

# Minute Virus of Mice Transcriptional Activator Protein NS1 Binds Directly to the Transactivation Region of the Viral P38 Promoter in a Strictly ATP-Dependent Manner

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The NS1 polypeptide of minute virus of mice (MVM) is a potent transcriptional activator of the MVM P38 promoter. The minimum region of this promoter required for transactivation has been identified and termed the transactivation region (*tar*). However, the function of *tar* and the biochemical steps involved in NS1-mediated transactivation are not well understood. Here we provide evidence that NS1 binds directly and specifically to *tar* in a strictly ATP-dependent manner. A DNA fragment containing *tar* was specifically coimmunoprecipitated with purified baculovirus-expressed MVM NS1, using antibodies directed against NS1 amino- or carboxy-terminal peptides. Using this immunoprecipitation assay, we found that the NS1-*tar* interaction was enhanced approximately 10-fold by ATP, but subsequent incubation at elevated temperatures in the presence, but not the absence, of MgCl<sub>2</sub> caused rapid loss of *tar* binding. This finding suggests that the *tar*-NS1 complex has a short half-life under assay conditions which favor ATP hydrolysis. Specific binding was efficiently inhibited by self-ligated oligonucleotides containing the core DNA sequence (ACCA)<sub>3</sub>, but the same nonligated 20- and 21-mer oligonucleotides were unable to compete effectively, indicating that NS1 only binds to its cognate site when this site is presented on DNA fragments of sufficient size. DNase I footprinting experiments performed in the presence of  $\gamma$ S-ATP revealed that NS1 protects a 43-bp sequence extending asymmetrically from the (ACCA)<sub>2</sub> sequence toward the TATA box of the promoter. NS1 footprints obtained at other sites in the MVM genome were similarly large and asymmetric, all extending approximately 31 bp 5' from the core (ACCA)<sub>2-3</sub> sequence. Surprisingly, no footprints were obtained in the absence of  $\gamma$ S-ATP even under low-stringency binding conditions. However, ATP could be omitted from the reactions if NS1 was first incubated with antibodies directed against its 16-amino-acid carboxy-terminal peptide. Since these antibodies probably create intermolecular cross-links, this finding suggests that NS1 may only bind its cognate site efficiently, or perhaps at all, if the transactivator is first induced to form oligomers. From these data, we hypothesize that ATP binding may also induce NS1 to oligomerize and that such assembly is required before the protein can bind effectively to the *tar* sequence. The functional implications of the NS1-*tar* interaction will be discussed.

The murine parvovirus minute virus of mice (MVM) has a linear DNA genome of approximately 5 kb in which a long single-stranded coding region is contained within short palindromic terminal sequences capable of folding into hairpin duplexes (12). The genome contains two overlapping transcription units, with promoters at map units 4 and 38 (P4 and P38) accessing two major blocks of open reading frame in the two halves of the genome. Alternatively spliced mRNAs transcribed from the P4 promoter encode about four nonstructural proteins, while the P38 promoter, which is itself embedded in the nonstructural gene, drives the synthesis of transcripts encoding the capsid polypeptides (3, 5, 24, 39). This limited coding potential makes MVM highly dependent on the synthetic machinery of the host cell for its own preferential replication. To divert the cellular DNA replication and transcription machinery toward viral expression, one of the nonstructural proteins, the 83-kDa nuclear phosphoprotein NS1, serves both as the initiator protein for viral DNA replication and as a potent transcriptional activator of the viral promoters. NS1 initiates DNA replication by binding to a specific sequence in a viral replication origin (10) and introducing a single-strand nick approximately 17 bases

away at a specific site (13, 37). It then appears to play an essential role in the establishment and maintenance of the replication fork, providing ATPase and helicase functions (13, 37, 47).

In contrast, the biochemical mechanisms by which NS1 activates transcription have been less well characterized. Co-transfection experiments using vectors expressing NS1 and the viral promoters fused to reporter genes revealed that while transcription from the P4 promoter could be upregulated somewhat by NS1 (9, 15), transcription from the P38 promoter could be enhanced approximately 100- to 1,000-fold (1, 9, 15, 44). A *cis*-acting upstream sequence which renders the P38 promoter of the closely related H-1 parvovirus responsive to NS1 was identified by 5' and 3' deletion analysis of P38 constructs fused to a reporter gene (41). This minimal 19-nucleotide (nt) region, designated the transactivation-responsive element or transactivation region, both termed *tar*, is located 116 nt upstream of the P38 transcription initiation site (20, 41), and mutational analysis revealed that single-nucleotide changes within this element did not abolish transactivation but did affect the level of activation achieved (20). Corresponding *tar* regions in MVM, canine parvovirus, and Aleutian mink disease virus (ADV) P38 promoters were subsequently shown to be required for NS1-mediated transactivation; however, additional upstream sequences were necessary for obtaining the full response to NS1 (44). Other elements in the vicinity of the

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promoter, including an Sp1 site, a TATA box (1, 20), and a less well characterized downstream element (27), have also been shown to influence promoter activity, but while these sequences may be important for basal activity, their role in transactivation is equivocal (1).

Transcriptional activators are generally of a modular nature, containing a DNA-binding domain involved in promoter recognition and an activation domain which interacts with the basic transcription machinery (40). Mutational analysis of NS1 showed that changes introduced into its carboxy-terminal region and into the A consensus sequence of its putative purine nucleotide-binding fold could inhibit its ability to activate transcription (29, 35, 43). Interestingly, it was recently reported that a peptide of 126 amino acids derived from the highly acidic carboxy-terminal region of NS1, when fused in frame to the DNA-binding domain of the *Escherichia coli* LexA protein, could transactivate a number of promoters harboring LexA operator sites (28). Thus, it appears that NS1 contains a potent activation domain, but how the molecule interacts with the P38 promoter, and why this interaction requires it to have an intact ATP-binding site, remains unclear. Being unable to demonstrate any direct interaction between NS1 and the viral promoter, a number of investigators have suggested that NS1 must contact this sequence indirectly by binding either to Sp1 (1, 26) or to an unidentified cellular *tar*-binding protein (20).

We recently developed an immunoprecipitation assay to study NS1-DNA interactions and were able to show that NS1 binds directly to the DNA motif (ACCA)<sub>2</sub> in the viral 3' replication origin (10). A complex cluster of similar sequences is found in the *tar* element, suggesting that NS1 may bind directly to this region in the promoter. In this report, we demonstrate that purified recombinant baculovirus-expressed MVM NS1 does bind specifically and directly to the *tar* region of the P38 promoter and that this binding is strictly ATP dependent.

## MATERIALS AND METHODS

**Chemicals and reagents.** Nucleotides, ribonucleotides, and protein A-Sepharose were obtained from Pharmacia (Uppsala, Sweden). RNase-free DNase I was from Promega Corp. (Madison, Wis.). Poly(dI)-poly(dC) was obtained from Sigma (St. Louis, Mo.). Ni<sup>2+</sup>-resin was from Diagen (Dusseldorf, Germany), plasmid pCRII was obtained from Invitrogen (San Diego, Calif.), and Sequenase was from U.S. Biochemical (Cleveland, Ohio).

