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Identification and Characterization of the orf Virus Type I Topoisomerase

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Vaccinia virus (VV) and Shope fibroma virus (SFV), representatives of the orthopox and leporipox genera, respectively, encode type I DNA topoisomerases. Here we report that the 957-nt F4R open reading frame of orf virus (OV), a representative of the parapox genus, is predicted to encode a 318-aa protein with extensive homology to these enzymes. The deduced amino acid sequence of F4R has 54.7 and 50.6% identity with the VV and SFV enzymes, respectively. One hundred forty amino acids are predicted to be conserved in all three proteins. The F4R protein was expressed in *Escherichia coli* under the control of an inducible T7 promoter, partially purified, and shown to be a *bona fide* type I topoisomerase. Like the VV enzyme, the OV enzyme relaxed negatively supercoiled DNA in the absence of divalent cations or ATP and formed a transient covalent intermediate with cleaved DNA that could be visualized by SDS–PAGE. Both the noncovalent and covalent protein/DNA complexes could be detected in an electrophoretic mobility shift assay. The initial PCR used to prepare expression constructs yielded a mutant allele of the OV topoisomerase with a G–A transition at nt 677 that was predicted to replace a highly conserved Tyr residue with a Cys. This allele directed the expression of an enzyme which retained noncovalent DNA binding activity but was severely impaired in DNA cleavage and relaxation. Incubation of pUC19 DNA with the wild-type OV or VV enzyme yielded an indistinguishable set of DNA cleavage fragments, although the relative abundance of the fragments differed for the two enzymes. Using a duplex oligonucleotide substrate containing the consensus site for the VV enzyme, we demonstrated that the OV enzyme also cleaved efficiently immediately downstream of the sequence CCCTT↓.

Replication and transcription of double-stranded DNA *in vivo* leads to the accumulation of torsional strain. This strain is resolved by topoisomerases which cleave one (type I enzymes) or both (type II enzymes) DNA strands, form a transient covalent enzyme/DNA complex and allow strand passage, and then rejoin the broken strand(s). The essentiality of these enzymes in prokaryotes and eukaryotes is underscored by their genetic redundancy. The biochemical properties of both type I and type II enzymes have been thoroughly reviewed (Wang, 1985; Osheroff, 1989; Champoux, 1990).

Vaccinia virus (VV), the prototypic orthopoxvirus, encodes a single type I topoisomerase (VV topo) that is packaged into virions (Bauer *et al.*, 1977; Fogelsong and Bauer, 1984; Shaffer and Traktman, 1987; Shuman and Moss, 1987). The enzyme has been well characterized and most closely resembles the eukaryotic type I topoisomerases (Bauer *et al.*, 1977; Fogelsong and Bauer, 1984; Shaffer and Traktman, 1987; Shuman *et al.*, 1988). The predicted and apparent molecular weights of the enzyme are 36,662 and 32,000, respectively (Shuman and Moss, 1987; Shaffer and Traktman, 1987), approximately one-third the size of the enzymes encoded by *S. cerevisiae, S. pombe,* and human cells (Uemura *et al.*, 1987; Thrash *et al.*, 1985; D'Arpa *et al.*, 1988). Nevertheless, the predicted amino acid sequence of the viral enzyme contains numerous regions of homology which are common to all of these enzymes (Shuman and Moss, 1987; Lynn *et al.*, 1989; Upton *et al.*, 1990). The inability to isolate VV recombinants lacking a topoisomerase gene has been taken as evidence that the enzyme is indispensable for viral replication (Shuman *et al.*, 1989a). Although the exact roles the enzyme plays in the viral life cycle have not been determined, it is likely to relieve the torsional stress engendered by transcription (Brill and Sternglanz, 1988; Liu and Wang, 1987; Schultz *et al.*, 1992) and DNA replication (Kim and Wang, 1989; Wang and Liu, 1990). Additional roles in intermolecular recombination (Shuman, 1991a, 1992) and in the resolution of concatemeric replication intermediates to mature genomes have been proposed (DeLange and McFadden, 1990).

Recombinant VV topo can be easily expressed in *Escherichia coli.* Like the enzyme produced during viral infections of mammalian cells (Shaffer and Traktman, 1987), the recombinant protein relaxes negatively su-

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percoiled DNA substrates in the absence of an energy cofactor (Shuman *et al.,* 1988; Klemperer and Traktman, 1993). Relaxation activity is stimulated by magnesium, manganese, calcium, cobalt, and spermidine but is inhibited by copper, zinc, and ATP. Unlike its cellular counterparts, the viral enzyme is resistant to the drug camptothecin (Shuman *et al.,* 1988). The viral enzyme binds to, and cleaves immediately downstream of, the specific target sequence 5′-$^{C}/_{T}$CCTT (Shuman and Prescott, 1990; Shuman, 1991b). The 3′-OH of the cleaved strand becomes covalently linked to Tyr^{274} of the polypeptide (Shaffer and Traktman, 1987; Shuman *et al.,* 1989b).

In conjunction with the ease of producing recombinant enzyme, the small size of the VV enzyme has made it an excellent target for genetic dissection. Isolation of mutants has been pursued primarily by two strategies: site-directed mutagenesis of conserved motifs or selection for alleles which have lost the toxicity associated with constitutive expression of the wild-type enzyme in *E. coli.* No mutants have yet been isolated which fail to bind noncovalently to the recognition sequence. However, a large number of amino acid substitutions have been associated with a reduction or elimination of the ability to form the cleaved complex: Gly^{132}-Asp, Gly^{132}-Ser, Thr^{147}-Ile, Gly^{166}-Glu, Lys^{220}-Asp, Lys^{220}-Ile, Lys^{220}-Asn, Arg^{223}-Gln, Arg^{223}-Glu, Arg^{223}-Gly, Arg^{223}-Lys, Thr^{224}-Gly, Thr^{224}-Pro, Tyr^{225}-Ser, and Tyr^{225}-Arg (Morham and Shuman, 1992; Klemperer and Traktman, 1993). To define residues which might be important in the DNA religation reaction, mutants which induce an SOS response in host bacteria have been sought; alterations of Gly^{226}-Asn appear to diminish rejoining activity (Gupta *et al.,* 1994).

As mentioned above, comparison of the VV enzyme with other type I topoisomerases has identified highly conserved motifs and has guided targeted mutagenesis studies. We considered that a comparison with the topoisomerase encoded by orf virus (OV), a parapoxvirus, might be particularly useful. The life cycle of OV parallels that of vaccinia virus. The genomes share many functional similarities, such as hairpin termini and concatemer resolution sequences, the presence of direct and inverted terminal repeats, and the overall gene content and localization (Mercer *et al.,* 1987; Fraser *et al.,* 1990; Fleming *et al.,* 1993). Indeed, the promoters regulating OV genes can be recognized by the transcriptional machinery encoded by vaccinia virus (Fleming *et al.,* 1992). However, the OV genome has a GC content of 63%, in sharp contrast to the 36% GC content of VV. We postulated that the OV and VV topoisomerases, while likely to be highly homologous, might have evolved differences conducive to manipulating such dissimilar DNA substrates in an optimal fashion.

In this report, we present the complete sequence of the OV type I topoisomerase (OV topo) and its successful expression in *E. coli.* A comparison of its properties with those of the vaccinia enzyme is shown, as is the isolation of a mutant allele of the OV enzyme which is impaired in its ability to cleave and relax DNA substrates.

MATERIALS AND METHODS

Preparation of DNA clones

Genomic OV clones. The cloning of the *Bam*HI F fragment of the OV strain NZ2 into pUC8 (pVU6) has been described (Mercer *et al.,* 1987). A 0.9-kb *Bam*HI/*Kpn*I fragment derived from the adjacent *Bam*HI C fragment was purified on glass beads (Vogelstein and Gillespie, 1979) and cloned into pTZ18R to give the clone pVUDL-TPI1.

