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# Identification of a vaccinia virus gene encoding a type I DNA topoisomerase

**(enzyme purification/protein sequence/gene mapping/sequence homology)**

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**ABSTRACT** **Vaccinia virus encapsidates a type I DNA topoisomerase (EC 5.99.1.2). The enzyme was purified from virus cores to apparent homogeneity, yielding a protein of $M_r$ 32,000. The amino-terminal sequence of the isolated $M_r$ 32,000 polypeptide was determined and used to map the putative structural gene for the vaccinia topoisomerase to the *H7r* open reading frame of the vaccinia genome. This gene encodes a 314-amino acid polypeptide containing a region homologous to a region of the type I topoisomerase from the yeast *Saccharomyces cerevisiae*.**

DNA topoisomerases are enzymes that modify the topological state of DNA via the breakage and rejoining of DNA strands (reviewed in ref. 1). A DNA topoisomerase was isolated from infectious vaccinia virus particles by Bauer *et al.* in 1977 (2), and, to our knowledge, this enzyme remains the only virus-associated DNA topoisomerase found in eukaryotic systems. As characterized initially, the enzyme relaxed both positively and negatively supercoiled DNAs, had a native $M_r$ of 44,000, and consisted of two major polypeptides of $M_r$ 24,000 and 37,000, the former being predominant (2). A more highly purified preparation, containing a major polypeptide of $M_r$ 37,000 and minor polypeptides of $M_r$ 31,000, 60,000, 75,000, and 90,000 (but lacking the $M_r$ 24,000 protein observed previously), was obtained by Fogelsong and Bauer, using a different purification protocol (3). More recently, Shaffer and Traktman (4) have purified the enzyme further, obtaining a topoisomerase with a native $M_r$ of 31,000–33,000 that contained a single predominant polypeptide of $M_r$ 30,000. Vaccinia topoisomerase was shown to be similar to other type I eukaryotic DNA topoisomerases (EC 5.99.1.2) in that (*i*) it catalyzes unit step changes in linking number; (*ii*) its action involves single-strand cleavage of DNA; and (*iii*) it forms a covalent bond between the enzyme and the 3′ end of the cleaved DNA (4).

Speculation as to the *in vivo* function of vaccinia topoisomerase has touched on roles in transcription, DNA replication, and DNA packaging (2). Progress has been hindered, however, by the lack of adequate *in vitro* systems for DNA replication as well as the absence of virus mutations that specifically affect topoisomerase activity. Indeed, while it has been presumed that the virus-encapsidated topoisomerase is also virus-encoded, there has been no direct demonstration that this is the case. As an initial step in a combined biochemical and genetic approach to this enzyme, we sought to purify the topoisomerase in sufficient quantity to permit structural analysis, to use such data to determine whether the enzyme is virus encoded, and if so to locate the topoisomerase structural gene within the virus genome. These objectives have been accomplished as described below.