**Plasmids and plasmid digests.** Plasmid pMVM<sub>tar</sub>, containing MVM nt 1838 to 2130 flanked by *Xba*I sites, was obtained by subcloning the *Xba*I-*Xba*I fragment from pCAT MVM<sub>1838-2130</sub> (44) into the *Xba*I site of pCRII. For DNA binding assays, plasmid pMVM<sub>tar</sub> was digested either with *Sau*3A and *Xba*I or with *Sau*3A, *Xba*I, and *Nco*I, and the 3' ends were filled in with Sequenase in the presence of three unlabeled nucleotides and [<sup>32</sup>P]dGTP. For DNase I footprinting analyses, pCAT MVM<sub>1838-2130</sub> was digested with either *Hind*III or *Xho*I and 3' end labeled on one strand by incubation with [<sup>32</sup>P]dATP or [<sup>32</sup>P]dCTP, respectively, in the presence of Sequenase and three unlabeled nucleotides. The labeled DNA was subsequently ethanol precipitated, digested with *Xho*I or *Hind*III, respectively, and purified from agarose gels. The *Hind*III enzyme restriction site is located in the polylinker of pCAT, and the *Xho*I site in the MVM sequence is located at nt 2070. Plasmid pTD7-2 contains 159 bp of MVM sequence between nt 2278 and 2436 isolated by PCR amplification and cloned into pCRII. Labeling of this sequence for DNase I protection analyses was performed as described for pCAT MVM<sub>1838-2130</sub> except that *Xba*I was used instead of *Xho*I. *Hind*III and *Xba*I enzyme restriction sites are both located in the pCRII polylinker.

**Cells and viruses.** *Spodoptera frugiperda* (Sf9) insect cells and the baculovirus *Autographa californica* nuclear polyhedrosis virus were grown as previously described (8). A recombinant baculovirus expressing MVM NS1 with an amino-terminal histidine tag was kindly provided by David Pintel, University of Missouri. The amino acid sequence fused to NS1 in this construct is MGG(H)<sub>6</sub>GGIEGR. When this NS1 gene construct was transferred into an infectious plasmid clone of MVM and used to transfect 324K cells, virus was recovered, confirming that this fusion protein was functionally competent *in vivo* (38a).

**Antisera.** Rabbit antisera directed against a fusion peptide containing the amino-terminal 91 amino acids of NS1, a 16-amino-acid peptide derived from the carboxy terminus of NS1 (αNSC), and a fusion protein containing NS1 amino acids 284 to 459 have been described elsewhere (11).

**Purification of recombinant NS1.** For expression of recombinant protein, Sf9 cells were plated in tissue culture dishes and infected at a multiplicity of infection of 1 to 2. The cells were harvested 40 to 44 h postinfection by being scraped into the medium and then centrifuged (500 × g for 5 min at 4°C). Cells were washed twice in phosphate-buffered saline and once in hypotonic buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-KOH [pH 7.8], 5 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA) and resuspended in 10 ml of hypotonic buffer containing 4 μg of leupeptin per ml. After 10 to 15 min on ice, the cells were lysed by Dounce homogenization (10 to 15 strokes with a B pestle) and incubated for a further 20 min. The lysate was adjusted to 300 mM NaCl, incubated for 20 min on ice, and cleared by centrifugation (20,000 × g for 30 min at 4°C). The cleared supernatant was applied to an Ni<sup>2+</sup> column equilibrated with buffer A (20 mM HEPES-KOH [pH 7.5], 5 mM MgCl<sub>2</sub>, 20% glycerol, 0.1 mM dithiothreitol, 300 mM NaCl, 0.01% Nonidet P-40, leupeptin) and washed sequentially with the 10 column volumes of the following buffers: buffer A; 20 mM phosphate-300 mM NaCl (pH 6.0); buffer A; buffer A substituted with 1% Nonidet P-40 and 50 mM NaCl; and then buffer A lacking MgCl<sub>2</sub> but substituted with 50 mM NaCl and containing first 4 mM and then 10 mM imidazole. The column was eluted with buffer A minus MgCl<sub>2</sub> containing 50 mM NaCl and 80 mM imidazole. Fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and stored at -80°C. In general, 5 × 10<sup>8</sup> Sf9 cells gave approximately 0.5 to 1.5 mg of NS1.

**NS1-DNA binding assays.** NS1-DNA binding assays were performed as previously described (10). Briefly, approximately 100 ng of purified baculovirus-expressed NS1 was incubated for 1 h with 10 ng of a mixture of <sup>32</sup>P-labeled DNA fragments derived from pMVM<sub>tar</sub> at 4°C in binding buffer [100 μl of 20 mM Tris-HCl (pH 8.0)-125 mM NaCl-10% glycerol-1% Nonidet P-40-5 mM dithiothreitol-2 μg of poly(dI)-poly(dC) per ml]. Nucleotides and double-stranded oligonucleotides for competition studies were added as indicated. The double-stranded oligonucleotides used as competitors had the following sequences: ori-core, (GATC)AGAAGTACCAACCATGTTCAC; ATF-box, (GATC)TTACGTAAGTAAGTGACGTGATGA [the (GATC) sequence is a four-base 5' overhang synthesized on each strand to promote ligation]; and ADV<sub>tar</sub>, GTATGGTTACTACAAGCAAT. These oligonucleotides were kinase treated and self-ligated so that they were present as mixtures of fragments ranging in size from 21 to approximately 500 bp. Titrated amounts of the appropriate anti-NS1 antiserum were then added, and the incubations continued for a further hour. Binding buffer (30 μl) containing 1.5 mg of swollen protein A-Sepharose was added, and the mixture was tumbled for 30 min at 4°C. Immune complexes were collected by centrifugation and washed twice with 1 ml of binding buffer. Samples were deproteinized with proteinase K in the presence of 0.5% SDS for 1 h at 55°C before analysis by electrophoresis on 2.5% agarose gels. The gels were fixed in 7% trichloroacetic acid, dried and exposed for autoradiography.

**DNase I protection assays.** DNA fragments labeled at the 3' end of one strand with <sup>32</sup>P-deoxynucleoside triphosphate (approximately 5 × 10<sup>4</sup> cpm) were incubated on ice for 1 h in a total volume of 50 μl with 500 ng of purified NS1 in binding buffer containing 100 mM NaCl and in the presence or absence of 0.5 mM γS-ATP. An equal volume of prewarmed binding buffer containing 4 mM MgCl<sub>2</sub> and variable amounts of DNase I (0.4 to 1.6 U per reaction) was then added, and the reactions were allowed to proceed for 2 min at 25°C. Reactions were terminated by the addition of 0.3 ml of 10 mM Tris-HCl (pH 8)-10 mM EDTA-0.5% SDS-300 μg of proteinase K followed by incubation at 50°C for 45 min. Samples were extracted with phenol-chloroform and precipitated with ethanol before being analyzed by electrophoresis through 6% denaturing acrylamide gels. Some samples were immunoprecipitated as described for DNA binding assays before being footprinted as described above. Samples of all DNA probes used for the footprinting assays were chemically cleaved at G residues by the procedure of Maxam and Gilbert (31) and electrophoresed in adjacent lanes to allow sequence alignment. Further alignments were performed with a DNA dideoxy sequence ladder as a size marker.

