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The Vaccinia Virus D5 Protein, Which Is Required for DNA Replication, Is a Nucleic Acid-Independent Nucleoside Triphosphatase

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The vaccinia virus D5 gene encodes a 90-kDa protein that is transiently expressed at early times after infection. Temperature-sensitive mutants with lesions in the D5 gene exhibit a fast-stop DNA$^-$ phenotype and are also impaired in homologous recombination. Here we report the overexpression of the D5 protein within the context of a vaccinia virus infection and its purification to apparent homogeneity. The purified protein has an intrinsic nucleoside triphosphatase activity which is independent of, and not stimulated by, any common nucleic acid cofactors. All eight common ribo- and deoxyribonucleoside triphosphates are hydrolyzed to the diphosphate form in the presence of a divalent cation. Implications for the role of D5 in viral DNA replication are addressed.

Vaccinia virus replicates in the cytoplasm of mammalian cells and is thought to encode all of the functions required for replication of its 192-kb double-stranded linear genome. Virally encoded enzymes with a known or presumed function in DNA replication include the DNA polymerase, thymidine kinase (TK), thymidylate kinase, ribonucleotide reductase, dUTPase, topoisomerase I, DNA ligase, and uracil DNA glycosylase (reviewed in references 16 and 48). Genetic analysis of complementation groups of temperature-sensitive (*ts*) mutants which display a strict DNA$^-$ phenotype at the nonpermissive temperature has led to the identification of three early viral genes required throughout DNA synthesis: E9L (DNA polymerase), B1R (serine/threonine protein kinase), and D5R (5, 6, 10, 11, 29, 38, 50). Although the function of the polymerase in replication is clear, the specific roles played by the B1 and D5 proteins during the viral life cycle remain a subject of ongoing inquiry.

The D5 gene has been sequenced and transcriptionally mapped, and synthesis of its 90-kDa protein product has been detected early in infection (12, 34, 39). Four *ts* mutants (*ts*17, *ts*24, *ts*69, and *ts*6389 [13, 39]) have been mapped within the D5 gene. These mutants are severely defective for DNA synthesis at the nonpermissive temperature and illustrate a requirement for D5 function throughout replication; shifting the mutants to the nonpermissive temperature at any point during replication causes a rapid and profound cessation of DNA synthesis. The three mutants with lesions affecting the amino terminus of the protein also display a deficit in homologous recombination at the nonpermissive temperature (39). Analysis of the predicted amino acid sequence of the D5 protein has failed to reveal any homology with known replication proteins but has identified a motif associated with ATP and GTP binding (15, 52). Further insight into the function of D5 has awaited its biochemical characterization. Here we describe the overexpression of D5 by using the hybrid T7/encephalomyocarditis virus (EMCV)/vaccinia virus expression system (32) and its purification to apparent homogeneity. We also present an analysis of its intrinsic nucleic acid-independent nucleoside triphosphatase (NTPase) activity and discuss the implications of this activity for the function of D5 during viral DNA replication.

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MATERIALS AND METHODS

Materials. DEAE-cellulose was obtained from Whatman BioSystems Ltd. (Kent, England). Heparin-agarose and 5′-bromo-2′-deoxyuridine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Bovine serum albumin (BSA) was from ICN Immunobiologicals (Lisle, Ill.). Ceramic hydroxyapatite (HAP; 40 μm; Asahi Optical Col. Ltd., Tokyo, Japan) was obtained from American International Chemical Inc. (Natick, Mass.). Superose 12 HR 10/30 and micrococcal nuclease were acquired from Pharmacia LKB Biotechnology (Piscataway, N.J.). Gel filtration standards and immunoblot reagents were purchased from Bio-Rad (Richmond, Calif.). Nitrocellulose was obtained from Schleicher & Schuell (Keene, N.H.). DNA modification enzymes were from New England Biolabs, Inc. (Beverly, Mass.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). ^{32}P-labeled NTPs were obtained from Dupont/NEN (Boston, Mass.). Polyethyleneimine-cellulose F plates were supplied by E. Merck (Darmstadt, Germany). Oligonucleotide primers were synthesized by using the Applied Biosystems (Foster City, Calif.) model 391 DNA synthesizer.

Cells and virus. BSC40 cells were originally provided by R. Condit. The recombinant vaccinia virus vTF7.5, human TK$^-$ 143 cells, and the DNA plasmid pTM1 (9, 32) were provided by B. Moss. Cells were cultured and viral stocks were prepared essentially as described previously (12).

Construction of the viral recombinant overexpressing the D5 ORF. Plasmid pTMD5 was constructed by subcloning the vaccinia virus D5R open reading frame (ORF) into the expression vector pTM1 (Fig. 1). PCR was used to introduce a novel *Nco*I site at the initiating ATG codon of the D5 ORF and to amplify the N′-terminal coding sequence from this initiating ATG codon until a *Nar*I site at position 317. This fragment was joined to cloned sequences encoding the remainder of the D5 gene from the *Nar*I site to an *Eco*RI site located 328 nucleotides after the translational termination of the D5 ORF. These fragments were ligated into a pTM1 vector previously digested with *Nco*I and *Eco*RI. The PCR-derived fragment was subjected to DNA sequence analysis to confirm its fidelity.

The pTMD5 construct was used to generate the recombinant vaccinia virus vTMD5. The inducible copy of the D5 gene was inserted into the nonessential viral TK locus by homologous recombination as diagrammed in Fig. 1A. Confluent monolayers of BSC40 cells were infected with wild-type (*wt*) vaccinia virus at a multiplicity of infection of 0.03. At 4 h postinfection (hpi), 10 μg of linearized pTMD5 was introduced into infected cells by calcium phosphate transfection (12), and cultures were incubated for 2 days. Following harvest, independent TK$^-$ isolates were twice plaque purified on TK$^-$ 143 cells in the presence of 25 μg of bromodeoxyuridine per ml (26). Viral genomic DNA was purified from bromodeoxyuridine-resistant isolates (49) and subjected to Southern blot analysis using vaccinia virus *Hin*dIII-D and TK gene sequences as DNA probes to