Cloning of OV topoisomerase I for expression in E. coli. The complete OV topoisomerase I (OV topo) gene was amplified by PCR using genomic DNA as a template and primers which placed *Eco*RI, *Hin*dIII, and *Nde*I sites at the 5′-end and an *Eco*RI site at the 3′-end of the gene. The PCR product was cloned into pUC7 and introduced into the DH5α strain of *E. coli.* Plasmid DNA was subsequently isolated from a transformed colony and subjected to DNA sequence analysis. The DNA sequence of this PCR-generated OV topo allele differed from that obtained for the genomic allele by a single nucleotide change at position 677 that was predicted to replace Tyr^{226} with a Cys residue. This OV topo allele was recloned as an *Nde*I/*Eco*RI^{blunt} fragment into a pET3 expression vector (Studier *et al.,* 1990) (prepared as an *Nde*I/*Bam*HI^{blunt} linear molecule). The blunted ends were generated by filling in the overhangs left by restriction digestion with the Klenow fragment of *E. coli* polymerase I and dNTPs. This construct, designated pET3c/OV^{Y226C}-topo, directed the synthesis of a mutant topoisomerase with minimal DNA relaxation activity. Generation of a wild-type (*wt*) expression construct was accomplished by replacing the 740-bp *Bam*HI–*Sac*I fragment from pET3c/OV^{Y226C}-topo with the corresponding fragment from the genomic clone pVUDL-TPI1. This pET3c/OV-topo construct was used successfully to produce active OV topo in *E. coli.*

Sequencing and enzymes. Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, the Klenow fragment of *E. coli* DNA polymerase I, Taq DNA polymerase, and Calf intestinal alkaline phosphatase were purchased from Boehringer-Mannheim (Mannheim, Germany and Indianapolis, IN) or New England Biolabs (Beverly, MA) and used according to the manufacturer's instructions. The sequence of the *Bam*HI F fragment which contains the promoter region and the first 109 nt of the OV topo gene has been reported (Fleming *et al.,* 1993). The remainder of the gene sequence was obtained from pVUDL-TPI1. Clones for sequencing were constructed by subcloning fragments of pVUDL-TPI1 into M13 vectors and sequenced by the dideoxy chain termination method of Sanger (Sanger *et al.,* 1977) in microtiter

plates using a T7 DNA polymerase kit supplied by Amersham Corp. (Arlington Heights, IL). Regions containing ambiguities and compressions were resolved by using kits containing Taq DNA polymerase (Pharmacia Biotech, Inc., Piscataway, NJ, and Amersham Corp.). Gel electrophoresis, autoradiography and sequence data analysis have been described previously (Mercer *et al.*, 1987; Fraser *et al.*, 1990). The consensus sequence was derived from individual sequences giving a redundancy of over 5. Sequence analysis of the PCR-derived topo allele used for expression purposes (see above) was performed using a Sequenase kit according to the manufacturer's instructions (United States Biochemical, Cleveland, OH).

Expression of *wt* and mutant alleles of OV topo and *wt* VV topo

The viral topoisomerases were overexpressed in *E. coli* HMS174 transformants carrying pET3c/VV-topo (Klemperer and Traktman, 1993), pET3c/OV-topo, or pET3c/OV^{Y226C}-topo. Cultures were grown in tryptone–phosphate (2% bacto-tryptone, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.8% NaCl, and 1.5% yeast extract) supplemented with 0.2% maltose and 50 μg/ml ampicillin until a density of 4×10^8 cells/ml was obtained. Expression of the topo genes from the upstream T7 promoter was initiated by infection with λCE6 (Studier *et al.*, 1990). After 20 min of absorption at room temperature (multiplicity of infection, 10 PFU/ml), infected cultures were shaken at 37° for 90 min. Cells were collected by centrifugation, washed in M9 buffer (42 m*M* Na_2HPO_4, 22 m*M* KH_2PO_4, 8.6 m*M* NaCl, 18.7 m*M* NH_4Cl, 2 m*M* $MgSO_4$, and 0.2% glucose) and then metabolically labeled for 5 min at 37° in M9 buffer supplemented with 625 μM of each amino acid excluding methionine. [^{35}S]Methionine (DuPont-NEN, Boston, MA) (specific radioactivity of 1200 Ci/mmol) was added to a final concentration of 23 n*M*. Cells were collected by centrifugation, subjected to a cycle of freeze–thawing, and resuspended at 20 times the original culture density in Buffer A (50 m*M* Tris, pH 7.4, 1 m*M* EDTA, 1 m*M* DTT, and 10% glycerol) containing 50 m*M* NaCl. The resulting lysates were sonicated five times for 30 sec each while on ice. Debris was removed by centrifugation for 30 min at 14,000 rpm in a Brinkmann microfuge. The topoisomerases were partially purified from the supernatants as described below. Whole cell extracts of uninduced (harvested just prior to λCE6 infection) or induced cultures were prepared as described above with omission of the final centrifugation. Aliquots representing 0.5 ml of the original culture were analyzed by SDS–PAGE (Laemmli, 1970). The total protein concentration within the whole cell and soluble extracts, and the phosphocellulose (p-cell) eluants described below, were determined with a colorimetric Bio-Rad (Hercules, CA) protein assay, using lysozyme as a standard.

Purification of type I topoisomerases

The topoisomerases were partially purified from bacterial lysates using a batch p-cell method of purification, as described previously (Klemperer and Traktman, 1993). The p-cell resin was equilibrated with Buffer A containing 0.25 *M* NaCl and a 50% slurry was prepared. Clarified bacterial lysates (see above) (0.6 ml) were mixed with an equal volume of the 50% slurry and incubated with end-over-end rotation for 15 min at 4°. The p-cell resin was collected by centrifugation at 2000 rpm for 2 min in a Brinkman microfuge and the supernatant was discarded. A similar elution with 0.6 ml of Buffer A containing 0.5 *M* NaCl was performed. Finally, topo was eluted into 0.6 ml of Buffer A containing 1 *M* NaCl. The salt content of this fraction was reduced to less than 2 m*M* NaCl by dialysis against Buffer A. This was used as the source of enzyme for all assays.

The partially purified fractions were resolved on SDS–polyacrylamide gels that were subsequently stained with Coomassie blue; the concentration of topoisomerase was estimated by comparison to known amounts of carbonic anhydrase. The concentration of VV, wt OV, and OV^{Y226C} topoisomerases in the final fractions was estimated to be 225, 150, and 19 ng/μl, respectively.

Topoisomerase assays

Relaxation of a supercoiled DNA substrate. Enzymatic assay of the VV and OV topoisomerases involved monitoring the relaxation of supercoiled plasmid (pUC19) DNA (Shaffer and Traktman, 1987; Klemperer and Traktman, 1993). To rid pUC19 of contaminating impurities prior to inclusion in relaxation assays, the DNA was passed through an Elutip membrane (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. Standard relaxation reactions (20 μl) contained 150 ng of pUC19 DNA, 40 m*M* Tris, pH 7.4, 0.1 *M* NaCl, 2.5 m*M* EDTA, and varying amounts of the partially purified topoisomerases. Dilutions of the p-cell eluants were prepared in a buffer containing 0.5 mg/ml BSA, 30 m*M* KPO_4, pH 7.0, 0.5 m*M* DTT, and 5% glycerol. Following a 15-min incubation at 37°, reactions were quenched with 5 μl of sample buffer containing 50% glycerol, 2% SDS, 0.1 *M* EDTA, and bromophenol blue. Samples were resolved by electrophoresis on horizontal 1.3% agarose gels cast and run in 0.5× TBE (50 m*M* Tris, 41.5 m*M* boric acid, and 0.5 m*M* EDTA) at 2.5 V/cm for 12 hr. Gels were stained with ethidium bromide and photographed under uv illumination.

Preparation of radiolabeled DNA substrates. For label transfer assays, plasmid DNA was nick translated (Rigby *et al.*, 1977) using all four α-^{32}P-labeled dNTPs to a specific radioactivity of $>5 \times 10^7$ cpm/μg. Oligonucleotide substrates for cleavage and gel shift assays were prepared with an Applied Biosystems 391 DNA synthesizer

(Foster City, CA). The 24-mer oligo duplex synthesized contains the VV topo consensus cleavage site ($^{C}/_{T}$CCTT$^{\downarrow}$) (Shuman and Prescott, 1990; Shuman, 1991b)

5′ CGT GTC GCC CTT$^{\downarrow}$ ATT CCG ATA GTG

GCA CAG CGG GAA TAA GGC TAT CAC.

The 5′ terminus of the upper (cleaved) strand was radiolabeled using [γ^{32}P]ATP and T4 polynucleotide kinase. The labeled oligonucleotide was subjected to gel purification prior to hybridization to the lower strand. To radiolabel the 3′ end of the upper (cleaved) strand of the DNA duplex, the following oligos were allowed to hybridize:

5′ CGT GTC GCC CTT$^{\downarrow}$ ATT CCG ATA G

GCA CAG CGG GAA TAA GGC TAT CAC.

The upper strand was radiolabeled using the Klenow fragment of DNA polymerase, [α-^{32}P]TTP and 0.2 m*M* dGTP. The duplex was gel-purified and used as substrate for the formation of covalent and noncovalent topoisomerase–DNA complexes, as described below.

For the generation of end-labeled pUC19 DNA, the restriction enzyme *Xba*I was used to cleave the plasmid at a unique site. The 3′-termini of both strands were then radiolabeled using the Klenow fragment of DNA polymerase, [α-^{32}P]dCTP and 0.2 m*M* TTP. Subsequent digestion with *Eco*RI allowed the purification of a singly radiolabeled DNA fragment of 2.66 kb.

