Poxvirus DNA topoisomerase knockout mutant exhibits decreased infectivity associated with reduced early transcription

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Vaccinia virus encodes a type I DNA topoisomerase that is highly conserved in all known poxviruses. Although the structure and catalytic activity of the enzyme were well studied, little was known about its biological function. The viral topoisomerase was thought to be essential, and roles in DNA replication, recombination, concatemer resolution, and transcription were suggested. Here, we demonstrated that the topoisomerase is not essential for replication of vaccinia virus in cultured cells, although deletion mutants formed fewer and smaller plaques on cell monolayers than wild-type virus. Purified mutant virus particles were able to bind and enter cells but exhibited reduced viral early transcription and a delay in DNA replication. Infecting with a high number of virus particles increased early mRNA and accelerated viral DNA synthesis. Processing of viral DNA concatemers into unit-length genomes was unimpaired at either a low or high multiplicity of infection. The data suggest that the primary, perhaps only, role of the poxvirus topoisomerase is to increase early transcription, which takes place within virus cores in the cytoplasm of infected cells. Because the topoisomerase functions early in infection, drugs capable of penetrating the virus core and irreversibly damaging DNA by trapping nicked DNA–topoisomerase intermediates could make potent antiviral agents.

Topoisomerases, by breaking and rejoining DNA strands, alleviate topological problems during replication, recombination, or transcription in prokaryotes and eukaryotes as well as some viruses (1). In addition, topoisomerases are excellent targets for anticancer and antimicrobial drugs. DNA topoisomerases are grouped into two families: type I enzymes break one DNA strand at a time, whereas type II enzymes break both strands simultaneously. Topoisomerases are divided further into subtypes A and B based on structural and mechanistic similarities. Vaccinia virus encodes a type 1B topoisomerase (2–4) that is highly conserved in all members of the poxvirus family. The poxvirus enzymes are distinguished from cellular topoisomerase I by their compact size, resistance to the drug camptothecin, and specificity for the pentapyrimidine (T/C)CCTT target sequence in DNA (4–8). The vaccinia virus topoisomerase has been well studied with regard to catalytic mechanisms (9, 10), yet virtually nothing is known about its biological role. Although the enzyme is expressed late during infection (4), it is packaged in virus particles (2) and therefore could act at the time of synthesis or during early stages of the next round of infection. The vaccinia virus topoisomerase can resolve Holliday junctions (HJs) *in vitro*, suggesting a role in the resolution of viral DNA concatemers to form unit genomes with hairpin telomeres (9, 11, 12). Biochemical and genetic studies, however, attribute this function to a recently discovered poxvirus HJ endonuclease with homology to bacterial RuvC (13, 14). Roles for poxvirus topoisomerase in DNA replication (15), recombination (16, 17), and compaction of the viral genome for packaging have also been considered. A role in early transcription, which occurs within virus cores after infection, would be consistent with the coordinate incorporation into virions of the topoisomerase, DNA-dependent RNA polymerase, and other viral enzymes involved in mRNA synthesis (18).

Genetic studies are required to distinguish among the many possible roles for the poxvirus topoisomerase. Poxviruses encode only one topoisomerase, and the failure to isolate a topoisomerase deletion mutant suggested that the enzyme is essential (19). The latter failure, however, can be attributed to the selection method because we have now succeeded in knocking out the vaccinia virus topoisomerase gene. The deletion mutant was viable but had low infectivity, which correlated with reduced early transcription rather than direct effects on the replication or processing of viral DNA. These findings have topical importance in view of current efforts to develop topoisomerase inhibitors as poxvirus therapeutics.

Materials and Methods

Construction and Isolation of Topoisomerase Deletion Mutants. The plasmid pFFH6RΔ was constructed by inserting the ORF encoding the enhanced GFP under control of the P11 vaccinia virus late promoter into pNEB193 (New England Biolabs). The GFP ORF then was flanked by a 511-bp DNA fragment from the 3′ end of the H5R gene and a 504-bp fragment from the 5′ end of the H7R gene of vaccinia virus strain Western Reserve (WR). Homologous recombination was carried out in BS-C-1 cells infected with vaccinia virus strain WR and transfected with pFFH6RΔ essentially as described (20). Diluted lysates were applied to BS-C-1 monolayers, and plaques containing recombinant virus were detected by fluorescence microscopy. Virus was recovered from fluorescent plaques, and the procedure was repeated until the virus was clonally pure. Deletion of the topoisomerase gene was confirmed by PCR and DNA sequencing.

