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Stabilization of type I topoisomerase–DNA covalent complexes by actinomycin D

(transcription/DNA-binding proteins/gene expression)

D. K. TRASK* AND M. T. MULLER†

Department of Molecular Genetics, The Ohio State University, Columbus, OH 43210

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ABSTRACT **The activity of the endogenous DNA topoisomerase type I (EC 5.99.1.2) can be quantified *in situ* by determining how efficiently the enzyme is trapped in a covalent complex with DNA upon lysis of nuclei with detergents. In this way, we can measure relative levels of topoisomerase binding to DNA at native sites in chromatin. Since the majority of topoisomerase I is localized in the nucleolus at rRNA genes, we have evaluated how low levels of actinomycin D, which terminate transcription of rRNA genes, affect the activity of topoisomerase I. *In vivo*, as well as *in vitro* with purified topoisomerase I, we have found that drug treatment extends the half-life of the covalent topoisomerase–DNA complex. Actinomycin D stabilizes the nicked intermediate in the cleavage and resealing reaction but otherwise does not significantly alter the strand-passing ability of topoisomerase I. Sequence-specific cleavages by topoisomerase I were stimulated by actinomycin D at identical sequences recognized by the enzyme in the absence of drug. The localization of topoisomerase I in the nucleolus, coupled with the observation that transcription in this organelle is highly sensitive to actinomycin D and camptothecin treatment, leads us to propose that topoisomerase I contributes to actinomycin D inhibition of transcription.**

A number of reports have suggested that type I DNA topoisomerase (topoisomerase I; EC 5.99.1.2) is involved in transcription based on its association with actively transcribed genes in chromatin (1–6). This association is clearly evident for rRNA genes in animals, yeast, and *Tetrahymena* (2, 3, 7). The enzyme is acting catalytically at genes characterized by a high rate of transcription and the heaviest enrichment of topoisomerase I is seen cytologically within the nucleolus (2). Topoisomerase I makes a transient single-strand break in the sugar–phosphate backbone of DNA (for reviews, see refs. 8 and 9), which introduces a site of rotational freedom in the template; thus, topoisomerase action may provide a swivel point to facilitate entry and/or progression of the bulky transcriptional apparatus. Alternatively, when nascent RNA chains are hybridized to the one strand of template, the topology changes and topoisomerase I may be required to return to (or adjust) the topological ground state. Studies of yeast topoisomerase I mutants suggest that topoisomerase I is not an essential gene (10, 11); however, it seems that topoisomerase II (EC 5.99.1.3) is complementing the defect since topoisomerase II (like topoisomerase I) has been associated with transcriptionally active regions in chromatin (ref. 12; M.T.M. and V. Mehta, unpublished data) and topoisomerase I and II double mutants display a defect in rRNA transcription (7).

A better understanding of the involvement of topoisomerase I in transcription can be obtained by analyzing the activity of topoisomerase I catalyzed reactions in a chromosomal setting. The covalent intermediate is a functional reaction intermediate in the process of breaking and rejoining the DNA substrate (13–15), and we have shown that the amount of DNA trapped in a covalent complex with topoisomerase I is a quantitative measure of the steady-state levels of topoisomerase activity (16). Therefore, the relative activity of endogenous enzyme can be determined by measuring the ratio between covalently bound and free topoisomerase.

Because of the close association between topoisomerase I and transcription, we determined whether inhibition of transcription had any effect on endogenous topoisomerase I activity. We report that actinomycin D results in an increase in formation of the covalent intermediate between topoisomerase I and DNA, and these findings may explain why rRNA transcription is particularly sensitive to low levels of the drug.