Label transfer: Visualization of the covalent protein–DNA intermediate. Two different DNA substrates, pUC19 and a 24-mer oligo duplex, were used for detection of the covalent DNA-topoisomerase I intermediate. Partially purified, dialyzed p-cell eluants were used as the source of topoisomerase. Reactions (20 μl) performed with the first substrate contained 2.5 pmol of each partially purified enzyme, 45 fmol of uniformly radiolabeled pUC 19, 20 m*M* Tris, pH 7.4, 0.4 m*M* EDTA, 0.4 m*M* DTT, and 4% glycerol. Following a 15-min incubation at 37°, DNA not covalently associated with topoisomerase was digested by addition of DNase I and $MgCl_2$ (15 μl) to final concentrations of 300 μg/ml and 15 m*M,* respectively (Shaffer and Traktman, 1987). After an additional 20 min of incubation at 37°, reactions were stopped by the addition of SDS-sample buffer, heated at 100° for 5 min, and fractionated by electrophoresis on SDS–12% polyacrylamide gels. Gels were fixed and dried and the DNA–topoisomerase I intermediates were visualized by autoradiography.

For the oligonucleotide duplex substrate, reactions (20 μl) containing 6 pmol of each partially purified topoisomerase, 100 fmol of 5′-^{32}P-labeled 24-mer oligo duplex, 20 m*M* Tris, pH 7.4, 12.5 m*M* NaCl, 0.4 m*M* EDTA, and 0.4 m*M* DTT were incubated at 37° for 15 min. Reactions were quenched by the addition of SDS-sample buffer, heated at 100° for 5 min, and fractionated by electrophoresis on SDS–12% polyacrylamide gels.

DNA cleavage assay. End-labeled pUC19 DNA was used to compare the cleavage specificities of the VV and OV topoisomerases (Shaffer and Traktman, 1987; Shuman and Prescott, 1990). Reactions (20 μl) containing either 12 pmol of OV topo or 8 pmol of VV topo, 14 fmol of 3′-labeled pUC19, 20 m*M* Tris, pH 7.4, 0.25 m*M* DTT, 0.25 m*M* EDTA, and 2.5% glycerol were incubated at 37° for 5 min. Following the addition of 1% SDS, samples were heated at 100° for 3 min, immediately cooled on ice and supplemented with NaOH (30 m*M*), EDTA (2 m*M*), and glycerol (10%). Samples were fractionated through horizontal alkaline agarose gels (1.2% agarose containing 30 m*M* NaOH and 2 m*M* EDTA) by electrophoresis at 2 V/cm. Gels were dried by blotting and examined by autoradiography.

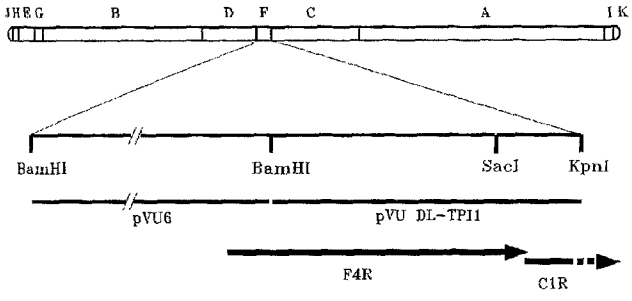
Visualization of covalent and noncovalent protein–DNA complexes by native gel electrophoresis. Reactions (20 μl) contained 11 and 16 pmol of OV and VV topo, respectively, 300 fmol 3′-^{32}P-labeled 24-mer oligo duplex, 10 m*M* Tris, pH 7.4, 0.4 m*M* DTT, 0.4 m*M* EDTA, and 4% glycerol. Following a 5-min incubation at 37°, glycerol was added to 10% and samples were fractionated at 4° through nondenaturing polyacrylamide gels (5% acrylamide, 0.25% bisacrylamide) cast and run in 0.25× TBE. Electrophoresis was performed in the cold at 15 V/cm. The gels were examined by autoradiography.

To compare the size of the cleaved products, 10-μl reactions containing 8.5 pmol of topo I, 300 fmol 3′-^{32}P-labeled 24-mer oligo duplex, 25 m*M* Tris, pH 7.4, 0.5 m*M* DTT, 0.5 m*M* EDTA, and 5% glycerol were incubated at 37° for 5 min. Following the addition of formamide (to 45%) and TBE (to 1×), samples were heated at 100° for 5 min and fractionated on urea–polyacrylamide gels (50% urea, 20% acrylamide, and 1% bisacrylamide, cast and run in 1× TBE). Gels were examined by autoradiography.

RESULTS

Mapping and sequence analysis of the OV topo gene

Open reading frame (ORF) F4R, potentially encoding the OV topo, had previously been identified at the right end of the centrally located *Bam*HI F fragment and the sequence of 109 bp at the 5′-end determined (Fleming *et al.,* 1993). The cleavage sites of a number of restriction endonucleases were mapped in the adjacent *Bam*HI C fragment and a convenient *Bam*HI/*Kpn*I fragment, potentially large enough to contain the remainder of the gene, was cloned and sequenced. The sequence of 157 nt of the *Bam*HI F fragment, and the 1024 *Bam*HI/*Kpn*I fragment, is shown in Fig. 1. To ensure that there was only a single *Bam*HI site (and no additional sequence) between the *Bam*HI F and C fragments, a fragment containing the gene was amplified using PCR. The resulting




```
      F3R >
221   K  A  A  A  C  K  K  *                                  F4R >
  1                                                          M  R  A  L
  1   AAGGCCGCCGCCTGCAAGAAGTAGGCGCACTAAATAGCGAGGCTCGGTATGCGGGCGCTG

  5   H  L  S  D  G  K  L  F  F  D  K  E  L  T  Q  P  V  P  D  D
 61   CACCTGTCAGACGGCAAACTTTTTTTTGACAAGGAGCTGACGCAGCCGGTCCCCGACGAC

 25   N  P  A  Y  A  V  L  A  K  I  R  I  P  P  H  L  S  D  V  V
121   AACCCCGCGTACGCTGTCCTTGCGAAGATCCGGATCCCACCGCACCTCTCGGATGTGGTC

 45   V  Y  E  Q  D  L  E  S  A  Q  Q  G  L  I  F  V  G  R  D  A
181   GTGTACGAGCAGGACCTCGAGTCTGCGCAGCAGGGCCTCATCTTCGTCGGGCGCGACGCC

 65   K  G  R  K  Q  Y  F  Y  G  R  G  H  V  E  R  R  T  A  V  R
241   AAGGGCCGAAAGCAGTACTTCTACGGGCGCGGACACGTGGAGCGGCGCACGGCCGTCCGC

 85   N  A  V  F  V  R  V  H  R  V  M  N  K  I  N  A  F  I  D  D
301   AACGCCGTGTTCGTGCGCGTGCACCGCGTCATGAACAAGATAAACGCCTTCATCGACGAC

105   H  L  A  S  G  S  E  A  E  A  Q  M  A  A  F  L  L  M  E  T
361   CACCTCGCCTCCGGCAGCGAGGCCGAGGCGCAGATGGCCGCCTTCCTGCTCATGGAGACG

125   S  F  F  I  R  V  G  K  T  R  Y  E  R  E  S  G  T  V  G  M
421   AGCTTCTTCATCCGCGTCGGCAAGACGCGCTACGAGCGCGAGAGCGGCACCGTGGGCATG

145   L  T  L  R  N  K  H  L  A  E  A  E  G  G  E  E  I  R  V  A
481   CTCACGCTGCGCAACAAGCACCTCGCCGAGGCCGAGGGCGGTGAGGAGATCCGCGTCGCC

165   F  V  G  K  D  R  V  A  H  E  F  A  V  R  E  G  Q  R  L  F
541   TTCGTGGGCAAGGACCGAGTCGCGCACGAGTTTGCCGTGCGCGAGGGGCAGCGGCTCTTC

185   A  A  L  R  R  L  W  D  P  G  A  P  D  R  L  L  F  D  R  L
601   GCGGCGCTGCGTCGGCTCTGGGACCCGGGCGCGCCCGACAGGCTGCTGTTCGACCGGCTG

205   S  E  R  R  V  Y  T  F  M  R  R  F  G  I  R  V  K  D  L  R
661   AGCGAGCGCCGCGTGTACACCTTCATGCGACGCTTCGGCATCCGCGTCAAGGACCTGCGC

225   T  Y  G  V  N  Y  T  F  L  Y  N  F  W  S  N  V  R  S  L  E
721   ACCTACGGCGTGAACTACACCTTCCTGTACAACTTCTGGTCCAACGTGCGCTCGCTGGAG

245   P  R  P  S  V  K  S  L  I  C  T  S  V  R  Q  T  A  E  T  V
781   CCGCGTCCCTCCGTGAAGTCGCTCATCTGCACCTCCGTGCGGCAGACCGCCGAGACGGTG

265   G  H  T  P  S  I  S  R  S  A  Y  M  A  T  A  V  L  E  L  V
841   GGGCACACGCCCTCGATCTCGCGCAGCGCCTACATGGCCACCGCGGTGCTCGAGCTCGTC

285   R  D  G  A  F  L  D  R  V  A  A  T  D  T  L  D  D  F  V  D
901   AGGGACGGCGCGTTCCTGGACAGAGTCGCCGCCACCGACACGCTCGACGACTTCGTGGAC

                                  C1R >
  1                               M  D  E  A  L  R  V  A
305   I  V  V  D  Y  V  N  N  S  E  Q  V  N  G  *
961   ATCGTCGTGGACTATGTAAATAACTCTGAGCAGGTAAATGGATGAGGCGCTGCGCGTGGC

  9   A  R  V  V  D  G  L  R  P  L  D  V  A  V  C  L  A  Q  L  R
1021  GGCGCGCGTCGTGGACGGGCTCCGGCCGCTGGACGTGGCCGTGTGTCTCGCGCAGCTGCG

 29   G  A  A  P  E  R  R  F  P  A  L  D  E  C  S  G  E  A  F  L
1081  CGGAGCCGCGCCCGAGCGCCGCTTCCCGGCGCTCGACGAGTGCTCCGGCGAGGCCTTCCT

 49   D  F  E  F  A  G  G  D  V  A  S  R  Y
1141  GGACTTTGAGTTCGCCGGCGGGGACGTGGCGTCGCGGTACC
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FIG. 1. Map location and sequence analysis of the OV F4R ORF. The *Bam*HI restriction endonuclease cleavage site map for OV strain NZ2 is shown at the top of the figure. The expanded map below it shows the relative positions of restriction endonuclease cleavage sites relevant to the DNA manipulations undertaken (see Materials and Methods). Below and to the same scale as the expanded map are the positions and designations of the plasmids used in the analyses and below that the relative locations of the F4R ORF and the 5′-end of the ORF designated C1R. The arrows indicate the orientation of transcription of these ORFs. The nucleotide sequence encompassing F4R and the 5′-end of C1R is shown below the maps. The sequence from nt 1 to 157, which represents the 3′-terminus of the OV *Bam*HI fragment, has been published previously (Fleming *et al.*, 1993) and is equivalent to nts 3192 to 3348 of the DNA sequence reported in that publication. The deduced amino acid sequences of the carboxy-terminus of ORF F3R, the complete F4R ORF, and the amino-terminus of ORF C1R are shown above the nt sequence. The putative late transcriptional initiation sites for F4R and C1R are underlined.

fragment was cloned and the sequence across the *Bam*HI junction confirmed.

The F4R ORF extends from the *Bam*HI F fragment into the *Bam*HI C and is 957 nt in length. The ORF is predicted to encode a protein of 318 aa, 4 aa larger than the topoisomerases encoded by the VV H6R and Shope fibroma virus (SFV) D2 genes (Shuman and Moss, 1987; Upton *et al.*, 1990). The predicted molecular weight of OV topo is 36,182. Alignment of the deduced amino acid sequences of the three poxviral genes using CLUSTAL (Higgins and Sharp, 1988) shows a high degree of homology, with 140 aa (44%) being invariant in all three proteins (Fig. 2). A comparison of the OV protein with that of VV gave an identity of 54.7% with conservative changes accounting for an additional 32.9%. With SFV, the identity was 50.6% with 34% conservative changes. The SFV and VV sequences are somewhat more closely related, being 61% identical with an additional 15% conservative changes (Upton *et al.*, 1990). The active site *Tyr* present at residue 274 in VV and 273 in SFV is conserved in OV at residue 275.

F4R is likely to be expressed late in infection as it is preceded by the late poxvirus transcription motif, TAAAT, 13 nt upstream of the predicted initiation codon. The sequence from the 3′-end of the putative topoisomerase I gene to the downstream *Kpn*I site was translated in all six frames, revealing one frame (C1R) predicted to encode 61 aa with 33% identity to the SFV ORF D2, and 16% identity to the VV (Copenhagen strain) ORF H7R. These SFV and VV ORFs also lie immediately downstream of the respective topoisomerase genes. A difference between C1R and its counterparts in VV and SFV is that its 5′-end overlaps the 3′-end of the topoisomerase gene by 5 nt as a consequence of the longer topoisomerase coding region.

Overexpression and purification of OV DNA topoisomerase

To demonstrate that the F4R ORF does indeed encode a DNA topoisomerase I, the gene was overexpressed in *E. coli* using the T7 RNA polymerase-driven pET vector system (Studier *et al.*, 1990). The topoisomerase genes were induced by infection of pET/VV-topo (Klemperer and Traktman, 1993) and pET/OV-topo transformants with λCE6. When soluble lysates of induced cultures were analyzed by SDS–PAGE, abundant protein species with