## RESULTS

**NS1 binds to the *tar* region of the MVM P38 promoter.** Plasmid pMVM<sub>tar</sub> (Fig. 1) was digested with *Sau*3A and *Xba*I, resulting in 23 fragments ranging in size from 5 to 1274 nt and including a 242-nt MVM fragment containing the *tar* element, the GC box, and the TATA box of the P38 promoter. The 3' ends of all the fragments were labeled with [<sup>32</sup>P]dGTP, and the mixture was incubated in the presence of 0.5 mM ATP with baculovirus-expressed NS1 purified by nickel ion chromatography (Fig. 2A). NS1-DNA complexes were immunoprecipitated with an antiserum directed against a 16-amino-acid peptide derived from the carboxy terminus of NS1 (αNSC). The 242-nt fragment containing the P38 promoter region was efficiently and specifically immunoprecipitated from the mixture (Fig. 2B, compare lanes 1 and 3). To locate the NS1 binding site within this minimal promoter region, the *Sau*3A- and

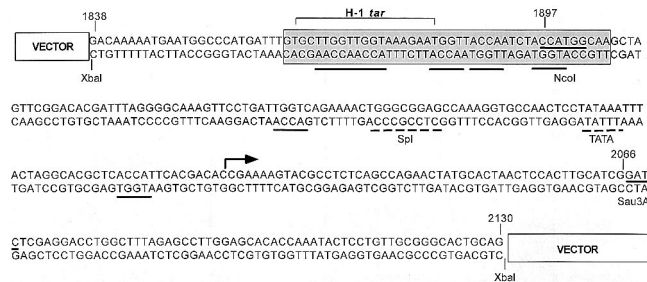


FIG. 1. Nucleotide sequence of the MVM P38 promoter fragment cloned into the pCRII vector. Locations of the *Sau3A*, *XbaI*, and *NcoI* restriction enzyme sites are shown. The previously proposed *tar* element of the H-1 parvovirus aligned with the corresponding sequence of MVM is indicated (41). The TATA box and GC box (Sp1 binding site) are indicated by dashed underlines (1). The putative RNA polymerase initiation site is marked by an arrow (6). The region protected by NS1 against DNase I digestion (results obtained for analysis with the  $^{32}\text{P}$  label on the upper strand) is indicated by a shaded box. Core tetranucleotides (ACCA or TGGT) are also underlined.

*XbaI*-digested pMVM $\text{tar}$  plasmid was further digested with *NcoI*, separating a DNA fragment of 68 nt containing the *tar* region from the 173-nt fragment harboring the Sp1 site and TATA box. In this analysis, the 68-nt DNA fragment containing the *tar* region was specifically immunoprecipitated (lane 6) compared with the corresponding fragment in the input lane (lane 4). In contrast, the 173-nt fragment containing the GC and TATA boxes did not bind purified NS1 (lane 4, fragment indicated by an asterisk). Thus, although the sequences of the *tar* region and the replication origin are functionally distinct and show only limited homology (see Fig. 7), in both cases NS1 binds specifically to the sequence containing the (ACCA) $_{2-3}$  motif. In the *tar* element, this sequence contains multiple tandem and inverted copies of the ACCA motif (Fig. 1) rather than a single copy of this sequence spaced appropriately from a nick site as seen in the 3' origin, raising the possibility that NS1 recognizes these sequences differently.

Next we incubated the *Sau3A*-, *XbaI*-, and *NcoI*-digested pMVM $\text{tar}$  fragments with NS1 in the presence of ATP and scavenged the complexes with antibodies directed against different regions of the NS1 molecule. Once again, the results obtained mirrored our previous observations with the 3' replication origin, with antibodies directed against both the amino- and carboxy-terminal peptides of NS1 precipitating NS1-*tar* complexes (Fig. 2C, lanes 3 and 4), while antibodies directed against NS1 amino acids 284 to 459 showed no NS1-DNA coprecipitation (lane 5). These results are of importance with respect to potential oligomeric forms of NS1 binding to *tar* and will be discussed later.

**NS1 binding to *tar* is stimulated by ATP.** To assay the influence of different nucleotides on the NS1-*tar* interaction, the standard immunoprecipitation assay was carried out with the  $\alpha\text{NSC}$  antibody in the presence of 0.5 mM ATP, GTP, CTP, UTP, or  $\gamma\text{S-ATP}$  at 4°C. NS1 bound to *tar* in the absence of ATP (Fig. 3, lane 3), but binding was stimulated approximately 10-fold in the presence of 0.5 mM ATP or  $\gamma\text{S-ATP}$  (lanes 4 and 8). The level of stimulation observed in several experiments varied between 4- and 10-fold and seemed to be dependent on the antibody/NS1 ratio. As observed in several experiments, binding was also stimulated to a certain degree by GTP, CTP, or UTP, although this effect ranged from almost no stimulation to about half the level of stimulation seen for ATP in the same experiment (lanes 5 to 7). Addition of  $\text{MgCl}_2$  to the assay generally resulted in lower levels of specifically precipitated DNA fragments if the temperature was allowed to rise even

moderately during the incubation or subsequent washing steps. Since NS1 possesses ATPase activity, this observation suggested that ATP hydrolysis might occur in the presence of  $\text{MgCl}_2$ , causing dissociation of the NS1-DNA complex.

To explore this possibility, DNA-NS1 immunoprecipitates were formed in the presence of ATP and then adjusted to 5 mM  $\text{MgCl}_2$ , or not adjusted, and incubated for a further 20 min at 25°C in DNA binding buffer. In the presence of 5 mM  $\text{MgCl}_2$ , the amount of NS1 bound to *tar* decreased under these conditions to the binding levels observed in the absence of ATP (Fig. 3; compare lanes 3 and 10), but no reduction in NS1-*tar* binding was observed after incubation at the elevated temperature in the absence of  $\text{MgCl}_2$  (compare lanes 4 and 9). Moreover, little if any reduction in binding was observed if  $\text{MgCl}_2$  was included in the binding reaction as long as the temperature was kept at 4°C or below throughout the assay procedure (data not shown). These results suggest that ATP may induce changes in the conformational or aggregation state of NS1 which lead to increased site-specific DNA binding, but that this binding is dynamic and easily reversed under conditions which favor ATP hydrolysis (i.e., in the presence of magnesium ions at elevated temperatures). Thus, a partial reduction in NS1-*tar* binding observed at elevated temperatures in the presence of  $\text{MgCl}_2$  and ATP may simply reflect hydrolysis (data not shown). This possibility is currently being explored.

