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NS1 Protein of Parvovirus B19 Interacts Directly with DNA Sequences of the p6 Promoter and with the Cellular Transcription Factors Sp1/Sp3

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The nonstructural proteins of parvovirus exert a variety of disparate functions during viral infection ranging from promoter regulation, involvement in DNA replication, and induction of apoptosis. Our interest was focused on the possible mechanism by which the NS1 protein mediates its effects on the p6 promoter of parvovirus B19. It is known that the p6 promoter is highly active in different cell lines and interaction with the viral NS1 protein results in a further increase of the activity. The protein may function by binding directly to the viral DNA or via an indirect binding through interaction with cellular transcription factors bound to the promoter. We examined the interaction of the NS1 protein with cellular transcription factors which are involved in regulating the promoter activity. After purified baculovirus-expressed NS1 protein in gel retardation assays was added, an altered complex formation was observed, indicating that NS1 protein interacts with Sp1/Sp3 transcription factors. Enzyme-linked immunosorbent assays verified these findings. The direct interaction of NS1 protein with p6 promoter elements was analyzed by a coprecipitation assay whereby labeled oligonucleotides spanning the entire promoter region were incubated with NS1 protein followed by an immunoprecipitation with NS1-specific antibodies. An eight-nucleotide-long, almost palindromic sequence (AGGGCGGA) was found as potential NS1-binding motif. Footprint analysis with oligonucleotides containing this DNA motif confirmed this result. Thus, transcriptional regulation by the NS1 protein may involve both the interaction with Sp1/Sp3 that binds to the promoter region and direct binding of NS1 to the promoter DNA.

INTRODUCTION

Parvovirus B19, the only member of the Parvoviridae that is pathogenic in humans, is a small nonenveloped icosahedral virus with a single-stranded linear 5.6-kb DNA genome. Both ends of the DNA genome are identical inverted terminal repeats (ITR) of 383 nucleotides in length. The distal 365 nucleotides of these repeats are imperfect palindromes that form hairpin structures which are necessary for priming DNA replication. The only functionally active promoter within the viral genome, the p6 promoter, is located at the 5′ palindrome and regulates the synthesis of all nine viral transcripts (Blundell *et al.,* 1987; Doerig *et al.,* 1990). Seven of these are mRNAs and are used for synthesis of a multifunctional protein, the so-called nonstructural protein 1 (NS1), two capsid proteins (VP1 and VP2), and several smaller polypeptides with still unknown function (Cotmore and Tattersall, 1984; Luo and Astell, 1993; Ozawa *et al.,* 1987).

The NS1 protein of parvovirus B19 is a multifunctional protein that performs many different functions during the virus life cycle. Besides trans-regulation of the p6 promoter, NS1 proteins influence the activity of other viral and cellular promoters (Doerig *et al.,* 1990; Sol *et al.,* 1993; Moffatt *et al.,* 1996; Liu *et al.,* 1991; Momoeda *et al.,* 1994; Gareus *et al.,* 1998). ATPase and DNA helicase activity have been predicted based on homology studies with proteins of other autonomous animal parvoviruses (Doerig *et al.,* 1990). Further enzymatic reactions during viral DNA replication were intensively studied for minute virus of mice supposed to be the initiation of replication by site-specific nicking within the origin of replication, generating free 3′ hydroxyls, which serve as primers for the DNA polymerase (Cotmore and Tattersall, 1994; Christensen *et al.,* 1997; Cotmore and Tattersall, 1998).

The strong cytopathic effect of parvovirus B19 leading to apoptosis in erythroid cells seems to be caused by an interaction between the NS1 protein and tumor necrosis factor α pathways (Sol *et al.,* 1999).

The mechanism by which the NS1 protein of B19 exerts its transcriptional regulation has not been investigated. The viral protein can interact either directly with specific promoter sequences or indirectly via interaction with DNA-binding proteins. We previously analyzed cellular transcription factors which are involved in the regulation of the promoter activity (Raab *et al.,* 2001). Based on these studies we investigated the interaction of the NS1 protein with cellular transcription factors using purified baculovirus-expressed NS1 protein in gel retardation assays and ELISA. The direct interaction of NS1 with

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p6 promoter elements was analyzed by incubating ^{32}P-labeled oligonucleotides spanning the entire promoter region with NS1 proteins followed by coimmunprecipitation with NS1-specific antibodies. Our results indicate that the NS1 proteins interact with the p6 promoter by binding to cellular transcription factors and by direct DNA binding. Both mechanisms may influence viral gene expression and promoter activity as well as genome replication and DNA synthesis.