MATERIALS AND METHODS

Isolation of Topoisomerase–DNA Complexes. After washing the cells (17) in buffer A (10 mM Tris·HCl, pH 7.6/0.15 M NaCl), the final pellet was resuspended in 4–6 pellet volumes of buffer B (50 mM Tris·HCl, pH 7.5/1 mM EDTA/5 mM $MgCl_2$/0.5% Nonidet P-40), and incubated on ice for 10 min. Nuclei were released with 5 or 6 strokes of a Dounce homogenizer using the A pestle, centrifuged for 5 min at 1500 × *g*, washed again with 5 pellet volumes of buffer B, and resuspended to 5×10^8 nuclei per ml in buffer B. After incubation at 37°C for 30 min, sarkosyl was added to 1% (vol/vol) and rapidly mixed. Two volumes of 9 M NaSCN were then added and the lysate was layered on a step CsCl gradient (18), which was centrifuged in an SW41 rotor (33,000 rpm; 18 hr) and fractionated into 0.4-ml fractions. The DNA peak was localized by absorbance at 260 nm, pooled, and dialyzed against buffer C (10 mM Tris·HCl, pH 7.6/1 mM EDTA) and digested with *Eco*RI. If the topoisomerase–DNA complexes were to be analyzed by the $KDodSO_4$ method (16), $NaDodSO_4$ was added to 1% followed by the addition of 0.1 vol of 2.5 M KCl. After 15 min on ice, the precipitate ($KDodSO_4$) was deposited by centrifugation (12,000 × *g*; 2 min). The pellet was washed three times by resuspension in 1 ml of buffer D (10 mM Tris·HCl, pH 7.5/1 mM EDTA/100 mM KCl) and centrifugation (12,000 × *g*; 2 min). The final pellet was resuspended in buffer E (same as buffer D but with NaCl replacing KCl). The DNA was precipitated with ethanol, washed with 70% ethanol, and resuspended in buffer E followed by digestion with proteinase K (50 μg/ml) for 30 min at 60°C. Immunoblot assays for topoisomerase I were performed on the CsCl gradient fractions exactly as described (2, 19).

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*Present address: Harvard Medical School, Dana–Farber Cancer Institute, Division of Cancer Genetics, Boston, MA 02115.
†To whom reprint requests should be addressed.

Topoisomerase Cleavage Reactions. Reaction mixtures (20 μl) contained 50 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml (topoisomerase I cleavage buffer), 5 ng of 5'-end-labeled DNA (20), and the specified amount of purified topoisomerase I (15). Topoisomerase II cleavage buffer contained 20 mM Tris·HCl (pH 8.0), 3 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 100 mM NaCl, 3 mM ATP, and 100 μg of bovine serum albumin per ml. Reaction mixtures were incubated for 20 min at 30°C, terminated by the addition of 2 vol of 1.5% $NaDodSO_4$, and digested with proteinase K. The DNA was purified by organic extractions, ethanol-precipitated, and resuspended in buffer for loading onto a 20% polyacrylamide sequencing gel (20, 21).

RESULTS

Actinomycin D Treatment of Nuclei Stimulates Formation of Covalent Complexes *in Situ*. Previous results have demonstrated that in MSB-1 cells topoisomerase I is localized predominantly in the nucleolus and is catalytically active on rRNA genes (2). These findings, in conjunction with the results from other investigators (3, 5, 7, 22), suggest that topoisomerase I is enriched at genes transcribed by RNA polymerase I, and we estimate that 60–80% of total cellular topoisomerase I is localized to the nucleolus. To explore the dependence of transcription on topoisomerase I activity, we used actinomycin D to terminate RNA chain elongation. MSB-1 cells were treated with either 0.08 or 1 μg of drug per ml and lysed with detergent to trap the covalent complexes (Fig. 1). The fraction of topoisomerase I trapped in covalent complexes was determined as described (2). Transcription of rRNA genes by RNA polymerase I is inhibited at the lower concentration, whereas polymerases II and III are affected only at the higher concentration (23, 24). The relative proportion of topoisomerase I in the DNA peak was greater in nuclei treated with actinomycin D. Intensity measurements were made with a soft laser densitometer and amounts of topoisomerase I at the density of DNA and free topoisomerase I were quantified relative to the total. In the absence of drug, 37% of the antigen (topoisomerase I) was associated with DNA, compared to 44% or 66% after treatment with 0.08 or 1 μg of actinomycin D per ml, respectively. As a control, nuclei were incubated at 4°C to terminate the endogenous topoisomerase I reaction. Essentially all of the topoisomerase I was released from DNA and found at the top of the gradient as free protein. Reconstruction experiments with purified topoisomerase I revealed that at 4°C the enzyme cannot initiate cleavages on DNA (data not shown), suggesting that upon shifting to 4°C, the enzyme can reseal preexisting nicks but cannot begin a new round of nicking (see *Discussion*).

