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Efficient Transactivation of the Minute Virus of Mice P38 Promoter Requires Upstream Binding of NS1

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The P38 promoter of the autonomous parvovirus minute virus of mice is strongly transactivated by the nonstructural protein NS1, a sequence-specific DNA-binding protein. In the context of the complete viral genome, the only unique *cis*-acting signals required for P38 transactivation by NS1 are the proximal Sp1 site and the TATA element. In the absence of additional upstream sequences, a dependence upon the NS1 binding site within the transactivation response region is observed. Addition of synthetic NS1 binding sites to transactivation response region deletion mutants can restore the ability of NS1 to transactivate P38, and NS1 transactivation has been directly correlated to its ability to bind upstream of the P38 promoter.

The 5-kb, single-stranded DNA genome of the autonomous parvovirus minute virus of mice (MVM) is organized into two overlapping transcription units, with promoters at map units 4 (P4) and 38 (P38) (3, 37). The P4 promoter generates mRNAs R1 and R2, which encode the nonstructural proteins NS1 and NS2, respectively (3, 10, 37), while the P38 promoter generates the R3 mRNAs, which encode the viral capsid proteins, VP1 and VP2 (see Fig. 1) (3, 29, 37). NS1, a nuclear 83-kDa phosphoprotein (10, 11), has been shown to possess ATPase and helicase activities (26, 49) and has recently been characterized as a sequence-specific DNA-binding protein (7). NS1 is also found covalently attached to the 5′ end of intracellular replicating viral DNA (13).

NS1 has essential roles in viral DNA replication (8, 9, 14), viral gene expression, and cytotoxicity (12, 30). It has been reported to have a modest stimulatory effect on P4 (15, 23) and has proven to be an extremely potent activator of P38 transcription (15–17, 40, 42, 45, 48). The weak basal level expression from P38, in the context of the viral genome, is such that P38 products are undetectable by RNase protection assays in the absence of NS1 (36, 51). Although P38 is located only 2 kb downstream of P4, mutational analysis demonstrates that wild-type regulation of the P38 promoter is not dependent upon transcription proceeding downstream from P4 (40–42, 45). The P38 promoter also exhibits wild-type patterns of expression when transfected on a supercoiled closed-circular plasmid.

Typically, transcriptional activators comprise at least two distinct functional domains: a promoter-targeting domain, which can consist of either a DNA binding domain or a protein recognition domain, and an activation domain (25, 39, 50). The amino- and carboxy-terminal domains of NS1, which contain numerous acidic residues (3), are required for its transcriptional activation properties (27, 30, 31, 42). NS1 has been reported to interact with Sp1 (27) and has also recently been shown to bind specifically to multiple $(ACCA)_2$ motifs within the MVM genome in a DNA sequence-specific manner (6, 7).

The MVM P38 promoter region contains several potential transcriptional regulatory elements, including the TATA element 23 nucleotides (nt) upstream of the RNA initiation site at nt 2005, a single GC box 17 nt upstream of the TATA element, and the transactivation response region (TAR), initially identified in the related parvovirus H1 (42), at nt −145 to −115. As was perhaps expected, previously published work addressing the regulation of P38 has shown that transactivation by NS1 is absolutely dependent upon the P38 TATA motif (2, 17). However, the extent to which the Sp1 site and the TAR element (which contains a consensus NS1 binding site) in particular were shown to be required depended upon the assay used and upon which flanking MVM sequences were present (17, 20, 40, 42, 45).

Recently, Christensen et al., utilizing a coimmunoprecipitation technique to detect specific NS1-DNA complexes, demonstrated that in addition to binding to numerous motifs throughout the MVM genome, NS1 binds specifically to an $(ACCA)_2$ motif which occurs within the TAR element (6). If NS1 binding is required for P38 transactivation, the existence of multiple NS1 binding sites throughout the MVM genome (4, 6) could explain why the TAR has been identified as a critical regulatory element under certain experimental conditions and not others. Although an interaction between Sp1 and NS1 had been previously demonstrated (27), it is not certain whether Sp1 targets NS1 to P38, since NS1 can be directed to P38 by its DNA binding motif in the absence of Sp1.

In this report we have performed a more comprehensive characterization of the *cis*-acting sequences required for NS1 transactivation of P38. Our results show that in the context of the complete viral genome, the TAR is dispensable; however, in abridged MVM constructs, which do not contain additional upstream NS1 binding sites, the NS1 binding site within the TAR is required. Addition of a synthetic NS1 binding site to TAR deletion mutants can restore the ability of NS1 to transactivate P38, and NS1 transactivation has been directly correlated to its ability to bind upstream of the P38 promoter.

MATERIALS AND METHODS

Cells and transfections. Murine A92L cells were propagated as described previously (48). All transient transfections were performed with DEAE-dextran as described previously (44).

Plasmid constructs. (i) pKONS1m. A modified version of pKONS1/2 (45), pKONS1m, was used to supply NS1. pKONS1/2 drives the NS1 and NS2 coding sequence from the simian virus 40 (SV40) early promoter. In pKONS1m, MVM nt 2016 to 2047 were altered (as previously described), such that the amino acid sequence of NS1 remains unchanged; the MVM sequence in this area was changed to 5′- CCGAAATAGACCCCGTTGTCGCAGAACTATGC-3′ (altered sequences are underlined). The nucleotide alterations in pKONS1m, while

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degenerate in the NS1 reading frame, introduce several radical changes in NS2 (changing the amino acid sequence EKYASQPELC to EIDPVVAELC) in a region which has been shown to be critical for NS2 function (36).

(ii) Linker-scanner mutants. The original mutations were constructed in David Ward's laboratory, Yale University, New Haven, Conn., and have been previously described (17). The *Bgl*II linker mutations introduce the sequence 5′-GAAGATCTTC-3′ between the following MVM sequences: LS1, nt 1842 to 1851; LS2, nt 1856 to 1866; LS3, nt 1867 to 1878; LS4, nt 1886 to 1897; LS5, nt 1891 to 1900; LS9, nt 1926 to 1938; LS11, nt 1953 to 1962; LS13, nt 1976 to 1987; LS14, nt 1987 to 1995; LS15, nt 2003 to 2012; LS16, nt 2012 to 2016. These linker-scanner mutants were cloned into the previously described p385UTT (36), which has a 15-nt addition, containing translation termination codons in all three reading frames (UTT) at the *Eco*RV site at nt 385 and a small deletion within the right-hand hairpin. p385UTT was constructed from a version of the original plasmid clone of MVM (pMVM, which contains a small deletion in the right-hand hairpin) cloned into pML-n, a 2.5-kb abbreviated version of pBR322 (36). These mutants also contain a 1,344-nt deletion between the *Hin*dIII sites at nt 2652 to 3996, which has been shown previously not to alter MVM transcription (51).

