was incubated in a medium salt buffer (50 mM KCl, 8% glycerol, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$) for 30 min at 30°C (normal growth temperature for *S. cerevisiae*), and the formation of higher multimers of topoisomerase II was monitored by sedimentation in an Eppendorf Microfuge. Soluble and sedimented fractions were analyzed by SDS-PAGE, and the Coomassie blue-stained gels were scanned. To calibrate the mass required for sedimentation in this assay, large native marker proteins (Pharmacia) were tested under identical conditions. Complexes of over 660 kDa were found to sediment, while smaller ones remained in the supernatant (data not shown).

We find that roughly 60% of the input DNA topoisomerase II forms multimers in the absence of added DNA (Fig. 1B, lanes 1). In different topoisomerase II preparations this value ranged from 40 to 70% but was consistent within a batch of topoisomerase II. There is no free or rapid exchange between the two fractions, for if we resuspend the pelleted fraction of topoisomerase II and repeat the sedimentation assay, 91% ± 4% of the resuspended topoisomerase II resediments (data not shown). To rule out the possibility that contaminating DNA causes aggregation of topoisomerase II during incubation, we treated the purified topoisomerase II fraction with nuclease. Neither digestion with DNase I nor digestion with micrococcal nuclease affected the formation of topoisomerase II multimers (data not shown). Quantitation of DNA in the topoisomerase II preparation by either random priming or a nick-translation method with radioactive precursors shows that the DNA contaminating our topoisomerase II is below our detectable level (<10 ng in 400 ng of topoisomerase II).

While nuclease does not affect sedimentation of topoisomerase II, short oligonucleotides could remain and lead to its aggregation. Indeed, it has been reported that guanine oligonucleotides inhibit topoisomerase II activity by promoting the formation of nucleotide-topoisomerase II complexes, which could be pelleted in a Microfuge (11). In our hands neither short oligo(dG) nor the double-stranded oligo(dT-dA)$_{14}$ affects the sedimentation of topoisomerase II in the absence of additional DNA (Fig. 1B, lanes 2 to 4), while even small quantities of poly(dA-dT) in the size range of 50 to 2,000 bp significantly enhanced the amount of topoisomerase II recovered in the pellet (Fig. 1B, lanes 5 to 7). This is consistent with previous observations that sufficiently long DNA with oligo(dA) stretches nucleate topoisomerase II binding (3), and it rules out the notion that residual short oligonucleotides are responsible for the observed multimerization of topoisomerase II.

Characterization of the topoisomerase II multimers. The topoisomerase II sedimentation could be due to a nonphysiological denaturation and precipitation. To examine this, we performed the assay in increasing salt concentrations, measured the size of the multimer by glycerol gradient centrifugation and assayed the ATP-dependent relaxation activity of the sedimented enzyme. After incubation of topoisomerase II in different concentrations of KCl, the efficiency of multimerization decreased with the increase in ionic strength (Fig. 2A). Thus, ionic interactions may be important in the presumed protein-protein interactions. Similarly, we studied the effect of protein concentration on multimerization by performing the sedimentation assay with both iodinated and phosphorylated topoisomerase II. As the labelled topoisomerase II is diluted from 24 to 0.8 ng/μl in our standard salt conditions, the fraction of sedimenting multimers diminishes from around 60 to 37% in a linear fashion (data not shown). Thus, multimerization is not restricted to highly concentrated solutions of topoisomerase II, yet it shows concentration dependence.

To determine the size of the complexes, 0.02% glutaraldehyde was added to the incubated topoisomerase II and the mixture was applied to a 15 to 40% linear glycerol gradient and centrifuged in a Kontron TST60 rotor. The distribution of topoisomerase II along the gradient fractions was determined by SDS-PAGE and silver staining (Fig. 2B). Whether cross-linked or not, no topoisomerase II is found with the size of a monomer (170 kDa), which suggests that topoisomerase II is isolated as a stable dimer. In addition to the major peak at about 360 kDa (presumably the dimeric enzyme), a second peak of topoisomerase II migrates beyond the 660-kDa marker and appears to correspond to multimers of four or more topoisomerase II monomers. Peaks corresponding to larger complexes are also observed (Fig. 2B). Gradient analysis of the cross-linked supernatant fraction from the sedimentation assay reveals a single peak of topoisomerase II of around 360 kDa. These results have been confirmed by migration on native agarose/polyacrylamide gels, in which we observe the majority of topoisomerase II migrating as dimeric enzyme, some forming larger complexes that barely enter the gel, but no monomeric form whatsoever (8a).

To demonstrate that these complexes of topoisomerase II larger than dimers do not reflect its inactivation, we have tested the pelleted and soluble fractions for ATP-dependent relaxation activity. Topoisomerase II from the sedimented fraction efficiently relaxes supercoiled pBR322 DNA in an ATP-dependent manner (Fig. 2C). Since equal amounts of topoisomerase II were assayed from both supernatant (lanes 3 to 5) and pellet (lanes 6 to 8) fractions, the relaxation assay indicates that the sedimented enzyme has a slightly higher specific activity than the soluble enzyme has. We conclude, therefore, that the topoisomerase II multimers are fully, if not preferentially, active.

Phosphorylation of the C terminus of topoisomerase II modulates multimerization. The phosphorylation of DNA topoisomerase II peaks in late G_2 or metaphase, and in both yeast cells and *Drosophila melanogaster*, casein kinase II appears to be the major kinase modifying topoisomerase II in vivo (2, 7; reviewed in reference 8). Since the phosphoacceptor sites of CKII are exclusively in the highly charged C-terminal 200 aa of *S. cerevisiae* topoisomerase II (7, 9, 11a), it seemed possible that this domain might influence a metaphase-specific topoisomerase II multimerization (see discussions in references 36 and 45). Again, topoisomerase II was incubated with its copurifying CKII and 10 μM ATP, with ^{32}P included to allow quantitation of the incorporated phosphate and to