The possibility that actinomycin D stabilized the covalent DNA–topoisomerase I intermediate was tested by using the $KDodSO_4$ precipitation assay (15, 16). $KDodSO_4$ precipitates only those DNA molecules with covalently bound topoisomerase and thus measures the concentration of DNA molecules trapped with protein (16). Reactions between topoisomerase I and simian virus 40 DNA were carried out in the presence or absence of the drug, $NaDodSO_4$ was added, and the complexes were precipitated by addition of KCl to measure formation of covalent complexes. The results (Fig. 2) reveal that actinomycin D treatment produced a 2-fold increase in the amount of protein–DNA covalent complexes.

The DNA molecules coupled to topoisomerase I were recovered from the $KDodSO_4$ precipitate and analyzed by gel electrophoresis. Based on the reaction mechanism of topoisomerase I (8, 9), each arrested covalent intermediate should contain DNA with at least one single-stranded nick because coupling of the enzyme to DNA via a 3'-phosphoryl linkage will break the deoxyribose–phosphate backbone. If actinomycin D were stabilizing the covalent intermediate, then after drug treatment the $KDodSO_4$-precipitated DNA should be enriched for open circular DNA (Fig. 3). Essentially all of the precipitable DNA from drug-treated reactions was open circular, and the increase in nicked DNA molecules parallels drug concentration.

