JOURNAL OF VIROLOGY, Jan. 1984, p. 1–8
0022-538X/84/010001-08$02.00/0

Vol. 49, No. 1

Effects of ATP and Inhibitory Factors on the Activity of Vaccinia Virus Type I Topoisomerase

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Received 15 July 1983/Accepted 29 September 1983

Vaccinia virus cores contain a type I topoisomerase which promotes the relaxation of superhelical DNA of either handedness (Bauer et al., Proc. Natl. Acad. Sci. U.S.A. **74**:1841–1845, 1977). The activity of partially purified vaccinia virus topoisomerase (VV-Topo I) was determined in the presence of ATP, dATP, GTP, ADP, and ATP analogs in which hydrolysis of the α,β or β,γ phosphate bond is restricted. Topoisomerase activity was stimulated 2.5-fold by the addition of 2 to 4 mM ATP or dATP to standard assay mixtures; 2 mM GTP produced no significant effect on enzyme activity. The addition of 2 mM β,γ-imido ATP or 2 mM γ-thiophosphate ATP reduced VV-Topo I activity by 80 and 65%, respectively. In contrast, 4 mM α,β-methylene ATP produced no significant change in topoisomerase activity compared to ATP itself. Assays performed in the presence of 4 mM ADP exhibited an 80% reduction in enzyme activity. The preparations of VV-Topo I used for these studies showed, however, no detectable DNA-dependent or -independent ATPase activity. The activity of VV-Topo I was similarly measured in the presence of the antibiotics novobiocin and coumermycin A_1, which inhibited enzyme activity by 50% at concentrations of 180 and 40 μM, respectively. Comparable inhibition of VV-Topo I activity was observed in the presence of 1 mM β,γ-imido ATP. We determined that novobiocin inhibits vaccinia core transcription at the same concentrations which inhibit vaccinia core topoisomerase I activity. These results suggest that the vaccinia DNA topoisomerase may play a role in the ATP-dependent transcription of viral genes from intact core particles.

The poxviruses represent an important group of animal viruses, having provided the first indication that a complex virus could contain its own enzymes and be capable of synthesizing RNA endogenously (16, 25). Vaccinia virus in particular has proved to be a very useful model system for the analysis of eucaryotic transcription in vitro, since the virus contains a full complement of enzymes required for mRNA synthesis. Several of these enzymes have been isolated from viral core particles, and their activities have been characterized. Among the enzymes clearly required for transcription are the DNA-dependent RNA polymerase (3, 33), guanylyltransferase and 7-methyltransferase (21, 23), 2′-*O*-methyltransferase (2), and polyadenylate polymerase (5, 24). A number of other virion encapsidated polypeptides which may function in transcription have been described: 5′-phosphate polyribonucleotide kinase (32), protein kinase (18), two nucleic acid-dependent ribonucleoside triphosphate phosphohydrolases (26), single-strand specific nuclease(s) (27, 28), two major DNA-binding proteins (4, 14), and vaccinia virus DNA topoisomerase I (VV-Topo I) (4).

Analysis of vaccinia core transcription in vitro has revealed a requirement for high concentrations of ATP (15, 17, 25). Apparently there are functions for ATP other than ribonucleotide polymerization, since purified preparations of RNA polymerase require substantially lower ATP concentrations. Furthermore, as noted above, vaccinia virions contain various other enzymes which require ATP. Finally, the substitution of the nonhydrolyzable ATP analogs β,γ-imido ATP (AMP-PNP) or γ-thiophosphate ATP (ATPγS) for ATP in transcription assays of vaccinia cores results in marked inhibition of RNA synthesis (11, 30). However, purified vaccinia RNA polymerase is not inhibited by AMP-PNP or ATPγS (30, 33).

The DNA topoisomerases may be classified into two broad categories: type I topoisomerases break and rejoin only one strand of the DNA duplex in the absence of an energy cofactor, and type II topoisomerases break and rejoin both strands of duplex DNA with concomitant hydrolysis of ATP to ADP and P_i (20, 37). The ATPase activity of the type II topoisomerases is inhibited, especially in procaryotes, by the antibiotics novobiocin and coumermycin A_1 (10, 13, 20). We have investigated the effects of ATP and ATPase inhibitors on the activity of VV-Topo I to gain insight into the reaction mechanism of the enzyme and as part of a more general investigation of the possible roles of the topoisomerase and ATP in vaccinia transcription.

MATERIALS AND METHODS

Materials. Unlabeled nucleotides and novobiocin were obtained from Sigma Chemical Co. [^{3}H]ATP and ATPγS were kindly provided by S. Shuman. Nalidixic acid and oxolinic acid were provided by R. Sternglanz. Coumermycin A_1 was a gift from F. Castora. AMP-PNP and α,β-methylene ATP (AMP-CPP) were purchased from Boehringer Mannheim, and ethidium bromide (EtdBr) was obtained from Calbiochem. All other chemicals were of reagent grade.

Virus. Vaccinia virus strain WR was purified from infected HeLa or L cells as described previously (12).

Preparation of nucleic acids. Supercoiled plasmid pSM1 was isolated as described previously (22). Phage φX174 viral DNA was kindly provided by J. Hurwitz.

