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Transcriptional Activation by the Parvoviral Nonstructural Protein NS-1 Is Mediated via a Direct Interaction with Sp1

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The nonstructural protein NS-1, encoded by the parvovirus minute virus of mice, is a potent regulator of viral gene expression. NS-1 does not bind DNA in a sequence-specific manner, and the mechanism by which it modulates viral promoter function is unclear. We have used Gal4–NS-1 fusion protein constructs to identify and characterize an activating domain encoded within the C-terminal 88 amino acids of NS-1 which competes effectively with the acidic activator domain of the herpes simplex virus VP16 protein. DNA affinity chromatography and immunoprecipitation experiments demonstrate that protein-protein interactions between the transcription factor Sp1 and NS-1 are required to bind NS-1 to promoter DNA in vitro. Cotransfection of Gal4–NS-1 and Sp1-VP16 acidic activator constructs into *Drosophila melanogaster* Schneider cells, which lack endogenous Sp1, stimulates transcription from a minimal promoter containing five Gal4 binding sites, while single-construct transfections do not. Cotransfection of Schneider cells with wild-type NS-1 and Sp1 constructs activates transcription from a simian virus 40 promoter 10- to 30-fold over that of either construct alone. Thus, Sp1–NS-1 interactions in vivo can stimulate transcription from a heterologous promoter containing Sp1 binding sites.

NS-1, an 83-kDa nuclear phosphoprotein, is the major nonstructural protein encoded by the parvovirus minute virus of mice (MVM). In addition to prominent roles in viral replication (32, 45) and the cytopathic effects associated with parvoviral infection (3, 5), NS-1 is an important regulator of viral gene expression. The MVM genome contains two transcription units; a promoter at map unit 4, termed P4, drives expression of the nonstructural proteins, including NS-1, while the downstream promoter, P38, controls expression of the structural capsid proteins (9). Regulatory effects on transcription from both promoters have been ascribed to NS-1. NS-1 transactivates the expression of viral structural proteins from the downstream P38 promoter (12, 31), enhancing expression approximately 1,000-fold over the low basal level (2). Effects of NS-1 on expression from its own promoter (P4) have been shown to range from activation to inhibition, presumably in a concentration-dependent manner (1, 11, 17, 33). NS-1 also exhibits a *trans*-inhibitory effect on expression from a number of heterologous promoters (21, 33), most notably the Rous sarcoma virus long terminal repeat promoter.

The mechanism by which NS-1 exerts this transcriptional regulation is currently not understood. Cellular and viral transcriptional regulatory proteins have generally been shown to contain two functionally separate domains (4, 30, 43). A binding domain, responsible for linking the protein to the appropriate promoter, allows an activation domain to interact with the transcriptional machinery to stimulate transcription. Activation domains tend to be acidic in nature although domains rich in glutamine or proline residues have also been described (26). Binding domains can interact either directly with specific promoter DNA sequences (26) or indirectly via protein-protein interactions with specific DNA-binding proteins (6, 15, 25, 29, 44). These domains appear to be modular in nature (14), and activation and binding domains from two separate proteins can be combined to generate a distinct, functionally active hybrid protein (4, 30).

The primary amino acid sequence of NS-1, a 672-amino-acid protein, exhibits two potential acidic domains. Both the amino- and carboxy-terminal portions of NS-1 contain a net negative charge. In addition, potential phosphorylation sites exist in both regions that, if utilized, would be expected to further add to the acidic nature of these regions (21, 40). Limited mutational analysis of the NS-1 protein has demonstrated that deletion of sequences at the carboxy terminus abolishes NS-1's ability to transactivate the P38 promoter (21, 40). The *trans*-inhibitory effects of NS-1 on expression from heterologous promoters, however, are stronger if the acidic domains at both the amino and carboxy termini are present (21). Work with the closely related H-1 parvovirus has shown that the mutation of a single amino acid (Lys-405→Ser) in the purine binding site of the NS-1 ATPase domain eliminates all known NS-1 functions (23). Previous studies have also suggested that the NS-1 protein is not a sequence-specific DNA-binding protein (2) and might therefore depend on interactions with one or more other proteins to target viral promoters. Mutational analysis of the MVM P38 promoter has shown that NS-1 transactivation is dependent only on the presence of functional TATA and GC box sequences in the promoter (2). The P4 promoter also contains a functional GC box as a major element in its promoter motif, and the transcription factor Sp1 is known to bind to the GC boxes present in both the P4 and P38 promoters (2, 28). Thus, transcriptional regulation by the NS-1 protein may involve an interaction with Sp1 bound to the promoter region. The work presented in this paper describes a series of experiments designed to test this hypothesis.

MATERIALS AND METHODS

Constructs. Constructs containing the wild-type NS-1 GalNS1(1–672) or N-terminal deletions GalNSdN360(360–672) and GalNSdN584(584–672) were constructed by inserting the appropriate NS-1 DNA fragment in frame to the

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Gal14(1–147) sequence in the vector pSG424 (36). The carboxy-terminally deleted clone, GalNSdC129, was generated by digestion of the GalNS1 clone with *Bst*EII and *Not*I, filling in of the resulting overhangs with Klenow fragment, and religation. This procedure leads to the removal of amino acids 543 to 672 of the NS-1 protein. The Sp1VP16 fusion was constructed by inserting the DNA sequence for the C-terminal 696 amino acids of the Sp1 protein in frame behind the acidic activating domain of the herpes simplex virus VP16 protein in the vector AAVP16 (46). The construct NS1dC129 was generated by digesting pKONS1/2 with the restriction enzyme *Bst*EII, filling in the overhangs with Klenow fragment, and religating. This results in the premature termination of the NS-1 transcript, resulting in a mutant form of NS-1 lacking the carboxy-terminal 129 amino acids. A similar mutant was previously described in work by Skiadopoulos et al. (40). The junctions of all fusion constructs were checked by DNA sequencing to confirm that fusions were in frame. The plasmid used to generate NS-1 protein in the reticulocyte lysate experiments was generated by cloning the NS-1 coding sequence downstream of the T7 promoter sequence in the pGEM3ZF+ vector.

pADHNS-1 contains the full-length NS-1 sequence cloned downstream of the *Drosophila melanogaster* alcohol dehydrogenase promoter in the vector pADH. Plasmid pAc-Sp1 has been previously described (10) and was a generous gift from A. Courey.

The G5EC (35, 46), GalVP16 (35, 46), P38CAT (2), and pSV2XCAT (2) reporter constructs as well as the wild-type NS-1 expression construct pKONS1/2 (2) have all been described elsewhere.

Cell culture and transfection. Mouse A92L fibroblast cells were maintained in Dulbecco modified Eagle medium, supplied by Gibco, containing 10% fetal calf serum and 1× glutamine-penicillin-streptomycin. A DEAE-dextran-mediated transfection method was used to introduce the plasmid DNA into the A92L cells (37). Cells were plated at a density of 1.5×10^6 cells per 100-mm plate 16 to 24 h prior to transfection. Quantities of DNA used in the transfection experiments are provided in the figure legends. To ensure equivalent transfection efficiencies, the total amount of DNA transfected was kept constant; DNA in the form of a pGEM vector was used as carrier DNA to adjust total DNA concentrations. Following transfection, the cells were grown for 40 to 48 h prior to harvesting. The protocol used for the chloramphenicol acetyltransferase (CAT) assays has been previously described (37). Following cell harvest, the cell pellets were resuspended in 150 μl of Tris-HCl (0.25 M, pH 7.9) and subjected to five rounds of freezing and thawing to lyse the cells. Following lysis, the cell supernatant was collected and the protein concentration was determined with Coomassie protein assay reagent purchased from Pierce. The CAT assays were run with equivalent amounts of protein based on these assays. CAT assays were run in duplicate, and in the case of the competition experiments, assays were run at least three separate times to ensure the results. Comparisons were done only on those assays that were in the linear range of CAT activity, i.e., between 0.5 and 75% acetylation. Quantitation of the CAT assays was done on a PhosphorImager (Molecular Dynamics).