(iii) Degenerate mutants. The Sp1-degenerate (MVM nt 1939 to 1969; 5′GG TCAGAAAATTGGGCGGAGCCAAAGT-3′), TATA-degenerate (MVM nt 1964 to 1996; 5′-GGTGCCAACTCCTATAAATTTACTAGGTTCGGC-3′), and RNA initiation site-degenerate (MVM nt 1983 to 2023; 5′-GGTTCGGCA CGTCACCATTTCACGACACCGAAAAG-3′) mutations (altered sequences are underlined) were introduced by M13 site-directed mutagenesis and cloned into pMVM.

(iv) TAR deletions. The p385UTT-LSΔ1/5 deletion was constructed from the p385UTT-LS1 and p385UTT-LS5 constructs, by standard techniques. This construct deletes the MVM nt 1842 to 1900. p385UTT-LSΔ5/9 was constructed similarly, with p385UTT-LS5 and p385UTT-LS9. This construct deletes the MVM nt 1891 to 1938.

(v) 5′ TAR deletions −/+ $(ACCA)_4$. p385UTT-LS1, -LS2, -LS3, -LS4, -LS5, and -LS9 were linearized with *Cla*I (pBR322 nt 23) and then partially digested with *Bgl*II. The synthetic NS1 binding sites were constructed by ligating two single-stranded oligonucleotides, 5′-CGATACCAACCAACCAACCAA-3′ and 5′-GATCTTGGTTGGTTGGTTGGTAT-3′, which contain *Bgl*II and *Cla*I overhanging ends. The annealed oligonucleotides were then cloned between the *Cla*I(pBR322 nt 23) and *Bgl*II sites of p385UTT.

(vi) bGH reporter constructs. A 2.5-kb bovine growth hormone (bGH) *Bam*HI fragment was inserted into p385UTT-LS14, -LS15, and -LS16, between the *Bgl*II sites at MVM nt 1987 (LS14), nt 2003 (LS15), or nt 2012 (LS16), and MVM nt 4212. These constructs were cloned into plasmids which contain a 5′ deletion upstream of nt 1866 or 1901.

(vii) pGST-NS1. The plasmid pGST-NS1 contains a genomic MVM fragment (nt 260 to 3521) cloned in frame with glutathione *S*-transferase (GST) in the polylinker of the vector plasmid, pGEX2TK (Promega, Madison, Wis.). This plasmid encodes full-length recombinant GST-NS1, as demonstrated by Western blot (immunoblot) analysis with antibodies directed to a peptide at the extreme C terminus of NS1 (a generous gift from S. Cotmore and P. Tattersall).

RNA isolation and characterization. Total RNA was isolated, and RNase protection assays were performed with MVM-specific probes as described previously (44). Quantitations were performed on a Molecular Dynamics PhosphorImager. pKONS1m was constructed such that when the wild-type MVM *Hae*III probe (MVM nt 1854 to 2378) was used (36, 44) for RNase protections, the nucleotide alterations of pKONS1m (MVM nt 2016 to 2046) generated a specific 234-bp breakdown product (nt 2046 to 2280) which could be quantitated. This allowed us to differentiate between transcripts originating from experimental templates and those from the cotransfected NS1-supplying plasmids. Quantitation of the relative accumulation of this RNA species, present in all cotransfection experiments, permitted the standardization of transfection efficiencies and allowed comparison of plasmids which lack MVM P4 sequences or contain the bGH reporter, since in all instances, an equivalent amount of pKONS1m was cotransfected.

p385UTT-LS1, -LS2, -LS3, -LS4, and -LS5 were analyzed with the wild-type *Hae*III probe, and p385UTT-LS13, -LS14, -LS15, and -LS16 were analyzed with homologous probes encompassing the same region, each having the appropriate mutations. The Sp1-, TATA-, and initiator region-degenerate mutants were also protected with homologous probes spanning nt 1854 to 2378.

The bGH RNase protection assays used an internal bGH-cDNA probe (22). The 338-bp bGH *Pst*I fragment spans the 5′ end of exon 1 to the 5′ end of exon 4 and was cloned into the *Pst*I site of the pGEM3Z (Promega) polylinker. To compare P38-bGH transcription with p385UTT P38 transcription, protected fragments were first standardized according to the number of uridines in each antisense probe. The P38 values were finally adjusted for their relative transfection efficiencies by the relative accumulation of the pKONS1m product, as described above.

For all constructs which contained a functional MVM P4 promoter, P38 activity was measured relative to the expression from the P4 promoter. All RNAs were assumed to be equally stable (44), and so steady-state levels were assessed. P4 and P38 products were quantitated and compared following standardization with the number of uridines present in the protected region of the antisense probe in each mRNA species. The full-length pMVM construct exhibited a P38/P4 ratio which was consistently between 3.3 and 2.8, similar to previously reported results (44). The P38/P4 ratio for p385UTT cotransfected with pKONS1m was consistently between 1.4 and 1.0, providing a consistent baseline from which to measure P38 activity. For all constructs which lacked a functional P4, P38 activity was measured relative to P38 expression from p385UTT cotransfected with pKONS1m. The R3M and R3m species for each sample were adjusted for the number of uridines present in the protected region of the antisense probe, totalled, and then standardized for their relative transfection efficiencies by the relative accumulation of the 234-bp pKONS1m breakdown species.

NS1 binding assays. Recombinant GST-NS1 was purified by glutathione agarose from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced *Escherichia coli* BL21 cells. Binding assays were performed on MVM subgenomic fragments derived from *Eco*RI-*Xho*I-*Hin*dIII triple restriction digests of p5′Δ2, p5′Δ5, and $p(ACCA)_4$5′Δ5. These digests leave the P38 promoter region on a 172- to 226-nt *Xho*I-*Eco*RI fragment. Glutathione-bound NS1 was incubated with 75 ng of [α-^{32}P]dATP-end-filled DNA, as described by Cotmore et al. (7), except that 150 mM NaCl was used, at 4°C for 2.5 h. The samples were washed three times in binding buffer, incubated in sodium dodecyl sulfate (SDS) loading dye and analyzed by 6% polyacrylamide gel electrophoresis (PAGE). GST-bound agarose beads alone exhibited no binding to DNA.