Assay for topoisomerase activity. The standard reaction mixture (25 μl) contained 10 mM potassium phosphate (pH 7.5), 0.1 M NaCl, 0.2 mM EDTA, 4.0 μg of pSM1 DNA I per ml, and various amounts of enzyme. After incubation at

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37°C for 30 min, reactions were terminated by freezing at −20°C. The thawed reaction mixtures were adjusted to 25% sucrose–0.01% bromphenol blue and subjected to electrophoresis on a 1% agarose slab gel 0.38 cm in thickness and 10 cm in length, containing 40 mM Tris-acetate (pH 8.3), 20 mM sodium acetate, 2.0 mM EDTA, and 0.5 μg of EtdBr per ml. Electrophoresis proceeded at 80 V for 3 h. The DNA bands were visualized by illumination from below with short-wave UV light and photographed with a Polaroid MP-4 camera using Kodak Royal Pan film. The developed negatives were scanned with a Joyce-Loebl microdensitometer, and the areas under the peaks were measured with the aid of a Numonics digital planimeter. An alternative assay employed the changes in fluorescence which accompany the reduction in the extent of dye binding due to relaxation by topoisomerase. The reaction proceeded and was terminated as described above; the fluorescence change was then determined with a Perkin-Elmer MPF-3L fluorescence spectrophotometer after a 1:10 dilution into 10 mM potassium phosphate–20 mM NaCl–0.1 μg of EtdBr per ml. Other details of the procedure have been described previously (36; D. Foglesong, Ph.D. Thesis, State University of New York, Stony Brook, 1980).

One unit of topoisomerase activity is defined as that amount of enzyme which converts 50% of a 1.0-μg sample of pSM1 DNA (22) from supercoiled to relaxed closed duplex DNA I_0 in 30 min under standard incubation conditions.

Purification of topoisomerase. The solubilization of type I topoisomerase from purified vaccinia virions labeled with [^{35}S]methionine and the purification of the enzyme through fraction V were performed as described previously (4). Fraction V topoisomerase was applied to a column of hydroxyapatite (0.9 by 6 cm) equilibrated with 0.25 M Tris-hydrochloride (pH 8.5)–0.2 NaCl–10% glycerol–0.1% Triton X-100–1.0 mM EDTA–2.0 mM dithiothreitol (buffer A). The column was washed with 10 bed volumes (40 ml) of buffer A and then washed with 10 bed volumes of 0.2 M potassium phosphate (pH 7.5)–0.2 M NaCl–10% glycerol–0.1% Triton X-100–1 mM EDTA–2 mM dithiothreitol (buffer B). The column was then eluted with a linear 0.2 to 1.0 M potassium phosphate gradient in buffer B programmed with the LKB 11300 Ultrograd gradient mixer. The flow rate for the washes and gradient was 12 ml/h, and fractions were collected every 12 min. After chromatography, a 0.1-ml sample of each fraction was trichloroacetic acid precipitated, and the ^{35}S radioactivity was determined. The topoisomerase activity of fraction VI was stable at 4°C for several months.

Assay for DNA-dependent ATPase. [^{3}H]ATP (26 cpm/pmol) at a concentration of 1 mM was incubated with 4 μg of pSM1 DNA I or 7.5 μg of φX174 viral DNA per ml, and various amounts of fraction VI topoisomerase under standard incubation conditions or in the presence of 3 mM $MgCl_2$. The reaction mixtures were incubated at 37°C for 30 min. A 5-μl portion of each sample was applied to polyethyleneimine thin-layer chromatography plates along with unlabeled ATP, ADP, and AMP markers. The plates were developed with 1 M LiCl and then dried. The ATP, ADP, and AMP spots were visualized with long-wave UV light, excised from the plate, and counted in 2.5 ml of Biofluor by scintillation counting.

Vaccinia core transcription assay. Transcription from intact vaccinia virus cores was measured by the incorporation of [^{3}H]UTP into acid-precipitable material. Reaction mixtures of 50 μl total volume contained 1 mM ATP, GTP, and CTP, 88 μM [^{3}H]UTP (170 cpm/pmol), 90 mM Tris-hydrochloride (pH 8.5), 50 mM NaCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. The above components were preincubated briefly at 37°C, and the reactions were started by the addition of 10^9 core particles prepared as described previously (4). The reaction mixtures were incubated at 37°C for 30 min. Transcription was terminated by chilling the tubes on ice and adding 0.2 ml of saturated sodium pyrophosphate, 0.1 ml of heat-denatured calf thymus DNA (1 mg/ml), and 5 ml of cold 5% trichloroacetic acid. Acid-precipitable material was collected on glass fiber filters, washed with 1% trichloroacetic acid and ethanol, dried, and scintillation counted.

RESULTS

Purification of topoisomerase by hydroxyapatite chromatography. Previous procedures for the purification of VV-Topo I involved chromatography on DEAE-cellulose followed by denatured DNA cellulose (4). This resulted in a preparation, designated fraction VI, which was heavily contaminated with a DNA-binding protein, previously shown to be a viral structural protein of M_r about 24,000 (4). We altered this protocol by substituting a hydroxyapatite column for the DNA cellulose, as described in detail above. The result is shown in Fig. 1. Topoisomerase activity eluted from hydroxyapatite as a single peak at 0.33 M potassium phosphate.