Purification of Vaccinia Virus. Wild-type and mutant vaccinia virus were propagated in HeLa cells and purified by sedimentation through a sucrose cushion and a sucrose gradient (20). The number of particles was determined from the OD at 260 nm by using the formula 1 OD = 1.2×10^{10} particles (20).

Fluorescence Microscopy. Cells were fixed with 4% paraformaldehyde for 20 min at 4°C followed by 40 min at room temperature. Cells were permeabilized in 0.05% saponin in PBS and incubated with a mixture of anti-A4 polyclonal antisera provided by M. Esteban (Centro Nacional de Biotecnologia, Madrid) (21) and anti-L1 mAb 7D11 provided by A. Schmalljohn (United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD) (22) in permeabilization buffer with 10% FCS followed by goat anti-mouse fluorescein-conjugated and goat anti-rabbit rhodamine red-conjugated antibodies (Jackson ImmunoResearch). DNA was visualized by staining with diamidino-2-phenylindole dihydrochloride (Molecular Probes) at 5 μg/ml for 5 min. Images were collected on a Leica

Abbreviations: HJ, Holliday junction; WR, Western Reserve; pfu, plaque-forming unit.

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TCS-NT/SP2 inverted confocal microscope with an attached argon ion laser (Coherent Radiation, Palo Alto, CA).

RNA Analysis. Total RNA was extracted from infected cells with the RNeasy mini kit (Qiagen, Valencia, CA), resolved by electrophoresis in 1% agarose gels, transferred to nylon filters, and hybridized to a DNA probe by using the NorthernMax-Plus kit (Ambion, Austin, TX). ^{32}P-labeled DNA probes were prepared by using the Random Primers DNA-labeling system (Invitrogen). Autoradiography was performed with a Typhoon 8600 PhosphorImager (Molecular Dynamics).

RNA Synthesis by Detergent-Permeabilized Virions. Purified virions were incubated in 20 μl of 50 mM Tris·HCl (pH 8.0)/5 mM DTT/10 mM $MgCl_2$/0.05% Nonidet P-40/5 mM ATP/1 mM GTP/1 mM CTP/0.02 mM UTP/1 μCi (1 Ci = 37 GBq) of [α-^{32}P]UTP (3,000 Ci/mmol). Incorporation of [α-^{32}P]UMP into trichloroacetic acid-insoluble material was determined.

Western Blotting. Analysis of proteins from infected cell lysates was carried out by SDS/PAGE and immunoblotting as described (23). S. Shuman (Sloan–Kettering Institute, New York) kindly provided antiserum to the vaccinia virus topoisomerase, and antibody to the H3 protein was as described (24).

Results

Isolation of a Vaccinia Virus Topoisomerase Deletion Mutant. The vaccinia virus genome contains nearly 200 ORFs that are likely to encode proteins. One of these, now designated H6R encodes a functional topoisomerase (4). We constructed a knockout plasmid containing a vaccinia virus promoter-GFP cassette flanked by genomic sequences present on either side of the topoisomerase ORF. Cells infected with wild-type vaccinia virus were transfected with the above-described plasmid, and independent plaques exhibiting green fluorescence were picked. After successive rounds of plaque isolation, several clones of vH6RΔ were obtained. Each clone made plaques that were smaller than those of the parental virus (Fig. 1*A*). The deletion of the topoisomerase ORF was confirmed by PCR (data not shown), and the absence of topoisomerase synthesis was demonstrated by Western blotting of infected cell lysates with antibody to the vaccinia virus topoisomerase (Fig. 1*B*). The presence in the infected cell lysates of another viral protein, encoded by the H3L ORF, was determined as a control (Fig. 1*B*).

