

Mutations in the NTP-binding Motif of Minute Virus of Mice (MVM) NS-1 Protein Uncouple ATPase and DNA Helicase Functions*

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The NS-1 protein of minute virus of mice (MVM) is required for viral DNA replication and transcriptional regulation. To define the domain structure of NS-1, we have generated point mutations in its putative NTP-binding/ATPase domain. We show that all mutants were unable to support replication of MVM DNA in a transient DNA replication assay. Furthermore, all mutants, except for the K405S substitution, were able to transactivate the P38 promoter in transient transfection experiments. NS-1 proteins bearing COOH-terminal deletions of 29 and 33 amino acid residues were also transcriptionally inert. Biochemical analysis of recombinant NS-1 expressed in insect cells shows that mutations in the putative NTP-binding/ATPase domain severely reduced helicase activity *in vitro*. However, affinity labeling experiments indicate that none of these mutations, except for K469T, impaired NTP-binding activity. Finally, all point mutants retained significant levels of ATPase activity, except for the E444Q mutant (1%).

These findings suggest that the replication and transcription activities of NS-1 reside in separate functional domains. In addition, NS-1 proteins with mutations in the putative nucleotide binding fold have lost helicase activity, whereas most retain nucleotide binding and ATPase functions, suggesting that the mutations have uncoupled the ATPase and helicase activities.

NS-1 is the major nonstructural protein encoded by the autonomous parvovirus, minute virus of mice (MVM).¹ It is an 83-kDa phosphorylated nucleoprotein (Cotmore and Tattersall, 1986), multifunctional in both replication and transcription. NS-1 is essential for replication (Tullis *et al.*, 1988). Current models propose that MVM DNA replication initiates by conversion of parental single-stranded viral DNA into a dimer replicative form. The dimer can be resolved to unit length replicative form molecules which function in the synthesis of progeny genomes (Astell *et al.*, 1985; Tattersall and Cotmore, 1990). As predicted (Astell *et al.*, 1985), NS-1 has been found covalently attached to the 5' ends of resolved concatemer junction fragments in infected cells, indicating that it acts as the site-specific nickase (Cotmore and Tattersall, 1992). Moreover, recombinant NS-1 purified from insect cells displays both helicase

and ATPase activities, *in vitro* (Wilson *et al.*, 1991). These functions are predicted to be necessary for resolution of junction fragments. The analogous Rep proteins of AAV have also been shown to have ATP-dependent site-specific endonuclease and helicase activities (Im and Muzyck, 1990; Snyder *et al.*, 1990). The MVM genome contains two overlapping transcription units (Pintel *et al.*, 1983), with promoter P4 directing transcription of the two nonstructural proteins, and promoter P38 controlling transcription of the capsid genes. NS-1 apparently regulates its own expression from P4 in a positive feedback loop (Doerig *et al.*, 1990), and also transactivates P38 (Rhode, 1985; Doerig *et al.*, 1988; Ahn *et al.*, 1992). It is not known how NS-1 achieves these effects since it has not been shown that NS-1 binds to either the P4 or P38 promoters. Furthermore, NS-1 appears to be cytotoxic to cells in tissue culture (Brandenburger *et al.*, 1990; Caillet-Fauquet *et al.*, 1990), the basis for which is not understood.

Previous studies suggest that, like other multifunctional proteins, the activities ascribed to NS-1 are located in discrete domains. Deletion of the NS-1 COOH terminus removes transactivating function in transient expression assays (Brandenburger *et al.*, 1990; Skiadopoulos *et al.*, 1992; Harris and Astell, 1993), in agreement with results obtained with the analogous NS-1 protein of H-1 virus (Rhode and Richard, 1987). NS-1 cytotoxicity has been found to be restricted to its NH₂- and COOH-terminal portions (Brandenburger *et al.*, 1990; Legende and Rommelaere, 1992). A region of NS-1 bears homology to the SV40 and polyomavirus large T antigens (Astell *et al.*, 1987), and this conserved region has been shown to contain ATPase and nucleotide binding functions in the latter proteins (Clermont *et al.*, 1984; Bradley *et al.*, 1984; Bradley, 1990; Weiner and Bradley, 1991). A consensus topography has been identified for the NTP-binding domains of these proteins (Bradley *et al.*, 1987). Furthermore, the domain encompasses three short amino acid motifs conserved in a superfamily of proteins involved in replication encoded by small DNA and RNA viruses (Gorbatenya *et al.*, 1990). These authors suggest that the conserved sequences are directly related to conservation of a helicase function.

In the present study, we have generated point mutations at residues in the putative MVM NS-1 domain which may be important for NTP-binding. We have analyzed these mutants for their ability to replicate MVM DNA and transactivate the P38 promoter. Furthermore, we have expressed these mutants using the baculovirus expression system to facilitate biochemical analysis of the intrinsic helicase and ATPase activities of NS-1 and examined the ability of each purified NS-1 protein to be affinity labeled with periodate oxidized [α -³²P]ATP using sodium borohydride (Clermont and Cuzin, 1982).

MATERIALS AND METHODS

Construction of Plasmids—Mutations were made in the NS-1 gene of pUCNS-1 (Wilson *et al.*, 1991). An EcoRI-BamHI fragment of NS-1

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¹ The abbreviations used are: MVM, minute virus of mice; AAV, adenoassociated virus; ox-ATP, oxidized ATP; PAGE, polyacrylamide gel electrophoresis; SV40, simian virus 40; bp, base pair.

coding sequence in pUCNS-1 (nucleotides 1084–2473 of MVM) was cloned into the polylinker of pSELECT-1 (Promega). Mutagenic oligos were used to introduce 1 or 2 bp changes in the NS-1 fragment by means of the Altered Sites *in vitro* mutagenesis system (Promega). All mutations were confirmed by DNA sequencing. The baculovirus transfer vector pAcYM1-NS-1 (Wilson *et al.*, 1991) was modified such that the *Eco*RI and *Bst*EII sites in the NS-1 coding sequence are unique. Each mutant NS-1 sequence was isolated from its pSELECT-1 background as an *Eco*RI-*Bst*EII fragment and substituted for the corresponding fragment in pAcYM1-NS-1. The resulting plasmids were used to construct recombinant baculoviruses. Construction of the MVM minigenome pPTLR and the NS-1 expression plasmid pCMVNS-1 have been described (Tam and Astell, 1993). Mutant NS-1 expression plasmids were generated by excising each mutant sequence from its pAcYM1 background as a 2.2-kilobase *Bam*HI fragment and ligating into the multiple cloning site of pCMV-5 (Andersson *et al.*, 1989). NS-1 deletion mutants were generated by Bal-31 nuclease treatment of pUCNS-1 linearized at *Stu*I (2375). Termini of digestion products were made blunt, ligated to *Bgl*II linkers, and recircularized. Two oligos which contained a TAA stop codon in all three reading frames were synthesized (see below). The 5' sticky ends 5' GATCTAAGTAACATAAGGATCCGAGCTC 3' and 3' ATTCAATTGATTCTAGGCTCGAGCTAG 5' enabled cloning into the novel *Bgl*II site of the pUCNS-1 deletion mutants. NS-1 truncations were isolated as *Bam*HI fragments and cloned into the polylinker of pCMV-5 to generate pCMVNS-1Δ2189C and pCMVNS-1Δ2176C. Construction of pP39CAT has been described (Skiadopoulos *et al.*, 1992). (P39 refers to the promoter at map unit P38. In other publications, including this communication, the P39 promoter is referred to as P38.)

