A DNA nicking-closing enzyme encapsidated in vaccinia virus: Partial purification and properties

(closed circular DNA/ethidium bromide/DNA complex winding changes)

WILLIAM R. BAUER, ERIC C. RESSNER, JOSEPH KATES, AND JAMES V. PATZKE

Department of Microbiology, School of Basic Health Sciences, State University of New York, Stony Brook, New York 11794

Communicated by Gary Felsenfeld, February 1, 1977

ABSTRACT Vaccinia virus cores contain an activity which is able to relax both left- and right-handed superhelical DNA. This virus-specific nicking-closing enzyme has been highly purified and differs from the corresponding host enzyme in salt optimum, in sedimentation coefficient, and in polypeptide composition as determined on sodium dodecyl sulfate/polyacrylamide gels. The enzyme is probably newly synthesized after the cessation of host protein synthesis which follows virus infection. The most highly purified preparation contains two polypeptides, one of molecular weight 24,000, and the other 35,000. The former polypeptide is a major constituent of the virus (7% of total protein by weight), whereas the latter is present in a much smaller amount (0.2%). Chromatography with denatured DNA-cellulose reveals that the activity is predominately associated with those fractions enriched in the polypeptide of greater molecular weight.

Many fundamental biological processes are associated with changes in the duplex winding of DNA. These include DNA replication; transcription; recombination; formation of nucleosomes; packaging of DNA in organelles, such as mitochondria or chloroplasts; formation of complex prokaryotic chromosomes (1); and encapsidation into viruses. Enzymes from several sources have recently been discovered which are able to relieve torsional stress on the DNA duplex. None of these appears to require a cofactor, and their primary function is to introduce a transient local swivel that allows the DNA to rotate in response to a torque.

The prokaryotic enzymes, termed omega proteins (2), have been purified from *Escherichia coli* 1100 (2, 3) and from *E. coli* B (4) and are able to catalyze duplex rotation in the right-handed (overwinding) direction only. The eukaryotic enzymes are able to catalyze rotation in both directions and have so far been discovered in several animal cell nuclei (5-9). In the present report we show that such an activity is encapsidated in mature vaccinia virions and is active in viral cores, which are able to carry out transcription as intact complexes. The activity is not identical to the corresponding host protein and is newly synthesized following infection. This result, representing a virus-specific nicking-closing enzyme1, confirms the widespread distribution of this activity.

The only presently available assay for nicking-closing activity is based upon the ability of the enzyme to remove superhelical turns from closed circular duplex DNA. Such DNA molecules are subject to the topological constraint (11) that the net interstrand winding, \( \alpha \), is a constant and is the sum of the number of duplex turns, \( \beta \), and the number of superhelical turns, \( \tau \). The free energy of superhelix formation (12) provides a driving force for the process catalyzed by the nicking-closing enzyme. In a closed DNA with \( \tau \neq 0 \), the enzyme alters \( \alpha \) so that \( \alpha = \beta \) and therefore \( \tau \) is reduced to zero. Such a change in tertiary structure is easy to detect by a variety of methods. The use of closed DNA for assay purposes should not, however, be regarded as a limitation on the substrate specificity of the enzyme. Vaccinia viral DNA, although apparently crosslinked at the termini (13), is essentially a linear duplex not subject to the above topological constraint.

MATERIALS AND METHODS

Reagents. Yeast alcohol dehydrogenase and horse heart cytochrome c were obtained from Worthington and Sigma, respectively. Ethidium bromide (EtBr) was obtained from Calbiochem, and \(^{35}\)S methionine was purchased from New England Nuclear. All other chemicals were of reagent grade.

Preparation of Virus. Vaccinia virus strain WR, grown in HeLa S cells, was purified by the method of Joklik (14). For the preparation of labeled virus, HeLa S cells were suspended at \( 10^7 \) cells/ml in 1:1 Puck’s saline/Eagle’s spinner medium (1/10 normal methionine concentration) and were infected with purified vaccinia virus at a multiplicity of infection of 4. After 30 min at 37°C, the suspension was diluted to \( 10^6 \) cells per ml with Eagle’s spinner medium (1/10 methionine) + 5% dialyzed calf serum. One hour after dilution, 3 mCi of \(^{35}\)S methionine (400 Ci/mm) was added. Labeling was allowed to proceed for 23 hr, after which the virus was purified as above.