Initial Characterization of the Topoisomerase Deletion Mutant. The titers of the topoisomerase deletion mutant vH6RΔ stocks were consistently ≈1 log lower than those of WR. The reduction in yield was confirmed by one-step growth studies (Fig. 1*C*). Subsequent experiments were designed to determine the stage of virus replication that was affected by the absence of the topoisomerase. However, when cells were infected with an equal number of plaque-forming units (pfu) of WR or vH6RΔ, viral protein and DNA synthesis were similar. Moreover, electron micrographs of cells infected with vH6RΔ showed typical immature (Fig. 2*A*) and mature (Fig. 2*B*) virions. Electron micrographs of sucrose gradient-purified vH6RΔ virions also appeared unremarkable (Fig. 1*C*). The specific infectivity, however, determined from the OD and pfu of purified vH6RΔ, was ≈10-fold lower than that of WR. This infectivity difference was confirmed by measuring viral DNA and protein of serial dilutions of purified virus by slot-blot hybridization and Western blot analysis, respectively (data not shown). The low infectivity of vH6RΔ, compared with WR, was also demonstrated by measuring the viral DNA and pfu in fractions from a CsCl gradient (Fig. 3).

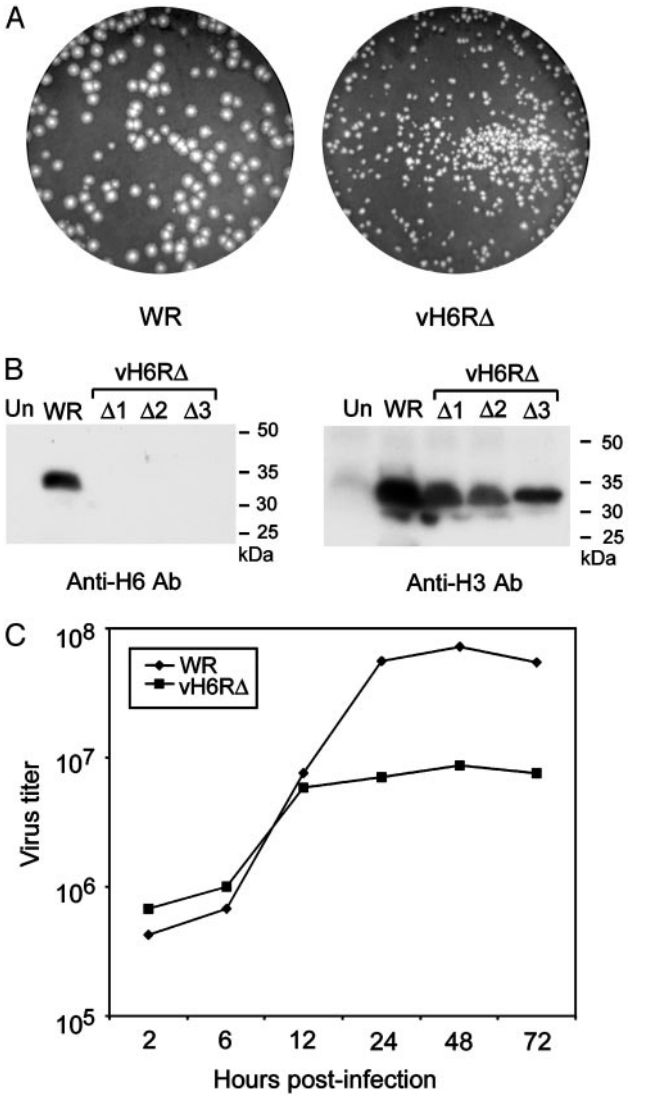

Fig. 1. Plaque formation and replication of a vaccinia virus topoisomerase knockout mutant. (*A*) BS-C-1 monolayers were infected with wild-type vaccinia virus (WR) or a topoisomerase deletion mutant (vH6RΔ). After 2 days the cell sheets were stained with crystal violet to visualize plaques. (*B*) BS-C-1 cells were uninfected (Un) or infected with 10 pfu per cell of WR or independent isolates (Δ1, Δ2, and Δ3) of vH6RΔ. After 24 h, cells were lysed and proteins were resolved by SDS/PAGE, transferred to a membrane, and detected by chemiluminescence after incubation with antibody to the topoisomerase (anti-H6 antibody) or the early protein encoded by the H3L ORF (anti-H3 antibody). Numbers on the right indicate positions of size markers in kDa. (*C*) BS-C-1 cells were infected with 10 pfu per cell of WR or vH6RΔ. At the indicated times, cells were lysed by freezing and thawing, and the virus titers were determined by plaque assay.