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## RESULTS

**Isolation of Homogeneous DNA Topoisomerase.** Initial steps in the purification of topoisomerase from vaccinia virions were as described previously for the preparation of RNA polymerase and capping enzyme (5). In brief, cores were solubilized with deoxycholate, this extract was passed over a column of DEAE-cellulose at high ionic strength to remove DNA, and the flow-through material was chromatographed on DEAE-cellulose at low ionic strength. Topoisomerase was not retained on this column, though RNA polymerase was bound quantitatively. The DEAE-II flow-through fraction was chromatographed on a column of heparin-agarose as described (5) and topoisomerase activity eluted between 0.52 M and 0.62 M NaCl, being well resolved from capping enzyme, nucleoside triphosphate phosphohydrolases I and II, and poly(A) polymerase at this step. Vaccinia DNase (6), a single-strand-specific endonuclease that might interfere with the assay of topoisomerase, was not retained by heparin-agarose and was thus also resolved from topoisomerase at this step. The heparin-agarose enzyme fraction was applied to a column of DNA-agarose, which was developed with a linear salt gradient. Column fractions were assayed for topoisomerase activity, manifest as the ability to convert negatively supercoiled plasmid DNA into the topologically relaxed form. As shown in Fig. 1, two peaks of topoisomerase activity were noted: a minor peak eluting at 0.16 M NaCl (fractions 10–12) and a major component eluting at 0.32 M NaCl (fractions 22–26). The polypeptide composition of the column fractions across the main peak of topoisomerase activity was analyzed by sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis with subsequent visualization of protein bands by silver staining. As shown in Fig. 2, a single polypeptide of $M_r$ 30,000 (relative to marker polypeptides) was seen in fractions 23 and 25, which contained peak topoisomerase activity. The relative abundance of this polypeptide correlated with topoisomerase activity across the column. Eluting at higher salt than topoisomerase (0.4 M NaCl, fractions 29–31) was a prominent polypeptide of $M_r$ 24,000 that was well resolved from the $M_r$ 30,000 protein. That the $M_r$ 30,000 polypeptide was indeed DNA topoisomerase was supported by the demonstration of a covalent DNA-protein cleavage intermediate. Aliquots of column fractions were mixed with $^{32}$P-labeled nick-translated λ phage duplex DNA, heated, then digested with nuclease P1 and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. As shown in Fig. 3, this reaction resulted in the specific labeling of a polypeptide of $M_r$ 32,000. The presence of this labeled species correlated with the activity profile of topoisomerase and with the presence of the $M_r$ 30,000 polypeptide seen in Fig. 2. The labeled $M_r$ 32,000 species was sensitive to digestion with proteinase K (not shown). These data are in agreement with those of Shaffer and Traktman (4), who reported labeling of a $M_r$ 37,000 protein in a similar assay. The

Abbreviation: ORF, open reading frame.

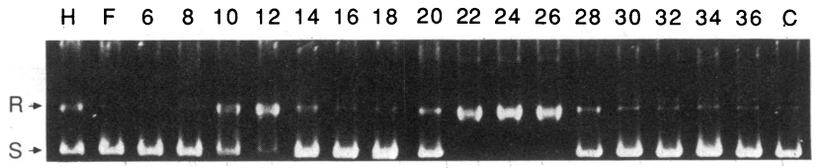


FIG. 1. DNA-agarose chromatography of vaccinia DNA topoisomerase. Virus cores were prepared from 1800 $A_{260}$ units of purified vaccinia virions and extracted with deoxycholate. The soluble extract was chromatographed on columns of DEAE-cellulose at high (0.2 M NaCl) and low (0.05 M NaCl) ionic strength as described (5). The DEAE-II flow-through fraction was chromatographed on a column of heparin-agarose and fractions containing topoisomerase eluting between 0.52 M and 0.62 M NaCl were pooled. This pooled fraction was diluted with 4 vol of buffer A [50 mM Tris·HCl, pH 8.0/1 mM EDTA/2.5 mM dithiothreitol/10% (vol/vol) glycerol/0.01% Nonidet P-40] and applied to a 6-ml column of single-strand DNA-agarose (Bethesda Research Laboratories) that had been equilibrated with 0.1 M NaCl in buffer A. The column was developed with an 80-ml linear gradient of 0.1 to 1.2 M NaCl in buffer A. Fractions (1 ml) were collected and assayed for topoisomerase activity. Reaction mixtures (20 μl) containing 40 mM Tris·HCl at pH 7.5, 0.1 M NaCl, 0.128 μg of pSC16 plasmid DNA (5), and 1 μl of the indicated column fraction were incubated for 15 min at 37°C, and the reaction was quenched by addition of a solution containing glycerol, xylene cyanol, bromphenol blue, and $NaDodSO_4$ (0.2% final concentration). The samples were analyzed by electrophoresis through horizontal 1.2% agarose gels in TBE buffer (50 mM Tris/50 mM boric acid/1 mM EDTA). After staining for 60 min with ethidium bromide at 0.5 μg/ml, the gel was photographed under short-wave UV illumination with Polaroid type 57 film. A photograph of the gel is shown. The DNA-agarose fraction assayed is indicated above each lane. H indicates the heparin-agarose pool; F indicates the flow-through fraction; and C indicates a control sample without added enzyme. The positions of supercoiled (S) and relaxed (R) forms of DNA are indicated by arrows. The salt profile of the column (not shown) was determined by using a Radiometer CDM3 conductivity meter.

slightly smaller size of the labeled species observed herein may reflect more extensive digestion of the covalently bound polynucleotide by nuclease P1 than by DNase 1 (7).

