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Vaccinia Virus Encapsidates a Novel Topoisomerase with the Properties of a Eucaryotic Type I Enzyme*

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Rose Shaffer‡ and Paula Traktman‡§¶

From the Departments of ‡Microbiology and §Cell Biology and Anatomy, Cornell University Medical College, New York, New York 10021

A DNA topoisomerase has been purified from vaccinia virus cores. The native enzyme is composed of a single subunit of 32,000 daltons. The enzyme relaxes both positively and negatively supercoiled DNA in the absence of an energy cofactor. Enzymatic activity is stimulated by magnesium ions and inhibited by ATP, and during relaxation the topoisomerase changes the linking number of the DNA substrate by steps of one. Trapping of the covalent DNA-enzyme intermediate has been achieved, and analysis of the cleavage of duplex DNA by the enzyme reveals that it makes a single-strand break and forms a covalent bond through the 3′-end of the broken strand. Enzymatic activity and formation of the trapped intermediate are inhibited by the drugs novobiocin, coumermycin A1, and berenil. The virally encapsidated enzyme has novel properties but most closely resembles a eucaryotic type I topoisomerase.

Few biological processes are as basic as the accurate replication of DNA and the regulation of its topology. The emerging picture of the enzymatic machinery required to replicate duplex DNA and to relieve the torsional stress accompanying replication and transcription is increasingly complex. The greatest strides in unraveling this process have come from procaryotic systems amenable to biochemical and genetic analysis. Our understanding of eucaryotic DNA metabolism has been hampered by the lack of such fruitful systems. In this context, our laboratory is developing vaccinia virus as a model system for the analysis of DNA replication and the regulation of DNA topology. Vaccinia, the prototypic member of the poxvirus family, replicates lytically in the cytoplasm of infected cells. Many viral enzymes, most notably those required for early viral transcription, are encapsidated within the virion. The 185-kilobase DNA genome appears to encode virtually all of the functions required for viral replication and transcription. The cytoplasmic localization of the virus and its unique genetic autonomy make this an excellent system in which to combine biochemical and genetic analysis. Relative to that of eucaryotes, the genome is simple and highly amenable to molecular biological analysis.

Several vaccinia enzymes with a role in DNA metabolism have been described; the thymidine kinase gene and enzyme (1, 2) and the DNA polymerase gene and enzyme (3–6) have been purified and extensively characterized. In addition, the existence of a viral ribonucleotide reductase has recently been demonstrated (7, 8), and reports of a novel DNA ligase activity have appeared. Several unique nucleases have been described as well as a putative viral histone-like protein and various DNA-binding proteins (reviewed in Ref. 9). Enzymes with activities limited to DNA replication are most typically synthesized early after infection; proteins with likely roles in transcription and replication have been isolated from virions. A DNA-dependent ATPase (10) and a topoisomerase (11, 12) have been described.

In this report, we describe further studies on the viral topoisomerase. We have partially purified this enzyme from virions and characterized its structure, enzymatic requirements, and mechanism of action. It is distinct from other known topoisomerases but most resembles a eucaryotic type I topoisomerase.

MATERIALS AND METHODS AND RESULTS[1]

Purification—The purification of a topoisomerase activity from vaccinia virions has been previously reported (11, 12). We have also purified this enzyme and have extended the characterization of its structure and mechanism of action. The enzyme purification and optimization of the assay are described in the Miniprint Supplement at the end of the paper.

Visualization of the Topoisomerase by Label Transfer—Type I topoisomerases are known to act by transiently breaking one strand of DNA and resealing the nick after changing the linking number of the supercoiled template by one (22). One of the hallmarks of this reaction is the formation of a covalent intermediate between the enzyme and the broken strand of the DNA (26). Identification of the polypeptide which can form this bond has been achieved by incubation of the enzyme with uniformly radiolabeled DNA and subsequent trapping of the intermediate (27). When preparations of vaccinia cores (Fig. 4, *lane 2*) or purified vaccinia topoisomerase (Fig. 4, *lane 1*) were incubated with radiolabeled DNA, treated with DNase I to remove excess DNA, and subsequently fractionated by SDS-PAGE,[2] a single polypeptide-DNA complex migrating as

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¶ To whom all correspondence should be addressed.

[1] Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1–3, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-4480, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; Me_2SO, dimethyl sulfoxide.

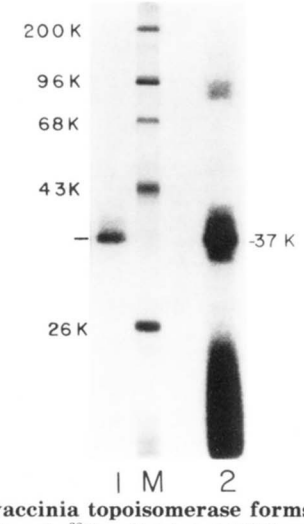

FIG. 4. **The vaccinia topoisomerase forms a covalent bond with DNA.** Uniformly ^{32}P-radiolabeled DNA was prepared by nick translation and used as the substrate in a label-transfer assay with the vaccinia topoisomerase as described under "Materials and Methods." Following reaction of the enzyme with the DNA and removal of the excess DNA by nuclease digestion, the samples were analyzed by SDS-PAGE and autoradiography. *Lane 1*, radiolabeled products of reaction containing 21 units of pool topoisomerase; *lane 2*, radiolabeled products of reaction containing that amount of vaccinia cores which contains 3 units of topoisomerase activity when assayed under standard conditions; *lane M*, molecular weight markers.

a 37-kDa species was seen after autoradiography. The intensity of this radiolabeled species corresponded to the level of topoisomerase activity seen in a given fraction (data not shown). Thus, we have identified a 37,000-dalton species as one or the only component of the vaccinia topoisomerase. The yield of enzyme-DNA intermediate is significantly increased by incubating the enzyme under suboptimal conditions (low salt). $MgCl_2$, which we have previously shown to increase the rate of the relaxation reaction, inhibits accumulation of the intermediate. ATP, which we have shown to inhibit enzyme activity, also interferes with the isolation of the intermediate (data not shown). We thus believe that ATP inhibition affects an early step in the enzymatic reaction which precedes the covalent bonding to, and breakage of, the DNA template.