Virus Binding and Entry. A defect in binding of virions to cells or entry of cores into the cytoplasm could account for the low infectivity of vH6RΔ. To evaluate these possibilities, infected cells were stained with antibody to the L1 envelope protein, which stains virions that have not entered the cell, or with antibody to the A4 core protein, which stains virions that have penetrated the cell (24), and with diamidino-2-phenylindole dihydrochloride to visualize nuclei. As expected, punctate L1 fluorescence was seen after incubation in the cold, and only occasional particles stained with antibody to A4 (Fig. 4). In contrast, there were many cell-associated particles that stained with antibody to A4 1 h after raising the temperature (Fig. 4).

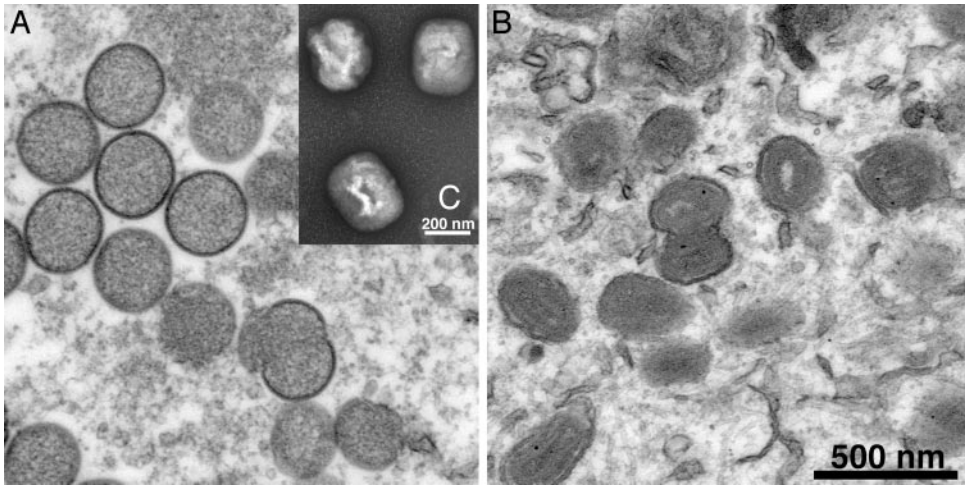

Fig. 2. Electron microscopy. BS-C-1 cells infected with vH6RΔ for 14 h were sectioned and examined by electron microscopy (23). Immature (*A*) and mature (*B*) virions are shown. (*C*) Sucrose gradient-purified vH6RΔ was deposited on a grid and negatively stained (23).

Similar numbers of intracellular cores were detected in cells infected with WR and cells infected with vH6RΔ, indicating that there was no defect in binding or entry steps.

Early Transcription. Poxvirus particles contain a complete early transcription system and can synthesize mRNA *in vitro* under appropriate conditions (25, 26). *In vitro* RNA synthesis by detergent-treated purified vH6RΔ particles was reduced only mildly when equal numbers of particles were compared (Fig. 5*A*) and the time course of synthesis was similar (data not shown). In infected cells, early mRNA synthesis begins almost immediately within virus cores and peaks in several hours (27). RNA was isolated from infected cells, resolved by gel electrophoresis, and detected with probes to early mRNAs encoding the DNA polymerase (E9L, 3,021 nt), the DNA polymerase processivity factor (A20R, 1,281 nt), and the recently characterized (F.D.F., unpublished data) G5R protein (1,305 nt). When cells were infected with the same number of OD units of vH6RΔ and WR, the amounts of all three vH6RΔ mRNAs were much lower than those of WR (Fig. 5 *B–D*), indicating an early transcription block. However, when the OD units of vH6RΔ were increased 10-fold over that of WR, to match the number of pfu, the difference in amounts of mRNA was much less (Fig. 5 *B–D*). The disparity between the *in vitro* and *in vivo* results probably relates to the disruption or loosening of the core structure by detergent treatment.