Isolation of Recombinant Baculoviruses—Isolation of the recombinant virus AcNS-1 has been described (Wilson *et al.*, 1991). To generate recombinants expressing mutant NS-1 proteins, we used the vector AcRP23lacZ (Possee and Howard, 1987). SF9 cells were transfected with a mixture of AcRP23lacZ DNA linearized with *Bsu* 36I, and plasmid pAcYM1-NS-1 mutant DNA, by calcium phosphate co-precipitation (Summers and Smith, 1987). Putative recombinants were identified as colorless plaques after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and screened for expression of NS-1 by Western blot analysis using monoclonal antibody CE10 (Yeung *et al.*, 1991) with the ECL detection system (Amersham Corp.). Isolated recombinants were plaque-purified twice, amplified, and used to obtain high titre virus stocks.

Purification of Recombinant NS-1 Proteins—SF9 cells were grown in TC-100 medium supplemented with 10% fetal bovine serum at 27 °C in spinner culture and were infected with recombinant baculovirus at a multiplicity of approximately 5. Cells were harvested at 72 h postinfection, washed, and used to prepare cell extract. NS-1 protein was purified by immunoaffinity chromatography (Wilson *et al.*, 1991).

Helicase Assays—Preparation of substrate and helicase assays were performed essentially as described (Wilson *et al.*, 1991).

Transfections, DNA Replication Assays, and Chloramphenicol Acetyltransferase Assays—COS-7 or LA9 cells were grown and transfected with plasmid DNA (Tam and Astell, 1993). Replication assays were performed on Hirt extracts at 72 h post-transfection (Tam and Astell, 1993). For chloramphenicol acetyltransferase assays (Gorman *et al.*, 1982), cell lysates were harvested 48 h post-transfection and assayed as described by Gill *et al.* (1990) using [¹⁴C]1-deoxycchloramphenicol (Amersham Corp.) as substrate. Results were quantitated by liquid scintillation.

Preparation of Oxidized ATP—The oxidized ATP (ox-ATP) was prepared by the method of Clermont and Cuzin (1982) with some modifications. Labeled ATP (12.5 μM, [α -³²P]ATP) was incubated in the dark in 0.5 mM HCl in the presence of sodium periodate (2 mM final concentration) for 30 min at room temperature. The reaction was terminated by adding 0.2 volume of 50% glycerol, followed by incubation for another 30 min. The mixture was then used immediately for the labeling reaction.

Covalent Affinity Labeling of NS-1 Protein with ox-ATP—Immunoaffinity purified NS-1 proteins (1.5–2 μg) were mixed at 4 °C with the ox-ATP preparation. Final concentrations were approximately 0.5–1.0 μM ox-ATP, 5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and 0.5 M KCl at pH 7.2. The reaction was allowed to proceed overnight at 4 °C in the presence of 10 mM sodium borohydride (NaBH₄) and stopped by the addition of an equal volume of SDS-PAGE sample buffer. Samples were boiled for 5 min and fractionated by 12% SDS-PAGE. Gels were dried for autoradiography.

ATPase Assay—The ATPase assay was monitored by the conversion of the ³²P label in [γ -³²P]ATP to a charcoal non-absorbable form. In a reaction volume of 20 μl about 500 ng of immunopurified NS-1 protein (wild type or mutant) was incubated with 50 mM Tris-HCl, pH 7.0, 100

μM NaCl, 5 mM MgCl₂, 5 mM dithiothreitol, 0.01% (v/v) Nonidet P-40, 30 μM unlabeled ATP, and 0.5 mCi of [γ -³²P]ATP (3000 Ci/mmol). After 30 min of incubation at 30 °C, 100 μl of a 7.5% (v/v) suspension of activated charcoal in 50 mM HCl, 5 mM H₃PO₄ were added, and the reaction mixture was allowed to stand on ice for 5 min. After pelleting the charcoal by centrifugation, 60 μl of supernatant were analyzed for free ³²P, by Cerenkov counting. Background radioactivity in the absence of NS-1 protein was subtracted from experimental results.

RESULTS

Rationale for Mutagenesis—The NTP-binding domain of SV40 T antigen is homologous to a region conserved among the Rep proteins of parvoviruses (Astell *et al.*, 1987) (Fig. 1A). The consensus topography of NTP-binding domains proposed by Bradley *et al.* (1987) is depicted for SV40 T and MVM NS-1 in Fig. 1, B and C, respectively. The model predicts at least three interactions between the nucleotide binding fold of the polypeptide and Mg²⁺ATP. For SV40 T (Fig. 1B), these interactions are: two negatively charged residues at the carboxyl end of the β -strand c (Glu-473 and Asp-474) complex to β - or γ -phosphate-Mg²⁺; a positively charged residue at the amino end of an α -helix (Lys-432) hydrogen bonds to the α -phosphate of the NTP; and a negatively charged residue at the carboxyl end of the β -strand d (Glu-510) hydrogen bonds to the 2'-OH or 3'-OH of the NTP ribose moiety. The corresponding residues for the putative NS-1 domain are indicated in Fig. 1, A and C. To examine the function of the region containing the NTP-binding motif, we generated specific amino acid substitutions at the locations shown (Fig. 1A) and tested their effects on NS-1 activities.