RESULTS

The disruption of putative P38 *cis*-acting elements in the context of the complete viral genome confirms that the Sp1 and TATA sites are the critical unique elements involved in NS1 transactivation of P38. Our first objective was to identify P38-proximal signals required for transactivation by NS1 in the context of the complete viral genome. Since the MVM P38 promoter region overlaps the NS1 coding sequence, mutational alteration of P38 in plasmids that also contain the P4 promoter would potentially alter any NS1 molecules produced from these test plasmids. To circumvent this problem, a translation termination signal was introduced into the NS1/2 coding sequence at MVM nt 385 (Fig. 1A) in the test plasmids which contain a P4 promoter. These plasmids were also nonreplicative because of a small deletion in the right-hand hairpin. Full-length, wild-type NS1, driven by the SV40 early promoter, was supplied in *trans*. For all cotransfection experiments, NS1 was supplied in a 1/2 ratio (NS1 to template). This ratio was determined to be the lowest concentration of NS1 which resulted in maximal P38 expression by a series of titration experiments (data not shown).

For all experiments described in this report we chose to analyze P38 transactivation by RNase protection assays. These assays permit direct identification and quantification of mRNAs which use authentic start sites and have the sensitivity to detect 1 to 10 specific mRNA molecules per cell (44). In the absence of NS1 supplied in *trans*, no P38-generated products were detected from p385UTT transiently transfected into A9 cells (Fig. 1B). When MVM NS1 was supplied by cotransfection, however, p385UTT generated nearly 50% of the P38 activity seen for pMVM alone (a nonreplicating MVM plasmid fully able to express NS1/2), as determined by the following equation: total P38 products/total P4 mRNA products (Fig. 1B). Transactivation of P38 mutants was subsequently compared with that of either of these two constructs.

To further examine the *cis*-acting elements required for P38 transactivation in this context, linker-scanner mutations proximal to the P38 promoter (originally constructed in D. Ward's laboratory, Yale University) were introduced into p385UTT. As previously observed, disruption of the proximal Sp1 site (p385UTT-LS11) and the P38 TATA motif (p385UTT-LS13) was seen to have significant inhibitory effects (Fig. 2). Since others have reported an even greater dependence upon the Sp1 site for P38 transactivation (2, 17) and since Sp1 sites have been shown to function over long distances (33, 46), an additional mutant was constructed which removed from the MVM molecule all potential Sp1 binding sites which might compensate for the Sp1 disruption at P38. There is a high-affinity Sp1

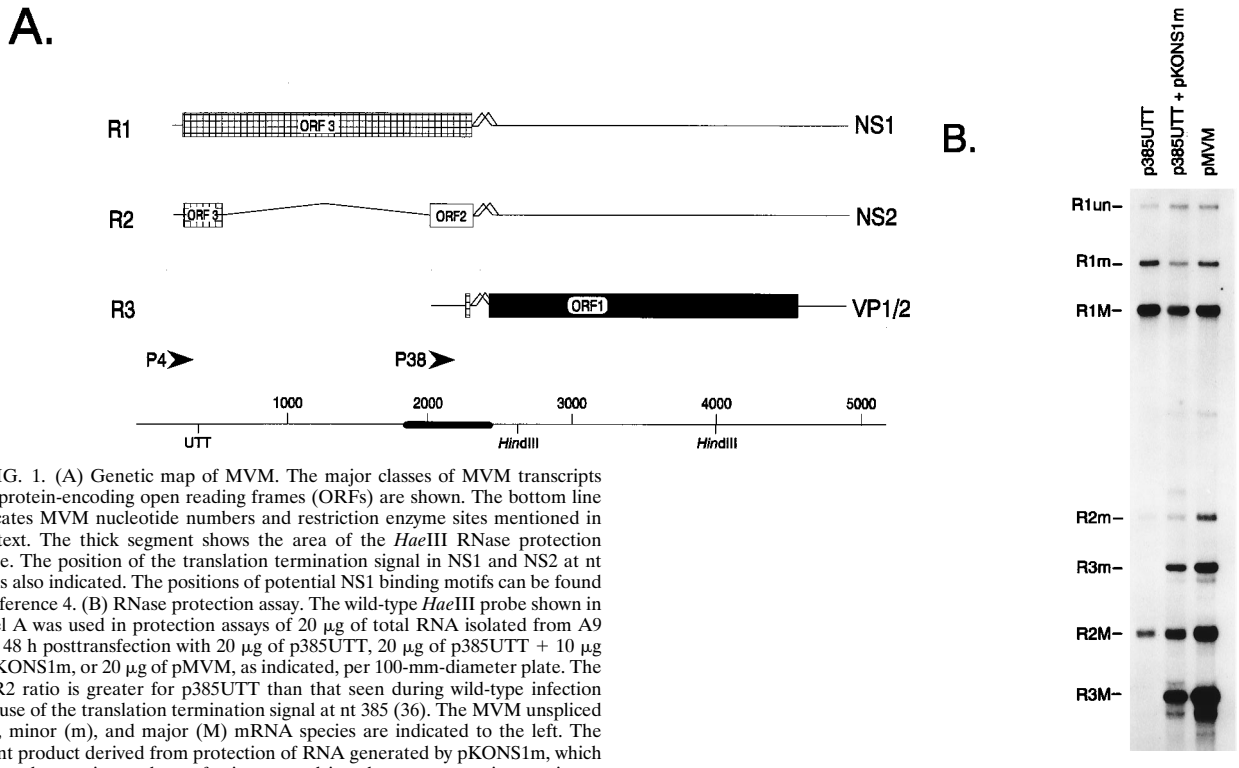

FIG. 1. (A) Genetic map of MVM. The major classes of MVM transcripts and protein-encoding open reading frames (ORFs) are shown. The bottom line indicates MVM nucleotide numbers and restriction enzyme sites mentioned in the text. The thick segment shows the area of the *Hae*III RNase protection probe. The position of the translation termination signal in NS1 and NS2 at nt 385 is also indicated. The positions of potential NS1 binding motifs can be found in reference 4. (B) RNase protection assay. The wild-type *Hae*III probe shown in panel A was used in protection assays of 20 μg of total RNA isolated from A9 cells 48 h posttransfection with 20 μg of p385UTT, 20 μg of p385UTT + 10 μg of pKONS1m, or 20 μg of pMVM, as indicated, per 100-mm-diameter plate. The R1/R2 ratio is greater for p385UTT than that seen during wild-type infection because of the translation termination signal at nt 385 (36). The MVM unspliced (un), minor (m), and major (M) mRNA species are indicated to the left. The 234-nt product derived from protection of RNA generated by pKONS1m, which was used as an internal transfection control in subsequent experiments, is not shown.

site immediately upstream of the MVM P4 promoter (38), and we have identified a sequence near the right-hand terminus of MVM which can also specifically bind Sp1 (5a). The mutant lacking all known Sp1 binding sites also retained approximately 30% of cotransfected p385UTT P38 activity (data not shown).

p385UTT-LS4, which contains a mutation adjacent to the NS1 binding site contained in the TAR, and p385UTT-LS14, which contains a mutation immediately downstream from the P38 TATA motif, were transactivated to levels nearly 1.5- and 2-fold that of p385UTT, respectively (Fig. 2). This increase has been seen by others (2, 17), although to varying degrees, and suggests that these regions may mediate an additional and as yet unidentified aspect of P38 regulation. p385UTT-LS3, which contains a mutation that disrupts the NS1 binding site that lies within the TAR, can be transactivated to levels near those of p385UTT (Fig. 2).