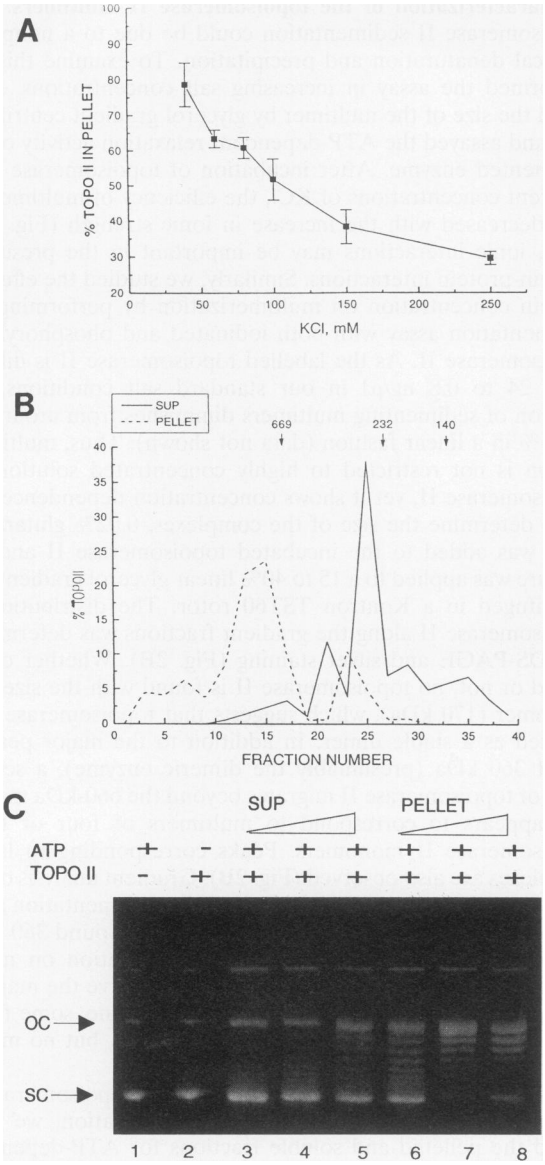

FIG. 2. Characterization of topoisomerase II multimers. (A) Two hundred nanograms of purified topoisomerase II was incubated in B1 buffer at 30°C for 30 min in the presence of increasing concentrations of KCl and was then centrifuged at 12,000 rpm (10,000 × *g*) at 4°C for 10 min in an Eppendorf Microfuge. The supernatant and pellet were collected, diluted with the SDS sample buffer, and separated on SDS-PAGE (23). The ratio of precipitated versus supernatant topoisomerase II was estimated by scanning the Coomassie blue-stained gels on a Hirschner Elscript 400 scanning densitometer for each salt concentration. The average and standard deviation were calculated from three experiments. (B) The topoisomerase II multimers formed as described above (incubation for 30 min at 30°C) were loaded onto a 4-ml linear gradient of 15 to 40% (vol/vol) glycerol in buffer B1 after cross-linking with glutaraldehyde. The gradient was centrifuged for 14 h at 35,000 rpm (120,000 × *g*) in a Kontron TST60 rotor. The fractions were collected starting from the bottom of the gradient and were separated by SDS-PAGE. Arrows indicate positions of native high-molecular-mass standards (thyroglobulin, 669 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa) which were centrifuged under similar conditions. Sup, supernatant. (C) Topoisomerase II multimers are catalytically active. Two hundred nanograms of purified topoisomerase II was incubated in B1 buffer in the absence of DNA at 30°C for 30 min and was then centrifuged in an Eppendorf Microfuge (10,000 × *g*). The supernatant and pellet were collected, adjusted to 20 μl with buffer B1, and incubated for 1 h at 30°C in 10 μl of a mixture containing different amounts of topoisomerase II, 1× decatenation buffer (see Materials and Methods), 1 mM ATP, and 0.2 μg of supercoiled pBR322 DNA. The reactions were stopped by the addition of SDS, EDTA, and proteinase K and were separated on a 1% agarose gel in Tris-acetate buffer. Lanes: 1, supercoiled pBR322 DNA; 2, DNA plus 200 ng of topoisomerase II without ATP; 3 to 5, reaction mixtures containing ca. 10, 25, and 60 ng topoisomerase II from the supernatant fraction, respectively; 6 to 8, reaction mixtures containing ca. 10, 25, and 60 ng of topoisomerase II from the pellet fraction, respectively. Sup, supernatant; OC, open circular DNA; SC, supercoiled DNA.

monitor the phosphorylated fraction of the enzyme by autoradiography.

The incubation of topoisomerase II with ATP and kinase slightly, but reproducibly, enhances the fraction of sedimenting topoisomerase II, compared with control incubations in its absence (Fig. 3, compare 1P with 2P; Fig. 4A and B). This difference is not very striking, perhaps because of a basal level of stable phosphorylation of topoisomerase II, as seen in the DNA precipitation assay. To check this, we have treated the phosphorylated topoisomerase II with CIP, and we reproducibly observe a decrease in the amount of multimeric topoisomerase II that can be pelleted in a Microfuge (Fig. 3, lanes 4). Importantly, when the CIP-treated samples shown in lanes 4 on a Coomassie blue-stained gel are exposed for autoradiography, the residual ^{32}P-labelled topoisomerase II is recovered entirely in the pellet fraction (Fig. 3, lanes 4′), showing that all the soluble topoisomerase II is in the dephosphorylated form. The longer the treatment with phosphatase, the less topoisomerase II we recover in the pellet (e.g., see Fig. 5, lanes 3). Again, to rule out a nonspecific effect of CIP or its buffer, topoisomerase II was incubated with an equivalent amount of heat-inactivated CIP or with BSA, and we find that neither has any influence on the observed sedimentation pattern (data not shown). When protein phosphatase 1, a conserved nucleus-localized phosphatase, was used instead of CIP, topoisomerase II again was recovered primarily in the supernatant fraction, and okadaic acid, an inhibitor of PP1, blocked this effect (Fig. 3, lanes 5 to 7).

The CKII tetramer that copurifies with yeast topoisomerase II is present in less than a 20:1 (wt/wt) ratio and cannot be detected even by silver staining of the topoisomerase II fraction (11a). Nonetheless, since CKII has been reported to form filament-like polymers and rings (40), it was important to rule out the possibility that CKII protein is itself responsible for the aggregation of topoisomerase II. To do this, yeast topoisomerase II was separated from the copurifying kinase on a glycerol gradient (9), and after dialysis, the kinase-free topoisomerase II was tested in the sedimentation assay. Multimers formed and pelleted in proportions similar to those observed prior to gradient purification from the kinase (see Fig. 4B, bars labelled "Control").

Effect of nucleotides on topoisomerase II multimerization. Lindsley and Wang (25) have shown that the binding of ATP and certain ATP analogs provokes a conformational change in yeast DNA topoisomerase II. Although Fig. 3 demonstrates that CKII-modified topoisomerase II is preferentially sedimented, it is important to determine whether nucleotide binding alone can enhance the precipitation of topoisomerase II, since ATP and ADP are both present during phosphorylation. We have incubated yeast topoisomerase II and its copurifying CKII with ATP, GTP, ADP, adenosine 5′-[β,γ-imido] triphosphate (AMP-PNP), and adenosine 5′-[γ-thio]triphosphate (ATPγS), prior to the centrifugation assay. In terms of