```
orftopo       1 MRALHLSDGKLFFDKELTQPVPDDNPAYAVLAKIRIPPHLSDVVVYEQDLESAQQGLIFV
vactopo       1 MRALFYKDGKLFTDNNFLNPVSDDNPAYEVLQHVKIPTHLTDVVVYEQTWEEALTRLIFV
shopetopo     1 MRAFTYKDGKLYEDKELTIPVHCSNPTYEILKHVKIPSHLTDVIVYEQTYEQSLSRLIFV
                ***.   ****. *...  **   **.* .*  ..** **.**.****  * .   ****

orftopo      60 GRDAKGRKQYFYGRGHVERRTAVRNAVFVRVHRVMNKINAFIDDHLA-SGSEAEAQMAAF
vactopo      60 GSDSKGRRQYFYGKMHVQNRNAKRDRIFVRVYNVMKRINCFINKNIKKSSTDSNYQLAVF
shopetopo    60 GLDSKGRRQYFYGKMHVQRRNSARDTIFIKVHRVIDKIHKFIDDTIEHKN-DVLFQLGVF
                * *.***.*****. **. * . *. .*..*  *. .*. **.  .     .  *.. *

orftopo     120 LLMETSFFIRVGKTRYERESGTVGMLTLRNKHLAEAEGGEEIRVAFVGKDRVAHEFAVRE
vactopo     121 MLMETMFFIRFGKMKYLKENETVGLLTLKNKHIEISP--DEIVIKFVGKDKVSHEFVVHK
shopetopo   120 MLMETSFFIRMGKVKYLKENDTVGLLTLKNKNIVREN--RKILIHFVGKDKIIHNFTVHS
                .**** **** ** .* .*. ***.***.**..       * . *****.. *.* *.

orftopo     180 GQRLFAALRRLWDPGAPDRLLFDRLSERRVYTFMRRFGIRVKDLRTYGVNYTFLYNFWSN
vactopo     179 SNRLYKPLLKLTDDSSPEEFLFNKLSERKVYECIKQFGIRIKDLRTYGVNYTFLYNFWTN
shopetopo   178 SNRLYKPLLRLIGRKEPDSFLFHKLSEKKVYKAVQQFGIRLKDLRTYGVNYTFLYNFWTN
                ..**. .* .* .   *. .**..***..**  ...****.*****************.*

orftopo     240 VRSLEPRPSVKSLICTSVRQTAETVGHTPSISRSAYMATAVLE-LVRDGAFLDRVAATDT
vactopo     239 VKSISPLPSPKKLIALTIKQTAEVVGHTPSISKRAYMATTILE-MVKDKNFLDVVSKT-T
shopetopo   238 VKSLNPIPPIKKMISTSIKQTADIVGHTPSISKRAYIANTVLEYLTHDSELINTIRDI-S
                *.*. * *. * .*  ...***. ********. **.* ..** .  *  ... .    .