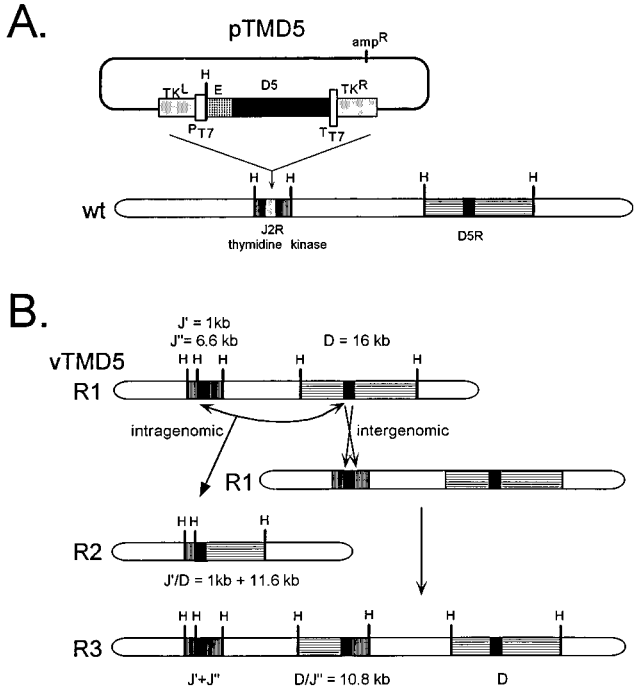

FIG. 1. Plasmid map of the expression vector pTMD5 and the genomic structures of the vaccinia virus recombinant vTMD5. (A) Plasmid map of the expression construct pTMD5, with relevant *Hin*dIII restriction sites and their positions indicated with the letter H. The components are as follows: TK^L and TK^R are the left and right halves, respectively, of the vaccinia virus TK gene; P_{T7} and T_{T7} are the promoter and terminator, respectively, for the T7 RNA polymerase; E is the translational control element from EMCV; D5R represents the coding region for the vaccinia virus D5 ORF; J2R represents the coding region for the vaccinia TK gene. (B) Partial genomic *Hin*dIII restriction map of the vaccinia virus recombinant vTMD5 and schematic representation of genomic recombination. The original isoform of the recombinant, R1, is shown at the top. The position of the endogenous D5 gene is indicated by the black box within the 16-kb *Hin*dIII D fragment (horizontal stripes) of the vTMD5 recombinant genome. The ectopic copy of the D5 gene in the TK locus is drawn as a black box lying within the *Hin*dIII J fragment (vertical stripes). The recombinant J sequences are 2.6 kb larger than the *wt* J fragment as a result of the insertion of the T7-regulated D5 cassette and form two novel restriction fragments designated J′ (1 kb) and J″ (6.6 kb). The rearranged isoforms of vTMD5 generated by intragenomic (R2) or intergenomic (R2 and R3) recombination are shown at the bottom and described in the text.

distinguish true viral recombinants from spontaneous TK^- mutants as described previously (28).

Overexpression of the D5 protein. Large-scale overproduction of the D5 protein was achieved by coinfecting BSC40 cells with vTMD5 and a second recombinant vaccinia virus, vTF7.5, that expresses the bacteriophage T7 RNA polymerase under the control of the constitutive vaccinia virus 7.5k promoter (32). Fifteen-centimeter-diameter dishes of confluent BSC40 cells were coinfected with each viral recombinant at a multiplicity of infection of 5 at 37°C. After infection was allowed to progress optimally at 37°C, cultures were shifted to 32°C at 4 hpi in order to increase the proportion of soluble recombinant D5. Infected cells were harvested at 30 hpi, washed with ice-cold isotonic buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 5 mM EDTA), and stored as cell pellets at −70°C. Single infections with *wt* virus or each recombinant alone were carried out on a smaller scale by using the same protocol.

Purification of the D5 protein. Infected cell pellets from large-scale coinfections (2×10^8 cells) were thawed in hypotonic buffer (10 mM Tris-HCl [pH 7.8], 10 mM KCl, 5 mM EDTA) and incubated on ice for 10 min. Cells were disrupted by Dounce homogenization, and the nuclei were removed by centrifugation at $1,000 \times g$ for 10 min at 4°C. The supernatant (cytoplasmic fraction) was saved, and the pellet was subjected to a second cycle of Dounce homogenization and centrifugation. The two supernatants were pooled and adjusted to 20 mM Tris (pH 7.4)–50 mM NaCl–1 mM dithiothreitol (DTT)–1 mM EDTA–10% glycerol (fraction I [FI]). Lysates were clarified prior to chromatographic fractionation by centrifugation at $15,000 \times g$ for 25 min at 4°C. Control extracts (*wt*, vTF7.5, and vTMD5) were prepared from 10^7 infected cells, using an appropriately scaled down protocol.

Purification scheme. All purification steps were performed on a Pharmacia FPLC apparatus at 4°C. FI (9 ml) was applied to a 5-ml DEAE-cellulose column equilibrated with buffer A (20 mM Tris HCl [pH 7.4], 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 50 mM NaCl. The column was washed with the starting buffer, and bound proteins were eluted with a 25-ml linear gradient of 50 mM to 1 M NaCl in buffer A. The profile of eluted proteins was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining as described previously (28). As necessary, the presence of the D5 protein within a fraction was detected by immunoblot analysis using a polyclonal anti-D5 antiserum (12, 47). Fractions containing D5 protein, eluting at approximately 500 mM NaCl, were pooled and diluted 1:1 with buffer A (fraction II [FII], 10.4 ml). FII was applied to a 5-ml heparin-agarose column that had been equilibrated with buffer A containing 250 mM NaCl. The column was washed with the starting buffer and then developed with a 25-ml linear gradient of 250 mM to 1 M NaCl in buffer A. Protein profiles were visualized by SDS-PAGE and silver staining, and peak D5-containing fractions (eluting at ≈880 mM NaCl) were pooled (fraction III [FIII], 8.8 ml). In preparation for the next column, ceramic HAP, FIII was diluted 1:4 with buffer B (1 mM $NaPO_4$ [pH 7.4], 1 mM DTT, 10% glycerol) lacking EDTA, and the remaining EDTA was chelated by adding $MgCl_2$ to 0.5 mM. The sample was immediately applied to a 1.5-ml HAP column that had been equilibrated with buffer B. The column was washed with buffer B and developed with a 10-ml linear gradient of 1 to 300 mM $NaPO_4$ (pH 7.4) in buffer B. Fractions were collected into tubes containing EDTA to a final concentration of 1 mM. Following SDS-PAGE analysis, peak D5 fractions (eluting at ≈250 mM $NaPO_4$ [pH 7.4]) were pooled and concentrated fivefold in an Amicon microconcentrator (fraction IV [FIV], 0.8 ml). Approximately 5% of each fraction was withdrawn during the purification for protein and enzyme assays. Fractions were stored on ice. For long-term storage of the purified protein, the HAP pool was dialyzed against a buffer containing 50 mM Tris HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 50% glycerol and kept at −20°C. Protein concentrations were determined with the Bradford method, using lysozyme as a protein standard.

NTPase assays. The hydrolysis of NTPs was measured by monitoring the conversion of rNTPs or dNTPs to the corresponding NDP form, using thin-layer chromatography (33). The standard 20-μl reaction mixture contained the protein fraction to be assayed in 50 mM Tris HCl (pH 7.4)–10 mM DTT–300 μg of BSA per ml–10 mM $MgCl_2$–150 mM NaCl–1 mM α-^{32}P-labeled NTP (50 μCi/μmol). In some cases, substrate and cofactor concentrations were varied as indicated. After incubation at 37°C for 1 h (or as indicated), reactions were stopped by the addition of EDTA to 20 mM or by rapid chilling to 0°C. One microliter of the reaction mixture was spotted onto a polyethyleneimine-cellulose plate, and the reaction products were separated by ascending chromatography in 0.8 M CH_3COOH–0.8 M LiCl. The positions of the NTP and NDP reaction products were identified by autoradiography or by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis, and their radioactivity was quantitated either by Cerenkov counting of the appropriately excised region of the thin-layer chromatography plate or by density scanning of the phosphor image with ImageQuant software. Numerical data were analyzed using the Excel spreadsheet (Microsoft Corporation) and represented graphically using Sigmaplot (Jandel Scientific). NTPase activity was calculated as the percent hydrolysis of the substrate, NDP/(NDP + NTP). A standard unit was defined as that amount of D5 protein capable of hydrolyzing 1 nmol of ATP in 1 h at 37°C.