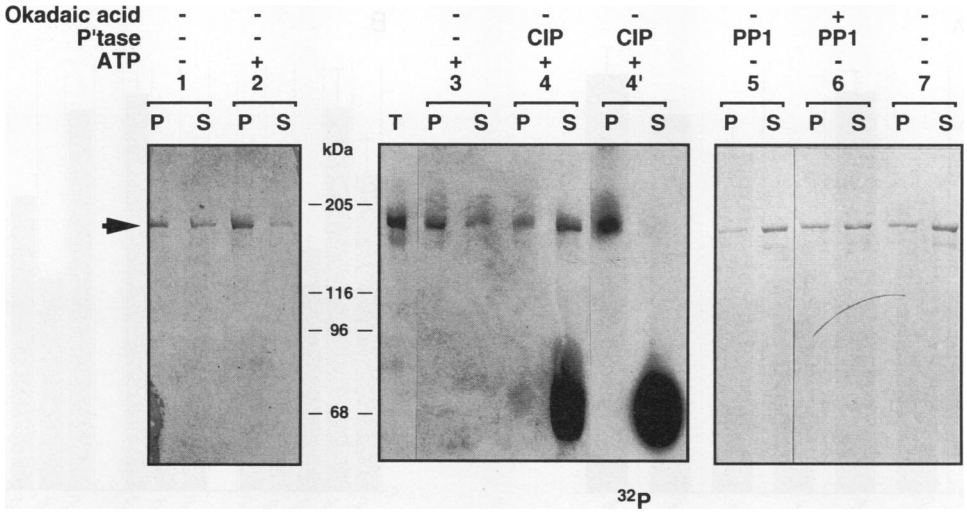

FIG. 3. Dephosphorylation reduces the sedimentation of topoisomerase II. For each pair of lanes, 200 ng of topoisomerase II was incubated either alone (lanes 1) or with 10 μM ATP and 1 μCi of [γ-^{32}P]ATP (lanes 2 to 4', +ATP) at 30°C for 30 min in buffer B1. To test the effects of dephosphorylation, 0.2 U of CIP (lanes 4 and 4', +CIP) was added and the incubation was continued at 37°C for 2 min before centrifugation. In an experiment using another preparation of topoisomerase II, 200 ng was incubated alone (lane 5), with 1 μl of PP1 (lane 5), or with both PP1 and 0.1 mM okadaic acid (lane 6) at 30°C for 30 min. All samples were centrifuged at 10,000 × *g* in an Eppendorf Microfuge for 10 min at 4°C, and pellet and supernatant fractions (P and S, respectively) were separated on an SDS–7% polyacrylamide gel. T indicates the total 200 ng of topoisomerase II denatured for the gel. Shown are Coomassie blue-stained gels and, for lanes 4' (indicated by ^{32}P) an autoradiograph of lanes 4 is shown. The large band at 68 kDa in lanes 4 and 4' is CIP itself. High-molecular-weight protein standards (Sigma) are indicated in the middle.

phosphorylation, GTP, ATP, and ATPγS are all donors for CKII-catalyzed phosphorylation, while AMP-PNP and ADP are not. It was previously reported that AMP-PNP and ATPγS cause conformational changes near the active domain of topoisomerase II, while ATP does not, presumably because bound ATP is rapidly hydrolyzed to ADP (25).

In Fig. 4A we summarize the effects of these various analogs on the sedimentation of topoisomerase II. The addition of ATP and both the hydrolyzable and the nonhydrolyzable ATP analogs enhances the precipitation of topoisomerase II, while ADP decreases it. At a concentration of 60 μM (which corresponds to the K_m of CKII for GTP), GTP also enhances precipitation (Fig. 4A), suggesting that both nucleotide binding (e.g., AMP-PNP) and phosphorylation (e.g., due to ATP, ATPγS, or GTP) can affect topoisomerase II multimerization.

To confirm that phosphorylation can enhance topoisomerase II interactions, topoisomerase II was incubated with CKII in the presence of ATPγS, prior to the treatment with CIP. ATPγS serves as a donor for CKII and forms a bond which is insensitive to phosphatase action, while mimicking a phosphate group. Preincubation with ATPγS, but not with AMP-PNP or ATP, renders topoisomerase II insensitive to the effects of CIP, i.e., ATPγS-treated topoisomerase II pellets equally in the presence or absence of CIP (Fig. 4B, ATPγS + CIP). Moreover, when AMP-PNP is added to CIP-treated topoisomerase II, it cannot restore the multimerization potential of the enzyme (Fig. 4B), suggesting that a basal level of phosphorylation is necessary for the nucleotide-binding effect. Finally, we dephosphorylated topoisomerase II, separated it from CIP by glycerol gradient sedimentation (as for removal of the kinase), and then rephosphorylated it by the addition of human CKII (Boehringer Mannheim) and ATP. We found partial restoration of the multimerization of topoisomerase II (Fig. 4B, last three columns). In conclusion, a basal level of phosphorylation seems important for multimerization of topoisomerase II, and phosphorylation by CKII beyond this level can enhance its aggregation by roughly 20%.

C-terminal truncation of topoisomerase II reduces multimer formation and is insensitive to kinase and phosphatase treatments. The fact that phosphorylation by CKII alters topoisomerase II multimer formation suggests either that the C-terminal domain mediates this interaction or that there is a conformational change upon phosphorylation that enhances protein-protein interaction. To test this, we truncated the yeast enzyme at aa 1235, which eliminates the C-terminal 200 aa and 10 major CKII phosphoacceptor sites (11a). The protein was overexpressed in yeast cells and was purified to homogeneity. In the standard sedimentation assay we found that at equal concentrations the truncated enzyme sediments less efficiently, with 17% of the enzyme recovered in the pellet fraction (Fig. 5, lanes 4), compared with 40% of the full-length protein in this particular preparation (Fig. 5, lanes 1). Consistent with the fact that the phosphorylation acts on sites removed from this truncated protein, we found that neither treatment with exogenously added CKII nor treatment with phosphatase affects the protein's sedimentation (Fig. 5, lanes 5 and 6). Under identical conditions they do influence that of full-length yeast topoisomerase II (lanes 2 and 3). Moreover, if we add bacterially expressed and purified C-terminal domain of topoisomerase II to the full-length enzyme, we find that the fraction which sediments diminishes, consistent with direct competition for sites of interaction (data not shown).

Topoisomerase II multimers can be visualized as aggregates and rosettes. Topoisomerase II purified by the method of Worland and Wang (44) is a stable dimeric enzyme that can be visualized by electron microscopy (Fig. 6). Rotary shadowing of the enzyme on electron microscopy grids usually reveals a V-shaped structure with three globular domains (Fig. 6a), which is reminiscent of the structure observed when the same technique is used with bacterial gyrase (22), to which topoisomerase II is evolutionarily and structurally related (45). Occasionally we observe a different shape, which resembles a more closed structure with four globular domains (Fig. 6c; see interpretation in Fig. 6h). If a negative staining technique is