orftopo     299 LDDFVDIVVDYVNNSEQVNG
vactopo     297 FDEFLSIVVDHVKSSTDG--
shopetopo   297 FDEFIRLITDYITNTQTV--
                .*.*. .. * . ..
```

FIG. 2. Alignment of the deduced amino acid sequences of poxvirus type I topoisomerases. The amino acid sequences of the OV (orftopo), VV (vactopo) (Rosel *et al.,* 1986; Goebel *et al.,* 1990; Shuman and Moss, 1987), and SFV (shopetopo) (Upton *et al.,* 1990) type I topoisomerases, deduced from the respective DNA sequences, were aligned using the program CLUSTAL (Higgins and Sharp, 1988). Amino acid residues common to all sequences (*) and residues that are similar (.) are marked below the alignments.

apparent molecular masses of 32 and 35.5 kDa were seen, representing the VV (closed circle) and OV topoisomerases (open circle), respectively (Fig. 3A). Unequivocal identification of these species as the overexpressed proteins was obtained when a comparison of cultures pulse-labeled pre- and postinduction with [^{35}S]-methionine was made (Fig. 3B). The anomolously rapid migration of the VV protein, which has a predicted molecular mass of 36.7 kDa, has been seen before (Shaffer and Traktman, 1987; Shuman *et al.,* 1988; Klemperer and Traktman, 1993).

The level of expression of OV topoisomerase was about half of that detected for the VV enzyme. Although VV and OV topoisomerases have 54.7% amino acid identity, the codon usage is quite different as a result of the vastly different GC content. This variable may affect the efficiency of translation within *E. coli.* Because of the reduced level of expression, we found it desirable to maximize the solubility of the OV topo. About 50% of the DNA topoisomerase produced was soluble when cultures were grown in LB medium. The use of an enriched growth medium, such as tryptone-phosphate, has elsewhere been shown to increase the solubility of overexpressed proteins by limiting inclusion body formation (Moore *et al.,* 1993). Culturing of our pET-topo transformants in this enriched medium similarly increased the fraction of target protein recovered in the soluble fraction (data not shown); this protocol was followed for all further experiments.

OV and VV topoisomerases were partially purified from soluble extracts by batch purification on p-cell resin. Both proteins bound tightly to the resin, with 1 *M* NaCl required for elution. The eluants were highly enriched for the respective topoisomerase proteins (Fig. 3A, lanes 4 and 8) and were used for all further biochemical analyses.

Comparison of VV and OV activities

Relaxation of supercoiled DNA. The ability to relax negatively supercoiled DNA is the definitive assay for topoisomerase activity. Supercoiled substrates migrate more rapidly than relaxed products, permitting the clear resolution of the two species on agarose gels. The inclusion of EDTA in our relaxation assays ensured that any activity observed reflected the VV and OV enzymes, because the *E. coli* enzymes have a strict requirement for a divalent cation (Wang, 1985; Champoux, 1990). The relative specific activities of the partially purified VV and OV enzymes were assessed by endpoint dilution: assays contained 2.5 pmol, 0.5 pmol, 0.1 pmol, or 20 fmol of topo per reaction (Fig. 4). The VV and OV topoisomerases were shown

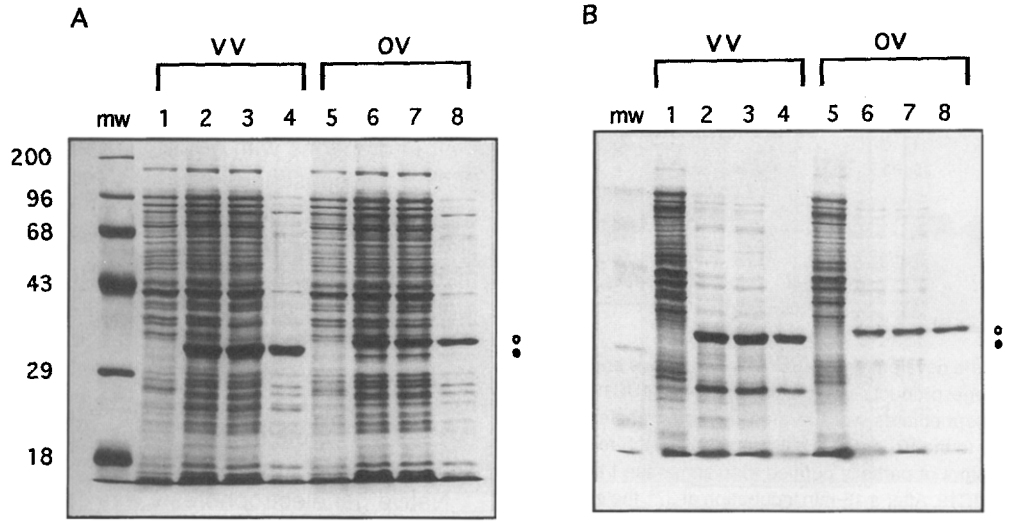

FIG. 3. Visualization of overexpressed and partially purified OV and VV topoisomerases. HMS174 transformants carrying the pET/VV-topo or pET/OV-topo plasmids were induced by infection with λCE6 and harvested as described under Materials and Methods. Lysates were analyzed on 12% SDS-polyacrylamide gels. (A) Proteins visualized by staining with Coomassie brillant blue dye; (B) an autoradiograph of lysates prepared from cultures metabolically labeled with [^{35}S]methionine as described under Materials and Methods. Whether lysates were prepared from cultures containing the pet/VV-topo or pet/OV-topo plasmid is indicated above the relevant lanes. The lanes contain the following samples: lanes 1 and 5, whole cell lysates from uninduced cultures (15 μg protein); lanes 2 and 6, whole cell lysates prepared at 90 min after induction with λCE6 (33 μg protein); lanes 3 and 7, soluble lysates prepared from induced cultures (29 μg protein); lanes 4 and 8, enriched topoisomerase preparations purified by phosphocellulose chromatography (8 μg protein). The concentrations of the VV and OV topoisomerases within the p-cell eluants were estimated to be 225 and 150 ng/μl, respectively. The VV (closed circle) and OV (open circle) topoisomerase species are indicated to the right of each panel. The lane marked *mw* contains protein standards whose molecular weights ($\times 10^{-3}$) are indicated at the left.

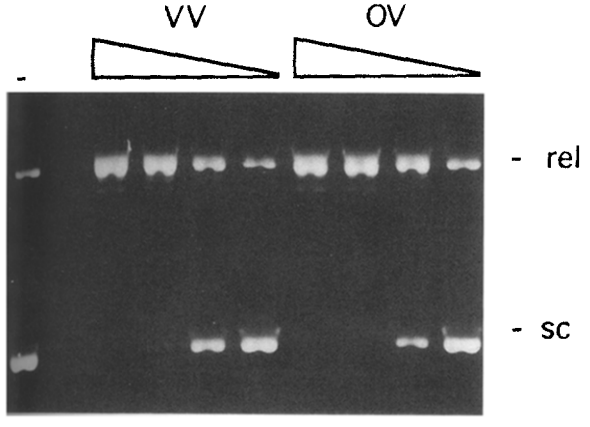

FIG. 4. Relaxation of supercoiled DNA by OV and VV topoisomerases. Partially purified preparations of the VV and OV DNA topoisomerases were assayed for their ability to relax supercoiled DNA. Reactions containing 150 fmol pUC19 and various dilutions of the phosphocellulose-purified enzymes were incubated at 37° for 15 min, quenched, and fractionated by electrophoresis through agarose gels. DNA was visualized by uv illumination after staining with ethidium bromide. A series of four reactions is shown for both the VV and OV topoisomerases. The amount of enzyme assayed decreases from left to right in each series; the four reactions shown contained 2.5, 0.5, 0.1, and 0.02 pmol of the relevant enzyme. A control in which no phosphocellulose eluant was added to the reaction (−) is also shown. The positions to which relaxed (rel) and supercoiled (sc) DNA species migrated are indicated to the right. The material seen in the control reaction that comigrates with the relaxed product represents small amounts of nicked, circular DNA that is present in the initial substrate.

to relax supercoiled DNA to a similar extent within this concentration range.

Visualization of the covalent DNA-topoisomerase intermediate using pUC19. Eukaryotic type I topoisomerases form a covalent complex with DNA in which the enzyme is covalently bound to the 3′ phosphoryl group at the site of cleavage (Champoux, 1990). VV topo is unusual among eukaryotic topoisomerases in that the covalent DNA/topoisomerase complex is stable enough to be trapped by the addition of a protein denaturant. This complex can be visualized by the transfer of radioactivity from a labeled DNA substrate to the enzyme (Shaffer and Traktman, 1987). In contrast, visualization of the covalent complex formed by the human topoisomerase requires the inclusion of pharmacological agents which inhibit the DNA religation reaction, forcing an atypical accumulation of the covalent protein/DNA intermediate. To assess the ability of the OV topo to form the covalent intermediate, the enzymes were incubated with uniformly ^{32}P-labeled pUC19. Proteins were fractionated by SDS-PAGE, and the protein/DNA adducts were visualized by autoradiography (Fig. 5A). The intermediates formed by both the VV and OV topo migrated as doublets. The OV intermediates migrated more slowly than those formed by the VV enzyme, consistent with the difference in the observed electrophoretic mobilities of the overexpressed proteins (Fig. 3). From these data we conclude that the OV enzyme is mechanistically similar to the VV topo in its ability to form a stable covalent intermediate.

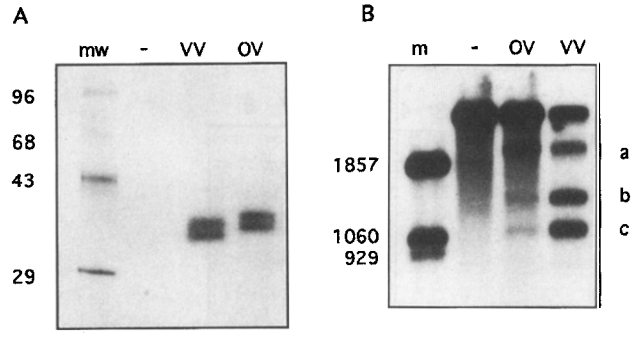