**NS1 binding to *tar* is inhibited by self-ligated oligonucleotides containing the core sequence (ACCA) $_{1-3}$ .** The specificity of NS1 binding to *tar* was examined by competitive inhibition using the standard DNA binding assay in the presence of different sets of 20- or 21-mer double-stranded oligonucleotides or the same oligonucleotides after they had been self-ligated. Monomeric duplex forms of the ori-core sequence containing the (ACCA) $_2$  sequence involved in NS1 binding to the MVM 3' replication origin (10) were unable to prevent NS1 binding to the *tar* fragment at a concentration of 10 or 50 ng/100  $\mu\text{l}$  (Fig. 4; compare lanes 4 and 5 with lane 3), although at higher concentrations, some competition was apparent. However, after self-ligation, the same oligonucleotide completely inhibited binding to *tar* at all concentrations tested (lanes 6 and 7). Similar results were obtained with a competitor oligonucleotide containing a part of the *tar* region from ADV, which contains only one ACCA sequence; once again, binding was inhibited only if the oligonucleotides were ligated (compare lanes 12 and 13 with lanes 14 and 15). As a negative control, we used an oligonucleotide containing the ATF-binding site from the MVM 3' replication origin. This oligonucleotide was unable to inhibit binding both as a monomer and after ligation (lanes 8 to 11). These results confirm the site specificity of NS1 binding and suggest that NS1 can bind to its cognate site only if this site is presented on a DNA fragment which is more than 22 nt in length. Since a single copy of the ori-core sequence cloned into an otherwise unrelated pCRII sequence provides a high-affinity template for NS1 binding (10), this finding suggests that the neighboring sequences can be more or less random.

**NS1 binds to *tar* only in the presence of ATP or NS1-specific antibodies.** Since NS1-*tar* complexes are labile in the presence of  $\text{MgCl}_2$  (and ATP) at elevated temperatures, we attempted to optimize the interaction for DNase I footprinting by using relatively low  $\text{MgCl}_2$  concentrations and short times of exposure to DNase I at 25°C. In the presence of ATP, NS1 recognized and protected a region of about 43 nt (MVM nt 1860 to 1902) over a wide range of DNase concentrations (Fig. 5A). As indicated in Fig. 1, this footprint projected from the direct (ACCA) $_2$  repeat motif toward the TATA box of the P38 promoter and covered most of the *tar* element, including the

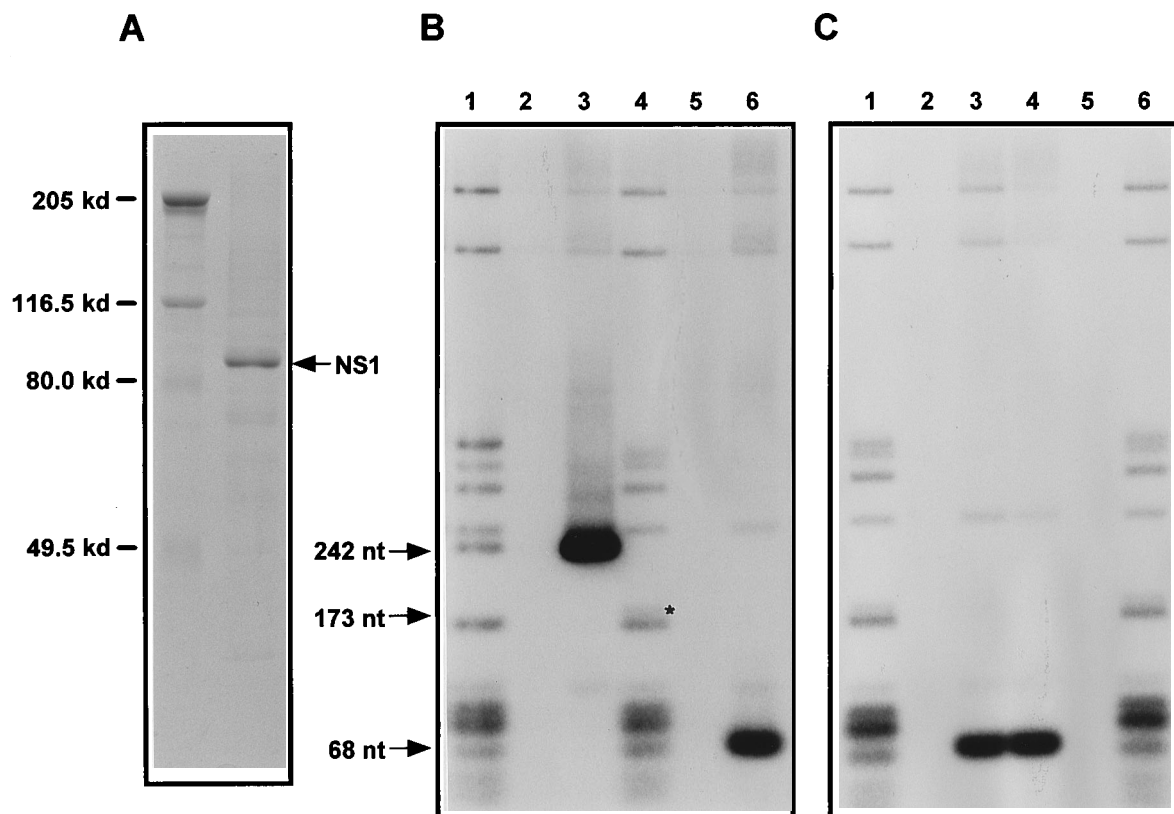


FIG. 2. Purified NS1 binds to the *tar* region of the MVM P38 promoter. (A) Protein molecular weight markers and an affinity-purified, histidine-tagged NS1 preparation (NS1 estimated at 2  $\mu$ g by comparison with standard proteins) purified from insect cells, electrophoresed on a Laemmli SDS-acrylamide gel, and stained with Coomassie brilliant blue. (B) Autoradiograph of an agarose gel in which lanes 1 and 4 contain the equivalent of 2% of the input [ $^{32}$ P]dGTP-labeled *Sau3A-XbaI* double digest and *Sau3A-XbaI-NcoI* triple-digested pMVM $_{tar}$  fragments, respectively, used in the NS1-DNA binding assays shown in the other lanes. Binding reaction mixtures containing the pMVM $_{tar}$  double digest (lanes 2 and 3) or the triple digest (lanes 5 and 6) were incubated with 100 ng of purified histidine-tagged NS1 and 0.5 mM ATP and subsequently precipitated with prebleed serum (lanes 2 and 5) or with an antiserum against the carboxy-terminal peptide of NS1 (lanes 3 and 6). Arrows indicate the positions of the 242-nt MVM P38 *XbaI-Sau3A* fragment and the 68-nt *XbaI-NcoI* *tar*-containing fragment associated with NS1 in the immunoprecipitates. The arrow at 173 nt indicates the position of the *NcoI-Sau3A* fragment containing the MVM Sp1 site and TATA box present in the input DNA in lane 4 (marked by an asterisk) but absent in the NS1-DNA immunoprecipitate (lane 5). (C) Autoradiograph of an agarose gel in which lanes 1 and 6 contain 2% of the input [ $^{32}$ P]-labeled *Sau3A-XbaI-NcoI* triple-digested pMVM $_{tar}$  fragments used in the NS1-DNA binding assay. Binding reactions were performed with this DNA and 100 ng of purified NS1 in the presence of 0.5 mM ATP and subsequently precipitated with antisera directed against different regions of NS1. Lane 2, prebleed serum; lane 3, antiserum against the amino terminus of NS1; lane 4, antiserum against the carboxy terminus of NS1; lane 5, antiserum raised against NS1 amino acids 284 to 459.

nearby decanucleotide element made up from inverted ACCA-containing pentamers. However, when ATP was omitted from the binding reaction, the DNase I pattern resembled that observed without any added NS1 (Fig. 5A), indicating that under these conditions, NS1 was not bound to the substrate. Similar results were obtained when the analyses were performed with substrates labeled at the 3' end of the other DNA strand. In this case, 42 nt (MVM nt 1864 to 1905) were protected from DNase I digestion in the presence of NS1 and ATP, while no protection was observed in the absence of ATP (Fig. 5B). NS1-binding sites located at other positions in the MVM genome also gave footprints in the presence, but not the absence, of ATP, and this result could not be modified by adding increasing amounts of NS1 to the reaction or by omitting Nonidet P-40 from the binding buffer and lowering the NaCl concentration to 50 mM (data not shown).