Photo-cross-linking. Cross-linking was performed essentially as described previously (4). Protein samples were incubated in 4.3 μM [α-^{32}P]ATP (0.23 Ci/μmol) in a 10-μl reaction mixture containing 50 mM Tris-HCl (pH 7.4), 10% glycerol, 10% dextrose, 10 mM $MgCl_2$, and 5 mM DTT at room temperature for 5 min, placed on ice, and UV irradiated for 30 min under a germicidal UV lamp. The samples were adjusted to contain 1% SDS, 1% β-mercaptoethanol, and 10% glycerol, boiled for 5 min, and subjected to SDS-PAGE analysis followed by silver staining. Gels were dried and exposed to Kodak XAR-5 film at room temperature.

Gel filtration analysis. A 200-μl sample containing 30 μg of D5 in 500 mM NaCl–50 mM Tris (pH 7.4)–1 mM DTT–1 mM EDTA–10% glycerol was injected onto a 25-ml Superose 12 column. The column was equilibrated and developed in the same buffer, and chromatography was performed at 0.25 ml/min on a Pharmacia FPLC apparatus. Fractions of 0.25 or 0.5 ml were collected. The profile of D5 within the fractions was determined by SDS-PAGE and silver staining. Chromatography was also performed with the following protein standards of known Stokes radii in order to calibrate the column: bovine thyroglobulin, 85 Å (1 Å = 0.1 nm); bovine gamma globulin, 52.3 Å; BSA, 36.1 Å; and chicken ovalbumin, 27.5 Å. Dextran blue and L-tryptophan were used to determine the column's void and total volumes, respectively. The Stokes radii and molecular weights of the D5 species observed were calculated by the method of Siegel and Monty (44).

RESULTS

Overexpression of the vaccinia virus D5R protein, using a vaccinia virus/T7 expression system. Because the D5 protein is made in relatively low amounts during the transient early phase of vaccinia virus gene expression (12), we sought to

develop an overexpression system to facilitate its purification and biochemical characterization in vitro. We initially explored several bacterial expression systems but were unable to induce accumulation of the intact D5 protein in *Escherichia coli* (13a). We therefore turned to the hybrid T7/EMCV/vaccinia virus expression system (9, 32), which has been used successfully to express many foreign proteins and a few vaccinia virus proteins, including the vaccinia virus DNA polymerase (28). The coding sequences for the D5R protein were amplified by PCR using oligonucleotide primers that introduced an *Nco*I site at the initiation codon of the D5 gene (see Materials and Methods). The D5 cassette was then inserted into the expression vector pTM1 to form pTMD5 carrying the D5 gene under the control of the bacteriophage T7 gene 10 promoter and the EMCV translational control element (Fig. 1A). The EMCV element renders the uncapped T7 RNA polymerase-directed transcripts translationally competent in mammalian cells by virtue of an internal ribosome entry site (9).

Plasmid pTMD5 was then used to create a recombinant vaccinia virus (vTMD5) that contains the T7 promoter-driven copy of the D5 gene within the nonessential viral TK locus. Following transfection of pTMD5 into *wt* vaccinia virus-infected cells, viral progeny were harvested and propagated in human TK^- 143 cells in the presence of BrdU to select for TK^- virus. Viral recombinants were identified among plaque-purified TK^- progeny by testing their ability to direct high levels of D5 protein expression upon coinfection with a second recombinant virus, vTF7.5, that expresses the bacteriophage T7 RNA polymerase from a constitutive vaccinia virus promoter (data not shown).

The anticipated structure of the recombinant virus (R1) generated by homologous recombination between the TK sequences of pTMD5 and the endogenous viral TK locus (contained within the *Hin*dIII J fragment) is diagrammed at the top of Fig. 1B. The endogenous D5R gene within the 16-kb *Hin*dIII D fragment is shown, as is the T7/ECMV-regulated copy of the D5 gene inserted ectopically into the 5-kb *Hin*dIII J fragment. Because of a *Hin*dIII restriction site within the EMCV leader, the insertion of the D5 cassette was expected to generate two novel *Hin*dIII restriction fragments of 1 and 6.6 kb, designated J′ and J″, respectively. From our prior experience in developing similar viral constructs (28), we also anticipated that the presence of two copies of the D5 gene in the same orientation in the viral genome could lead to further inter- and intragenomic recombination, thereby generating the rearranged genomic forms R2 and R3 depicted at the bottom of Fig. 1B.

The existence of the recombinant genomic structures diagrammed in Fig. 1 was verified by *Hin*dIII restriction analysis and Southern hybridization of encapsidated viral genomes. Genomic DNA was isolated from *wt* virus and vTMD5 recombinants, digested with *Hin*dIII, fractionated on 0.7% Tris-agarose-EDTA agarose gels, transferred to nitrocellulose, and hybridized with viral DNA probes representing the *Hin*dIII J or D genomic fragment (data not shown). A radiolabeled DNA probe derived from sequences internal to the D5 gene hybridized to the 16-kb *Hin*dIII D fragment in both *wt* and vTMD5 genomic digests. In addition, the recombinant contained three novel fragments of 6.6 (J″), 11.6 (J′/D junction fragment), and 10.8 (D/J″ junction fragment) kb which hybridized to the D5 probe and were not seen in parallel analysis of *wt* genomic DNA. These fragments were present in nearly equimolar amounts, as judged by ethidium bromide staining. A probe derived from the *Hin*dIII J fragment recognized the J′ (1-kb) and J″ (6.6-kb) fragments within the recombinant as well as the novel J′/D and D/J″ junction fragments. As further confirmation, parallel filters were probed with DNA sequences derived from the *Hin*dIII D fragment upstream of (and not including) D5 which hybridized to the unrearranged copy of *Hin*dIII D fragment (16 kb) and the 10.8-kb D/J″ fragment but not the J′/D fragment. Likewise, a probe derived from D sequences downstream of (and not including) D5 recognized only full-length D and the 11.6-kb J′/D fragment. This pattern of hybridization is consistent with the recombinant genomic forms R1, R2, and R3 depicted in Fig. 1. Moreover, intact genomic isoforms consistent with these structures were observed during our analysis of a similar recombinant virus containing similarly positioned D5 alleles but lacking the EMCV leader. Encapsidated genomes were analyzed by contour-clamped homogeneous electric field gel electrophoresis. Three distinct DNA species were clearly resolved; their electrophoretic mobilities corresponded well to the predicted sizes of the three recombinant isoforms (R1 [195 kb], R2 [178 kb], and R3 [215 kb] [13a]). Interestingly, although the small form represents a defective virus which is deleted in essential genes in the *Hin*dIII H fragment (lying between J and D), stocks of vTMD5 grow to high titer and do not appear to be encumbered by the presence of the rearranged forms.