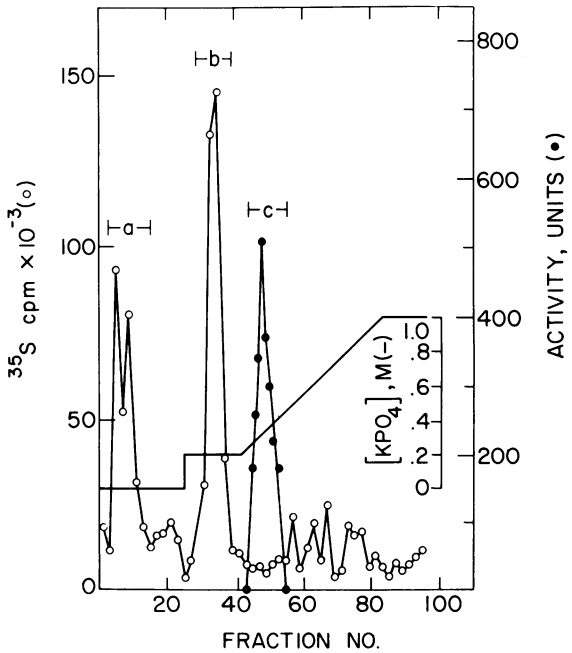

FIG. 1. Purification of VV-Topo I by hydroxyapatite chromatography. Starting with 9.5 mg of purified vaccinia virus, 2.5 ml of fraction V (4) containing 32,000 U of activity was layered onto an hydroxyapatite column (0.9 by 6.0 cm). Chromatography proceeded as described in the text. The wash with buffer A removed protein VV-11K (region a), and polypeptide VV-24K and the guanylyltransferase were removed with 0.2 M potassium phosphate (region b). The column was then eluted with a 0.2 to 1.0 M potassium phosphate linear gradient. Approximately 40% of the input topoisomerase activity (13,000 U) was recovered from the column. The peak of activity (region c) elutes at 0.33 potassium phosphate. Activity was determined by dilution series for each peak fraction using the fluorescence assay as described in the text (●). The profile of [^{35}S]methionine-labeled protein is shown by open circles (○).

The polypeptide composition of the peak fraction of topoisomerase activity from a hydroxyapatite column has been examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (E. Ressner, unpublished data). The results show that the peak fraction contains predominately a polypeptide of M_r 37,000 and is devoid of both VV-11K and VV-24K. The fraction contains small amounts (<20% total) of polypeptides of M_rs 31,000, 60,000, 75,000, and 90,000.

In contrast to the DNA cellulose-based purification, resolution of topoisomerase activity from VV-24K was essentially complete. The DNA-binding proteins VV-11K (14) and VV-24K (4) eluted from hydroxyapatite in the flowthrough and in the 0.2 M potassium phosphate wash, respectively. Fractions containing topoisomerase activity were pooled and designated fraction VI. The pooled fractions containing topoisomerase activity exhibited no detectable ribonucleoside triphosphate phosphohydrolase or RNA polymerase activities (data not shown).

Effects of ATP and ATP analogs on activity of VV-Topo I. Fraction V VV-Topo I was assayed in the presence of 4 mM ATP, NAD, AMP-CPP, and AMP-PNP and compared with control assays performed under standard incubation conditions as described above. Assays were performed over a range of enzyme concentrations to measure accurately the amount of topoisomerase required to convert 50% of closed, underwound DNA I to the closed, relaxed DNA I_0. After incubation at 37°C for 30 min, reactions were terminated, and the DNA was subjected to agarose gel electrophoresis in the presence of 0.5 μg of EtdBr per ml, as described above. The results are presented in Fig. 2, which shows the extent of conversion of supercoiled to relaxed closed duplex DNAs as a function of the amount of enzyme added to reaction mixtures. The extent of conversion is roughly proportional to enzyme activity over the range of 30 to 60% conversion; beyond 60%, the extent of conversion increasingly underestimates the activity. Topoisomerase activity was enhanced by the addition of 4 mM ATP or 4 mM NAD. However, the addition to standard reaction mixtures of 4 mM AMP-PNP, an ATP analog with a nonhydrolyzable γ-phosphate, completely inhibited topoisomerase activity. In contrast, assays containing 4 mM AMP-CPP, an ATP analog in which hydrolysis between the α- and β-phosphates is prevented by the methylene bridge, exhibited about the same activity as control assays. These data suggest that ATP hydrolysis between β- and γ-phosphates may be required for VV-Topo I activity.

