

Repression of Vaccinia Virus Holliday Junction Resolvase Inhibits Processing of Viral DNA into Unit-Length Genomes

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The vaccinia virus A22R gene encodes a protein that is homologous to the bacterial enzyme RuvC and specifically cleaves and resolves four-way DNA Holliday junctions into linear duplex products. To investigate the role of the vaccinia virus Holliday junction resolvase during an infection, we constructed two recombinant viruses: vA22-HA, which has a short C-terminal epitope tag appended to the A22R open reading frame, and vA22i, in which the original A22R gene is deleted and replaced by an inducible copy. Polyacrylamide gel electrophoresis and Western blot analysis of extracts and purified virions from cells infected with vA22-HA revealed that the resolvase was expressed after the onset of DNA replication and incorporated into virion cores. vA22i exhibited a conditional replication defect. In the absence of an inducer, (i) viral protein synthesis was unaffected, (ii) late-stage viral DNA replication was reduced, (iii) most of the newly synthesized viral DNA remained in a branched or concatemeric form that caused it to be trapped at the application site during pulsed-field gel electrophoresis, (iv) cleavage of concatemer junctions was inhibited, and (v) virion morphogenesis was arrested at an immature stage. These data indicated multiple roles for the vaccinia virus Holliday junction resolvase in the replication and processing of viral DNA into unit-length genomes.

Poxviruses encode many of the proteins involved in the expression, replication, and packaging of their DNA genomes in the cytoplasm of infected cells (33). The mature genome of vaccinia virus (VV), the prototype member of this large family of vertebrate and invertebrate viruses, is a linear double-stranded molecule of about 185,000 bp with covalently linked hairpin termini (2, 18). By mapping of temperature-sensitive conditional lethal mutations, five viral genes that are expressed early in infection and that are required for DNA replication were identified. These genes encode a DNA polymerase (11, 23, 47), a nucleic acid-independent nucleoside triphosphatase (15, 16), a serine/threonine protein kinase (36), a uracil DNA glycosylase (14, 31, 45, 48), and a protein that is a product of the A20R open reading frame (ORF) (22) and that has been referred to as a processivity factor (46). Although early-gene expression is sufficient for VV DNA replication and recombination (26), concatemeric forms of VV DNA accumulate in the absence of late-gene expression, suggesting that a viral late-protein functions as a resolvase (7, 30) or that late transcription per se is needed (20, 44) or both.

The DNA that connects unit-length genomes is known as the concatemer junction (3, 34). When transfected into infected cells, plasmids containing the concatemer junction are resolved into minigenomes with hairpin ends (8, 9, 27, 29). Because the resolution sequence contains a functional late promoter, components of the transcription system may be involved in DNA processing (20, 44). In this regard, conditional lethal capping enzyme mutants are defective in concatemer resolution (5, 19). The concatemer junction contains an inverted repetition, which in supercoiled plasmids can form a cruciform structure

resembling a four-way Holliday junction (HJ) recombination intermediate (10, 28). Extracts of poxvirus-infected cells contain an HJ resolving activity (43). In addition, poxvirus-encoded topoisomerase (40, 42) can cleave and ligate a variety of DNA structures, including an HJ (35, 39). Recently, a specific VV HJ resolvase was identified (17). No genetic data are yet available, however, to determine if either the topoisomerase or the HJ resolvase is involved in concatemer resolution *in vivo*.

The encoding of an HJ resolvase by the VV A22R ORF was suggested by amino acid similarities to the bacterial enzyme RuvC and confirmed by expression and characterization of the activity of a recombinant A22R protein (17). Database searches indicated that RuvC homologs are present in all poxviruses as well as an iridovirus. Here we demonstrate the synthesis of the HJ resolvase during VV infection and describe the construction and characterization of a recombinant VV (rVV) with a stringently regulated A22R gene. Repression of A22R expression resulted in decreased late-stage VV DNA replication, accumulation of unprocessed DNA molecules with uncleaved concatemer junctions, and interruption of virus morphogenesis prior to the acquisition of infectivity.

MATERIALS AND METHODS

Cell lines and viruses. BS-C-1 cells were grown in Eagle's minimal essential medium (E-MEM) supplemented with 10% fetal bovine serum (FBS). HeLa S3 and STO cells were grown in Dulbecco's minimal essential medium with 10% FBS. All rVV's were derived from strain WR. The rVV vT7lacO1 (1) was propagated in HeLa S3 cells. The rVV vA22/A22i (derived from vT7lacO1), containing a guanine phosphoribosyltransferase gene (*gpt*), was isolated and propagated in BS-C-1 cells in the presence of E-MEM containing mycophenolic acid, hypoxanthine, xanthine, and 2.5% FBS (13). The inducer-dependent rVV vA22i was isolated and grown in BS-C-1 cells in E-MEM containing 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and 2.5% FBS. The rVV vA22-HA (derived from vA22i) was isolated in STO cells by reverse *gpt* selection (21) and propagated in HeLa S3 cells.

Plasmid construction and PCR. For construction of rVV's, we needed to insert an inducible copy of the A22R gene into the VV hemagglutinin (HA) A56R

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locus and then delete the endogenous A22R gene. However, because the A22R ORF in the VV genome overlaps the A20R ORF by 71 nucleotides and the predicted A23R gene promoter region by 23 nucleotides, the entire A22R gene could not be deleted. We considered that if the 5' and 3' ends of the A22R ORF remained, then the full-length ORF might be restored by recombination with the inducible copy. To avoid sequence duplication, we made silent mutations (designation "smt") in codons 152 to 187 of the inducible A22R ORF. For isolation and detection purposes, we also added a C-terminal histidine tag. Plasmid transfer vector pVOTE.1 (49) was modified by deleting the encephalomyocarditis virus leader and was named pVOT. Plasmid pcDNA-A22R-his containing the VV A22R ORF with a six-histidine tag (17) was used as a template to make the silent mutations and to include the six-histidine tag by PCR. The resulting PCR product, A22Rsmt-his, was digested with *Nco*I and *Bam*H I, gel purified, and inserted into pVOT, resulting in pVOT-A22Rsmt-his.

To remove the endogenous A22R gene, a 499-bp DNA segment that preceded the A22R gene and a 556-bp DNA segment that followed it were amplified by PCR using VV genomic DNA and two pairs of oligonucleotide primers. A copy of the *gus* color selection gene with a synthetic early-late viral promoter was amplified by PCR using pZippy-gus/neo (a gift from T. Shors) as a template and the oligonucleotide primers. The three PCR products were joined by a two-step recombinant PCR procedure, resulting in a 2,907-bp PCR product called 5'/3'A22-gus.

PCR was used to replace the six-histidine tag of pVOT-A22Rsmt-his with the influenza virus HA epitope tag. A 416-bp DNA segment that preceded the endogenous A22R ORF and a 562-bp segment that followed it were amplified by PCR using VV genomic DNA and two pairs of oligonucleotide primers. The three products were joined by recombinant PCR, resulting in a 1,562-bp product. The latter fragment was gel purified and ligated into vector pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen).

rVV construction. vA22i was constructed in two steps, insertion of an inducible A22R gene and deletion of the endogenous one. Approximately 10⁶ BS-C-1 cells were infected with vT7lacOI at a multiplicity of 0.2 for 1 h at 37°C. The infected cells were washed twice with Opti-MEM (Life Technologies) and transfected overnight with 2 µg of pVOT-A22Rsmt-his using Lipofectamine (Life Technologies). The transfection mixture was replaced with E-MEM containing 2.5% FBS, and the cells were harvested at 48 h. Lysates were prepared by freezing and thawing three times and sonicating for 30 s. Recombinant virus vA22/A22i was plaque purified three times in selection medium containing mycophenolic acid, hypoxanthine, and xanthine. Insertion of the A22Rsmt-his gene in the VV HA locus was verified by PCR and gel electrophoresis. To remove the endogenous A22R gene, BS-C-1 cells were infected with vA22/A22i at a multiplicity of 1 and then transfected with 1 µg of 5'/3'A22-gus DNA. Cells were harvested at 24 h, and lysates were prepared. The rVV plaques were selected in the presence of 50 µM IPTG and identified by staining with 0.1 µg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; Clontech Laboratories)/ml, and vA22i was plaque purified three times. Small rVV stocks were prepared and tested for a conditional lethal phenotype by plaque assays in the absence of IPTG. Replacement of the endogenous A22R gene with the *gus* marker was confirmed by PCR and gel electrophoresis.

vA22-HA was constructed in two steps, introduction of an A22R gene with the influenza virus HA epitope tag at the C terminus into the A22R locus of vA22i and deletion of the inducible copy. First, 10⁶ BS-C-1 cells were infected with vA22i at a multiplicity of 1; after 1 h, they were transfected with 2 µg of pCR2.1-A22Rsmt-HA as described above. After 24 h, the cells were harvested and cell lysates were prepared. A plaque assay was carried out in the absence of IPTG, and rVV plaques were identified by the absence of X-Gluc staining. vA22-HA/A22i was plaque purified three times, and the presence of the A22Rsmt-HA gene in the A22R locus was confirmed by PCR and gel electrophoresis. Next, BS-C-1 cells were infected with vA22-HA/A22i and transfected with 2 µg of a 1,976-bp PCR product containing an intact A56R gene. vA22-HA was isolated using STO cells in the presence of 0.1 mM 6-thioguanine by a reverse *gpt* selection procedure (21). Small rVV stocks were prepared, and the replacement of the inducible A22Rsmt-his gene with the A56R gene was confirmed by PCR and gel electrophoresis.