FIG. 5. Formation of the covalent enzyme/DNA intermediate and generation of DNA cleavage products using a radiolabeled pUC19 substrate. (A) Covalent intermediates were formed by label transfer from nick-translated pUC19 to the VV or OV topoisomerase I. Each reaction (20 μl) contained 2.5 pmol of partially purified topoisomerase I and 45 fmol of radiolabeled pUC19. After a 15-min incubation at 37°, the excess DNA was digested with DNase I. The samples were fractionated by SDS–12% PAGE and visualized by autoradiography. A control in which no phosphocellulose eluant was added (–) is also shown. The lane marked mw contains ^{14}C-labeled protein standards whose molecular weights ($\times 10^{-3}$) are indicated at the left. (B) Analysis of VV and OV topoisomerase cleavage specificities using a linearized pUC19 DNA substrate containing a 3′-radiolabel on one DNA strand. Reactions containing 14 fmol of radiolabeled pUC19 DNA and either 12 pmol of OV topoisomerase or 8 pmol of VV topoisomerase were incubated at 37° for 5 min. Products were resolved by electrophoresis under denaturing conditions and visualized by autoradiography. The source of the partially purified enzyme is indicated above each lane (OV and VV); cleavage products denoted a, b, and c are indicated to the right. A control reaction in which no phosphocellulose eluant was added is also shown (–). Lane m represents radiolabeled DNA standards whose sizes in nucleotides are indicated to the left.

OV and VV DNA topoisomerases yield similar cleavage patterns when incubated with end-labeled pUC19 DNA. Because of the difference in GC content between the OV and VV genomes, we considered it worthwhile to determine whether or not the two viral enzymes cleave DNA at different recognition sequences. To locate the sites of strand cleavage, 3′-radiolabeled pUC19 DNA was used as a substrate. Upon cleavage at a given site, the topoisomerase remains covalently associated with the nonradiolabeled fragment, and therefore the mobility of the labeled fragments is not affected. Following a brief incubation of substrate and enzyme, SDS was added to trap the cleaved complexes (and prevent religation) and the cleavage products were analyzed on denaturing alkaline agarose gels. Incubation with either poxviral DNA topoisomerase resulted in the production of three main cleavage products (a, b, and c) which migrated more rapidly through the gel than the uncleaved substrate (Fig. 5B). The products generated by both enzymes had indistinguishable mobilities, suggesting that the enzymes bind to and cleave the same sites within the DNA substrate. Product "a" was most abundant when cleavage was performed with OV topo, whereas "b and c" were the predominant products generated by VV topo.

OV topo binds to and cleaves an oligonucleotide duplex containing the VV topo consensus cleavage site. The apparent commonality of the preferred cleavage sites of the VV and OV topos was investigated further by studying their interactions with an oligonucleotide substrate containing the consensus site for VV topo recognition and cleavage. The autoradiograph of an SDS–PAGE gel shown in Fig. 6A depicts the labeled protein/DNA intermediates (arrows) formed during cleavage of this 5′-radiolabeled oligo duplex. It is apparent that the OV enzyme cleaves this substrate with high efficiency. As was seen when the assay was performed with a radiolabeled plasmid substrate (Fig. 5A), the covalent intermediate formed by the OV enzyme migrates more slowly than that generated by the VV topo.

Native gel electrophoresis was also used to visualize the interactions between the enzymes and the oligo substrate. This gel-shift assay permits the visualization of both the covalent DNA/protein intermediate and the noncovalent complex whose formation precedes catalysis (Morham and Shuman, 1992; Klemperer and Traktman, 1993). When an oligonucleotide duplex is radiolabeled at the 3′-end of the cleavable strand, the labeled DNA fragment is released upon cleavage and migrates more rapidly than the noncovalent complex or the free substrate. The autoradiograph shown in Fig. 6B illustrates the formation of both types of complex by the VV and OV enzymes. Although the apparent molecular weight of the OV protein is greater than that of the VV protein, the noncovalent complex formed by the OV topoisomerase migrated more rapidly than the VV complex. The structure of the noncovalent complexes formed by the two proteins may therefore be somewhat different, affecting their electrophoretic mobilities under these assay conditions. The radiolabeled strand released upon cleavage and covalent complex formation is indicated near the bottom of the gel. Although a lower level of cleavage product was seen with the OV enzyme in the experiment shown in Fig. 6B, cleavage levels comparable to that seen with VV topo were more usual. To confirm that the OV and VV enzymes cleaved at precisely the same position within the oligonucleotide substrate, the radiolabeled cleavage fragments were examined on high resolution denaturing gels (Fig. 6C). The 12-nt fragments released by the two enzymes had indistinguishable electrophoretic mobilities. In sum, these data demonstrated that the OV enzyme recognized and cleaved the optimal target sequence of the VV topoisomerase.

OV^{Y226C} contains a point mutation which alters the enzymatic activity of the enzyme

As described above (see Materials and Methods), our initial cloning of a PCR-derived OV-topo allele yielded a mutant with a reduced ability to relax supercoiled DNA.

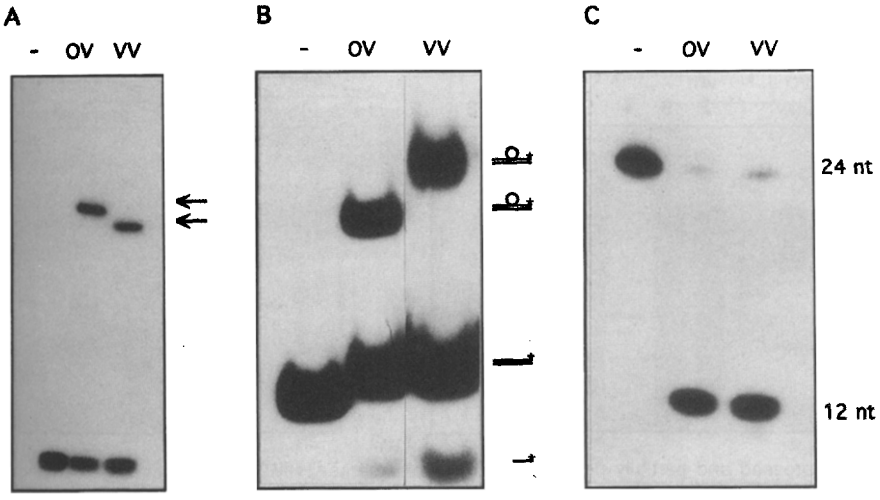

FIG. 6. Noncovalent and covalent interactions between the OV and VV topoisomerases and a radiolabeled oligonucleotide substrate. A 24-mer oligonucleotide duplex which contains the VV topoisomerase I consensus cleavage site (CCTTT↓) was radiolabeled at the 5′- or 3′-terminus of the upper (cleaved) strand and used in reactions containing partially purified VV or OV topoisomerases (indicated above each lane). Lanes marked – represent controls in which no enzyme was added. (A) DNA–topoisomerase covalent intermediates were formed between the 5′-labeled oligo duplex and topoisomerase I and visualized following fractionation on SDS–12% polyacrylamide gels. Reactions contained 6 pmol of each partially purified topoisomerase and 100 fmol of 5′-radiolabeled 24-mer oligo duplex. The arrows to the right of the autoradiograph denote the covalent DNA–topoisomerase complexes formed by the two enzymes. (B) Covalent and noncovalent DNA–topoisomerase complexes were formed between the 3′-radiolabeled oligo duplex and topoisomerase I and visualized following resolution through nondenaturing 5% polyacrylamide gels cast and run in 0.25× TBE. Reactions contained 300 fmol of 3′-radiolabeled 24-mer oligo duplex and 11 pmol of the OV topoisomerase or 16 pmol of the VV topoisomerase. Symbols to the right of the autoradiograph identify each species as representing the radiolabeled (*) substrate with or without bound topoisomerase (circle) and before or after topoisomerase-mediated cleavage. (C) The 3′-radiolabeled fragments released upon cleavage of the oligonucleotide substrate by the OV and VV topoisomerase were examined by high-resolution urea–acrylamide electrophoresis. Reactions contained 8.5 pmol of VV or OV topoisomerase, as indicated, and 300 fmol of 3′-radiolabeled 24-mer oligo duplex. The sizes of the labeled DNA strands are indicated to the right of the autoradiograph.

Both the *wt* (OV) and mutant allele of the OV topoisomerase (OV^{Y226C}) were shown to produce a protein of apparent molecular weight 35,500 (Fig. 7). The mutation within the OV^{Y226C} allele reduced both the solubility and the accumulation of the protein, although sufficient soluble enzyme was purified using the batch p-cell protocol to allow a comparative analysis (Fig. 7). OV^{Y226C} topoisomerase showed an approximately 25-fold reduction in its ability to relax supercoiled DNA relative to the *wt* OV enzyme (Fig. 8A). A similar decrease in activity was seen when the enzymes were assayed for their ability to form a covalent intermediate with radiolabeled pUC 19 DNA Fig. 8B). The OV^{Y226C} enzyme was not deficient in its ability to bind DNA noncovalently, however, as demonstrated both by gel shift and filter binding analyses (data not shown). Therefore, the mutation affects the ability of the enzyme to cleave DNA, reducing the amount of covalent enzyme/DNA intermediate formed. The mutation in this allele is an A to G transition at nt 677 of the open reading frame. The Tyr^{226} residue affected lies within a region which is highly conserved in many type I topoisomerases (Fig. 8C). The analogous domain of VV topo, including the corresponding Tyr residue, has been extensively mutagenized and shown to be involved in DNA cleavage/covalent intermediate formation (Klemperer and Traktman, 1993).