Native Molecular Weight—As described above, we have detected a 37,000 M_r species in our topoisomerase preparation which makes a covalent bond with DNA under the conditions of the topoisomerase assay. We wished to determine whether the native enzyme contained additional polypeptide species and whether it was monomeric or multimeric in structure. The native molecular weight of the enzyme was determined using two experimental approaches, glycerol gradient sedimentation and gel filtration. The topoisomerase pool was sedimented through a 5–20% glycerol gradient along with native ^{14}C-labeled protein standards. The topoisomerase activity sedimented at 2.9 S which, for a globular protein, can be calculated to correspond to a molecular weight of 31,000 (Fig. 5, *panel A*). This corresponds well to the species detected in the label transfer studies, making an allowance for the DNA fragment bound to the labeled protein-DNA intermediate. The enzyme was also analyzed by gel filtration (Fig. 5, *panel B*). Comparison of the elution profile of topoisomerase activity to that of native molecular weight standards led to the calculation of a Stokes radius of 27.5 Å and a molecular weight of approximately 33,000 for the topoisomerase. The gel filtration and glycerol gradient data are consistent in revealing a native molecular weight of 31,000–33,000 for the enzyme. The peak fraction from the Superose column was fractionated on an SDS-PAGE gel, which was then silver-stained, and appeared to contain predominantly a single polypeptide which co-migrated with a 30,000 M_r standard (Fig. 2*B*). Thus, the label-transfer experiment, the sedimentation analysis, and the gel filtration data, in sum, indicate that the enzyme is monomeric with a molecular weight of approximately 32,000.

Mechanism of DNA Cleavage—An intermediate step in topoisomerization by type I enzymes is the transient cleavage of one strand of the duplex. Procaryotic and eucaryotic type I enzymes have been shown to differ in their cleavage mechanism. Procaryotic enzymes cleave DNA and form a covalent linkage through the 5′-phosphate of the broken strand (28), whereas eucaryotic enzymes cleave DNA and form a bond through the 3′-phosphate of the broken strand (26). To investigate the mechanism used by the vaccinia topoisomerase, the enzyme was incubated with linear duplex DNA fragments radiolabeled at a single 5′ or 3′ terminus. After incubation, the mix was fractionated by electrophoresis on a denaturing agarose gel and examined by autoradiography. *Lanes 1–4* of Fig. 6 display the products after incubation of the enzyme with DNA radiolabeled at a 3′ terminus. The DNA probe is shown in *lane 1*; in *lane 2*, the predominant 3′-radiolabeled cleavage product seen after incubation of the DNA with vaccinia topoisomerase is clearly visible. To determine whether the radiolabeled cleavage fragment contained bound topoisomerase, a portion of the reaction shown in *lane 2* was treated with proteinase K prior to electrophoresis. Proteinase K treatment of the DNA fragment alone causes no alteration in mobility (data not shown). As shown in *lane 3*, the mobility of the cleavage product was not altered by protease treatment. Thus, topoisomerase was not bound to the free 5′-end of the broken DNA. In *lanes 5–8*, the products of incubating the enzyme with DNA radiolabeled at a single 5′ terminus are displayed. *Lane 5* represents the DNA fragment alone, and in *lane 6* the two predominant 5′-radiolabeled cleavage products seen after incubation with topoisomerase are indicated. A portion of the reaction shown in *lane 6* was treated with proteinase K prior to electrophoresis, and as shown in *lane 7*, the migration of the cleavage products was altered significantly. Thus, the enzyme was bound to the free 3′-end of the broken strand. Both experiments indicate that vaccinia topoisomerase cleaves DNA and forms a bond through the 3′-end of the broken strand, in a manner analogous to eucaryotic topoisomerases.

Additional experiments were performed to probe the mechanism of DNA cleavage mediated by the vaccinia topoisomerase. To date, topoisomerase-DNA cleavage intermediates have been seen only after exposure of the enzyme-DNA reaction to the protein denaturants SDS or alkali (22, 29). Because our identification of the protein intermediate relied on SDS-PAGE (Fig. 4), and visualization of the DNA cleavage products was accomplished on alkaline agarose gels (Fig. 6, *lanes 1–3, 5–7*), we wished to test whether we could indeed detect the enzyme-DNA intermediate in the absence of denaturants. In the reactions shown in Fig. 6, *lanes 2* and *6*, DNA cleavage could have occurred during the reaction itself or might have been induced by the addition of alkali. In *lane 7*, the increased migration of the cleavage product reflects the removal of the topoisomerase from the DNA by proteinase K prior to the addition of alkali in preparation for electrophoresis. Therefore, the DNA cleavage must have occurred during the reaction with topoisomerase or upon the addition of proteinase K/SDS. To distinguish between these possibilities, the enzyme-DNA complex was disrupted without denaturants