Plaque assays and one-step growth curve. BS-C-1 monolayers in six-well plates were infected with 10-fold serial dilutions of VV for 1 to 2 h. After adsorption, inocula were removed, E-MEM containing 0.5% methylcellulose and 5% FBS and with or without 50 µM IPTG was added, and cells were incubated at 37°C for 2 days. The cell monolayers were stained with crystal violet, and plaques were counted.

BS-C-1 cells in six-well plates were infected at a multiplicity of 5 for 1 h at 37°C. After absorption, inocula were removed, cells were washed twice with E-MEM containing 2.5% FBS, and E-MEM with or without 50 µM IPTG was

added. At various times after infection, cells were harvested and lysates were prepared by freezing and thawing three times followed by sonication for 1 min. Virus titers were determined by plaque assays in the presence of 50 µM IPTG if required.

Western blot analysis. Protein samples in 0.06 mM Tris-HCl (pH 6.8)-2% (wt/vol) sodium dodecyl sulfate (SDS)-10% (vol/vol) glycerol-0.001% (wt/vol) bromophenol blue-2.5% (vol/vol) β-mercaptoethanol were analyzed by electrophoresis through an SDS-4 to 20% gradient polyacrylamide gel (Owl Scientific) and transferred to nitrocellulose (Protran BA85; Schleicher & Schuell). After blocking was done with phosphate-buffered saline (PBS) containing 5% powdered milk and 0.1% (vol/vol) Tween 20 for 1 h, membranes were incubated with various primary antibodies for 2 h, followed by washes in PBS containing 0.1% Tween 20. The membranes were then incubated with appropriate secondary antibodies conjugated with horseradish peroxidase for 45 min. Proteins were detected by chemiluminescence (SuperSignal West Dura extended-duration substrate; Pierce). The primary antibodies used were as follows: antitetratristidine monoclonal antibody (MAb; Qiagen), anti-HA MAb conjugated to horseradish peroxidase (Roche Molecular Biochemicals), anti-H3L peptide antiserum (6a), D12L antiserum (a gift from S. Shuman), and A3L peptide antiserum. The secondary antibodies used were anti-mouse or anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham).

Virus purification and detergent extraction. Approximately 1.5 × 10⁹ HeLa S3 cells grown in a suspension culture were infected with vA22-HA at a multiplicity of 8 for 30 min at 37°C. At 72 h after infection, cells were harvested, sedimented, resuspended in 10 mM Tris-HCl (pH 9.0), and Dounce homogenized. VV in the cytoplasmic fraction was purified by sedimentation through a 36% sucrose cushion followed by two successive sucrose gradient sedimentations as previously described (12). The concentration of the viral suspension was determined from the optical density (OD) at 260 nm using the formula 1 OD unit equals 64 µg of virion protein/ml.

Approximately 50 µg of purified vA22-HA was incubated in 100 µl of 0.5% (vol/vol) Nonidet P-40 (NP-40)-50 mM Tris-HCl (pH 8.0) with or without 50 mM dithiothreitol (DTT) for 30 min at 37°C. The soluble membrane-associated proteins and insoluble core protein fractions were collected by centrifugation at 20,000 × g for 30 min at 4°C. Viral cores were resuspended in 0.2% (wt/vol) deoxycholate-100 mM Tris-HCl-250 mM NaCl-10 mM DTT and incubated on ice for 30 min. The insoluble proteins were pelleted by centrifugation, and the soluble core protein fraction was collected. The various protein fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting or silver staining.

Amino acid pulse-labeling. BS-C-1 cell monolayers in 12-well plates were infected with VV at a multiplicity of 5. After 1 h at 37°C, the cells were washed twice and incubated in the presence or absence of 50 µM IPTG. At various times after incubation, the cells were washed twice with methionine- and cysteine-free medium, incubated in fresh medium of the same composition but containing 2.5% dialyzed FBS for 30 min, and then labeled with 100 µCi of a mixture of [³⁵S]methionine and [³⁵S]cysteine/ml for 30 min at 37°C. Cells were harvested and incubated in hypotonic buffer (10 mM Tris-HCl [pH 8.0], 10 mM KCl, 0.5 mM EDTA)-0.2% (vol/vol) NP-40-20 mM β-mercaptoethanol-1 mM CaCl₂-0.2 mM phenylmethylsulfonyl fluoride-10 µg of micrococcal nuclease per ml for 30 min on ice. Nuclease digestions were stopped by adding 0.06 mM Tris-HCl (pH 6.8)-2% (wt/vol) SDS-10% (vol/vol) glycerol-0.001% (wt/vol) bromophenol blue-2.5% (vol/vol) β-mercaptoethanol.

Electron microscopy. BS-C-1 cell monolayers were infected with vA22i at a multiplicity of 10 at 37°C and washed twice 1 h later with complete medium. Cells were incubated for 24 h in the presence or absence of 50 µM IPTG. Cells were fixed with 2% glutaraldehyde and embedded in Epon resin, and ultrathin-sectioned samples were viewed on a Philips CM100 electron microscope (50).

Analysis of viral DNA synthesis. BS-C-1 cells in 12-well dishes were infected with VV at a multiplicity of 5 for 1 h at 37°C and washed twice with complete medium. At various times after infection, cells were harvested, sedimented, washed in PBS, resuspended in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])-1 M ammonium acetate, and lysed by freezing and thawing three times to prepare total DNA. The DNA was either spotted directly onto a BrightStar-Plus nylon membrane (Ambion) in duplicate using a slot blot apparatus (Hoeffer) or digested with proteinase K, sonicated, and denatured by incubation in 0.4 M NaOH-10 mM EDTA in a boiling water bath for 10 min prior to being spotted onto the membrane. After being washed twice with 10× SSC, the membrane was soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl), in neutralizing solution (1.5 M NaCl, 1 M Tris base), and in 5× SSC. DNA samples were UV cross-linked to the nylon membrane with a Stratalinker 2400 (Stratagene). VV DNA was detected using the Quik-Hyb protocol (Stratagene) with VV genome *Hind*III A and D fragments that were ³²P labeled using a