The maintenance and transfection of *Drosophila* embryo Schneider cells (line SL2) were carried out by a previously described protocol (10). Amounts of the various DNA constructs transfected are shown in the figure legends. The amount of DNA transfected was normalized to 20 μg per 100-mm plate with pGEM plasmid DNA. CAT assays were performed and analyzed as described above.

NS-1 retention studies. The P38 promoter fragment was isolated by digestion of plasmid p38RH (2) with *Nco*I and *Xho*I. The 180-bp fragment was gel purified and biotinylated by Klenow fragment fill-in of the restricted ends with a Bio-dUTP nucleotide analog. The fragment was incubated with streptavidin agarose resin. A total of 1 pmol of the DNA fragment was bound per 50 μl of resin. [^{35}S]methionine-labelled NS-1 protein was made with a rabbit reticulolysate system (Promega). The plasmid used had the NS-1 sequence cloned in front of the T7 promoter in the pGEM3ZF vector. Crude nuclear extract (CNE) was prepared from the murine fibroblast cell line A92L. The protocols used have previously been described in detail (28). CNE was depleted of Sp1 by the incubation of 1 ml of CNE with 5 μl of a polyclonal antibody to Sp1 (a generous gift from S. Jackson and R. Tjian) for 2 h at 4°C, followed by treatment with protein A-Sepharose to clear the antigen-antibody complex. The antibody used is one that is known to recognize both human and murine forms of the Sp1 protein. The protein A-Sepharose antigen-antibody complex was pelleted by brief centrifugation, and the cleared supernatant was used as CNE (without Sp1). Binding reactions were performed in a final volume of 500 μl and were set up as follows. A total of 50 μl of CNE, with or without Sp1 (10 mg of protein per ml), 10 μl of labelled NS-1 (20,000 cpm), and 10 μl of poly(dI-dC) (2 mg/ml) were incubated with 50 μl of the DNA resin on a nutator for 30 min at room temperature. Final buffer conditions for the binding were 60 mM KCl–6 mM $MgCl_2$–12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9])–12% glycerol–2% polyvinyl alcohol–10 μM $ZnCl_2$. In addition, all binding reaction mixtures and washes contained proteinase inhibitors. Final concentrations for these inhibitors were 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 50 μg each of aprotinin, leupeptin, and pepstatin per ml. Following binding, the resin was washed five times with 1 ml of buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 0.1% Nonidet P-40, 10 μM $ZnCl_2$) containing 0.1 M KCl and eluted with 500 μl of buffer D plus 0.2 M KCl followed by 500 μl of buffer D plus 1.0 M KCl. Proteins present in the final wash and the two KCl eluates were precipitated by trichloroacetic acid and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prior to autoradiography, the gel was fixed and treated with Amplify (Amersham).

Protein staining of P38-bound proteins. Binding reaction mixtures were identical to those described above except that the labelled NS-1 was not included. Proteins present in the eluted fractions were biotinylated by procedures outlined previously (16). Briefly, 1 μl of 5 N NaOH was added to the 500 μl of eluate to raise the pH above 8.0. NHS-X-Biotin (Pierce) was dissolved at a concentration of 250 mM in dimethyl formamide and added to the eluates at a final concentration of 25 mM. The solution was nutated for 30 min at room temperature. Following incubation, lysine, at a final concentration of 100 mM, was added to stop the reaction. Proteins in the solution were precipitated by treatment with trichloroacetic acid and resuspended, and samples were run on SDS-polyacrylamide gels. Following electrophoresis, the gel was electroblotted onto an Immuno-Lite membrane (Bio-Rad). Streptavidin-alkaline phosphatase complex at a 1:5,000 dilution was bound to the biotinylated proteins present on the blot and detected by chemiluminescence, according to the manufacturer's procedure (Bio-Rad).

Coimmunoprecipitation of labelled NS-1 with antibody to Sp1. Protein extracts and binding conditions were the same as those described in the preceding sections with the following exceptions. Immunoprecipitations done with the NS-1–bovine serum albumin (BSA) reaction mixture contained BSA at a protein concentration equivalent to that in the CNE. The purified Sp1 extract was prepared from CNE following a previously described protocol (28) and was incubated at a concentration of 50 ng of protein per binding reaction mixture. Experiments were also performed in which the binding reaction mixture was treated with ethidium bromide and nucleases prior to coimmunoprecipitation. In these reaction mixtures, the concentration of ethidium bromide was 100 μg/ml and the nucleases, RNase A and DNase, were at 20 μg/ml. Binding reaction mixtures contained the NS-1 and protein extracts in the same buffer system as described above. The P38 promoter DNA fragment was not included in the binding reaction mixture. Following a 15-min incubation at room temperature, 2 μl of the Sp1 antibody was added to the reaction mixture, which was then further incubated for 2 h at 4°C. Protein A-Sepharose was added to the reaction mixture, and the incubation was continued for another 30 min. The protein A-Sepharose complex was then pelleted by brief centrifugation, and the pellet was washed extensively in buffer D containing 0.1 M KCl. SDS-PAGE and autoradiography were performed as described above.

RESULTS

In order to identify regions of the NS-1 protein that might play important roles in transcriptional activation, amino- and carboxy-terminally deleted portions of NS-1 were cloned in frame after the Gal4 DNA binding domain in plasmid pSG424. These constructs were then cotransfected into mouse fibroblast (A92L) cells along with a CAT reporter construct (G5EC) containing five Gal4 binding sites in front of a minimal promoter. Following transient transfection for 40 to 48 h, lysates were prepared and analyzed for CAT activity.

NS-1 contains a potent activation domain. GalNS1 containing the wild-type NS-1 protein was assayed for its ability to activate transcription from the G5EC reporter construct. Figure 1 shows the results of these assays. CAT expression was enhanced approximately 200-fold over G5EC plus the Gal4 binding domain alone [pSG424/Gal4(1–147)]. The GalVP16 construct which contains the activation domain of the herpes simplex virus VP16 protein fused to the Gal4 DNA binding domain was used as a positive control. The activation domain of the VP16 protein is the most potent acidic activator currently known (35). The GalNS1 construct exhibited activation levels that were 30 to 50% of those of the GalVP16 construct. When cotransfected with the G5EC construct, the wild-type NS-1 without the Gal4 binding domain (plasmid pKONS1/2) exhibited no activity, indicating that NS-1 must be targeted to the promoter to be effective. The ability of the GalNS1 plasmid to activate transcription of a P38CAT reporter construct was also tested to ensure that fusion to the Gal4 DNA binding domain did not inhibit NS-1 activity. Results of these analyses demonstrated that the GalNS1 plasmid can transactivate P38CAT to the same levels as that of the wild-type pKONS1/2 plasmid. The GalVP16 plasmid has no effect on activation from the P38 promoter. Taken together, our results suggest that the NS-1 protein contains a potent activation domain and