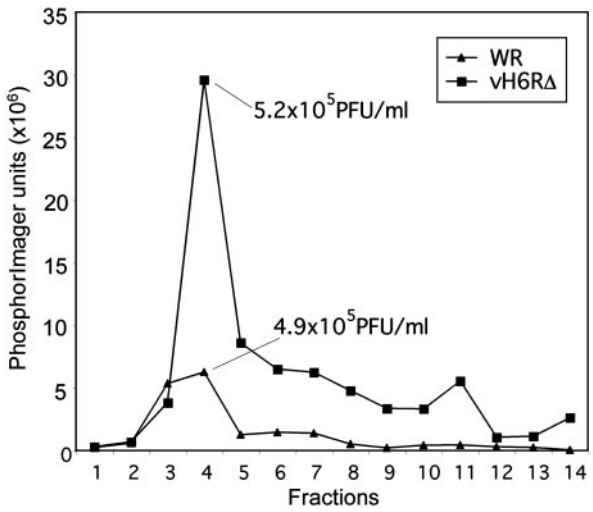

Fig. 3. CsCl gradient sedimentation. Equal pfu of sucrose gradient-purified WR and vH6RΔ were analyzed by CsCl gradient centrifugation as described (23). Fractions were collected from the bottoms of the tubes, virus particles were diluted and pelleted by centrifugation, and aliquots were used for slot-blot hybridization to a ^{32}P-labeled viral DNA probe. Radioactive material is indicated in arbitrary PhosphorImager units. The virus titers in the peak fractions were determined by plaque assay.

Viral DNA Synthesis and Processing. Next we compared viral DNA replication in cells infected with WR and vH6RΔ. When the same number of OD units was used for infection, vH6RΔ DNA accumulated more slowly than that of WR (Fig. 6*A*), consistent with the lower levels of early mRNAs encoding proteins required for DNA synthesis. The lag was mostly eliminated, however, when the number of vH6RΔ particles used for infection was increased 10-fold to equalize the pfu with WR (Fig. 6*B*).

Vaccinia virus DNA replicates as large head-to-head or tail-to-tail concatemers that are resolved rapidly into unit-length genomes of nearly 200,000 bp, which can be characterized by pulse-field gel electrophoresis (28, 29). To determine whether the vaccinia virus topoisomerase is required for efficient resolution of concatemers, we analyzed viral DNA in cells infected with equal numbers of WR and vH6RΔ particles. In each case, unit-length genomes were predominant at all times examined (Fig. 7*A*). In addition, vH6RΔ DNA did not accumulate in the wells, which would have indicated branched structures unable to penetrate the gel.

Each concatemer junction consists of an imperfect palindrome that is resolved into two hairpin termini. When viral DNA is digested with the restriction endonuclease *Bst*EII, 2.6- and 1.3-kbp fragments representing the concatemer junction and hairpin terminus, respectively, are formed and can be detected by using a ^{32}P-labeled oligonucleotide probe (30). Previous experiments had shown that the concatemer junction fragment is barely detectable because of its transient nature during infection with wild-type vaccinia virus but accumulates when total late gene expression is inhibited (28, 29) or when the HJ endonuclease is specifically repressed (14). As shown in Fig. 7*B*, the 1.3-kbp terminal fragment was detected at all times after infection with low or high numbers of particles. Similar results were obtained with WR (data not shown). Thus, expression of the vaccinia virus topoisomerase is not necessary for efficient concatemer resolution.