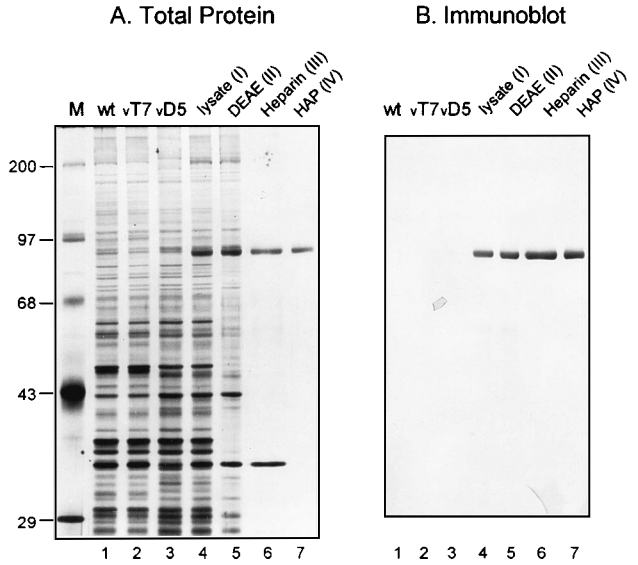

FIG. 2. Purification of the recombinant D5 protein. (A) SDS-PAGE analysis of protein fractions representing sequential steps of purification of the 90-kDa D5 protein. Lane M represents protein molecular weight standards, with their corresponding molecular masses indicated in kilodaltons at the left. Four-microgram samples of the following cytoplasmic extracts were fractionated on SDS–10% polyacrylamide gels and visualized by silver staining: lane 1, *wt*-infected cells; lane 2, vTF7.5-infected cells; lane 3, vTMD5-infected cells; lane 4, cells coinfected with vTF7.5 and vTMD5 (FI). In lanes 5 to 7, the amount of protein applied was adjusted to represent approximately equal amounts of D5 protein. Lane 5 contains 3 μg of the peak eluate from DEAE-cellulose (FII); lane 6 contains 0.8 μg of the peak eluate from heparin-agarose (FIII); lane 7 contains 0.4 μg of the peak eluate from ceramic HAP (FIV). (B) Immunoblot analysis of fractions representing the sequential steps of D5 purification. A gel parallel to the one shown in panel A was transferred to nitrocellulose and incubated with a polyclonal anti-D5 antiserum. Filters were developed colorimetrically after incubation with a second antibody.

Overexpression and purification of the D5 protein. Overexpression of the 90-kDa D5 protein was achieved by coinfecting BSC40 cells with vTMD5 and a second recombinant, vTF7.5, that directs expression of the T7 phage RNA polymerase (32). As seen by SDS-PAGE analysis in Fig. 2, coinfections initiated at a multiplicity of infection of 5 PFU per cell for each recombinant virus resulted in the accumulation of substantial

TABLE 1. Purification profile of vaccinia virus D5 protein

Fraction	Total protein		D5 protein			ATPase activity		Enrichment	
	mg	% yield	μg	% yield	Specific protein (μg of D5/μg of total protein)	Total U[a]	Sp act (U/μg of total protein)	D5 protein	Activity
FI (lysate)	10.07	100	646	100	0.06	201,400	20	1	1
FII (DEAE)	4.05	40	460	71	0.11	127,348	31.4	1.8	1.6
FIII (heparin)	0.56	5.6	368	57	0.66	35,392	63.2	11.0	3.2
FIV (HAP)	0.19	1.9	190	29	1	15,006	79.0	16.7	4.0

[a] One unit = 1 nmol of ATP hydrolyzed per h at 37°C. FIV activity = 118.5 nmol of ATP hydrolyzed per min/nmol of D5.

amounts of the 90-kDa protein (Fig. 2A, lane 4) which reacted with the D5 antiserum (Fig. 2B, lane 4). The small amount of D5 made during a comparable infection with *wt* virus (lane 1) or with either recombinant virus alone (lanes 2 and 3) is at the limit of detection. Relative to the levels seen in *wt* infections, these conditions of overexpression led to a 20-fold increase in the level of accumulated D5 protein, as determined by comparisons of known amounts of purified D5 (see below) with serial dilutions of uninduced and induced extracts (data not shown).

Shifting the coinfected cultures to 32°C at 4 hpi was found to increase the fraction of recombinant D5 recovered in the soluble hypotonic lysate; similar results had been obtained during our expression and purification of the viral DNA polymerase (17). This modified induction protocol was therefore adopted for the preparation of large-scale lysates for D5 purification. A hypotonic lysate from 2×10^8 cells was subjected to the purification scheme described in Table 1, using a Pharmacia FPLC apparatus. The presence of the D5 protein was monitored over the course of purification by immunoblot analysis using a polyclonal anti-D5 antiserum (12). The proteins present in each fraction following sequential chromatography on DEAE-cellulose, heparin-agarose, and HAP were visualized by SDS-PAGE, silver staining, and immunoblotting; a representative purification in shown in Fig. 2. FIV contained a single protein of approximately 90 kDa (Fig. 2A, lane 7) which reacted with the anti-D5 antiserum (Fig. 2B, lane 7). The yield of D5 protein during the purification was estimated by comparing serial dilutions of each extract with known amounts of purified D5 protein (as determined by analysis of FIV with the Bio-Rad protein assay) in an immunoblot assay. The results of a representative purification are summarized in Table 1. Overall, there was a 16.7-fold enrichment of D5 protein, with a 29% yield.

The D5 protein has an intrinsic NTPase activity. Because the predicted amino acid sequence of the D5 protein possesses a type A motif found in many nucleotide hydrolases involved in DNA replication (15, 22, 52), we tested the induced extracts and purified protein for NTPase activity. Proteins were incubated with 1 mM [α-^{32}P]ATP, and the conversion of ATP to ADP was monitored by thin-layer chromatography under conditions in which NMPs, NDPs, and NTPs are easily resolved. The positions of the radiolabeled NTP substrate and the more rapidly migrating products were detected by autoradiography or PhosphorImager analysis, and their identities were determined by comparison with known standards run in parallel.