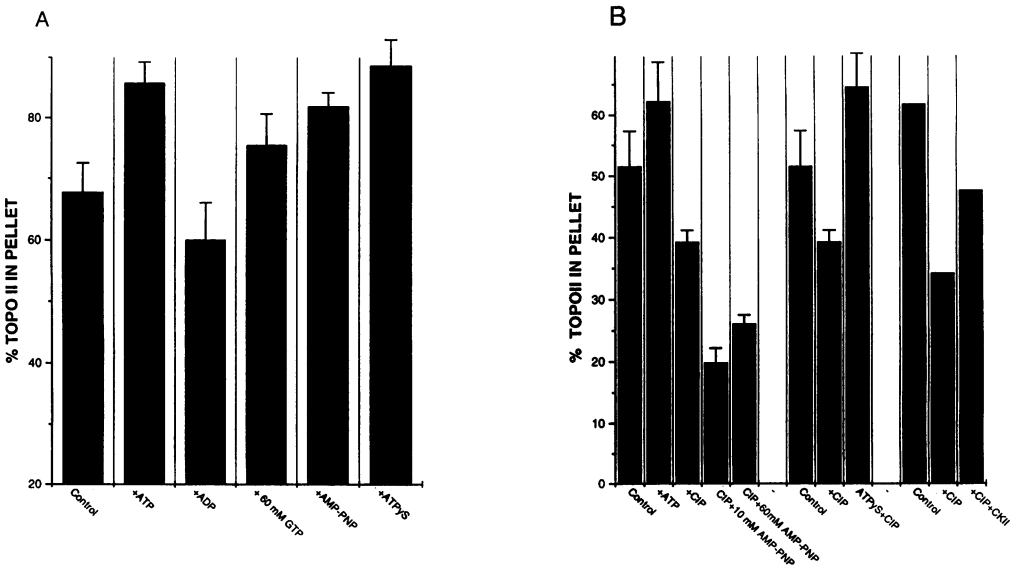

FIG. 4. Nucleotide binding and phosphorylation enhance topoisomerase II multimerization. Two hundred nanograms of topoisomerase II (Control) and, where indicated, 10 μM ATP, ADP, GTP, ATPγS, and AMP-PNP were incubated at 30°C for 30 min in buffer B1 and were then centrifuged in an Eppendorf Microfuge. The pellet and supernatant fractions were separated on an SDS–7% polyacrylamide gel and were then stained with Coomassie blue. The ratio of precipitated versus supernatant topoisomerase II was estimated by scanning the Coomassie blue-stained gels on a Hirschner Elscript 400 densitometer. Two different preparations of topoisomerase were used, which accounts for the difference in sedimentation in the control lanes. The bars indicate the averages of two experiments done with the same topoisomerase II preparation, and the thin bar indicates standard deviation. To test the effects of phosphorylation and nucleotide binding, topoisomerase II was additionally incubated at 37°C for 2 min with 0.2 μl of CIP, and then AMP-PNP was added and the mixture was incubated for 30 min at 30°C before centrifugation (CIP+AMP-PNP). To test the blocking effect of ATPγS, topoisomerase II, incubated in the presence of 10 μM ATPγS, was treated with CIP for 2 min at 37°C before centrifugation (ATPγS+CIP). The effect of rephosphorylation of CIP-dephosphorylated DNA was studied by incubating 200 ng of CIP-treated and then glycerol gradient-purified topoisomerase II (+CIP) with 1 U of human CKII in the presence of 10 mM ATP for 30 min at 30°C prior to the sedimentation assay (+CIP+CKII). The control topoisomerase II was purified through the glycerol gradient but was not CIP treated. (A) Effects of nucleotides on topoisomerase II multimer formation. (B) Effects of dephosphorylation and rephosphorylation on topoisomerase II multimer formation.

used, we again observe predominantly two images, either four globular regions with an apparent hole in the middle (Fig. 6d, arrows), or U-shaped molecules, which resemble the three-globed images seen with rotary shadowing (Fig. 6b, arrows; see interpretation in Fig. 6h). Since in both preparations the yeast topoisomerase II used was at least 90% pure by Coomassie blue staining (9) and was mainly dimeric, the two different images most certainly correspond to the dimer viewed either from different angles or in two conformational states, as suggested earlier by the analysis of proteolysis patterns (25).

Electron microscopy confirms that tetramers and perhaps higher multimers can be seen in higher frequency after the addition of 250 μM ATP, as shown in Fig. 6e. Quantitation of the dimeric form, as opposed to higher multimers, reveals an increase in multimer frequency from 45 to 75% (n = 500) upon incubation with ATP and the copurifying kinase, prior to spreading the enzyme preparation (Fig. 7). In this case, the spreading of the enzyme on mica was also done in fairly high salt to reduce nonspecific aggregation (see Materials and Methods). If DNA is added (in this case a linearized replicating yeast vector), large rosettes of DNA and multimeric topoisomerase II are seen (Fig. 6f and g). Consistent with the observation of looped-out regions of DNA in these aggregates, recent work has documented that topoisomerase II prefers to bind to sites at which two double helices cross over (31, 46).

Topoisomerase II does not interact with the majority of soluble nuclear proteins. If topoisomerase II plays a role in long-range chromosomal organization, its multimerization should not be impeded by the presence of other nuclear proteins, and in fact it might be expected to complex other nuclear proteins as it sediments. To check this, we incubated topoisomerase II in the presence of 10 μg either of a soluble yeast nuclear transcription extract (41) or of a resolubilized nuclear scaffold fraction (20), followed by the standard sedimentation assay. Most nuclear proteins in the transcription extract remain in the supernatant fraction, in either the presence or the absence of topoisomerase II, with or without exogenous ATP (data not shown). Nor is the multimerization of topoisomerase II in any way impeded by the nuclear extract (data not shown). However, the preparation of the yeast transcription extract involves centrifugation to remove insoluble proteins and is somewhat depleted for endogenous topoisomerase II (41). Therefore we turned to a renatured extract of proteins from the yeast nuclear scaffold (20) to look for potential interactions between topoisomerase II and other proteins with putative roles in nuclear substructure.

Topoisomerase II was incubated under standard conditions in the presence of 2.5 μg of a nuclear scaffold extract or, alternatively, each component was incubated alone and then centrifuged in an Eppendorf Microfuge. A limited number of scaffold fraction proteins were found to sediment from the extract in the absence of exogenous topoisomerase II (Fig. 8A, lanes 1 and Fig. 8B, lanes 1). Among these were several in the range of 70 to 90 kDa and topoisomerase II itself, which migrates at 170 kDa (Fig. 8A, lanes 4). Western blot analysis with antitopoisomerase II shows that approximately 40% of the endogenous topoisomerase II sediments even after the

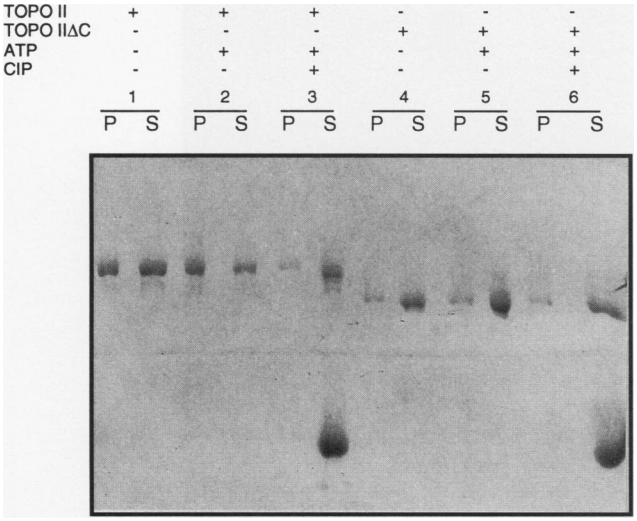

FIG. 5. A C-terminally truncated topoisomerase II does not readily form multimers. For each pair of lanes, 200 ng of full-length topoisomerase II or of C-terminally truncated topoisomerase II (aa 1235; see Materials and Methods) was incubated alone (lanes 1 and 4, respectively) or with 10 μM ATP and 1 μCi of [γ-^{32}P]ATP (lanes 2 and 5, respectively, +ATP) at 30°C for 30 min in buffer B1. To test the effects of dephosphorylation, 0.2 U of CIP (lanes 3 and 6, respectively, +CIP) was added and the incubation was continued before centrifugation. All samples were centrifuged in an Eppendorf Microfuge for 10 min at 4°C, and pellet and supernatant fractions (P and S, respectively) were separated on an SDS–7% polyacrylamide gel. Bars indicate migration of high-molecular-weight protein standards (Sigma).

urea denaturation/renaturation process required for preparation of the solubilized scaffold extract (20).

