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Inhibition of Parvovirus Minute Virus of Mice Replication by a Peptide Involved in the Oligomerization of Nonstructural Protein NS1

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The large nonstructural protein NS1 of the minute virus of mice and other parvoviruses is involved in essential steps of the viral life cycle, such as DNA replication and transcriptional regulation, and is a major contributor to the toxic effect on host cells. Various biochemical functions, such as ATP binding, ATPase, site-specific DNA binding and nicking, and helicase activities, have been assigned to NS1. Homo-oligomerization is a prerequisite for a number of proteins to be fully functional. In particular, helicases generally act as homo-oligomers. Indirect evidence of NS1 self-association has been recently obtained by a nuclear cotransport assay (J. P. Nüesch and P. Tattersall, Virology 196:637–651, 1993). In order to demonstrate the oligomerizing property of NS1 in a direct way and localize the protein region(s) involved, the yeast two-hybrid system was used in combination with deletion mutagenesis across the whole NS1 molecule, followed by high-resolution mapping of the homo-oligomerization domain by a peptide enzyme-linked immunosorbent assay method. This study led to the identification of a distinct NS1 peptide that contains a bipartite domain involved in NS1 oligomerization. Furthermore, this isolated peptide was found to act as a specific competitive inhibitor and suppress NS1 helicase activity in vitro and parvovirus DNA replication in vivo, arguing for the involvement of NS1 oligomerization in these processes. Our results point to drug targeting of oligomerization motifs of viral regulatory proteins as a potentially useful antiviral strategy.

Parvoviridae is a family of small single-stranded DNA viruses with genome sizes in the range of 5 kb. Vertebrate autonomous parvoviruses, including the prototype minute virus of mice (MVMp), are characterized by similar genomic organizations. Because of the smallness of their genomes, parvoviruses rely on various genetic strategies to optimize coding potential. These include the use of two promoters, overlapping open reading frames, alternative mRNA splicing, polyadenylation signals, posttranslational modifications, and multifunctional proteins (7, 18, 32, 53). The genome can basically be divided into two parts, with the left-hand portion encoding two types of nonstructural proteins, NS1 and NS2, and the right-hand portion coding for virus structural proteins (7, 16, 22, 32). Nonstructural proteins NS1 and NS2 are phosphorylated and show localization that is mainly nuclear (NS1) or both cytoplasmatic and nuclear (NS2) (17, 20). NS2 seems to be required in a cell type-dependent manner for efficient viral DNA and protein synthesis and for virus production (43, 44). NS1 plays essential roles in viral DNA replication (7, 18, 19), viral gene expression (18, 25, 29), and cytotoxicity (12, 42).

Biochemical studies have shown that the NS1 proteins of autonomous parvoviruses and the analogous Rep78 and Rep68 products of adeno-associated viruses (a group of defective members of *Parvoviridae*) possess intrinsic ATP-dependent helicase, ATPase, and site-specific endonuclease activities (7, 15, 18, 31). These functions are involved in viral DNA synthesis, particularly in the resolution of telomeres and concatemeric intermediates leading to the covalent attachment of NS1 to the 5′ ends of DNA replication products (21). On the other hand, the NS1 protein contains a transcription-activating domain and controls viral gene expression, modulating the activity of its own promoter (P4) to a small extent and strongly inducing the P38 promoter that drives the structural transcription unit (18, 25, 29). NS1 binds to DNA in a sequence-specific manner through a motif $(ACCA)_{2-3}$ that is highly represented in the parvoviral genome (15) but not restricted to it (61). Multiple *cis*-acting sequences, including a transactivation-responsive upstream motif, GC and TATA boxes, and a downstream element, were reported to be important for the activity and NS1 induction of promoter P38 (29).

NS1 is a DNA-binding protein with replicational and transcriptional functions. It is noteworthy that a number of proteins exhibiting either one of these activities proved to act as homo- and/or hetero-oligomers. This is the case particularly for most helicases (64), transcription factors (5, 35, 39, 62), endonucleases (3, 49), and multifunctional proteins, such as large T antigen (LTAg) of simian virus 40 (SV40) (40). In the latter case, tetrameric or smaller-order LTAg homo-oligomers bind to a 35-bp sequence within the SV40 DNA origin of replication in the absence of ATP, whereas DNA binding is enhanced 10-fold and a 70-bp sequence containing the entire origin is protected by two hexamers of LTAg when ATP is added (38, 40). It is tantalizing to speculate that NS1 also binds to DNA through an interaction of this type, since this protein induces the formation of a large footprint that extends over 43 nucleotides on both strands around the origin of replication but only in the presence of ATP or antibody that is assumed to act as a cross-linker (15). The same conditions are required for NS1 to bind to the transactivation-responsive region of promoter P38, which also results in a large and asymmetric footprint (14). Indirect evidence of NS1 homo-oligomerization has

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been obtained by a cotransport assay (47). Mutant forms of NS1 protein from MVMp lacking the nuclear localization signal (NLS^-) were found to be transported into the nucleus when they were coexpressed with wild-type (wt) NS1, presumably through association of the former with the latter NS1 polypeptide. NLS^- mutant forms of NS1 lacking amino acids (aa) 95 to 254 or the 67 C-terminal aa were still competent for nuclear translocation in the presence of wt NS1, suggesting that the homo-oligomerization domain is narrowed down to a distinct region(s) of the protein. However, direct evidence of NS1-NS1 interaction and a role for homo-oligomerization in NS1 functioning was lacking. This prompted us to investigate these questions by using the NS1 proteins of the closely related MVMp and H1 viruses as models.

We first determined whether NS1 homo-oligomers were physically detectable and whether a defined continuous amino acid stretch(es) could be identified as being necessary to their formation. Should this be the case, the role of oligomerization could then be tested by overloading cells or in vitro reaction mixtures with the corresponding peptide to prevent full-length NS1 molecules from interacting with each other and by assessing the consequences for NS1 functioning. Initial demonstration and mapping of an NS1 linear domain involved in homo-oligomerization were attempted by using the yeast two-hybrid system (6). This approach is based on the modular nature of the yeast GAL4 protein, which comprises separable domains responsible for DNA binding and transcriptional activation (33). Plasmids encoding two hybrid proteins, with one consisting of the GAL4 DNA-binding domain (BD) fused to intact NS1 and the other consisting of the GAL4 activation domain (AD) fused to a mutant form of NS1, were constructed and introduced together into yeast. This allowed NS1 mutant forms retaining a functional oligomerization motif to be identified through interaction with intact NS1. Indeed, this interaction led to reconstitution of a functional GAL4 activator capable of inducing the expression of a reporter gene that was placed under the control of a regulatory region containing multiple recognition sites for the GAL4 BD. This system is highly sensitive and offers an in vivo alternative to in vitro approaches for studying protein-protein interactions. In a second step, mapping of the NS1 homo-oligomerization domain was refined by using a synthetic peptide library that covered an NS1 region found to be essential for oligomerization in the two-hybrid system and by testing their interaction with NS1 by a recently described enzyme-linked immunosorbent assay (ELISA) (63).

The results presented here indicate that a 20-aa peptide of NS1 is sufficient to bind full-length NS1. This peptide comprises a bipartite oligomerization motif that is able to inhibit NS1 oligomerization and helicase activities in vitro. When it was microinjected into host cells, the interaction peptide suppressed parvovirus DNA replication, arguing for a role of oligomerization in essential NS1 function(s). Furthermore, these data show that antiviral agents can be designed in the form of short synthetic peptides that target the oligomerization domains of viral regulatory proteins.

MATERIALS AND METHODS

Construction of NS1 vectors for expression in yeast. The GAL4-NS chimeric plasmids used (Fig. 1A; Table 1) were constructed by standard procedures (57). The mutations introduced (see below) were confirmed by double-strand sequencing by the dideoxy method. With the exception of one case that was noted and discarded, no spontaneous alterations were observed. All plasmids were amplified in the bacterial strain *Escherichia coli* sure (Stratagene). NS1 coding sequences were derived from the genome of H1 or MVMp virus.

(i) H1 virus NS1-expressing plasmids. The full-length NS1 coding sequence of parvovirus H1 DNA was produced by PCR from the infectious molecular clone pSR19 (27) in the form of a 5′ *Bam*HI-*Sal*I 3′ fragment. This fragment was inserted into the pGAD424 and pGBT9 vectors (Clontech) downstream from and in-frame with the GAL4 coding sequence (23). Different strategies were used to generate plasmid derivatives harboring specific deletions.