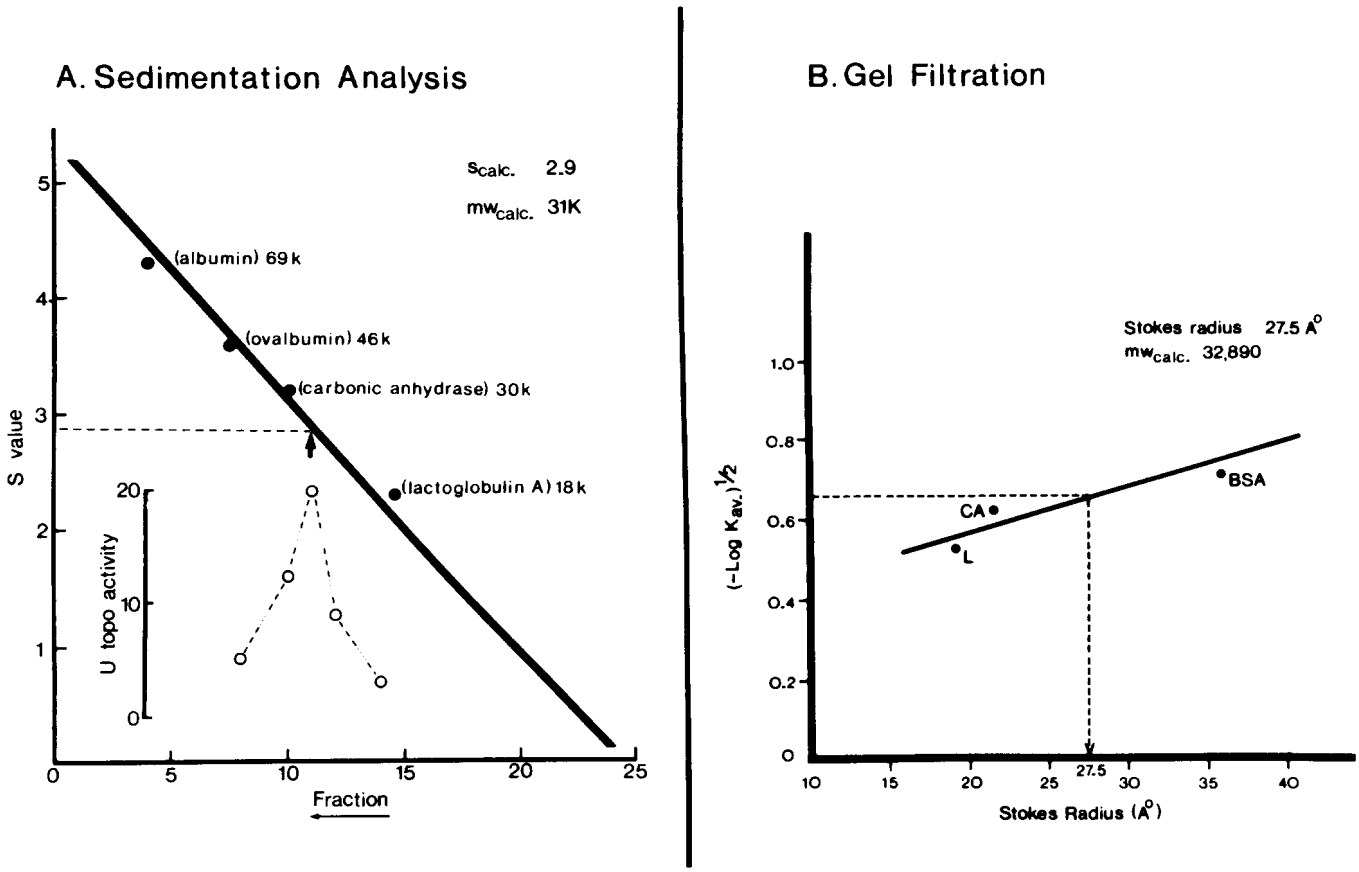

FIG. 5. **Glycerol gradient sedimentation and gel filtration analyses of the vaccinia virus topoisomerase.** *A*, the sedimentation coefficient of the vaccinia topoisomerase was determined by sedimenting the enzyme through a 5–20% glycerol gradient with four proteins of known S values (bovine serum albumin, 4.3 S; ovalbumin, 3.6 S; carbonic anhydrase, 3.2 S; and β-lactoglobulin A, 3.0 S) as described under "Materials and Methods." Using the experimentally obtained sedimentation coefficient and the known molecular weights of the internal protein markers, a crude estimate of the molecular weight of the topoisomerase was determined using the method of Martin and Ames (20). *B*, to determine the Stokes radius of the vaccinia topoisomerase, the protein was analyzed by gel filtration chromatography through a Superose 12 resin on a fast protein liquid chromatography apparatus as described under "Materials and Methods." The column was calibrated with three proteins of known Stokes radii (bovine serum albumin, 35.5 Å; carbonic anhydrase, 21.4 Å; lysozyme, 19.1 Å), and the results were analyzed using the methods of Siegel and Monty (21).

prior to electrophoresis. In *lanes 4* and *8*, DNA was reacted with topoisomerase, and then the reaction was made 0.5 M with respect to NaCl to disrupt noncovalent enzyme-DNA interactions. Clearly, no DNA cleavage products are seen, indicating that the cleavage intermediate can only be visualized if the DNA-enzyme complex is treated with a denaturant such as alkali or SDS.

A further experiment was performed to test whether the denaturant-induced cleavage intermediate is formed by a single-strand or double-strand break. Type I and type II topoisomerases are in part defined by their utilization of single-strand and double-strand cleavage mechanisms, respectively (22). In *panel C* of Fig. 6, the interaction of the topoisomerase and the 5′-labeled substrate is analyzed on nondenaturing gels. *Lane 9* represents the DNA fragment alone; the reaction of DNA and topoisomerase is shown in *lane 10*. No cleavage is seen, a finding consistent with the requirement for a denaturant illustrated in *panels A* and *B* and discussed above. The sample shown in *lane 11* was treated with proteinase K/SDS following incubation with enzyme. If the SDS-induced cleavage was a double-stranded break, the cleavage fragments should be apparent after nondenaturing electrophoresis. However, if the break was a single-stranded nick, then hybridization of the broken strand to the intact strand should maintain the integrity of the DNA duplex, and no cleavage fragments should be seen after nondenaturing electrophoresis. No cleavage fragments are seen in *lane 11*, consistent with the topoisomerase causing a single-strand break. To confirm that cleavage fragments could be seen on nondenaturing gels, two additional controls were performed and are shown in *lanes 12* and *13*. DNA was treated with topoisomerase and was then directly (*lane 12*) or after treatment with proteinase K/SDS (*lane 13*) denatured with NaOH, neutralized with HCl, and examined by nondenaturing electrophoresis. Cleavage fragments with (*lane 12*) and without (*lane 13*) bound protein are clearly visible. Thus, the data indicate that the vaccinia topoisomerase causes single-strand breaks and that the protein-DNA cleavage intermediate must be trapped by a denaturant in order to be visualized. Although visualization requires trapping, it is generally acknowledged that normal relaxation of DNA by type I topoisomerases proceeds through a transient breakage intermediate (22).

Topoisomerase-mediated Change in Linking Number—The experiments shown in Fig. 6 indicate a single-strand breakage mechanism for the vaccinia topoisomerase, a hallmark of a type I enzyme. Another consistent feature of type I enzymes is that the linking number of the substrate changes in steps of one during relaxation. Type II enzymes, which cause dou-

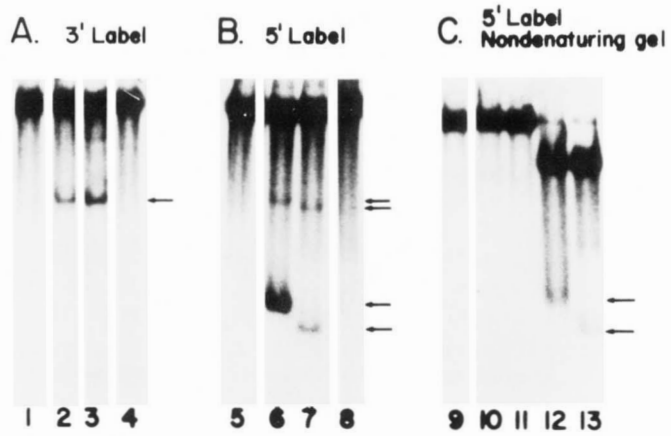

FIG. 6. **Cleavage of duplex DNA by the vaccinia topoisomerase involves a single-strand break, and visualization of the enzyme-DNA intermediate requires trapping by a denaturant.** Cleavage reactions were carried out and analyzed as described under "Materials and Methods." Each reaction contained DNA labeled at a single 3′ (*panel A*) or 5′ (*panels B* and *C*) terminus, with or without vaccinia pool topoisomerase. *Panels A* and *B*, analysis on denaturing gels. *Lanes 1* and *5*, DNA only; *lanes 2* and *6*, DNA with topoisomerase; *lanes 3* and *7*, DNA with topoisomerase, proteinase K digestion; *lanes 4* and *8*, DNA with topoisomerase, addition of 0.5 M NaCl. *Panel C*, analysis on nondenaturing gels. *Lane 9*, DNA only; *lane 10*, DNA with topoisomerase; *lane 11*, DNA with topoisomerase, proteinase K digestion; *lane 12*, DNA with topoisomerase, denatured/neutralized; *lane 13*, DNA with topoisomerase, proteinase K digestion, denatured/neutralized.