Purification of Nicking-Closing Activity. The procedure is that of Moss et al. (15), with minor modifications. Cores were prepared from 20 mg of purified virus by resuspension at \( 2 \times 10^{11} \) particles per ml in 0.05 M Tris-HCl (pH 8.5)/0.05 M 2-mercaptoethanol/0.2% (vol/vol) Triton X-100, and incubation at 37°C for 30 min. Cores were centrifuged at 20,000 X g for 15 min and resuspended at \( 10^{12} \) particles per ml by two or three 15 sec periods of sonication in 0.25 M NaCl/0.3 M Tris-HCl (pH 8.5)/0.1 M dithiothreitol (DTT). The suspension was adjusted to contain 0.1% sodium deoxycholate and incubated for 45 min at 4°C. Released DNA was sheared by a 10 sec sonication, and a supernatant fraction was obtained from a 135,000 X g spin at 4°C for 60 min. This fraction was adjusted to contain 10% (vol/vol) glycerol/0.1% Triton X-100/1 mM EDTA (dissodium salt). DNA was removed by passage through a 0.7 x 4 cm DEAE-cellulose column equilibrated with 0.2 M NaCl, 0.25 M Tris-HCl (pH 8.5)/10% glycerol/0.1% Triton X-100/1 mM EDTA/2 mM DTT.

A column of denatured DNA (dDNA)-cellulose, 0.9 x 7 cm, was equilibrated with 0.15 M Tris-HCl (pH 8.5)/10% glycerol/0.1% Triton X-100/1 mM EDTA/2 mM DTT (dDNA-cellulose buffer) + 0.05 M NaCl. The flow-through fraction

Abbreviations: EtBr, ethidium bromide; DTT, dithiothreitol; dDNA, denatured DNA; EDTA, disodium salt of ethylenediaminetetraacetic acid; DNA L, native covalently closed circular duplex DNA; DNA L, relaxed closed circular duplex DNA; DNA II, nicked (containing a single-strand break) circular duplex DNA.

1 This activity has variously been termed omega (2, 3, 8, 9), untwisting activity (5), relaxing activity (6, 7), and nicking-closing activity (10). We have arbitrarily chosen to employ the latter designation.
from the DEAE-cellulose column was diluted with 3 volumes of dDNA-cellulose buffer and applied to the dDNA-cellulose column. After washing with 8 ml of dDNA-cellulose buffer +0.05 M NaCl, the activity was eluted in a 0.05–0.50 M NaCl gradient in dDNA-cellulose buffer, collecting fractions every 15 min with a flow rate of 15 ml/hr.

Assay for Nicking-Closing Activity. Bacteriophage PM-2 was grown and the DNA, which was used for all assays, was extracted by the method of Salditt et al. (16). The assay mixture contained 1 μg of PM-2 native covalently closed circular duplex DNA (DNA I) in 250 μl of 0.2 M NaCl/0.01 M Tris-HCl (pH 8.5)/0.2 mM EDTA, except where noted. To this was added 5 μl of the solution containing nicking-closing activity followed by a 30 min incubation at 37°. The reaction was stopped by chilling on ice except as otherwise noted, and the samples were adjusted to contain EtBr at 0.5 μg/ml, 6% sucrose, and 5% saturated bromophenol blue. Aliquots containing approximately 0.05 μg of DNA were subjected to electrophoresis on a 1% agarose slab gel, 0.38 cm thick and 10 cm long, at 50 V for 6 hr. The gel and chamber buffer was 0.04 M Tris-acetate (pH 8.3)/0.02 M sodium acetate/20 mM EDTA/EtBr at 0.5 μg/ml. The DNA bands were visualized by illumination from below with a short-wavelength UV light and were photographed with Polaroid type 55 F/N film through a Tiffar 23A filter. The extent of conversion was estimated by cutting out and weighing the peaks produced by scanning the negative with a Joyce–Loebl recording microdensitometer. The peak areas were found to vary linearly with the amount of DNA in the sample up to approximately 0.2 μg. One unit of activity is defined as that amount of enzyme which converts 1/2 of a 1.0 μg sample of PM-2 DNA from I to II, (the relaxed closed circular duplex DNA) in 30 min under the conditions of the assay.

RESULTS

Purification of Activity. The purification of nicking-closing activity is summarized in Table 1. No activity was detected after even prolonged incubation with whole virions or with the supernatant from disrupted virus, precluding simple surface adsorption of host enzyme to the viral envelope. Activity first appears in preparations of whole cores and significantly increases upon disruption of cores. Total activity increases by 40% following DEAE-cellulose chromatography, which probably reflects the removal of competitive inhibition by vaccinia DNA (7). The amount of activity associated with the pellet after disruption of cores and centrifugation at 135,000 × g for 60 min was approximately 20% of that in the solubilized fraction, as assayed in 0.2 M NaCl. Fig. 1 presents the results of affinity chromatography with dDNA-cellulose. This procedure yields a single peak of nicking-closing activity which co-elutes with the major peak of bound protein as gauged by trichloroacetic-acid-precipitable radioactivity. The major peak fraction elutes at 0.23 M NaCl at 4° and the amount of protein under the peak of enzyme activity represents 15% of the input radioactivity.