A series of terminal and internal deletions were obtained by endonuclease digestion and religation with convenient restriction sites and insertion of double-stranded adapters to keep the NS1 reading frame. Deletion of the sequence encoding the 69 C-terminal amino acids of NS1 was achieved by restriction digestion of pGAD-NS1 and pGBT9-NS1 with *Xba*I and *Sal*I endonucleases and insertion of adapter 5′ CTAGACTAACCCGGG 3′, generating pGAD-dlC69 and pGBT9-dlC69, respectively. pGAD-dl543:605 was produced by digestion with *Bst*EII and *Xba*I and insertion of adapter 5′ GTTACCAATCTGCT 3′. pGAD-dl504:543 was produced by digestion with *Bsm*I and *Bst*EII and insertion of adapter 5′ CAACAAAGGAG 3′. pGAD-dl360:509 was digested with *Nhe*I and *Bsm*I and religated with adapter 5′ CTAGCACCAGAATGCT 3′.

Another set of NS1 deletion mutants was produced by PCR technology. Oligonucleotides AP1 (5′ CACTGTCATGTGCTG 3′) and AP4 (5′ TGGCATGGCAGACTT 3′), which anneal to the NS1 template at codons 128 and 381 in the forward and reverse senses, respectively, served as external primers for PCR mutagenesis. Oligonucleotides causing a defined sequence of the template to loop outward was used as overlapping internal primers. To generate mutant pGAD-dl307:353 (deletion of NS1 codons 307 to 353), oligonucleotide pairs AP1 and AP6 (5′ GTTTGCTCATCATCATCCAGTC 3′) and AP4 and AP5 (5′ GACTGGATGATGATGAGCAAACTAGCCAAC 3′) were used to amplify deleted derivatives of sequences encompassing codons 128 to 356 and 303 to 381, respectively. Both PCR products were purified, denatured, annealed, and amplified again with outside primers AP1 and AP4. The final PCR product was substituted for the equivalent wild-type DNA fragment. Similarly, plasmid pGAD-dl349:358 was generated with primer pairs AP1 and AP8 (5′ TCTGGTGCTAGCCATAGCTTTTTCCAGAAT 3′) and AP4 and AP7 (5′ ATTCTGGAAAAAGCTATGGCTAGCACCAGA 3′). Other small deletion mutants were created by using chimeric PCR primers that contained an *Eco*RI site (underlined in sequences below) as a substitute for the extruded sequence from the viral DNA template. The following pairs of primers were used: for pGAD-dl226:275, AP1 and AP2 (5′ TCTAGTTTGAATTCTGTCCCTTGGTG GACT 3′); for pGAD-dl278:350, AP4 and AP3 (5′ CGCGGCAGAATTCCA AAGCAAACTAGCCAAC 3′); and for pGAD-dl278:305, AP4 and AP9 (5′ CGCGGCAGAATTCAAATGATGATGCAGCCA 3′).

(ii) MVMp NS1-expressing plasmids. The NS1 coding region from MVMp DNA was isolated as a *Nco*I-*Sal*I fragment from vector pTM1-NS1 (47) and introduced into pGAD424 and pGBT9 with the help of an adapter encompassing the *Nco*I site (5′ AATTCCCCATGGA 3′), creating pGAD-NS1(MVMp) and pGBT9-NS1(MVMp), respectively. The deletion giving rise to the previously described *dl*158 mutant form of NS1 was isolated as an *Eco*RV-*Xho*I fragment from vector pTHisNS1 dl158 (46) and inserted into *Eco*RV-*Sal*I-digested pGAD-NS1(MVMp) to generate double mutant pGAD-dl158:C67. The mutation responsible for the previously described NS1 K405M substitution (45) was transferred through an *Eco*RI-*Bst*EII fragment into pGAD-NS1(MVMp) and pGAD-NS1(H1) to give rise to pGAD-MVMp 405M and chimera pGAD-H1/MVMp 405M, respectively. The same fragment encoding the original K at position 405 was used to produce chimera pGAD-H1/MVMp.

(iii) MVMp NS2-expressing plasmids. The NS2 coding region was isolated as a *Nco*I-*Bgl*II fragment from vector pQE-NS2p (48) and introduced into pGBT9 and pGAD424 with the help of an adapter that encompassed the *Nco*I site (8).

Yeast two-hybrid analysis. Yeast genetic manipulations were performed as previously described (6) with *Saccharomyces cerevisiae* HF7c and SFY526 and shuttle vectors pGBT9 and pGAD424 (Clontech). Plasmid pGBT9 harbors the coding sequence for the first 149 aa of the GAL4 protein, comprising the BD, as well as the yeast selectable gene *TRP1*. Vector pGAD424 contains the sequence encoding the GAL4 AD (aa 768 to 881) and the yeast selectable gene *LEU2*. Both plasmids harbor the 2μm origin of replication. The expression of GAL4 fusion proteins is driven by the constitutively active yeast *ADH1* promoter.

Recombinant vectors were introduced into SFY526 and HF7c cells as previously described (26). Yeast cultures were maintained at 30°C in SD minimal medium (0.67% [wt/vol] Bacto yeast nitrogen base, 2% [wt/vol] dextrose) containing the appropriate amino acid supplements.

β-Galactosidase assays. Protein-protein interactions in the two-hybrid system can be observed on plates in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or quantitated in liquid cultures by measuring the cell-associated β-galactosidase activity. Plate assays were performed by the filter lift method (10). Quantitative liquid assays were carried out as follows. Aliquots (0.5 or 1 ml) from exponentially growing cultures (optical density at 600 nm [OD_{600}] of ~1) were spun and resuspended in 0.5 ml of Z buffer (100 mM phosphate buffer [pH 7.0], 10 mM KCl, 1 mM Mg_2SO_4, 50 mM β-mercaptoethanol). After chloroform (50 μl) and 0.1% sodium dodecyl sulfate (SDS; 50 μl) were added, samples were vortexed for 30 s, supplemented with *o*-nitrophenyl-β-D-galactopyranoside (160 μl of a 4 mg/ml solution in sodium phosphate buffer), and incubated at 30°C for various times. Reactions were stopped with 250 μl of 1 M sodium carbonate. OD_{420}s were read. Background values (obtained with non-transformed cells) were subtracted, and the results were expressed in OD_{420} units/min of reaction for 1 ml of culture at an OD_{600} of 1. Assays were done in duplicate for at least 10 independent transformants.