RESULTS

Interaction between the viral NS1 protein and the transcription factors Sp1/Sp3

To analyze how the multifunctional NS1 protein of parvovirus B19 mediates its strong activity to transactivate the p6 promoter, electrophoretic mobility shift assays (EMSA) using baculovirus-expressed NS1-protein were performed. As published previously, the interactions of DNA-binding proteins of different nuclear cell extracts with three p6 promoter regions were characterized in detail (Raab *et al.,* 2001). Thus we could demonstrate binding of the transcription factor Sp1 to three GC boxes within the p6 promoter and specific interaction of the factor Sp3 preferentially to two of these GC motifs in the direct vicinity of the TATA box. After addition of purified NS1 protein produced by recombinant baculovirus into samples containing oligonucleotides of all three promoter regions and nuclear extracts from HeLa cells, the DNA–protein complexes were analyzed by gel retardation assays. A change in complex formation was observed exclusively for the oligonucleotide spanning region H. The two major and two minor retarded bands identified as complexes formed of Sp1 and Sp3 factors were almost totally inhibited in the presence of NS1 protein (Fig. 1A, region H), revealing that the viral protein influences the binding of both transcription factors to the DNA fragments. Interestingly, we observed that the formation of the Sp1/DNA complex with region D is not altered by the presence of NS1 protein. Controls using the Sp1 consensus sequence in the retardation assays confirmed that the NS1 protein interacts with the transcription factors Sp1 and Sp3, both of which are involved in the formation of the major retarded bands because the formation of both DNA–protein complexes was inhibited. These results indicate that the NS1 protein interferes exclusively with the binding of Sp1 and Sp3 to the GC boxes present in promoter region H. The binding of Sp1 to the GC box in region D was not altered.

To validate complex formation between NS1 and Sp1/Sp3 proteins, an enzyme-linked immunosorbent assay (ELISA) was performed using NS1 protein bound to the solid phase and HA-tagged Sp1 or Sp3, both produced with recombinant baculovirus as potential binding partners. Comparison of the expression levels of Sp1 and Sp3 by Western blot analysis revealed that nearly identical amounts of protein were produced (data not shown). Monoclonal antibodies directed against the HA tag fused to factors Sp1 and Sp3, and peroxidase-conjugated secondary antibodies were used to detect the bound transcription factors. As a control, Sf9 cell extracts infected with wild-type baculovirus were applied. The results demonstrate that both transcription factors Sp1 and Sp3 bind specifically and with similar affinity to NS1 protein and thus confirm the data obtained by EMSA (Fig. 1B). Background values observed for the control using crude extracts of Sf9 cells infected with wild-type baculovirus were negligible (less than 0.01).

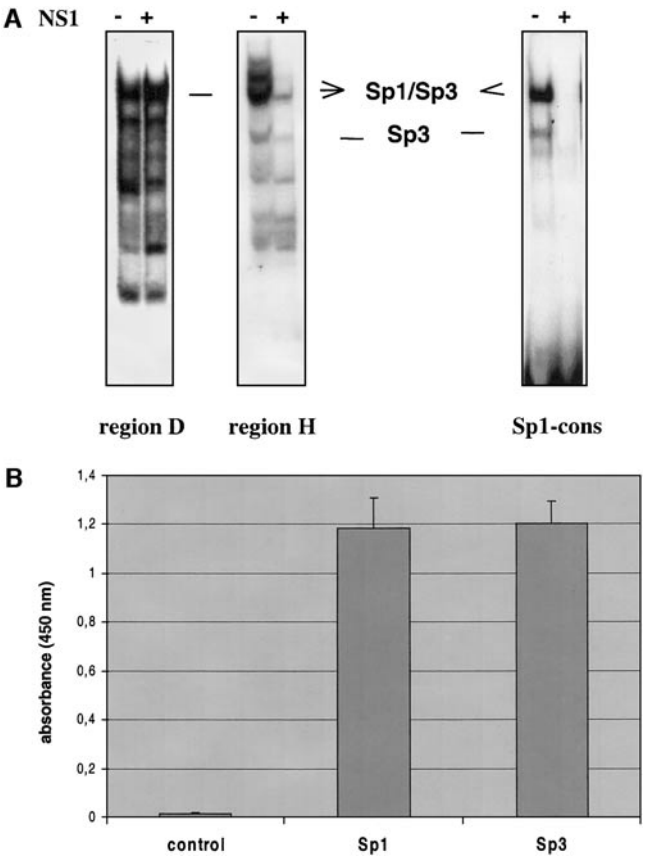

FIG. 1. NS1 protein interacting with the transcription factors Sp1 and Sp3. (A) NS1 inhibits the binding of cellular transcription factors Sp1/Sp3 to region H of the p6 promoter. EMSAs were performed using ^{32}P-labeled oligonucleotides (region D, region H, and Sp consensus sequence), 5 μg of nuclear extracts prepared from HeLa cells, and 1.25 μg of baculovirus-expressed NS1 protein as indicated. After electrophoresis, the gels were exposed to X-ray films. The assignments of the transcription factors that were identified as parts of the complexes are given. (B) Purified baculovirus-expressed NS1 protein immobilized on ELISA plates was incubated for 1.5 h with crude extracts of Sf9 cells expressing HA-tagged SP1 or Sp3. After washing, bound transcription factors were detected by a monoclonal anti-HA antibody followed by peroxidase-conjugated second antibody. Bound Sp1 or Sp3 was quantitated spectrophotometrically. Control wells were incubated with crude extracts of Sf9 cell infected with wild-type baculovirus (control).