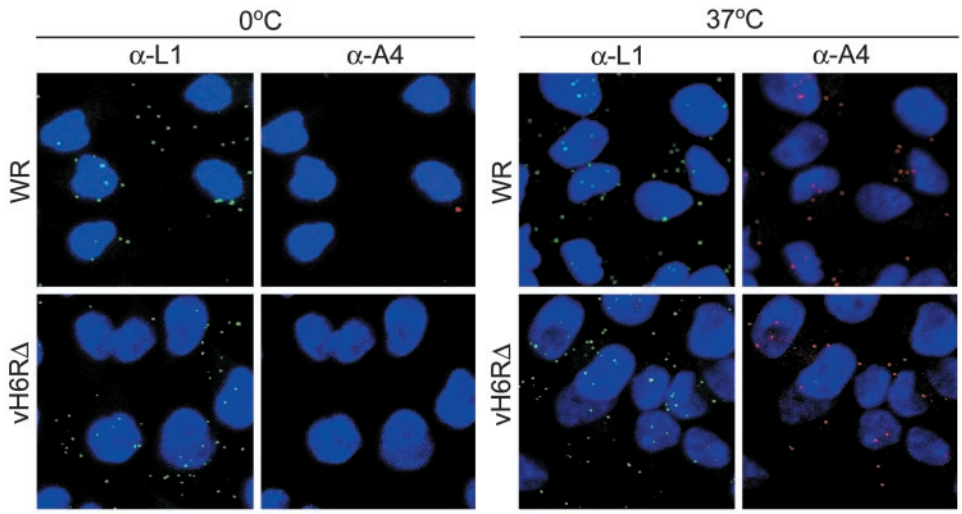

Fig. 4. Confocal microscopy. HeLa cells on coverslips were infected at 0°C with equivalent numbers of particles of WR or vH6RΔ. After 1 h, some coverslips were fixed and others were incubated for an additional 1 h at 37°C before fixing. The cells were stained with anti-A4 polyclonal antisera (α-A4) and anti-L1 mAb (α-L1) followed by goat anti-mouse fluorescein-conjugated and goat anti-rabbit Rhodamine red-conjugated antibodies (Jackson ImmunoResearch). Nuclear DNA was visualized by staining with diamidino-2-phenylindole dihydrochloride. Green, L1 protein; red, A4 protein; blue, diamidino-2-phenylindole dihydrochloride staining nucleus.

Discussion

Our finding that vaccinia virus does not require its topoisomerase for replication in cell culture was surprising, because the gene is conserved in all sequenced poxviruses. Although a prior attempt to delete the vaccinia virus topoisomerase gene was unsuccessful (19), there is a reasonable explanation. The previous selection method was based on acquisition of resistance to mycophenolic acid by insertion of the *Escherichia coli gpt* gene into the target gene locus (31). In this scheme, antibiotic resistance depends on viral synthesis of xanthine-guanine phosphoribosyltransferase to overcome the induced block in purine metabolism. But, because vaccinia virus early gene expression is severely reduced in the absence of topoisomerase, we suggest that deletion mutants would be unable to express enough xanthine-guanine phosphoribosyltransferase to overcome the block and form visible plaques. In contrast, the present method used GFP expression as a sensitive reporter, which allowed us to pick small plaques without selection. It may be useful to reassess failed attempts to isolate other viral mutants by similar antibiotic-selection schemes.

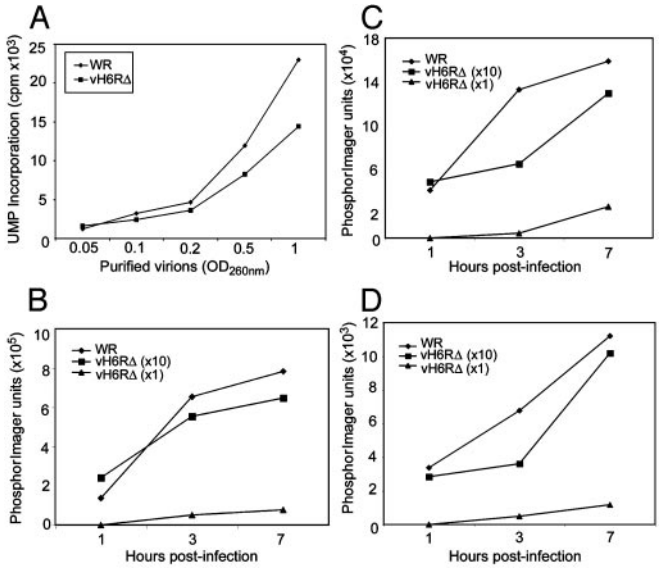

Fig. 5. RNA synthesis. (*A*) Sucrose gradient-purified WR and vH6RΔ particles were treated with detergent and incubated with [α-^{32}P]UTP and other ribonucleoside triphosphates at 37°C. Incorporation of [α-^{32}P]UMP was determined by trichloroacetic acid precipitation and scintillation counting. (*B–D*) BS-C-1 cells were infected with 10 pfu per cell of WR or an equivalent number of OD units (×1) or pfu (×10) of vH6RΔ. At the indicated times after infection, RNA was extracted, resolved by agarose gel electrophoresis, transferred to a membrane, hybridized to a ^{32}P-labeled DNA probe containing sequences of the G5R (*B*), E9L (*C*), or A20R (*D*) ORFs, and analyzed on a PhosphorImager.