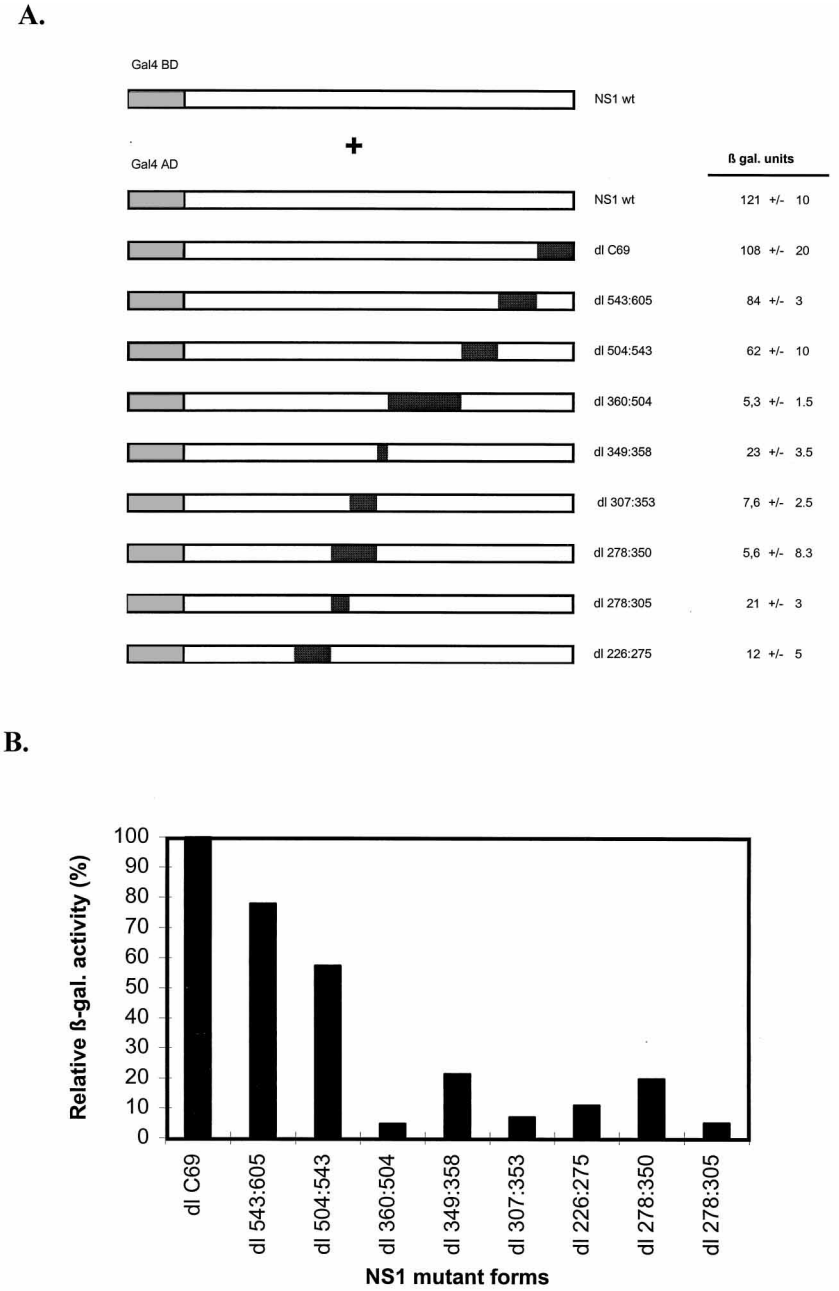

FIG. 1. Mapping of an NS1 homo-oligomerization domain by using the two-hybrid system. (A) H1 virus wt NS1 protein and deleted derivatives were produced as fusion polypeptides with the GAL4 AD and tested in cotransformation experiments for the ability to interact with wt NS1 fused to the GAL4 BD. In-frame deletions in GAL4 AD-NS proteins are shown by black boxes and lie between the amino acids indicated in the respective designations. β-Galactosidase (β gal.) activities were determined in duplicate for more than 10 individual colonies and are averages from four independent experiments (standard deviations were less than 20%). The background value obtained with untransformed cells was less than 0.1 β-galactosidase units. The inducing activity of the GAL4 BD-wt NS1 fusion protein alone (~6 β-galactosidase units) was subtracted. (B) β-galactosidase (β-gal.) activities exhibited by cells cotransformed with the indicated NS1 deletion mutant proteins are expressed as percentages of the NS1 *dl*C69 value. Each bar represents one deletion mutant form of NS1; the order is determined by the central positions of the respective deletions along the NS1 amino acid sequence. Data are averages and standard deviations from at least four independent assays.

Peptides and ELISA. Peptides linked at their N termini to a biotin residue through a 4-aa SGSG spacer were synthesized (Chiron Mimotopes, Clayton, Australia) to cover the wt NS1 protein with 16- or 5-aa overlaps. An additional set of peptides, in which each residue from aa 261 to 280 was substituted in turn by alanine, was generated. Lyophilized peptides were dissolved in dimethyl sulfoxide at 13 μg/μl and stored at −20°C.

Whole-protein extracts were prepared from A9 cells at 20 h after mock treatment or MVMp inoculation (multiplicity of infection, 10 PFU/cell). Briefly, cells were collected in ice-cold phosphate-buffered saline (PBS), washed twice, and resuspended in 1 volume of lysis buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 20 min, the cell suspension was frozen at −70°C, thawed on ice, and vigorously vortexed. After centrifugation (18,000 × *g*, 10 min, 4°C), the supernatant was frozen in liquid nitrogen. The protein concentrations of extracts were determined with a ready-to-use solution provided by Bio-Rad. His-tagged NS1 protein was produced from recombinant baculovirus and purified as previously described (4). Streptavidin-coated plates (Chiron Mimotopes) were blocked by incubation for 1 h at room temperature with PBS containing 1% sodium caseinate and 0.5% Tween 20 (100 μl/well). After four washes with PBS containing 0.1% Tween 20, peptides (650 ng in 100

TABLE 1. NS1 oligomerization and transcription activation in yeast[a]

GAL4 AD fusion	β-Galactosidase activity[b]				
	BD	H1 NS		MVMp NS	
		BD-wt NS1	BD-NS1 *dl*C69	BD-wt NS2	BD-wt NS1
AD	<0.1	6	<0.1	<0.1	60
H1 NS					
AD-wt NS1	<0.1	127	7	ND[c]	ND
AD-NS1 *dl*C69	<0.1	114	0.9	ND	ND
MVMp NS					
AD-wt NS1	<0.1	ND	ND	<0.1	230
AD-*dl*158:C67	<0.1	ND	ND	ND	190
AD-NS1 Met405	<0.1	ND	ND	ND	120
AD-wt NS2	<0.1	ND	ND	<0.1	60
H1-MVM chimeric NS					
AD-wt NS1	<0.1	200	ND	ND	ND
AD-NS1 Met405	<0.1	205	ND	ND	ND

[a] NS1 was used both as bait and prey by fusing the protein with the separate BD and AD of GAL4. A *lacZ* gene placed under the control of GAL4 recognition motifs served as the reporter to reveal the capacity of NS1 for activating transcription by itself (cell transformation with BD-NS1 alone) or for homo-oligomerization and reconstitution of a functional GAL4 activator (cell cotransformation with BD-NS1 and AD-NS1). The NS1 proteins tested were the wt and mutant derivatives that harbored an amino acid substitution in the ATP-binding site (Met405), a C-terminal deletion encompassing the AD (NS1 *dl*C69 and *dl*158:C67), or an internal deletion affecting the BD (*dl*158:C67). NS1 proteins of MVMp and H1 virus origins, as well as chimeric polypeptides consisting of the H1 virus wt protein in which the sequence from aa 276 to 543 (comprising the ATP-binding site) was replaced by the equivalent portion from MVMp wt NS1 or NS1 Met405, were analyzed. The NS2 protein of MVMp, sharing 85 N-terminal aa with NS1, was tested in a similar way for self-association and cross-interaction with NS1.

[b] *lacZ* gene expression was measured and quantified in international β-galactosidase units (41). Data are averages from at least four independent experiments (standard errors were less than 20%). In each experiment, β-galactosidase assays were performed in duplicate for at least 10 independent transformants.

[c] ND, not determined.

μl of PBS supplemented with 0.1% sodium caseinate) were added individually to appropriate wells and allowed to bind for 1 h at room temperature. All subsequent incubations were carried out in a total volume of 100 μl for 1 h at room temperature, with extensive washing of plates with PBS containing 0.5% Tween 20 between each step. Peptide-coated wells were treated with 10 μg of total protein extract or 300 ng of purified His-tagged NS1 protein. SP8 anti-NS1 antiserum (11) was added at a 1/1,000 dilution in PBS containing 0.1% sodium caseinate and 1% sheep serum, followed by incubation with horseradish peroxidase-conjugated sheep anti-rabbit antibodies (Dianova) at a 1/2,000 dilution. Bound antibodies were detected by using the chromogenic substrate 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonate) as a 0.5 mg/ml solution in 0.1 M Na_2HPO_4–0.08 M citric acid–0.01% (wt/vol) hydrogen peroxide. Plates were read at 405 and 492 nm.

Mammalian cell lines and viruses. A9 cells were cultured in minimal essential medium (MEM) supplemented with 5% fetal calf serum. In order to obtain cell extracts containing NS1 protein, A9 cells on 15-cm-diameter dishes were incubated for 1 h at 37°C in 5 ml of MEM supplemented with cesium chloride-purified MVMp (multiplicity of infection, 5 PFU/cell). After removal of the inoculum, cultures were washed and further incubated with fresh complete MEM for 24 h.

Helicase assay. Helicase assays were performed as previously described (46). The M13-VAR substrate was constructed by reverse primer annealing to single-stranded M13 DNA and extension for 5 min at room temperature in the presence of all four deoxynucleoside triphosphate (dNTP) precursors (including [α-^{32}P]dATP) and Sequenase. ^{32}P-labeled fragments of various lengths were obtained by the addition of ddGTP and further incubation for 20 min at room temperature. Purified NS1 (10 ng) produced from recombinant vaccinia virus in HeLa cells (45, 46) was incubated with various amounts of NS1-derived peptides for 5 min at room temperature before M13-VAR substrate (20 ng) was added. The helicase reaction was carried out in 20 mM HEPES-KOH (pH 7.5)–5 mM $MgCl_2$–5 mM KCl–0.1 mM DTT–2 mM ATP for 30 min at 32°C. The reaction was stopped by the addition of SDS and EDTA, and the products were analyzed by 7% nondenaturing polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS.