Intriguingly, the addition of topoisomerase II enhances the sedimentation of several proteins, again primarily in the molecular mass range of 70 to 90 kDa (Fig. 8A, lanes 1P and 2P). To identify these proteins, we probed with antibodies that recognize scaffold proteins in other species. One of the factors is recognized by antisera specific for the *S. pombe* Nuc2p, a protein implicated in nuclear segregation at mitosis. Another cross-reacts with affinity-purified antibodies against the 135-kDa chromosomal scaffold protein 2 of chickens (Sc-2) (40a). Cloning and peptide analysis will be necessary to evaluate whether the proteins in question are true *S. cerevisiae* homologs or simply contain cross-reacting epitopes. Consistent with results reported above, treatment of topoisomerase II with CIP prior to incubation with the scaffold extract significantly decreases the amounts of topoisomerase II and of associated scaffold proteins that aggregate and sediment (Fig. 8A, lanes 3).

Conditions that enhance or disrupt interactions between topoisomerase II dimers. We have demonstrated an effect of both phosphorylation and nucleotides on the sedimentation of topoisomerase II alone in vitro. Since the presence of topoisomerase II in nuclear scaffold and matrix fractions may reflect a similar multimerization or aggregation of the enzyme, we screened a variety of factors known to affect the formation of the nuclear matrix in situ, including metals, heat, DNA cleavage, thiol reagents, and RNase A (discussed in references 14 and 21).

(i) Copper and calcium. The metaphase scaffold is usually isolated from HeLa chromosomes after the addition of low concentrations of Cu^{2+} or Ca^{2+}, followed by extraction with lithium-diiodosalicylate or high concentrations of NaCl, which efficiently remove histone and many other soluble proteins (16, 24, 28). In solution, however, salt concentrations beyond 150 mM clearly impede the sedimentation of topoisomerase II as multimers (Fig. 3A), as does the addition of $CaCl_2$ (data not shown). In contrast, the addition of $CuSO_4$ increases the fraction of sedimenting topoisomerase II (Fig. 8B, lanes 4, and Fig. 8C). Lewis and Laemmli reported a stabilizing effect of copper for metaphase scaffold isolation, which was reversible by strong copper chelators (24). Similarly, we see that the $CuSO_4$ effect on topoisomerase II sedimentation is reversed by β-mercaptoethanol (Fig. 8C, lanes 5). Other antioxidants, such as thiodiglycol or $NaBH_4$, have no effect (data not shown). Surprisingly, the addition of $CuSO_4$, like phosphorylation, stimulates topoisomerase II decatenation activity.

Copper does not generally affect the sedimentation of proteins in the scaffold extracts (Fig. 8B, lanes 1), although the addition of Cu^{2+} with topoisomerase II enhances cosedimentation of a subset of scaffold proteins (Fig. 8B, lanes 6). Intriguingly, the loss of interactions apparently due to the dephosphorylation of topoisomerase II (Fig. 8A, lanes 3) was overcome by adding 0.5 mM $CuSO_4$ (data not shown). Since there is not covalent modification of these proteins, Cu^{2+} may be complexed either by sulfhydryl groups or by nitrogen and hydroxyl groups of two polypeptides, mediating a stable, but copper-dependent, interaction (e.g., reference 35).

(ii) Temperature. It has been noted by several workers that nuclear matrices can be stabilized by the incubation of nuclei at 37°C (14, 21, 27, 28) and that the association of certain proteins, such as p53 and p62$^{c\text{-}myc}$, with this fraction is dependent on the temperature shift (26). Topoisomerase II multimerization in solution is not affected by incubation at 37°C (Fig. 8B, lanes 5), although the solubility of certain scaffold proteins is dramatically affected (Fig. 8B, lanes 2). If topoisomerase II is added to the scaffold extract, it sediments along with the subset of proteins that appear to aggregate at 37°C (Fig. 8B, lanes 7), perhaps mimicking events that occur in isolated nuclei during the nuclear matrix stabilization by temperature shift.

(iii) β-glycerophosphate. Recently it has been reported that 100 mM NaCl removes >95% of the topoisomerase II from chromosomes formed in vitro by incubation of sperm nuclei in *Xenopus* extracts (19). It was suggested on this basis that topoisomerase II had no role in the organization of these chromosomes. The total extraction of topoisomerase II at 100mM NaCl is unlike the behavior of the enzyme in natural chromosomes (13, 14, 16, 38) and contradicts the facts that the optimal ionic strength for decatenation is around 160 mM (29) and that topoisomerase II-DNA interactions are observed in bandshift and filter-binding assays at 0.4 to 1 M KCl (11a, 31). To resolve this discrepancy we tested the effects on topoisomerase II multimerization of the buffer used in *Xenopus* extracts to create and wash in vitro-condensed chromosomes (20 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and 80 mM β-glycerophosphate) (19). The addition of EGTA has little effect on the solubility of topoisomerase II, but as low a concentration as 20 mM β-glycerophosphate counteracts or prevents the interactions that lead to topoisomerase II multimerization (Fig. 9A, lanes 2 and 3). While β-glycerophosphate is a disodium compound, its effect of keeping topoisomerase II a soluble dimer is significantly more pronounced than that of the bimolar equivalent of salt (Fig. 9A, lanes 4).

To examine this further, we tested the effects of EGTA and β-glycerophosphate on ATP-dependent DNA relaxation by topoisomerase II. While increased KCl clearly stimulates re-