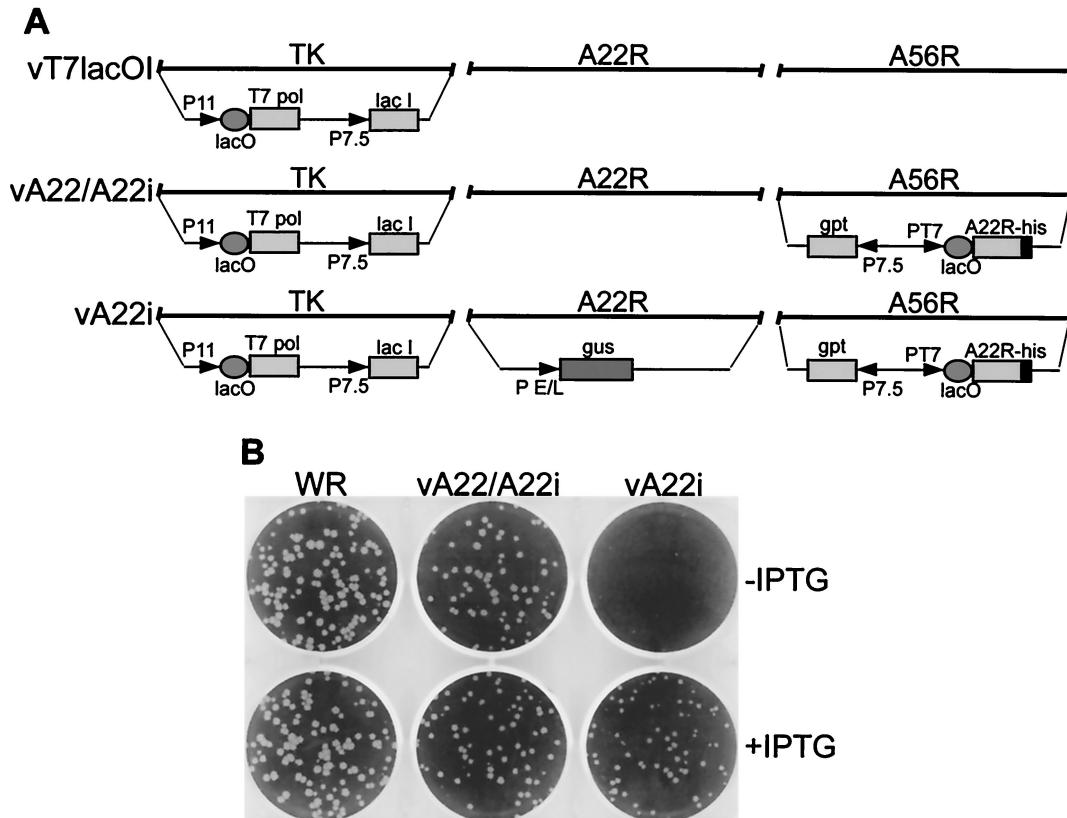


FIG. 1. Construction of a conditional lethal mutant with an inducible A22R gene. (A) Representations of relevant portions of the genomes of vT7lacOI, vA22/A22i, and vA22i corresponding to the J2R (thymidine kinase [TK]), A22R (HJ resolvase), and A56R (HA) genes. Insertions of exogenous genes into these regions are depicted below the diagrams. Abbreviations: PT7 and T7 pol, bacteriophage T7 promoter and RNA polymerase gene, respectively; lac I and lacO, *E. coli* lac repressor gene and lac operator element, respectively; gus, color marker gene; gpt, mycophenolic acid resistance gene; P11, P7.5, and P E/L, late, early-late, and synthetic early-late VV promoters, respectively. (B) Plaque formation. BS-C-1 cell monolayers were infected with wild-type VV (WR), vA22/A22i, or vA22i in the absence (−) or presence (+) of 50 μM IPTG. After 48 h, monolayers were stained with crystal violet and photographed.

random priming kit (Life Technologies) and autoradiography. Radiolabeled DNA was quantified with a PhosphorImager (Storm 860; Molecular Dynamics) and ImageQuant software.

Analysis of viral DNA by pulsed-field gel electrophoresis and restriction endonuclease digestion. BS-C-1 cell monolayers in six-well plates were infected with VV at a multiplicity of 5 for 1 h, washed twice with complete E-MEM, and incubated in fresh medium of the same composition but with or without 50 μM IPTG. At various times after infection, cells were harvested, sedimented, washed with PBS, dispersed at a concentration of 10⁷ per ml in suspension buffer (CHEF genomic DNA plug kit; Bio-Rad), warmed to 50°C, mixed with Clear-cut agarose (Bio-Rad) to a final concentration of 0.75%, and formed into 100-μl agarose plugs. Agarose plugs were incubated in a proteinase K reaction mixture (Bio-Rad) at 50°C for 24 h and then equilibrated in wash buffer (Bio-Rad). One-third of an agarose plug (~33 μl) was loaded into a 1% agarose gel (pulsed-field certified agarose; Bio-Rad), and electrophoresis was carried out in 0.5× TBE (44.5 mM Tris-borate, 1 mM EDTA [pH 8.3]) using a CHEF-DRII apparatus (Bio-Rad). Pulsed-field electrophoresis was performed at 5.8 V with a switching time gradient of 50 to 90 s for 22 h at 14°C. The agarose gel was stained with 0.5 μg of ethidium bromide per ml and UV irradiated on a transilluminator for 10 min, followed by soaking in 0.25 M HCl for 30 min to nick DNA. DNA was transferred to an Immobilon-Ny+ nylon membrane (Millipore) and detected by Southern blot hybridization using a 5' ³²P-end-labeled oligonucleotide complementary to the 70-bp repeats at the ends of the VV genome (6).

with ethanol. After drying, DNA pellets were resuspended in 10 mM Tris-HCl (pH 8.0) and passed through a 25-gauge needle. The DNA concentration was calculated by determining the absorbance at 260 nm, based on 1 unit equaling 50 μg of DNA/ml. A 2-μg sample of DNA was digested with *Bst*II at 37°C, subjected to electrophoresis through a 0.7% agarose gel (SeaKem ME; FMC), and transferred to an Immobilon-Ny+ nylon membrane. DNA was detected by Southern blot hybridization using a 5' ³²P-end-labeled oligonucleotide complementary to the 70-bp repeats at the ends of the VV genome (6).

RESULTS

Construction of a conditional lethal rVV with an inducible A22R gene. To gain insight into the role of the VV HJ resolvase during infection, a mutant with an A22R gene regulated by a bacteriophage T7 promoter and the *Escherichia coli* lac repressor was generated. The starting virus for this construction, vT7lacOI (1), contained an inducible bacteriophage T7 RNA polymerase gene and a constitutive *E. coli* lac repressor gene (Fig. 1A). Inducible mutant virus vA22i was constructed in two steps. First, a transfer plasmid containing an inducible A22R gene with a six-histidine tag at the C terminus (A22R-his) and the gpt selectable marker was inserted into the VV HA gene (ORF A56R) of vT7lacOI by homologous recombination. The resulting intermediate virus, vA22/A22i, was selected in the presence of mycophenolic acid and contains

Concatemer junctions were analyzed by restriction enzyme digestion and Southern blotting. Pellets of infected cells were suspended in 50 μl of 150 mM NaCl–20 mM Tris-HCl (pH 8.0)–10 mM EDTA and then lysed in 250 μl of 20 mM Tris-HCl (pH 8.0)–10 mM EDTA–0.75% SDS–0.4 mg of proteinase K/ml. The reaction mixtures were incubated for ~6 h at 37°C. The samples were extracted with phenol, phenol-chloroform, and chloroform before precipitation

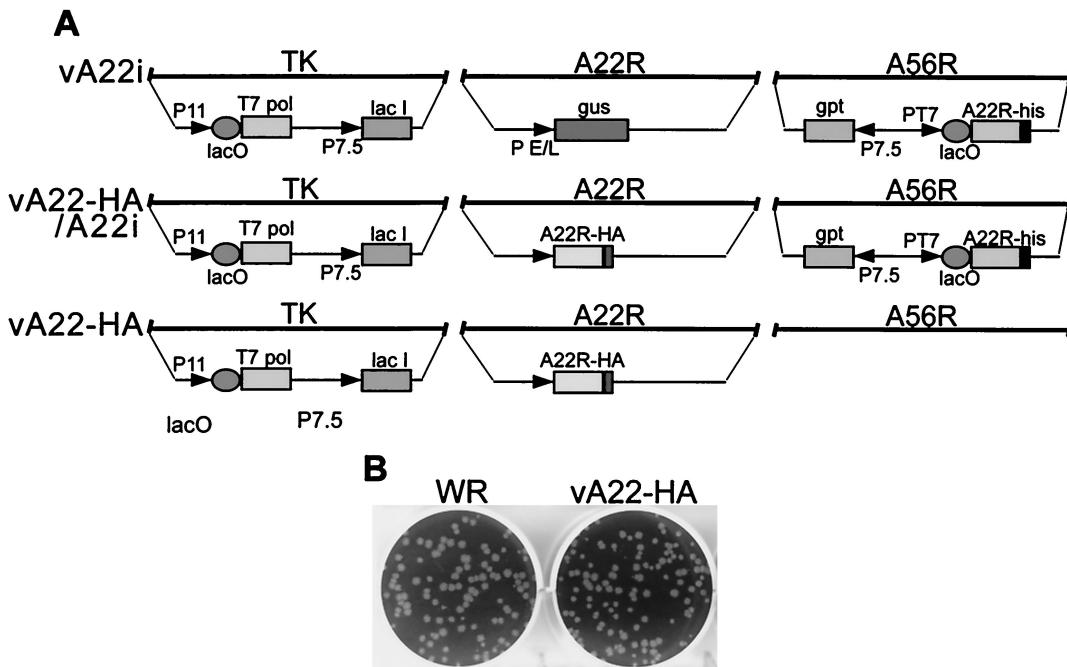


FIG. 2. Construction of an rVV with an HA epitope-tagged A22R gene. (A) Representations of relevant portions of the genomes of vA22i, vA22-HA/A22i, and vA22-HA. Abbreviations are the same as those used in Fig. 1. (B) Plaque formation. BS-C-1 cell monolayers were infected with wild-type VV or vA22-HA. After 48 h, monolayers were stained with crystal violet and photographed.