The native size of the major topoisomerase was investigated by using velocity sedimentation. An aliquot of DNA-agarose fraction 25 was applied to a 4.8-ml 15–30% (vol/vol) glycerol gradient and centrifuged for 39 hr in a Beckman SW 55 rotor. Under these conditions, DNA topoisomerase sedimented as a single component with a sedimentation coefficient of 3.1 S relative to reference proteins (hemoglobin and cytochrome *c*) that were sedimented in a parallel gradient (Fig. 4*A*). Analysis of the polypeptide composition of the glycerol gradient fractions (Fig. 4*B*) correlated topoisomerase activity with the presence of a single polypeptide of $M_r$ 32,000. The gel mobility of the $M_r$ 32,000 polypeptide shown in Fig. 4*B* was identical to that of the polypeptide shown in Fig. 2 when the DNA-agarose- and glycerol gradient-purified topoisomerase preparations were analyzed on the same gel (not shown), and the slight difference in assigned $M_r$ was due to use of different marker proteins. These data are consistent with vaccinia topoisomerase being a monomer of a $M_r$ 32,000 polypeptide. The minor component of topoisomerase (Fig. 1) has not yet been characterized.

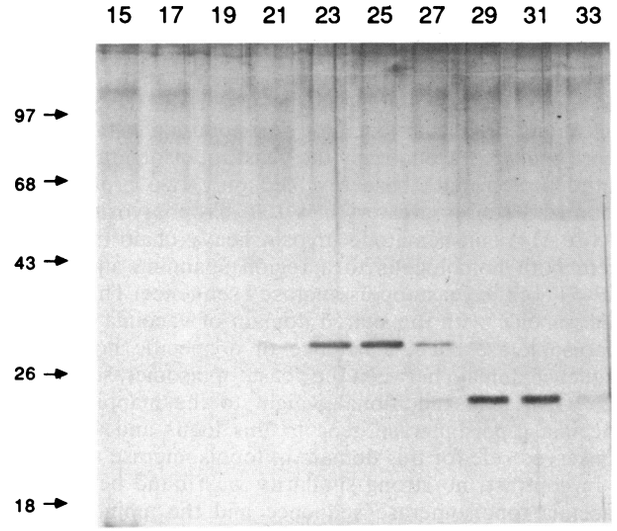


FIG. 2. Polypeptide composition of DNA topoisomerase. Aliquots (20 μl) of the indicated DNA-agarose fractions were made 1% in $NaDodSO_4$ and electrophoresed on a 4% polyacrylamide stacking gel/10% polyacrylamide running gel containing 0.1% $NaDodSO_4$. Protein bands were visualized by using the Bio-Rad silver stain kit. The positions and sizes ($M_r \times 10^{-3}$) of marker proteins (Bethesda Research Laboratories) are indicated by arrows.

**Amino-Terminal Sequence and Gene Identification.** DNA-agarose fractions 23–26, which contained the major topoisomerase activity, were pooled, concentrated by vacuum centrifugation, and electrophoresed in a 10% polyacrylamide gel containing $NaDodSO_4$. The gel contents were electroblotted onto a polyvinylidene difluoride membrane, which was then stained with Coomassie blue. The membrane containing the $M_r$ 32,000 polypeptide was excised and the amino-terminal sequence of the bound protein was determined by William S. Lane and David Andrews of Harvard University, using an Applied Biosystems 470A protein sequencer equipped