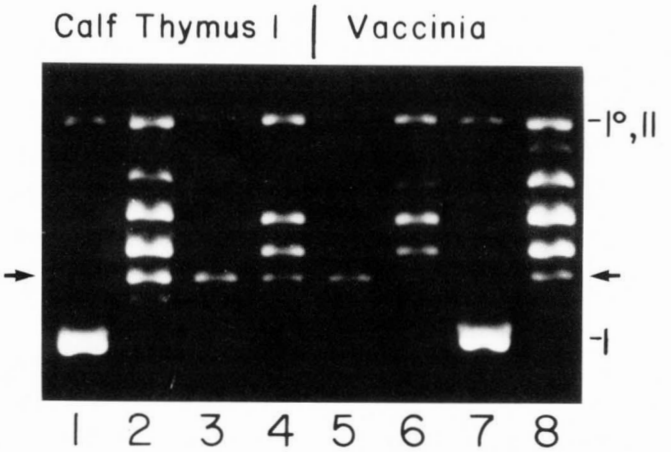

FIG. 7. **Vaccinia virus topoisomerase changes the linking number of a purified topoisomer in steps of one.** A purified topoisomer of pSP65 plasmid DNA was prepared and then partially relaxed with calf thymus topoisomerase I or with vaccinia topoisomerase as described under "Materials and Methods." The samples were then fractionated on Tris borate-EDTA-agarose gels and the DNA visualized with UV light after ethidium bromide staining. *Lane 1*, pSP65 DNA; *lane 2*, pSP65 partially relaxed with calf thymus topoisomerase I; *lane 3*, purified pSP65 isomer; *lane 4*, purified isomer partially relaxed with calf thymus topoisomerase I; *lane 5*, purified pSP65 isomer; *lane 6*, purified isomer partially relaxed with vaccinia topoisomerase; *lane 7*, pSP65 DNA; *lane 8*, pSP65 DNA partially relaxed with vaccinia topoisomerase.

ble-stranded breaks, change the linking number of the substrate in steps of two (30). Partial relaxation of supercoiled plasmid DNA with calf thymus topoisomerase I yielded a population of topoisomers whose linking number differs by one, as shown in Fig. 7, *lane 2*. From this ladder, a single isomer was purified (shown in *lanes 3* and *5*) and partially relaxed by calf thymus topoisomerase I (*lane 4*) and the vaccinia topoisomerase (*lane 6*). In each case, relaxation of the purified topoisomer regenerated the population of isomers shown in *lane 2*, each differing in linking number by steps of one. These data confirm the identification of the vaccinia topoisomerase as a type I enzyme.

TABLE II
Drug inhibition

Drug	Concentration	Inhibition[a]
		%
Novobiocin	8 μM	11
	40 μM	39
	200 μM	81
	1 mM	100
Coumermycin A1	8 μM	22
	40 μM	87
	200 μM	100
Berenil	800 nM	25
	4 μM	65
	20 μM	92
	100 μM	100
Nalidixic acid	8 μM	4
	40 μM	8
	200 μM	12
	1 mM	35
	5 mM	87

[a] Sufficient "pool" topoisomerase was incubated with 200 ng of plasmid DNA to achieve 90% relaxation under standard conditions. Drugs were included as indicated, and samples were analyzed by gel electrophoresis and densitometry. Percent inhibition of DNA relaxation relative to a control reaction was determined; the values shown represent the average of two independent experiments.

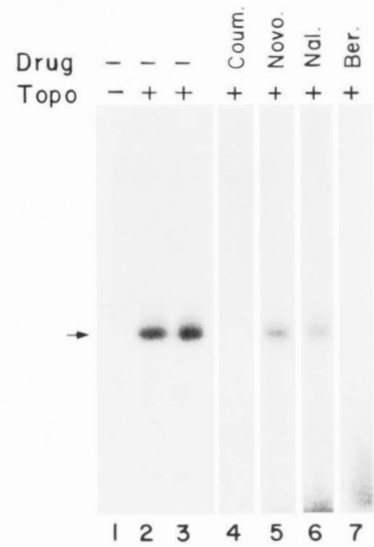

FIG. 8. **Novobiocin, coumermycin A1, berenil, and nalidixic acid inhibit the formation of a covalent intermediate between the vaccinia topoisomerase and DNA.** 40 units of vaccinia pool topoisomerase activity were reacted with uniformly ^{32}P-labeled DNA as described under "Materials and Methods" for the label transfer assay. Inhibitors of the relaxation reaction were included in the reaction at the concentrations indicated below; after digestion with nuclease to remove excess DNA, the samples were fractionated on SDS-PAGE and analyzed by autoradiography. *Lane 1*, no enzyme; *lane 2*, 40 units of enzyme; *lanes 3–7*, 40 units of enzyme with the following additions: *3*, dimethyl sulfoxide to 14% (v/v); *4*, 200 μM coumermycin A1 and dimethyl sulfoxide to 14%; *5*, 200 μM novobiocin; *6*, 5 mM nalidixic acid; *7*, 500 μM berenil.

Drug Inhibition—The effect of various topoisomerase inhibitors on the vaccinia enzyme was monitored in two ways: inhibition of conversion of supercoiled DNA to relaxed circles and the effect of the drugs on the formation of the protein-DNA intermediate. Novobiocin, coumermycin A1, berenil, and nalidixic acid were tested. The effect of the drugs on the relaxation reactions is summarized in Table II. Coumermycin

A1 was an extremely effective inhibitor, whereas novobiocin, a related compound, was less potent. These data confirm the previously reported inhibition of the vaccinia enzyme by these two compounds (12). The most potent inhibitor was berenil, a known inhibitor of trypanosome replication and of mammalian mitochondrial topoisomerase (31). Nalidixic acid induced inhibition only at much higher concentrations. The effect of the drugs on the label-transfer assay (Fig. 8) closely paralleled their effect on the enzymatic reaction, suggesting that these drugs may act at an early step in the enzyme-DNA interaction. The inhibition of the vaccinia enzyme by these drugs is especially interesting in light of the previous classification of novobiocin and coumermycin as type II topoisomerase inhibitors (30).