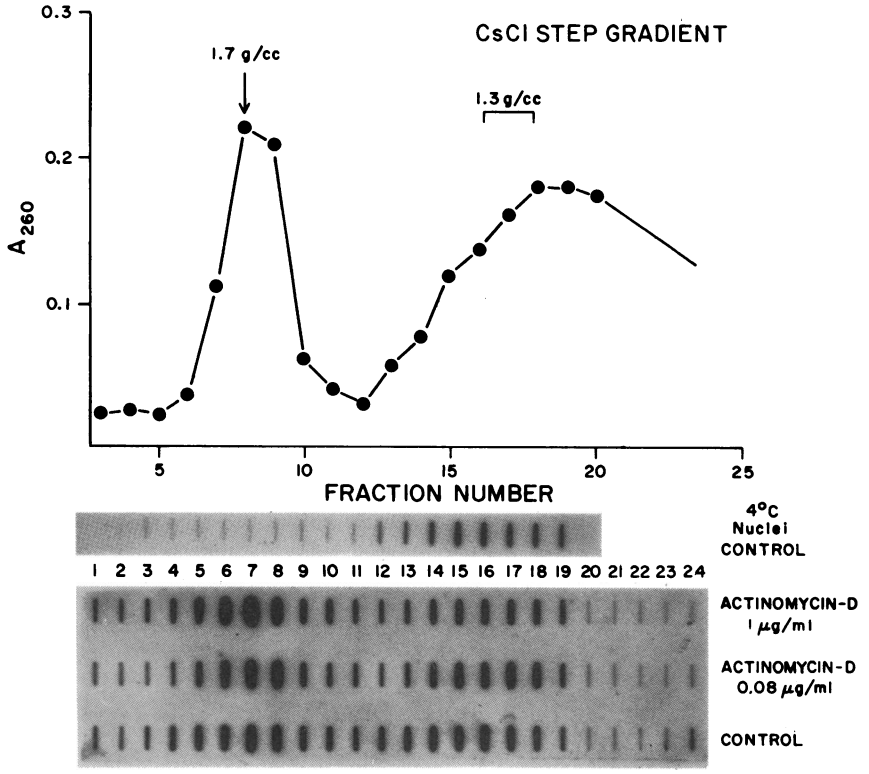

FIG. 1. Effect of actinomycin D on endogenous topoisomerase I activity. Nuclei were prepared from exponentially growing MSB-1 cells (labeled with [^{3}H]thymidine) and were incubated for 30 min at 37°C in buffer B in the indicated amount of actinomycin D. The controls were reaction mixtures without drug either at 37°C or at 4°C. The reactions performed at 37°C contain 1×10^8 nuclei each, whereas the 4°C control contained 2.5×10^7 nuclei. Reactions were terminated by addition of detergent followed by CsCl gradient fractionation and immunoblotting with anti-topoisomerase I IgG. The gradient profile was taken from a single representative gradient. The densities are indicated and the UV absorbing material at 1.7 g/ml was coincident with the [^{3}H]thymidine-labeled material.

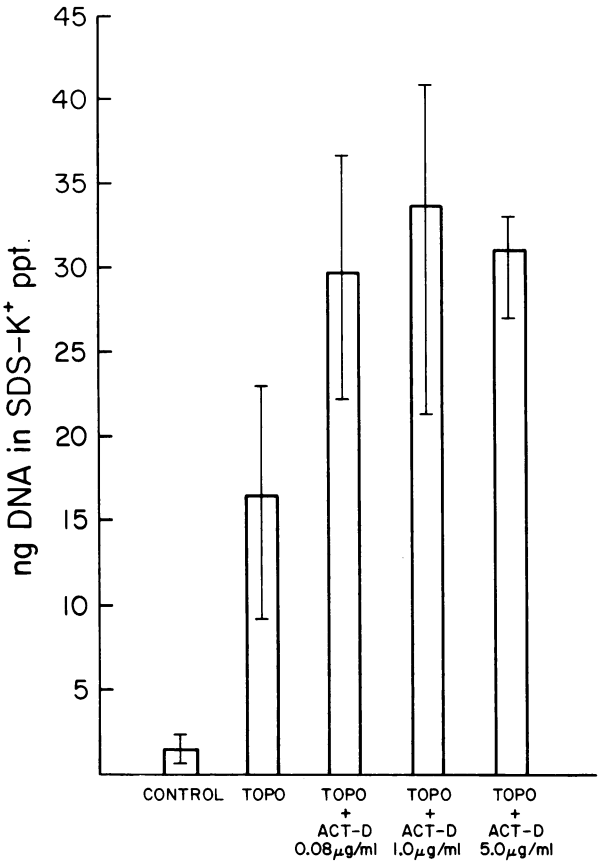

FIG. 2. Actinomycin D stimulates formation of covalent topoisomerase–DNA complexes *in vitro*. Reaction mixtures contained 0.1 μg of [^{3}H]thymidine-labeled simian virus 40 DNA, 20 ng of purified avian topoisomerase I (15) in a final vol of 0.1 ml in buffer B, and the indicated amount of actinomycin D. Reactions were carried out in triplicate. After 30 min at 37°C, the reactions were terminated with $KDodSO_4$ ($SDS\text{-}K^+$) and precipitable DNA was measured by the filter method as described (16). A control reaction mixture contained 5 μg of actinomycin D per ml and no topoisomerase I.