We next chose to analyze three mutants in which the Sp1 site, the TATA site, or the region of R3 initiation was disrupted by several nucleotide changes but in which the amino acid coding sequence for NS1 is unchanged. This approach obviated the need for cotransfection of NS1 and so perhaps reflects a ratio of NS1 to MVM template which more closely mimics wild-type MVM infection. These mutants gave results similar to those of mutants requiring cotransfection, described above. The Sp1-degenerate mutant (pMVMSp1) exhibited approximately 25% of the pMVM activity, the TATA-degenerate mutant (pMVMTATA) abolished detectable P38 transcription, and the initiation region-degenerate mutant (pMVMInr) exhibited approximately 70% of the P38 activity expressed from pMVM (Fig. 3).

The NS1 binding site contained within the TAR is dispensable in the context of the complete viral genome. The data presented so far demonstrate that the Sp1 and TATA elements are critical for P38 transactivation. Surprisingly, however, linker-scanner mutations in the TAR (p385UTT-LS2, -LS3, and -LS4) had little negative effect upon P38 regulation in the context of the complete viral genome. The TAR has been shown to contain a strong NS1 binding site, $(ACCA)_2$, which is disrupted by LS3. To more thoroughly assess the role of the TAR within the context of the complete viral genome, we chose to delete this region entirely (p385UTT-LSΔ1/5, which deletes MVM nt 1842 to 1900). Upon cotransfection with pKONS1m, transactivation of the p385UTT-LSΔ1/5 P38 promoter is not impaired, but rather is activated to almost twice the relative level seen for the P38 promoter of p385UTT (Fig. 4). A similar-sized deletion immediately downstream of the TAR (p385UTT-LSΔ5/9, which deletes MVM nt 1891 to 1938) has little effect upon P38 transactivation (Fig. 4).

These experiments suggest that in the context of the complete viral genome, the NS1 binding site within the TAR is not necessary for P38 transactivation. Since previously published work, which analyzed the TAR's role in abridged MVM genomes, demonstrated a requirement for the TAR, it is quite likely that the NS1 binding site within the TAR is functionally redundant and that when it is deleted or disrupted it can be compensated for by the numerous potential NS1 binding sites remaining in the full-length MVM genome. The increased P38 expression from the TAR deletion mutant, p385UTT-LSΔ1/5, is consistent with the linker-scanner mutation analysis presented above, further suggesting that LS4 may identify an additional site for regulation of P38.

The NS1 binding site within the TAR is critical when no upstream MVM sequences are present. To further resolve the role of the TAR in P38 transactivation, a linker-scanner-based series of 5′ deletion mutants which extended into the P38 promoter region and in which all upstream MVM sequences