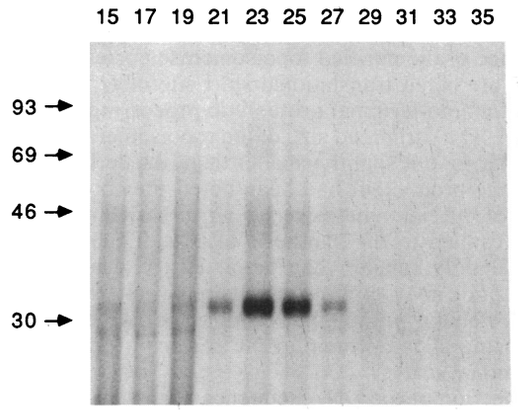


FIG. 3. Formation of a covalent DNA-topoisomerase complex. λ DNA (digested with *Hin*dIII restriction endonuclease) was labeled with [α-$^{32}$P]dCTP (6000 Ci/mmol, Amersham; 1 Ci = 37 GBq) by nick-translation. Reaction mixtures for DNA-topoisomerase complex formation contained (in 10 μl) 50 mM Tris·HCl at pH 7.5, approximately 10 ng of $^{32}$P-labeled nick-translated DNA, and 1 μl of DNA-agarose column fraction. After incubation for 20 min at 37°C the samples were heated for 2 min at 100°C, then made 0.1 M in sodium acetate, pH 5.5. Nuclease P1 (5 μg, Boehringer Mannheim) was added, and the mixture was further incubated for 2 hr at 37°C. The samples were denatured with $NaDodSO_4$ buffer and electrophoresed on a 4% polyacrylamide stacking gel/10% polyacrylamide running gel in 0.1% $NaDodSO_4$ until the dye had migrated out of the bottom of the gel. The gel was dried and autoradiographed as shown. The DNA-agarose column fraction assayed is indicated above the lane. The positions and sizes ($M_r \times 10^{-3}$) of $^{14}$C-labeled marker proteins (Amersham) are indicated by arrows.