Since NS1 was able to bind to *tar* in the absence of ATP but in the presence of antibody, in the immunoprecipitation assay shown in Fig. 3, we also carried out DNase I footprinting analysis in the presence of the  $\alpha$ NSC antibody. Standard immunoprecipitation assays were performed in the presence or absence of ATP, and the immunoprecipitates then subjected to

DNase I footprinting analysis. Since the immunoprecipitation assay was much more effective in the presence of ATP, the amounts of [ $^{32}$ P]-labeled DNA in the resulting samples were equalized before the samples were subjected to DNase I digestion. Under these conditions, the same region of *tar* was protected from DNase I digestion, but the level of protection was no longer influenced by ATP (Fig. 5C). Thus, the  $\alpha$ NSC antibody can effectively mimic the function of ATP in this assay.

The footprints obtained in the presence of  $\alpha$ NSC were subtly different in several ways from the corresponding protected regions obtained in the absence of antibody. First, the footprints were less dramatic because faint background bands were invariably observed within the antibody-mediated footprint (Fig. 5C), and second, a thymidine-guanidine dinucleotide at nt 1874 and 1875 (boxed) within the footprint became more sensitive to DNase I digestion than the rest of the protected nucleotides, while at the side proximal to the TATA box, the protected region was approximately 4 nt shorter. We do not know what causes these differences, but it seems probable that dimers created by antibody cross-links would not be sterically identical to multimers which we presume form by self-associ-

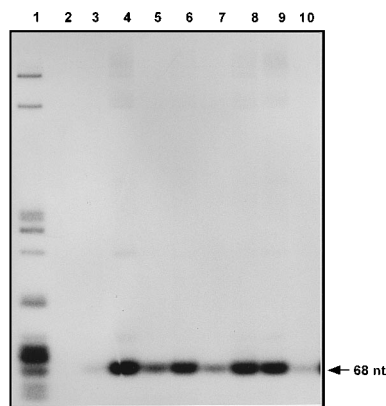


FIG. 3. NS1 binding to the *tar* region is stimulated by ATP. Autoradiograph of an agarose gel showing the  $^{32}$ P-labeled, pMVM*tar* triple digest described in Fig. 2 incubated with 100 ng of NS1 in the presence of a panel of nucleoside triphosphates, analyzed after immunoprecipitation with an antiserum against the carboxy terminus of NS1. Lanes: 1, 2% of the equivalent  $^{32}$ P-labeled pMVM*tar* input fragments present in each NS1-DNA binding assays as analyzed in lanes 2 to 10; 2, negative control (a binding assay was performed in the presence of NS1 and 0.5 mM ATP but with prebleed serum substituted for anti-carboxy-terminal NS1 antiserum); 3 to 8, no ATP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, and 0.5 mM  $\gamma$ S-ATP, respectively, included in the binding reaction. For Lane 9, 0.5 mM ATP was included in the binding reaction, but after immunoprecipitation, the sample was further incubated for 20 min at 25°C in DNA binding buffer. For lane 10, the conditions were the same as for lane 9 except that the sample was adjusted to 5 mM  $MgCl_2$  after immunoprecipitation.

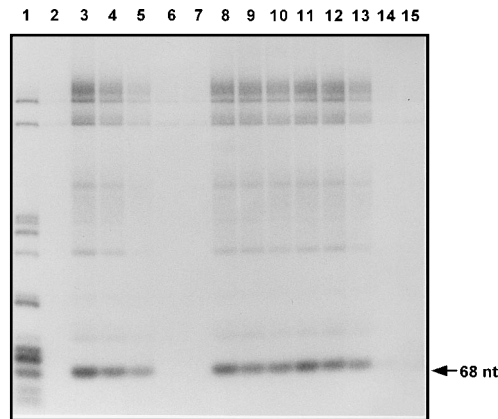


FIG. 4. NS1 binding to *tar* is inhibited by self-ligated oligonucleotides containing the core sequence (ACCA)<sub>1-3</sub>. The autoradiograph represents an agarose gel showing the  $^{32}$ P-labeled pMVM*tar* triple digest incubated with 100 ng of NS1 and 0.5 mM ATP in the presence of different ligated or unlabeled double-stranded oligonucleotides (see Materials and Methods) and analyzed after immunoprecipitation with an antiserum against the carboxy terminus of NS1. Lane 1, 2% of the equivalent  $^{32}$ P-labeled pMVM*tar* input fragments present in each NS1-DNA binding assay; 2, negative control (a binding assay was performed in the presence of NS1 and 0.5 mM ATP but with prebleed serum substituted for anti-carboxy-terminal NS1 antiserum); 3, no competitor oligonucleotide; 4, 10 ng of unlabeled ori-core double-stranded oligonucleotide; 5, 50 ng of unlabeled ori-core double-stranded oligonucleotide; 6, 10 ng of ligated ori-core double-stranded oligonucleotide; 7, 50 ng of ligated ori-core double-stranded oligonucleotide; 8, 10 ng of unlabeled ATF box double-stranded oligonucleotide; 9, 50 ng of unlabeled ATF box double-stranded oligonucleotide; 10, 10 ng of ligated ATF box double-stranded oligonucleotide; 11, 50 ng of ligated ATF box double-stranded oligonucleotide; 12, 10 ng of unlabeled AD*Vtar* double-stranded oligonucleotide; 13, 50 ng of unlabeled AD*Vtar* double-stranded oligonucleotide; 14, 10 ng of ligated AD*Vtar* double-stranded oligonucleotide; 15, 50 ng of ligated AD*Vtar* double-stranded oligonucleotide.

ation in the presence of ATP and thus that the antibody-mediated complexes may not bind as intimately to the DNA. Alternatively, the antibody may physically block part of the NS1-*tar* interaction, allowing enhanced entry of DNase I. Whatever its cause, the characteristics of the antibody-mediated footprint are dominant, so that addition of ATP cannot restore the somewhat larger and more distinct protected regions seen with NS1-ATP in the absence of antibody.