Optimum Conditions for Activity. The most highly purified enzyme preparation (Fraction VI) exhibits a broad pH dependence, with optimal activity at pH 7.5. The activity is reduced to approximately 25% of the maximum at pH 5.0 and 8.5, and no activity could be detected at pH values above 9.0 or below 4.0. The temperature dependence of Fraction VI showed an optimum at 37°. Approximately 80% of the activity was retained when the assay was performed at 22° and at 41°.

The salt optimum (NaCl) of the specific activity was found to shift to progressively lower values as purification proceeded. Table 1 lists specific activity determinations at three NaCl concentrations for each fraction. The eukaryotic nicking-closing activities reported previously (6–9) exhibit an ionic strength optimum of 0.2 M and are nearly inactive at 0.05 M, whereas the reverse is true of the vaccinia enzyme. The salt optimum for Fractions II–IV lies at 0.15 M NaCl, and the range of activity broadens and moves towards lower salt concentrations as purification proceeds. Fraction VI is most active at 0.05 M NaCl, and the specific activity is reduced to 20% at 0.15 M and at 0.025 M NaCl. Virus-associated deoxyribonuclease activity, known to convert simian virus 40 Form I DNA to Form II

Table 1. Purification of nicking-closing activity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Preparation</th>
<th>Protein, mg</th>
<th>Specific activity, units/mg</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05 M</td>
<td>0.15 M</td>
</tr>
<tr>
<td>I</td>
<td>Whole virus</td>
<td>19.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>Cores</td>
<td>12.3</td>
<td>&lt;100</td>
<td>3,200</td>
</tr>
<tr>
<td>III</td>
<td>Disrupted cores</td>
<td>12.3</td>
<td>&lt;100</td>
<td>5,100</td>
</tr>
<tr>
<td>IV</td>
<td>Supernatant, 135,000 × g</td>
<td>5.7</td>
<td>3,600</td>
<td>5,900</td>
</tr>
<tr>
<td>IVp</td>
<td>Pellet, 135,000 × g</td>
<td>6.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>V</td>
<td>DEAE-cellulose flowthrough</td>
<td>3.7</td>
<td>7,700</td>
<td>12,500</td>
</tr>
<tr>
<td>VI</td>
<td>dDNA-cellulose peak fraction</td>
<td>0.29</td>
<td>400,000</td>
<td>81,000</td>
</tr>
</tbody>
</table>

The amount of protein was assessed by scintillation counting of incorporated [35S]methionine, based upon a specific radioactivity of 1.1 × 10⁶ cpm/mg for whole virus. The enzyme activity was assayed at 37° as described in Materials and Methods and represents the average of four determinations at the indicated NaCl concentrations. The purification factor is the ratio of the specific activity of each preparation at its salt optimum to that of cores at 0.15 M NaCl.
(nicked circular duplex) (17), interfered with assays of Fractions II–V conducted in 0.05 M NaCl but not in 0.15 M NaCl. No DNase activity was detectable in Fraction VI preparations at either ionic strength.

Characterization of Activity by Centrifugation and Chromatography. The centrifugation of Fraction VI results in substantial loss of enzyme activity compared to storage at 4°C for an equivalent time period, for reasons not yet understood. Fig. 2 presents the sedimentation velocity profile of a mixture of radioactive Fraction VI and unlabeled Fraction IV; parallel experiments (data not shown) demonstrated that addition of Fraction IV affects neither the shape nor the position of the peak of radioactive activity from Fraction VI. The peaks of radioactivity and of enzyme activity are displaced by approximately 0.13 S, corresponding to a molecular weight difference of 2700 for two globular proteins (18). The sucrose gradient sedimentation results shown in Fig. 3 compare the sedimentation of the activity from Fraction V with that of two marker proteins. By comparing the ratios of distances sedimented, the Fraction V activity was determined to have a sedimentation coefficient of 3.3 S. Applying the relationship $S_1/S_2 = (M_1/M_2)^2/3$ (18) yields a value of 44,000 for the molecular weight ($M$) of the nicking-closing activity. If the enzyme is asymmetric the true molecular weight might be substantially greater.