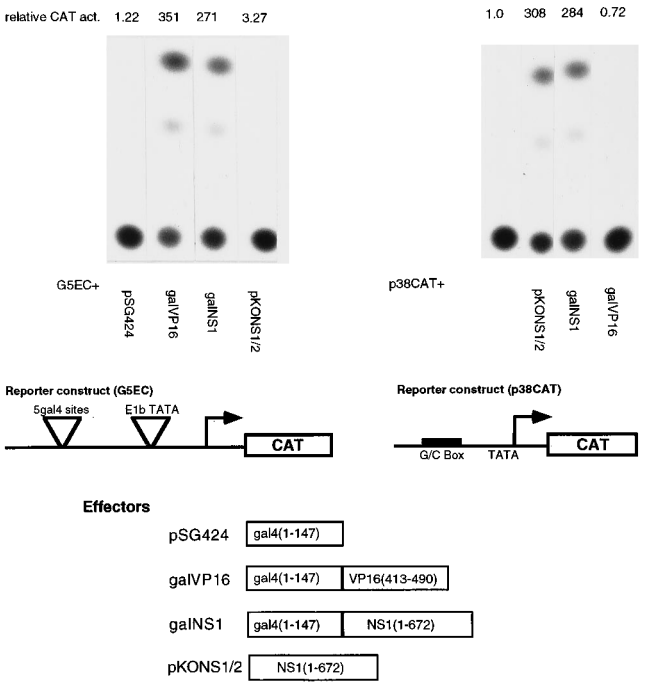

FIG. 1. GalNS1 can activate transcription from the G5EC reporter construct and is similar to wild-type NS-1 in activating the P38 promoter. Four micrograms of the reporter construct G5EC containing five Gal4 binding sites upstream of a minimal promoter or the P38CAT reporter construct was used to transfect A92L cells along with either 500 ng of the pSG424 construct, 300 ng of the GalVP16 plasmid, 500 ng of the GalNS1 plasmid, or 500 ng of the pKONS1/2 plasmid. Cells were subsequently lysed and assayed for CAT activity according to standard protocols (37). Relative CAT activity was determined on the basis of the average percent acetylation values obtained with a PhosphorImager from at least three separate experiments. Activity of the reporter construct alone was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this activity. An autoradiogram of a representative thin-layer chromatography plate is shown.

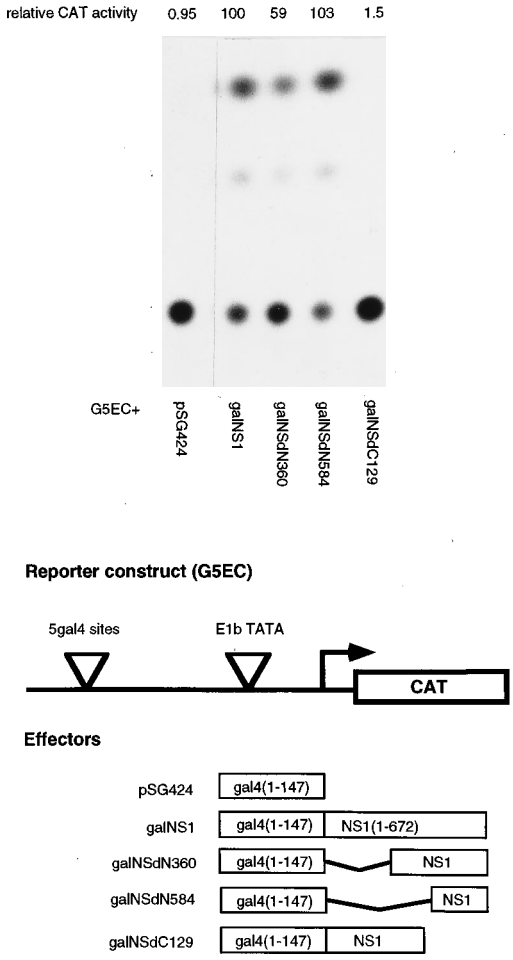

FIG. 2. The ability of various NS-1 regions fused to the Gal4 binding domain to activate transcription from the G5EC reporter construct. Five micrograms of the reporter plasmid G5EC was used to transfect A92L cells with either 4 μg of the pSG424 plasmid (lane 1), 4 μg of the GalNS1 plasmid (lane 2), 4 μg of the plasmid GalNSdN360 (lane 3), 4 μg of the plasmid GalNSdN584 (lane 4), or 4 μg of the plasmid GalNSdC129 (lane 5). Cells were subsequently lysed and assayed for CAT activity as described in the legend to Fig. 1. An autoradiogram of the resulting thin-layer chromatography plate along with the relative CAT activity based on a GalNS1 activity of 100 is shown.

that the ability of NS-1 to activate transcription is dependent on its ability to come into close proximity with the promoter region.

The activation domain of NS-1 is located in the carboxy terminus of the protein. To further delineate the region of the NS-1 protein responsible for transcriptional activation, deletions were made in both the amino and carboxy termini of the NS-1 protein and cloned in frame behind the Gal4 DNA binding domain. The effects of these GalNS1 deletion mutants on transcriptional activity are shown in Fig. 2. Deletion of the carboxy-terminal 129 amino acids of NS-1 (plasmid GalNSdC 129) reduces the ability of the GalNS1 fusion construct to activate transcription from the G5EC reporter plasmid by a factor of 70-fold compared with the GalNS1 construct. Activity of the GalNSdC129 construct was only slightly higher than transfection with the G5EC reporter construct alone. Deletions in the amino terminus of NS-1 did not markedly affect activation of CAT activity by the GalNS1 constructs. For example, the GalNSdN360 plasmid, in which the N-terminal 360 amino acids were deleted, gave activation that was only slightly less than the wild-type (GalNS1) construct. Furthermore, the construct GalNSdN584, in which all but the carboxy-terminal 88 amino acids of the NS-1 protein were deleted, activates CAT expression from the G5EC reporter construct to the same levels as those seen with the full-length GalNS1 construct. These results indicate that the NS-1 protein contains a strong activation domain that is localized within the carboxy-terminal 88 amino acids.

The activation domain in NS-1 acts in a fashion similar to that of the activation domain of VP16. Previous studies have shown that the C-terminal region of NS-1 exhibits a net negative charge, and it has been suggested that it might encode a potential acidic activation domain (21, 40). Therefore, experiments were designed to examine whether the NS-1 activation domain and the activation domain of a member of the acidic activator class, VP16, could compete with each other for target proteins important in the activation of transcription by acidic activators.