**NS1 footprints at other sites in the MVM genome are highly asymmetric.** In the 3' replication origin of MVM, the NS1 footprint was highly asymmetric, extending about 31 nt 5' to the core ACCA sequence but only 4 nt 3' to this motif (10). However, 20 nucleotides 5' to the ACCA sequence lies the specific consensus sequence CTWWTCA, at which NS1 is known to recognize and nick the DNA in order to initiate viral DNA replication (13). Thus, it could be that the size of the protected region and the asymmetry of binding to the ACCA motif in the origin are dictated by second-site interactions between NS1 and the nick site. However, the NS1 footprint at the *tar* site is the same size as the one on the origin, and although there are no potential nick sites within the protected region, there are multiple ACCA motifs of various sizes and arranged in different patterns, suggesting that, once again, there could be multiple interactions between NS1 and the DNA. To clarify this situation, we analyzed another putative NS1-binding site containing the core ACCA motif located in the small intron sequence of MVM starting at nt 2333. This sequence contains a single copy of (ACCA)<sub>3</sub> and no obvious nick site sequences. Using the immunoprecipitation assay, we demonstrated that a DNA fragment containing this site was efficiently coprecipitated with NS1 (data not shown). Once again, in the presence of ATP, the NS1 footprint extended over approximately 43 nt (MVM nt 2328 to 2370) on both DNA strands (Fig. 6) and was highly asymmetric, extending 38 nt 5' from the end of the ACCA sequence. Thus, asymmetric binding about the ACCA core sequence is an intrinsic property of

NS1 and is not dictated by the surrounding DNA sequence. Alignment of the protected regions in *tar*, the 3' replication origin, and the intron sequence (Fig. 7) thus allows us to predict that the primary interaction between NS1 and *tar* is mediated by the direct (ACCA)<sub>2</sub> motif, so that the inverted ACCA pentamers lie within the asymmetric NS1 extension that projects toward the TATA box. Whether these secondary sequences influence the affinity or avidity of the NS1-*tar* interaction is not clear at this point, but preliminary competition assays between different sites suggest that this is likely.

## DISCUSSION

**ATP-dependent recognition of ACCA-containing sites by NS1.** In this report, we demonstrate that the MVM NS1 polypeptide binds directly and specifically to the *tar* region of the MVM P38 promoter through a core (ACCA)<sub>2-3</sub> recognition sequence. A remarkable feature of this interaction is that it can be demonstrated only in the presence of either ATP or antibodies directed against terminal NS1 sequences, while conditions which favor ATP hydrolysis promote dissociation of the complex. Antibodies could theoretically induce intramolecular conformational changes which facilitate NS1 binding to DNA, but it seems unlikely that polyclonal antibodies directed against opposite molecular termini could both produce exactly the same effect. However, both antisera could efficiently create intermolecular cross-links, suggesting that monomeric NS1 may bind DNA poorly, if at all, and that the antibodies produce their effects simply by cross-linking NS1 into active multimers. DNase I footprints obtained in the presence of antibodies are very similar to those obtained with ATP alone,

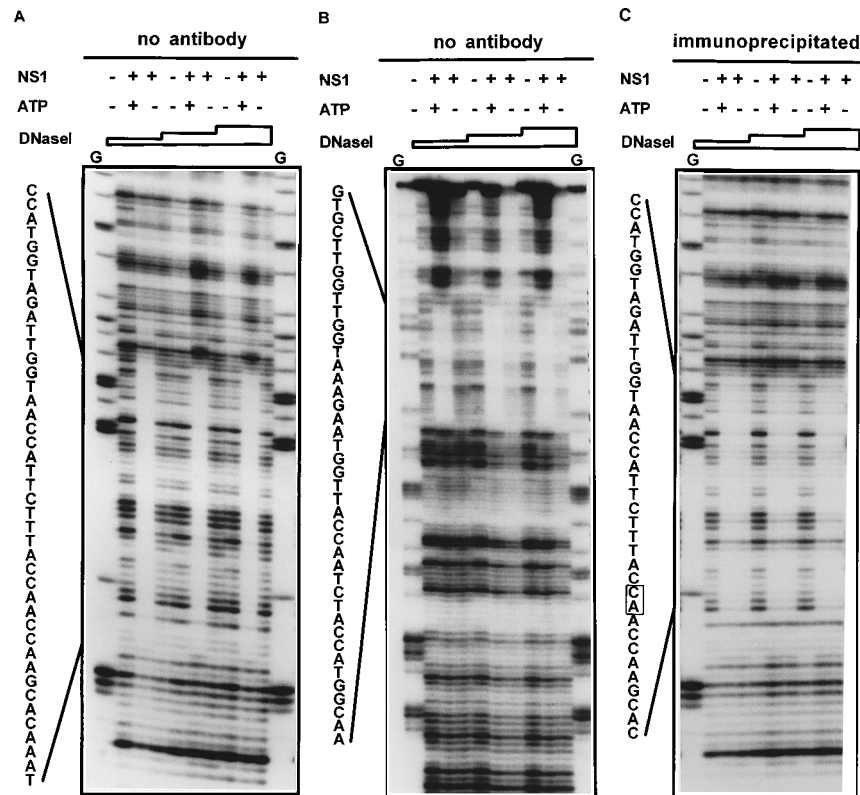


FIG. 5. NS1 binds to *tar* only in the presence of ATP or NS1-specific antibodies. (A) DNase I protection analysis of the NS1-MVM P38 promoter interaction ( $3'$   $^{32}$ P label on the lower strand) was performed with purified NS1 in the absence or presence of ATP as indicated. The DNase I protection assays were performed with 0.4, 0.8, and 1.6 U of DNase I. Lane G is a Maxam-Gilbert G sequence ladder. (B) DNase I protection analysis of the NS1-MVM P38 promoter interaction was performed as described for panel A except that the  $3'$   $^{32}$ P label was on the top strand. (C) DNase I protection analysis of the NS1-MVM P38 promoter interaction ( $3'$   $^{32}$ P label on lower strand) was performed after immunoprecipitation of the NS1-MVM P38 complex with anti-carboxy-terminal NS1 antibodies in the presence or absence of ATP as indicated.

indicating that the NS1 structures generated in these two situations interact with the DNA in similar ways and suggesting that ATP may not be inducing major changes in the DNA-binding domain of individual NS1 molecules. Nuclear cotransport studies have shown that NS1 does assemble into oligomers *in vivo* and that mutations at a critical lysine residue (K-405) in its ATP-binding fold prevent such self-association (36). This finding suggests that NS1 may be able to assemble into multimeric forms only if it can bind and/or hydrolyze ATP normally and together with the antibody results, suggests that the role of ATP in the present study may also be to induce multimer formation.

The  $\alpha$ NSC antibody used in this study recognizes epitopes expressed in the terminal 16 amino acids of NS1, and it is thus likely that binding is mostly restricted to one antigen combining site per NS1 molecule. This means that the limited *tar* binding observed in the absence of ATP in immunoprecipitation assays is most likely mediated by NS1 dimers, although larger complexes could form if, for example, NS1 self-associates after dimer formation. However, these analyses show that in the absence of ATP, *tar* binding is relatively weak and can be enhanced 10-fold by ATP addition. Thus, ATP must induce additional conformational changes in NS1 which enhance dimer-mediated site-specific DNA binding, or it may promote the formation of higher-order NS1 multimers which bind DNA with higher avidity. Mutations at lysine 405 in the NS1 ATP-binding fold do not prevent it from recognizing and binding to DNA containing the (ACCA)<sub>2-3</sub> motif *in vitro* in immunopre-

cipitation assays in which it is exposed to anti-NS1 sera, but this minimal binding cannot be enhanced by ATP addition (10). These same mutants are unable to assemble into multimers *in vivo* (36), which suggests that ATP does indeed induce the formation of higher-order multimers.