Sequence-Specific Cleavage by Topoisomerase I Is Stimulated by Actinomycin D. To determine whether actinomycin D alters the sequence specificity of the cleavage reaction, an end-labeled fragment was incubated with purified chicken topoisomerase I or II and cleavages were induced by addition of $NaDodSO_4$ followed by analysis of DNA products on a sequencing gel. The DNA sequence that was tested for cleavage contained a strong topoisomerase II cleavage site. The sequence, therefore, has an internal control to test the possibility that site-specific topoisomerase II cleavages might be stimulated by actinomycin D. The results (Fig. 4) show that addition of drug at 0.04 μg/ml did not stimulate topoisomerase I cleavage, whereas 0.5 and 2.5 μg/ml resulted in a 2- or 4-fold stimulation in cleavage, respectively. The topoisomerase I cleavage site TI_a (Fig. 4) that was barely detectable in the absence of drug (lane 2) was clearly stimulated by actinomycin D (lanes 4 and 5). Therefore, actinomycin D stimulated cleavages at the same sites, and it does not appear to alter sequence specificity of topoisomerase I. (The conclusion applies to this particular test sequence, which we presume to represent the general case.) In contrast, camptothecin, a potent inhibitor of topoisomerase I (25), stimulated cleavages at a number of different sites (lane 6), which did not match the cleavages seen in the absence of drug. Similar results with camptothecin were recently reported by Thomsen *et al.* (26). Based on the intensity of bands, camptothecin was more effective than actinomycin D in stimulating the cleavage reaction; however, camptothecin was tested at a significantly higher concentration than actinomycin D. The location of cleavage sites is shown in the sequence at the bottom of Fig. 4. Note that the topoisomerase I cleavage of 5′-end-labeled DNA yields a residual peptide coupled through the 3′-phosphoryl end. As a result, a mobility shift of up to 5 base pairs (bp) is encountered (27); therefore, the actual cleavage sites must be inferred.

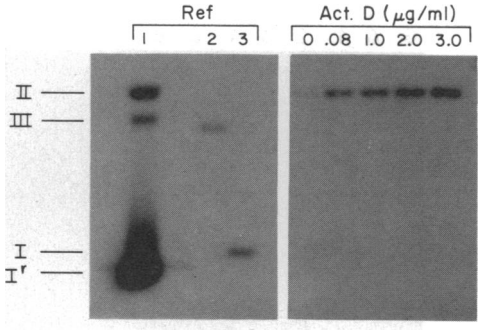

FIG. 3. Analysis of $KDodSO_4$-precipitated DNA from actinomycin D-treated reaction mixtures. The reactions described in the legend of Fig. 2 were carried out and the radioactive DNA in the $KDodSO_4$ precipitate was recovered and analyzed by gel electrophoresis in a 1% agarose gel (11 × 14 cm). Electrophoresis was performed at 2 V/cm for 16 hr in TAE buffer (40 mM Tris/25 mM Na acetate/1 mM EDTA, pH 8.3) containing 0.5 μg of ethidium bromide per ml. Lane 1 contains three DNA markers: form II, open circular DNA; form III, linear DNA; form I^r, relaxed DNA. Lane 2 contains a linear DNA marker and lane 3 contains a supercoiled (form I) DNA marker. The remaining reaction mixtures contained topoisomerase I and the amount of actinomycin D indicated above each lane.

Topoisomerase II cleavages in the test fragment were not stimulated by actinomycin D (Fig. 4, lanes 8–12). A strong topoisomerase II cleavage site (see sequence at bottom of Fig. 4) was clearly detected in the absence of drugs (lane 8) and cleavage at this site was stimulated by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (lane 9). Addition of either 0.5 or 2.5 μg of actinomycin D per ml did not stimulate topoisomerase II cleavages but suppressed the cleavage reaction at that sequence. Additional cleavage specificities on this particular 72-bp fragment were not revealed in the presence of actinomycin D.

Covalent Complex Formation in the Presence of Actinomycin D Is Reversible. The following experiment was carried out to determine whether actinomycin D treatment induces the formation of an irreversible covalent (nicked DNA) intermediate. We show in Fig. 1 that incubating nuclei at 4°C prior to adding the protein denaturant resulted in a >80% release of the topoisomerase from the DNA. At the suboptimal temperature, the enzyme seals the nicks and does not reinitiate subsequent rounds of breaking and rejoining. To determine whether actinomycin D is permanently freezing a nicked intermediate (as measured by increased formation of covalent complexes induced by $NaDodSO_4$), we incubated nuclei in the presence or absence of drug at 37°C and then shifted the temperature to 4°C prior to trapping the covalent complexes with the detergent. The formation of the covalent intermediate was then measured by CsCl gradients and immunoblotting followed by densitometry of the blot to ascertain the proportion of topoisomerase I that was covalently coupled to DNA (Table 1). Lowering the temperature resulted in the enzyme resealing the nicks both in the presence and absence of actinomycin D as attested by the finding that significantly less topoisomerase I is covalently bound to DNA at 4°C. Formation of covalent complexes was