NS-1 Mutants Are Unable to Support DNA Replication—To determine whether mutations in the NTP-binding motif affect NS-1 replication functions, we tested the ability of each mutant to effect replication of an MVM minigenome, pPTLR (LR). The pPTLR minigenome contains 411 nucleotides of the left terminus fused to the 807 nucleotides of the right terminus. This minigenome has previously been shown to replicate in the presence of wild type NS-1 (Tam and Astell, 1993). Each mutant NS-1 was cloned into the SV40 origin-based expression vector pCMV-5 and co-transfected with pPTLR into COS-7 cells (Fig. 2). Transfection with pPTLR alone results in a single band of unreplicated input DNA (*lane 2*). Co-transfection of pCMVNS-1 (wild type NS-1) with pPTLR results in the generation of monomer (*mLR*) and dimer (*dLR*) replicative forms (*lane 3*). The heterogeneous composition of the *mLR* and *dLR* has been shown to reflect the alternative covalently closed or extended duplex hairpin terminus at either end of the minigenome (Tam and Astell, 1993). However, co-transfection with mutant NS-1 expressing plasmids did not produce detectable MVM replicative intermediates (Fig. 2, *lanes 4–10*), indicating mutations in the NTP-binding/ATPase domain impair one or more of the activities required for replication. Western blot analysis of lysates from transfected COS-7 cells show that all constructs produced full length NS-1 proteins at similar levels (data not shown).

NS-1 NTP-binding Site Mutants Are Able to Transactivate the P38 Promoter—To measure the effect of NTP-binding/ATPase motif mutations on NS-1 transcription activity, each mutant was tested in a chloramphenicol acetyltransferase assay (Gorman *et al.*, 1982). The mutant pCMVNS-1 derivatives were cotransfected with the pP39CAT construct. (The middle promoter of the MVM genome is referred to as either the P39 or P38 promoter.) Transfections were carried out in LA9 cells, and total cell lysates were assayed for chloramphenicol acetyltransferase activity at 48 h post-transfection (Fig. 3). In the absence of NS-1, there is a low basal chloramphenicol acetyltransferase activity (*lane 1*). Wild type NS-1 activated the P38 promoter and resulted in 34% acetylation of the chloramphenicol sub-

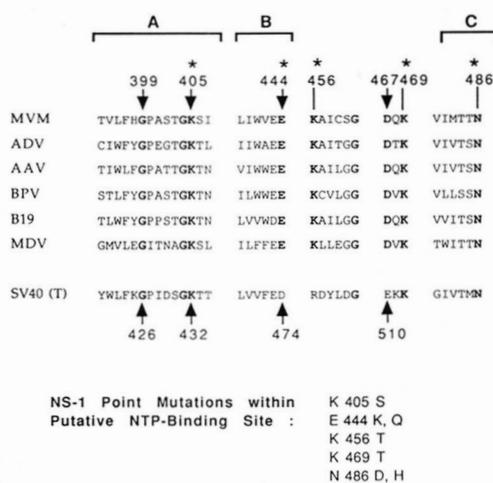
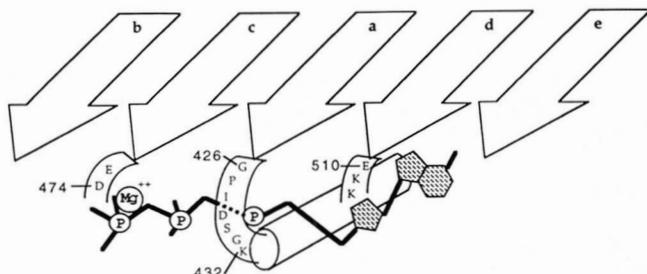
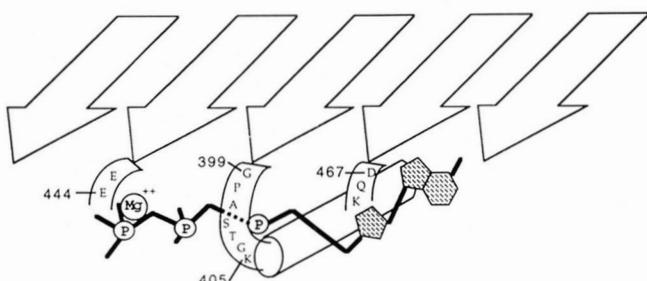
A**B****C**

Fig. 1. *A*, amino acid sequence homology of parvovirus Rep proteins to the NTP-binding region of SV40 T antigen. Sequences are listed with amino termini to the left and are not contiguous. The locations of the A, B, and C motifs conserved among replication proteins of small DNA and RNA viruses are indicated above the aligned sequences. Highly conserved residues appear in boldface. MVM sequence corresponds to the NS-1 protein. Residues believed to constitute important features of the NTP-binding domain of SV40 T and NS-1 (putative) are indicated with arrows. Locations of NS-1 point mutations generated for this study are denoted by * and are listed below the figure. ADV, aleutian disease virus; BPV, bovine parvovirus; B19, human parvovirus B19; MDV, mosquito densoencephalitis virus. Adapted from Gorbatenko *et al.* (1990). *B*, model of the tertiary structure of the NTP-binding fold of SV40 T antigen, proposed by Bradley *et al.* (1987). The binding domain comprises amino acids 418–528. Arrows represent five parallel β -strands pointing to their C termini and labeled alphabetically according to their primary sequence order. The cylinder represents an α -helix with its amino terminus toward the front of the model. An MgATP molecule is depicted with its α -phosphate within the glycine loop extending from the COOH terminus of β -strand *a* to the NH₂ terminus of the α -helix. Other residues