There are similarities between NS1 and the homologous proteins Rep68 and Rep78, encoded by the helper-dependent parvovirus adeno-associated virus type 2. Like NS1, Rep78 and Rep68 are pleiotropic replication initiator proteins which also transregulate viral promoters (22, 23, 32). These proteins recognize the sequence (GAGC)<sub>3</sub> in the stem region of the viral hairpin and more or less degenerate forms of this sequence in the viral P5 promoter and at other sites both within and outside the viral genome (7, 33, 34, 46). As suggested here for NS1, DNA binding involves homodimers if not higher-order multimers of Rep, and such complexes can bind simultaneously to more than one DNA-binding site (34).

However, unlike NS1, Rep68 and Rep78 bind their recognition sequence in the absence of ATP, as demonstrated by both footprinting and gel shift assays (34, 38). In the present study, the NS1 footprints were mapped on DNA fragments which lacked obvious secondary structure, while for Rep, three different footprint patterns which, at least in part, reflect the different secondary structures of the substrates used have been reported (7, 21, 34). On linear (nonhairpinned) substrates, and in the absence of ATP, Rep footprints are much smaller than their NS1 counterparts and do not extend over the nick site where Rep must certainly interact with the origin to initiate

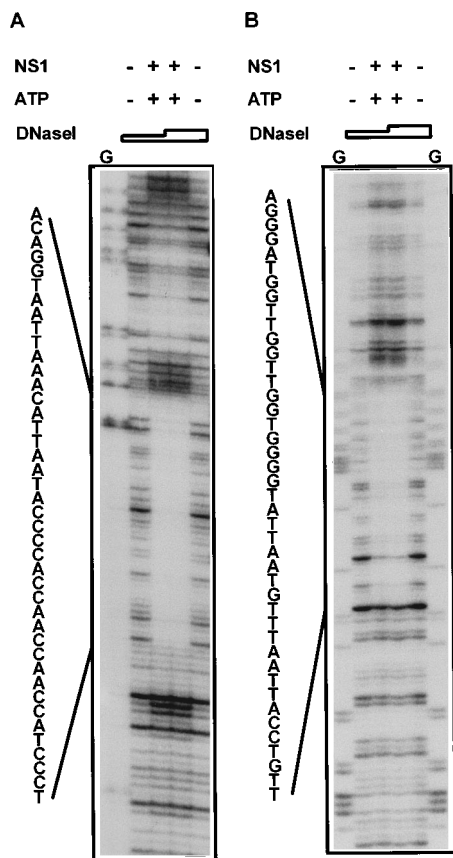


FIG. 6. NS1 footprints at other sites in the MVM genome are highly asymmetric. (A) DNase I protection analysis of the NS1 interaction with a binding site containing the core (ACCA)<sub>3</sub> motif starting at MVM nt 2333 (3' <sup>32</sup>P label on the lower strand) was performed with purified NS1 in the presence of ATP as indicated. The DNase I protection assays were performed with 0.4 and 0.8 U of DNase I. Lane G is a Maxam-Gilbert G sequence ladder. (B) DNase I protection analysis of the NS1 interaction with a binding site containing the core (ACCA)<sub>3</sub> motif starting at MVM nt 2333 was performed as described above with 3' <sup>32</sup>P label on top strand.

replication (7, 34). Thus, although one might suspect that addition of ATP may increase the size of these Rep footprints in the required direction, it is nonetheless remarkable that Rep can assemble into multimers and form stable interactions with DNA in its absence.

There are many similarities between the way NS1 recognizes and interacts with its cognate site and the way in which T antigen (TAg) binds to the simian virus 40 (SV40) replication

Tar: ATTGTGCTTGGTTGGTTAAAGAAATGGTTACCAATCTACCATGGCAAAGCTA  
TAAACACGAACCAACCATTTCTTACCAATGGTTAGATGGTACCGTTTCGAT

Intron: TTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTACCTGTTTAA  
AAATTCCTTACCAACCAACCAACCCCATTAATTACAAATTAATGGACAAAT

Left ori: ACGTGAACATGGTTGGTCAGTTCTAAATAATGATAAGCGGTTACAGGGAGTT  
TGCGCTTGTACCAACAGTCAAGATTTTACTATTTCGCCAAGTCCCTCAA

FIG. 7. Alignment of the regions in the MVM P38 promoter, MVM 3' replication origin, and a sequence at MVM nt 2328 to 2370 protected from DNase I digestion by NS1 in the presence of ATP. The regions protected from DNase I digestion in the MVM P38 promoter, MVM 3' replication origin (10), and the sequence at MVM nt 2328 to 2370 were aligned and are shown boxed. The conserved motifs within the protected regions are depicted in a shaded box. The sequence designated *tar* includes MVM nt 1860 to 1909, the intron sequence includes MVM nt 2324 to 2373, and the 3' replication origin (Left ori) includes MVM nt 30 to 1 and 1 to -21.

origin (16). At temperatures in excess of 30°C, TAg will give in vitro footprints on the SV40 origin only in the presence of ATP, and in this situation, ATP is known to be involved in the assembly of TAg into a double-hexamer structure which protects the entire 70-bp origin core. Unlike our findings with NS1, TAg footprints can be obtained in the absence of ATP at lower temperatures, but under these conditions, the largest structure found is a tetramer, and protection extends over a smaller region of 35 bp from the center of the core. TAg-origin complexes formed in the presence of ATP have a short half-life, and this rapid dissociation makes it impossible to demonstrate DNA binding in conventional gel shift assays, although such complexes can be detected if they are cross-linked with glutaraldehyde prior to electrophoresis (14). The same is true for NS1, which gives retarded NS1-*tar* complexes only if these complexes are fixed with glutaraldehyde, although binding can be detected easily without prior fixation as a supershifted complex in the presence of anti-NS1 antibodies (data not shown). Amino acid sequence alignment of NS1 and TAg has identified a surprisingly conserved region in these otherwise rather disparate proteins which extends through the predicted P-loop structure associated with ATP binding, hydrolysis, and helicase activity (2, 4). Since both proteins appear to respond to the binding of ATP by forming multimers, it seems possible that this common domain is also responsible for initiating oligomer formation.

There is one potentially important difference in the ways NS1 and papovavirus DNA-binding proteins appear to interact with their cognate sites which could be of structural significance. While ATP-dependent binding of SV40 and polyomavirus large T antigens (30) and bovine papillomavirus E1 proteins (42) is markedly enhanced by the presence of low levels of MgCl<sub>2</sub>, complex formation between NS1 and its recognition sequence appears to proceed equally well without this cation. However, this apparent dissimilarity will need to be evaluated further by using the type of nitrocellulose filter binding assays employed for SV40 and polyomavirus proteins (30), since the immunoprecipitation assays used here to demonstrate NS1-DNA interactions cannot provide the rapid kinetic analyses at different ATP and magnesium concentrations which are required to explore this point.