If NS-1 and VP16 activate transcription through similar mechanisms, then increased expression of either should inhibit, by a squelching mechanism, not only their own activational ability but the activational ability of the other as well. To test whether the activation domains of NS-1 and VP16 could inhibit each other's activational capacity, experiments were designed in which the G5EC reporter construct was cotransfected along with either the GalNS1 or GalVP16 activator

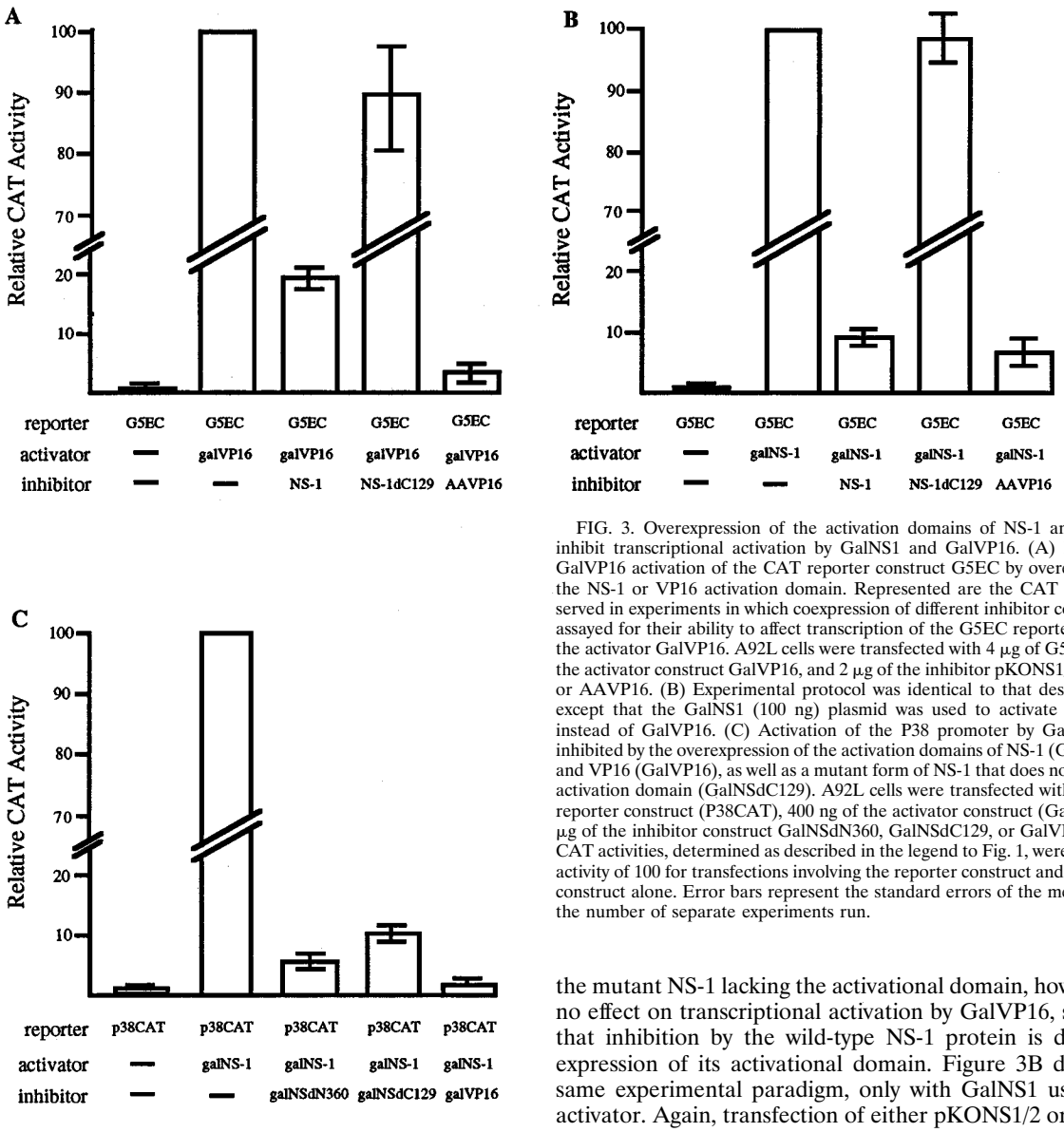

FIG. 3. Overexpression of the activation domains of NS-1 and VP16 can inhibit transcriptional activation by GalNS1 and GalVP16. (A) Inhibition of GalVP16 activation of the CAT reporter construct G5EC by overexpression of the NS-1 or VP16 activation domain. Represented are the CAT activities observed in experiments in which coexpression of different inhibitor constructs was assayed for their ability to affect transcription of the G5EC reporter plasmid by the activator GalVP16. A92L cells were transfected with 4 μg of G5EC, 20 ng of the activator construct GalVP16, and 2 μg of the inhibitor pKONS1/2, NSdC129, or AAVP16. (B) Experimental protocol was identical to that described above except that the GalNS1 (100 ng) plasmid was used to activate transcription instead of GalVP16. (C) Activation of the P38 promoter by GalNS1 can be inhibited by the overexpression of the activation domains of NS-1 (GalNSdN360) and VP16 (GalVP16), as well as a mutant form of NS-1 that does not contain the activation domain (GalNSdC129). A92L cells were transfected with 4 μg of the reporter construct (P38CAT), 400 ng of the activator construct (GalNS1), and 2 μg of the inhibitor construct GalNSdN360, GalNSdC129, or GalVP16. Relative CAT activities, determined as described in the legend to Fig. 1, were based on an activity of 100 for transfections involving the reporter construct and the activator construct alone. Error bars represent the standard errors of the mean based on the number of separate experiments run.

alone or with one of three inhibitor constructs. The inhibitor constructs used expressed either wild-type NS-1 (pKONS1/2), the activational domain of VP16 (AAVP16), or an NS-1 mutant protein in which the activational domain has been deleted (NS-1dC129). In the experiment whose results are depicted in Fig. 3A, GalVP16 was cotransfected into A92L cells along with the reporter construct G5EC and CAT activity was subsequently assayed. This CAT activity was assigned a relative value of 100. Similar transfections were then performed in which in addition to transfection with G5EC and GalVP16, one of the three inhibitor constructs was also transfected. Assays were then performed to determine what effect expression of these inhibitors had on the ability of GalVP16 to activate CAT expression from the G5EC reporter construct. CAT activity from these experiments was expressed in relation to the activity observed with transfection of G5EC and GalVP16 alone. Figure 3A shows that cotransfection of either pKONS1/2 or AAVP16 dramatically inhibits the activation of CAT expression by GalVP16. Expression of the NS-1 protein inhibited activation 5-fold while expression of the VP16 activational domain inhibited activation 20-fold. Transfection of the mutant NS-1 lacking the activational domain, however, had no effect on transcriptional activation by GalVP16, suggesting that inhibition by the wild-type NS-1 protein is due to the expression of its activational domain. Figure 3B depicts the same experimental paradigm, only with GalNS1 used as the activator. Again, transfection of either pKONS1/2 or AAVP16 can inhibit GalNS1 transcriptional activation of the G5EC reporter construct whereas transfection of the NS-1 activational domain mutant does not. Inhibition by both NS-1 and the VP16 activation domain was on the order of 20-fold. None of the constructs used as inhibitors had any appreciable effect on transcription from a simian virus 40 (SV40) promoter-driven CAT reporter construct (pSVXCAT), suggesting that the inhibition is not the result of a generalized inhibition of transcription by these constructs (data not shown). Taken together, these results suggest that the NS-1 and VP16 activational domains utilize a similar set of cofactors in their mechanism of activation.

Regions outside the carboxy terminus appear to be important in targeting NS-1 to the promoter region. We also performed a series of experiments designed to look for regions of NS-1 that may play a role in targeting the NS-1 protein to specific promoter regions. These experiments involved assaying the ability of the carboxy-terminally deleted GalNS1 fusion construct (GalNSdC129) to inhibit transactivation of the P38CAT construct by GalNS1 (Fig. 3C). As controls, an amino-terminally deleted GalNS1 fusion construct (GalNSdN360)

and GalVP16 were also assayed for their ability to inhibit transcriptional activation of P38CAT by the GalNS1 activator. As expected, expression of both the activational domain of NS-1 (GalNSdN360) and the activational domain of VP16 (GalVP16) was found to inhibit the ability of GalNS1 to activate CAT expression from the P38CAT reporter construct. It was also shown, however, that expression of a mutant form of the NS-1 protein lacking the activation domain (GalNSdC129) was also able to inhibit activation of the P38 promoter by GalNS-1. Because the GalNSdC129 construct has no ability to activate transcription, we believe that the inhibition is not due to the sequestering of activation domain target proteins but instead is the result of inhibiting the ability of the full-length, active NS-1 protein to interact with proteins responsible in targeting the NS-1 protein to the P38 promoter region. While this interpretation is speculative, it did provide the impetus for experiments designed to search for such interacting factors.