NS1 protein binds to an 8-nt sequence element within the p6 promoter

To examine whether the NS1 protein interacts directly with p6 promoter elements, an approach similar to that used to demonstrate the direct binding of the NS1 protein of MVM to the p38 promoter was used (Cotmore *et al.,* 1995). The eight oligonucleotides spanning the entire promoter region (see Fig. 2A) were labeled with [γ-^{32}P]ATP and incubated with NS1 protein. The protein–DNA complexes were immunoprecipitated with NS1-specific human monoclonal antibodies (Gigler *et al.,* 1999). Oligonucleotides spanning regions A and D were precipitated efficiently and specifically. A third NS1–DNA complex (region F) was immunoprecipitated, however, to a lesser extent (Fig. 2B).

After the sequences represented by oligonucleotides A, D, and F were analyzed, a common sequence element, an almost palindromic octamer motif 5′-*AGGGCGGA*-3′ could be identified in region A, whereas regions D and F contained the complementary sequence 5′-TCCCGCCT-3′.

To determine if the NS1 protein interacts with this octamer, we used short oligonucleotides (14 nt) containing the potential NS1-binding motif, the mutated form of the binding motif with 3 nucleotide exchanges in the center of the motif, and an oligonucleotide (21 nt) comprising the Sp1 consensus sequence into the immunoprecipitation assay. In this analysis, the DNA fragment with the NS1-binding motif was exclusively precipitated and complex formation with the mutated oligonucleotide or the Sp1 consensus sequence was not observed (Fig. 2C). The specificity of NS1 binding to the octamer motif was examined by competitive inhibition using a DNA binding assay with ^{32}P-labeled oligonucleotides of region A together with the NS1 motif or the mutated fragment (NS1-mut) as competitor oligonucleotides. Binding could be inhibited by a 50-fold excess of the NS1 motif as competitor (data not shown). These results confirm the sequence specificity of the interaction and suggest that NS1 proteins recognize this motif even if it is present in a short DNA fragment.

NS1 proteins protect oligonucleotides from digestion with DNase I

DNase I protection assays were carried out using an oligonucleotide of 77 nt of length comprising region A and partly region B and purified preparations of NS1 protein. These assays showed a footprint of 55 bp present in both DNA strands (Fig. 3). The DNA sequence protected by NS1 proteins extends from the nucleotide at position 9 up to the nucleotide at position 65. Because this represents a relatively large region, it may be assumed that NS1 proteins exert their DNA-binding activity in an oligomeric form. Furthermore, the footprint is rather asymmetric with respect to the potential NS1-binding site. It extends 14 nucleotides 5′ to the NS1 motif and 33 nucleotides into the 3′ direction. These results confirm our previous observations that NS1 protein binds directly to the promoter DNA.

Helicase activity of NS1 proteins

Preparations of recombinant NS1 proteins produced in S2 cells were analyzed for potential helicase activity. The helicase assay used a [γ-^{32}P]ATP-labeled oligonucleotide annealed to single-stranded M13mp18 DNA as substrate.

The advantage of the S2 expression system is that the recombinant NS1 protein is secreted into serum-free medium. Therefore, the protein could be applied into the helicase assays after concentration without a further purification step that may destroy the structure of the protein and the enzyme activity. As shown in Fig. 4 (lanes 2, 3, 4) NS1 protein is able to displace the labeled oligonucleotides as part of the double-stranded substrate. The level of activity to unwind DNA fragments from a circular M13 template increased with increasing amount of NS1 protein up to 900 ng.