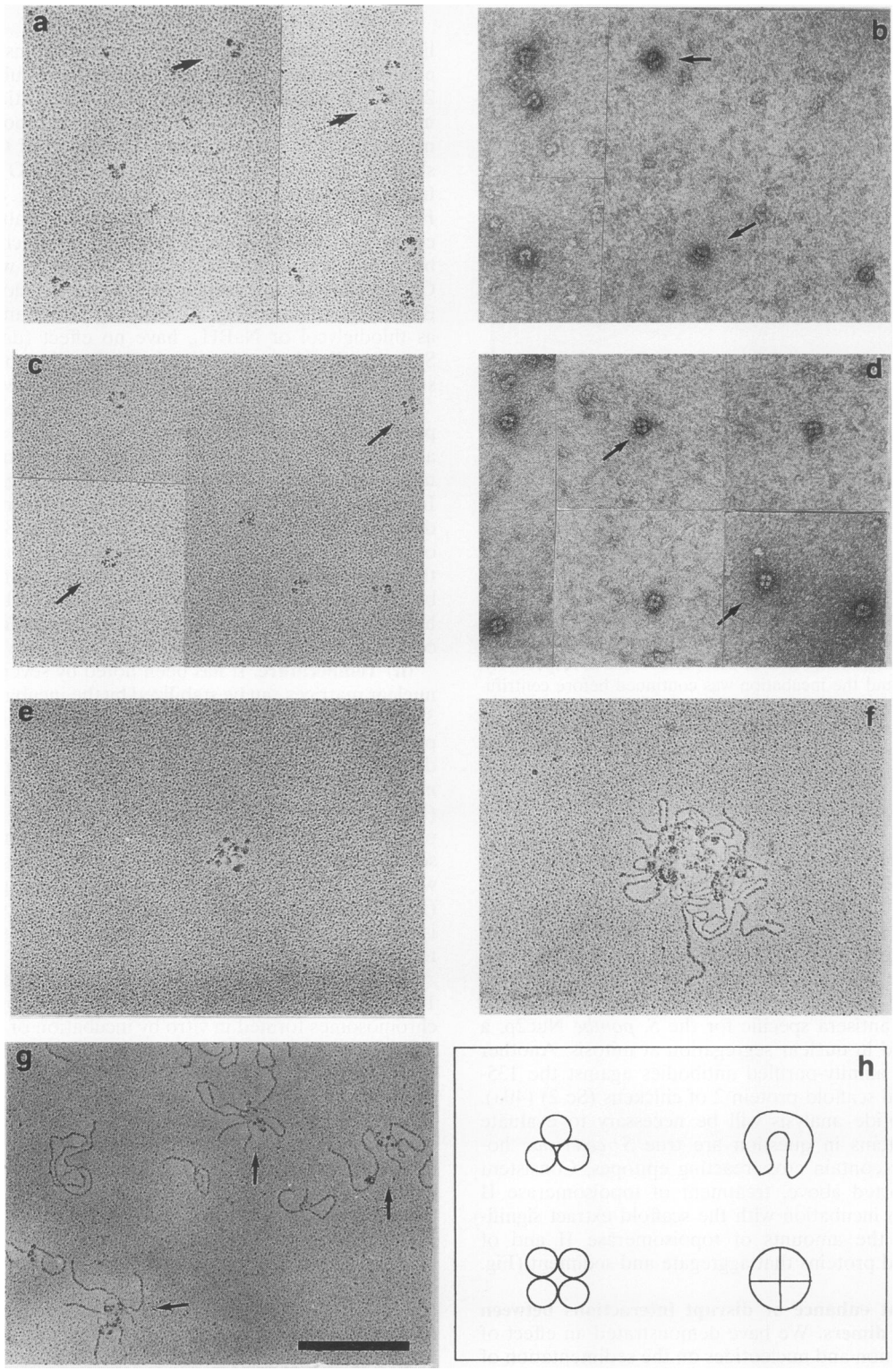

FIG. 6. Visualization of topoisomerase II by electron microscopy. (a to e) Yeast DNA topoisomerase II containing copurifying CKII (44) was mounted for electron microscopy as described below, after incubation in the presence (e) or absence (a to d) of 250 μM ATP. Images in panels a, c, and e were shadowed with platinum, while samples in panels b and d were made to directly adhere to grids and were negatively stained with uranyl acetate. (f and g) Six hundred nanograms of topoisomerase II was incubated with 100 ng of *Eco*RI and *Hin*dIII-digested plasmid pSW301 for 15 min at 30°C; this was immediately diluted to 100 mM ammonium acetate and 60% glycerol, sprayed on a freshly cleaved mica surface, and shadowed with platinum at a 7° angle. A carbon replica was made at a 90° angle, floated off on a water surface, and observed under a JEOL 100× electron microscope. (a to d) Incubation of topoisomerase II without DNA or ATP, viewed after shadowing (a and c) or negative staining (b and d); (e) incubation of topoisomerase II with ATP without DNA; (f and g) incubation of topoisomerase II with ATP and DNA, viewed after shadowing. The bar variously indicates 100 nm (in panels e to g), 110 nm (in panels b and d), and 125 nm (in panels a and c). (h) Interpretations of the shapes observed.

laxation activity, the enzyme is inhibited in buffers containing from 10 to 80 mM β-glycerophosphate (Fig. 9B, lanes 7 to 10). Either the combination of 20 mM EGTA with 40 mM β-glycerophosphate or 80 mM β-glycerophosphate alone completely abolishes the DNA relaxation activity of topoisomerase II (Fig. 9B, lanes 11 and 12). In parallel experiments, we show that similar concentrations of β-glycerophosphate interfere with topoisomerase II-DNA interactions as measured by bandshifts and with the binding of topoisomerase II to phosphocellulose columns (data not shown). Thus, although the mechanism is unclear, β-glycerophosphate blocks topoisomerase II interaction with itself and with DNA and therefore allows the enzyme to be readily extracted from chromosomes reconstituted in β-glycerophosphate-containing buffers (19).

DISCUSSION

Both genetic results (39) and biochemical evidence from in vitro systems for chromosome condensation (4, 19, 43) suggest that topoisomerase II activity is necessary for proper chromosome condensation. Little is known about how this might be achieved. In most models for mitotic chromosome structure, protein-protein interactions are proposed to aid in the compaction of DNA. In view of the fact that DNA topoisomerase II is an abundant protein in purified metaphase chromosomes from a number of species (12, 16), it is important to evaluate the possibility that topoisomerase II interacts with itself or with other proteins, while binding chromosomal DNA.

Until now most studies of topoisomerase II have concentrated on its interaction with DNA and its mode of enzymatic activity. Here we show for the first time that enzymatically active yeast topoisomerase II can form complexes the size of tetramers and apparently larger. Using an in vitro sedimentation assay, we have been able to define parameters that influence this interaction, including nucleotide binding and phosphorylation within the C-terminal 200 aa of topoisomerase II by CKII.

Both electron microscopy and glycerol gradient centrifugation confirm the formation, in the absence of nucleic acids, of larger complexes containing yeast DNA topoisomerase II dimers. In images that clearly resolve the dimeric enzyme, we show for the first time that the overall shape of the eukaryotic topoisomerase II is very similar to that of *Escherichia coli* DNA gyrase (22). DNA topoisomerase II and gyrase are closely related, and the single eukaryotic topoisomerase II gene can be considered a fusion between *gyrA* and *gyrB* subunit genes. The C-terminal domain of topoisomerase II, the target for CKII and other phosphorylating kinases, is not, however, present in prokaryotic type II enzymes (45).

Our results on topoisomerase II sedimentation yield two additional observations. First, some of the reagents that have been used in assays of chromosome condensation in vitro, and for the isolation of the chromosomal and nuclear scaffolds, influence topoisomerase II aggregation. Even in the absence of such reagents, however, topoisomerase II interacts with and causes the cosedimentation of certain polypeptides in a nuclear scaffold extract, including a factor that cross-reacts with affinity-purified antibodies to the *S. pombe* protein Nuc2p (18, 40a). Finally, we show that the 40 to 80 mM β-glycerophosphate present in the *Xenopus* extracts used for in vitro condensation assays (19) can inhibit both topoisomerase II decatenation activity and protein-protein interactions. Thus, the apparent lack of stable interactions involving topoisomerase II in reconstituted chromosomes must be interpreted with caution (19).