Sp1 is required for the binding of NS-1 to the promoter region. To test for proteins that may be required for bringing the NS-1 protein in contact with specific promoter regions, a 180-bp fragment of DNA containing the P38 promoter was isolated by restriction digestion and the 5′ overhangs were labelled with a biotinylated dUTP nucleotide analog with a Klenow fragment fill-in procedure. The biotinylated DNA fragment was then bound to a streptavidin-agarose resin. The resin was incubated, in batch, with CNE prepared from mouse A92L fibroblast cells and in vitro-synthesized [^{35}S]methionine-labelled NS-1. The resin was then washed extensively with binding buffer containing 0.06 M KCl and eluted, in two separate steps, with binding buffer containing 0.2 M and 1.0 M KCl. The 1.0 M KCl wash is sufficient to remove all proteins bound to the DNA fragment. The unbound supernatant and eluted fractions were then analyzed by SDS-PAGE and autoradiography. Initial results showed that NS-1 could be retained on the column in the presence of proteins found in the CNE (Fig. 4). A number of control experiments were carried out to determine if this interaction was specific. When BSA protein was used in place of the CNE, the NS-1 protein was no longer retained on the column following the washes (data not shown), indicating a specific interaction of NS-1 with proteins present in the CNE. NS-1 was also not retained if the promoter fragment was not included in the binding reaction mixture or if the promoter fragment was not previously biotinylated (data not shown), ruling out the possibility that the NS-1 protein was interacting nonspecifically with the DNA resin.

To test whether NS-1 was interacting with Sp1 present in the CNE, the extract was cleared of Sp1 by incubation with an antibody to Sp1 and subsequent binding of the antibody-antigen complex to protein A-Sepharose. This Sp1$^-$ CNE was then tested for its ability to retain NS-1 on the P38-containing DNA resin (Fig. 4). CNE containing Sp1 is shown to retain the NS-1 protein whereas the protein extract cleared of Sp1 does not. The differences in retention between the two extracts were on the order of 30-fold. The small amount of NS-1 retained in the incubations with Sp1$^-$ CNE is comparable to the amount retained when BSA is used instead of CNE and probably indicates a small amount of nonspecific binding. Both major bands present in the autoradiograms react with antibody to the carboxy terminus of NS-1 (data not shown). The bottom band most likely represents a proteolytic breakdown product of the full-length NS-1 protein. The proportions of these two bands varied between translation reactions. Figure 4B shows the proteins present in CNE that bind to the P38 promoter under our binding conditions. Four proteins with apparent molecular masses of 175, 150, 110, and 95 kDa are retained by the P38 promoter fragment. While the identities of these proteins are

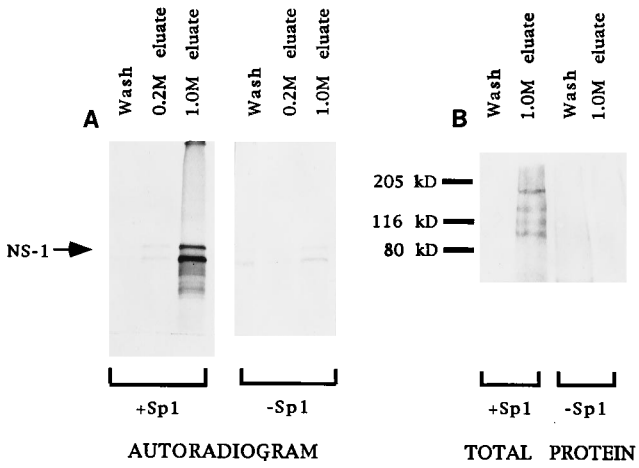

FIG. 4. NS-1 is bound to the P38 promoter in the presence of Sp1. (A) Radiolabelled NS-1 is retained by a biotinylated P38 promoter DNA resin in the presence of CNE containing Sp1 but not CNE depleted of Sp1. The autoradiogram shows the result of experiments in which [^{35}S]methionine-labelled NS-1 is incubated with biotinylated P38 promoter DNA linked to streptavidin-agarose and CNE either containing Sp1 or depleted of Sp1. Following binding, the resin was washed extensively with binding buffer and bound proteins were eluted with buffer containing increasing KCl concentrations. Increasing concentrations of KCl elute the labelled NS-1 protein when the incubations are carried out with CNE with Sp1. Incubations performed with CNE depleted of Sp1 fail to retain NS-1 in the resin, and no protein is detected by autoradiography from eluted protein fractions. (B) Proteins present in various KCl washes were biotinylated with NHS-X-Biotin, run on SDS-polyacrylamide gels, and electroblotted onto membranes. Streptavidin-alkaline phosphatase was bound to the biotinylated proteins present on the blot and detected by a chemiluminescence procedure. The autoradiograph depicts the proteins that bind to the P38 promoter under our binding conditions when either CNE or the Sp1$^-$ CNE is used.

unknown, two of the proteins exhibit molecular masses similar to those of the Sp1 doublet (105 and 95 kDa). Incubation of the CNE with antibody to Sp1 and the subsequent clearing of the antibody-antigen complex by protein A-Sepharose eliminate almost all of the proteins present in the CNE that bind to the P38 promoter fragment. These results suggest the possibility of a direct interaction between the Sp1 and NS-1 proteins, although an indirect interaction between the two proteins through a separate Sp1-binding protein cannot be ruled out.

Antibodies to Sp1 can coimmunoprecipitate the NS-1 protein. As a further test of the interaction between Sp1 and NS-1, we asked whether an antibody to the Sp1 protein could coimmunoprecipitate the NS-1 protein from a complex mixture of proteins. CNE from A92L cells containing Sp1 was incubated with ^{35}S-labelled NS-1 in binding reaction mixtures similar to those described above. The extract–NS-1 mixture was incubated with an antibody to Sp1 and then further incubated with protein A-Sepharose. The protein A-Sepharose antibody-antigen complex was spun down and washed extensively in binding buffer containing 0.1 M KCl. Protein present in the washed pellet was then analyzed by SDS-PAGE and autoradiography. As a control, immunoprecipitations were done with preimmune serum instead of the Sp1 antibody to eliminate the possibility of the NS-1 protein interacting nonspecifically with immunoglobulins. Coimmunoprecipitations were also performed on mixtures of NS-1 and BSA, NS-1 and purified Sp1 protein, and NS1 and CNEs previously cleared of Sp1 in the manner described previously. Immunoprecipitations were also performed subsequent to the treatment of the binding mixtures with ethidium bromide and nucleases to eliminate the possibility that NS-1 and Sp1 interact indirectly through binding to nucleic acid moieties. Immunoprecipitations involving the pre-