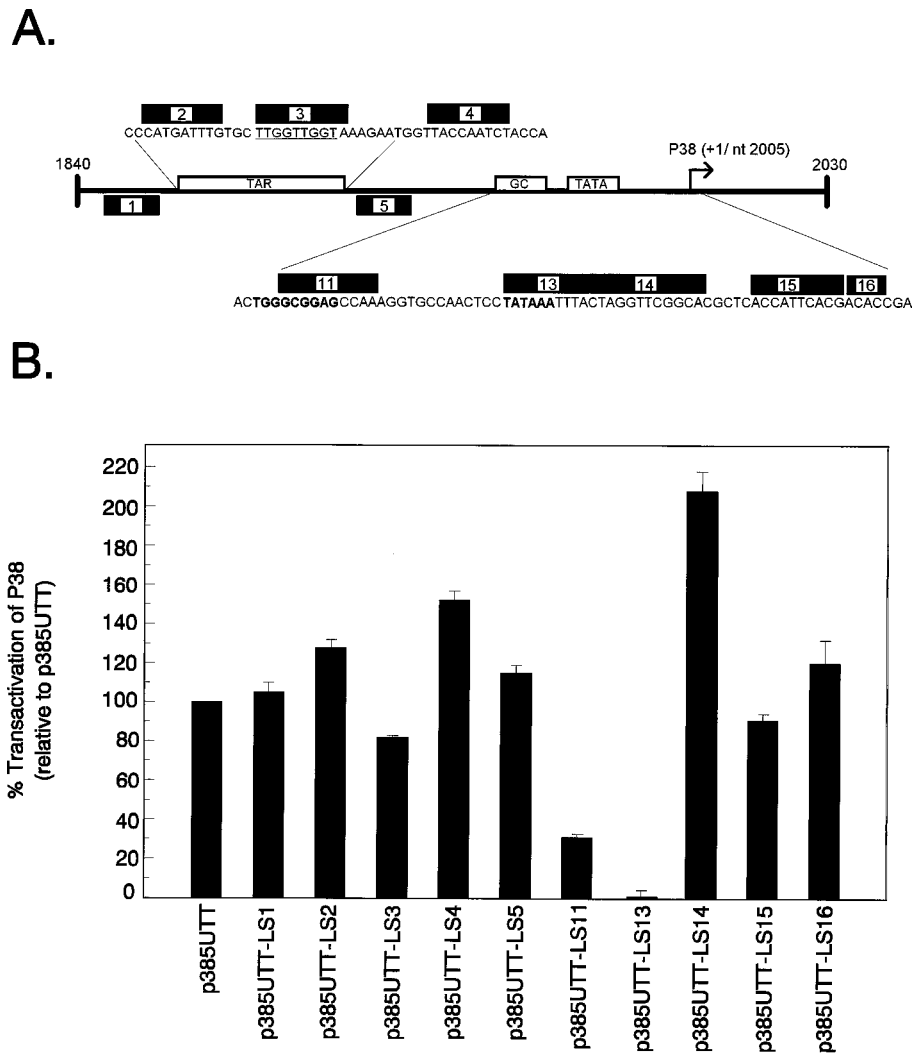

FIG. 2. (A) Genetic map of the linker-scanner mutations surrounding the P38 promoter region. The positions of the various linker-scanner mutations are indicated by darkened boxes, and previously identified *cis*-acting regulatory elements are indicated in relation to the P38 start site. The MVM sequences which are disrupted by the linkers are indicated. The NS1 binding site within the TAR is underlined, and the P38, Sp1, and TATA elements are in boldface type. (B) NS1 P38 transactivation of linker-scanner mutants. All linker-scanner mutations were evaluated in the p385UTT background and compared with the p385UTT parent. Values were obtained from RNase protection assays with either the wild-type *Hae*III probe (for p385UTT-LS1 to -LS5) or homologous *Hae*III probes (for p385UTT-LS11 to -LS16) as described in Materials and Methods, with 20 μg of total RNA isolated from A9 cells 48 h posttransfection with 20 μg of each p385UTTLS mutant and 10 μg of pKONS1m per 100-mm-diameter plate. P38 transactivation of each linker-scanner construct is presented relative to p385UTT transactivation as described in Materials and Methods, and values are as follows (including 95% confidence limits): p385UTT-LS1, 105% ± 5%; p385UTT-LS2, 128% ± 4%; p385UTT-LS3, 82% ± 1%; p385UTT-LS4, 152% ± 5%; p385UTT-LS5, 115% ± 4%; p385UTT-LS11, 31% ± 2%; p385UTT-LS13, 0.1% ± 0.1%; p385UTT-LS14, 208% ± 10%; p385UTT-LS15, 91% ± 2%; p385UTT-LS16, 120% ± 12%. The values represent the means of five (p385UTT-LS1 to -LS5) or four (p385UTT-LS11 to -LS16) separate experiments.

are removed was constructed. The magnitude of transactivation of these mutants is compared with transactivation of P38 in the context of the complete viral genome (p385UTT). p5′Δ1 and p5′Δ2 retain the NS1 binding site within the TAR; the other mutants tested delete the NS1 binding site and additional downstream sequences (Fig. 2A). Although a search of the plasmid sequences revealed that they contain no consensus NS1 binding motifs, $(ACCA)_2$, and that only three ACCA monomers were brought within 700 nt, these experiments were done with the expectation that plasmid sequences brought proximal to P38 by such deletions could contain cryptic NS1 binding sites. p5′Δ1, a mutant in which MVM sequences upstream of the TAR have been deleted, could be transactivated to the same level as full-length p385UTT. Deletion of sequences upstream of LS2 led to a modest decrease in P38 transactivation; however, when the MVM NS1 binding site within the TAR was also deleted (p5′Δ3), P38 could be transactivated only to levels less than 40% that of p385UTT (Fig. 5A). This decrease demonstrates that the TAR and its NS1 binding site are a critical component of the P38 promoter when no additional upstream MVM NS1 binding sequences are present. p5′Δ3, p5′Δ4, and p5′Δ5 exhibit similar levels of activity, suggesting that no other component critical for P38 transactivation is lost between nt 1867 and 1900. When additional sequences upstream of nt 1938 were deleted (p5′Δ9), however, an additional threefold decrease in transactivation was observed (compare p5′Δ5). This suggests that this region (nt 1891 to 1938) is required for full transactivation when upstream sequences have been deleted. Whether this region functions as a target for the binding of participating factors or

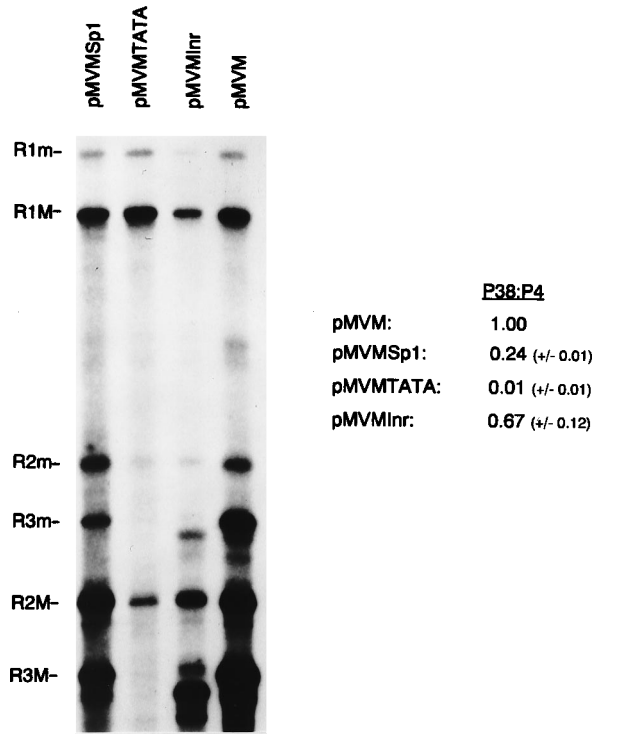

FIG. 3. Mutants in three P38 regulatory motifs and which express wild-type NS1 exhibit levels of P38 expression relative to the corresponding linker-scanner mutations when cotransfected with pKONS1m. To the left is shown an RNase protection assay, with homologous *Hae*III-like probes for each construct, of 20 μg of total RNA taken 48 h posttransfection of A9 cells with 20 μg of the following plasmid DNAs: pMVM, pMVMSp1, pMVMTATA, pMVMInr. Values to the right were determined as described in Materials and Methods and are the means of four separate experiments. 95% confidence values are indicated in parentheses. The 234-nt product generated by protection of RNA from pKONS1m, which was used as an internal transfection control as described in Materials and Methods, is not shown for this experiment. P38 transcripts from pMVMInr utilize an aberrant start site, and protected species migrate slightly faster than those from correctly initiated P38 transcripts.

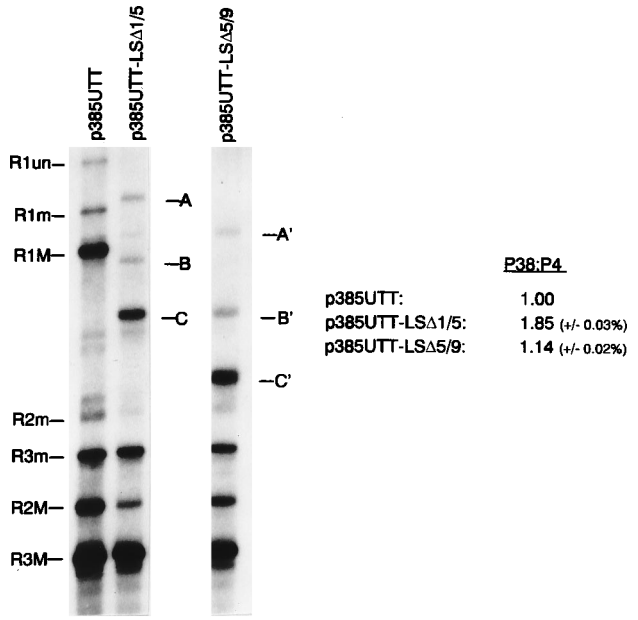

FIG. 4. The TAR is dispensable for P38 transactivation in the context of the complete viral genome. To the left is shown an RNase protection assay, with the wild-type *Hae*III probe, of 20 μg of total RNA taken 48 h after transfection of A9 cells with 10 μg of pKONS1m, 20 μg of p385UTT, or plasmids in which a region containing the TAR (nt 1842 to 1900 in p385UTT-LSΔ1/5) or a region immediately downstream of the TAR (nt 1891 to 1938 in p385UTT-LSΔ5/9) was deleted. A, B, and C derived from R1un, R1m, and R1M, respectively, generated by p385UTT-LSΔ1/5, and A′, B′, and C′ derived from R1un, R1m, and R1M, respectively, generated by p385UTT-LSΔ5/9, are shown. The fragments protected with R1 generated by the deletion mutants are smaller because of the deletion within the protected region. The R1/R2 ratio for plasmids containing a translation termination signal at nt 385 is greater than that seen for the wild type (36). P38 transactivation is measured relative to that of p385UTT + pKONS1m, as described in Materials and Methods. Values to the right represent the means of three separate experiments, and 95% confidence limits are indicated in parentheses. The 234-nt product produced by protection of RNA generated by pKONS1m, used as an internal transfection control in this experiment, is not shown.