DISCUSSION

The autonomy of poxvirus replication from host functions is reflected in the cytoplasmic localization of the infectious cycle. Poxviruses encode most, if not all, of the proteins required for the regulated transcription and replication of the viral genome (Moss, 1990; Traktman, 1990a,b, 1991). Because both of these processes perturb DNA topology, the discovery that the prototypic *Orthopoxvirus,* vaccinia, encodes and encapsidates a type I topoisomerase was not unanticipated (Bauer *et al.,* 1977; Shaffer and Traktman, 1987; Shuman and Moss, 1987). Shope fibroma virus, a *Leporipoxvirus,* encodes a type I topoisomerase with 61% identity to that of VV (Upton *et al.,* 1990). Here we report the DNA sequence, overexpression, and biochemical characterization of the topoisomerase encoded by orf virus, a *Parapoxvirus.* The enzyme is encoded by the 957-bp F4R ORF, which initiates within the *Bam*HI F fragment of the OV genome and extends into the adjacent *Bam*HI C fragment. The gene is predicted to encode a protein of 318 aa with a molecular weight of 36,182.

Extensive homology is apparent upon comparison of the topoisomerases encoded by the representatives of these three poxvirus genera. OV and VV topoisomerases display 54.7% identity and 34% conservative changes. OV

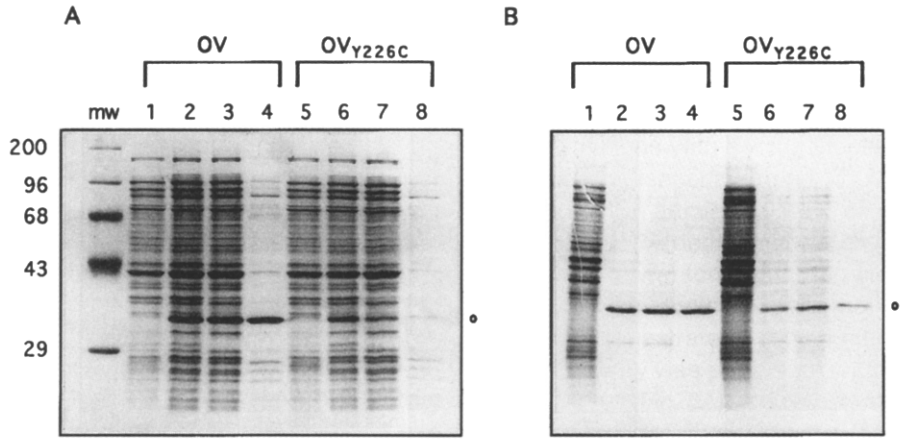

FIG. 7. Visualization of overexpressed and partially purified OV^{Y226C} topoisomerase. HMS174 transformants carrying the pET/OV-topo or pET/OV^{Y226C}-topo plasmids were induced by infection with λCE6 and harvested as described under Materials and Methods. Lysates were analyzed on 12% SDS–polyacrylamide gels; whether samples were prepared from cultures carrying the wild-type (OV) or mutant (OV$_{Y226C}$) topoisomerase alleles is marked above the relevant lanes. (A) Proteins visualized by staining with Coomassie brillant blue dye; (B) an autoradiograph of lysates prepared from cultures metabolically labeled with [^{35}S]methionine as described under Materials and Methods. The lanes contain the following samples: lanes 1 and 5, whole cell lysates from uninduced cultures; lanes 2 and 6, whole cell lysates prepared at 90 min after induction with λCE6; lanes 3 and 7, soluble lysates prepared from induced cultures; lanes 4 and 8, enriched topoisomerase preparations purified from soluble lysates of induced cultures by phosphocellulose chromatography. Lanes 5–8 contained approximately 11, 29, 26, and 6 μg of protein, respectively. The concentration of OV^{Y226C} topoisomerase within the enriched preparation (lanes 8) was estimated to be 19 ng/μl. The amount of protein analyzed from the lysates obtained from pET/OV-topo transformants (lanes 1–4) was the same as that described in the legend to Fig. 3. The wild-type and mutant OV topoisomerase species are indicated to the right of each panel (open circle). The lane marked mw contains protein standards whose molecular weights (×10^{-3}) are indicated at the left.

and SFV topoisomerases show 50.6% identity and 34% conservative changes. A comparison of the amino acid compositions of the OV and VV enzymes provides a vivid illustration of how genomic GC content affects codon usage and amino acid composition. For example, the OV enzyme has only 11 Lys residues, encoded by AA$^{A}/_{G}$, whereas the VV enzyme has 34. In contrast, the OV enzyme contains 34 Arg residues, encoded by CGN (31 examples) or AG$^{A}/_{G}$, whereas the VV enzyme only contains 15. The OV enzyme contains 29 Ala residues (GCN), whereas only 9 are found within the VV enzyme; 20 Gly residues (GGN) are contained within the OV enzyme, but only 11 in the VV enzyme. Conversely, the VV enzyme has 20 Ile (AU^{A}/C/$_{U}$) and 20 Asn (AA$^{C}/_{U}$) residues, whereas the OV enzyme contains only 11 of each. Despite these dissimilarities, the proteins share several blocks of extensive identity which may elucidate which regions of the protein are essential for DNA binding and/or catalysis. A role for these regions in catalysis has in some cases been confirmed by mutational analysis (Morham and Shuman, 1990, 1992; Klemperer and Traktman, 1993). By analogy to other enzymes (Lynn *et al.*, 1989; Shuman *et al.*, 1989b; Upton *et al.*, 1990), we can predict that Tyr^{275} of the OV DNA topoisomerase I is the residue involved in covalent linkage to the cleaved DNA substrate. The motif surrounding this residue is SRSAY; the VV and SFV enzymes contain a SKRAY motif, whereas the *S. cerevisae* active site is contained within the sequence SKINY.

As is the case with the VV and SFV topoisomerase genes, the F4R ORF is preceded by a late poxvirus promoter. No F4R transcripts have yet been detected in OV-infected cells. A topoisomerase-specific transcript has only recently been found in VV-infected cells (Soos and Traktman, unpublished observations) after earlier attempts were unsuccessful (Rosel *et al.*, 1986), suggesting that this enzyme may in general be expressed at very low levels. The DNA sequence downstream of the OV ORF F4R is predicted to encode the NH_2-terminus of an ORF denoted C1R. Given the parallels in the organization of the OV and VV genomes (Fleming *et al.*, 1993), it is not unexpected that this ORF shows some homology to the VV H7R and SFV D2 ORFs, which likewise lie downstream of the respective topoisomerase genes.

Definitive proof that the putative OV topoisomerase gene encodes a type I topoisomerase was demonstrated upon overexpression of the OV F4R protein in *E. coli*. The protein produced had an apparent molecular weight of 35,500, in good agreement with that predicted from the deduced amino acid sequence. The recombinant protein was partially purified and shown to catalyze the relaxation of supercoiled DNA with a specific activity equivalent to that seen for the VV enzyme.

The covalent protein/DNA intermediate formed by the OV enzyme during DNA relaxation could be visualized without the addition of pharmacological inhibitors of DNA religation. This property of forming a stable intermediate has previously been a distinguishing feature of the VV

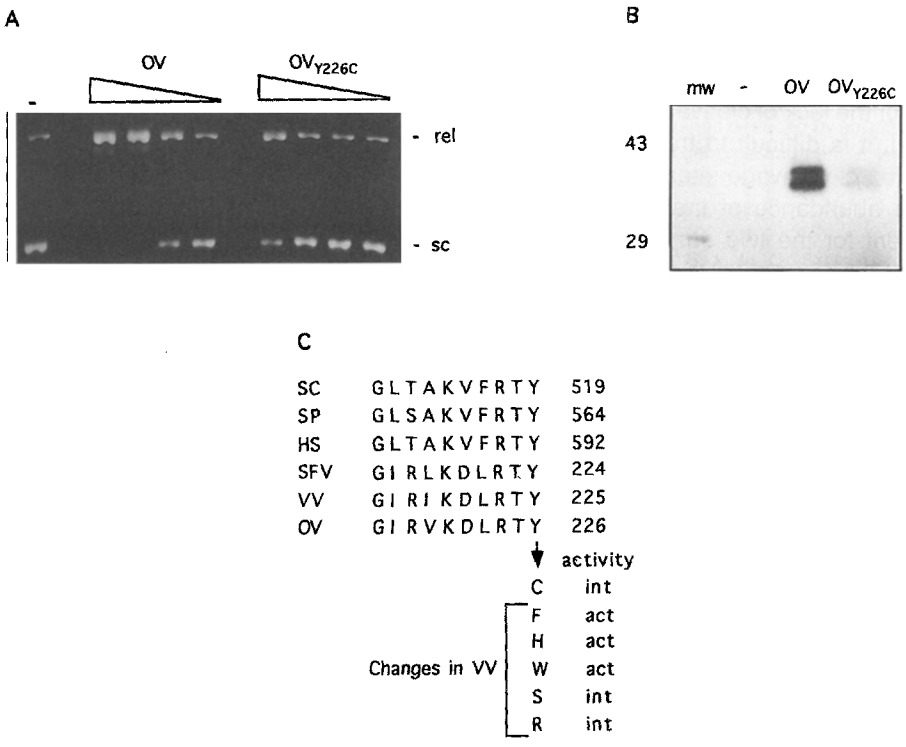

FIG. 8. Analysis of the DNA relaxation activity of the Y226C mutant of OV topoisomerase. (A) Partially purified preparations of the wild-type (OV) and mutant OV (OV^{Y226C}) DNA topoisomerases were assayed for their ability to relax supercoiled DNA. Reactions were performed as described in the legend to Fig. 4. For each enzyme, a series of four dilutions were assayed; from left to right, the reactions shown contained 4, 0.8, 0.16, and 0.03 pmol of enzyme. Reaction products were fractionated by electrophoresis through agarose gels, stained with ethidium bromide, and visualized by uv illumination. Photographs of the stained gels are shown. A control in which no phosphocellulose eluant was added to the reaction, −, is also shown. The positions to which relaxed (*rel*) and supercoiled (sc) DNA species migrated are indicated. (B) Covalent intermediates were formed by label transfer from nick-translated pUC19 to the enzyme. Briefly, each reaction contained 4.3 pmol of partially purified topoisomerase and 22 fmol of radiolabeled pUC19. After a 15-min incubation at 37°, the excess DNA was digested with DNase I. Samples were fractionated by SDS−12% PAGE and visualized by autoradiography. The enzyme used is indicated above each lane. Lane mw contains ^{14}C-labeled protein standards whose molecular masses ($\times 10^{-3}$) are indicated at the left. (C) The mutation in OV^{Y226C} topoisomerase maps within a region whose amino acid sequence is highly conserved among eukaryotic DNA topoisomerases. Shown is an amino acid alignment of topoisomerases as previously described (Shuman and Moss, 1987; Lynn *et al.