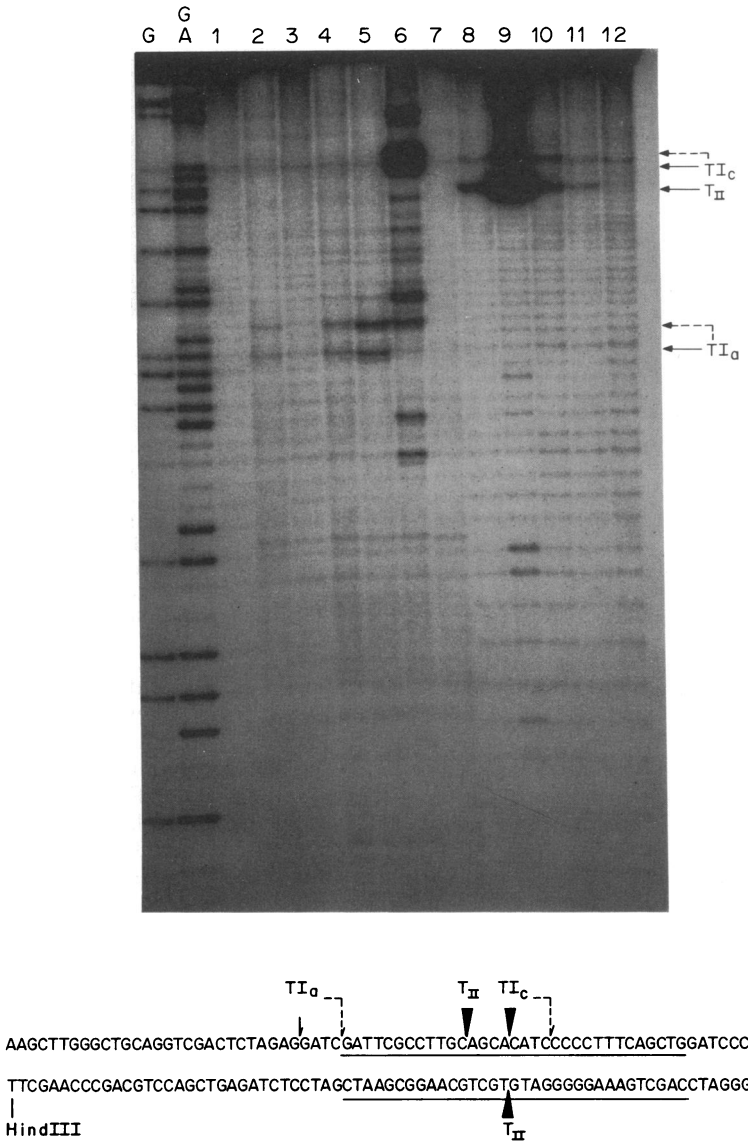

FIG. 4. Effect of actinomycin on sequence-specific cleavages of topoisomerase I and II. Topoisomerase I or II cleavage reactions were carried out as described in *Materials and Methods* and in the presence or absence of various inhibitors. The fragment (72 bp, containing a strong topoisomerase II site that was cloned into pUC12) was uniquely 5'-end-labeled at the *Hin*dIII site. The chemical sequence ladders are indicated (leftmost lanes) and the cleavage sites are marked to the right of the figure and on the DNA sequence displayed at the bottom. Cleavages marked on the sequence were derived from an analysis of the top strand cleavages. On the bottom strand, the topoisomerase II site was derived from a different experiment (not shown). The underscored sequence denotes the region synthesized as an oligonucleotide. TI_a corresponds to cleavages induced by topoisomerase I in the presence of actinomycin D, TI_c are topoisomerase I cleavages due to camptothecin, and TII marks the topoisomerase II cleavage site. Lane 1 shows the intact fragment. Topoisomerase I reactions correspond to lanes 2–7 (30 ng of topoisomerase I per reaction and 5 ng of DNA) as follows: lane 2, topoisomerase I alone; lane 3, 0.04 μg of actinomycin per ml; lane 4, 0.5 μg of actinomycin per ml; lane 5, 2.5 μg of actinomycin per ml; lane 6, 0.25 mg of camptothecin per ml (lactone form); lane 7, 20 μg of 4'-(9-acridinylamino)methanesulfon-*m*-anisidide per ml. Reaction mixtures in lanes 8–12 contained 50 ng of purified topoisomerase II and the following constituents: lane 8, topoisomerase II alone; lane 9, 20 μg of 4'-(9-acridinylamino)methanesulfon-*m*-anisidide per ml; lane 10, 0.04 μg of actinomycin per ml; lane 11, 0.5 μg of actinomycin per ml; lane 12, 2.5 μg of actinomycin per ml.

stimulated at least 2-fold by drug at 1 μg/ml; however, the lower concentration (0.08 μg/ml) was identical to the control without drug. A cross-linking efficiency of 5.9% was very close to the detection limit with the method (see Fig. 1, 4°C control) and, therefore, these values are not reliable.