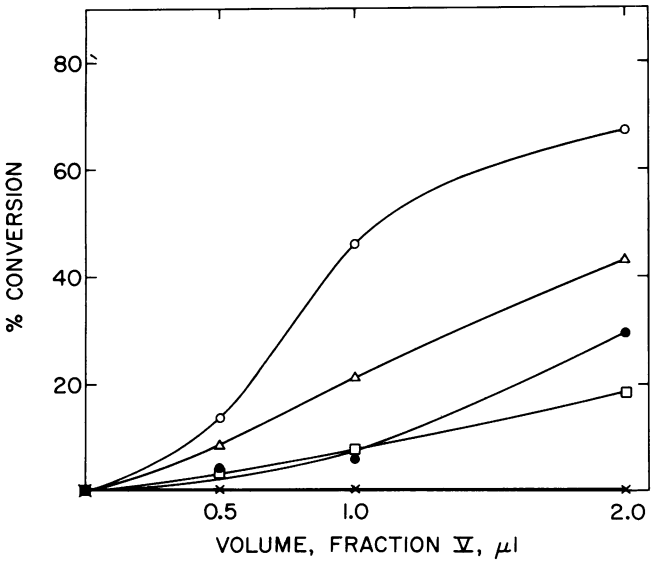

FIG. 2. Effects of 4 mM ATP, NAD, AMP-CPP, and AMP-PNP on the activity of VV-Topo I. Portions of 0.5, 1.0, and 2.0 μl of fraction V topoisomerase were assayed using 0.1 μg of pSM1 DNA I in the presence of 4 mM ATP (○), NAD (△), AMP-CPP (□), or AMP-PNP (×) or in the absence of effectors (●) using EtdBr-agarose gel electrophoresis as described in the text.

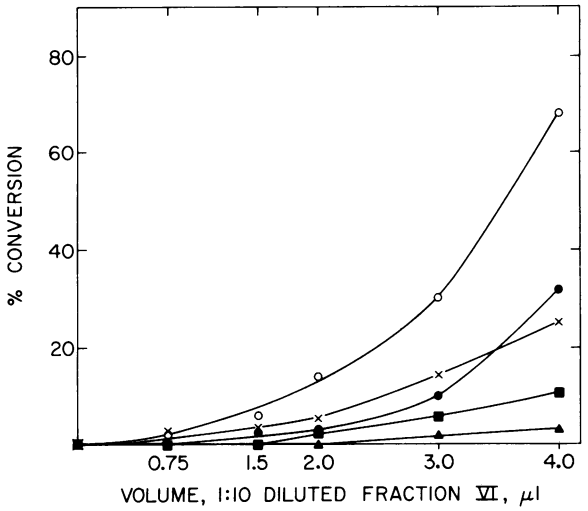

FIG. 3. Effects of 2 mM ATP, GTP, ATPγS, and AMP-PNP on the activity of VV-Topo I. Portions of 0.75, 1.5, 2, 3, and 4 μl of 1:10 diluted fraction VI topoisomerase were assayed in the presence of 2 mM ATP (○), GTP (×), ATPγS (■), or AMP-PNP (▲) or in the absence of effectors (●) using 0.1 μg of pSM1 DNA I analyzed by EtdBr-agarose gel electrophoresis as described in the text.

Further studies of the effects of ATP and its analogs on the activity of the topoisomerase have been conducted with more highly purified fraction VI enzyme preparations. Assays of fraction VI topoisomerase were performed over a range of enzyme concentrations in the presence of 2 mM ATP, GTP, AMP-PNP, and ATPγS and compared with control assays in the absence of added nucleotides or their derivatives. The conversion of supercoiled pSM1 DNA to its relaxed form was determined by EtdBr-agarose gel electrophoresis as described above. The results are presented in Fig. 3. The addition of 2 mM ATP to assays resulted in a stimulation of topoisomerase activity over the entire range of enzyme concentrations tested. However, the presence of 2 mM GTP in the assays did not significantly affect enzyme activity. The addition of 2 mM ATPγS to these enzyme assays produced a 65% inhibition of activity. The hydrolysis of the γ-phosphate of ATPγS by most ATPases is suppressed (30). The addition of 2 mM AMP-PNP correspondingly reduced topoisomerase activity by about 80%.

Fraction VI topoisomerase was also assayed over a range of enzyme concentrations in the presence of 4 mM ATP, dATP, and ADP and compared to standard assay conditions in the absence of exogenous nucleotides. After standard incubation, the DNA was analyzed by EtdBr-agarose gel electrophorsis. The results are presented in Fig. 4. The addition of 4 mM ATP to the assays resulted in stimulation of topoisomerase activity by at least 2.5-fold, as did the addition of 4 mM dATP. The enhancement by 4 mM ATP of the topoisomerase activity of fraction VI (250%) is significantly greater than that observed for fraction V (40%), as shown in

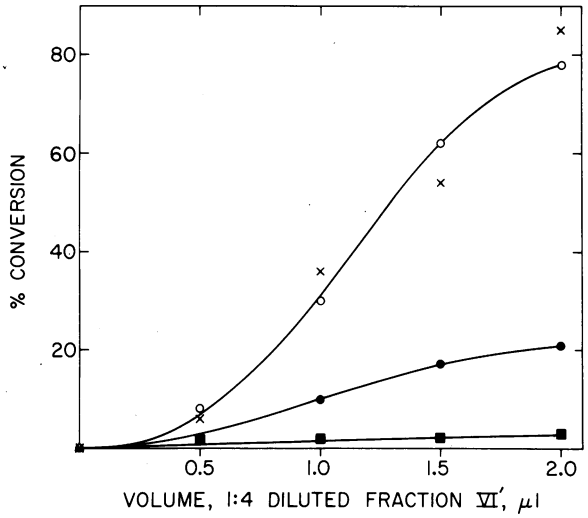

FIG. 4. Effects of 4 mM ATP, dATP, and ADP on the activity of VV-Topo I. Portions of 0.5, 1.0, 1.5, and 2.0 μl of 1:4 diluted fraction VI topoisomerase were assayed in the presence of 4 mM ATP (○), dATP (×), or ADP (■) or in the absence of effectors (●) using 0.1 μg of pSM1 DNA I analyzed by EtdBr-agarose gel electrophoresis.