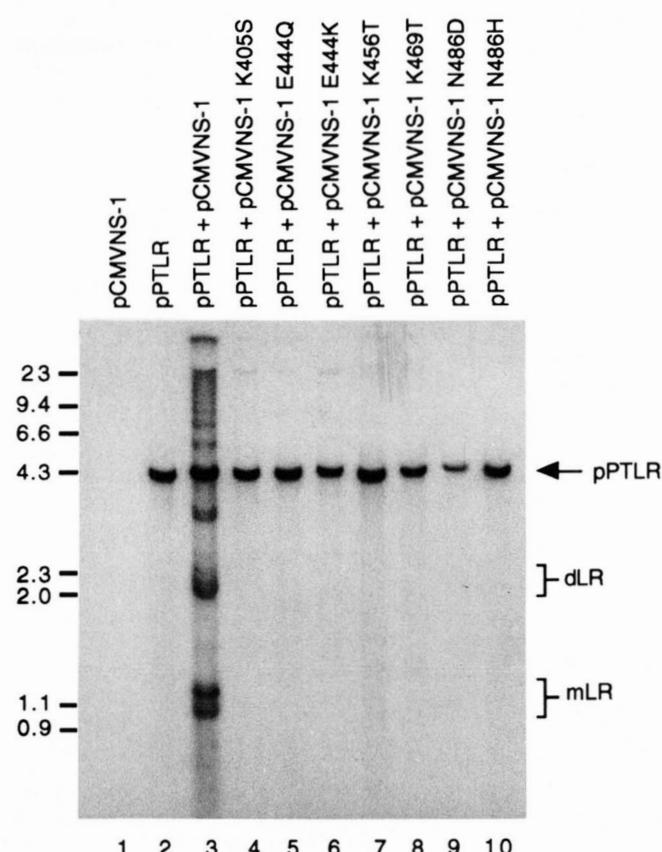


FIG. 2. Putative NTP-binding mutants of NS-1 do not support the DNA replication of the MVM minigenome pPTLR. Plasmid pPTLR was linearized with EcoRI before transfection. Five micrograms of each plasmid (pPTLR and pCMV plasmids containing wild type or mutant NS-1 genes) were transfected into COS-7 cells. Hirt DNA extracts were isolated 72 h post-transfection and electrophoresed on 1.0% agarose gel. The DNA was blotted to a nylon filter and probed with ³²P-labeled BamHI insert of pPTLR. The bracketed regions labeled mLR and dLR correspond to the monomer and dimer replicative forms of the minigenome of MVM replicated in COS-7 cells.

strate (*lane 2*). Transactivation is reduced to background levels by the K405S mutation (*lane 3*), and reduced to approximately half the wild type level by the E444K mutation (*lane 5*). However, transactivation is unaffected or slightly enhanced by the other amino acid substitutions (*lanes 4 and 6–9*). Previous studies have indicated that the COOH-terminal residues are essential for NS-1 transactivation (Rhode and Richard, 1987; Brandenburger *et al.*, 1990; Legendre and Rommelere 1992; Harris and Astell, 1993) and that the amino-terminal 543 amino acids are insufficient to maintain transactivation (Skiadopoulos *et al.*, 1992).² To verify this, we constructed plasmids that express truncated NS-1 proteins lacking either 29 (pCMVNS-1Δ2189C) or 33 COOH-terminal residues (pCMVNS-1Δ2176C) and tested these for transactivation. Both deletion mutants failed to activate the P38 promoter (Fig. 3, *lanes 10 and 11*). Expression of these truncated NS-1 proteins was confirmed by Western blotting analysis (data not shown). Here we note that mutants in the NTP-binding/ATPase domain, except for the K405S substitution, clearly were able to transactivate the P38 promoter but are replication defective.

² C. Harris, unpublished results.

dues extending from the C termini of the β -strands that are believed to make important contacts with MgATP are shown (see text). After Weiner and Bradley (1991). *C*, model of the putative MVM NS-1 NTP-binding site corresponding to the domain shown for SV40 in *B*.

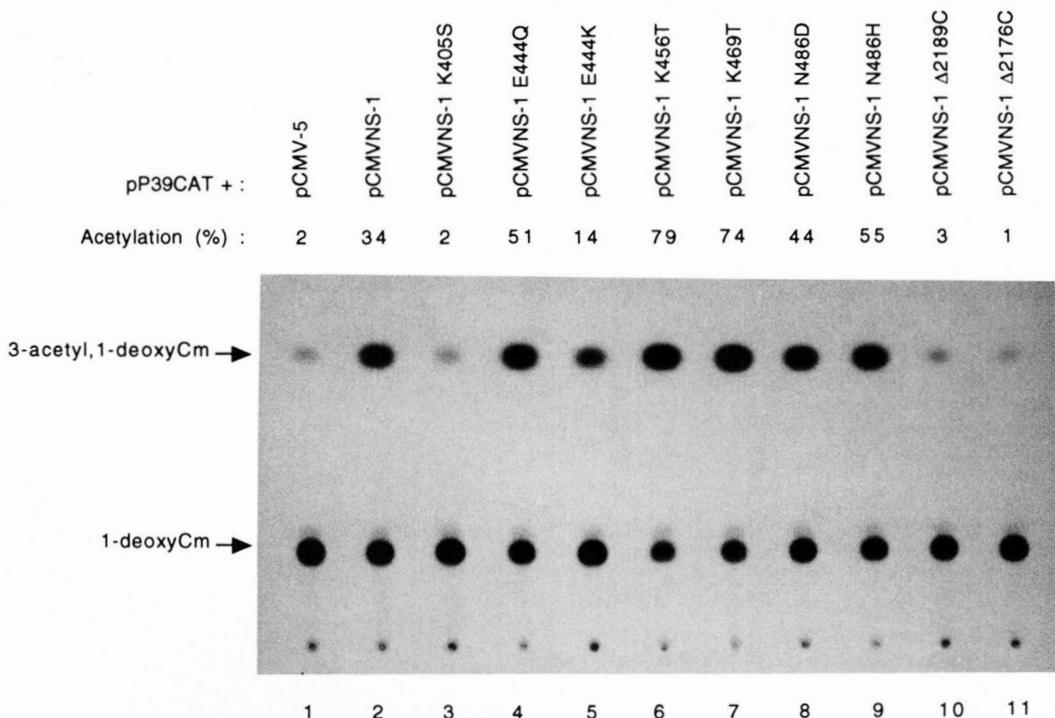


FIG. 3. Transactivation of pP39CAT by pCMVNS-1 and mutant derivatives. LA9 cells were co-transfected with pP39CAT (5 µg) and pCMV-5 (5 µg) or pCMVNS-1 wild type and mutant derivatives (5 µg) as indicated at the top of the figure. Crude cell extracts were prepared at 48 h post-transfection, and chloramphenicol acetyltransferase enzyme activity was assayed as described under "Materials and Methods." The percentage of acetylation is indicated above each lane. Cm, chloramphenicol.

The inactivity of the K405S substitution is in agreement with results obtained with the same mutation in the analogous NS-1 protein of the parvovirus H-1 (Li and Rhode, 1990). Since the K405S mutant is both replication and transcription defective, this substitution may disrupt tertiary structure resulting in a non-functional polypeptide. However, other properties of the K405S mutant suggest it is not grossly misfolded (see "Discussion").

Expression of NS-1 Mutants in Insect Cells—NS-1 is believed to provide several activities needed for MVM replication, including ATP binding and ATPase activity presumably required for the helicase activity (Wilson *et al.*, 1991). Hence we sought to determine whether these activities were affected by the mutations that had abrogated NS-1 replication activity (Fig. 2). We elected to overexpress each mutant using recombinant baculoviruses to test purified mutant NS-1 activities *in vitro*. Recombinants expressing NS-1 mutants were isolated and propagated in Sf9 cells (see "Materials and Methods"). The Western blot in Fig. 4 shows lysates of Sf9 cells infected with each recombinant baculovirus probed with monoclonal antibody specific to NS-1. Wild type NS-1 from a nuclear extract of MVM-infected LA9 cells appears as a single band of approximately 83 kDa (lane 1). Each recombinant baculovirus generated a single band (lanes 3–11) which comigrated with MVM NS-1, indicating that full-length wild type and mutant NS-1 are expressed at similar levels in the infected insect cells.