Table 1. Effect of temperature shifts on actinomycin D stimulation of covalent complexes in nuclei

Experimental treatment	% topoisomerase I associated with DNA
No drug, 37°C for 15 min, followed by sarkosyl lysis	32
No drug, 37°C for 15 min, 4°C for 30 min, followed by sarkosyl lysis	5.9
Actinomycin (0.08 μg/ml), 37°C for 15 min, 4°C for 30 min, followed by sarkosyl lysis	5.9
Actinomycin (1.0 μg/ml), 37°C for 15 min, 4°C for 30 min, followed by sarkosyl lysis	10.3

Nuclei were prepared from exponentially growing MSB-1 cells and 1×10^8 nuclei were incubated as indicated prior to lysis with detergent. The lysates were layered on the step CsCl gradients and the distribution of topoisomerase I in the various fractions was analyzed by immunoblotting on a slot-blot device as described in *Materials and Methods*. The fraction of topoisomerase I coupled covalently to DNA was determined by densitometry of the immunoblot. The sum of intensities of antigen in fractions 6–9 (which correspond to the DNA peak, see Fig. 1) were divided by the total intensities of antigen in all fractions.

Actinomycin D Does Not Inhibit Strand-Passing Activity of Topoisomerase I. Actinomycin D intercalates and unwinds DNA (28, 29). The addition of actinomycin D to relaxed DNA results in the introduction of positive supercoils, and in the presence of purified topoisomerase I these positive supercoils are then relaxed. After removal of the drug, the DNA will become more negatively supercoiled if the topoisomerase has strand-passing activity in the presence of the drug. As shown in Fig. 5 (lanes 4–9), the topoisomerase I must be active in the presence of actinomycin D. The same results were obtained with topoisomerase II (data not shown).

DISCUSSION

Actinomycin D inhibits transcript elongation (not initiation) by RNA polymerase, and DNA intercalation could explain the basis for inhibition of transcription *in vivo* (29–31). Our results indicate that actinomycin D stimulates the formation of covalent (or nicked) topoisomerase I–DNA intermediates *in vivo* and *in vitro*. These findings suggest a mechanism by which actinomycin D may contribute to the observed inhi-