DISCUSSION

We have demonstrated two mechanisms by which the NS1 protein of parvovirus B19 may influence the activity of the p6 promoter: It binds directly to the p6 promoter through the DNA sequence element which contains an almost palindromic motif *AGGGCGGA,* and it binds indirectly via interaction with the cellular transcription factors Sp1/Sp3. Based on previous studies where we identified various transcription factors binding to the promoter of parvovirus B19 using electrophoretic mobility shift assays, we have now analyzed the interactions of the viral NS1 protein with the complexes formed by the promoter DNA and the cellular transcription factors. These results suggest that the interaction is mediated via binding of the NS1 protein to the transcription factors Sp1 and Sp3. The observation that NS1 protein interacts with the factors Sp1/Sp3 is supported by previous data indicating that the transactivation mediated by the viral protein was significantly reduced by the introduction of mutations into the GC boxes used as binding sites for Sp1/Sp3 proteins (Gareus *et al.,* 1998). Interestingly, the NS1 protein seems to interact preferentially with Sp1 proteins bound to the GC boxes located in region H, the only region where factor Sp3 could be identified as an interaction partner. In region D containing one GC box, binding of Sp3 proteins was not observed and NS1-proteins did not influence the complex formation. It may be speculated that the protein interactions depend on individual flanking sequences that are necessary for the formation of the Sp1/Sp3–NS1 protein complexes with the promoter or that the presence of Sp3 is indispens-

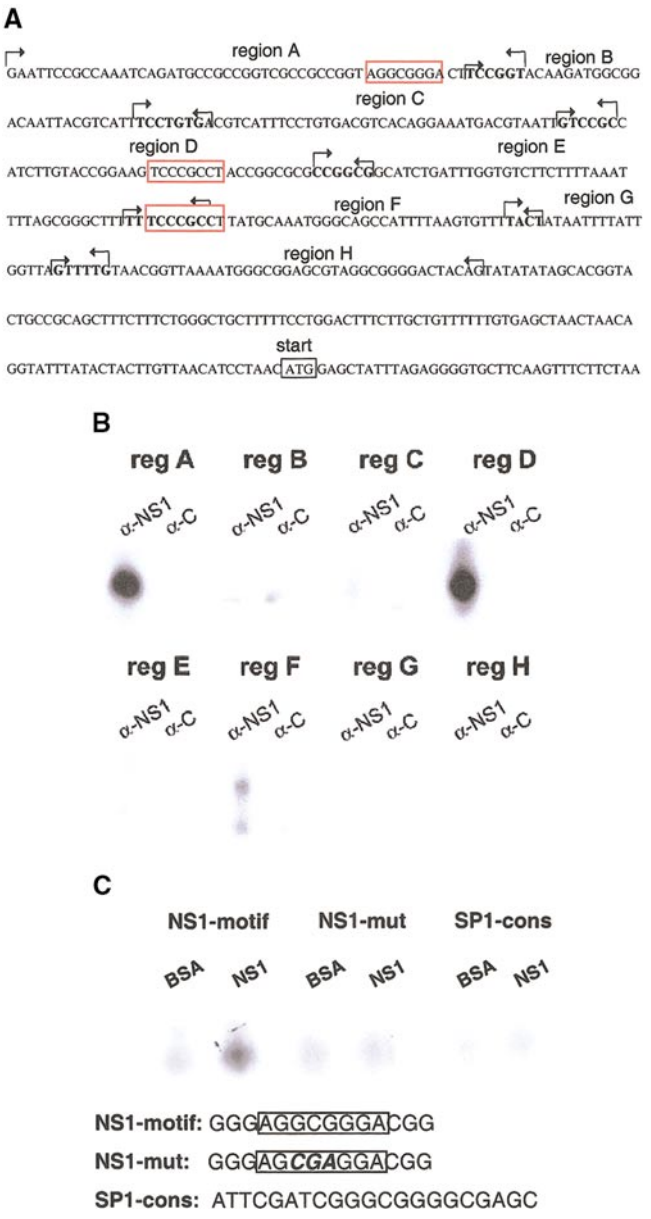

FIG. 2. NS1 binding to regions A and D of the p6 promoter. (A) Sequence of the p6 promoter of parvovirus B19 subdivided into eight regions (A–H). The overlapping nucleotide sequences of the eight oligonucleotides used for the protein–DNA immunoprecipitation are marked by bold letters. The potential NS1-binding motif is marked by red boxes. (B) Immunprecipitation assays were performed using ^{32}P-labeled oligos (regions A–H) incubated with 500 ng of purified baculovirus-expressed NS1 protein and precipitated with a monoclonal anti-NS1 antibody or with a monoclonal anti-VP1 antibody (control). Samples were deproteinized by incubation with proteinase K and subsequently analyzed on an agarose gel. After electrophoresis the gels were exposed to X-ray films. (C) NS1 binding to NS1 motif *AGGCGGGA*. Autoradiography of an agarose gel showing a immunoprecipitation with the ^{32}P-labeled oligonucleotides (NS1 motif, NS1 mut, Sp1 cons) treated as described above. BSA was used as control protein.