A second surprise is that the primary and perhaps only role of the vaccinia virus topoisomerase is related to early gene expression. The key to this discovery was finding that the infectivity of purified mutant virus particles was ≈10-fold lower than that of wild-type virus. Although the mutant particles were capable of infecting cells and releasing cores in the cytoplasm, there was a severe reduction in viral early mRNAs. By infecting cells with 10 times more mutant virus particles, however, the amount of early mRNA approached that of wild-type virus. Thus, the impairment of viral early mRNA synthesis could account for the 10-fold lower infectivity of mutant virus particles. *In vitro* transcription by mutant virus particles was reduced only mildly, presumably because the detergent treatment disrupted or loosened the core structure. A role of the topoisomerase in early gene expression was anticipated by the finding that the topoisomerase is inserted into virus cores coordinately with enzymes and factors required for mRNA synthesis (18).

Although RNA polymerases are powerful motors that can overcome substantial frictional resistance (32), a topoisomerase may be needed to relieve supercoiling during transcription of a linear duplex template when the polymerase or an associated macromolecule is attached to a large structure or when the motion of a nascent RNA chain with associated proteins is severely hindered because of the milieu (33). The poxvirus topoisomerase presumably enhances transcription by relieving torsional stress within the constrained environment of the intact core. Topological problems may be aggravated when the transcripts are very long. In this context, poxviruses use a stringent termination mechanism only for synthesis of early mRNAs (34),

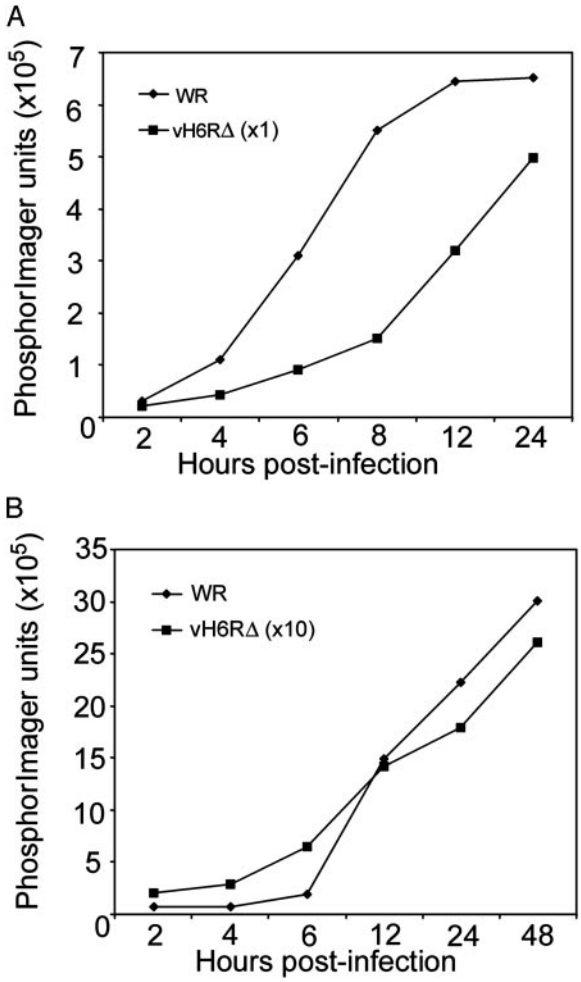

Fig. 6. Viral DNA synthesis. (*A*) BS-C-1 cells were infected with 10 pfu of WR or an equivalent number of OD units of vH6RΔ (×1). At indicated times, DNA was extracted, applied to a membrane, and analyzed by hybridization to a ^{32}P-labeled viral DNA probe as described (14). The radioactivity was measured with a PhosphorImager. (*B*) As described for *A* except that BS-C-1 cells were infected with 10 pfu per cell of WR or an equal number of pfu (×10) of vH6RΔ.