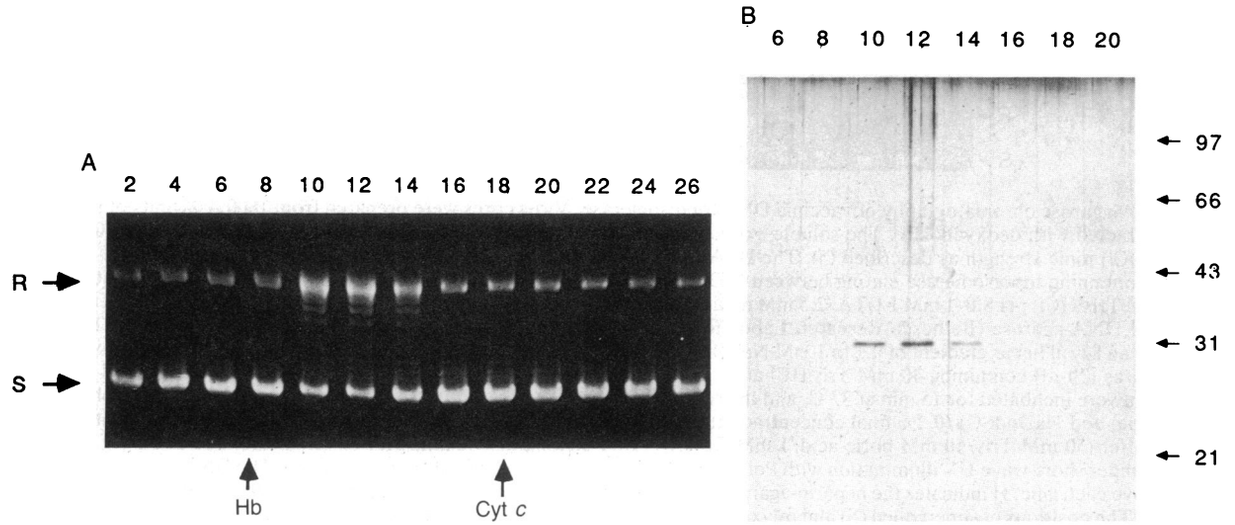


FIG. 4. Glycerol gradient sedimentation of topoisomerase. (*A*) An aliquot (0.16 ml) of DNA-agarose fraction 25 was applied to a 4.8-ml 15–35% glycerol gradient containing 0.25 M NaCl in buffer A and centrifuged in a Beckman SW 55 rotor at 55,000 rpm for 39 hr at 4°C. Fractions (0.2 ml) were collected from the bottom and an aliquot (2 $\mu$l) of every even-numbered fraction was assayed for topoisomerase activity as described for Fig. 1. Each reaction mixture contained 0.42 $\mu$g of pSC16 DNA and incubation was for 30 min. Products were analyzed in a 0.7% agarose gel in TBE. A photograph of the gel is shown. The fraction assayed is indicated above each lane. The positions of reference proteins (hemoglobin and cytochrome *c*) sedimented in a parallel gradient are indicated by arrows. The positions of relaxed (R) and supercoiled (S) forms of pSC16 DNA are indicated by arrows. (*B*) Aliquots (20 $\mu$l) of the indicated glycerol gradient fractions were made 1% in $NaDodSO_4$ and electrophoresed in a 4% polyacrylamide stacking gel/11% polyacrylamide running gel. A photograph of the silver-stained gel is shown. The positions and sizes of marker proteins (Bio-Rad) are indicated by the arrows.

with an on-line model 120A phenylthiohydantoin analyzer. The first 22 amino acid residues were determined to be Met-Arg-Ala-Leu-Phe-Tyr-Lys-Asp-Gly-Lys-Leu-Phe-Thr-Asp-Asn-Asn-Phe-Leu-Asn-Pro-Val-Ser-. A vaccinia protein sequence library, derived from open reading frames (ORFs) predicted from available DNA sequences, was screened for homology to this amino-terminal sequence. A perfect alignment was found for the *H7r* ORF in the *Hin*dIII H fragment, which had been sequenced by Rosel *et al.* (8). The protein sequence encoded by this ORF is shown in Fig. 5. It specifies a 314-residue polypeptide of $M_r$ 36,700. The amino-terminal sequence of the purified topoisomerase corresponds exactly to the predicted translational start site of *H7r*, indicating a lack of amino-terminal proteolytic processing in the mature protein. The estimated size of the topoisomerase, $M_r$ 32,000, is similar to, but slightly smaller than, the derived size of the *H7r* gene product. It had been noted previously that translation of mRNA synthesized *in vitro* that contained the *H7r* ORF resulted in the synthesis of a $M_r$ 32,000 polypeptide, again slightly smaller than predicted (8). Thus, the mature polypeptide may migrate anomalously on $NaDodSO_4$ gels used for analysis, though it cannot be excluded that there is processing of the protein at the carboxyl terminus, both *in vivo* and *in vitro*.

Inspection of the DNA sequence near the amino terminus of the topoisomerase gene indicates that the translation initiation codon is preceded by TAA. The 5′ TAAATG motif is a characteristic feature of vaccinia genes expressed late in infection (8–11). Contained within the ORF of the topoisomerase gene are six copies of the sequence TTTTTNT, which has been shown to be a signal for termination of early transcription and is excluded from the coding sequences of early genes (12). These observations lead to the prediction that vaccinia topoisomerase is encoded by a late viral gene. This prediction is consistent with the observation of Poddar and Bauer (13) that topoisomerase does not appear in vaccinia-infected cytoplasts until 3 hr after infection, a time at which the rate of viral DNA synthesis is maximal.