To facilitate biochemical analysis, we have used immunoaffinity chromatography to isolate NS-1 from crude lysates of infected Sf9 cells (Wilson *et al.*, 1991). A Coomassie Blue-stained protein gel indicated that all NS-1 proteins have been purified to at least 85% (Fig. 5). Purified NS-1 K469T appears as two equally abundant species (83 and 79 kDa), in contrast to the single NS-1 band present in Sf9 cell lysates prior to purification (Fig. 4, lane 8). The cause of the two different mobilities is unknown, although the K469T substitution may render the polypeptide more susceptible to proteolytic cleavage during purification. Western blot analysis has shown that the band mi-

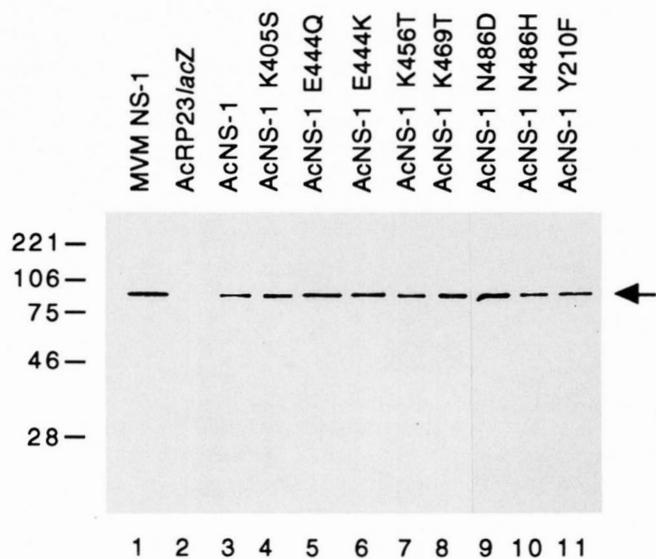


FIG. 4. Expression of NS-1 proteins in insect cells by recombinant baculoviruses. Sf9 cells were grown in monolayer culture (6×10^6 cells/25 cm 2 flask) and infected with the baculovirus AcRP23/lacZ or NS-1 expressing recombinants at a multiplicity of 5. Cells were harvested at 48 h postinfection in 400 µl of SDS reducing buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β -mercaptoethanol, 0.05% bromophenol blue) and boiled for 2 min. LA9 cells were infected with MVM at a multiplicity of 10 and used to prepare a nuclear extract at 48 h postinfection by the method of Dignam *et al.* (1983). Sf9 crude lysates and LA9 nuclear extract (11.6 mg/ml) were diluted 1/20 in sample buffer and fractionated by 12% SDS-PAGE, 5 µl/lane. Proteins were blotted onto nitrocellulose and probed with the monoclonal antibody CE10 specific for NS-1 (Yeung, *et al.*, 1991) using the ECL detection system (Amersham Corp.). Lane 1, LA9 nuclear extract containing MVM NS-1 protein control; lane 2, Sf9 lysate after infection with AcRP23/lacZ control; lanes 3–11, Sf9 lysates after infection with recombinant baculovirus expressing wild type or mutant NS-1 protein as indicated above each lane. A single band appears in all lysates of cells infected with NS-1 expressing viruses (arrow). Approximate positions of molecular weight standards are shown on the left.

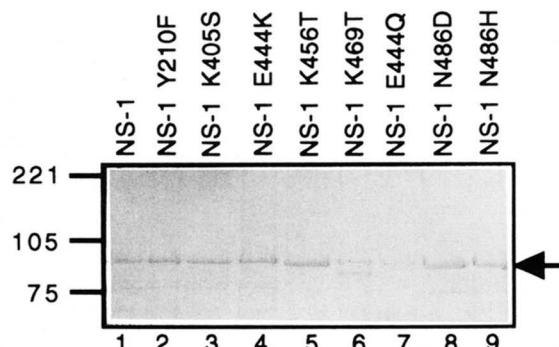


FIG. 5. SDS-polyacrylamide gel electrophoresis of immunoaffinity purified wild type and mutant proteins. Fractions from the immunoaffinity column showing reactivity with Mab-CE10 were pooled and subjected to electrophoresis on a 12% polyacrylamide separating gel with 4% stacking gel containing SDS. The gel was stained with Coomassie Blue. Lanes 1–9 show the resolution of the immunoaffinity purified wild type and mutant NS-1 proteins as indicated above each lane. Position of the ($\times 10^{-3}$) molecular weight are indicated. The K469T mutant migrates as two bands, 83 and 79 kDa. In addition, most of the other minor bands present are also detected by NS-1 monoclonal antibodies and are due to proteolytic degradation which occurs during the purification procedure.

grating at approximately 83 kDa (and both the 83- and 79-kDa bands in the case of NS-1 K469T) is recognized by monoclonal antibodies to NS-1.

Mutations in the NTP-binding Region Impair Helicase Activity—None of the NTP-binding motif NS-1 mutants are capable of effecting MVM DNA replication (Fig. 2). Replication is believed to require several activities provided by NS-1, including site-specific nicking, ATPase and helicase activities, and hence one or more of these activities may be impaired in the point mutants. To determine whether the mutations affect helicase function, we tested for helicase activity associated with the immunoaffinity purified NS-1 proteins (Fig. 6). Incubation of the substrate with purified wild type NS-1 results in release of free primer (lane 3), demonstrating intrinsic NS-1 helicase activity. However, purified NS-1 proteins bearing mutations within the NTP-binding motif showed substantially less helicase activity (lanes 5–11). By contrast, a mutant bearing a point mutation outside of the NTP-binding region, NS-1 Y210F, displayed helicase activity at wild type levels (lane 4). It is significant that NS-1 Y210F has also been found to be incapable of supporting MVM DNA replication (Skiadopoulos and Faust, 1993). These results strongly suggest that the mutations in the NTP-binding region have impaired NS-1 helicase function leading to its inability to replicate MVM DNA.