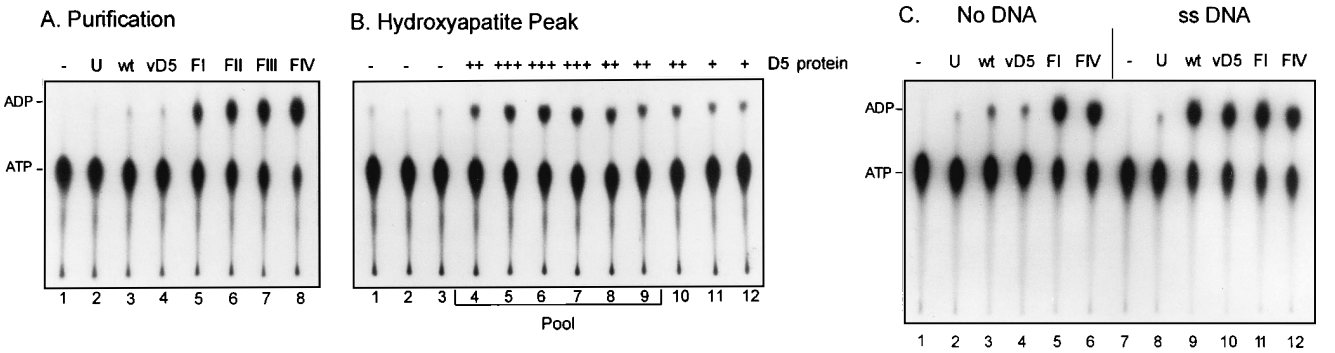

FIG. 3. DNA-independent ATPase activity is correlated with D5 overexpression and purifies with D5 protein. (A) ATPase activity in cytoplasmic extracts was measured in reactions mixtures containing 1 mM [α-^{32}P]ATP (50 μCi/μmol), 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT, and BSA at 150 μg/ml. Reaction mixtures contained buffer alone (lane 1) or 0.4 μg of cytoplasmic extracts prepared from cells treated as follows: lane 2, uninfected (U) BSC40 cells; lane 3, *wt* vaccinia virus infection; lane 4, vTMD5 infection; lane 5, coinfection with vTMD5 and vTF7.5 (FI); lane 6, fractions pooled after DEAE-cellulose chromatography (FII); lane 7, fractions pooled after heparin-agarose chromatography (FIII); lane 8, fractions pooled after HAP chromatography (FIV). After incubation for 1 h at 37°C, 1 μl of each reaction mixture was spotted onto polyethyleneimine-cellulose F and resolved by thin-layer chromatography. An autoradiogram of the chromatography plate is shown. (B) ATPase activity copurifies with the D5 protein. Four-microliter samples of fractions collected during development of the HAP column with a gradient of 0 to 300 mM $NaPO_4$ were tested in ATPase assays as described for panel A. The level of D5 protein present in each fraction, as visualized by SDS-PAGE and silver staining (not shown), is indicated by the number of plus signs shown at the top. The fractions represented in lanes 4 to 9 were pooled to form FIV. Although some D5 persisted in fractions shown in lanes 10 to 12, these fractions were excluded from the pool because of the coelution of an additional 35-kDa protein. (C) The ATPase activity associated with D5 is not stimulated by the addition of DNA. Reactions were carried out as described for panel A except that NaCl was omitted from the reaction mixtures. To remove endogenous nucleic acids, each cytoplasmic extract was pretreated with micrococcal nuclease (0.08 U/μl) in the presence of 1 mM $CaCl_2$ for 15 min at 22°C. The nuclease was inactivated by the addition of EGTA to 2 mM. Treated extracts were combined with reaction buffer lacking DNA (lanes 1 to 6) or containing denatured single-stranded (ss) salmon sperm DNA at 25 μg/ml (lanes 7 to 12). The reaction mixtures contained buffer alone (lanes 1 and 7) or 1.5 μg of extracts prepared from cells treated as follows: lanes 2 and 8, uninfected BSC40 cells; lanes 3 and 9, *wt* vaccinia virus infection; lanes 4 and 10, vTMD5 infection; lanes 5 and 11, coinfection with vTMD5 and vTF7.5 (FI); lanes 6 and 12, 0.4 μg of the fractions pooled after HAP chromatography (FIV).

The radiolabel present in each species was quantitated as described in Materials and Methods. As seen in Fig. 3, cytoplasmic extracts derived from vTF7.5-vTMD5 coinfection (FI extract, lane 5) were approximately fivefold more active at hydrolyzing ATP to ADP than were similarly prepared extracts derived from uninfected cells or cells infected with *wt* virus or vTMD5 alone (lanes 2 to 4).

To verify that the induced ATPase activity that we observed was due to the overexpression of D5 and not simply a consequence of the abortive nature of this type of coinfection (see references 1 and 32 for discussions), we tested extracts prepared from a *wt* infection blocked with the DNA replication inhibitor cytosine arabinoside and from cells induced to overexpress the viral DNA polymerase by coinfection with vTF7.5 and vTMDNApol (28). Little if any NTPase activity was seen in these extracts (data not shown), indicating that a failure to complete the late phase of infection does not by itself lead to elevated ATPase activity.

Because many NTPases involved in DNA replication are dependent on or stimulated by the presence of nucleic acid (22, 24) cofactors, we investigated whether such cofactors had an impact on the ATPase activity of control and coinfected extracts. Extracts were pretreated with micrococcal nuclease in the presence of 1 mM $CaCl_2$ to circumvent the possibility that endogenous DNA within the extracts might mask stimulation by exogenously added factors. Following inactivation of the nuclease by the addition of EGTA to 2 mM (37), NTPase activity was assayed with or without the addition of denatured salmon sperm DNA (Fig. 3C). While uninfected cells contained little cytoplasmic activity under any conditions, *wt* and vTMD5 infections induced measurable ATPase activity in the cytoplasm which was stimulated approximately fivefold by the addition of single-stranded DNA (Fig. 3C; compare lanes 3 and 4 with lanes 9 and 10). The DNA-dependent activity in these extracts is probably catalyzed by nucleotide phosphohydrolase I (NPH I [D11]) and NPH II (I8, RNA helicase), which are expressed at late times. Strengthening this conclusion is our observation that the stimulation of ATPase activity by DNA was abrogated by the addition of 150 mM NaCl (data not shown), in keeping with the salt sensitivity reported for these two viral enzymes (36, 43). In contrast, extracts prepared from cells coinfected with vTMD5 and vTF7.5 (FI) exhibited elevated levels of ATPase activity which were only slightly stimulated (approximately 10%) by the addition of DNA (Fig. 3C; compare lanes 5 and 11). The ATPase activity of the purified D5 enzyme (FIV) was not stimulated by DNA (lanes 6 and 12). Similar results were obtained when various amounts of the purified enzyme were assayed with either ATP or dATP substrates in the presence of double-stranded or activated salmon sperm DNA, single-stranded M13 circular DNA, poly(dA), tRNA, or early vaccinia virus mRNA at concentrations of up to 250 μg/ml (data not shown). Evidently, the D5 ATPase activity does not require, and is not stimulated by, any common nucleic acid cofactor.

On the basis of these findings, reaction conditions were optimized for ATP hydrolysis by the purified protein (data not shown). A titration was performed in the presence of 1 mM ATP at 37°C to determine the range of enzyme concentrations giving a linear increase in ATP hydrolysis. A time course was then performed with an enzyme concentration in the mid-linear range, and the rate of hydrolysis was found to be linear for up to 2 h under these conditions. The reaction was largely insensitive to changes in salt concentration from 0 to 300 mM, with a slight optimum at 150 mM NaCl. Activity was strictly dependent on the presence of a divalent cation, with optimum activity seen in the presence of 10 mM $MgCl_2$. Substitution of Mn^{2+} or Ca^{2+} for Mg^{2+} reduced the activity by more than 2- or 10-fold, respectively.