merely optimizes the spacing between essential promoter elements is not yet clear (the same region is dispensable for P38 transactivation in the context of the full genome, as shown by the deletion mutant p385UTT-LSΔ5/9 [Fig. 4]). These experiments suggest that an NS1 binding site upstream of the P38 TATA motif is essential for efficient transactivation of P38 by NS1. Interestingly, the abundant $(ACCA)_2$ motifs downstream of P38 could not compensate for the absence of the required upstream sites.

Deletion mutants which either retained or lost the TAR were tested for their ability to drive a reporter construct placed at various distances downstream from the P38 TATA element. The bGH gene was selected for these assays, and a specific RNase protection assay was developed which could compare bGH levels with MVM R3 production from constructs which retained MVM sequences downstream of P38 when transfected in parallel. When bGH was fused at three positions immediately downstream of the P38 TATA (MVM nt 1987 [LS14], nt 2003 [LS15], and nt 2012 [LS16]) in constructs which retained the TAR element (p5′Δ2-14bGH, p5′Δ2-15bGH, and p5′Δ2-16bGH, respectively), full transactivation of P38 was observed and in fact reached levels approximately twofold higher than those of analogous constructs which retained MVM sequences downstream (Fig. 5B). However, when the bGH was fused at the same three positions in constructs in which the TAR is deleted (p5′Δ5-14bGH, p5′Δ5-15bGH, and p5′Δ4-16bGH), transactivation was significantly reduced, again demonstrating a role for an upstream NS1 binding site.

The bGH sequences brought adjacent to the P38 TATA contain at least 11 consensus $(ACCA)_n$ NS1 binding motifs, and so it remains possible that wild-type levels of transactivation of P38 require NS1 binding sites both upstream and downstream of the P38 TATA. The reporter fusion experiments described do demonstrate, however, that sequences downstream of the P38 TATA element do not need to be of MVM origin for regulated expression and suggest that if downstream sequences are required for efficient P38 transactivation, it is likely that only NS1 binding sites are sufficient, rather than some other MVM-specific element which affects RNA initiation, processing, or procession of the transcription machinery downstream. It is also interesting that none of the minimal promoter/reporter constructs tested acquired a detectable level of basal expression in the absence of NS1 (data not shown).

P38 activity can be partially restored to 5′ TAR deletion mutants by the addition of a synthetic NS1 binding site. Addition of an $(ACCA)_4$-containing, double-stranded oligonucleotide to p5′Δ2 (which retains MVM sequences upstream of the TAR NS1 binding site) or to p5′Δ3 (which deletes the TAR NS1 binding site) increases their responsiveness to NS1 ap-