Our current data suggest that ATP hydrolysis leads to the dissociation of the NS1-DNA complex. SV40 TAg is a DNA-dependent ATPase (17), and it has been shown recently (8) that the highly purified NS1 polypeptide of parvovirus ADV also has a low specific ATPase activity which is stimulated 5- to 10-fold by double-stranded and single-stranded DNA. If the same is true for MVM NS1, this would suggest that oligomers formed in solution may be relatively stable, while those formed on the DNA, or having bound to the DNA, may hydrolyze ATP rather rapidly and thus disrupt the complex. This notion suggests that the interaction between NS1 and DNA is highly dynamic. Candidate NS1-binding sites are distributed at fairly regular intervals throughout the MVM genome, so that restriction fragments in excess of 100 bp generally bind to NS1 in immunoprecipitation assays (data not shown). Together with the surprisingly large size of the NS1 footprints, this repetitive distribution means that most of the genome could potentially be covered with NS1 and suggests that NS1 may play a structural role in the viral chromatin. Although this might be energetically rather expensive, such a dynamic scaffolding could be particularly suited to the viral life cycle, since not only would it not impede transcription or replication forks, but it would actively sequester a pool of NS1 close to the viral origins and promoters which are themselves dependent on activation by NS1. Preliminary studies suggest that NS1-binding sites at crit-



ical positions in the genome, such as *tar*, can effectively compete with other sites under limiting NS1 concentrations (data not shown), but presumably such critical interactions are also stabilized in initiation complexes by secondary interactions between NS1 and other DNA-binding proteins (13, 26).

The nature of the optimal NS1 DNA recognition sequence remains uncertain. NS1 binds efficiently to single copies of *tar* when *tar* is presented as part of a larger plasmid fragment, but such binding can be inhibited by the appropriate oligonucleotides only if they are first ligated into fragments of more than 21 nt. Thus, NS1 can make stable contact with its recognition site only when this site is presented in the context of flanking DNA, as might be expected given the invariably large size (43 bp) of its DNase I footprints. Alignment of the footprints obtained for *tar*, the MVM 3' replication origin, and an (ACCA)<sub>3</sub> sequence located in the small MVM intron (Fig. 7) shows that there is no obvious consensus in the flanking sequences but that protected regions are always positioned asymmetrically with respect to the recognition sequence, extending 38 or 39 bases 5' of the first nucleotide in the (ACCA)<sub>2-3</sub> motif but only 4 to 8 nt 3' of this site. At present, we do not know how these flanking sequences influence NS1 binding to the (ACCA)<sub>2-3</sub> core, although preliminary competition experiments using immunoprecipitation assays suggest that they are significant. Just as the nick site which is located in the protected region of the 3' replication origin must interact directly with NS1 to initiate DNA replication, it is tempting to speculate that the inverted ACCA pentamer element located in the *tar* footprint may also promote binding by allowing specific secondary interactions. Although we do not know the size of the minimum core sequence required for binding, an (ACCA)<sub>2</sub> motif is clearly sufficient, and there are indications that single nucleotides in this sequence are not critical. Thus, methylation interference experiments failed to identify specific NS1-guanine contacts, suggesting either that methylation does not interfere with the interaction or, more likely, that a single methylated residue still allows efficient binding (data not shown). Functional analysis of H-1 *tar* tends to support this suggestion, since single substitutions in the core sequence did not abolish transactivation but simply reduced the level achieved (19).

**Mechanism of NS1-mediated transactivation of the P38 promoter.** There are three important regulatory elements, a TATA box, an Sp1-binding site, and the *tar* element, which appear to be required for transcription from the P38 promoters of MVM and H-1 and the P36 promoter of ADV (19, 44). However, the role played by each of these elements, and in particular the route and mechanism by which NS1 transactivates such promoters, remains controversial (1, 26). Unable to detect any direct interactions between NS1 and the promoter elements, some authors suggested that NS1 must influence transcription by interacting with cellular intermediates. Moreover, while some of these investigators believed that transactivation was mediated through the *tar* element (19, 41, 44), others have suggested that the *tar* sequence plays no role in P38 regulation and that transactivation is mediated through a specific interaction with the Sp1 polypeptide (1, 26).

Despite the body of evidence implicating *tar* in this interaction (19, 41, 44), its role has been questioned because promoter constructs in which *tar* is internally deleted still show measurable transactivation by NS1 in cotransfection experiments (1). However, 5' and 3' deletion studies suggest that *tar* is simply a minimal element required for transactivation (19, 41, 44), while sequences upstream of *tar* (MVM nt 1349 to 1836) which do not influence basal transcription are responsible for about 80% of the observed transactivation level (44). These sequences are particularly well endowed with (ACCA)<sub>n</sub>

elements to which NS1 would be expected to bind, and during construction of the critical internal deletion used in a previous study (1), a potential NS1-binding site with the sequence CAACCAAACCAA (MVM nt 1839 to 1828) was positioned close to the now-deleted *tar* region. Subsequent experiments in which linker-scanning mutations were introduced into the Sp1 site, reducing transcription to undetectable levels, led the authors to suggest that NS1 might function through Sp1 (1). However, while basal transcription from the both the MVM and H-1 P38 promoters is so low that it is difficult to measure in the absence of competent Sp1 sites, it can still be detected in the most sensitive assays and is transactivated by NS1 in constructs containing *tar* (19, 28, 44). Together, these observations suggest that multiple NS1-binding sites upstream of the Sp1 site and TATA box, including *tar*, influence P38 transactivation and that the latter may simply be a particularly potent NS1-binding site which is located near the promoter at an appropriate distance from binding sites for other essential transcription factors. According to this interpretation, the only role of Sp1 is to sequester the basal transcription machinery at the promoter.

Mutations introduced into both the purine-binding fold and the carboxy-terminal peptide of NS1 inhibit the protein's ability to activate transcription (28, 29, 35). However, such studies are difficult to interpret because it is not clear whether these mutations compromise the ability of NS1 to translocate to the cell nucleus, bind to the promoter, or activate transcription. The results presented here show that mutations in the ATP-binding fold are likely to impair transactivation by preventing NS1 self-association and subsequent site-specific DNA binding. This interpretation is strengthened by the recent finding that if the carboxy-terminal domain of NS1 is fused to the DNA-binding domain of the *E. coli* LexA protein, the resulting chimera can function as a transcriptional activator for promoters containing LexA operator sites (28). This finding indicates that mutations in the NS1 carboxy terminus are likely to interfere with transcriptional activation by impairing the protein's interaction with the basal transcription machinery. Interestingly, transactivation by LexA-NS1 fusion proteins occurred only if several LexA operator sites were introduced upstream of the TATA box, tending to support our contention that oligomeric forms of NS1 must be presented to the transcription machinery through the interaction between NS1 and the *tar* sequence (28). The LexA chimera experiments also indicate that the helicase activity of NS1 is not required for transcriptional activation, thus suggesting that NS1 is not required to unwind adjacent sequences in order to enhance transcription but binds near the promoter simply in order to deliver multiple copies of its acidic carboxy terminus. Like the potent transcriptional activator VP16 encoded by herpes simplex virus, the carboxy-terminal domain of NS1 is acidic and proline rich. It also contains multiple copies of the sequence motif (S/T)PXX (where X represents lysine, arginine, serine, threonine, alanine, leucine, or proline), which was first identified by computer-aided sequence analyses as a motif that is frequently reiterated in gene-regulatory proteins (45) and was subsequently implicated in this regulation by functional analysis of the CCAAT-binding transcription factor CTF1 (25). In general, acidic transcriptional activators have more than one target in the basal transcription machinery of a promoter, and VP16 has been shown to interact with TATA-binding protein, TFIIB, TAF<sub>II</sub>40, and recently TFIIF (18). This finding suggests that the carboxy terminus of NS1 may also activate transcription by interacting directly with multiple target proteins in the basal transcription machinery.



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