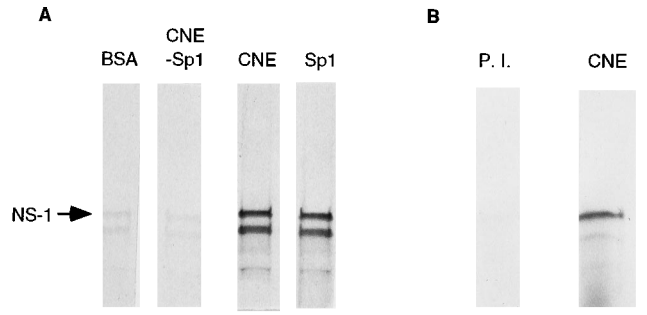

FIG. 5. Antibody to Sp1 can coimmunoprecipitate NS-1. Autoradiogram showing the coimmunoprecipitation of labelled NS-1 from various protein solutions. The Sp1 antibody can precipitate labelled NS-1 from binding reaction mixtures with normal CNEs or purified Sp1. Binding reaction mixtures devoid of Sp1 fail to precipitate NS-1 out of solution, indicating that NS-1 does not react nonspecifically with the Sp1 antibody. When NS-1 is incubated with protein extracts containing Sp1, the Sp1 antibody can coimmunoprecipitate the NS-1. Treatment of the binding reaction mixtures with 100 μg of ethidium bromide per ml and 20 μg each of DNase and RNase A per ml prior to immunoprecipitation did not affect the NS-1–Sp1 interaction, suggesting that nucleic acid moieties do not play a role in the interaction. Binding reaction mixtures were incubated with an antibody to Sp1 for 2 h at 4°C; incubation was followed by pelleting of the antigen-antibody complex by treatment of the reaction mixture with protein A-Sepharose. The protein A-Sepharose complex was washed extensively with binding buffer prior to analysis of the complexed proteins by SDS-PAGE and autoradiography. (A) Untreated extract; (B) extract treated with ethidium bromide and nucleases. P. I., preimmune serum.

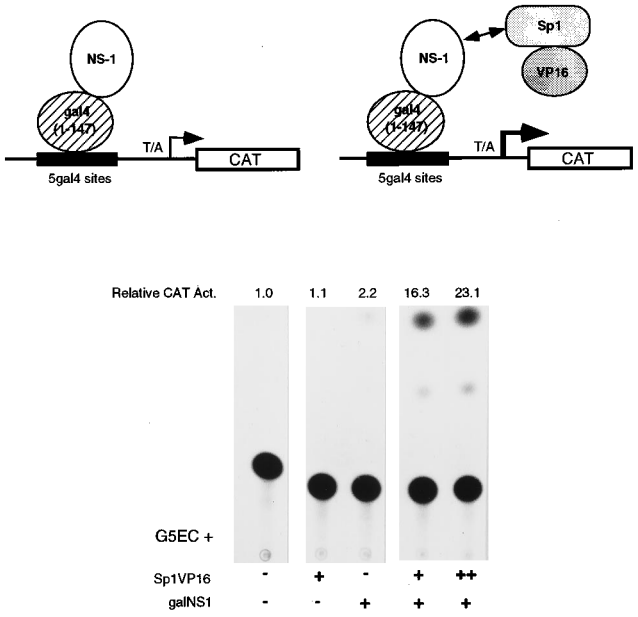

FIG. 6. NS-1 and Sp1 can interact in vivo. Constructs in which the NS-1 protein was fused to the Gal4 DNA binding domain and Sp1 sequences were cloned behind those encoding the activation domain of VP16 were cotransfected into Schneider cells along with a CAT reporter construct containing five Gal4 binding sites upstream of a minimal promoter. The top panel shows the experimental paradigm diagrammatically. The bottom panel depicts an autoradiogram of the resulting CAT assays. The GalNS1 and Sp1VP16 constructs have little effect on transcription from the G5EC reporter construct when transfected individually. When the fusion constructs are cotransfected, however, a significant increase in CAT expression is produced, suggesting an interaction between the two proteins in vivo. In lanes 2 to 4, 2 μg of each construct was used; lane 5 results were obtained with 2 μg of GalNS1 and 4 μg of Sp1VP16. Relative CAT activity (Act.) was determined as described in the legend to Fig. 1. Activity of the reporter construct alone was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this activity.

immune sera, BSA, or the CNE depleted of Sp1 failed to coimmunoprecipitate the NS-1 protein (Fig. 5). However, immunoprecipitations with CNE containing Sp1 or the highly purified Sp1 did coimmunoprecipitate the NS-1 protein. Prior treatment of the binding reaction mixture with ethidium bromide, DNase, and RNase A also had no effect on the coimmunoprecipitation of NS-1 by the Sp1 antibody. NS-1 was coimmunoprecipitated out of mixtures containing Sp1 at a 30-fold greater level than that seen with mixtures not containing Sp1. The coimmunoprecipitation of NS-1 from solutions containing highly purified Sp1 also suggests that this is a direct interaction between NS-1 and Sp1. Because the binding reaction mixtures used in this experiment did not contain promoter fragment DNA, the interaction between Sp1 and NS-1 was also shown to be independent of Sp1 being bound to the promoter.

Sp1 can interact with NS-1 in vivo. The in vitro experiments demonstrated an ability of Sp1 and NS-1 to interact under controlled conditions of salt and temperature; however, these types of experiments cannot predict whether an interaction can take place in the cellular environment. To test for an interaction in vivo, expression constructs were made in which the NS-1 protein was fused, in frame, behind the Gal4 DNA binding domain, and the Sp1 protein was fused, in frame, behind the potent acidic activating region of the protein VP16. These constructs were transfected into a Schneider cell line along with a CAT reporter construct containing five Gal4 binding sites upstream of a minimal promoter. Schneider cells were chosen because of their lack of endogenous Sp1 (10, 38), which could hinder interpretation of any possible interactions. Additionally, the GalNS1 fusion protein has only a weak transcriptional activation activity in this cell line. Interaction between the NS-1 and Sp1 proteins would be expected to activate transcription from the reporter construct by bringing the potent VP16 activating region in contact with the transcriptional machinery. Figure 6 shows the results of experiments done with these constructs. By itself, the GalNS1 construct has only a small effect on transcription from the reporter construct while transfection of the Sp1VP16 construct alone has no effect on transcription. Cotransfection of the two constructs increased transcription 10- to 20-fold depending on the amounts transfected. Transfection of GalNS1 and the VP16 activation domain or GalNS1 and a number of other VP16 protein fusions showed no activity (data not shown), further suggesting a specific interaction between the NS-1 and Sp1 proteins. These results indicate that the NS-1 and Sp1 proteins do interact in vivo and that the interaction is specific.