Is phosphorylation a physiological modulator of topoisomerase II interactions? Topoisomerase II is a substrate for several types of protein kinases (reviewed in reference 8). Phosphorylation of topoisomerase II by any of a variety of kinases (CKII, $p34^{cdc2}$, Ca^{2+}/calmodulin-dependent kinase, and protein kinase C) results in the stimulation of topoisomerase II activity, although to date only CKII or a closely related kinase have been shown to reactivate the fully dephosphorylated enzyme (1, 7, 9, 32, 33, 36). The 10 major CKII acceptor sites in yeast topoisomerase II are located in the C-terminal 200 aa, i.e., downstream of aa 1235 (7, 9, 11a). Truncations that remove this C-terminal domain of either the yeast or the *Drosophila* enzymes, however, do not interfere with the enzyme's activity in vitro and support mitotic growth of yeast cells, albeit with a reduced efficiency of plasmid decatenation and slightly enhanced chromosome breakage (10, 17, 36). One model that accounts for the stimulation of activity in a phosphorylated enzyme and the behavior of the truncation is that the C-terminal region is a negative regulatory domain which undergoes a conformation change when phosphorylated, thus activating the enzyme (8). The results presented here show that the dephosphorylated enzyme does not readily form multimers and that this activity is stimulated by phosphorylation. Consistently, the truncated enzyme is insensitive to either kinase or phosphatase treatments, and its sedimentation properties are similar to those of CIP-treated full-length topoisomerase II (Fig. 5, compare lanes 3 and 4). This not only may implicate the C terminus in a conformational change that encourages multimerization but also suggests that the C terminus itself is a site of interaction. This is reinforced by the observation that the addition of the bacterially expressed C-terminal domain of topoisomerase II competes for topoisomerase II interactions, while yeast transcription extracts do not (data not shown).

Since CKII is apparently not cell cycle regulated (reviewed in reference 8), topoisomerase II should have a basal level of phosphorylation as soon as it is synthesized, as well as a certain

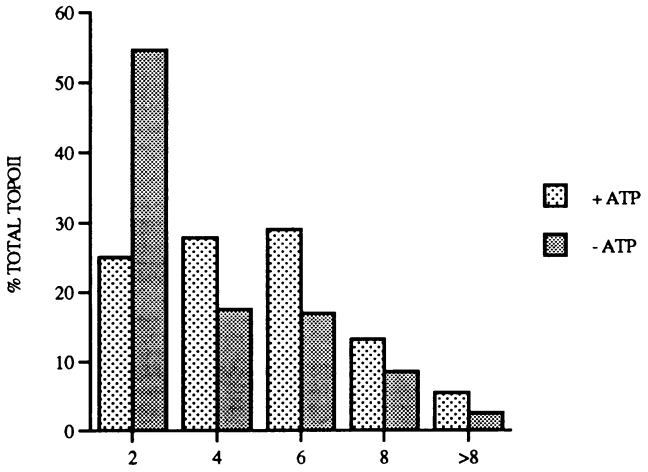

FIG. 7. Distribution of topoisomerase II complexes as assayed by electron microscopy. Ten micrograms of topoisomerase II containing the copurifying CKII was incubated with or without ATP (final concentration, 160 μM) for 30 min at 30°C. The samples were immediately diluted to 600 mM ammonium acetate in 66% glycerol and were sprayed on mica as described in Materials and Methods. A total of 500 topoisomerase II molecules or complexes were scored in each experiment. The same experiment in low salt gives an increase from 22% multimers to 91% multimers (four or more molecules per multimer upon the addition of ATP. Numerals on the abscissa indicate the number of topoisomerase II molecules in a multimer.

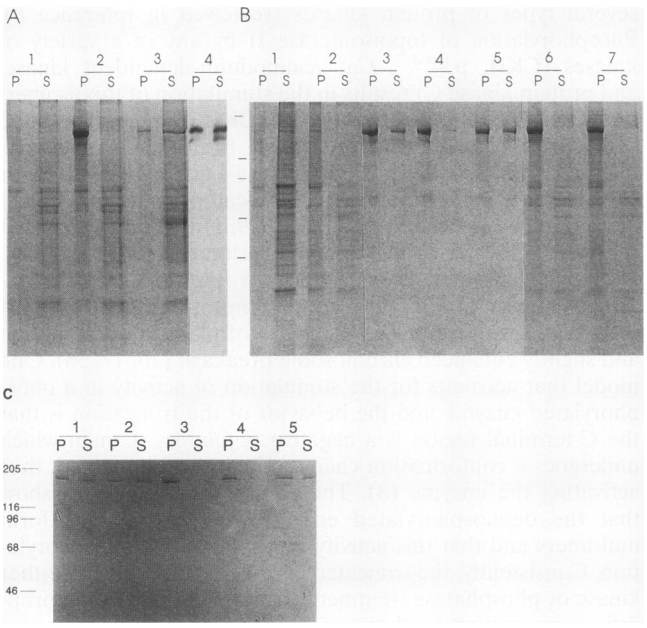

FIG. 8. Topoisomerase II sedimentation assay in the presence of yeast nuclear scaffold extract. Two hundred nanograms of purified yeast topoisomerase II and/or 2.5 μg of yeast nuclear scaffold extract (see Materials and Methods) was incubated in B1 buffer at 30°C for 30 min and was then centrifuged at 12,000 rpm (10,000 × *g*) at 4°C for 10 min. The supernatant and pellet were collected, diluted with the SDS sample buffer, and separated on SDS-PAGE (23). P and S, pellet and supernatant fractions, respectively. (A) Lanes 1, scaffold extract without topoisomerase II; lanes 2, topoisomerase II with scaffold extract; lanes 3, same as lanes 2, except that topoisomerase II was treated with CIP before incubation with the scaffold extract; lanes 4, same samples as in lanes 1 but blotted onto a nitrocellulose filter and probed with antitopoisomerase II antibodies. (B) Lanes 1, scaffold extract without added topoisomerase II was incubated in the presence of 0.5 mM $CuSO_4$; lanes 2, scaffold extract without added topoisomerase II was incubated at 37°C; lanes 3, topoisomerase II was incubated in the standard conditions; lanes 4, topoisomerase II was incubated in the presence of 0.5 mM $CuSO_4$; lanes 5, topoisomerase II was incubated at 37°C rather than at 30°C; lanes 6, topoisomerase II with scaffold extract was incubated in the presence of 0.5 mM $CuSO_4$; lanes 7, topoisomerase II with scaffold extract was incubated at 37°C. Bars indicate positions of high-molecular-weight marker proteins (Sigma) (see Fig. 9). (C) Purified topoisomerase II was sedimented as described in Materials and Methods after incubation with no $CuSO_4$ (lanes 1), 0.1 mM $CuSO_4$ (lanes 2), 0.5 mM $CuSO_4$ (lanes 3), 0.5 mM $CuSO_4$ with 1 mM β-mercaptoethanol (lanes 4), or 0.5 mM $CuSO_4$ with 5 mM β-mercaptoethanol (lanes 5).