able. An alternative explanation is that NS1 proteins interact with complexes of at least two Sp1/Sp3 molecules, indicating that the assembly to oligomers may facilitate the interaction of NS1 proteins with these transcription factors. A supershifted band containing Sp1/Sp3 and NS1 proteins cannot, as yet, be demonstrated. This may be because EMSA is not the appropriate system for the resolution of multicomponent complexes. Sp1 proteins are ubiquitously synthesized and stimulate the transcription activity of a variety of cellular and viral promoters (Lania *et al.,* 1997). In competition with Sp1, binding of the factor Sp3 can repress or activate gene expression as it has been shown for several human and viral genes (Hagen *et al.,* 1994; Kwon *et al.,* 1999; Qin *et al.,* 1999; Tsai *et al.,* 1999). Our ELISA data allow speculation that binding of NS1 proteins to Sp1/Sp3 occurs independently of binding to viral DNA. Therefore, the overexpression of the NS1 gene that has been observed in cells nonpermissive for parvovirus B19 infection could result in a deregulation of the compatibility of Sp1/Sp3 in regulation of critical cellular promoter. Similar to our experiments for parvovirus B19, interaction of the NS1 protein of MVM (minute virus of mice) with factor Sp1 has been demonstrated by DNA affinity chromatography and

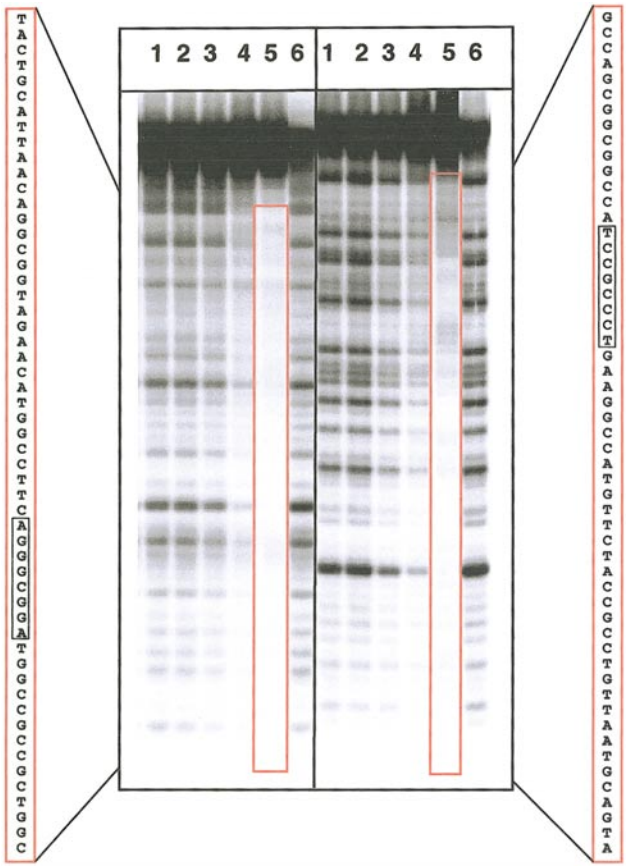

FIG. 3. NS1 protects a 56-bp fragment within region A of the p6 promoter. DNase I protection analysis (left, lower strand; right, upper strand) was performed with different amounts of purified NS1 (lane 1, 0.25 μg; 2, 0.5 μg; 3, 2.5 μg; 4, 3.75 μg; 5, 5 μg; 6, 5 μg control protein) in the presence of γ S-ATP and 1 U of DNase I. At the left and right side of the footprint the sequence of the protected region is shown. The potential NS1-binding site is marked by boxed area.

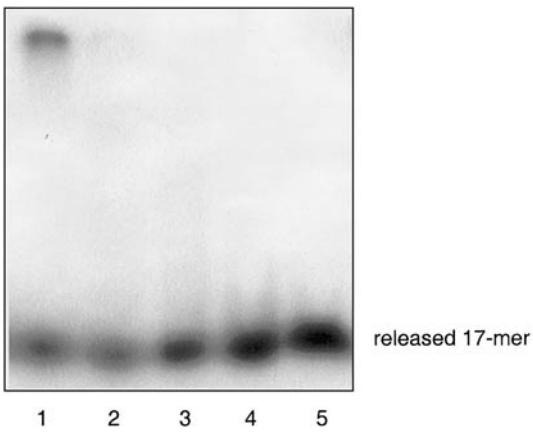

FIG. 4. NS1 protein expressed in D2 cells exhibits DNA helicase activity. M13 primer helicase substrate (see Materials and Methods) was incubated without NS1-protein (lane 1) or 300 ng (lane 2), 600 ng (lane 3), or 900 ng (lane 4) of NS1 protein for 30 min at 37°C. Lane 5 shows unreacted helicase substrate boiled prior to electrophoresis. The reaction mixtures were analyzed on 10% nondenaturing acrylamide gels.

immunoprecipitation experiments (Krady and Ward, 1995).