Protein immunoprecipitation. The complete MVMp NS1 coding sequence was isolated from vector pRSV-NS1 (60), filled in with Klenow DNA polymerase, and introduced in the blunted *Eco*RI site of vector pX (50), giving rise to pX-NS1. To create pX-dlC67 (encoding a truncated NS1 protein lacking the 67 C-terminal aa), *Xho*I digestion and religation were performed.

Full-length NS1 and *dl*C67 NS1 were independently produced from plasmids pX-NS1 and pX-dlC67 with a TNT transcription-translation kit from Promega according to the instructions of the manufacturer in the presence of [^{35}S]methionine (translabel [1,000 Ci/mmol]; ICN Pharmaceuticals). Reaction products (10^6 cpm) were diluted either separately or together in 400 μl of immunoprecipitation buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 U of aprotinin per ml, and 1 mM NaF), and different amounts of inhibitor peptides were added. Immunoprecipitation took place at 4°C overnight in the presence of a 50% suspension of protein A-G Sepharose beads (Pharmacia). After four washes with immunoprecipitation buffer, immunocomplexes were separated by electrophoresis on an 8% polyacrylamide gel containing 1% SDS and visualized by autoradiography.

Antiserum. The SP8 polyclonal rabbit antiserum is directed against a carboxy-terminal peptide of NS1 and is equivalent to the previously described SP7 serum (11).

Microinjection. A9 cells were plated on 12-mm-diameter coverslips (CELLocate; Eppendorf) on individual 35-mm-diameter dishes to give half-confluent monolayers after 24 h of incubation. About 500 cells within a marked area in the center of each coverslip were microinjected in less than 30 min with 0.1 pl of an aqueous solution of an infectious MVMp molecular clone (a kind gift of P. Tattersall) (10 μg/ml) supplemented (or not) with a synthetic NS1 peptide (6.5 to 65 ng/ml). The replication-defective MVM ΔNS DNA clone (24) was used as a negative control. Microinjection was performed with a Zeiss-AIS computer-controlled system. Coverslips were further incubated in normal growth medium (MEM enriched with 5% fetal calf serum) at 37°C. As an internal control for monitoring cells after microinjection, fluorescein isothiocyanate-dextran (final concentration, 0.5%) was used as a coinjection marker. At 24 h after microinjection, fluorescent cells were counted and the coverslip was discarded if survivor cells did not reach 75% of the total number of microinjected cells.

DNA hybridization. At 48 h after microinjection, cells were washed twice with PBS. A 25-mm-diameter nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) was gently applied to the cell layer and pressed two times (2 min each) with Whatman 3MM paper presaturated with 0.5 M NaOH–1.5 M NaCl. After neutralization with 3MM paper saturated with 1.5 M NaCl–0.5 M Tris-HCl (pH 7.4), the membrane was backed for 1 h at 80°C in order to immobilize transferred DNA. Prehybridization and hybridization were performed in 50 mM HEPES-NaOH (pH 7.5)–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5× Denhardt's reagent–1% SDS–100 μg of salmon sperm DNA per ml–50% formamide at 42°C. A radioactive probe spanning the whole genome of MVMp was obtained in the form of a *Bam*HI DNA fragment that was excised from plasmid pMM984, purified by agarose gel electrophoresis, and labeled with [^{32}P]dCTP by using a Megaprime kit (Amersham). After overnight hybridization, the membrane was washed first in 2× SSC–2% SDS at room temperature for 20 min and then in 0.2× SSC–0.2% SDS at 65°C for 20 min.

RESULTS

NS1 homo-oligomerization in yeast. Yeast strains SFY526 and HF7c contain the *E. coli lacZ* gene under the control of GAL4 DNA-binding sites in distinct promoters and were used to reveal the binding of a transcriptional activator to the DNA recognition motif for GAL4. Similar results were obtained with both strains; the results for SFY526 cells are shown in Table 1. Data are β-galactosidase activities above the background value given by cells cotransformed with plasmids that expressed separately the GAL4 BD and AD alone (preventing the former from directing the latter to the target promoter).

Cells coexpressing AD and a BD fusion with the wt NS1 protein of either H1 or MVM virus (BD-wt NS1) showed a significant increase in β-galactosidase activity compared with that of the AD-plus-BD control (Table 1). This induction is likely to result from both BD-mediated targeting of the *GAL1* promoter driving the *lacZ* gene and the intrinsic capacity of NS1 for activating transcription, as demonstrated previously in mammalian cells (29, 37, 54, 55). In agreement with the assumption that NS1 retains this capacity to some extent in yeast,

no stimulation of β-galactosidase production was detected with the BD-NS1 *dl*C69 fusion comprising a C-terminally deleted mutant form of NS1 previously shown to lack the AD (36).

A dramatic increase in β-galactosidase activity was observed when BD-wt NS1 was introduced with AD-wt NS1 instead of AD alone (Table 1). This result argued for the occurrence of NS1 homo-oligomerization in this system, which bridged the GAL4 AD and BD and allowed the former to induce the target promoter. The NS1 proteins of both MVM and H1 viruses proved able to oligomerize not only with themselves but also with each other, in keeping with the high (95%) amino acid homology of the NS1 products of these closely related viruses (18). The NS1 capacity for oligomerization exhibited some specificity since no significant interaction was detected between BD-NS1 and AD fusions with other proteins, such as lamin A, p53, and SV40 LTAg (data not shown). Furthermore, the two-hybrid system failed to reveal the formation of oligomers between NS1 and the other parvovirus nonstructural products, NS2 (Table 1). Similarly, NS1-NS2 complexes were not detected in parvovirus-infected mammalian cells by coimmunoprecipitation (11). NS1 and NS2 proteins share 85 N-terminal aa that are accessible to antibodies (SP12 antiserum) (11) in both polypeptides. Altogether, these data suggest that the NS1 oligomerization domain lies downstream from this region within the NS1-specific amino acid sequence. This is consistent with the apparent inability of NS2 to form homo-oligomers in yeast (Table 1). It should also be stated that the C-terminally deleted mutant NS1 *dl*C69, which lacks the transcription-activating region, retained the capacity for homo-oligomerization (Table 1), pointing to the involvement of distinct NS1 domains in these respective functions. This conclusion was further tested with the reciprocal set of effector plasmids by fusing the NS1 *dl*C69 mutant form to the BD and determining its ability to oligomerize with full-length NS1 fused to the AD. A positive signal was also obtained with this combination of constructs, confirming that the NS1 *dl*C69 mutant form kept the capacity for physically interacting with NS1 (1). Therefore, these results argued against the requirement of either the N- or C-terminal portion of NS1 for the homo-oligomerization of this protein. This tentative conclusion was sustained by more refined mapping (see below).

Disruption in the NLS prevents NS1 from migrating into the nucleus, unless the mutant protein is coexpressed with wt NS1 (47). This rescue was ascribed to the cotransport of NLS^- NS1 mutants with wt NS1, providing indirect evidence of NS1 homo-oligomerization. The capacity of wt NS1 for the cotransport of NLS^- derivatives was found to be abolished by an amino acid substitution at position 405 (K405M or K405R) within the NTP-binding site of the protein (45). It was therefore of interest to determine the effect of this substitution at position 405 on NS1 homo-oligomerization in yeast. In contrast with its interference with cotransport in mammalian cells, the K405M substitution failed to prevent NS1 from interacting with itself in the two-hybrid system (Table 1). This was true for both the NS1 protein of MVMp and a chimeric NS1 polypeptide in which aa residues 276 to 543 from MVMp wt NS1 or K405M were substituted for the equivalent sequence of H1 virus NS1. The discrepancy in the requirement of oligomerization for NTP binding between the two-hybrid and cotransport assays may be due to the fact that dimer formation is enough for positive scoring in the former but multimers are conceivably involved in the latter. Indeed, the SV40 LTAg, which shares structural (A-type purine nucleotide-binding motif and helicase domain) and functional (replicative and transcriptional activities) features with NS1 (2, 31), was reported to depend on ATP fixation for the formation of DNA-binding hexamers but not for dimerization or tetramerization (38, 40). Furthermore, similar amino acid substitutions in Rep78 and Rep68 proteins, the equivalent of NS1 in the adeno-associated virus group of *Parvoviridae*, were reported to exert a dominant negative effect on wt Rep78 and Rep68 helicase and DNA replication activities (13, 34), arguing for the occurrence at least to some extent of oligomerization in the absence of ATP binding.