Fig. 2 and 4. Fraction V topoisomerase preparations contain numerous proteins known to bind and/or hydrolyze ATP (5, 18, 26, 30, 32, 33) which are not present in fraction VI. It should be recalled that the specific activity is substantially underestimated by the extent of conversion in the region above about 60% conversion, which is the case here. In contrast, the addition of 4 mM ADP to assays of fraction VI topoisomerase resulted in an 80% inhibition of enzyme activity. The stimulation of VV-Topo I activity by ATP, along with the observed inhibition of the enzyme by ADP, AMP-PNP, and ATPγS, is especially noteworthy in view of the lack of an absolute ATP requirement and the absence of hydrolysis of added ATP (see below). ATP is required for the activity of DNA gyrase and the other type II DNA topoisomerases (19, 34). Experiments are currently in progress to ascertain the results of the addition of various divalent cations on the above stimulatory effects.

Effects of various concentrations of AMP-PNP on activity of VV-Topo I. The effects of AMP-PNP on the activity of fraction V vaccinia topoisomerase were investigated over a range of concentrations, using pSM1 DNA as substrate. Topoisomerase activity at each AMP-PNP concentration was determined by incubation with increasing amounts of enzyme in the presence and absence of AMP-PNP. The percent conversion of supercoiled pSM1 DNA to the relaxed form was determined by EtdBr-agarose gel electrophoresis, and the results are presented in Fig. 5. Percent inhibition is expressed as 100 $(1\text{-}U/U_c)$, where U and U_c are units of activity in the samples with and without inhibitor, respectively. Somewhat surprisingly, the activity of the enzyme appears to have been slightly stimulated in the presence of 500 to 750 μM AMP-PNP. Similar results have been reported for DNA gyrase in the presence of low concentrations of AMP-PNP and for the T4 topoisomerase in the presence of low concentrations of ATPγS (19, 35). Higher concentrations of AMP-PNP result in pronounced inhibition of topoisomerase activity. Fifty percent inhibition of activity was observed upon the addition of 1 mM AMP-PNP. The addition of 4 mM AMP-PNP to standard assays resulted in no detectable remaining topoisomerase activity. It should be noted that the sensitivity of the assay is insufficient to preclude the existence of a low level of activity (<10% of control values) in the presence of 4 mM AMP-PNP.

Assays of VV-Topo I ATPase activity. To test the hypothesis that hydrolysis of ATP to ADP and P_i is required for VV-Topo I activity, fraction VI enzyme was incubated with 1 mM [^{3}H]ATP (26 cpm/pmol) (i) in the standard 25-μl reaction mixtures, (ii) in the absence of DNA, or (iii) in the presence of 3 mM $MgCl_2$. Samples of fraction VI topoisomerase, corresponding to 0.1 and 0.5 U of enzyme activity, were tested for ATPase activity under each experimental condition. After incubation at 37°C for 30 min, a 5-μl portion of each reaction mixture was analyzed by polyethyleneimine thin-layer chromatography. The percent radioactivity as ATP, ADP, and AMP was calculated for each incubation. No ATP hydrolysis was detected for any of the reaction conditions tested. The remaining 20 μl of the reaction mixtures containing pSM1 DNA was electrophoresed on a 1% agarose gel containing 0.5 μg of EtdBr per ml, confirming that 50 and 100% of the supercoiled pSM1 DNA was converted to relaxed DNA for assays containing 0.1 and 0.5 U of topoisomerase activity (data not shown). Thus, VV-Topo I was active under the above reaction conditions in which no ATP hydrolysis was detected. No hydrolysis of ATP could be detected in similar experiments in which 7.5 μg of φX174 viral DNA per ml was substituted for pSM1 DNA I (data not shown). The results of the above three sections taken together raise the possibility of nonhydrolytic, perhaps allosteric, involvement of ATP in VV-Topo I activity.

Effects of novobiocin and coumermycin A_1 on activity of VV-Topo I. The antibiotic novobiocin has been characterized as a competitive inhibitor of the ATPase activity of *Escherichia coli* DNA gyrase (35). Novobiocin has also been shown to inhibit some type II DNA topoisomerases, which also hydrolyze ATP to ADP and P_i (20). The effect of novobiocin on the activity of VV-Topo I was determined by assaying the fraction VI topoisomerase in the presence of