Until now, direct binding of NS1 proteins to DNA elements in the p6 promoter could not be shown for parvovirus B19. With respect to the NS1 protein of MVM it has been suggested that rather unstable complexes of NS1 protein and DNA may form in the presence of ATP. Due to the rapid dissociation of the complex, it was impossible to directly demonstrate the interaction of NS1 proteins with DNA by standard gel shift assays. The binding of NS1 protein to the transactivation region of the p38 promoter of MVM was finally shown by using a DNA–protein binding assay (Christensen *et al.,* 1995). Based on a similar approach, we were able to identify a NS1-binding octamer motif (Fig. 2B). An alternative approach using biotinylated oligonucleotides containing the NS1 motif or NS1 mut on a streptavidin-coated matrix that was incubated with NS1 protein and subsequently with NS1-specific antibodies confirmed the coprecipitation results (data not shown). Competition assays using a 50-fold excess of unlabeled oligonucleotides with NS1 motif inhibited the precipitation and therefore could verify the specificity of NS1 binding (data not shown). The octamer motif *AGGGCGGA* is present in the left- and in the right-hand hairpin regions of the viral genome (nt 39 to nt 46 and nt 5080 to nt 5088; referring to plasmid pYT103 (Shade *et al.,* 1986) and two times in a complementary form *TCCCGCCT* at positions nt 148 to nt 155 and 216 to nt 223. In contrast to our results, the site binding the NS1 protein of MVM is distributed at regular intervals throughout the entire MVM genome, suggesting that the binding of NS1 proteins may additionally play a structural role in the formation of viral chromatin in the infected cells. A further significant difference is that NS1 proteins of MVM only bind its recognition site when it is present in the context of flanking DNA sequences and inhibition of the binding can only be achieved when the appropriate oligonucleotide is ligated into a fragment of more than 21 nt (Christensen *et al.,* 1995).

During parvovirus replication, two steps exist that require DNA unwinding activity. The first is during terminal resolution when the DNA polymerase is copying the sequences of the terminal repeat. The second event occurs during the next step in genome replication when the terminal palindrome has to be unwound to reform the terminal hairpin for the displacement of the priming strand. NS1 protein exhibits ATP-dependent helicase activity. It was striking that in the helicase and all other described assays we had to use 5- to 10-fold amounts of protein to show any protein function compared to the published data of other parvoviral nonstructural proteins (Wilson *et al.,* 1991; Christensen *et al.,* 1995; Im and Muzyczka, 1990). This may be due to the rapid degradation and the high instability of NS1 proteins independent of expression either via recombinant baculovirus or in S2 cells.

NS1 proteins of MVM form oligomers and mutations in ATP-binding fold prevent the self-association, indicating that ATP binding may induce the formation of multimers that facilitate binding to viral DNA (Pujol *et al.,* 1997; Nuesch and Tattersall, 1993). The poor binding of NS1 proteins of MVM to the promoter in the absence of ATP can be increased 10-fold by addition of ATP. For the NS1 protein of parvovirus B19 we cannot demonstrate such a strong dependence on ATP, although the binding of the protein to the NS1 motif is impaired slightly in the absence of ATP (data not shown). Similar to the NS1 protein of the B19 virus, the nonstructural proteins Rep 68 and Rep 78 of helper-dependent parvoviruses AAV 2 bind to their respective recognition sites in the absence of ATP (McCarty *et al.,* 1994; Owens *et al.,* 1991). Nevertheless, the large size of the NS1 footprint and the failure to demonstrate the binding of the NS1 protein of parvovirus B19 to DNA in EMSAs indicate the formation of NS1 oligomers in our system.

At present, relatively few details about the proteins associated with replicating DNA of parvovirus B19 *in vivo* are known due to the lack of an appropriate cell culture system. Furthermore, it is not clear if indirect binding of the NS1-proteins to Sp1/Sp3 oligomers or the direct interaction with the viral DNA is the primary step for gene expression and which one is the potentiating mechanism of transactivation. On the basis of our results, both regulatory elements appear to be required for efficient transactivation of the p6 promoter.

MATERIALS AND METHODS

Oligonucleotides

The oligonucleotides containing the consensus binding site for Sp1/Sp3 and the respective nonspecific oli-

gonucleotide were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were ready for use. The double-stranded probes were radioactively labeled by the use of T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) to a specific activity of ~100 cpm/μg and subsequently purified by chromatography (Micro Bio-Spin 6, Bio-Rad, Munich, FRG). The sequences of the eight oligonucleotides used in NS1-DNA binding assays have been published elsewhere (Raab *et al.,* 2001).

Antibodies

Monoclonal antibodies specific for Sp1 and Sp3 were obtained from Santa Cruz Biotechnology. Monoclonal antibodies directed to viral NS1 and VP1 proteins have been described elsewhere (Gigler *et al.,* 1999). The monoclonal anti-HA antibody was obtained by Roche Diagnostics (Mannheim, Germany).