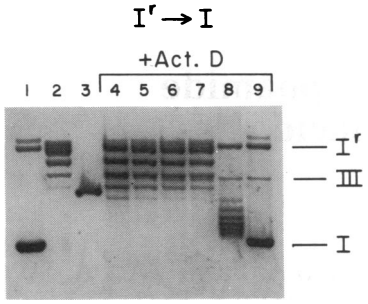

FIG. 5. Relaxation of plasmid DNA by topoisomerase I in the presence of actinomycin D. Reaction mixtures contained pXP1, which is a 5S RNA gene cloned into pSP64 (courtesy of D. Brown). Lanes: 1, supercoiled DNA; 2, relaxed DNA; 3, *Hin*dIII-digested DNA (controls). In lanes 4–9, supercoiled DNA was incubated with 1 unit of purified topoisomerase I (unit definition in ref. 16) for 60 min at 37°C followed by the addition of 0, 0.025, 0.05, 0.1, 1, or 5 μg of actinomycin D per ml, respectively. Reactions were continued for an additional 60 min. A small amount of contaminating nuclease from bovine serum albumin produced linear DNA during the first 60 min of incubation. Relaxed (I^r), supercoiled (I), and linear DNA positions (III) are marked.

bition of transcription by the drug and the following arguments support this possibility.

First, rRNA genes are extremely sensitive to relatively low concentrations of actinomycin D, whereas there is little effect on the transcription by RNA polymerase II or III (23, 24). Topoisomerase I is heavily concentrated in the nucleolus and catalytically active on rRNA genes (2); thus, high sensitivity to actinomycin D may reflect the requirement for topoisomerase in transcription of rRNA genes (7). Second, the sensitivity of transcription to actinomycin D *in vivo* is not observed *in vitro*: Concentrations of the drug required to arrest transcription *in vitro* are considerably higher than *in vivo* concentrations (32). Evidently, actinomycin D blocks *in vitro* transcription because of the unfavorable interaction between the drug–DNA complex and RNA polymerase (33). Studies in this laboratory (unpublished data) and others (34) have indicated that topoisomerases are not required for *in vitro* transcription utilizing RNA polymerase II or III. In contrast, topoisomerase I is essential for *in vitro* transcription of supercoiled rRNA plasmids (35). Third, addition of actinomycin D to cells results in the production of DNA breaks (36) with attached proteins (37). We have used monospecific antibodies to demonstrate *in situ* that topoisomerase I is a logical candidate for a protein that is coupled to DNA at the site of these breaks. In addition, actinomycin D alone does not cause strand breakage *in vitro*, demonstrating that intercalation by itself is not sufficient to cause this effect (38).

The *in vitro* cleavages with purified topoisomerase I and end-labeled DNA fragments directly show that actinomycin D stimulates topoisomerase I cleavages at the same sites observed in the absence of drug. Camptothecin yielded cleavages at many different sites and stimulated cleavage to a much greater extent. Topoisomerase II-induced cleavages in a 72-bp test sequence were not stimulated by actinomycin D; however, with a plasmid DNA substrate of 5 kilobases, we find that topoisomerase II cleavages are stimulated (although weakly) by actinomycin D at sites that are different from those sites stimulated by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (data not shown). Our data indicate that actinomycin D stabilizes a covalent intermediate that is detected after denaturing the active topoisomerase. The mechanism is not clear but stabilization could be due to either accelerated cleavage of DNA or impaired ability of the topoisomerase to reseal transient breaks. While actinomycin D treatment of nuclei extends the half-life of the covalent complex, it appears that topoisomerase I can still carry out the resealing step in the presence of drug; thus, actinomycin does not freeze the topoisomerase–DNA intermediate into a permanent and irreversible complex. As shown in Table 1, lowering the temperature in the presence of the drug significantly decreased the number of covalent complexes. Therefore, the enzyme must have resealed the existing nicks but failed to reenter the nicking and closing cycle. We also note that at 4°C, purified topoisomerase I will not initiate new rounds of cleavage. These findings suggest that at the suboptimal temperature, the resealing activity of the enzyme was slightly impaired by actinomycin at 1 μg/ml. The data suggest, but do not prove, that actinomycin D impairs the ability of topoisomerase I to reseal the single-stranded breaks in the process of strand passing.

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