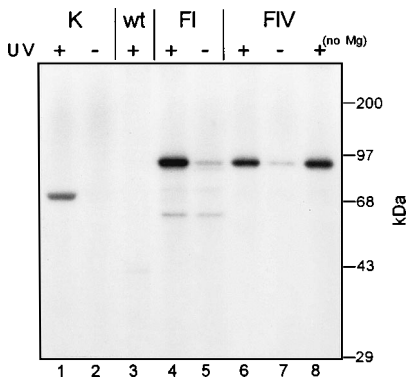

FIG. 4. UV cross-linking of the D5 protein to ATP. Fractions FI and FIV from the chromatographic purification were incubated with 4.3 μM [α-^{32}P]ATP (0.23 Ci/μmol) in a 10-μl reaction mixture in the presence (lanes 1 to 7) or absence (lane 8) of 10 mM $MgCl_2$ and, where indicated, subsequently irradiated for 30 min under a germicidal UV lamp (lanes 1, 3, 4, 6, and 8). Reaction mixtures contained the following: lanes 1 and 2, the Klenow (K) fragment of the *E. coli* DNA polymerase (1.5 μg); lane 3, cytoplasmic extract from cells infected with *wt* vaccinia virus (2.3 μg); lane 4 and 5, FI extract from cells coinfected with vTMD5 and vTF7.5 (2.3 μg); lanes 6 to 8, the peak eluate from HAP chromatography (FIV, 0.25 μg). Following treatment, the samples were boiled in SDS-PAGE sample buffer and fractionated on an SDS–10% polyacrylamide gel. An autoradiograph of the gel is shown, with the electrophoretic positions of molecular weight standards indicated at the right.

Using the optimized reaction conditions, we measured DNA-independent ATPase activity throughout the purification process and found that it copurified with the immunoreactive D5 protein (Fig. 3A and B). The yield of activity is summarized in Table 1, with 1 U of enzyme defined as the amount required to hydrolyze 1 nmol of ATP to ADP in 1 h at 37°C. Overall, there was a fourfold enrichment in specific activity during the purification, and 1 nmol of purified D5 was capable of hydrolyzing 119 nmol of ATP per min at 37°C. The specific ATPase activity is therefore approximately 140 fmol of D5 protein per unit.

D5 can be UV cross-linked to ATP. Our electrophoretic analyses of FIV indicated that the D5 protein had been purified to apparent homogeneity. Nevertheless, we sought additional verification that the ATPase activity of FIV was indeed attributable to the presence of D5 and did not reflect trace levels of any of the many proteins with nucleotide-hydrolyzing activity present in cellular extracts. Purified *E. coli* Klenow polymerase, a lysate prepared from *wt*-infected cells, and the FI and FIV fractions described above were incubated with 4 μM [α-^{32}P]ATP with or without subsequent exposure to a germicidal UV lamp for 30 min on ice. Samples were adjusted to contain 1% SDS, 1% β-mercaptoethanol, and 10% glycerol, boiled for 5 min, and then resolved by SDS-PAGE followed by silver staining and exposure for autoradiography. As seen in Fig. 4, the Klenow enzyme (75 kDa), which binds ATP noncovalently, retained the radiolabel only upon exposure to UV light (lanes 1 and 2). In the induced extracts (FI), a faint radiolabeled species of 90 kDa, corresponding to D5, was seen in the absence of exposure to UV (lane 5); the level of radiolabeled D5 was dramatically enhanced by exposure to UV irradiation (lane 4). Samples containing D5 protein purified to apparent homogeneity (FIV) showed a similar pattern (lanes 6 and 7). D5 was the only radiolabeled species seen in the purified fraction, and this species reacted with the D5 antiserum in immunoblot analysis (not shown). Cross-linking to ATP was

also evident when $MgCl_2$ was omitted from the reaction (lane 8). This was somewhat surprising since the NTPase activity of D5 is strictly dependent on the presence of a divalent cation. Cross-linking of D5 to the radiolabeled ATP can also be seen in the *wt*-infected extracts (lane 3) upon longer exposure of the autoradiogram. The additional labeled species in the *wt* and FI fractions are likely to be other viral proteins known or presumed to bind to ATP, such as the 63-kDa DNA ligase (20), the large subunit of the capping enzyme (95 kDa [31]), NPH I (72 kDa), NPH II (78 kDa [36]), vETF (74 kDa [3]), and the catalytic subunit of the poly(A) polymerase (55 kDa [14]). The DNA ligase would be expected to bind both covalently and noncovalently to ATP and is probably the prominent species of ≈66 kDa seen in lanes 4 and 5. The T7 RNA polymerase (98 kDa [46]) should be present in FI extracts and would also be expected to bind to ATP noncovalently.

Characterization of the NTPase activity of purified D5. Copurification of a nucleic acid-independent NTPase activity with the D5 protein and the demonstration of a direct physical interaction between the protein and ATP clearly indicate that D5 encodes a nucleotide-hydrolyzing enzyme. To further characterize the kinetic parameters of the reaction, we determined the apparent K_m of the enzyme for ATP. The relationship between the initial reaction velocity and the concentration of ATP was measured by varying the concentration of ATP from 0 to 4 mM. We observed a hyperbolic relationship between the initial enzyme velocity and substrate concentration, and from these data (not shown), the K_m value was approximated by determining the ATP concentration required to obtain half the maximum enzyme velocity (1/2 V_{max}). We performed a second experiment in which the ATP concentration was varied from approximately one-half to twice the estimated K_m. Data collected in duplicate from these studies were plotted as a Lineweaver-Burk double-reciprocal plot (Fig. 5A). From these analyses, we determined the apparent K_m value for ATP hydrolysis to be 380 μM.

Although our initial characterization of the NTPase activity of D5 was performed with ATP as a substrate, the in vivo substrate is not known. We therefore compared the utilization of the common rNTPs and dNTPs under standard reaction conditions with the nucleotide present at 1 mM. The efficiency of hydrolysis of each nucleotide by purified D5 is presented in Fig. 5B. D5 could use all eight of the nucleotides tested as substrates; the greatest activity was measured with CTP and TTP, and the least was measured with GTP and dGTP.

D5 appears to form higher-molecular-weight complexes. The native molecular weight of both partially and fully purified D5 was investigated by gel filtration chromatography. Two main species of D5 were observed, with apparent Stokes radii of 71.1 ± 5.7 and 39.8 ± 2.1 Å. These values correspond to molecular weights of 243,000 ± 27,000 and 96,000 ± 10,000, respectively, consistent with the presence of a dimeric or trimeric form of D5 as well as the monomer form. The multimeric species was observed even in the presence of 500 mM NaCl.