DISCUSSION

The encapsidation of a topoisomerase in vaccinia virions has been reported previously (11, 12). We have extended these observations by purifying the topoisomerase extensively and characterizing its physical structure and its mechanism of action. Our analysis of the native enzyme by gel filtration and glycerol gradient sedimentation indicated that the enzyme had an approximate molecular weight of 32,000. Characterization of the trapped intermediate formed by the covalent linkage of the enzyme to radiolabeled DNA identified a single polypeptide-DNA species of approximately 37 kDa. These data, in sum, indicate that the enzyme is a 32-kDa monomeric protein. This is unusually small for a topoisomerase, as most of those previously characterized have molecular weights in the range of 100,000. Nevertheless, we have absolutely no evidence of proteolytic degradation, and the 32-kDa species is the only topoisomerase-related species found in our initial and purified preparations. It should be stressed that in keeping with vaccinia virus' extreme stability, the topoisomerase and other enzymes we have purified from virions have shown no degradation or diminution of activity after months of storage at 4 °C. The enzymatic activity, as defined by the relaxation of negatively supercoiled DNA to covalently closed relaxed circles, has a salt optimum of 125 mM NaCl and is stimulated by magnesium ions. Magnesium appears to increase the rate of the relaxation reaction; in keeping with this finding, the presence of magnesium inhibits the accumulation of the trapped enzyme-DNA intermediate. The enzyme does not require an energy cofactor, and indeed ATP inhibits the enzyme, slowing the rate of DNA relaxation. ATP also inhibits the formation of the enzyme-DNA intermediate, and we believe that it may destabilize protein-DNA interactions. To address the point that previous authors found a stimulation of the vaccinia enzyme by ATP, we cite the caution of Wang (22) in interpreting the effect of ATP on topoisomerases not purified to homogeneity. ATP might exert an indirect effect on the enzyme via interaction with kinases whose action might modify topoisomerases (32, 33) or by chelation of ions within the reaction buffers. Our data indicate inhibition of enzymatic activity and enzyme-DNA intermediate formation by the drugs berenil, coumermycin A1, and novobiocin. It is extremely interesting that the latter two drugs, classified as exemplary inhibitors of type II enzymes, inhibit the vaccinia topoisomerase. We are interested in defining the mode of inhibition of the enzyme by these drugs, in light of previous data suggesting that their interaction with type II enzymes involves the ATP-binding site (34, 35). We have confirmed the previous finding (11) that the enzyme relaxes both positively and negatively supercoiled DNA (data not shown) and have further shown that during relaxation of a negatively supercoiled plasmid the linking number of the DNA is changed in steps of one. The cleavage of duplex DNA by the enzyme has been investigated, and we have determined that the enzyme makes single-stranded breaks and forms a covalent bond through the 3′-end of the broken strand. Trapping of the cleaved intermediate requires the use of a protein denaturant such as SDS or alkali. By all of these criteria, the vaccinia enzyme most closely resembles a eucaryotic type I enzyme. The finding that the viral enzyme mechanistically resembles eucaryotic topoisomerases makes vaccinia an even more valuable model system for the analysis of the role of the topoisomerase in transcription and replication. Our purification and characterization of the enzyme open the way for our identification of the gene encoding the enzyme, a goal currently being pursued in our laboratory. Detection of the enzyme in extracts of vaccinia-infected cells, but not of uninfected cells, has been achieved by the incubation of infected cell extracts with radiolabeled DNA to form the trapped covalent intermediate (data not shown). Our observation that the enzyme level increases early after infection (data not shown) is in agreement with the results of others (36). Rigorous proof that the enzyme is indeed virally encoded awaits the identification of the relevant gene or the isolation of a viral mutant with an altered topoisomerase. The biology of the virus, however, strongly predicts that this will be the case. Having characterized the structure of the viral topoisomerase, our goal is to proceed to identify the gene encoding the enzyme and to perform a genetic analysis of its role in viral transcription, recombination, and replication.

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Supplement to:
"Vaccinia Virus Encapsidates a Novel Topoisomerase with the Properties of a Eucaryotic Type I Topoisomerase", Rose Shaffer and Paula Traktman

Materials and Methods

Virus and Cells. Wild-type vaccinia virus of the WR strain was obtained from Bryan Roberts. Mouse L cells grown as monolayer or suspension cultures were obtained from Richard Condit and Joseph Kates, respectively. Monolayers were grown in DME supplemented with 5% fetal calf serum; suspension cultures were grown in Joklik-modified MEM supplemented with 2.5% calf serum, 2.5% horse serum. For the preparation of viral stocks, 3 liter cultures at 5 x 10^5 cells/ml were infected at a multiplicity of infection of 1 and harvested at 48 hours post infection. Cells were collected by centrifugation and broken by Dounce homogenization. Nuclei were removed by centrifugation, and virus was purified from cytoplasm by sucrose gradient sedimentation as previously described (13). Yield was determined by absorbance at 260 nm; the calculation used to quantitate virus was 1 A_{260} unit unit = 48 µg of virions (14). [^{35}S]-methionine labeled virions were prepared by infecting cells in 70% methionine-free Joklik's MEM, 25% Joklik's MEM, 2.5% dialyzed calf serum and 2.5% dialyzed horse serum. Radiolabelled methionine was used at 1.5 mCi/l. The specific activity of the final virus preparation used for topoisomerase purification was 1.6 x 10^7 cpm per mg of viral protein.

Topoisomerase Purification. To prepare viral cores, 30 mg of virus was incubated at 37°C for 30 min in the presence of 50 mM Tris pH 8.3, 50 mM DTT and 0.5% Nonidet-P40. Cores were collected by centrifugation, and then incubated on ice for 45 min in the presence of 300 mM Tris pH 8.5, 250 mM NaCl, 100 mM DTT, 0.1% sodium deoxycholate to release a soluble enzyme fraction. After sonication to shear the viral DNA, the solubilized fraction of the cores was freed of insoluble structural components by ultracentrifugation. The soluble enzymes were adjusted to contain 10% glycerol, 0.1% Triton X-100 and 1 mM EDTA and then applied to a 1 ml DEAE-cellulose column which had been equilibrated with buffer A (250 mM Tris pH 8.5, 200 mM NaCl, 1 mM EDTA, 0.1% Triton X100, 2mM DTT, 10% glycerol). The column was washed with buffer A, and 0.5 ml fractions were collected and assayed for protein by monitoring ^{35}S-cpm in the presence of Biofluor in a Packard scintillation counter. The pooled protein-containing fractions were loaded directly on to a 5 ml hydroxylapatite column which had been equilibrated in Buffer A. The column was washed with 10 column volumes of Buffer A and then 10 column volumes of Buffer B (200 mM NaCl, 0.1% Triton X100, 2 mM DTT, 1 mM EDTA, 10% glycerol) containing 180 mM potassium phosphate, pH 7.4. A 50 ml linear gradient of potassium phosphate (180 mM to 800 mM) in Buffer B was then applied. The column was run at 12 ml/hr and 2.5 ml fractions were collected. The ionic concentrations of the fractions was determined using a conductivity monitor, and topoisomerase activity was assayed as described below. All manipulations were performed in the cold, and fractions were stored on ice in the cold room or at -80°C.