both inducible and endogenous A22R gene copies (Fig. 1A). The inducible A22R-his ORF is preceded by the bacteriophage T7 promoter and the *E. coli lac* operator, but the encephalomyocarditis virus leader of the original system (49) was eliminated to prevent cap-independent translation of potential upstream read-through transcripts. The natural A22R gene was then deleted from vA22/A22i by homologous recombination using DNA containing the *gus* color selection marker gene. Plaques that formed in the presence of IPTG and stained blue with X-Gluc were picked to yield vA22i (Fig. 1A). PCR was used to confirm the structure of the rVV at each stage of construction.

The ability of the rVVs to replicate and infect adjacent cells in the presence and absence of IPTG was determined by plaque assays. vA22/A22i, which contains both inducible and unregulated A22R genes, produced plaques in the presence or absence of IPTG (Fig. 1B). In contrast, vA22i, which contains just an inducible A22R-his gene, produced plaques only in the presence of IPTG, indicating that the HJ resolvase was stringently regulated and essential for virus replication. Previous studies had shown that the plaques formed by vT7lacOI and derivatives were smaller than those of the parental VV strain WR, presumably due to the insertions in the thymidine kinase and HA genes, consistent with the small difference in plaque sizes between WR and the new rVVs (Fig. 1B).

Construction of an rVV with an epitope-tagged A22R gene. To facilitate the characterization of the HJ resolvase, we constructed a second recombinant VV, in which the A22R ORF was engineered to contain the influenza virus HA epitope tag at the C terminus while retaining the authentic A22R genome location and transcriptional regulatory signals. Construction of vA22-HA was performed in two steps, starting with IPTG-

dependent vA22i as the parental virus (Fig. 2A). This protocol provided a selection scheme and, by isolating a revertant virus, also assured us that vA22i had no spurious gene defects. The A22R-HA ORF was inserted into the A22R locus of vA22i by homologous recombination, and plaques that formed in the absence of IPTG and did not stain with X-Gluc were picked. The resulting intermediate virus (vA22-HA/A22i) contained the epitope-tagged A22R gene but still had the inducible A22R-his gene (Fig. 2A). Homologous recombination was then used to restore the VV HA gene and thereby delete the inducible A22R-his gene as well as the *gpt* gene from vA22-HA/A22i. Recombinant vA22-HA (Fig. 2A) was isolated using a reverse *gpt* selection procedure (21) and characterized by PCR. Plaques formed by vA22-HA were similar in size to those formed by WR (Fig. 2B), indicating that the epitope-tagged HJ endonuclease was fully functional.

Expression of the HJ resolvase during VV infection. The HA epitope tag on the A22R gene of vA22-HA provided a way to detect the A22R protein by SDS-PAGE and Western blotting of infected cell extracts. A doublet band was detected with an anti-influenza virus HA MAb at 4 h after infection, and its intensity progressively increased with time (Fig. 3). As expected for a late protein, the bands were not detected even after 24 h in the presence of cytosine arabinoside, an inhibitor of DNA replication. The mass of the upper band was estimated to be 23 kDa, similar to that expected for the product of the A22R-HA ORF. The lower A22R-HA protein species apparently resulted from internal initiation at an in-frame methionine 16 residues downstream of the start of the ORF. The late expression of the HJ resolvase suggested involvement in the processing rather than the synthesis of viral DNA.

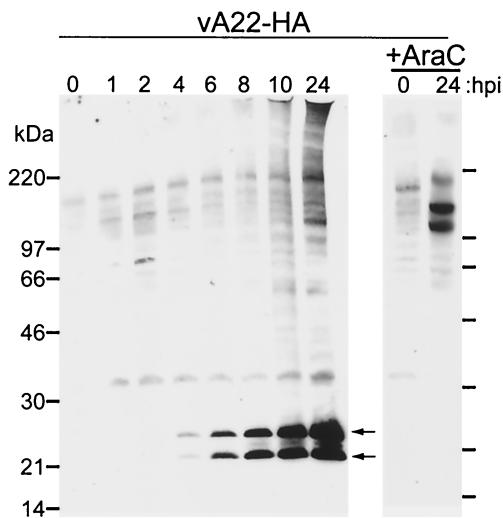


FIG. 3. HJ resolvase synthesis during virus infection. BS-C-1 cells were infected at a multiplicity of 5 in the absence or presence of 44 μ M cytosine arabinoside (AraC). At the indicated hours postinfection (hpi), cells were lysed in hypotonic buffer containing 0.2% NP-40 and 10 μ g of micrococcal nuclease per ml and analyzed by Western blotting using a horseradish peroxidase-conjugated anti-HA antibody. Proteins were detected by chemiluminescence. The positions and masses of marker proteins are indicated. Arrows point to the A22R-HA doublet.

The HJ resolvase is packaged in virion cores. Virus from cells infected with vA22-HA was purified by successive sucrose gradient sedimentations, and individual fractions from the second sedimentation were analyzed by OD measurement (Fig. 4A), SDS-PAGE and silver staining (not shown), and Western blotting with an anti-HA MAb (Fig. 4A). The fractions containing the A22R-HA doublet corresponded to those containing virus particles. The purified virus particles were extracted with 0.5% NP-40 in the absence or presence of 50 mM DTT. The membrane and core fractions were then separated by centrifugation and analyzed by SDS-PAGE and Western blotting (Fig. 4B) or silver staining (Fig. 4C). None of the A22R protein was extracted into the supernatant under these conditions, indicating that it was entirely associated with the core (Fig. 4B). In contrast, considerable amounts of the H3L protein (Fig. 4B) and other membrane proteins (Fig. 4C) were released by the nonionic detergent. The core was treated with deoxycholate and DTT and separated into soluble and insoluble fractions. The A22R protein and the major 4b (A3L) core structural protein were found entirely in the insoluble fraction, whereas some capping enzyme (D12L) (Fig. 4B) and other proteins (Fig. 4C) were found in the supernatant. We concluded that the HJ resolvase is associated with virions and tightly packaged in cores.

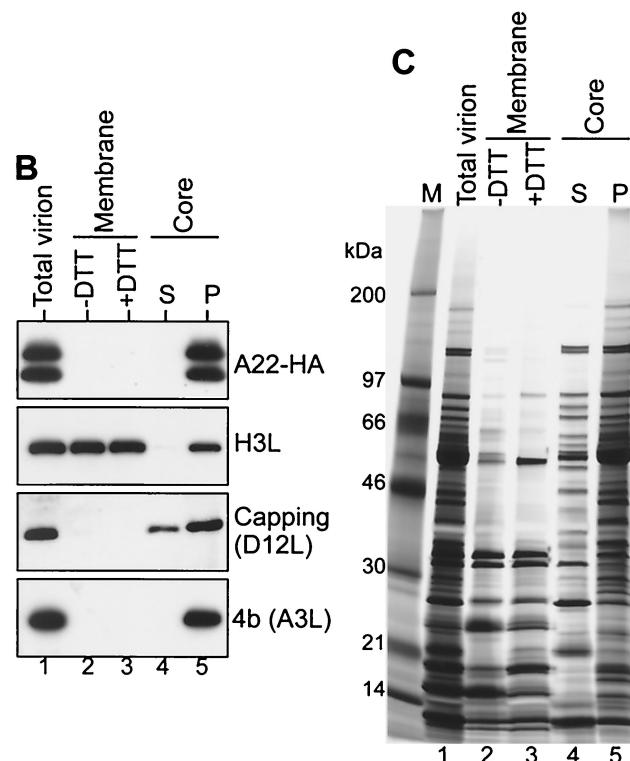
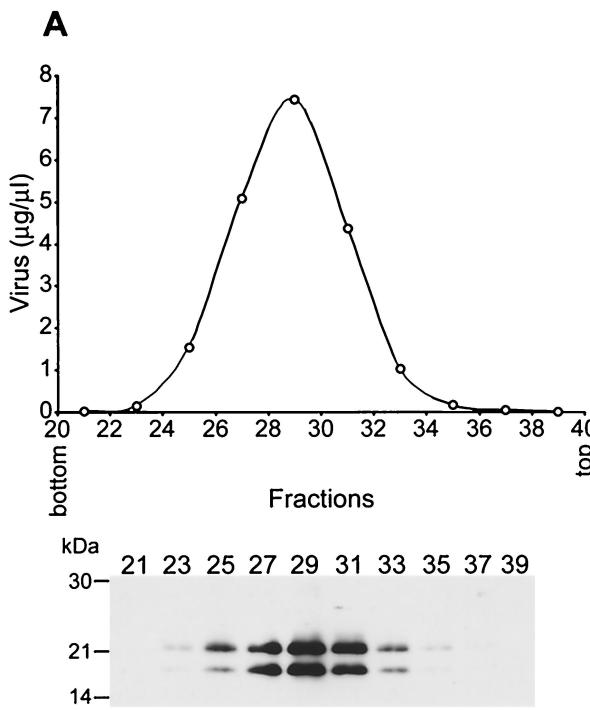