NS-1 and Sp1 can interact to activate transcription from the SV40 promoter in Schneider cells. To determine if the interaction between Sp1 and NS-1 can lead to changes in the transcriptional regulation of promoters containing Sp1-binding GC boxes, we examined the effect that coexpression of NS-1 and Sp1 has on transcription from the SV40 early/late promoter. The SV40 promoter contains five Sp1 binding sites known to be important in its regulation (13). Again, to avoid the effects of endogenous Sp1, Schneider cells were used. Constructs expressing NS-1 and Sp1 were transfected separately or together with an SV40 CAT reporter construct. Following transient transfection, the cells were harvested and cell lysates were analyzed for CAT activity. Previous work has shown that Sp1 alone can activate transcription from the SV40 promoter in Schneider cells; the level of activation, however, is less than that seen in mammalian cells (10). As can be seen in Fig. 7, expression of Sp1 activates a moderate level of CAT expression from the SV40 promoter as expected, while expression of NS-1 by itself has no effect on transcription. However, when the NS-1 and Sp1 proteins are both expressed, a significant

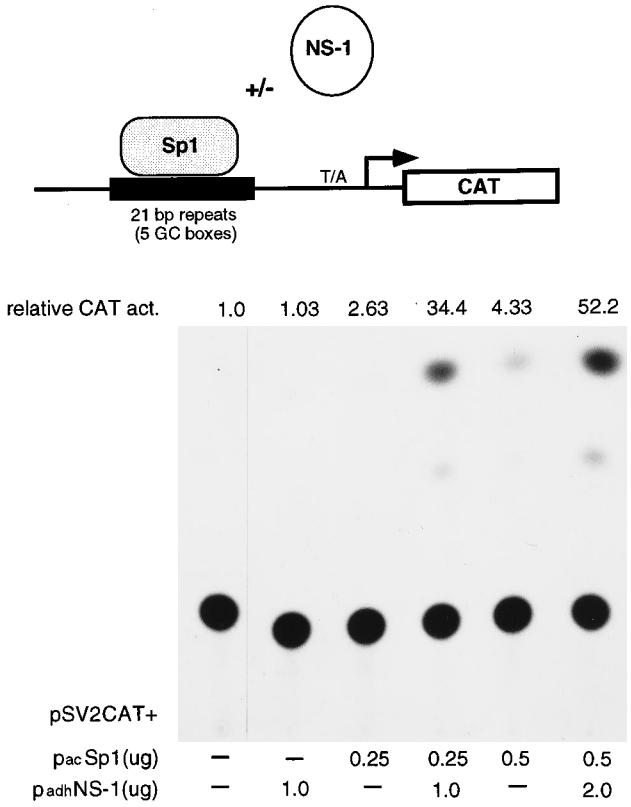

FIG. 7. NS-1 and Sp1 can interact in vivo to activate transcription from a reporter construct containing multiple Sp1 binding sites. Constructs expressing NS-1 and Sp1 were transfected into Schneider cells individually or together along with a reporter construct driven by the SV40 promoter. The SV40 promoter contains five potential Sp1 binding sites and is known to be regulated by Sp1. The top panel shows the experimental design in diagrammatic form. The bottom panel shows an autoradiogram of the various CAT assays. NS-1 transfected alone fails to activate expression from the SV40 promoter (pSV2XCAT). When Sp1 is transfected alone, a moderate level of activation is observed. Cotransfection of NS-1 and Sp1 constructs leads to a substantial activation of expression from the SV40 promoter. These results suggest that NS-1 can interact with Sp1 bound in the promoter region to upregulate transcription. Relative CAT activity (act.) was determined as described in the legend to Fig. 1. Activity of the reporter construct alone was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this activity.

activation in transcription is observed. This activation occurred over a wide range of Sp1 concentrations, although the extent of activation did appear to decrease slightly at higher Sp1 concentrations. Activation ranged from 10- to 30-fold depending on the initial amount of Sp1 transfected. The two concentrations of Sp1 DNA transfected in the experiments represent concentrations previously shown to result in in vivo levels of Sp1 protein in the range normally found in mammalian cells (10). These experiments indicate that NS-1 and Sp1 can interact in vivo and that this interaction can affect the transcriptional regulation of the SV40 promoter.

DISCUSSION

The results presented here indicate that NS-1 exhibits separate domains important in both activation of transcription and the targeting of the activation domain to the appropriate promoter region. Domain swapping experiments were used to demonstrate a potent activational domain encoded within the terminal 88 amino acids of the NS-1 protein. Binding of NS-1 to specific promoter regions was shown to occur through an interaction of NS-1 with the general transcription factor Sp1. Results of both in vitro binding experiments and cotransfection experiments in vivo show that the NS-1 protein interacts specifically with the Sp1 protein and that this interaction can lead to a dramatic increase in expression from a promoter containing Sp1 binding sites.

The acidic activation domain of NS-1. Previous studies designed to characterize specific regions of the NS-1 protein important in transcriptional regulation used in vivo cotransfection assays to measure the abilities of various mutant forms of NS-1 to transactivate a P38CAT reporter construct (23, 33, 40). While providing some important insights into NS-1 function, the design of these studies prevented a clear analysis of specific domain regions within the NS-1 protein. In addition, the use of a P38 promoter-driven reporter construct did not permit the differentiation between mutational effects that altered the ability of NS-1 to target the promoter from those that affect NS-1's activational domain. The domain swapping experimental paradigm used here eliminates these problems. When the terminal 88 amino acids of NS-1 are fused to the Gal4 DNA binding domain, these amino acids were able to activate transcription from the G5EC reporter construct to the same levels as those of the full-length GalNS1 construct. A third construct, GalNSdN360, did exhibit a slightly lower activation potential than either the GalNS1 or GalNSdN584 construct. The reason for this is not readily apparent, but it might be the result of spatial constraints. It may be that with this particular construct the length of the fusion protein was not appropriate for ideal binding to factors in the transcriptional complex. Our results also suggest that this is the only activational domain present in the NS-1 protein. The lack of activity in the carboxy-terminally deleted construct, GalNSdC129, coupled with the equivalent activity of the GalNS1 and GalNSdN584 constructs would argue against a possible activation domain in the amino terminus. While it is possible that the fusion of the Gal4 sequences to the amino terminus of the NS-1 protein might inhibit a potential activity in this region, our data indicating that the GalNS1 construct can activate transcription from the P38CAT reporter construct to the same levels as wild-type NS-1 suggests that any masking effect due to the fusion of the Gal4 DNA binding domain to NS-1 is minimal. Experiments were also done that indicate that the activation domain of NS-1 acts in a fashion similar to that of the activational domain of the herpesvirus VP16 protein. Overexpression of either protein was shown to inhibit the ability of both NS-1 and VP16 to activate transcription. The ability of the two proteins to compete for factors important in transcriptional activation lends additional support to existing sequence data suggesting that this region of NS-1 might encode an acidic activation domain. Further work is required, however, to understand exactly how NS-1 mediates transcriptional activation and what cofactors or transcription-associated factors (TAFs) it utilizes in its activation.

How does NS-1 interact with appropriate promoter regions? Research to date has been unable to show an interaction of NS-1 with specific DNA sequences. Our experiments measuring the ability of the P38 fragment to retain NS-1 in defined binding conditions provide further evidence that in the absence of specific DNA-binding proteins the NS-1 protein does not interact with sequences present in the P38 promoter. NS-1 is not retained by the P38 fragment when incubated with protein extracts not containing Sp1. Activity of the herpes simplex virus VP16 protein depends on its ability to interact with proteins bound at Oct-1 sites present in herpes simplex virus promoters (15, 20, 42), while recent evidence suggests that one mode by

which the adenovirus E1a protein can activate transcription involves an interaction with the ATF-2 DNA-binding protein (24, 25). Our data indicate that one mode by which NS-1 can target specific promoter regions involves an interaction with Sp1.