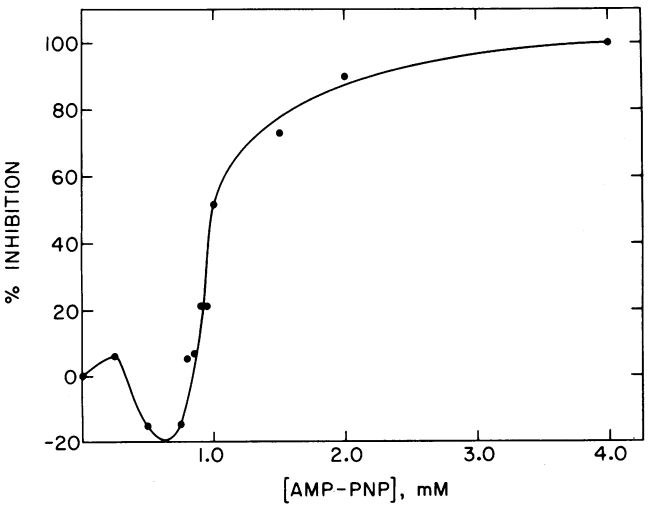

FIG. 5. Concentration dependence of AMP-PNP inhibition of VV-Topo I. Fraction V topoisomerase was assayed in the presence of various concentrations of AMP-PNP over a range of enzyme concentrations analyzed by the EtdBr-agarose gel assay as described in the text. Percent inhibition is 100 − percent control activity.

various novobiocin concentrations. For each novobiocin concentration, VV-Topo I activity was measured over a range of enzyme concentrations, and the enzyme concentration corresponding to 1 U (50% conversion) was ascertained. The results are presented in Fig. 6. Novobiocin strongly inhibits the activity of VV-Topo I. In particular, 50% inhibition was observed in the presence of 180 μM novobiocin, and essentially complete inhibition was observed in the presence of 650 μM novobiocin. A similar result has been reported for the *Drosophila* type II DNA topoisomerase (13).

The effects of the antibiotic coumermycin A_1 on the activity of VV-Topo I were also determined. Coumermycin A_1, which is structurally related to novobiocin (29), inhibits *E. coli* DNA gyrase by competitive inhibition of the gyrase ATPase activity (10). Fraction VI VV-Topo I was assayed over a range of coumermycin A_1 concentrations as described above, except that the reaction mixtures contained 8% (vol/vol) dimethyl sulfoxide to maintain coumermycin A_1 in solution. The presence of 8% dimethyl sulfoxide in reaction mixtures did not significantly affect topoisomerase activity. For each coumermycin A_1 concentration the activity of the topoisomerase was measured by an enzyme dilution series and EtdBr-agarose gel analysis of DNA. The results are presented in Fig. 7. Low concentrations of coumermycin A_1 produced a significant stimulation of topoisomerase activity, up to a maximum of 65% stimulation at 15 μM coumermycin A_1. Assays performed at higher concentrations of coumermycin A_1 resulted in a strong inhibition of topoisomerase activity. Inhibition of 50% of enzyme activity was observed in the presence of 40 μM coumermycin A_1, in close agreement with the levels of coumermycin A_1 required to inhibit the type II DNA topoisomerases (13). Complete inhibition of topoisomerase activity was observed in the presence of 75 μM coumermycin A_1. Thus, on a molar basis, coumermycin A_1 exhibits the most potent inhibition of the VV-Topo I activity of the inhibitors characterized in this report.

Effects of other reagents on the activity of VV-Topo I. The activity of the topoisomerase in the presence of three other reagents was investigated in less detail. Fraction V topoisomerase was assayed over a range of enzyme concentrations in the presence and absence of nalidixic and oxolinic acids. The activity of fraction VI topoisomerase was similarly determined in the presence of *N*-ethylmaleimide. Topoisomerase activity was found to be insensitive to nalidixic acid, a DNA gyrase inhibitor (9), at a concentration of 860 μM but sensitive to oxolinic acid, a more potent analog of nalidixic acid, at the relatively high concentration of 460 μM. The topoisomerase is 50% inhibited by *N*-ethylmaleimide at a concentration of 1 mM, indicating that a reduced sulfhydryl group on the enzyme is required for activity.

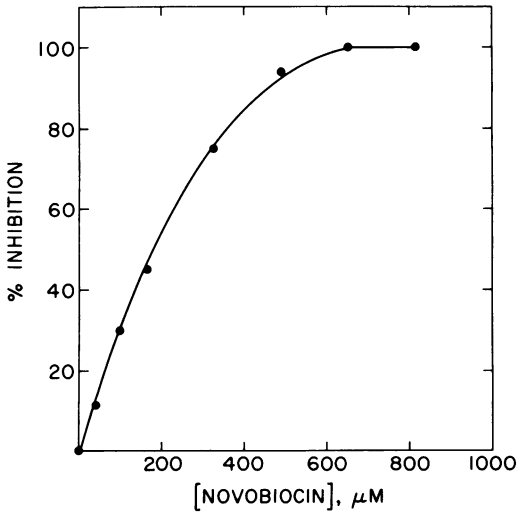

FIG. 6. Concentration dependence of the inhibition of VV-Topo I by novobiocin. Fraction VI topoisomerase was assayed in the presence of various concentrations of novobiocin over a range of enzyme concentrations by the EtdBr-agarose gel assay described in the text. Percent inhibition is 100 − percent control.