Mutations in the Putative NTP-binding Domain Can Be Covalently Labeled with Oxidized [α - 32 P]ATP—To define the sites for the NTP-binding in NS-1 protein, we attempted affinity labeling of wild type and mutant NS-1 proteins with periodate-oxidized [α - 32 P]ATP. It is evident from Fig. 7 that all NS-1 proteins were labeled with oxidized [α - 32 P]ATP to almost the same extent, except for the K469T mutant which consistently labeled to a substantially lower level than the wild type. This suggests that the ϵ -NH₂ group on lysine 469 may form a Schiff's base with the ox-ATP and be covalently linked to the affinity label in the presence of NaBH₄. Fig. 7 demonstrates that mutant NS-1 Y210F (which as far as we know does not lie in the NTP-binding domain) exhibits a low level of affinity labeling with oxidized [α - 32 P]ATP as well. NS-1 Y210F has recently been reported to be incapable of supporting MVM DNA replication (Skiadopoulos and Faust, 1993). However, this mutation does not appear to affect its helicase activity. The reduced level of ATP binding may be sufficient to maintain helicase activity in an *in vitro* assay, but this mutation may block other func-

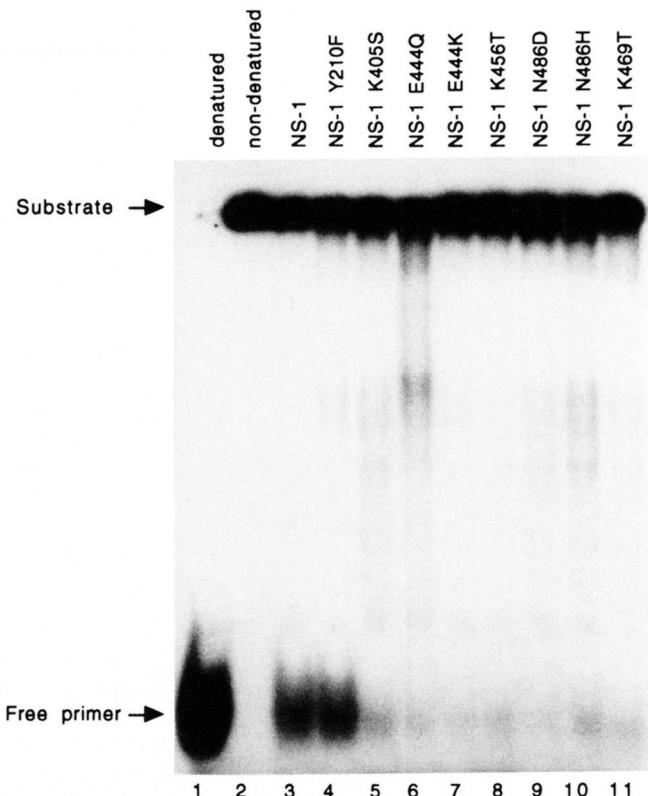


FIG. 6. Mutations in the putative NTP-binding domain impair NS-1 helicase activity *in vitro*. In a reaction volume of 20 μ l, the immunoaffinity purified wild type or mutant NS-1 proteins (300 ng) were incubated with M₁3DNA annealed with a labeled oligonucleotide primer ($2-4 \times 10^4$ cpm) in the presence of 20 mM Tris-HCl buffer, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol, 2 mM ATP, 0.1 mg/ml bovine serum albumin for 60 min at 37 °C. The reaction was stopped by the addition of 10 μ l of a mixture of 0.1 M EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol (v/v), and 2% SDS. The reaction products were analyzed on a nondenaturing 6% polyacrylamide gel (200 \times 400 \times 3 mm) by electrophoresis for 90 min at 200 V. Gels were dried and exposed for autoradiography at -70 °C. Lane 1, the control reaction performed in the absence of NS-1 (wild type) shows primer released from heat denatured substrate; lane 2, substrate incubated in the absence of NS-1 protein (wild type). Subsequent reactions contained wild type or mutant NS-1 protein as indicated above each lane (lanes 3–11).

tions required for replication (e.g. resolution of the dimer bridge). No label was observed in the wild type NS-1 after incubation with [α - 32 P]ATP that had not been previously oxidized with periodate.

Wild Type as Well as Mutant NS-1 Proteins Have ATPase Activity—Immunoaffinity purified NS-1 proteins were examined for their ability to hydrolyze ATP. Wild type and all the mutant NS-1 proteins are able to hydrolyze [γ - 32 P]ATP, although to varying degrees (Table I). Mutants E444Q, N486D, N486H, and K456T showed reduced ATPase activity (1, 40, 47, and 72%, respectively) compared to the ATPase activity exhibited by wild type NS-1, whereas ATPase activity associated with mutants K405S, E444K, and K469T was comparable to wild type levels. Presumably the mutations which reduce ATPase activity involve residues involved in hydrolyzing ATP, or alter the tertiary structure of the protein sufficiently to reduce ATPase activity. Although the K469T mutant is affinity labeled with ox-ATP at a low level, this is probably due to the lack of an adjacent reactive ϵ -NH₂ group rather than the inability to bind ATP.

DISCUSSION

When we began these studies, our goal was to define NTP-binding/ATPase and helicase domains of NS-1. By comparison