**Homology of Vaccinia Topoisomerase to the Type I Topoisomerase from Yeast.** The derived amino acid sequence of vaccinia topoisomerase was compared to the sequences of other topoisomerases by using the FASTP program (14). The greatest similarity (optimized score = 125; optimized score for self-comparison = 1571) was noted in the case of the yeast gene encoding type I topoisomerase. The derived amino acid sequence of yeast topoisomerase I indicates a $M_r$ 90,020 protein containing 769 amino acids (15), consistent with the structure of the enzyme ($M_r$ 90,000) as purified from *Saccharomyces cerevisiae* (16). As shown in Fig. 6, a 117-amino acid overlap is found between vaccinia topoisomerase (residues 116–228) and yeast topoisomerase I (residues 406–522) in which 26% amino acid identity is seen. The level of homology increases to 70% when conservative amino acid changes are taken into account. A comparison of the *H7r* ORF to the entire National Biomedical Research Foundation data base* revealed no other optimized homology score higher than 68. Conversely, when the yeast topoisomerase I sequence was screened against the vaccinia sequence library (currently including about 50 ORFs) only the *H7r* ORF registered significant homology (second-highest optimized score being 62). Comparing the yeast topoisomerase I to the entire protein data base revealed only two proteins with optimized scores greater than 100. Tropomyosin $\alpha$ chain (score 114) and nematode myosin heavy chain (score 130) were both homologous to a region spanning amino acids 522–717 of the yeast topoisomerase I sequence. This area did not coincide with the shared domain of vaccinia and yeast topoisomerases. The existence of a specific homologous sequence domain between the yeast topoisomerase I and the *H7r* ORF lends additional weight to the mapping of the vaccinia topoisomerase gene to this locus and suggests a conserved role for this domain in topoisomerase activity.

In contrast, no strong similarity was found between the vaccinia topoisomerase sequence and the amino acid sequence derived for the type I topoisomerase from *Escherichia coli* (17). This is not surprising, insofar as the bacterial enzyme is mechanistically distinct from its eukaryotic counterparts. *E. coli* topoisomerase I relaxes negatively super-