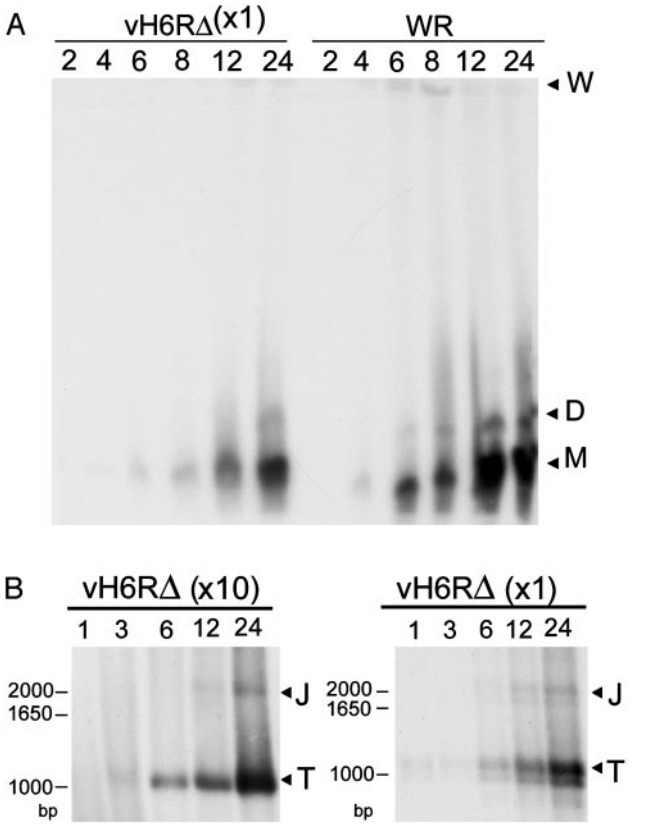

Fig. 7. Processing of viral DNA into unit-length genomes. (*A*) BS-C-1 cells were infected with 10 pfu per cell of WR or an equivalent number of OD units of vH6RΔ. The viral DNA was analyzed by pulse-field gel electrophoresis, transferred to a membrane, and hybridized to a ^{32}P-labeled DNA probe. M, genome monomer; D, genome dimer. (*B*) BS-C-1 cells were infected with 10 pfu per cell of WR (data not shown) or an equivalent number of OD units (×1) or pfu (×10) of vH6RΔ. T, 1.3-kbp telomere fragment; J, 2.6-kbp concatemer junction fragment.

possibly to limit the movement of the transcription complex or the length of the mRNAs in the core environment.

Does the vaccinia virus topoisomerase also have a role in viral DNA synthesis or recombination? There are no data to support this. Known poxvirus DNA replication proteins are not packaged in virus particles and must be synthesized *de novo* early in infection. Therefore, the delayed viral DNA synthesis in cells infected with the mutant virus can be attributed to the reduction in early transcription. Moreover, when the transcription defect was overcome by infecting cells with a larger number of mutant particles, there was no longer a delay in DNA synthesis. Participation of the poxvirus topoisomerase in DNA synthesis also seems unlikely because the known replication enzymes and factors are synthesized early in infection and the topoisomerase is expressed late. A role of the poxvirus topoisomerase in homologous DNA recombination is also unlikely on the grounds that late gene expression is not required (28, 35). Our experiments do not exclude a role for a cellular topoisomerase in transcription or replication, and the inhibition of vaccinia virus RNA and DNA synthesis by camptothecin, a specific inhibitor of cellular topoisomerase, has been reported (36).

The resolution of poxvirus DNA concatemers is an important processing step and, unlike DNA synthesis and recombination, depends on viral late gene expression (28, 29). The topoisomerase was the only candidate resolvase (12) before the discovery of the poxvirus HJ endonuclease (13). However, whereas concatemers accumulated when synthesis of the HJ endonuclease was repressed (14), we found no such accumulation in the absence of topoisomerase. Nevertheless, a backup role of the topoisomerase could explain why repression of the HJ resolvase did not entirely prevent concatemer resolution. Alternatively, delayed concatemer resolution could have resulted from incomplete repression of the HJ endonuclease.

The present study has important implications for the development of poxvirus topoisomerase inhibitors as antiviral drugs. Although it may seem counterintuitive, our findings may make the topoisomerase a more attractive drug target even though the enzyme is not essential for virus replication in cell culture. Most topoisomerase-targeting drugs in clinical use damage DNA by trapping covalent DNA–enzyme intermediates (37). Because our data suggest that the poxvirus topoisomerase is active at a very early stage of infection, a DNA-damaging topoisomerase drug that penetrates the core might block replication totally. Although topoisomerase null mutants might escape such antiviral effects, the large decrease in infectivity of topoisomerase knockout viruses would probably make that inconsequential.

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