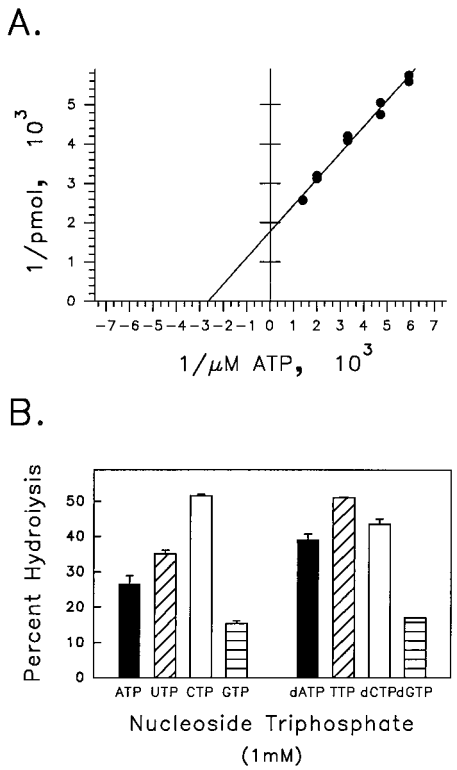

FIG. 5. Analysis of the kinetic parameters and substrate specificity of the vaccinia virus D5 protein. (A) The K_m of the vaccinia virus D5 protein for ATP was determined by using assay conditions described for Fig. 2A except that substrate concentration was varied as indicated. Each reaction mixture contained 20 U of D5 protein (see Materials and Methods). The relationship between initial velocity and substrate concentration was first examined by using a broad range of substrate concentrations. After estimating the ATP concentration that resulted in 1/2 V_{max} (i.e., the K_m), duplicate reactions were performed with a narrower range of ATP concentrations that spanned this value. The data were plotted on Lineweaver-Burk reciprocal plots, and linear regression analysis was used to determine the *x*-axis intercept ($-1/K_m$). (B) Utilization of eight NTPs by the purified recombinant D5 protein was examined under reaction conditions described in the legend to Fig. 2A. Twenty units of D5 protein (FIV) was added to parallel reactions, each containing one of the indicated NTPs at 1 mM (50 μCi/μmol). Standard errors were calculated for data obtained from three independent sets of reactions.

DISCUSSION

Overexpression and purification of vaccinia virus D5R. We present here the first report of the overexpression, purification, and biochemical characterization of the 90-kDa protein encoded by the vaccinia virus D5R gene. This early gene product had previously been shown genetically to be essential for ongoing viral DNA synthesis. Overexpression was accomplished within virally infected mammalian cells by using the hybrid T7/EMCV/vaccinia virus system; production of the D5 protein in its native context was intended to allow potential posttranslational modifications to occur normally.

As we had seen when the vaccinia virus DNA polymerase was overexpressed in this system (28), the recombinant genomes containing the endogenous and ectopic copies of the D5 genes underwent a series of further recombination events. Because recombination between other duplicated alleles has not been reported, it is possible that the sizes of the genes involved (both D5 and DNA polymerase are ≥3 kb in length) and the distance between the endogenous and ectopic alleles influence these genomic rearrangements. In the case of the DNA polymerase, the opposite orientations of the ectopic and endogenous alleles meant that recombination between them led to the inversion of the intervening 25.5 kb. This recombinational inversion would not be expected to affect either the regulation or the primary structure of any genes (28). In contrast, the endogenous and ectopic alleles in our recombinant construct (R1) are present in the same orientation (Fig. 1B). Intragenomic recombination between the two D5 alleles joined the left half of the *Hin*dIII J fragment to the right half of the *Hin*dIII D fragment (J′/D chimera), with the concomitant loss of the

right half of *Hin*dIII-J, all of *Hin*dIII-H, and the left half of *Hin*dIII-D. The existence and structure of the resultant genome (R2, 178 kb) was confirmed both by contour-clamped homogeneous electric field electrophoresis and by Southern blot analysis of *Hin*dIII-digested genomic DNA. This genomic isoform would clearly be noninfectious as a result of the deletion of a large number of essential genes. Intergenomic recombination between the endogenous and ectopic D5 alleles in the R1 recombinant led to the formation of a larger genomic isoform (R3, 215 kb) carrying tandemly repeated copies of the *Hin*dIII H fragment separated by a chimeric D/J″ junction fragment (as well as the reciprocal product, R2). It is assumed that this genomic form would also be unstable and would undergo further recombinational events. All three of these isoforms appear to be encapsidated, since genomic analysis was performed on DNA isolated from sedimentable particles. Viral stocks of vTMD5 are therefore in a recombinational equilibrium in which the R1, R2, and R3 isoforms are continuously generated and resolved.

The vTMD5 stock grew to high titer, and therefore it does not appear that the genomic instability of this recombinant compromises its infectivity. Moreover, the levels of D5 overexpression achieved upon coinfection with vTF7.5 were comparable to those observed for the DNA polymerase (28) and other target proteins (32) produced with this expression system. It is worth noting that both the R2 and R3 recombinant forms include a T7-regulated copy of D5 and so should serve as templates for D5 overexpression.

Upon coinfection of BSC40 cells with vTMD5 and vTF7.5, the quantity of D5 which accumulated was approximately 20-fold greater than that observed during a *wt* infection. By 30 hpi, the D5 protein represented 6 to 10% of the total cellular protein, depending on the preparation; this value is in agreement with the reported efficacy of the overexpression system (32). As previously observed (28), the solubility of the overexpressed protein was enhanced when inductions were performed at 32°C. Beginning with approximately 10 mg of protein derived from 2×10^8 coinfected cells, sequential chromatography on DEAE-cellulose, heparin-agarose, and HAP resins enriched the D5 protein 16.7-fold and yielded 190 μg of highly purified recombinant D5 protein.

D5 is a nucleic acid-independent NTPase. The D5 protein possesses a characteristic extended type A site of the purine nucleotide binding motif found in NTP-hydrolyzing enzymes such as the vaccinia virus thymidine and thymidylate kinases (extended type A site), NPH I and NPH II (short type A site), and diverse helicases, nucleoside kinases, and other NTP-utilizing proteins (15, 22, 23, 52). The ATP binding pockets of these sites are generally bipartite, composed of A and B elements. The A sequence forms a loop which binds one of the phosphates of the nucleotide. The B sequence, which contains a conserved aspartate residue within a stretch of hydrophobic residues, binds a second phosphate via Mg^{2+} (15). The A site of the D5 protein is found at amino acids 498 to 518 of the predicted sequence. Three potential B sites lie downstream of the A site, and it is not known which is functional. These sites lie within a region extending from 84 amino acids upstream to 42 amino acids downstream of the residue affected within the mutant *ts*E69 (13). An amino acid substitution proximal to the actual B site could contribute to the mutant phenotype, as suggested for *ts* DNA^- mutants of herpes simplex virus with lesions in the UL5 helicase (55).

The functional significance of the predicted nucleotide binding site was borne out by the demonstrated increase in ATPase activity upon overexpression of D5. The majority of the ATPase activity within extracts prepared from *wt*-infected cells was DNA dependent and most probably reflects the activity of the late viral proteins NPH I (D11) and NPH II (RNA helicase, I8). Extracts prepared from D5-overexpressing cells had a ≥5-fold increase in DNA-independent ATPase activity and very little DNA-dependent activity. This altered profile reflects the overexpression of D5 and the diminished synthesis of the majority of late viral proteins known to occur with this overexpression system (1, 32) as a result of domination of the cellular machinery by the abundant T7 RNA polymerase-regulated transcripts.

This induced DNA-independent ATPase activity copurified with D5. In each step of the purification, fractions representing the peak of immunoreactive D5 protein demonstrated ATPase activity (Fig. 3); the purified D5 protein not only was enzymatically active but could easily be cross-linked to radiolabeled ATP by UV irradiation (Fig. 4). The activity of the purified protein was relatively insensitive to changes in salt concentration. Although the noncovalent binding of the enzyme to ATP did not require Mg^{2+} (Fig. 4), nucleotide hydrolysis was strictly dependent on the presence of a divalent cation (data not shown). DNA cofactor analysis of the purified protein confirmed that the ATPase activity of D5 neither requires nor is stimulated by the addition of single- or double-stranded DNA or RNA cofactors. This nucleic acid independence was observed in reactions performed in the absence or presence of 150 mM NaCl. Although DNA clearly has no effect on the enzymatic activity of D5, filter binding assays demonstrate that the protein does bind to double-stranded DNA in ≤50 mM NaCl (data not shown).