FIG. 4. Association of the HJ resolvase with purified virions. (A) Cosedimentation of the A22-HA protein with virus particles. Sucrose gradient fractions were collected and analyzed for particles by light scattering and for the A22-HA protein by Western blotting as described in the legend to Fig. 3. (B) Detergent extraction of purified vA22-HA virions. Virions were incubated with 0.5% NP-40 in the absence or presence of 50 mM DTT, and the released membrane-associated and insoluble core proteins were separated by centrifugation. The viral cores were then resuspended in deoxycholate-containing lysis buffer and separated by centrifugation into supernatant (S) and pellet (P) fractions. The samples were analyzed by SDS-PAGE and Western blotting using antibodies to the HA epitope tag, the H3L protein, the large subunit of capping enzyme (D12L), and major core protein 4b (A3L). (C) The fractions described in panel B were analyzed by SDS-PAGE and silver staining.

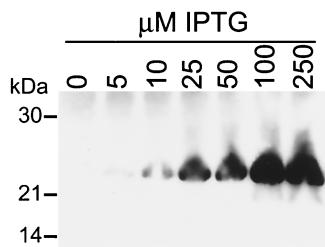


FIG. 5. Inducer-dependent expression of the HJ resolvase. BS-C-1 cells were infected with 5 PFU of vA22i per cell in the absence (0) or presence of 5 to 250 μ M IPTG. Cells were harvested after 20 h and analyzed by SDS-PAGE and Western blotting using an antitetrathistidine antibody. Proteins were detected by chemiluminescence. The positions and masses of marker proteins are indicated.

Inducible expression of the HJ resolvase during infection with vA22i. The addition of six histidines to the C terminus of the A22R ORF in vA22i allowed us to detect the HJ resolvase with antibody to the tag. Cells were infected in the presence or absence of IPTG and harvested after 20 h. In the absence of IPTG, no protein band was seen by SDS-PAGE and Western blotting (Fig. 5). A faint band of the expected size was detected at the lowest concentration of IPTG (5 μ M), and its intensity increased with IPTG concentration (Fig. 5). We attributed the finding of a single 23-kDa A22R-his band, instead of a doublet, to an optimal Kozak translation initiation sequence (25) engineered in the vA22i construct. In vA22-HA, the A22R-HA ORF retained the natural translation initiation context, which was suboptimal. Evidently, translation initiation at the downstream site was not necessary for the replication of vA22i.

Inducer-dependent replication of vA22i. Plaque assays had indicated that the replication of vA22i was stringently repressed in the absence of IPTG (Fig. 1B). One-step growth yield assays were carried out to determine the IPTG dependence under conditions to be used for biochemical experiments. BS-C-1 cells, in the presence or absence of 50 μ M IPTG, were infected with vA22i, vA22/A22i, or WR at a multiplicity of 5. In the presence of IPTG, the yield of vA22i started to plateau by 12 h, and the final yield was similar to that of vA22/A22i and only slightly lower than that of WR (Fig. 6). In the absence of IPTG the yield of vA22i at 12 h was more than 2 log units lower than that in the presence of the inducer. At later times, however, there was a gradual increase in the titer of vA22i so that the difference was less than 2 log units at 24 h and approximately 1 log unit at 48 h.

Effect of A22R repression on viral DNA synthesis. Considering that the known viral proteins required for DNA replication are encoded by early genes, the late expression of the A22R gene seemed to make such a role for the HJ resolvase unlikely. Nevertheless, to evaluate the effect of repression of the A22R gene on viral DNA synthesis, BS-C-1 cells were infected with vA22i in the absence and presence of 50 μ M IPTG or with vA22/A22i or WR as a control. At various times, the infected cells were harvested and total DNA was prepared from the infected cell lysates. Viral DNA accumulation was quantified by spotting the samples directly onto membranes followed by denaturation and hybridization in situ with a 32 P-labeled viral DNA probe. For the first 6 h, viral DNA accumulated at similar rates under all conditions (Fig. 7A). How-

ever, at subsequent times, viral DNA accumulated more slowly in cells infected with vA22i in the absence of IPTG than in its presence. We considered that the decrease might have been an artifact due to the formation of branched DNA molecules that were difficult to denature for hybridization while bound to the membrane. However, a similar result was obtained when the DNA samples were deproteinized, sheared, and denatured in alkali at 100°C prior to slot blot analysis (Fig. 7B). These results indicated that the HJ resolvase was needed to maintain a high rate of viral DNA replication.

Inhibition of viral DNA processing under nonpermissive conditions. Because of the ability of the A22R protein to resolve HJs, we were most interested in examining the viral DNA that formed under nonpermissive conditions. DNA from cells infected with WR or vA22i in the presence or absence of IPTG was analyzed by pulsed-field gel electrophoresis and Southern blotting. Genome-length (185-kbp) DNA was detected at 2 h and accumulated along with smaller amounts of putative dimer and trimer species with time (Fig. 8B). Some viral DNA also remained at the top of the gel (Fig. 8B) along with high-molecular-weight cellular DNA detected by ethidium bromide staining (not shown). A similar pattern of DNA accumulation occurred in cells infected with vA22i in the presence of IPTG, although the DNA bands were first detected at 4 h and there was an additional band between the monomer and the dimer species (Fig. 8A). In contrast, when cells were infected with vA22i in the absence of IPTG, nearly all the viral DNA remained at the top of the gel for the first 12 h (Fig. 8A). At 24 h after infection, however, monomer, dimer, and trimer species were detected, although at levels achieved within 6 h in the presence of the inducer. The appearance of some unit-length genome DNA at 24 h in the absence of IPTG correlated with the rise in infectivity noted earlier (Fig. 6).

Accumulation of viral concatemer junctions under nonpermissive conditions. The DNA trapped at the top of the pulsed-field gel could represent branched or concatemeric species. Restriction enzyme analysis of DNA from infected cells has

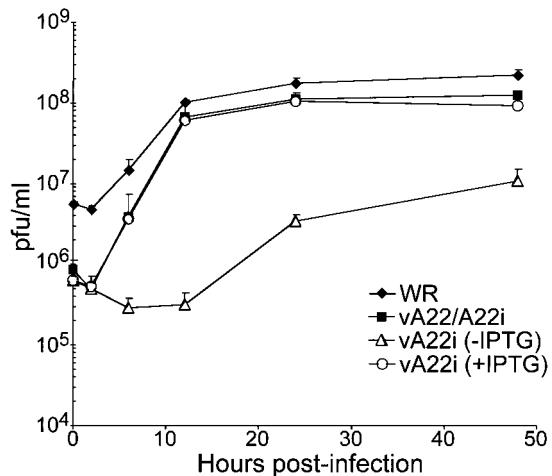


FIG. 6. Inducer-dependent replication of vA22i. BS-C-1 cells were infected with 5 PFU of WR, vA22/A22i, or vA22i in the absence or presence of 50 μ M IPTG. At the indicated times, the cells were harvested and virus titers were determined by plaque assays in the presence of IPTG. Upper bars of standard deviations are shown.