Purification of recombinant NS1 protein

Recombinant baculovirus encoding the NS1 gene was used to infect Sf9 cells at a multiplicity of infection of 2. Infected cells (1×10^9) were harvested 4 days postinfection and lysed in the presence of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, and 2 μg/ml leupeptin) by the addition of phosphate-buffered saline–0.5% (w/v) sodium deoxycholate. After centrifugation (3000 *g* for 5 min), the insoluble fraction containing recombinant NS1 protein and nuclei was resuspended in 25 mM HEPES, 5 mM $MgSO_4$, pH 7.4, and subjected to DNase treatment (final concentration: 10 μg/ml) (Sigma, Poole, UK) for 1 h at room temperature. Following a brief centrifugation and wash step (3000 *g,* 3 min), the insoluble NS1 protein was resuspended in 0.5–1.0 ml 6 M guanidinium thiocyanate at a concentration of 4–5 mg/ml. This preparation was diluted to 0.5 mg/ml in 50 mM $NaHCO_3$, pH 9.4, containing 8 M urea and dialyzed against 50 mM $NaHCO_3$, pH 9.4, to remove denaturing agents prior to binding assay.

Cloning of the NS1 gene into pMT/BIP/V5-His

PCR was used to amplify the NS1 coding sequence from pJB by using the primers 5′-ATTAGAATTCCCCATGGAGCTATTTAGAGGG-3′ and 5′-ATTAAGATCTGTCGACTTACTCATAATCTACAAAGC-3′) (Mori *et al.,* 1987) creating two restriction sites, *Eco*RI and *Sal*I. The resulting 2-kb fragment was digested with *Eco*RI and *Sal*I and cloned into the *Eco*RI and *Sal*I sites of the pSVK3 vector. Subsequently, the NS1-coding region was subcloned as an *Eco*RI–*Sal*I restriction fragment into the expression plasmid pMT/BiP/V5/His (Invitrogen, Groningen, Netherlands) using the *Eco*RI and *Xho*I sites.

NS1 gene expression in S2 cells

S2 cells were maintained and induced to produce recombinant NS1 protein according to manufacturers' instructions (Invitrogen). S2 cells (kindly provided by Dr. Gabler, University of Regensburg) were maintained at 24°C in DES Expression Medium (Invitrogen), supplemented with 10% fetal calf serum. Cells were cotransfected with 1 μg of pCoHygro (Invitrogen), encoding the gene for hygromycin resistance and 19 μg of pMT-NS1 using the $CaPO_4$ precipitation method. Five days post-transfection, hygromycin was added to a final concentration of 300 μg/ml. Cells were passaged approximately every 4–5 days. A stable population of hygromycin-resistant cells was obtained after 4 weeks. S2 cells were induced to produce NS1 proteins by addition of 500 μM $CuSO_4$.

Nuclear extracts

Preparation of the nuclear extracts was performed as previously described (Raab *et al.,* 2001). Briefly, 1×10^9 HeLa cells/ml were washed twice in PBS and once in hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$). The cells were resuspended in 1 vol of hypotonic buffer and incubated for 10 min on ice. The suspension was transferred into a dounce homogenizer and cells were lysed by a minimal number of strokes (20×–30×) until most of the cells were broken. Nuclei were recovered by centrifugation (5 min, 750 *g*), suspended in 1 ml of hypotonic buffer, and collected by centrifugation. The nuclei were suspended in one volume (100–300 μl) of high-salt extraction buffer (20 mM HEPES (pH 7.9), 25% glycerol, 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and incubated for 30 min at 4°C with gentle rocking. Following this incubation, the mixture was centrifuged (30 min, 100,000 *g*). The supernatant was dialyzed at 4°C for 3 h (10 mM HEPES (pH 7.9), 10% glycerol, 100 mM NaCl, 1 mM EDTA). The protein concentration in the extracts was determined as 2 μg/μl.

Electrophoretic mobility shift assays (EMSAs)

Gel retardation assays were performed using 5 μg nuclear extracts of HeLa cells, 1 μg poly(dI-dC), and 20 fmol labeled DNA in a final volume of 20 μl containing 8 μl of the binding buffer (100 mM Tris/HCl, pH 7.5, 500 mM NaCl, 5 mM dithiothreitol), 5 mM $MgCl_2$, 10% glycerol, 0,05% NP40. For shift assays in the presence of NS1 protein 2 μl of binding buffer containing 3 mM $MgCl_2$ was added. For controls, nonradiolabeled competitor oligonucleotides were added to the reaction prior to the addition of labeled DNA probes. The samples were subjected to electrophoresis on native 5% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) containing 0.5× TBE. Gels were subsequently dried for autoradiography.