In the course of purification, the enrichment of ATPase activity was about fourfold less than the enrichment of the D5 protein itself, as assessed by immunoblot analysis (Table 1). This is not surprising, since the protein was purified on the basis of its recognition by the antiserum, not by enzyme activity. Possible explanations for this discrepancy include the presence of additional ATPases in the FI fraction or, more interestingly, the possible loss of factor(s) that stimulate the D5 NTPase activity during the purification. The purified enzyme was capable of hydrolyzing 119 nmol of ATP per min per nmol of D5 at 37°C. D5 has an apparent K_m of 380 μM for its ATP substrate (Fig. 5A). This value lies between those reported for the vaccinia virus NPH I and NPH II (140 and 640 μM, respectively [35]) and is in the upper range of reported K_m values for DNA-dependent ATPases involved in DNA replication and repair (measured in the presence of their nucleic acid cofactors) such as simian virus 40 large T antigen (20 μM [53]), *E. coli* DnaB (106 μM [2]), human DNA helicase α (28 μM [40]), *Saccharomyces cerevisiae* DNA helicase III (200 μM [41]), and *S. cerevisiae* replication factor C (15 μM [54]) as well as enzymes involved in precursor metabolism such as rat adenosine kinase (14 μM for ATP in various concentrations of adenosine [30]), human adenylate kinase (110 μM with AMP at 5 mM [21]), various thymidylate kinases (1.2 to 6 mM with 0.5 mM TDP [19]), and NDP kinases (*Dictyostelium discoideum* NDP kinase, 370 μM [25]; HBx, 95 μM [7]). In addition to ATP, D5 is able to use the other common rNTPs and dNTPs (Fig. 5B). The functional significance of this broad substrate specificity is not known.

Possible functions of the D5 protein in vaccinia virus replication. Analysis of the four viral *ts* mutants with lesions in D5 indicated a clear requirement for D5 function during viral DNA synthesis. No detectable DNA was synthesized in infections maintained at the nonpermissive temperature, and further DNA synthesis ceased within 5 min when cultures were shifted to the nonpermissive temperature in the midst of DNA synthesis. Moreover, the phenotype manifested by the three

mutants with N′-terminal lesions was also indicative of a role for D5 in facilitating homologous recombination (39). The demonstration of NTPase activity could suggest possible roles that the protein might play in viral DNA metabolism. For example, replicative DNA helicases are NTPases required to unwind double-stranded DNA templates at replication origins and/or in conjunction with a moving replication fork. All known helicases possess NTPase activity that either depends on or is stimulated by DNA binding (24). Many DNA helicases bear distinct signature sequences, while others exhibit little relationship to one another beyond the possession of the nucleotide binding consensus (15). Of the nine vaccinia virus proteins exhibiting the NTP-binding sequence pattern, four proteins, D6R (vETF subunit), D11L (NPH I), I8R (NPH II), and A18R possess helicase motifs (23), and the latter two have demonstrated RNA or DNA helicase activity (4a, 42, 43). In contrast, D5 does not possess significant homology to any known helicase, and its DNA independence argues against helicase activity. However, we do not rule out a helicase function since the mechanism and cytoplasmic location of vaccinia virus DNA metabolism might allow for replication functions that have diverged from their nuclear counterparts.

The fast-stop phenotype of the D5 *ts* mutants suggests that D5 may play a role in DNA elongation (39). We have recently established conditions in which infected cell lysates direct the processive conversion of a singly primed, *E. coli* single-stranded DNA-binding protein-coated M13 templates to the double-stranded RFII form (28a). Whereas extracts from DNA polymerase mutant-infected cells are inactive in this assay, extracts from cells infected with a D5 mutant under nonpermissive conditions appear to retain full activity. We conclude that D5 might not be involved in primer extension per se but might be required for the utilization of a double-stranded DNA template. Its apparent link to DNA recombination would also support a role for D5 in the unwinding and/or annealing of DNA strands. D5 might conceivably be a component of a viral single-stranded DNA-binding protein or might interact with and activate a viral helicase.

Another possible role for D5 is involvement in deoxyribonucleotide metabolism. Sequence comparisons performed by Gorbalenya and Koonin (15) tentatively placed the D5 protein in the family of thymidylate kinase enzymes. Furthermore, D5 inactivation was shown to affect the thymidine pathway in studies on the profile of TK activity exhibited by various *ts* mutants (39). Permissive infections performed with *ts*17 and *ts*24, two D5 mutants, displayed the characteristic pattern of activity associated with *wt* infection. Activity peaked early in infection and then declined, presumably as a result of feedback inhibition by accumulated TDP and TTP. In contrast, nonpermissive infections showed sustained levels of TK activity which continued late into infection, suggesting that feedback inhibition of the enzyme failed to occur. This observation was specific to the D5 mutants and not simply a consequence of the absence of DNA and late protein synthesis, since a *ts* mutant with a lesion in the DNA polymerase did not exhibit deregulated TK activity.

These observations suggest that the loss of D5 influences, and may have a direct role in, DNA precursor metabolism. In addition to TK (ORF J2), vaccinia virus encodes an active thymidylate kinase (ORF A48) and a heterodimeric ribonucleotide reductase (ORFs F4 and I4). These enzymes are conserved among poxviruses but are nonessential for viral growth in tissue culture. In addition, vaccinia virus ORF A57 has 30% amino acid similarity to guanylate kinase of *S. cerevisiae* but is separated from its putative nucleotide binding site by a frameshift mutation (45). Like many vaccinia virus enzymes, those involved in precursor metabolism share clear functional and sequence homologies with their counterparts in other organisms. D5 apparently lacks any broad homology to nucleotide biosynthetic enzymes. Nevertheless, we have considered the possibility that D5 possesses nucleoside mono- or diphosphokinase activity, since certain (although not all [e.g., reference 18]) of these enzymes display intrinsic DNA-independent NTPase activity with broad substrate specificity (7, 8). Although our preliminary experiments have failed to demonstrate nucleotide kinase activity for D5, this possibility remains worthy of consideration.

Several of the replicative functions described above are carried out in the context of multiprotein complexes. Particular activities associated with particular subunits may be revealed only in conjunction with partner proteins. For example, herpes simplex virus UL5 helicase activity is detectable only in the presence of its binding partner UL52 (56); the ATPase activity of human helicase α is evident only in the presence of a single-stranded DNA-binding protein on the DNA primer-template (40). Likewise, the ATPase activity of human replication factor C is stimulated by proliferating cell nuclear antigen (51), and DNA precursor biosynthesis may occur within a multienzyme complex (27). Given these complexities, our analysis of the biochemical properties of the purified D5 protein will be complemented by exploring the interaction of D5 with other viral proteins. In concert, these lines of investigation should help to elucidate the role of D5 in vaccinia virus replication.

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