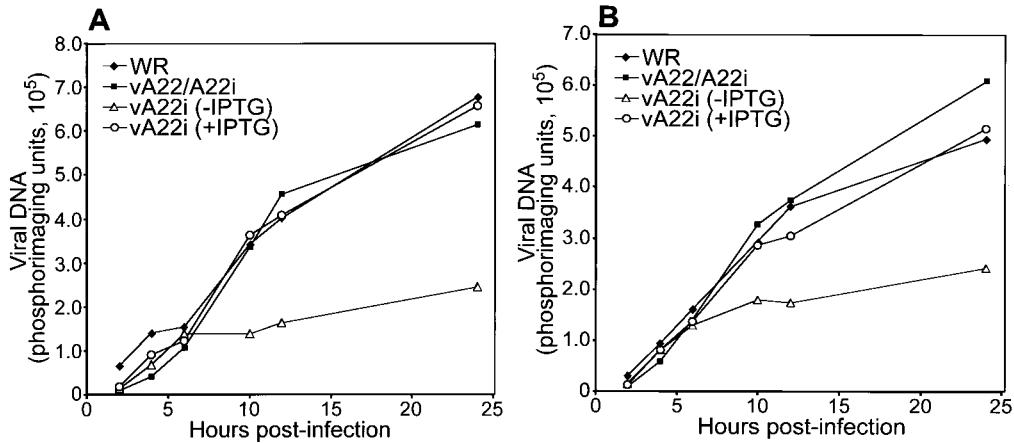


FIG. 7. Synthesis of viral DNA. BS-C-1 cells were infected with 5 PFU of WR, vA22/A22i, or vA22i in the absence or presence of 50 μ M IPTG. At the indicated times, the cells were harvested and lysed in a high-salt solution. (A) Duplicate DNA samples were spotted directly onto a membrane and hybridized to 32 P-labeled VV DNA. (B) DNA samples were deproteinized, sonicated, and denatured with alkali at $\sim 100^\circ\text{C}$ prior to spotting onto a membrane and hybridization. Radioactivity was measured with a PhosphorImager and quantified with ImageQuant software.

been used to detect concatemer junctions. The hairpin ends of mature VV genomes with two sets of tandem repeats separated by a *Bst*EII site are depicted in Fig. 9A. An unresolved concatemer junction is shown above the hairpin ends (Fig. 9A). *Bst*EII digestion of the concatemer junction and hairpin termini would yield 2.6- and 1.3-kbp fragments, respectively, that would hybridize to a 32 P-labeled oligonucleotide containing repeat sequences. Previous experiments had shown that the 2.6-kbp concatemer junction fragment was faintly and transiently detected early in infection with wild-type VV (3). In contrast, the junction accumulated when cells were infected with mutant viruses that were defective in concatemer resolu-

tion (30). Resolved hairpin termini were detected in DNA from cells infected for 4 or more hours with wild-type VV (Fig. 9B), consistent with the time of appearance of unit-length genomes. The concatemer junction fragment could barely be detected. A similar pattern occurred when cells were infected with vA22/A22i in the absence of IPTG (Fig. 9C). The junction fragment was seen at 4 h after infection with vA22i in the presence of IPTG but was barely discerned at 6 h and was undetectable at later times (Fig. 9C). Correspondingly, the resolved hairpin ends accumulated in the presence of IPTG, as shown by intense bands (Fig. 9C). In contrast, the junction fragment persisted and accumulated with time in cells infected

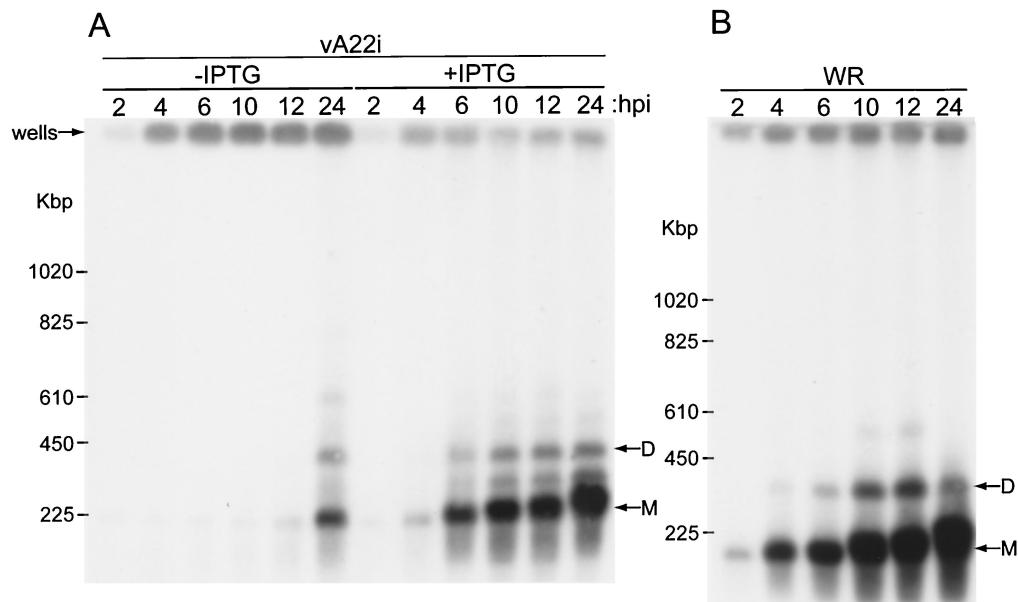


FIG. 8. Analysis of viral DNA by pulsed-field gel electrophoresis. BS-C-1 cells were infected with a 5 PFU of vA22i in the absence or presence of 50 μ M IPTG (A) or WR (B). Cells were harvested at the indicated times (hpi, hours postinfection) and embedded in agarose. The DNA was subjected to pulsed-field electrophoresis, transferred onto a nylon membrane, and hybridized with 32 P-labeled VV DNA. An autoradiograph is shown with the position of the wells and DNA size markers on the left. The positions of monomer (M) and dimer (D) viral genomes are indicated by arrows on the right.

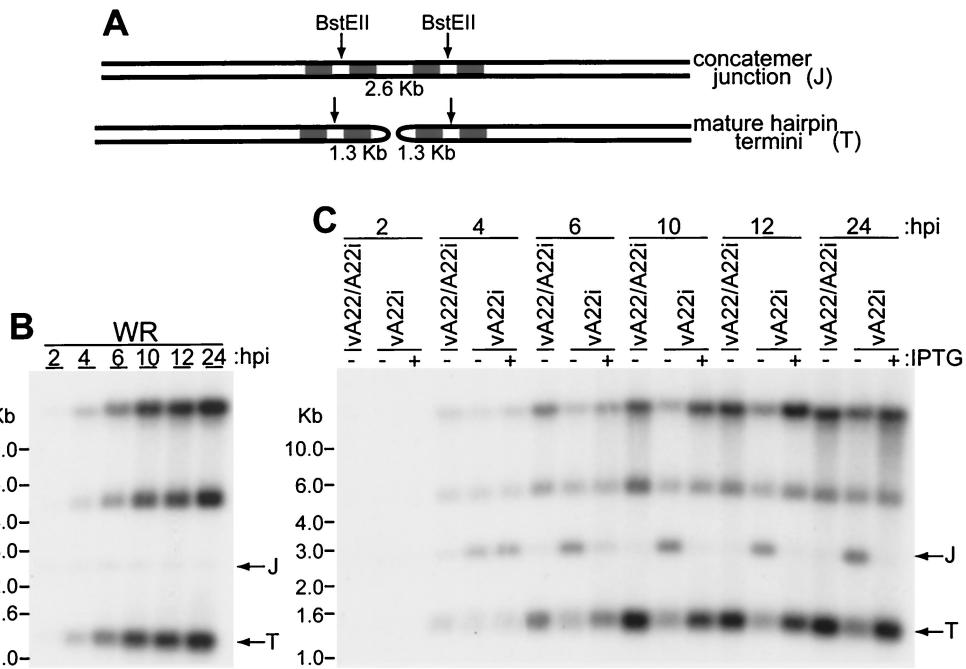


FIG. 9. Analysis of concatemer junctions. (A) Diagram of a concatemer junction (J) and mature hairpin termini (T). *Bst*EII cleavage sites are shown. (B and C) BS-C-1 cells were infected with WR (B) or with vA22/A22i or vA22i in the absence (−) or presence (+) of 50 μ M IPTG (C). At the indicated times (hpi, hours postinfection), DNA was purified; 2 μ g was digested with restriction enzyme *Bst*EII, electrophoresed through an agarose gel, transferred onto a nylon membrane, and hybridized with a 32 P-labeled oligonucleotide complementary to the repeated sequence near the ends of the genome. The positions of monomer termini (T) and concatemer junction (J) intermediates are indicated by arrows.

with vA22i in the absence of IPTG (Fig. 9C), even though total viral DNA synthesis was reduced under these conditions. A fragment corresponding to the hairpin ends was faint but accumulated slightly by 24 h in the absence of IPTG (Fig. 9C). These data indicated that the expression of A22R is required for the efficient resolution of concatemer junctions. Further studies are needed to determine whether the DNA also contains branches.