Mapping of an NS1 region necessary for self-association. NS1 proteins from MVMp deleted at either an internal (aa 95 to 254; NS1 *dl*158) or C-terminal (from aa 605; mutant C67) position were still able to interact with wt NS1 in the cotransport assay (47). This was confirmed in the present work by constructing the double mutant NS1 *dl*158:C67 and showing its competence to associate with wt NS1 and to give a positive signal in the two-hybrid system (Table 1). Knowing that NS1 proteins are unlikely to bind to each other through their N termini (see above), this result suggests that the region from aa 255 to 605 of NS1 comprises an oligomerization domain(s).

This possibility was tested by introducing deletions scanning this whole region (Fig. 1A) and by measuring the residual abilities of corresponding GAL4AD-NS1 deletion fusions to interact with GAL4BD-wt NS1. As shown in Fig. 1, all deletions within the region from aa 225 to 504 strongly reduced the formation of oligomers in yeast, allowing the NS1 region necessary for self-association to be narrowed down to this internal amino acid sequence.

No further conclusions could be drawn from these data with regard to the position of the NS1-NS1 interaction motif(s). Indeed, NS1 mutant proteins scoring negative in the two-hybrid system may be altered in other regions besides the dimerization motif itself, e.g., at sites that affect the accessibility of this motif or the overall stability of the polypeptide. Therefore, an alternative peptide mapping method was used in an attempt at circumventing these intricacies and identifying the critical NS1 element(s) that is sufficient to bind wt NS1.

Peptide mapping of an NS1 motif sufficient for interaction with wt NS1. In order to refine the localization of the NS1 oligomerization motif(s), synthetic peptides scanning the region of interest (aa 221 to 529) were tested for the ability to trap NS1 proteins extracted from infected cells. A set of peptides was used to this end; each comprised 20 aa, with a 16-aa overlap between adjacent peptides (Fig. 2A). Bound NS1 was revealed by an ELISA with SP8 anti-NS1 antibodies (11) directed against the C terminus that is not involved in oligomerization (see above). Of 79 peptides, only one (peptide 11) was able to bind NS1 when it was incubated with MVMp-infected cell extracts (Fig. 2B). A similar result was obtained with purified, baculovirus-produced NS1 protein (data not shown), indicating that the interaction was direct rather than mediated by a cellular bridging protein. The sequence of NS1-binding peptide 11 is shown in Fig. 2A. The fact that the flanking peptides (peptides 10 and 12) failed to interact with NS1 argued for the requirement of amino acid residues both upstream and downstream from the peptide 10 and 12 overlapping sequence, i.e., outside the frame (Fig. 2A), for binding. It should be stated that a similar screening was done with 20-aa-long peptides (with 5-aa overlaps) covering the rest of the NS1 protein on either side of the region from aa 221 to 529. None of these peptides was able to capture NS1 (data not shown), in agreement with the aforementioned dispensability of NS1 N- and C-terminal portions for dimerization in the two-hybrid system.

With the object of identifying the residues of peptide 11 that are critical for interaction with NS1, each amino acid was replaced in turn by alanine (with the exception of A268, for

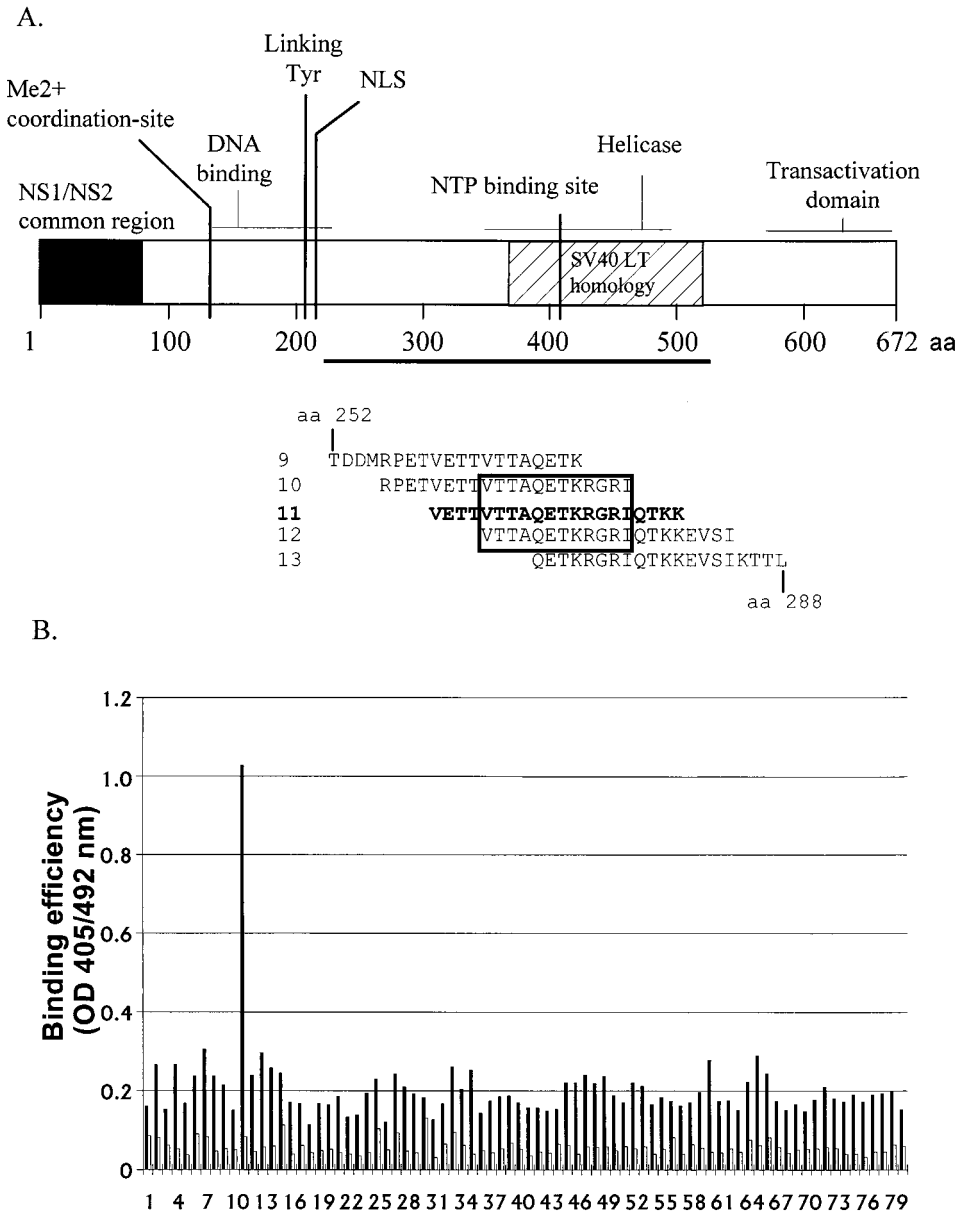

FIG. 2. Direct interaction of the full-length NS1 protein with NS1-derived peptides. (A) Schematic representation of the NS1 protein, showing the common NS1/NS2 N terminus (filled), the linking tyrosine for covalent DNA attachment, NLS, Me^{2+} coordination site, the homology region with SV40 LTAg (LT) containing the NTP-binding site (hatched), and the domains involved in DNA binding, helicase function, and transactivation (36, 46, 65). Peptides (20-mers) derived from the MVMp NS1 sequence were synthesized to cover the region from aa 221 to 529 (solid bar) in an overlapping fashion (16-aa overlap) and were numbered from 1 to 79. The amino acid sequences of peptides 9 to 13 are given, with a frame showing the overlap among peptides 10, 11, and 12. (B) Plates coated with the indicated peptides were incubated with MVMp (filled) or mock-infected (open) A9 cell extracts. NS1 binding was revealed by ELISA with an NS1-specific antiserum (SP8) and was expressed as the OD_{405} and OD_{492}.

which T was substituted). As illustrated in Fig. 3A, the NS1 binding profile of this set of substituted derivatives of peptide 11 showed two distinct regions in which amino acid changes caused strong reductions in the capacity for trapping NS1, compared with that of the original peptide. As indicated in the amino acid sequence (Fig. 3A), these results defined a bipartite motif ($VETTVTX_9IQT$) that appeared to be sufficient for interaction with NS1. This structure accounts for the failure of both flanking peptides, peptides 10 and 12, to bind NS1, presumably due to the lack of one part of the motif. It is worth noting that the bipartite motif is strictly conserved within the parvovirus genus (Fig. 3B).