Fraction VI was subjected to cation-exchange chromatography on carboxymethyl-cellulose, as shown in Fig. 4, with the result that activity and radioactivity virtually co-eluted. Retention on this column is characteristic of basic polypeptides. This result demonstrates that the enzyme activity is closely associated with a major viral protein, but the lack of sedimentation coincidence shown in Fig. 2 suggests that they are not identical.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. The dDNA-cellulose chromatographic fractions comprising the peak of enzyme activity were analyzed under denaturing conditions by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Fig. 5 shows the results obtained after visualization of the polypeptide bands by fluorography (22). On a separate gel (data not shown) the molecular weights of the two principal components of Fraction VI were determined using six standard molecular weight markers. The smaller and overwhelmingly more prevalent species is a 24,000 dalton polypeptide which appears to correspond to viral polypeptide (vp) 8 (23). These fractions contain, in addition, a polypeptide of 35,000 daltons. This latter component was not detected by Sarov and Joklik (23), but was reported as a core component by McCrea and Szilagyi (24), who labeled it vp 6b. In order to avoid confusion with the envelope polypeptide labeled vp 6b by Sarov and Joklik (23), we will term the present component vp 6b'. These are the only two polypeptides detectable by either staining (data not shown) or autoradiography that appear to be correlated with enzyme activity, and Fig. 5B indicates that this correlation is more significant for vp 6b' than for vp 8. The peak

![Fig. 2. Sedimentation velocity profile of partially purified nicking-closing activity in a 5–20% glycerol density gradient. An aliquot of 0.2 ml of Fraction VI containing 15,000 cpm was mixed with 0.1 ml of unlabeled Fraction IV, layered onto a 4.5 ml preformed gradient, and centrifuged at 65,000 rpm for 2 hr at 4°C in an SW 65 rotor. Sedimentation is to the left as shown, and the fraction size was 0.175 ml. The sedimentation solvent contained 0.01 M Tris-HCl (pH 8.5)/0.2 mM EDTA/0.2 M NaCl/0.1% Triton X-100/2 mM DTT.](image)

![Fig. 3. Sedimentation velocity profile of nicking-closing activity contained in a DEAE-cellulose flowthrough (Fraction V). Centrifugation was through a preformed 5–20% linear sucrose gradient in an SW 50.1 rotor at 41,400 rpm, for 19 hr, at 4°C. The markers are yeast alcohol dehydrogenase (O), $s_{20,w} = 7.4$ S (19), and horse heart cytochrome c ($A$, $s_{20,w} = 2.2$ S (20)). The fraction volumes were 92 µl. The sedimentation solvent contained 0.01 M Tris-HCl (pH 8.5)/1 mM EDTA/0.2% NaCl/2 mM DTT.](image)

![Fig. 4. Chromatography on carboxymethyl-cellulose of purified (Fraction VI) nicking-closing activity. The input to the column consisted of pooled peak fractions from a dDNA-cellulose column diluted 1.4 with buffer, yielding final concentrations of 0.05 M NaCl/0.03 M Tris-HCl (pH 8.5)/0.1% Triton/1 mM EDTA/2 mM DTT. The salt gradient was calculated as described in the legend to Fig. 1, and fractions of 3.8 ml were collected.](image)

![Fig. 5. A, Electrophoretic analysis of the principal polypeptide bands from Fraction VI. B, Autoradiograph of a gel of the same composition as in A, except that the fluorographs were obtained with NaI.](image)
of vp 8 is considerably broader and extends well outside the region of significant activity. It should be noted, however, that no activity was detected in fractions lacking vp 8. The analysis of Fig. 3 suggests that the enzyme activity can be attributed neither to vp 8 nor to vp 6b' in their monomeric forms; some degree of interpolypeptide association is necessary to account for the observed sedimentation rate.

Activity on Positively Supercoiled DNA. PM-2 I DNA was first converted (Incubation 1) with the nicking-closing enzyme to PM-2 I, which has a topological winding number, \( \alpha \), equal to one-tenth the number of base pairs. Starting with this completely relaxed DNA, EtBr was added to various final concentrations (Incubation 2) so as to generate a series of increasingly positively supercoiled DNA molecules. If the enzyme accepts these species as substrates (i.e., reduces \( \alpha \) to zero in the presence of bound dye), the values of \( \alpha \) for the final products will be less than one-tenth the number of base pairs. After removal of both enzyme and dye, these products will be negatively supercoiled to a degree that reflects the concentration of dye in the second incubation.