Synthesis of viral protein under nonpermissive conditions. Previous studies had shown that the resolution of concatemer junctions was inhibited when cells were infected with mutant viruses that have a global block in viral late-protein synthesis (7, 30). We were therefore curious to determine whether viral late-protein synthesis was also blocked in cells infected with vA22i under nonpermissive conditions. This was of particular concern because of decreased viral DNA replication which occurred at late times after nonpermissive infection with vA22i. To investigate viral protein synthesis, cells were infected with WR, vA22/A22i, or vA22i in the presence or absence of IPTG. The cells were labeled with [35 S]methionine and [35 S]cysteine for 30 min at various times after infection. Under all conditions, the patterns of viral protein synthesis were very similar, and viral late proteins were detected at 5 h and later times (Fig. 10). The only difference between cells infected with vA22i and other viruses was the appearance of a 65-kDa band that probably represented the *gus* protein.

The proteolytic processing of certain core proteins is dependent on virus maturation (24). Pulse-chase experiments indicated that cleavage of P4a and P4b to 4a and 4b was reduced in cells infected with vA22i in the absence of IPTG compared

to that in cells infected with WR, vA22/A22i, or vA22i in the presence of IPTG (data not shown), consistent with the inhibition of virus morphogenesis documented in the next section.

Effect of A22R repression on virus morphogenesis. Cells infected with vA22i in the presence or absence of IPTG were examined by electron microscopy. Typically, virus assembly occurs asynchronously, and immature and mature virions can be detected in the same cell. This was the case for cells infected in the presence of IPTG, where circular immature virions as well as brick-shaped mature virions were seen at 24 h (Fig. 11). In the absence of IPTG, there were mainly immature virus particles, some of which had nucleoids, and dense spherical particles (Fig. 11). The latter resembled aberrant virions lacking DNA that were previously found in cells infected with a DNA-packaging mutant (6). The “eye-ball” appearance of some structures that appeared to be detached from the cell is actually dense spherical particles with a ring of cytoplasm in tangentially cut microvilli. However, there were additional dense spherical particles without the ring of cytoplasm that were probably externalized.

DISCUSSION

It was previously reported that the A22R ORF encodes an *E. coli* RuvC homolog with specific HJ resolvase activity (17). In this follow-up study, we have shown that the HJ resolvase is expressed late in infection, packaged in virus particles, and required for efficient late-stage DNA replication and processing of DNA concatemers into unit-length genomes. These im-

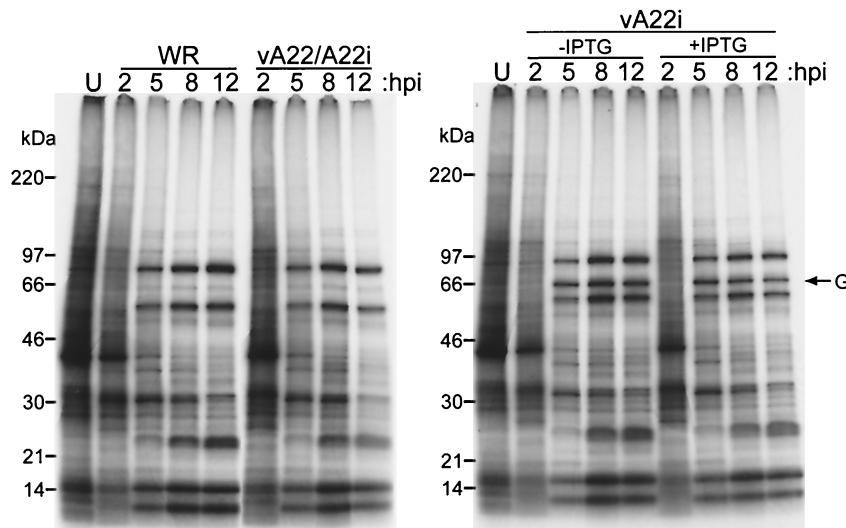


FIG. 10. Viral protein synthesis under permissive and nonpermissive conditions. BS-C-1 cell monolayers were infected with 5 PFU (per cell) of WR, vA22/A22i, or vA22i in the absence (−) or presence (+) of 50 μ M IPTG. At the indicated hour postinfection (hpi), cells were labeled with a mixture of [35 S]methionine and [35 S]cysteine for 30 min. The labeled proteins were analyzed by SDS-PAGE and autoradiography. Lanes U contain proteins from uninfected cells. G, position of the band presumed to be the *gus* protein, which is present only in vA22i samples. The positions and masses of marker proteins are indicated.

portant functions of the HJ resolvase are consistent with the presence of A22R orthologs in all sequenced poxviruses.

To facilitate studies of the HJ resolvase, we made an rVV in which the A22R ORF had a C-terminal epitope tag but remained under the control of its natural promoter. Synthesis of the HJ resolvase was prevented by an inhibitor of DNA replication but was detected at about 4 h after a normal infection and then increased with time, as predicted from the late promoter consensus sequence. Like many late proteins, the HJ resolvase was associated with the cores of purified virus particles. This localization makes it necessary to consider whether the resolvase has a role in DNA packaging or at an early step in infection, although it may simply reflect the abundance of the HJ resolvase in viroplasm that is incorporated into assembling virus particles. As discussed below, the presence of the enzyme in virions affects the interpretation of some experiments.

A genetic approach was taken to determine the role of the HJ resolvase. vA22i, an inducible conditional lethal mutant VV that expresses bacteriophage T7 RNA polymerase and the *E. coli lac* repressor, was constructed. The stringency of the system is based on the use of two *E. coli lac* operators: one regulating the expression of T7 RNA polymerase and the other regulating the activity of a T7 promoter adjacent to the A22R gene (49). The original system was designed for high-level expression and contains part of the encephalomyocarditis virus leader sequence to allow cap-independent translation. In a modified version of the expression system used here, we removed this leader sequence to preclude cap-independent translation of late RNAs that initiated upstream of the T7 promoter and continued past the operator. In cells infected with vA22i, expression of the HJ resolvase was dependent on IPTG, and the protein could not be detected by Western blotting in the absence of the inducer. Consistent with this finding, vA22i plaque formation was stringently repressed under these conditions. Under one-step virus growth conditions, vA22i rep-

lication started to plateau at 12 h in the presence of IPTG. In the absence of the inducer, virus replication was undetectable at the latter time but occurred at a low rate over the next 36 h. We considered several possible explanations for the delayed virus production. One is that a low level of the HJ resolvase was made but not detected by Western blotting. Another possibility is the relatively high multiplicity used for the one-step growth experiment (5 PFU, or ~100 particles/cell), compared to 1 particle in a plaque assay, coupled with the fact that HJ resolvase is packaged in virus particles. A third possibility is that the requirement for the HJ resolvase is not absolute and that there are other DNA-processing mechanisms involving other viral or cellular proteins, such as topoisomerase, which is known to cleave HJs in vitro (35, 39). The latter explanation would imply the possibility of isolating a viable, although poorly replicating, deletion mutant. An effort to obtain such a virus is under way.