Inhibition of NS1 oligomerization by peptide 11. As mentioned above, oligomerization of NS1 from H1 virus with a C-terminally truncated version of the protein (*dl*C69) was readily detectable in the two-hybrid assay. A similar mutant form of NS1 from MVMp also scored positive for association with the wt protein in the cotransport assay (47) and retained a significant capacity for viral DNA amplification and cytotoxicity (37). With the aim of ascertaining whether the NS1-NS1 interaction from MVMp could be competed by peptide 11, we used a transcription-translation system (TNT; Promega) to coexpress ^{35}S-labeled wt NS1 and *dl*C67 molecules from a mixture of vectors pX-NS1 and pX-dlC67 (Fig. 4, lanes 11 to

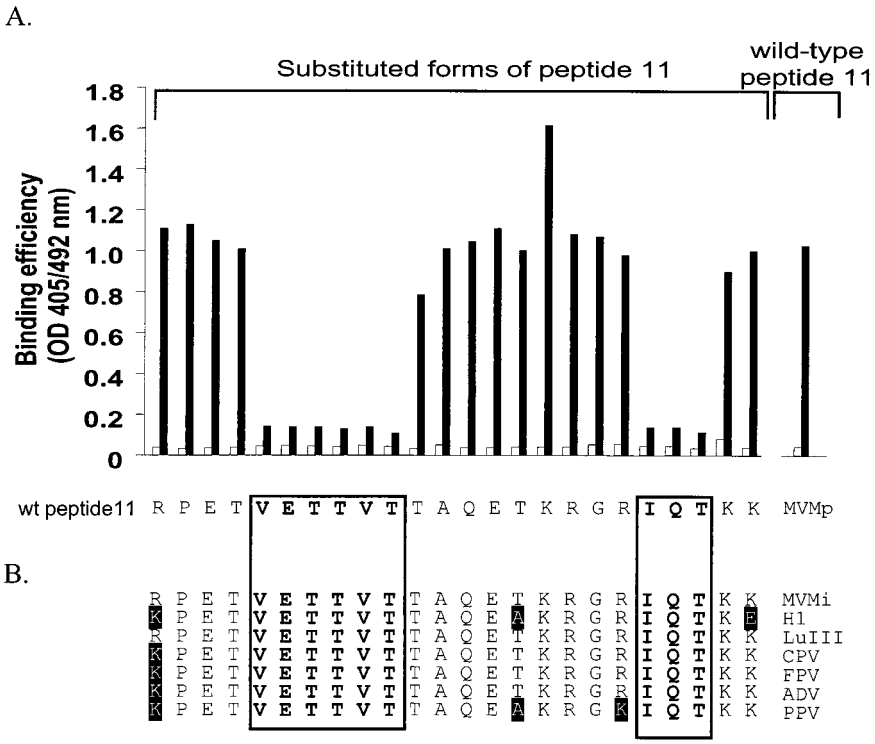

FIG. 3. Substitution analysis of peptide 11 residues required for NS1 binding. (A) Substituted derivatives of peptide 11 from MVMp NS1 were produced with each carrying an amino acid change at one position (A for all residues, except for A268, which was replaced by a T). Substituted derivatives and wt peptide 11 were compared for the ability to bind recombinant baculovirus-produced NS1 of MVMp (filled) or an equivalent preparation from wt baculovirus-infected cell extract (open) by ELISA as described in the legend to Fig. 2. Each pair of columns in the histogram corresponds to a substituted form of peptide 11 and is positioned along the wt sequence (*x* axis) according to the location of the amino acid change. Data obtained with wt peptide 11 are shown on the right for comparison. The amino acids whose replacement impaired NS1 binding are indicated in bold type in the peptide 11 sequence. (B) Equivalent sequences from NS1 proteins of various autonomous parvoviruses are aligned. Amino acid differences with respect to the MVMp sequence are shown by black boxes and do not concern the framed bipartite binding motif. CPV, canine parvovirus; FPV, feline parvovirus; PPV, porcine parvovirus; ADV, Aleutian disease virus.

13). The reaction products were subjected to immunoprecipitation in the presence of different synthetic NS1 peptides with the SP8 antiserum directed against the C terminus of NS1, and immunocomplexes were analyzed by SDS-PAGE (Fig. 4, lanes 1 to 10). When mutant and wt forms of NS1 were produced separately, wt NS1 (Fig. 4, lane 10) but not *dl*C67 (lane 9) was immunoprecipitated with SP8 antibodies. This is in keeping with the fact that the SP8 antiserum is directed against an NS1 portion which is absent from *dl*C67. In addition, the SP8 antiserum recognized a species slightly smaller than the *dl*C67 polypeptide that was presumably produced from an internal start site in the NS1 transcript. In contrast, a small but significant amount of NS1 *dl*C67 was present in the immunoprecipitate when the mutant form was coexpressed with the wt protein (Fig. 4, lane 1), arguing for the interaction of both polypeptides (wt NS1 and *dl*C67) under the conditions tested. The small amount of NS1 *dl*C67 coimmunoprecipitated with wt NS1 may indicate that the *dl*C67 product could not effectively compete with wt NS1 in oligomer formation. In addition, the SP8 antiserum may precipitate wt homo-oligomers preferentially, since multiple copies of the carboxy terminus are available. As illustrated in Fig. 4, lanes 2 to 4, peptide 11 abolished the coimmunoprecipitation of *dl*C67 with wt NS1 in a dose-dependent manner. This effect was specific since peptides 2, 10, and 12 failed to suppress the association of both NS1 polypeptides (Fig. 4, lanes 5 to 8).

Inhibition of NS1 helicase activity by peptide 11. By analogy with other helicases (64), particularly SV40 LTAg, which is structurally related to NS1 in the helicase domain (2), the oligomerization of NS1 can be expected to be required for helicase activity. In order to investigate this possibility, we determined whether blocking of the NS1-NS1 interaction by peptide 11 prevented NS1 from exerting its helicase function. To this end, NS1 was incubated in the presence of various amounts of synthetic NS1 peptide 11 (specific for the oligomerization domain) or peptide 2 (unrelated before the M13-VAR substrate was added. The helicase reaction was allowed to take place for 30 min, and the products were analyzed by nondenaturing PAGE. The helicase-deficient K405R mutant of NS1 (45) served as a negative control. As shown in Fig. 5, lanes 1 to 8, peptide 11 led to suppression of the NS1 helicase activity, ranging from a significant reduction to a complete inhibition at the lowest (2.75 ng/μl) and highest (65 ng/μl) peptide concentrations tested, respectively. This suppression was specific for the interacting peptide, since no inhibition was observed when unrelated peptides were used up to a concentration of 65 μg/μl, as shown for peptide 2 (GGYFLSSDSGWKTN FLKEGE) (Fig. 5, lanes 9 to 12). In addition, no inhibition occurred when peptide 11 was added after the onset of the reaction (data not shown). This observation, together with the fact that peptide 11 is located outside of the helicase domain of NS1 (aa 398 to 470 [31]), also makes it unlikely that inhibition of the unwinding reaction results from a direct block of the helicase at its active center. Therefore, our results suggest that the motif from aa 261 to 280 is essential for the NS1 helicase activity in an indirect way, most likely by mediating the forma-