Preparation and Radiolabeling of Plasmid DNA. pBR 322, pUC 8 and pSP65 plasmids were prepared by the alkaline lysis procedure (15). Restriction digestions were performed according to the manufacturer's instructions. Linearized DNA was radiolabeled at the 5' end with γ-[^{32}P]-ATP and polynucleotide kinase after dephosphorylation with calf intestinal alkaline phosphatase. DNA was radiolabeled at the 3' end with α-[^{32}P]-dNTP's and the Klenow fragment of DNA polymerase I. DNA was nick-translated according to the method of Rigby et al. (16). DNA fragments were separated by TAE-agarose electrophoresis (40 mM Tris, 20 mM acetate, 1 mM EDTA) and purified by binding to glass powder (17).

Topoisomerase Assay. The standard assay reaction for topoisomerase activity contained 200 ng of pUC 8 plasmid DNA in 40mM potassium phosphate pH 7.4, 150 mM NaCl, 2.3% glycerol, 0.02% Triton X-100, 0.2 mM EDTA and 0.4 mM DTT and various sources of topoisomerase as indicated in the text. Reactions were allowed to proceed for 30 min at 37°C before being stopped by the addition of SDS to a final concentration of 0.2% and adjusted to 1 X TBE (50 mM Tris base, 50 mM Boric acid, 1 mM EDTA). Samples were electrophoresed on horizontal 1% agarose gels in 1X TBE buffer at 4 volts/cm for 3 hours. After staining in 1 µg/ml of EtBr for 15 minutes and destaining in water for 30 min, gels were photographed under short-wave UV illumination with Polaroid 665 film. Negatives were scanned with a Quick Scan densitometer (Helena laboratories). To quantitate enzyme activity, the percent of supercoiled DNA converted to relaxed circular DNA was determined by cutting out and weighing the supercoil peaks from densitometric scans of reactions performed with a series of enzyme dilutions. One unit of topoisomerase activity is defined as the amount of enzyme which converts 50% of the supercoils in 0.2 µg of pUC 8 DNA to the relaxed circular form in 30 min at 37°C. (90% of the DNA in the pUC 8 preparations was found to be in the supercoiled form.) To confirm that the supercoils were converted to the relaxed circular form and not to co-migrating nicked circles, duplicate samples were sometimes electrophoresed on 1% agarose gels cast and run in the presence of 0.5 µg/ml EtBr. Under these conditions, nicked circular DNA migrates in the same position as in standard gels, whereas the relaxed circular DNA migrates with supercoiled DNA.

Label-transfer Assay. The standard reaction mixture for detection of the covalent topoisomerase/DNA intermediate contained 40 mM potassium phosphate pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 0.4 mM DTT, 2% glycerol, 0.02% Triton-X100, 63 ng of ^{32}P-nick translated pUC 8 DNA and 40 units of vaccinia topoisomerase. After 8 min of incubation at 37°C, $MgCl_2$ and DNase I were added to 5 mM and 100 µg/ml, respectively and the sample was returned to 37°C for an additional 10 min. SDS-PAGE sample buffer was added (final concentrations of 1% SDS, 50 mM Tris pH 6.8, 1% β-mercaptoethanol, 6% glycerol), the samples were heated at 90°C for 5 min and then electrophoresed on 12% SDS polyacrylamide gels (18). Gels were fixed, dried and visualized by autoradiography.

DNA-Cleavage Assay. To prepare DNA fragments labeled on one strand at a single terminus, pBR 322 was digested with Hind III. Half of the digested DNA was dephosphorylated with calf intestinal alkaline phosphatase and then phosphorylated with Polynucleotide kinase in the presence of γ-[^{32}P]-ATP. The other half of the digested DNA was labeled at the 3' terminus with the Klenow fragment of E. coli Polymerase I in the presence of α-[^{32}P]-dATP. Both samples were then digested with Bam HI and fractionated by electrophoresis on 1% horizontal agarose gels in TAE buffer. The 4 kb Hind III-Bam HI fragment was visualized by UV illumination of the gel following EtBr staining and then purified by binding to glass powder. Each DNA cleavage reaction contained 25 ng of end-labeled DNA and 22 units of vaccinia topoisomerase in a final reaction which contained 14 mM potassium phosphate pH 7.4, 16 mM NaCl, 0.8 % glycerol, 0.008% Triton X-100, 0.08 mM EDTA and 0.16 mM DTT. After 30 min of incubation at 37°C, appropriate samples received proteinase K to 100 µg/ml and were incubated for an additional 10 min at 37°C as indicated in the text. Those reactions to be examined by alkaline agarose electrophoresis received alkaline sample buffer (final concentrations of 2 mM EDTA, 30 mM NaOH) and were then fractionated by electrophoresis through 1.2% vertical alkaline agarose gels. The gels were cast and run in 2 mM EDTA, 30 mM NaOH at 5 volts/cm for 4 hours. Gels were neutralized, dried, and examined by autoradiography. Reactions to be analyzed by nondenaturing agarose electrophoresis either directly received TAE sample buffer or were first denatured in 250 mM NaOH and then neutralized by the addition of HCl to 250 mM. These samples were then fractionated on 1.2 % vertical TAE-agarose gels run at 5 volts/cm for 4 hours; the gels were dried and examined by autoradiography.