*, 1989; Upton *et al.*, 1990): *S. cerevisiae* (SC), *S. pombe* (SP), human (HS), Shope fibroma virus (SFV), vaccinia virus (VV), and orf virus (OV). The position of the relevant Tyr residue within the amino acid sequence of each topoisomerase is shown to the right. The descending arrow denotes the amino acid substitutions at this residue which have been studied. The Cys substitution shown is the OV topoisomerase mutation described in this report. The other substitutions shown were introduced into the VV topoisomerase by site-directed mutagenesis. Enzymes containing the Phe (Shuman *et al.*, 1989b), His, and Trp (Klemperer and Traktman, 1993) substitutions maintain wt levels of relaxation and cleavage activity (act), while the Ser and Arg (Klemperer and Traktman, 1993) substitutions lead to reduced, intermediate (int) levels of activity. The Cys substitution reported here for the OV enzyme also leads to an intermediate level of relaxation and cleavage activity. Within the figure, all amino acids are designated according to the single letter code.

enzyme which is not shared by other eukaryotic enzymes (Shaffer and Traktman, 1987). The impact of adding a variety of cations on the formation of the covalent intermediate was investigated (data not shown). Covalent adduct formation was abolished for both the OV and VV topoisomerases upon the inclusion of 5 m*M* $ZnSO_4$. Inclusion of other divalent cations had slight but equivalent effects on the two enzymes. These data reinforce the conclusion that the two poxviral enzymes have indistinguishable mechanisms of action.

Our demonstration that the two enzymes preferentially recognized and cleaved the same DNA sequence provided the most compelling evidence of the similarity between these enzymes. Both enzymes recognized and cleaved immediately 3′ of the sequence CCCTT within a 24-mer oligonucleotide duplex substrate. That the two enzymes cleaved at precisely the same position was shown by high resolution electrophoresis of the cleavage products. The sequence within this substrate conforms to the previously defined consensus sequence for the VV enzyme: $^{C}/_{T}CCTT^{\downarrow}$ (Shuman and Prescott, 1990; Shuman, 1991b). This experiment indicates that the OV enzyme can utilize this sequence, but does not address whether such a sequence is optimal. An indication of the cleavage sites preferred by the enzyme was obtained by utilizing an end-labeled, 2.66-kb DNA substrate. Three major cleaved fragments were observed after incubation of the substrate with either the OV or VV enzymes. That the

same products were seen provides strong evidence that the enzymes share a preferred recognition site. Because detection of a given fragment can reflect cleavage at a high affinity site and/or the lack of cleavage at sites proximal to the radiolabel, it is difficult to translate the data obtained into a ranking of cleavage sites. Therefore, the fact that the relative abundance of the three products observed was different for the two enzymes is difficult to interpret precisely. Our conclusion is that the core recognition site of the OV and VV enzymes is the same, but that the flanking sequences which provide an optimal context may be distinct for the two enzymes.

The initial step in generating our OV topo expression construct was the cloning of a PCR-derived copy of the F4R ORF into a pUC vector and the introduction of this clone into the DH5α strain of *E. coli.* The plasmid recovered from such a transformant contained a point mutation at nt 677 which was predicted to replace Tyr^{226} with a Cys residue. The protein encoded by this allele was subsequently shown to have severely reduced enzymatic activity. Based on previous experience, we believe that the recovery of this allele was not fortuitous but reflected the toxicity of the wild-type allele when expressed at a basal level from a promoter within the pUC plasmid. We and others (Upton *et al.,* 1990; Fernandez-Beros and Tse-Dinh, 1992; Countryman and Traktman, unpublished observations) have shown that even low levels of constitutive expression of the VV or SFV topoisomerases can be inhibitory to growth. We have also shown that pUC/topo plasmids do direct the expression of some active enzyme within *E. coli* even when no obvious promoter lies immediately upstream of the topo gene; the same is true for pET plasmids in uninduced bacteria. Activity was evinced by the predominance of relaxed DNA isomers within plasmid preparations; such relaxed DNA is not found within plasmids containing no insert or truncated or mutant alleles of the enzyme (Klemperer and Traktman, 1993). We therefore believe that the G–A transition within the OV^{Y226C} allele arose either during PCR or spontaneously within *E. coli* and was selected for on the basis of reduced toxicity.

The Tyr residue affected in this mutant, OV-$topo^{Y226C}$, lies within a region which is highly conserved among eukaryotic type I topoisomerases (Fig. 8C). This region, including the analogous Tyr residue of the VV topoisomerase (Tyr^{225}) has been the target of extensive site directed mutagenesis (Klemperer and Traktman, 1993). Replacement of Tyr^{225} with a Phe (Shuman *et al.,* 1989b), His, or Trp (Klemperer and Traktman, 1993) residue did not alter enzyme activity *in vitro,* implying that an aromatic residue at this position may be sufficient. However, replacement with either a Ser or Arg residue resulted in an enzyme with altered activity (Klemperer and Traktman, 1993). Although relaxation activity was $\geq$50% of that of the *wt* enzyme, partially relaxed isomers were visible. These enzymes also displayed a temperature-sensitivity: the ratio of the DNA relaxation activity seen at 50 vs 25° was reduced by 25-fold relative to the *wt* enzyme. In addition, covalent adduct formation was reduced to 10–20% of *wt* adduct formation when pUC19 was used as the DNA substrate. The alteration of OV Tyr^{226} to a Cys residue in the mutant described in this report impairs enzyme activity more than other mutations described, since relaxation activity was shown to be reduced by more than 25-fold at 37°. Covalent adduct formation was severely inhibited in this mutant. Minimal covalent adduct was visible when pUC 19 was used as the substrate and adduct formation was reduced relative to the *wt* OV topo when the 24-mer oligonucleotide duplex substrate was used. The ability of this enzyme to bind DNA noncovalently was not affected, however, consistent with our previous studies of VV topo alleles carrying mutations at this residue or within this conserved region.

The studies reported here underline the structural and functional similarities between the type I topoisomerases encoded by three genera of poxviruses. The overall sequence homology is widespread and extensive, and the most conserved regions are likely to highlight catalytically active domains. Given the almost complete divergence of the nucleotide composition of the VV and OV genomes, it was unanticipated that the VV and OV topoisomerases would target the same consensus sequence for recognition and cleavage. It is tempting to speculate that the stretch of pyrimidines which constitute the topoisomerase recognition sequence, $^{C}/_{T}CCTT^{\downarrow}$, has been conserved among poxviruses of different genera to accommodate the GC vs AT richness of the *Parapoxvirus* and *Orthopoxvirus* genera, respectively. Likewise, the conservation of mechanistic features as well as the shared target sequence provides a further stimulus to unravel the specific role(s) of these enzymes within the poxviral life cycle.

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