The nature of the NS-1–Sp1 interaction. The data presented here strongly suggest that the proteins exhibit a direct interaction. Highly purified extracts of murine Sp1 when incubated with NS-1 and precipitated with antibody to Sp1 were able to coimmunoprecipitate the NS-1 protein. The Sp1 preparation used was purified from CNE by DNA affinity chromatography with oligonucleotides complementary to the MVM P4 promoter GC box as the DNA binding target (28). The GC box present in MVM's P4 promoter has previously been shown to bind Sp1 with strong avidity (28). The protein bound to this affinity column ran on SDS-polyacrylamide gels as a 95-kDa–105-kDa doublet that reacted on Western blot (immunoblot) analysis to antibodies of Sp1. Furthermore, the Sp1 doublet was the only protein visible on a silver-stained gel, indicating that it is a highly purified Sp1 sample. While a low level of one or more protein contaminants may be present in this Sp1 preparation, the comparable results seen with this purified Sp1 sample and CNE lend support to the hypothesis that the interaction is a direct one. This interpretation is further supported by additional mutagenesis studies described below. The observation that the interaction between NS-1 and Sp1 was not affected by treatment of the binding reaction mixtures with either ethidium bromide or nucleases also suggests that nucleic acid moieties do not play a role in the interaction.

Experiments have been performed in which a series of glutathione-*S*-transferase–Sp1 fusion proteins have been overexpressed and isolated from bacterial cultures. These fusion proteins were constructed by cloning various Sp1 mutant sequences in frame behind the glutathione-*S*-transferase coding sequences present in pGEX vectors. These glutathione-*S*-transferase–Sp1 fusion proteins were then bound to glutathione-agarose beads and assayed for their ability to retain labelled NS-1 in binding experiments. Preliminary data from these experiments indicate that the NS-1 protein is interacting with the portion of the Sp1 protein that encodes Sp1's DNA binding domain (unpublished data). Similar experiments using glutathione-*S*-transferase–NS-1 fusion proteins and labelled Sp1 in binding assays demonstrate that sequences present in the amino-terminal half of the NS-1 protein are responsible for the NS-1–Sp1 interaction (unpublished data). The results of these experiments correlate nicely with the experiments detailed in this paper and lend additional support to the argument that NS-1 and Sp1 interact directly.

What role does the Sp1–NS-1 interaction play in transcriptional regulation from promoters containing GC boxes? We have shown that the SV40 CAT reporter construct is dependent on the presence of both NS-1 and Sp1 for high-level expression in Schneider cells. The existence of Sp1 binding sequences in many of the promoters known to be affected by NS-1 expression suggests that this may be the primary method by which NS-1 comes in contact with specific promoters. The in vitro work done with the P38 promoter fragment also demonstrated that the presence of the Sp1 protein was required for retention of the NS-1 protein in the P38 promoter fragment. These results lead to the conclusion that the interaction between NS-1 and Sp1 represents an important aspect in the ability of NS-1 to regulate transcription. It should be noted that experiments designed to look for transactivation of a P38CAT construct in Schneider cells by cotransfection of Sp1 and NS-1 failed to show a substantial increase in CAT expression. While we are not sure why such an activation would be seen with the SV40CAT construct and not that of the P38 promoter, one possible explanation might be related to the decreased activity of NS-1's activation domain in *Drosophila* cells relative to murine cells. The GalNS1 construct is a potent activator of transcription in mouse fibroblast A92L cells but is a weak activator in Schneider cells (unpublished results). The activation domain in NS-1 contains a number of putative phosphorylation sites, and it may be that the differential utilization of these sites in the two cell lines accounts for the different levels of activation observed. If the putative acidic nature of NS-1's activation domain is decreased in Schneider cells, possibly through changes in the phosphorylation state of the activation domain, then a single NS-1 unit might not possess enough of a negative charge density to measurably affect transcription. However, because the SV40 promoter contains five GC boxes compared with the one GC box present in the P38 promoter, the SV40 promoter potentially has the ability to bring more NS-1 units in contact with the transcriptional complex. This increased number of NS-1 molecules at the promoter could result in an increased negative charge density in the promoter region, leading to an effect on transcriptional activation.

Could the interaction of NS-1 with Sp1 be an important factor in other NS-1 functions? Recent work has shown a correlation between the cytotoxicity observed with NS-1 expression and the ability of NS-1 to *trans*-inhibit heterologous promoters (21), suggesting that NS-1's cytotoxicity may result from a deregulation of transcription from critical promoters. Legendre and Rommelaere (21) postulated that acidic regions within the NS-1 protein, if overexpressed, could inhibit, by a squelching effect, the proper functioning of other transcriptional activators utilizing acidic domains. Our inhibition results with the activation domain of the herpes simplex virus VP16 protein demonstrate that this is indeed possible. Our results indicating that NS-1 and Sp1 can interact independently of Sp1 binding to DNA sequences also raise the possibility that high levels of NS-1 expression might effectively sequester Sp1, preventing it from activating important cellular promoters. Alternatively, increased NS-1 expression might result in the overactivation of Sp1 at GC box-containing promoters, leading to changes in transcriptional regulation. Parvovirus infection has also been shown to inhibit tumor formation in animals (5, 34, 47). This oncosuppressive activity has been demonstrated in cell culture, for cells transformed by a number of viruses, including SV40. The transformed cells appear to be able to express a higher level of the NS-1 protein (7, 8, 41, 47), suggesting that the oncosuppressive nature of parvovirus infection in certain transformed cell lines may reflect an oncolytic effect of increased NS-1 expression. NS-1's activity as a transcriptional regulatory protein might therefore provide a mechanism for both its cytotoxic effects and the oncosuppressive nature of parvoviral infection.

Does NS-1 interact with other transcription factors? The activating domain of Sp1 has been shown to be rich in glutamine residues (10), and its ability to activate transcription appears to be the result of an interaction with the TAF 110 protein, a cofactor in the TFIID complex of *D. melanogaster* (19). The TAF 110 protein bears some structural similarity to the Sp1 protein, notably in the glutamine and serine/threonine-rich regions. Sp1 forms homomultimers (27), and it appears that the regions utilized by Sp1 in forming multimers are the same as those used in Sp1's interaction with TAF 110 (19). Recently, Sp1 has been shown to interact with both the initiator binding protein YY1 (39) and the bovine papillomavirus E2 protein (22). Thus, Sp1 appears capable of interactions with a number of proteins to elicit effects in transcriptional regulation. By comparison, little is known about the cellular proteins

with which NS-1 interacts. The parvovirus genome encodes only a small number of proteins (9), and a number of viral functions, including viral DNA replication, are known to be dependent on cellular factors provided by replicating cells. The prominent role of NS-1 in a number of viral functions suggests that interactions between NS-1 and some of these cellular factors are likely. The well-characterized adenovirus E1a protein relies on interactions with a number of cellular proteins to elicit its multiple functions (18, 48, 49). It may be that as NS-1 is characterized further, multiple interactions between it and other cellular proteins will also be observed.

Potential models for transcriptional activation following NS-1–Sp1 interaction. An interesting aspect of the NS-1–Sp1 interaction is that Sp1 is itself a potent transcriptional activator. This suggests a number of models for how the interaction of these two proteins could effect transcription. NS-1 might interact with Sp1 and activate transcription through its own strong activation domain. Alternately, the interaction may permit the use of both the NS-1 and the Sp1 activation domains. The activation domain in Sp1, being rich in glutamine residues, would be expected to use a different set of cofactors in its activation than the acidic domain of NS-1. This use of separate cofactor populations would reduce the effects of squelching, enabling an additive effect on expression by the two proteins. It is also possible that the binding of NS-1 to Sp1 might increase Sp1's affinity for GC box sequences, specifically those that normally bind Sp1 weakly. If so, this could explain the low basal activity of the P38 promoter in the absence of NS-1 expression. Further work is required to determine which, if any, of these models are employed.

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