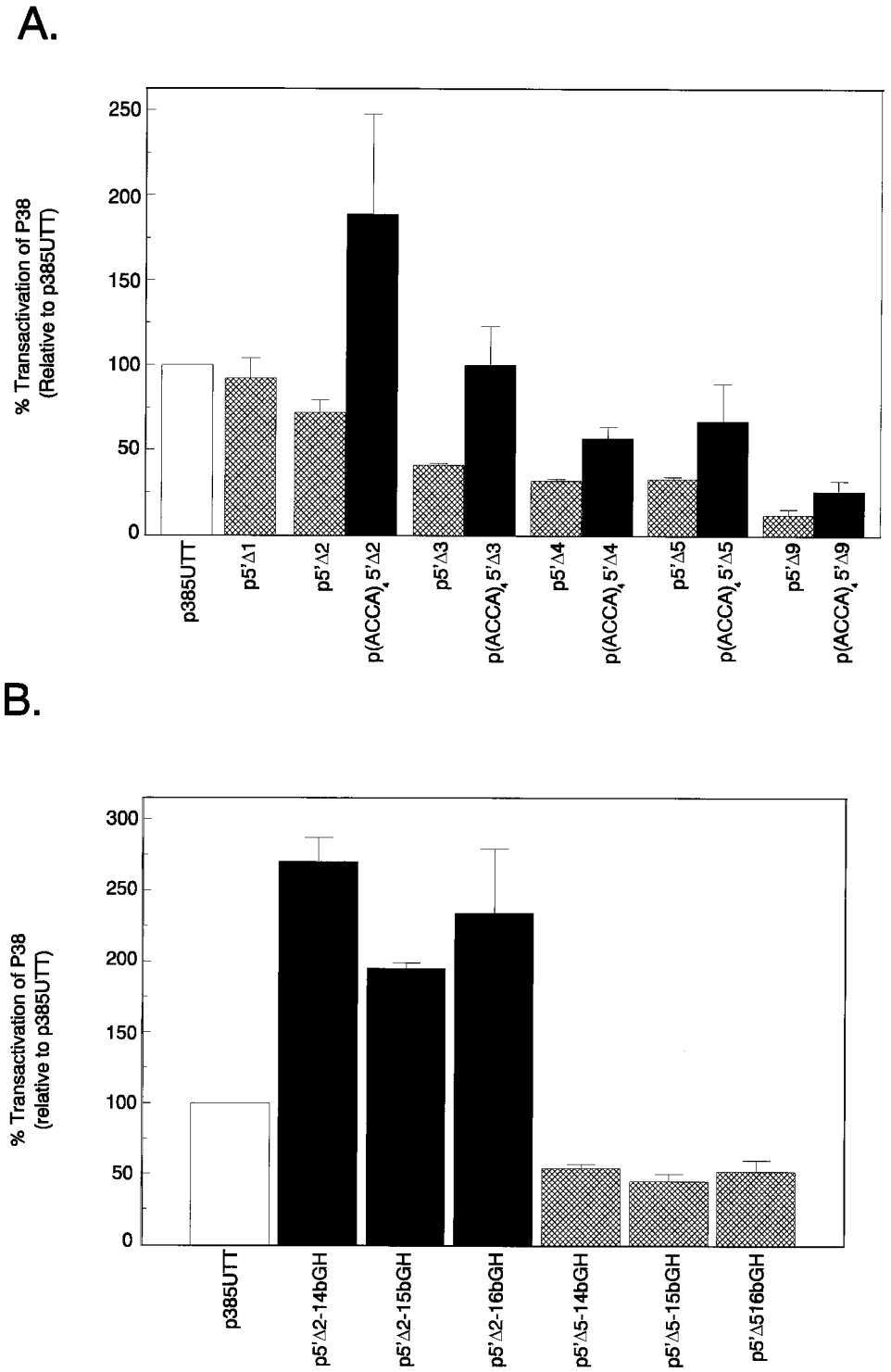

FIG. 5. The NS1 binding site within the TAR is essential in 5′ deletion mutants. The map which indicates the position of the linker-scanner mutations which were used to construct the 5′ deletions is shown in Fig. 2A. (A) Relative P38 expression values for 5′ deletion mutants (hatched bars), 5′ deletion mutants with a synthetic NS1 binding site (solid bars), and p385UTT (open bar) are shown. P38 values, from four (5′ deletion mutants) or five (deletion mutants with the synthetic NS1 binding site) separate experiments, are reported relative to that of p385UTT + pKONS1m, as described in Materials and Methods, and are as follows (including 95% confidence limits): p5′Δ1, 92% ± 12%; p5′Δ2, 72% ± 7%; p5′Δ3, 41% ± 1%; p5′Δ4, 32% ± 1%; p5′Δ5, 33% ± 1%; p5′Δ9, 12% ± 1%; p(ACCA)$_4$5′Δ2, 189% ± 59%; p(ACCA)$_4$5′Δ3, 100% ± 23%; p(ACCA)$_4$5′Δ4, 67% ± 22%; p(ACCA)$_4$5′Δ9, 26% ± 6%. Although the standard deviations (bars) of p(ACCA)$_4$5′Δ2 and p(ACCA)$_4$5′Δ5 are large, their P38 activities are still significantly greater than those of the corresponding deletion mutants without synthetic NS1 binding sites. (B) P38 bGH reporter constructs. Constructs have the bGH genomic sequence fused to the LS14, LS15, or LS16 position (Fig. 2A), with their 5′ end at either LS2 (solid bars) or LS5 (hatched bars). p385UTT expression is indicated by an open bar. Values from three separate experiments, determined from RNase protections of 20 μg of total RNA isolated from A9 cells transfected with 20 μg of experimental plasmid and 10 μg of pKONS1m per 100-mm-diameter plate, are as follows (including 95% confidence limits): p5′Δ2-14bGH, 270% ± 17%; p5′Δ2-15bGH, 195% ± 4%; p5′Δ2-16bGH, 234% ± 45%; p5′Δ5-14bGH, 54% ± 3%; p5′Δ5-15bGH, 45% ± 5%; p5′Δ5-16bGH, 52% ± 8%.

proximately twofold, resulting in levels of P38 transactivation which either exceed or achieve levels seen for p385UTT [see p(ACCA)$_4$5′Δ2 and p(ACCA)$_4$5′Δ3, Fig. 5A]. Addition of the (ACCA)$_4$ oligonucleotide to p5′Δ4, p5′Δ5, and p5′Δ9 also results in a twofold increase of P38 transactivation compared with each parent deletion; however, these mutants cannot be transactivated to p385UTT levels [see p(ACCA)$_4$5′Δ4, p(ACCA)$_4$5′Δ5, and p(ACCA)$_4$5′Δ9, Fig. 5A]. These results are consistent with the hypothesis that NS1 binding proximal to the P38 promoter facilitates its full transactivation. However, the inability of a synthetic binding site to restore transactivation of p(ACCA)$_4$5′Δ4, p(ACCA)$_4$5′Δ5, and p(ACCA)$_4$5′Δ9 to wild-type levels suggests either that an additional element upstream of LS4 is required for full transactivation of P38 or that these large mutations sufficiently alter the structure of the P38 promoter such that even a strong NS1 binding site cannot fully rescue P38 activity. Alternatively, since NS1 has been shown to have a footprint which extends 31 nt downstream from its binding motif (6), NS1 binding to these 5′ deletion mutants may also begin to sterically interfere with other activator proteins (Sp1, TAFs, etc.) (5, 19) important for P38 transactivation.

P38 transactivation by NS1 correlated with NS1's ability to bind to the TAR. The abilities of NS1 to directly bind to the promoter regions of p5′Δ2, which can be efficiently transactivated by NS1, and of p5′Δ5, which is only poorly transactivated, were compared by a modified version of the assay recently developed by Cotmore et al. (7). As seen in Fig. 6, the P38 promoter fragment of p5′Δ2, which contains the NS1 binding site, can bind to NS1, while the analogous fragment from p5′Δ5, which lacks the TAR NS1 binding site, binds very poorly (at least 10-fold less well, based on the ratio of the bound promoter fragment to bound fragment B [Fig. 6]). Furthermore, binding to the P38 promoter fragment is restored substantially by the addition of a synthetic NS1 binding site, p(ACCA)$_4$5′Δ5 [compare the ratio of the bound promoter fragment to bound fragment B for p(ACCA)$_4$5′Δ5 relative to those of p5′Δ2 and p5′Δ5 (Fig. 6)]. This addition also results in a twofold increase in transactivation (see above). These experiments demonstrate that transactivation of P38 directly correlates with the ability of NS1 to bind upstream of P38 and suggest that such binding may be required for its activity.