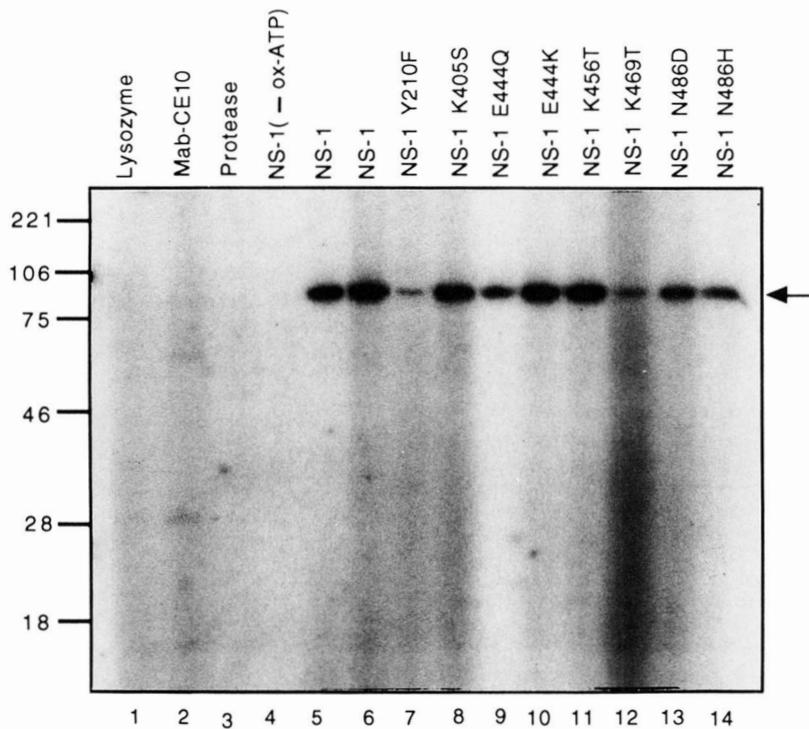


FIG. 7. Covalent labeling by oxidized ATP of NS-1 proteins. Immunoaffinity purified wild type or mutant NS-1 proteins (1.7 µg each) were incubated with periodate-oxidized [α -³²P]ATP (1.0 µM, 800 Ci/mmol) followed by treatment with NaBH₄ (10 mM final concentration) in a total volume of 90 µl (see "Materials and Methods"). After covalent labeling with oxidized ATP, the samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis followed by autoradiography. Lane 1, lysozyme; lane 2, Mab-CE10; lane 3, protease (*S. griseus*); lane 4, wild type NS-1 protein incubated with [α -³²P]ATP (not oxidized with periodate); lanes 5 and 6, wild type NS-1 incubated with oxidized [α -³²P]ATP; lanes 7 to 14, mutant NS-1 proteins (as indicated above each lane) with oxidized ATP.

TABLE I
Immunoaffinity purified wild type NS-1 and mutant NS-1 proteins have ATPase activity

500 ng of immunoaffinity purified wild type or mutant NS-1 proteins were incubated for 30 min at 30 °C in a reaction volume of 20 µl containing 30 mM ATP and 0.5 µCi of [γ -³²P]ATP (see "Materials and Methods"). The released ³²P was determined by Cerenkov counting following precipitation of unreacted ATP with acid-washed activated charcoal. ATPase activity as observed with wild type NS-1 protein was considered 100%. One unit of ATPase activity is defined as the hydrolysis of 1 nmol of ATP in 1 h at 30 °C.

Protein	Activity
	%
NS-1 (wild type)	100
NS-1 Y210F	14
NS-1 K405S	87
NS-1 E444Q	1
NS-1 E444K	88
NS-1 K456T	72
NS-1 K469T	95
NS-1 N486D	40
NS-1 N486H	47

of the sequence of parvovirus NS-1 proteins with that of SV40 T antigen, we predicted key residues of the nucleotide binding fold. These residues fall within the A, B, and C consensus motifs for nucleotide binding folds.

The mutations constructed were K405S; E444K, E444Q; and N486D, N486H (Fig. 1A). (All numbers for parvovirus amino acids correspond with the NS-1 protein from MVM.) In addition, we had noted earlier that 2 lysine residues (456 and 469) are conserved within six parvovirus NS-1 proteins (Astell *et al.*, 1987) and one of these may correspond with the lysine (SV40 T 513) conserved in papillomavirus proteins. Both of these NS-1 lysine residues were also mutated (K456T, K469T).

Our initial experiments with the mutant NS-1 proteins were to test if each supported replication of an MVM minigenome

and was capable of transactivating the P38 promoter. We did these studies first as we did not wish to spend time isolating recombinant baculovirus clones if a particular mutant did not affect either of these activities. As seen in Fig. 2, all the mutant NS-1 proteins were unable to support replication of the minigenome. However, all (except K405S) were able to transactivate the P38 promoter (Fig. 3). We expected that these mutants would be replication defective as the NTP binding fold is predicted to bind ATP and hydrolysis of this ATP is the likely source of energy for the helicase function associated with NS-1 (Wilson *et al.*, 1991). In addition, we targeted the K405S mutation because an identical mutation made in the NS-1 gene of H1 virus had previously been shown to be defective in both replication and transactivation (Li and Rhode, 1990). Indeed, our studies confirmed this observation. Because this residue is well outside the COOH-terminal region identified by several groups as the transactivation domain (Rhode and Richard, 1987; Brandenburger *et al.*, 1990; Legendre and Rommelaere, 1992; Harris and Astell, 1993), either this residue is important for transcriptional regulation because it is located near the transactivation domain in the three-dimensional structure, or this mutation may cause a disruption of the tertiary structure resulting in a non-functional polypeptide due to incorrect folding. We would argue that the latter explanation is unlikely as the recombinant NS-1 K405S protein is expressed at equivalent levels in transfected COS-7 and infected insect cells (grossly misfolded proteins are usually degraded rapidly), and the mutant protein was purified by an identical procedure to that of wild type NS-1. Furthermore, the K405S mutation in NS-1 from H1 is translocated to the nucleus (Li and Rhode, 1990). In addition, MVM NS-1 K405R and K405M mutants have been shown to be capable of transactivation and are translocated to the nucleus (Nuesch *et al.*, 1992).

It should be noted that mutant E444K was consistently half

as effective in the transactivation assay as the wild type protein. What is surprising is that substitution of an acidic residue (E) with a larger basic one (K) only reduced transactivation by half. This observation suggests that mutations well outside the accepted domain can influence transactivation, although how these mutations are effective will require sophisticated tertiary structure information.

One striking difference between the NS-1 protein of MVM and Rep 78 protein of AAV-2 is that while we (this report) and others (Nuesch *et al.*, 1992) have found that most point mutations within the NS-1 NTP-binding motif are replication negative and transactivation positive, McCarty *et al.* (1992) found that all replication defective mutants of Rep 78 are also unable to transactivate the AAV promoters. McCarty suggested that loss of transactivation by a double mutant T341I and N342Y (the TN amino acids are part of the A consensus sequence, GPATTGKTN (Fig. 1A)) indicates that ATP is required for transactivation and cites loss of transactivation by the H1 NS-1 K405S mutant to support this conclusion. We have shown here that while a K405S mutation is transactivation negative, the protein can be affinity labeled with ox-ATP (*i.e.* bind ATP) and retains 87% of wild type ATPase activity. Hence we believe it is unlikely that ATP binding is required for transactivation.

Because the nucleotide binding fold is expected to bind ATP and hydrolyze it for energy to drive the helicase function, we anticipated that all of our mutants would be helicase negative. Wild type and each mutant NS-1 proteins were expressed in insect cells, purified by immunoaffinity, and tested for helicase activity (Wilson *et al.* 1991). As expected, each mutant was reduced in helicase activity by more than 20-fold (Fig. 6). In contrast, NS-1 bearing a mutation well outside the nucleotide binding fold, Y210F, retained 100% helicase activity. This mutant NS-1 has been shown to be replication negative and transactivation positive (Skiadopoulos and Faust, 1993).