Glycerol Gradient Analysis. The sedimentation rate of the vaccinia topoisomerase was determined relative to native ^{14}C-labeled bovine serum albumin (4.3S, 69,000 mw), ovalbumin (3.6S, 46,000 mw), carbonic anhydrase (3.2S, 30,000 mw) and β-lactoglobulin A (3.0S, 18,367 mw). These radiolabeled proteins served as internal markers when 50 µl (270 units) of pool vaccinia topoisomerase was sedimented through a linear gradient of 5 to 20% glycerol in 180 mM potassium phosphate pH 7.4, 200 mM NaCl, 0.1% Triton X-100, 1 mM EDTA and 2 mM DTT. Centrifugation was for 28 hours in a Beckman SW 50.1 rotor at 50,000 rpm (rcf = 240,000). 200 µl fractions were collected; 20 µl were assayed for topoisomerase activity (in a 40 µl reaction containing 90 mM potassium phosphate pH 7.4, 100 mM NaCl, 4 mM $MgCl_2$, 5% glycerol, 0.05% Triton X100, 0.5 mM EDTA, 1 mM DTT) and 20 µl were fractionated by SDS-PAGE and analyzed by fluorography (19) to determine the position of the marker proteins. The topoisomerase sedimentation coefficient was obtained by interpolation relative to these proteins; an estimate of molecular weight was derived using the method of Martin and Ames (20).

FPLC. 540 units of vaccinia topoisomerase activity was applied to a pre-packed Superose 12 column (10 mM x 30 cm) equilibrated in Buffer B containing 180 mM potassium phosphate pH 7.4 and chromatographed on a Pharmacia FPLC apparatus. The column was developed with Buffer B at a flow rate of 0.25 ml/min and 0.5 ml fractions were collected. Standards of bovine serum albumin (Stokes radius, 35.5 A°), carbonic anhydrase (21.4 A°) and lysozyme (19.1 A°) were chromatographed under identical conditions. All fractions were monitored for absorbance at 280 nm and assayed for topoisomerase activity; calculations of Stokes radius and molecular weight were determined by the method of Siegel and Monty (21).

Purification and Partial Relaxation of Individual Topoisomers. DNA topoisomers were prepared by incubating 10 µg of pSP65 plasmid DNA with 25 units of calf thymus topoisomerase I (BRL) in a 100 µl reaction containing 50 mM Tris pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 µg/ml BSA at 15°C for 30 min. Reactions were stopped by the addition of SDS to 0.4% and the DNA was fractionated by electrophoresis in a 1% horizontal TAE-agarose gel at 0.8 v/cm for 14 hrs. After staining in EtBr, the DNA was visualized with long-wave UV light and individual isomers excised from the gel. Isolated DNA was recovered by binding to glass powder; subsequently, each isomer was freed of EtBr by organic extraction and then further purified on an Elutip-d matrix according to the manufacturer's (Schleicher and Schuell) instructions. Purified DNA was concentrated by ethanol precipitation. Partial relaxation of each isomer with vaccinia topoisomerase was accomplished by incubating 100 ng of isomer with 7 units of vaccinia topoisomerase for 30 min at 15°C under standard buffer conditions. For comparison, the same quantity of each isomer was reacted with 0.5 units of calf thymus topoisomerase I (BRL) for 30 min at 15°C under the conditions described above for this enzyme. Reactions were stopped by the addition of SDS to 0.4% and the DNA was analyzed on TBE-agarose gels electrophoresed at 1 volt/cm for 14 hours.

Drug Inhibition Studies. A 10 mM stock of novobiocin was prepared in 10 mM Tris pH 7.4; 10 mM coumermycin A1 was prepared in Me_2SO_4. A 35 mM stock of nalidixic acid was prepared in 50 mM KOH; an aqueous stock of 10 mM berenil was used. Inhibition experiments contained 200 ng of pUC 8 DNA, sufficient vaccinia topoisomerase to achieve 90% relaxation under standard conditions and the appropriate drug as indicated in the text. The pH of the reactions was shown to be unaffected by the presence of the drug. The inhibitory effect of several concentrations of each drug on the standard relaxation reaction was quantitated.

Reagents. Novobiocin, berenil, coumermycin A1, nalidixic acid and ethidium bromide were obtained from Sigma. DNAse I was obtained from Cooper Biomedical; calf thymus topoisomerase I and protein molecular weight standards for SDS-PAGE electrophoresis were purchased from Bethesda Research Labs. ATP, proteinase K, restriction enzymes, calf intestinal alkaline phosphatase, polynucleotide kinase, the Klenow fragment and DNA Polymerase I were obtained from Boehringer Mannheim. Radiolabeled nucleotide triphosphates, the silver-staining kit and native ^{14}C-labeled protein standards were from New England Nuclear. DEAE-cellulose was obtained from Whatman; hydroxylapatite was purchased from BioRad.

RESULTS

Purification

Our topoisomerase assay is based on the ability of the enzyme to convert supercoiled DNA (Form I) to relaxed circular DNA (Form I^0). These forms can be resolved by their distinct electrophoretic mobilities on agarose gels. Topoisomerization was distinguished from nicking activity by electrophoresing duplicate samples in the presence of EtBr; under these conditions, Form I^0 becomes positively supercoiled and migrates rapidly with Form I, whereas the migration of nicked circular DNA is unaffected. This assay was used to monitor topoisomerase activity during purification. Soluble enzyme extracts were prepared from 30 mg of ^{35}S-labeled virions. The protein eluate from a DEAE-cellulose column was fractionated on a 5 ml hydroxylapatite column (12). The elution profile is shown in Figure 1; the peak activity eluted at 220 mM potassium phosphate. Peak fractions were pooled and used for most of the characterizations described below (designated "pool"). As shown in Table 1, we have clearly achieved a highly significant purification of the enzyme. Analysis of the purified fraction on SDS-PAGE revealed approximately ten polypeptides, as shown in Figure 2, panel A, lane 8. A portion of the peak eluate from the hydroxylapatite column was further purified by gel filtration on an FPLC apparatus. This fraction is significantly more pure, containing one predominant band when analyzed on silver-stained SDS-PAGE (Figure 2, panel B). By several experimental criteria, the activity of the "pool" enzyme is indistinguishable from the more highly purified FPLC fraction, and hence was used for the majority of our studies.

VV Topoisomerase Purification

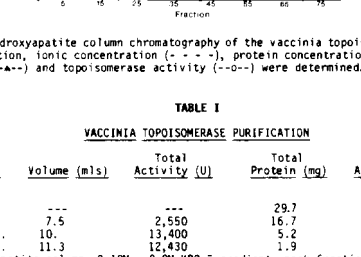

Figure 1. Hydroxyapatite column chromatography of the vaccinia topoisomerase. For each fraction, ionic concentration (- - - -), protein concentration as reflected by ^{35}S-cpm (--▲--) and topoisomerase activity (--○--) were determined.