Fig. 6 shows the locations of the product DNA species after migration through a gel containing EtBr at 0.5 \( \mu \)g/ml. The sample that was relaxed at a total EtBr concentration of 2 \( \mu \)g/ml migrates approximately as a relaxed circle in the gel. The critical total EtBr concentration (that concentration in which the DNA behaves as a relaxed circle) shifts from approximately 2 \( \mu \)g/ml under the assay conditions to 0.5 \( \mu \)g/ml under the lower temperature and ionic strength electrophoresis conditions; this is expected on the basis of the known salt-temperature-dependence of the intrinsic binding constant (25). Those samples relaxed at higher dye concentrations remain negatively supercoiled during electrophoresis, while those relaxed at lower dye concentrations have bound sufficient EtBr to become positively supercoiled during the electrophoresis. Both types of species will migrate faster than the relaxed circle. It is therefore clear that the vaccinia enzyme accepts both positively and negatively supercoiled DNAs as substrates.

DISCUSSION

The vaccinia virion contains an enzyme which is able to relax superhelical DNA of either twist direction. This enzyme is distinct from that found in human cell nuclei by several criteria: ionic strength optimum (0.05 M NaCl versus 0.20 M); sedimentation coefficient (3.3 S versus 4.4 S); and polypeptide composition as revealed on sodium dodecyl sulfate/polyacrylamide gels. In this latter case we observe two bands, a major species of 24,000 daltons (vp 8), comprising 7\% of the mass of the virus, and a minor species of 35,000 daltons (vp 6b'), comprising 0.2\% of the virus. The nuclear enzyme from human KB cells, on the other hand, has been reported to be a single polypeptide chain of 60,000 daltons (46). The vaccinia-associated activity is probably newly synthesized after infection, because \( { }^{35}S \) methionine is incorporated into the only detectable polypeptides in Fraction VI. Host protein synthesis is rapidly shut off following vaccinia infection (26).

The highest specific activity which we have obtained is that of Fraction VI, 400,000 units/mg. This value corresponds to the relaxation of one DNA molecule per vp 8 monomer or two per dimer, and to 45 DNA molecules per vp 6b' monomer or 90 per dimer. Because the activity sediments with a minimum molecular weight of 44,000 and is much more likely to be associated with vp 6b' than with vp 8, it is highly probable that the action is catalytic, as is the case with other nicking-closing enzymes.

The biological function of the vaccinia enzyme remains an open question. It has been previously suggested that nicking-closing activity is involved in either DNA replication (2) or transcription (9). It would appear unlikely that the vaccinia virion protein is involved only in DNA replication. During the course of vaccinia infection early enzymes involved in DNA metabolism are synthesized from transcripts of the incoming viral genome (27). If a nicking-closing enzyme were needed only for DNA replication, there would be no need for its en-
capsidation. In this event, ample opportunity would be available for de novo enzyme synthesis prior to DNA replication.

It appears to us to be likely that this enzyme is involved in the process of transcription from the viral cores. Such a role would be consistent with the presence in the virion of a variety of other enzymes required for transcription and modification of the transcript, including RNA polymerase (29), poly(A) polymerase (29), and enzymes involved in capping and methylating mRNA (30). If vp 6b' is active as a dimer of molecular weight 70,000, we calculate that each viral core contains approximately 125 such enzymes. This compares with the estimate of 110 molecules of poly(A) polymerase per core (31) and would correspond to about two or three nicking-closing enzymes per transcriptional growing point (32), an amount consistent with a role in transcription from cores. A requirement for nicking-closing activity might well be exhibited by spatially constrained systems such as chromatin, mitochondria, and vaccinia cores because of the ability to relieve torsional stress.

Yet a third possibility must be considered, that the nicking-closing activity is required for virus assembly. This function would allow the DNA to interact with the virion proteins with maximal freedom from torsional distortion. This hypothesis predicts that nicking-closing activity will be associated with complex viruses in general, regardless of whether or not they carry other virion enzymes or are able independently to support transcription.

The association of vp 6b' with vp 8, which is present in amounts (7% of the virus) expected for a viral structural protein, is possibly of significance in terms of both the architecture of the virus and the mechanism of transcription. The nuclear nicking-closing activity of mouse L cells appears, in an analogous manner, to be closely associated with the basic protein, histone H1 (33). The vaccinia enzyme (vp 6b') thus appears to associate with a major basic structural protein (vp 8) on the one hand, and to interact with DNA on the other. The detailed nature and significance of both these interactions remains to be elucidated.

We wish to thank M. Krinsky and M. McLeod for assistance in the growth of HeLa cells and of vaccinia virus, and Mrs. Lenora Pong for her invaluable help in the preparation of the manuscript. This work was supported by National Institutes of Health Grants GM-21176 (to W.R.B.) and AI-11839 (to J.K.). E.R. is the recipient of traineeships from the National Cancer Institute under Grants CA-05243 and CA-09176. Facilities for the growth of animal cell cultures were partially supported by National Science Foundation Grant GB-7505578.

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.