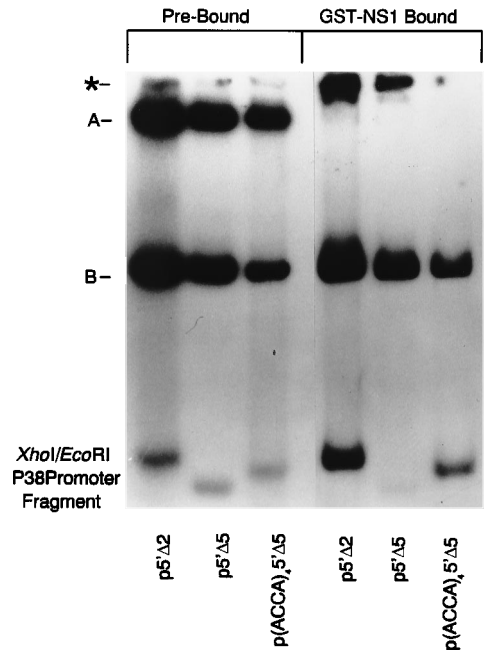

FIG. 6. NS1 binding directly correlates with P38 transactivation. p5′Δ2, p5′Δ5, and p(ACCA)$_4$5′Δ5 were digested with *Eco*RI, *Xho*I, and *Hin*dIII, generating three fragments: the *Eco*RI-*Hin*dIII fragment (A, 3,554 bp), the *Hin*dIII-*Xho*I fragment (B, 580 bp), and the P38 promoter fragment [226 bp from p5′Δ2, 172 bp from p5′Δ5, or 194 bp from p(ACCA)$_4$5′Δ5]. Digests were incubated with recombinant GST-NS1 as described in Materials and Methods, incubated in SDS loading dye, and analyzed by 6% PAGE. Bands A and B both contain several strong NS1 binding sites; however, only B is brought down on beads, because in this assay, large DNA molecules are poorly retained (33a). Band B can therefore be used as an internal control when comparing samples. Note that there is more target DNA (compare bands B) in the p5′Δ5 samples versus p(ACCA)$_4$5′Δ5. GST-bound agarose beads alone exhibited no DNA binding (data not shown). The asterisk shows radioactivity remaining in the wells. Approximately 2 to 5% of each labelled sample was also directly loaded without subsequent NS1 binding (Pre-Bound).

DISCUSSION

The results presented in this report demonstrate that in the context of the complete viral genome, the P38 TATA element and proximal Sp1 site are the only unique *cis*-acting components required for NS1 transactivation of P38. However, when 5′ deletion mutants in which all upstream MVM sequences have been deleted are analyzed, a strict dependence upon the NS1 binding site within the TAR is observed. Transactivation can be restored to TAR deletion mutants by the addition of a synthetic NS1 binding site and can be directly correlated to the ability of NS1 to bind this region. The most simple resolution of these results, as has been speculated by others (6, 45), is that the critical component of the TAR, namely the NS1 binding site, is functionally redundant and, if deleted or disrupted, can be compensated for by the remaining (ACCA)$_2$ motifs present in the 5′-flanking MVM sequences. This report explains previously published work which analyzed P38 transactivation utilizing numerous different assays and in the context of a variety of abridged MVM genomes (1, 2, 17, 21, 45).

There are between 40 and 50 binding sites clustered in the MVM genome (4, 6). Our results also suggest that an NS1 binding site that is in the opposite orientation to that in the TAR retains its function when upstream of P38. A more complete picture of the orientation requirement of this motif will require further characterization. In addition, the P38 promoter in a construct in which all sequences upstream of the TAR were deleted could be transactivated to the same level as P38 in the context of the complete viral genome, indicating that sequences upstream of the TAR are dispensable for activity. This is in contrast to what has been reported for Aleutian mink disease parvovirus, for which a greater dependence upon upstream sequences was observed (45). In our experiments, only wild-type full-length NS1 was supplied via the cotransfecting plasmid, pKONS1m. However, it has previously been demonstrated that NS1 alone can mediate wild-type levels of P38 transactivation (34, 35).

Christensen et al. employed an NS1-DNA coprecipitation technique which demonstrated direct NS1 binding to the TAR, mediated by the (ACCA)$_2$ motif (6). Our results suggest that when the TAR alone is deleted (p385UTT-LSΔ1/5), at least some of the numerous NS1 binding motifs present in the MVM 5′-flanking sequences are capable of mediating P38 transactivation. However, it appears that only upstream NS1 binding sites are capable of compensating for a TAR disruption or deletion. The 5′ deletion mutants, all of which retained downstream MVM sequences, exhibited a threefold decrease when the NS1 binding site within the TAR was disrupted, compared with those 5′ deletions which retain the NS1 binding

site. Therefore, even though numerous consensus NS1 binding motifs are present in the 3′-flanking sequences, they cannot compensate for the absence of upstream binding motifs.

Although downstream NS1 sites cannot compensate for the loss of upstream NS1 sites, they may still participate in maximal P38 transactivation. In our experiments, the bGH fusion reporter constructs exhibit a significant increase in P38 transactivation; the bGH sequences contain numerous strong potential NS1 binding sites, which may facilitate the increased transcriptional transactivation of P38 in these constructs. Krauskopf et al. (28) have previously described an element downstream of P38, termed the downstream promoter element, that is important for transactivation in vitro. The region which contains the *cis*-acting downstream promoter element was protected from nuclease attack in the presence of NS1, and its footprint, although not identified in the original report, is consistent with NS1 binding to the $(ACCA)_2$ site at nt 2691. Taken together, these results imply that NS1 binding sites downstream may affect transactivation of P38. As mentioned above, however, our results demonstrate that if sequences downstream of the P38 TATA element are required for transactivation of P38, they need not be of MVM origin, which further suggests that only an NS1 binding site is necessary, in contrast to an MVM-specific sequence which is important for a critical feature of RNA initiation, processing, or procession of the transcription machinery downstream (20).

The system we describe cannot compare basal with transactivated levels of P38 because, by RNase protection analysis, basal levels are undetectable. Therefore, we cannot identify an element(s) which is required solely for transactivated expression. Any transactivated expression will also require the basal transcription elements, and therefore it is not surprising that mutations of the TATA and Sp1 motifs negatively affect transactivated transcription from P38. P38 activity during infection, however, may only exist in response to NS1 (44). Whether this implies that the P38 promoter is intrinsically deficient or under active repression (24) is not known. Preliminary experiments in which individual elements of P38 have been modified to resemble elements from constitutively expressing promoters suggest that we cannot confer detectable basal levels of expression to P38 simply (32a). Also, it is of note that we have been unable to identify a minimal promoter element which acquires detectable basal activity in our assays. These results strongly suggest that NS1 likely affects the basal transcription machinery itself.

The C terminus of NS1, which mediates transcriptional activation as well as at least some aspect of cytotoxicity (30–32), contains a proline-rich region and possesses a net negative charge (3). As has been proposed by others, the recently identified specific DNA binding properties of NS1 suggest a model in which cytotoxicity results when NS1 is directly targeted to a variety of cellular promoters which contain NS1 binding motifs and subsequently activates transcription in an unregulated manner (30, 31). It is also possible that NS1 squelches components of the cellular transcription machinery if NS1 binds cellular factors yet fails to direct them to the appropriate promoters (18, 39, 47). Krady and Ward (27) have recently shown that NS1 coimmunoprecipitates with the ubiquitous eukaryotic transcription factor, Sp1. At high concentrations of NS1, this may result in decreased transcription from GC-dependent promoters which lack NS1 binding sites. NS1 may also interact with a number of other cellular transcriptional components, just as the well-characterized, highly acidic transcriptional activator VP16 interacts with TBP, TFIIB, $TAF_{II}40$, and TFIIH (25, 43, 50). An interaction such as these could explain NS1's reported ability to *trans* inhibit a range of heterologous promoters (30, 42).

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