In further experiments we tested whether if NS-1 could be affinity labeled with oxidized [α -³²P]ATP in the presence of NaBH₄. According to the model proposed by Bradley *et al.* (1987) for SV40 T antigen, glutamic residue 510 is predicted to interact via hydrogen bonding with the 2'- or 3'-OH of the ribose. Although we have not mutated the corresponding residue (Asp-467) in NS-1, we did alter the lysine at 469 (K469T) and a second conserved lysine (K456T). Surprisingly, in the presence of sodium borohydride the wild type and all of the NTP-binding motif mutant proteins were labeled with [α -³²P]ox-ATP although the K469T mutant is labeled at a very low level. We interpret these results to indicate that the mutant proteins are not grossly misfolded but retain a reasonable nucleotide binding fold in which ATP is able to bind, although the binding affinities may vary greatly. Because the affinity labeling reaction proceeds over an extended period of time, cross-linking of [α -³²P]ATP to the mutant enzymes may appear to occur as efficiently as with the wild type. We consistently observed that the K469T mutant was labeled at a reduced level (<10% of wild type). This lysine residue is located very near (two amino acids further along the polypeptide chain) to the conserved aspartic residue predicted to hydrogen bond with the 2'- or 3'-OH of the ribose (Bradley *et al.*, 1987); hence the K469T mutation probably cannot be cross-linked to the ox-ATP because it cannot form a Schiff's base. It should be noted that the 79-kDa form of the K469T mutant (Fig. 7) was not labeled by ox-ATP.

The Y210F mutation is not predicted to be part of the NTP-binding fold although it may be in close proximity to this region in the tertiary structure. Of interest is that although the Y210F mutant has low ATPase activity (14%) it appears to have full helicase activity. While the *Escherichia coli* REP protein hydrolyzes 2 ATP molecules/bp unwound (Lohman, 1992) many DNA-dependent helicases can carry out a futile hydrolysis of ATP, hydrolyzing as much as 4000 ATP molecules per bp unwound (Tuteja *et al.*, 1993). Hence, the low amount of ATPase activity associated with Y210F mutant (14%) may be sufficient to support the helicase activity we observe.

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REFERENCES

- Ahn, J. K., Pitluk, Z. W., and Ward, D. C. (1992) *J. Virol.* **66**, 3776–3783
- Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264**, 8222–8229
- Astell, C. R., Chow, M. B., and Ward, D. C. (1985) *J. Virol.* **54**, 171–177
- Astell, C. R., Mol, C. D., and Anderson, W. F. (1987) *J. Gen. Virol.* **68**, 885–893
- Bradley, M. K. (1990) *J. Virol.* **64**, 4939–4947
- Bradley, M. K., Hudson, J., Villaneuva, M. S., and Livingston, D. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 6574–6578
- Bradley, M. K., Smith, T. F., Lathrop, R. H., Livingston, D. M., and Webster, T. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 4026–4030
- Brandenburger, A., Legendre, D., Avalosse, B., and Rommelaere, J. (1990) *Virology* **174**, 576–584
- Caillet-Fauquet, P., Perros, M., Brandenburger, A., Spegelaere, P., and Rommelaere, J. (1990) *EMBO J.* **9**, 2989–2995
- Clermont, P., and Cuzin, F. (1982) *J. Biol. Chem.* **257**, 6300–6305
- Clermont, P., Gaudray, P., May, E., and Cuzin, F. (1984) *J. Biol. Chem.* **259**, 15196–15203
- Cotmore, S. F., and Tattersall, P. (1986) *Virus Res.* **4**, 243–250
- Cotmore, S. F., and Tattersall, P. (1992) *J. Virol.* **66**, 420–431
- Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
- Doerig, C., Hirt, B., Beard, P., and Antonietti, J. P. (1988) *J. Gen. Virol.* **69**, 2563–2573
- Doerig, C., Hirt, B., Antonietti, J. P., and Beard, P. (1990) *J. Virol.* **64**, 387–396
- Gill, G., Sadowski, I., and Ptashne, M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2127–2131
- Gorbalyena, A. E., Koonin, E. V., and Wolf, Y. I. (1990) *FEBS Lett.* **262**, 145–148
- Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051
- Harris, C., and Astell, C. R. (1993) in *IUBMB Symposium on Nucleic Acids and Membranes*, University of British Columbia, pp. 24, Vancouver, Canada
- Im, D. S., and Muzychka, N. (1990) *Cell* **61**, 447–457
- Legendre, D., and Rommelaere, J. (1992) *J. Virol.* **66**, 5705–5713
- Li, X., and Rhode, S. L. I. (1990) *J. Virol.* **64**, 4654–4660
- Lohman, T. M. (1992) *Mol. Microbiol.* **6**, 5–14
- McCarty, D. M., Ni, T.-H., and Muzychka, N. (1992) *J. Virol.* **66**, 4050–57
- Nuesch, J. P., Cotmore, S. F., and Tattersall, P. (1992) *Virology* **191**, 406–416
- Pintel, D., Dadachanji, D., Astell, C. R., and Ward, D. C. (1983) *Nucleic Acids Res.* **11**, 1019–1038
- Possee, R. D., and Howard, S. C. (1987) *Nucleic Acids Res.* **15**, 10233–10248
- Rhode, S. L. I. (1985) *J. Virol.* **55**, 886–887
- Rhode, S. L. I., and Richard, S. M. (1987) *J. Virol.* **61**, 2807–2815
- Skiadopoulos, M. H., and Faust, E. A. (1993) *Virology* **194**, 509–517
- Skiadopoulos, M. H., Salvino, R., Leong, W. L., and Faust, E. A. (1992) *Virology* **188**, 122–134
- Snyder, R. O., Samulski, R. J., and Muzychka, N. (1990) *Cell* **60**, 105–113
- Summers, M. D., and Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*. Texas Agricultural Experiment Station, Bulletin No. 1555
- Tam, P., and Astell, C. R. (1993) *Virology* **193**, 812–824
- Tattersall, P., and Cotmore, S. F. (1990) in *CRC Handbook of Parvoviruses*, pp. 123–140, CRC Press Inc., Boca Raton, FL
- Tullis, G. E., Labieniec-Pintel, L., Clemens, K. E., and Pintel, D. (1988) *J. Virol.* **64**, 2736–2744
- Tuteja, N., Khalilur, R., Tuteja, R., and Falaschi, A. (1993) *Nucleic Acids Res.* **21**, 2325–2329
- Weiner, B. M., and Bradley, M. K. (1991) *J. Virol.* **65**, 4973–4984
- Wilson, G. M., Jindal, H. K., Yeung, D. E., Chen, W., and Astell, C. R. (1991) *Virology* **185**, 90–98
- Yeung, D. E., Brown, G. W., Tam, P., Russnak, R. H., Wilson, G., Clark, L. I., and Astell, C. R. (1991) *Virology* **181**, 35–45.