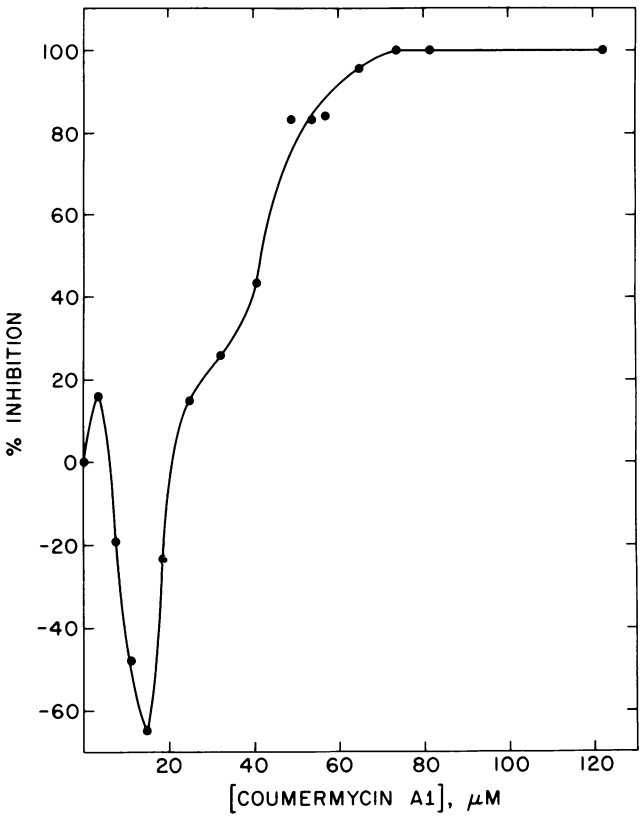

FIG. 7. Concentration dependence of the inhibition of VV-Topo I by coumermycin A_1. Fraction VI topoisomerase was assayed in the presence of various concentrations of coumermycin A_1 over a range of enzyme concentrations by the EtdBr-agarose gel assay described in the text. Percent inhibition is 100 − percent control.

Effect of novobiocin on topoisomerase activity and transcription of vaccinia cores. The preparation of intact vaccinia core particles was performed as described previously (4). Topoisomerase activity of cores was measured by the gel assay method at the optimal NaCl concentration of 0.15 M (4). Transcription of core particles was measured by the incorporation of [^{3}H]UTP into acid-precipitable material as described above. Assays of core topoisomerase activity and transcription were performed at various concentrations of novobiocin, and the results are shown in Fig. 8. Novobiocin inhibits transcription dramatically, with 50% inhibition observed at 550 μM novobiocin. A very similar inhibition of core topoisomerase activity was observed over the same novobiocin concentration range with 50% inhibition at 600 μM (data not shown). These results are consistent with involvement of the topoisomerase in transcription from viral cores. The concentration of novobiocin (600 μM) required for 50% inhibition of core topoisomerase activity is signifi-

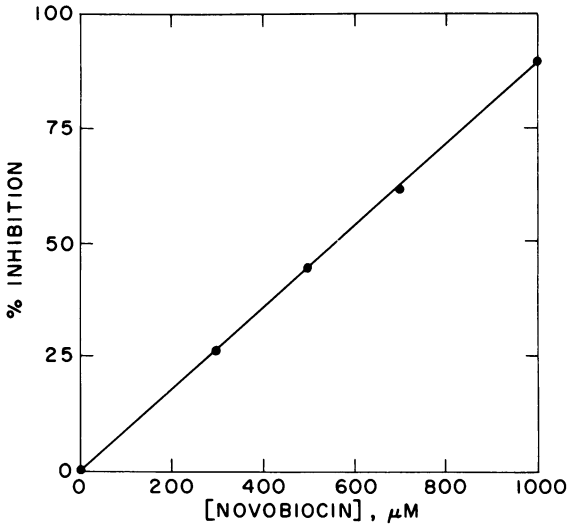

FIG. 8. Concentration dependence of novobiocin inhibition of vaccinia core transcription. Transcription of intact vaccinia cores was measured by the incorporation of [^{3}H]UTP into acid-precipitable material as described in the text in the presence of various concentrations of novobiocin. Percent inhibition is 100 − percent control.

cantly higher than that required for 50% inhibition of the purified topoisomerase (180 μM), as presented in Fig. 6. Intact viral cores may not be fully permeable to novobiocin.

DISCUSSION

In recent years a number of enzymes which insert transient breaks into the phosphodiester-sugar backbone of DNA have been isolated and characterized from both procaryotic and eucaryotic cells. These enzymes have been designated DNA topoisomerases, since they promote the interconversion of topological isomers of closed duplex DNA in vitro (37). The DNA topoisomerases have been categorized into two broad groups. Type I DNA topoisomerases react with closed duplex DNA by a mechanism of breakage and rejoining of one strand of the DNA duplex (37). The type I topoisomerases do not require an energy cofactor such as ATP, since the energy of the broken phosphodiester bond is conserved in a covalent linkage with the enzyme and is thus available to restore that bond (6, 7). The type II DNA topoisomerases are catalytically inactive in the absence of ATP and are also strongly inhibited by ATP analogs which restrict hydrolysis between the β- and γ-phosphates, e.g., AMP-PNP and ATPγS (13, 19, 34). The type II topoisomerases from T4-infected *E. coli* and eucaryotic cells are inhibited by novobiocin and coumermycin A_1, although at relatively high concentrations (13, 20).