To investigate the defect in the formation of infectious virus, we analyzed viral DNA synthesis and processing. In cells infected with wild-type VV or vA22i under permissive or nonpermissive conditions, viral DNA accumulation was similar up to 6 h after infection. However, at later times, viral DNA accumulated much more slowly in cells infected with vA22i in the absence of the inducer than in the presence of the inducer. This result was reminiscent of bacteriophage T4, in which origin initiation of DNA replication is programmed to cease and recombinational mechanisms become important at late times (32). In circumstances where the DNA primase is deficient, bacteriophage T4 endonuclease VII facilitates the priming of DNA synthesis by a mechanism involving the cleavage of recombinant junctions. Such a pathway may be relevant for VV, as no poxvirus primase has been identified and the nuclear location of the cellular primase could preclude its use for cytoplasmic viral DNA replication. In this model, the 3' ends produced by the VV HJ resolvase might prime DNA synthesis. The accumulation of extensively branched DNA molecules,

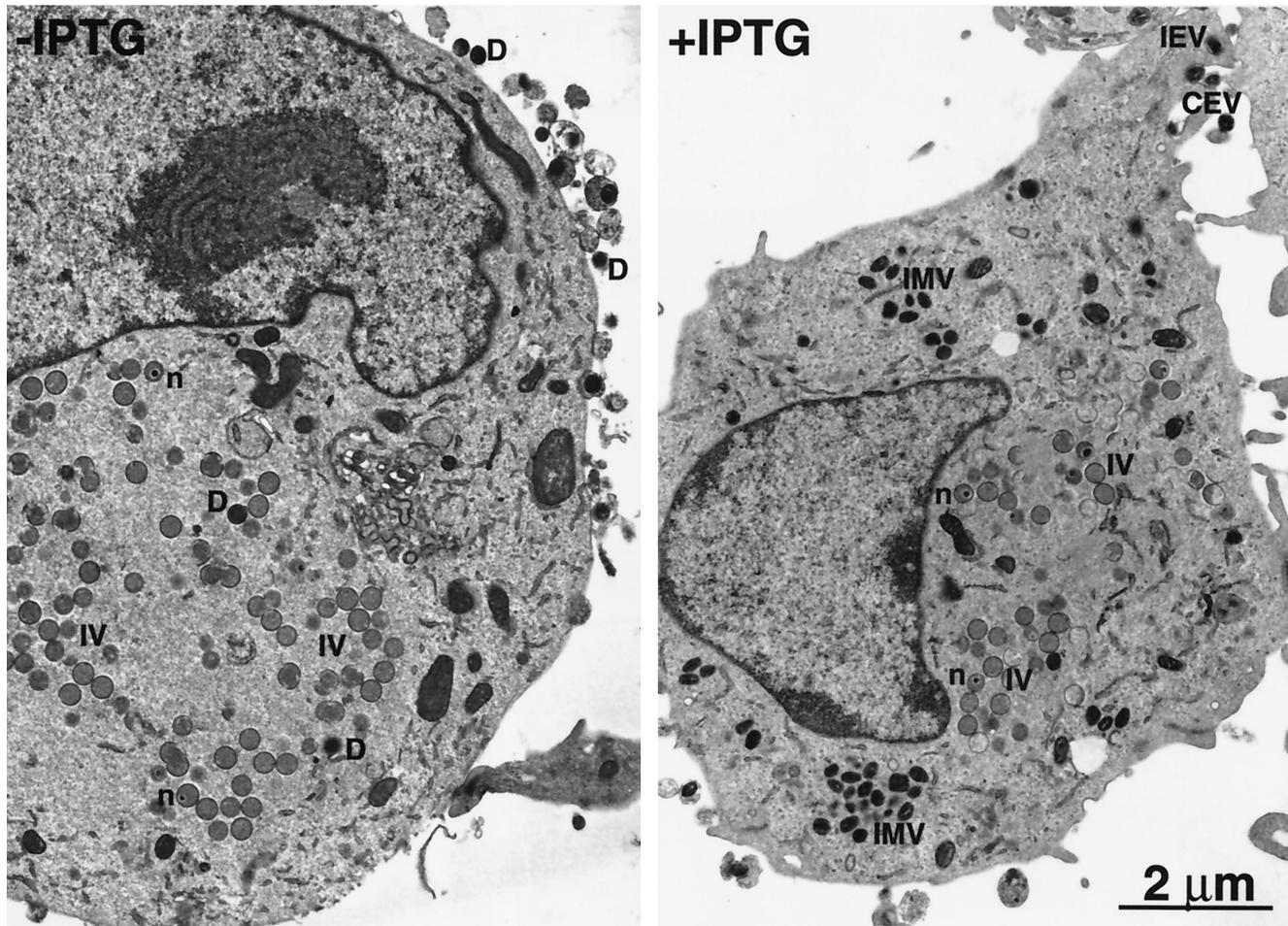


FIG. 11. Electron microscopy of cells infected with vA22i. BS-C-1 cells were infected with vA22i at a multiplicity of 10 in the absence (−) or presence (+) of 50 μ M IPTG. After 24 h, the cells were harvested, fixed, and embedded in Epon. Ultrathin sections were prepared for electron microscopy. Abbreviations: IV, immature virion; n, nucleoid; D, dense immature virion; IMV, intracellular mature virion; CEV, cell-associated enveloped virion; IEV, intracellular mature enveloped virion.

discussed below, also could contribute to the slowing down of VV DNA synthesis under nonpermissive conditions.

A previous study had demonstrated that sufficient viral DNA is made by 6.5 h after infection for a nearly normal yield of VV (37). Therefore, it seemed unlikely that the observed defect in DNA synthesis could entirely account for the inhibition of vA22i yield under nonpermissive conditions. Based on the *in vitro* activity of the HJ resolvase, we suspected that there was also a block in the processing of the replicated viral DNA. We used pulsed-field gel electrophoresis, a technique previously used to resolve full-length and concatemeric forms of poxvirus DNA (7, 30), to examine this idea. Under conditions of permissive infection, genome-length viral DNA species were detected at 4 h after infection; however, they were not detected until 24 h under nonpermissive conditions. Prior to that time, the viral DNA was found exclusively at the top of the gel, corresponding to the application site. The late appearance of unit-length genomes correlated with the delayed rise in infectivity in the absence of the inducer.

Nonmigrating DNA species are also seen under normal conditions and have been thought to represent branched, concatemeric networks (7, 30). To further characterize the role of the

HJ resolvase, we specifically analyzed the viral DNA by restriction endonuclease analysis for the presence of concatemer junctions (3). Under permissive conditions, uncleaved concatemer junctions were detected at 4 h after infection but were difficult to see at later times. In contrast, concatemer junctions increased in amount and persisted for at least 24 h under nonpermissive conditions. This result could be readily explained if the concatemer junction is a substrate for the HJ resolvase. Supporting such an idea are data indicating that the concatemer junction can assume an HJ-like structure in a supercoiled plasmid (28) and that the HJ endonuclease can resolve such a structure *in vitro* (A. Garcia, unpublished data). Nevertheless, other explanations were considered. One was a general defect in viral late-gene expression, which is known to inhibit concatemer resolution (7, 30), perhaps as a result of the reduced DNA replication at late times. However, metabolic labeling of vA22i-infected cells revealed a normal level of viral late-protein synthesis under nonpermissive conditions. It will be important to determine whether the DNA made under nonpermissive conditions is highly branched and whether the HJ resolvase has debranching activity. Although we favor the idea that the HJ resolvase directly cleaves concatemer junc-

tions, it is also possible that the primary role of this enzyme is to resolve DNA networks and that another enzyme, such as topoisomerase (35, 38), resolves concatemer junctions. Failed attempts to delete the topoisomerase gene from VV suggested that it is essential (41), but the absence of conditional lethal topoisomerase mutants has prevented efforts to determine its role. Temperature-sensitive mutations in either the small or the large subunits of capping enzyme result in a defect in DNA concatemer resolution that is not understood but appears to be independent of a general defect in viral protein synthesis (5, 19).

A defect in viral morphogenesis occurred when cells were infected with vA22i in the absence of the inducer. Brick-shaped mature virions were rare, and the predominant structures were immature virions and dense spherical particles similar to ones previously shown to lack DNA and to form when cells were infected with an A32L mutant defective in DNA packaging (6). Under normal conditions of VV infection, unit-length genomes are packaged and branched or concatemeric forms are apparently excluded. For bacteriophage T4, DNA processing is associated with assembly; endonuclease VII debranches replicated DNA, and a capsid-associated endonuclease cleaves the concatemers (4). In contrast, the resolution of VV DNA concatemers occurs even when assembly is blocked at an early stage by the drug rifampin (30) or at a later stage with a DNA-packaging mutant (6).

In summary, the results presented here indicate that the A22R protein is involved in late-stage DNA replication, resolution of concatemer junctions, formation of unit-length genomes, and morphogenesis of virus particles. If bacteriophage T4 is a guide, then the replication, recombination, and processing of VV DNA may be interconnected with alternative pathways.

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