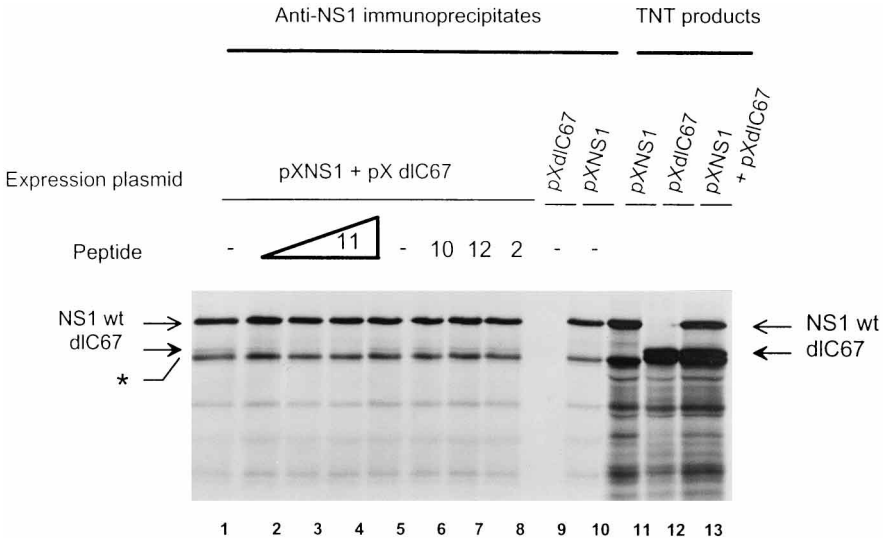

FIG. 4. Interference of NS1 peptides with coimmunoprecipitation of wt NS1 and *dl*C67 proteins. ^{35}S-labeled wt NS1 and *dl*C67 proteins were produced with an in vitro transcription-translation system (TNT; lanes 11 to 13). wt NS1 (lane 10) and *dl*C67 (lane 9) preparations or mixtures (lanes 1 to 8) were incubated with antiserum SP8, which is directed against the C terminus of NS1 and fails to recognize the *dl*C67-terminally deleted protein. Immunoprecipitation was carried out in the absence (−; lanes 1, 5, 9, and 10) or presence of 2.6 (lane 2), 12 (lane 3), or 26 (lanes 4 and 6 to 8) ng/μl of the indicated peptide. Immunoprecipitates were recovered and analyzed by SDS-PAGE. *, truncated NS1 translation product.

tion of NS1 oligomers that are the actual effectors of the helicase function.

Inhibition of viral DNA replication in vivo by peptide 11. The multifunctional NS1 protein is essential for parvovirus DNA replication (7) and may associate with the 3′ viral DNA replication origin in the form of an oligomer, as recently hypothesized from footprinting data (15). This led us to further investigate whether in vitro-identified peptide 11 was able to interact with NS1 and suppress parvovirus DNA replication in target cells, presumably as a result of competition for NS1 oligomerization. To this end, an infectious MVMp DNA clone was microinjected with or without increasing amounts of peptide 11 into A9 cell nuclei. Parallel cultures were treated in a similar way with overlapping peptides 10 and 12 or unrelated peptide 2 (all were unable to bind NS1 in an ELISA [Fig. 2]) and served as negative controls for nonspecific effects of injected synthetic peptides. An automated device that allowed the routine microinjection of around 500 nuclei in a confined area in the center of a coverslip for each dose of peptide was used. Two days after injection, total DNA was denatured in situ and transferred to a nitrocellulose membrane that was hybridized with a ^{32}P-labeled MVMp DNA probe. In the absence of any peptide, a strong hybridization signal was detected (Fig. 6A). Since DNA was extracted at 48 h after microinjection, it cannot be ruled out that some progeny virus spread occurred and contributed to enlargement of the corresponding autoradiographic spot. In the presence of peptide 11, only a residual signal was visible; this represented input viral DNA since it was equivalent to the signal produced when the replication-defective MVM ΔNS molecular clone was microinjected (Fig. 6B). This led us to conclude that peptide 11 is indeed capable of inhibiting intracellular MVMp DNA replication. This effect was dependent on the peptide concentration (Fig. 6A) and was specific, since no reduction of the hybridization signal was observed in cells treated with peptide 2, 10, or 12 at the concentration (70.9 μM) at which peptide 11 abolished MVM DNA replication (Fig. 6B). Therefore, these data argued for both the accessibility and functional importance of this domain in allowing NS1 to interact with constituent peptide 11 under physiological conditions.

DISCUSSION

NS1 homo-oligomerization. Nuclear cotransport studies provided indirect evidence of NS1 homo-oligomerization (47). The present study substantiates this conclusion in a direct way by showing that NS1-NS1 interaction takes place both in yeast (two-hybrid system) and in mammalian cell extracts (coimmunoprecipitation). This property is in line with the known roles played by the multifunctional NS1 protein in the control of DNA replication and transcription (7, 18). Indeed, a number of proteins involved in DNA metabolism have been found to act as homo-oligomers; these include transcription factors (5, 35, 39, 58, 62), replication factors (28), and multifunctional polypeptides, such as SV40 LTAg (40). The interactions of some of these factors with DNA appear to be site specific. Similarly, NS1 was recently reported to bind to DNA sequences containing the motif $(ACCA)_{2-3}$ (15). This NS1 responsive element is present in particular in the viral 3′ replication origin, where its interaction with NS1 generates a large footprint that is suggestive of higher-order oligomer formation (15). The degree of homo-oligomerization achieved by NS1 could not be estimated in the present study since the formation of homodimers would be sufficient to give rise to positive signals in the assays used. ATP binding was found to be dispensable for SV40 LTAg antigen dimerization and tetramerization but necessary for the formation of functional DNA-binding double hexamers (40). This may account for our finding that an amino acid substitution in the NTP-binding site of NS1 (Met405) did not impede NS1-NS1 interaction in the two-hybrid system, whereas it abolished NS1 cotransport (47), which may require conformational changes that involve ATP binding and multimerization. Another hint at the capacities of parvoviral nonstructural proteins for self-association to some extent in the absence of ATP is given by the dominant negative effects of NTP-binding site mutant forms of adeno-associated

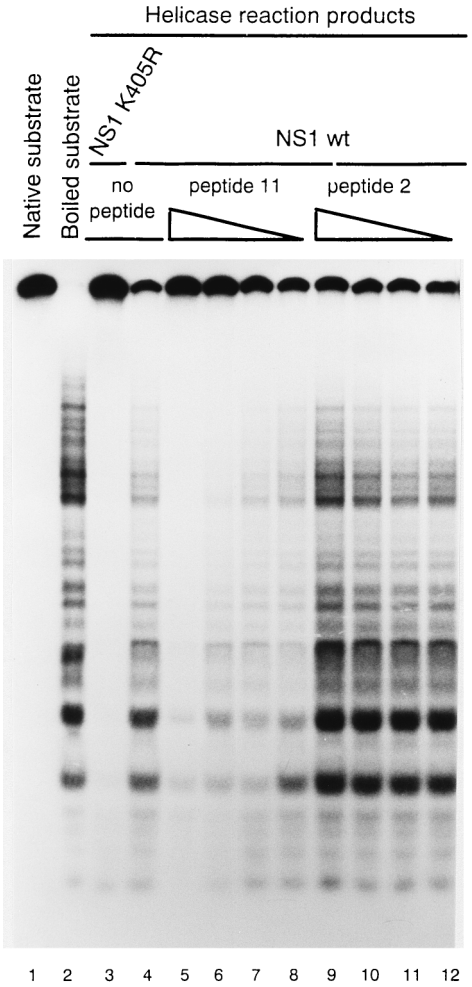

FIG. 5. Interference of NS1 peptides with the in vitro helicase activity of full-length NS1. Recombinant vaccinia virus-produced NS1 of MVMp was tested for its ability to displace ^{32}P-labeled oligonucleotides annealed to circular M13 single-stranded DNA. Reaction products were fractionated by PAGE and visualized by autoradiography. The profile of the heat-denatured DNA substrate (Boiled substrate) is shown in lane 2 for comparison. All other reactions were carried out with native substrate in the absence of NS1 (lane 1) or in the presence of either the replication-deficient mutant protein NS1 K405R (lane 3) or wt NS1 (lanes 4 to 12). The helicase activity of wt NS1 was tested in the absence of synthetic peptide (lanes 3 and 4) or in the presence of 1.3 (lanes 5 and 9), 0.4 (lanes 6 and 10), 0.165 (lanes 7 and 11), or 0.055 (lanes 8 and 12) μg of peptide 11 (lanes 5 to 8) or peptide 2 (lanes 9 to 12).