TABLE I

VACCINIA TOPOISOMERASE PURIFICATION

Fraction	Volume (mls)	Total Activity (U)	Total Protein (mg)	Specific Activity(U/mg)
I.virus	---	---	29.7	---
II.cores	7.5	2,550	16.7	153
III.sol. enz.	10.	13,400	5.2	2,577
IV.DEAE F.T.	11.3	12,430	1.9	6,542
V.Hydroxyapatite column, 0.18M - 0.8M KPO_4^- gradient, peak fractions.			(µg prot.)	
G5	2.5	1,750	3.8	
G6	2.5	7,500	4.2	
G7	2.5	11,125	5.5	
G8	2.5	14,450	6.8	2,100,000
G9	2.5	8,550	6.8	
G10	2.5	7,150	6.8	

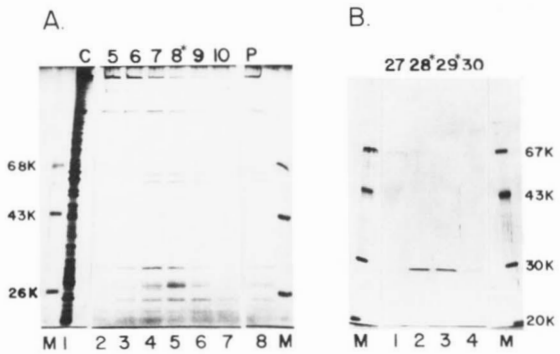

Figure 2. SDS-PAGE Analysis of Fractions Containing Topoisomerase Activity. Panel A. Samples of fractions from the hydroxyapatite column containing topoisomerase activity were electrophoresed on a 10% SDS-polyacrylamide gel which was then silver-stained to detect proteins. Lanes M, molecular weight markers (bovine serum albumin 68 kDa, ovalbumin 43 kDa, α-chymotrypsinogen 26 kDA); lane 1, 2 µl cores, 0.7 units topoisomerase activity; lane 2, 30 µl gradient fraction 5 (G-5), 21 units activity; lane 3, 30 µl G-6, 90 units activity; lane 4, 30 µl G-7, 135 units activity; lane 5, 30 µl G-8, 174 units activity; lane 6, 30 µl G-9, 102 units activity; lane 7, 30 µl G-10, 87 units activity; lane 8, 30 µl pool (G-6,7,8,9), 159 units. Peak fraction is indicated with an asterisk; arrow indicates polypeptide most likely to represent topoisomerase.
Panel B. Samples of fractions from the FPLC Supose 12 gel filtration chromatography which contained topoisomerase activity were electrophoresed on a 12% SDS-polyacrylamide gel; proteins were visualized after silver-staining. Lanes M, molecular weight markers (bovine serum albumin 67 kDa, ovalbumin 43 kDA, carbonic anhydrase 30 kDa, soybean trypsin inhibitor 20 kDa); lane 1, 30 µl fraction 27, 0.1 units activity; lane 2, 30 µl fraction 28, 16 units activity; lane 3, 30 µl fraction 29, 15 units activity; lane 4, 30 µl fraction 30, 6 units activity. Peak fractions are indicated with an asterisk.

Optimization of the Relaxation Assay

Experiments were performed to determine the salt optimum of the enzyme, the need for divalent cations and the role of an energy cofactor. Figure 3 A illustrates the enzyme activity over a broad salt range; the enzyme has an optimum of 150 mM NaCl. Although this result differs from previously published findings (11), it is highly reproducible in our laboratory. Figure 3b illustrates the stimulation of the enzyme by $MgCl_2$. Although the enzyme is active in the absence of magnesium and the presence of 0.2 mM EDTA, the activity is stimulated approximately three-fold by the addition of 1 mM $MgCl_2$ (Figure 3b). As illustrated by the time-course of relaxation shown in Figure 3c, the rate of the reaction is increased significantly in the presence of magnesium. Lastly, the effect of ATP on enzyme-mediated DNA relaxation was investigated. Type I topoisomerases classically do not require an energy cofactor, whereas type II enzymes require ATP, whose hydrolysis accompanies DNA isomerization (reviewed in 22). The addition of ATP at concentrations of 0.1 - 5 mM has never stimulated our preparations of vaccinia topoisomerase. Conversely, ATP slows the rate of the reaction significantly. This again differs from previously published results of others (12); however, our results are reproducible and consistent with our further characterization of the vaccinia enzyme as a type I topoisomerase. Inhibitory effects of ATP on type I topoisomerases have been reported for the Ustilago maydis (23), Hela (24) and chicken erythrocyte (25) topoisomerases.

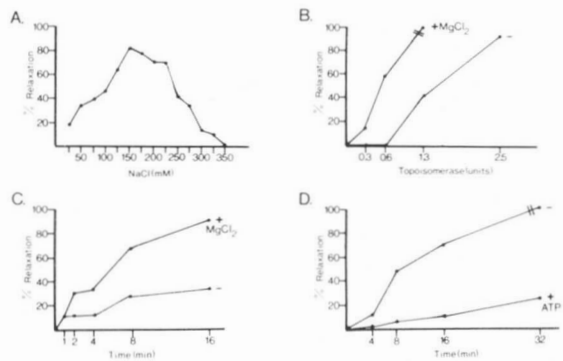

Figure 3. Optimization of the Topoisomerase Reaction. A. Vaccinia Topoisomerase has maximal activity at 150 mM NaCl. 0.2 µg of pUC 8 DNA was reacted with 21 units of vaccinia virus topoisomerase in a reaction containing 25 mM potassium phosphate pH 7.4, 1% glycerol, 0.01% Triton X-100, 0.1 mM EDTA, 0.23 mM DTT and various amounts of NaCl. After incubation at 37°C for 30 min the reactions were stopped by the addition of SDS and the degree of relaxation of the DNA was determined by agarose gel electrophoresis and densitometry as described in Materials and Methods.
Panels B and C. Magnesium stimulates vaccinia topoisomerase activity. In Panel B, 0.2 µg of plasmid DNA was reacted with various amounts of "pool" topoisomerase and then analyzed as described in panel A. The same series of reactions was carried out in the presence of sufficient $MgCl_2$ to give a concentration of 1 mM above the chelating capacity of the EDTA present in the reaction. Panel C illustrates a comparison of the time course of DNA relaxation in the absence and presence of 3 mM $MgCl_2$. Each reaction contained 0.3 units of "pool" topoisomerase and was terminated and analyzed as described above.
Panel D. ATP inhibits the vaccinia topoisomerase. Panel D represents a comparison of the time course of DNA relaxation by the vaccinia topoisomerase in the absence or presence of 4 mM ATP. All reactions contained 1 unit of pool topoisomerase and 0.2 µg of plasmid DNA and were analyzed as described above.