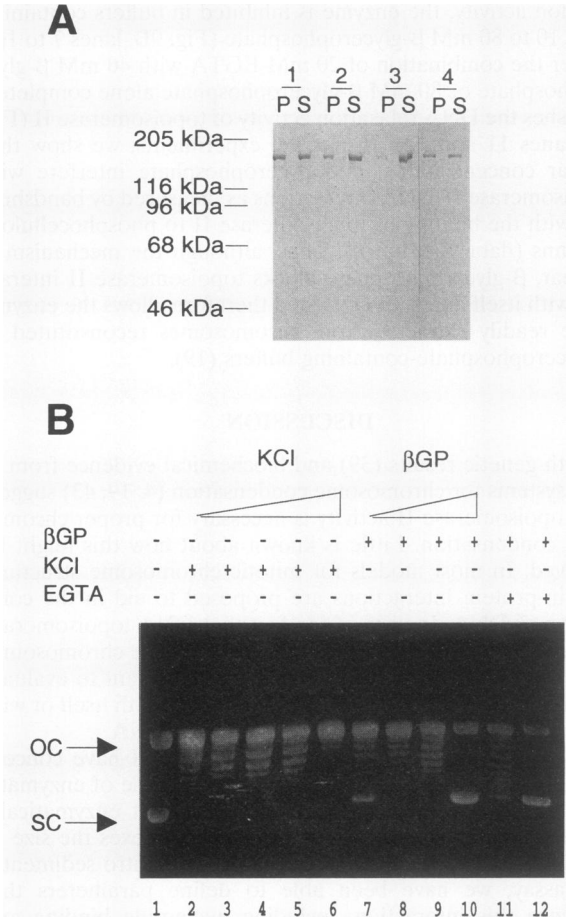

FIG. 9. Effects of β-glycerophosphate on topoisomerase II multimerization and activity. (A) Two hundred nanograms of purified topoisomerase II was incubated in B1 buffer at 30°C for 30 min in the presence of increasing concentrations of β-glycerophosphate and was then centrifuged at 12,000 rpm (10,000 × *g*) at 4°C for 10 min in an Eppendorf Microfuge. The supernatant and pellet were collected, diluted with SDS sample buffer, and separated on SDS-PAGE (23). Lanes 1 to 3, topoisomerase II incubated in 0 mM, 20 mM, and 80 mM β-glycerophosphate, respectively; lanes 4, topoisomerase II incubated in 160 mM KCl. Bars indicate positions of high-molecular-weight marker proteins (Sigma). P and S, pellet and supernatant fractions, respectively. (B) One hundred nanograms of purified topoisomerase II was incubated in decatenation buffer (see Materials and Methods), 1 mM ATP, and 0.2 μg of supercoiled pBR322 DNA in the presence of increasing concentrations of KCl or β-glycerophosphate for 15 min at 30°C. Reactions were stopped by the addition of EDTA, SDS, and proteinase K and were analyzed on a 1% agarose gel in Tris-acetate buffer. Lane 1, supercoiled pBR322 DNA; lanes 2 to 6, reaction mixtures containing 20, 40, 80, 160, and 320 mM KCl, respectively; lanes 7 to 10, reaction mixtures containing 10, 20, 40, and 80 mM β-glycerophosphate, respectively; lane 11, reaction mixture containing 40 mM β-glycerophosphate and 10 mM EGTA; lane 12, reaction mixture containing 80 mM β-glycerophosphate and 20 mM EGTA. OC, open circular DNA; SC, supercoiled DNA.

level of constitutive activity. However, the level of phosphorylation is greatly enhanced in late G_2 and M phase. This should stimulate decatenation activity and could perhaps promote interactions of topoisomerase II with itself and/or other proteins, facilitating chromosome condensation. CKII is the major kinase-modifying yeast topoisomerase II in vivo, yet a role for other kinases is not excluded, and other kinases may also promote protein-protein interactions. Candidates for other ligands for topoisomerase II may be the coprecipitating nuclear scaffold proteins or the second major nonhistone protein in metaphase chromosomal scaffolds, Sc-2 (24).

Recently it has been shown that the MPM2 antibody, which recognizes mitosis-specific phosphorylated epitopes, specifically stains topoisomerase II in Indian muntjac chromosomes (38). A distinctive axial staining results, much like that observed with antitopoisomerase II in swollen human chromosomes (13, 16). A similar staining was observed with the MPM2 antibody in reconstituted chromosomes (19). These results are consistent with earlier proposals that a subset of topoisomerase II (i.e., a phosphorylated form) plays a role in mitotic chromosome organization. It thus appears likely that the different populations of topoisomerase II observed for *Dro-*

sophila embryos may also represent differentially modified forms of the enzyme (37).

Are scaffold stabilization and matrix stabilization results of topoisomerase II multimerization? We observe a profound effect of copper and of β-glycerophosphate on the solubility of topoisomerase II. These reagents have been used in the past in the isolation of scaffold or matrix structures and are often used in chromosome condensation assays. In early experiments on chromosome structure it was observed that if chromosomes were isolated in polyamine-EDTA buffers, it was necessary to add a low concentration of Cu^{2+} or Ag^{2+} during histone extraction to stabilize the residual scaffold against disruption (24). The stabilization was readily reversed by chelators but not by reducing agents; this suggests that the metals were complexed by chromosomal proteins, rather than that they acted catalytically to promote disulfide bond formation. Although Cu^{2+} is present in isolated chromosomes (24), there is no convincing evidence either for or against a physiological role for copper in chromosome structure. Since the addition of copper overcomes the effects of phosphatases, which reduce topoisomerase II multimerization, it is unlikely that phosphorylation and copper ions function through a common pathway. It is noteworthy, however, that the decatenation activity of topoisomerase II is stimulated by both copper and phosphorylation.

A limited number of other nuclear proteins cosedimented in our assay, and most of these will not sediment in the absence of topoisomerase II, unless the extracts are shifted temporarily to 37°C. Effects of temperature shift and the cross-linking of sulfhydryl groups have been studied in attempts to understand the isolation of residual nuclear structures or matrices (24, 26, 27, 28; see reviews in references 13 and 21). Not only the stabilization but also its disruption by β-mercaptoethanol and is mimicked by our simple sedimentation assay. Clearly, further molecular and genetic evidence is required to prove whether the interphase nuclear matrix reflects an underlying network of structural proteins or simply a preferentially insoluble subset of nuclear proteins.

Perhaps the most important point in our study in respect to this question is to show that topoisomerase II, which has been implicated in chromosome condensation by genetic studies, also forms readily sedimenting multimers without any obvious stabilizing conditions or agents. Moreover, the observation that all topoisomerase II is extracted from chromosomes reconstituted in *Xenopus* extracts with 100 mM NaCl is likely to be explained by the presence of 80 mM β-glycerophosphate in these extracts (19). Thus, while our study underscores the risks of artefacts inherent in matrix isolation and chromosome reconstitution procedures, it also provides a mechanistic model to explain how topoisomerase II might influence chromosome condensation at the G_2-M transition through regulated protein-protein interactions.

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