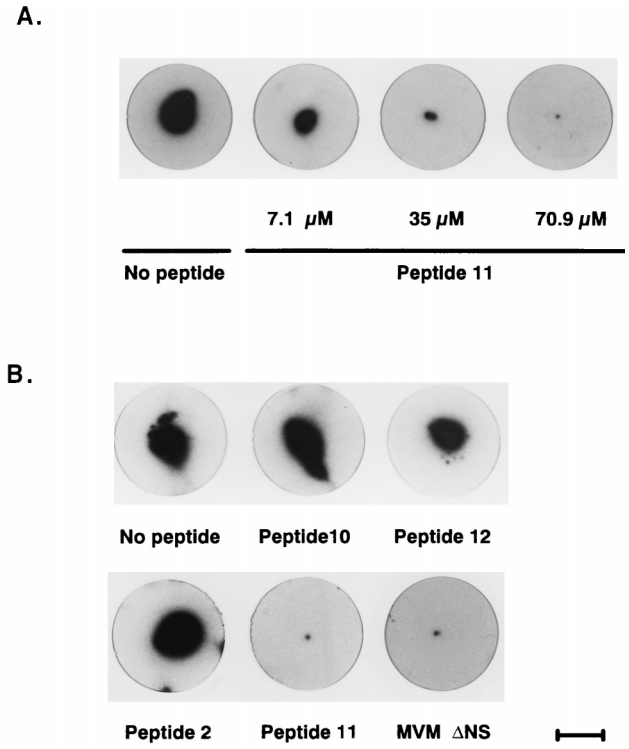

FIG. 6. Interference of specific NS1 peptides with MVMp DNA replication in permissive cells. A9 cells were microinjected intranuclearly with an infectious MVMp DNA clone in the presence or absence (No peptide) of the indicated amounts of NS1 peptides. At 48 h postinjection, cells were transferred under denaturing conditions to the center of each circular nitrocellulose membrane. Viral DNA amplification was measured by hybridization with a ^{32}P-labeled MVMp DNA probe. As a control in panel B, a replication-defective MVMp molecular clone (MVM ΔNS), instead of infectious MVMp DNA, was microinjected in the absence of any peptide. Bar, 10 mm.

virus Rep78 and Rep68 proteins on the helicase and viral DNA replication functions of the wt polypeptide (13, 34). Alternatively, the GAL4 AD fusion may lead NS1 to undergo a conformational change that compensates for the Met405 substitution with regard to NS1-NS1 interaction.

NS1 interaction motif. By using synthetic NS1 peptides in an ELISA-type protein-binding assay (30, 63), we identified a discrete NS1 element, VETTVTTAQETKRGRIQTKK (aa 261 to 280), which is sufficient for interaction with full-length NS1 under in vitro conditions. Critical residues in this element define a putative bipartite interaction motif within the sequence VETTVTX$_9$IQT. This motif is located in a region that is essential for NS1 oligomerization in yeast. Furthermore, a peptide encompassing the interaction motif was found to act as a specific competitor and to inhibit NS1-NS1 association in coimmunoprecipitation experiments with reticulocyte extracts. Altogether, these data argue for the involvement of this motif in NS1 dimerization.

This conclusion was substantiated by functional assays. Since most helicases are known to work as oligomers, the intrinsic helicase activity of NS1 (65) was tested for its sensitivity to competitive inhibition by free NS1 peptides comprising (or not) the interaction motif. A dose-dependent reduction in NS1 helicase activity was indeed observed in the presence of the interaction peptide but not other NS1 peptides, suggesting that homo-oligomerization through the interaction motif plays a role in at least some NS1 functions. This possibility was further supported by showing that the interaction peptide was able to abolish in a specific way MVMp DNA replication after microinjection into permissive cells, whereas other NS1-derived peptides were inactive in this respect. It is worth noting that the amino acid sequence of the NS1 interaction motif is fully conserved among a number of autonomous parvoviruses, in keeping with its importance for virus replication. Altogether, these data indicate that a potent suppression of virus replication can be achieved by blocking a domain required for the oligomerization of an essential viral nonstructural protein. Our results point to oligomerization motifs of viral regulatory proteins as targets for the design of new peptide-mimetic antiviral agents.

It should also be stated that although a single NS1 peptide was able to trap the wt NS1 protein in the in vitro binding assay, NS1 oligomerization in the two-hybrid system was impaired by deletions not only in this element but also in neigh-

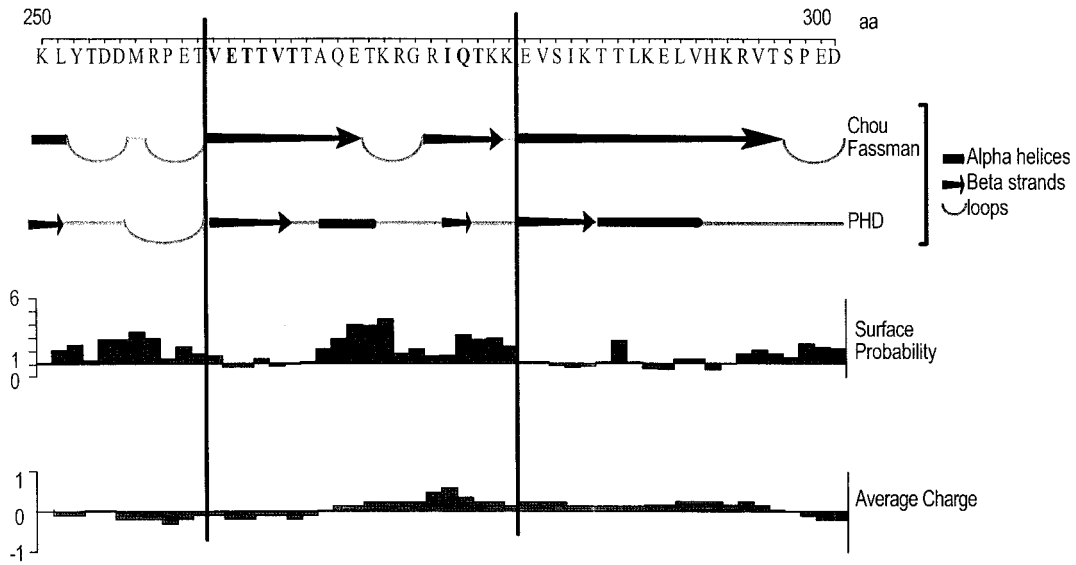

FIG. 7. Structural features of the NS1-NS1 interaction region. The sequence of the NS1 region from aa 250 to 300 is shown, with the interaction peptide delimited by vertical lines and the residues essential for interaction in bold. Linear plots of computer-predicted secondary structure (Chou-Fassman and PHD algorithms), surface probability, and charge distribution (Emini algorithm) in this region were determined by using DNAstar and PHD (EMBL) softwares.

boring regions on either side of the interaction motif. These results suggest that the identified motif lies in a conformational domain which has to be preserved for proper interactions between full-length NS1 molecules. Moreover, there may be additional domains that are involved in NS1 self-association but fail to be revealed by the peptide ELISA method, e.g., due to their lack of confinement to the peptides used as baits or posttranslational modification constraints. Thus, the ability of the interaction peptide on its own to trap NS1 in vitro does not rule out the possibility that in the context of the whole protein, this motif takes part in intramolecular interactions that make NS1 competent for homo-oligomerization through other domains. Finally, there is a possibility that NS1 dimerization leads to structural modifications which uncover additional interaction motifs, allowing the formation of higher-order multimers.

NS1 structure in the region of the interaction motif. In the absence of available X-ray crystallographic or nuclear magnetic resonance data and given our failure to identify potential folding patterns in NS1 by comparison with reference polypeptides from data banks, the DNAstar and PHD (European Molecular Biology Laboratory [EMBL], Heidelberg, Germany) softwares were used to predict the secondary structure of the NS1-NS1 interaction region. As illustrated in Fig. 7, both elements of the bipartite interaction motif are assigned to β-strands that can be distinguished by the degree of hydrophilicity and overall charge. The contribution of β-sheets to the formation of protein dimer interfaces is well documented (9, 52). Therefore, it can be speculated that the NS1 region containing the bipartite motif interacts with itself in homo-oligomerization. A similar type of association has previously been reported for a number of homo-oligomeric proteins (51, 56, 59). Additional experiments particularly with NS1 clones modified by site-directed mutagenesis are needed to assess the role of this putative β-strand structure in NS1-NS1 interactions.

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