The topoisomerase isolated from vaccinia virions is the first example of a eucaryotic virus-encapsidated topoisomerase (4). The vaccinia enzyme is similar in its activity to the eucaryotic type I topoisomerases in that it relaxes both positively and negatively supercoiled DNA in the absence of a divalent cation and in the absence of exogenous ATP. However, in this report we demonstrate that the activity of the vaccinia topoisomerase is stimulated by ATP. The superhelix relaxation activity of a type II DNA topoisomerase from *Drosophila* embryos requires ATP or dATP; GTP and other nucleoside triphosphates cannot substitute for ATP (13). *E. coli* DNA gyrase can also utilize dATP instead of ATP, but GTP and other nucleoside triphosphates cannot replace ATP (34). Furthermore, VV-Topo I activity is completely inhibited by 4 mM AMP-PNP, an ATP analog in which hydrolysis between the β-and γ-phosphates is blocked by an imido linkage. The experiments with AMP-PNP reveal a pattern of increasing inhibition of topoisomerase activity with increasing concentration of AMP-PNP. Fifty percent inhibition of VV-Topo I activity was observed in the presence of 1 mM AMP-PNP, and complete inhibition of enzyme activity was observed at 4 mM AMP-PNP. It should be noted that the sensitivity of the assay cannot preclude the existence of a low level of activity (less than 10% of control values) in the presence of 4 mM AMP-PNP. At a concentration of 250 μM AMP-PNP, a small but reproducible stimulation of VV-Topo I activity was observed. Similar findings have been reported for DNA gyrase (35) and, at low levels of ATPγS, with the T4 topoisomerase (19). In contrast to the results with AMP-PNP, enzyme activity was largely unaffected by 4 mM AMP-CPP, an ATP analog in which hydrolysis between the α- and β-phosphates is blocked by a methylene linkage. Inhibition of VV-Topo I activity was also observed in the presence of 2 mM ATPγS, another ATP analog in which hydrolysis between the β- and γ-phosphate bonds is inhibited. Inhibition of DNA gyrase and the T4 DNA topoisomerase by ATPγS have been reported (19, 34). VV-Topo I is also inhibited by 4 mM ADP, which inhibits both DNA gyrase and the *Drosophila* type II topoisomerase (13, 34).

The mechanism of the ATP-associated stimulation is still unclear. No detectable ATPase activity was observed in the presence or absence of pSM1 supercoiled DNA or of φX174 viral DNA as effector or in the presence or absence of 3 mM $MgCl_2$. The sensitivity of the assay was adequate to detect picomole levels of ATP hydrolysis, as confirmed by parallel assays of vaccinia phosphohydrolase I. Since the pSM1 DNA in the ATPase assays was determined to be relaxed by the action of the vaccinia topoisomerase, it appears that ATP hydrolysis per se is not required for VV-Topo I activity. We cannot exclude the possibility of a very low level of ATP hydrolysis (less than picomole levels of hydrolysis), but these results indicate that VV-Topo I does not catalyze ATP hydrolysis to the extent observed for *E. coli* DNA gyrase or other type II topoisomerases, which hydrolyze several hundred picomoles of ATP under similar conditions (19, 34). An alternative explanation for the effects of ATP and ATP analogs on VV-Topo I is that ATP and its analogs exert an allosteric effect on the enzyme, as has been suggested for DNA gyrase (35). Further studies to characterize precisely the reaction mechanism of the vaccinia topoisomerase are in progress and should clarify the involvement of ATP in the reaction.

Since DNA topoisomerase is encapsidated by vaccinia virus along with RNA polymerase and other enzymes required for transcription (but not DNA polymerase, DNA ligase or other replicative enzymes), it has been proposed that the topoisomerase may be required for transcription of viral genes within vaccinia cores (4). Two possible models could account for such a requirement. The topoisomerase might act as a "swivelase" to relax positive supercoiling in advance of a transcriptional fork, as has been suggested for topoisomerase function in DNA replication. Alternatively, the topoisomerase might act in concert with DNA-binding proteins to induce negative supercoiling in DNA, as has been observed in vitro for the *Xenopus laevis* topoisomerase (1). It is noteworthy that DNA-protein complexes isolated from vaccinia virions exhibit the sedimentation properties of

negatively supercoiled DNA in the presence of EtdBr (8, 31), and greater activity of vaccinia RNA polymerase is obtained with supercoiled DNA templates than with the corresponding relaxed forms (33).

If VV-Topo I is in fact required for transcription, one would predict an inhibition of vaccinia core transcription by novobiocin, a potent inhibitor of the topoisomerase. We find that novobiocin inhibits vaccinia core transcription 50% at a concentration of 550 μM, approximately the same concentration of novobiocin required for 50% inhibition of core topoisomerase activity. Higher concentrations of novobiocin are required to inhibit the encapsidated topoisomerase than are required for comparable inhibition of the solubilized topoisomerase, perhaps due to limited permeability of viral cores to novobiocin. The inhibition of transcription by novobiocin supports the hypothesis of an involvement of the topoisomerase in vaccinia transcription. However, it is not certain that novobiocin is specific for the topoisomerase in the vaccinia system. Assays of vaccinia RNA polymerase, phosphohydrolase I, and phosphohydrolase II in the presence of 1 mM novobiocin failed to reveal any significant inhibition (D. Foglesong, unpublished data). The apparent involvement of VV-Topo I in core transcription may reflect a general requirement for DNA topoisomerase activity for transcription of genes within DNA-protein complexes, including chromatin.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant GM-21176 from the National Institutes of Health.

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