*Protein Identification Resource (1986) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 10.0.

```
                                        10                                  20
Met Arg Ala Leu Phe Tyr Lys Asp Gly Lys Leu Phe Thr Asp Asn Asn Phe Leu Asn Pro

                                        30                                  40
Val Ser Asp Asp Asn Pro Ala Tyr Glu Val Leu Gln His Val Lys Ile Pro Thr His Leu

                                        50                                  60
Thr Asp Val Val Val Tyr Glu Gln Thr Trp Glu Glu Ala Leu Thr Arg Leu Ile Phe Val

                                        70                                  80
Gly Ser Asp Ser Lys Gly Arg Arg Gln Tyr Phe Tyr Gly Lys Met His Val Gln Asn Arg

                                        90                                 100
Asn Ala Lys Arg Asp Arg Ile Phe Val Arg Val Tyr Asn Val Met Lys Arg Ile Asn Cys

                                       110                                 120
Phe Ile Asn Lys Asn Ile Lys Lys Ser Ser Thr Asp Ser Asn Tyr Gln Leu Ala Val Phe

                                       130                                 140
Met Leu Met Glu Thr Met Phe Phe Ile Arg Phe Gly Lys Met Lys Tyr Leu Lys Glu Asn

                                       150                                 160
Glu Thr Val Gly Leu Leu Thr Leu Lys Asn Lys His Ile Glu Ile Ser Pro Asp Glu Ile

                                       170                                 180
Val Ile Lys Phe Val Gly Lys Asp Lys Val Ser His Glu Phe Val Val His Lys Ser Asn

                                       190                                 200
Arg Leu Tyr Lys Pro Leu Leu Lys Leu Thr Asp Asp Ser Ser Pro Glu Glu Phe Leu Phe

                                       210                                 220
Asn Lys Leu Ser Glu Arg Lys Val Tyr Glu Cys Ile Lys Gln Phe Gly Ile Arg Ile Lys

                                       230                                 240
Asp Leu Arg Thr Tyr Gly Val Asn Tyr Thr Phe Leu Tyr Asn Phe Trp Thr Asn Val Lys

                                       250                                 260
Ser Ile Ser Pro Leu Pro Ser Pro Lys Lys Leu Ile Ala Leu Thr Ile Lys Gln Thr Ala

                                       270                                 280
Glu Val Val Gly His Thr Pro Ser Ile Ser Lys Arg Ala Tyr Met Ala Thr Thr Ile Leu

                                       290                                 300
Glu Met Val Lys Asp Lys Asn Phe Leu Asp Val Val Ser Lys Thr Thr Phe Asp Glu Phe

                                       310
Leu Ser Ile Val Val Asp His Val Lys Ser Ser Thr Asp Gly
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FIG. 5. Amino acid sequence of vaccinia DNA topoisomerase. The amino acid sequence was derived from the *H7r* ORF of the virus genome. The experimentally determined amino-terminal sequence of vaccinia topoisomerase is enclosed in the box.

coiled DNA, and strand cleavage involves covalent linkage to the 5′ end of the DNA chain (18). Neither the gyrA nor the gyrB subunit of *Bacillus subtilis* topoisomerase II (DNA gyrase) displayed homology to vaccinia topoisomerase. A short region of similarity between vaccinia topoisomerase and yeast topoisomerase II was noted, consisting of a 26-residue region, corresponding to residues 128–153 in vaccinia topoisomerase and residues 1130–1155 in yeast topoisomerase II, that displayed 39% identity and gave a homology score of 46. The yeast topoisomerase II is itself homologous with bacterial type II topoisomerase; the amino-terminal portion of the $M_r$ 164,000 yeast topoisomerase II is homologous to the bacterial gyrB subunit, while the carboxyl-terminal portion of yeast topoisomerase II is homologous to the gyrA subunit (19, 20). However, the short region of similarity between yeast topoisomerase II and vaccinia topoisomerase does not occur within a region of homology shared by yeast topoisomerase II and bacterial DNA gyrase. Thus the existence of significant homology between vaccinia topoisomerase and yeast topoisomerase II remains uncertain.

## CONCLUSIONS

As described above, the vaccinia-encapsidated DNA topoisomerase was isolated from virus cores in homogeneous form and in sufficient quantity for microsequence analysis. The experimentally determined amino-terminal sequence corresponded exactly to that specified by the *H7r* ORF, suggesting strongly that the enzyme is virus encoded and that the *H7r* ORF comprises the topoisomerase structural gene. The conclusion is predicated on the assumption that the $M_r$ 32,000 polypeptide is, in fact, the DNA topoisomerase—an assumption supported by our purification data and by those of Shaffer and Traktman (4). The designation of the *H7r* ORF as the topoisomerase gene was supported further by the homology between the *H7r* ORF sequence and the amino acid sequence of the type I topoisomerase from yeast.

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                    420                 440                 460
ytopo   QKAVAIYLIDVFALRAGGEKSEDEADTVGCCSLRYEHVTLKPPNTVIFDFLGKDSIRF
        : :: . . ..: .: :  :  .:..:::  .:. .:... .:.....:.:::...
vtopo   QLAVFMLMETMFFIRFGKMKYLKENETVGLLTLKNKHIEI-SPDEIVIKFVGKDKVSH
                    130                 150                 170

                    480                 500                 520
ytopo   YQEVEVDKQVFKNLTIFKRPPKQPGHQLFDRLDPSILNKYLQNYMPGLTAKVFRTYNAS
           :. .....: :  ..  .. :.. ::..:. . . .....  :.  : .:::...
vtopo   EFVVHKSNRLYKPLLKLTDDSS-PEEFLFNKLSERKVYECIKQF--GIRIKDLRTYGVN
                    190                 210
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FIG. 6. Homology between yeast topoisomerase I and vaccinia topoisomerase. Alignment of the yeast (ytopo) and vaccinia (vtopo) amino acid sequences by using the FASTP program (14) revealed the homology shown above. Identical amino acids are bridged by two dots; conservative amino acid changes are indicated by a single dot. The numerical positions of residues within the protein sequences are indicated above the ytopo sequence and below the vtopo sequence. The number denotes the position of the residue at the last digit of the number.

We anticipate that the identification of the topoisomerase gene will facilitate biochemical and genetic analyses. Expression of the enzyme in active form in bacteria should circumvent the limitations inherent in dealing with such small quantities of protein as are obtained from virion extracts. It will be of interest to determine the location of the active site for DNA strand cleavage within the protein molecule, with particular attention to whether the active site lies within the sequence domain shared with yeast topoisomerase I. In addition, mutagenesis of the vaccinia topoisomerase gene should provide insight into the role of this enzyme in vaccinia replication.

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