NS1 protein/DNA-binding assay

Binding assays were performed as described elsewhere (Cotmore *et al.,* 1995). Briefly, approximately 800 ng of purified baculovirus-expressed NS1 protein was

incubated for 10 min in a final volume of 100 μl binding buffer B (20 mM Tris/HCl, pH 7.5, 125 mM NaCl, 10% glycerol, 1% NP40, 5 mM dithiothreitol, 200 ng poly(dI-dC), 0.5 mM ATP) before 1.5 pmol of ^{32}P-labeled oligonucleotides was added. Samples were incubated for another 30 min on ice before 2 μl of antibody was added and the incubation continued for an hour. Binding buffer (30 μl) containing 1.5 mg of swollen protein A–Sepharose beads was added and the mixture were gently shaken and left overnight at 4°C. After removal of the supernatant, immunoprecipitates were washed three times with 1 ml binding buffer. Samples were deproteinized by incubation 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. Gels were dried and exposed for autoradiography.

DNase I protection analysis

Footprinting was performed as described (Niller and Hennighausen, 1991): Both strands of oligonucleotide 5′-GATGCCGCCGGTCGCCGCCGGTAGGCGGGACTTCCGGTACAAGATGGCGGACAATTACGTCATTTGCTGTGACGTCA-3′, 5′-TGACGTCACAGCAAATGACGTAATTGTC CGCCATCTTGTACCGGAAGTCCCGCCTACCGGCGGCGACCGGCGGCATC-3′ were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. After end-labeling, either labeled strand was hybridized with its unlabeled counterpart. For footprinting, protein was incubated with 0.1 μg of poly(dI-dC) at room temperature (RT) for 10 min followed by an incubation of 15 min with 8 ng of end-labeled fragments (20,000 cpm) in 12 mM HEPES/KOH, pH 7.9, 10% glycerol, 1.6 mM dithiothreitol, 0.12 mM EDTA, 60 mM KCl, and 6 mM $MgCl_2$ in a reaction volume of 50 μl. γ-S-ATP was included as indicated. The probe was digested with 1 unit of DNase I (RNase free, Roche) at RT for 1 min. The DNase I digestion was stopped by addition of 100 μl of stop solution (100 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS, 10 μg proteinase K, 1 μg tRNA), incubation for 15 min at 37°C, and denaturation at 90°C for 2 min. Digestion products were phenol extracted, ethanol precipitated, and separated on a 6% sequencing gel. Maxam and Gilbert A+G sequencing markers (Maxam and Gilbert, 1980) of the corresponding probes were used to locate the footprints.

Helicase assay

A 17-base oligonucleotide (universal primer, Amersham Pharmacia Biotech, Freiburg, Germany) was radioactively labeled with [γ-^{32}P]dATP by the use of T4 polynucleotide kinase at 37°C for 1 h. Unincorporated ATP was removed by chromatography (Micro Bio-Spin 6, Bio-Rad, Munich, FRG). Subsequently, the radioactively labeled oligos were annealed to 1 μg of single-stranded M13mp18 DNA (Amersham). The DNA helicase assay was performed in a reaction mixture of 30 μl containing 5 μl of "one-phor-all"-buffer (10 mM Tris–acetate, pH 7.5, 10 mM Mg acetate, 50 mM K-acetate, 1 mM ATP, 4 mM DTE), 20 ng of substrate, and indicated amounts of protein. The reaction was incubated for 30 min at 37°C and stopped by addition of gel-loading buffer. Products of the reaction were analyzed on an 8% polyacrylamide gel in 1× TBE. Following electrophoresis, gels were dried and autoradiographed.

ELISA

Purified NS1 protein produced by recombinant baculovirus was diluted to a final concentration of 2 μg/ml in 10 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6. One hundred microliters of the solution was incubated per well of ELISA plates (4°C, overnight). The wells were washed three times with PBS, 0.05% Tween, and blocked with PBS containing 2% BSA at RT for 1 h and washed three times with PBS, 0,05% Tween. HA-tagged Sp1 produced by recombinant baculovirus (kindly provided by Dr. Horowitz, North Carolina State University) was added into the wells (crude Sf9 cell extracts of 10^4 infected cells/well), incubated for 1.5 h at 37°C, and washed three times. After incubation with 100 μl of anti-HA rat monoclonal antibodies (1.5 h at 37°C, dilution 1:1000), the wells were washed followed by an incubation with 100 μl of 1:1000 diluted horseradish peroxidase-conjugated rabbit anti-rat antibody (1.5 h at 37°C). After washing, 100 μl of TMB reagent substrate (BD Pharmingen, Heidelberg, Germany) was added and incubated for 15 min. H_2SO_4 (2 N) was added and the optical density was determined at 450 nm.

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