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Characterization of the Minute Virus of Mice P38 Core Promoter Elements

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While the minute virus of mice (MVM) P4 promoter, which drives the viral nonstructural genes, is highly active in the absence of viral proteins, P38, the capsid gene promoter, is strictly dependent on the viral nonstructural protein NS1. Once fully transactivated, however, P38 mediates twice the steady-state level of expression achieved by P4. In this report, we address the discrepancy between the ability of P38 to mediate very high levels of activated transcription yet only low levels of basal expression, and we investigate the determinants that govern P38 basal expression. The isolated P38 core promoter elements (the P38 Sp1-binding site and TATA element) are at least as transcriptionally competent as the analogous P4 promoter elements. Proximally positioning P4 enhancer factor-binding sequences (nucleotides [nt] 57 to 157) upstream of isolated P38 core transcription regulatory elements or upstream of a native, though abbreviated, P38 cassette (MVM nt 1938 to 2072) confers significant levels of expression to P38 in the absence of NS1, while the full left-end hairpin sequences (nt 1 to 133) elevate basal P38 activity to levels equivalent to P4 basal levels. In the context of the complete viral genome, however, proximally positioned enhancer sequences are unable to confer significant levels of expression to P38, suggesting that low P38 basal levels are a consequence not only of a lack of proximal enhancer elements but also of additional positional regulatory constraints which can be overcome by NS1.

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, 9, 28). The P4 promoter generates mRNAs R1 and R2, which encode the nonstructural proteins NS1 and NS2, respectively (3, 7, 28), while the P38 promoter generates the R3 mRNAs, which encode the viral capsid proteins, VP1 and VP2 (3, 21, 28). NS1 is a nuclear 83-kDa phosphoprotein (7, 8) that has been shown to possess ATPase and helicase activities (19, 26, 38) and sequence-specific DNA-binding properties (5, 6). NS1 is also found covalently attached to the 5′ hairpin of MVM DNA replicative intermediates (10). NS1 has essential roles in viral DNA replication (reviewed in reference 11) and viral gene expression and cytotoxicity (reviewed in reference 37). It has been reported to have a modest stimulatory effect on P4 and has proven to be an extremely potent activator of P38 transcription (12, 13, 16, 20, 22, 23, 30, 35). At late times after release from a highly synchronized infection, transcription from the P38 promoter occurs approximately twice as efficiently as transcription from the P4 promoter (33).

Transcription of P4 in transfected cells, like that in infected cells, occurs in the absence of viral proteins; however, P38 exhibits a strict dependence on NS1 for detectable expression (12, 13, 16, 20, 23, 30, 35). P38 is a relatively simple promoter, containing only a TATA box, a single Sp1-binding site, and a putative initiator sequence (2, 16). Although P38 is located only 2 kb downstream of P4, the activity of the P38 promoter seems neither to be stimulated nor inhibited by transcription elongating downstream from P4 (23). The P38 promoter exhibits wild-type patterns of expression when transfected on a supercoiled closed-circular plasmid or following excision from plasmid sequences (24).

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Here we report a comparative analysis of the core promoter elements of P38 and the constitutively expressing P4 promoter. Replacement of P38 core promoter elements with those from constitutively expressing MVM P4 or simian virus 40 (SV40) promoters did not confer detectable levels of basal expression to P38 as assayed by RNase protection assay. This finding suggested that the reason for extremely low P38 basal expression was not specific to the P38 core promoter elements themselves. When isolated and assayed by more sensitive means, the core elements of P38 (Sp1 and TATA) mediated levels of basal activity similar to that of the analogous P4 elements, suggesting that neither the P38 TATA nor Sp1 motif is intrinsically deficient and that the differing constitutive levels of P38 and P4 might be due to activator-binding sequences upstream of P4. The introduction of P4 activator-binding sequences immediately upstream of P38 in such minimal constructs or in a native though abbreviated P38 cassette conferred substantial levels of basal expression; insertion of such sequences at similar positions relative to P38 within the full-length viral genome, however, had a negligible effect. The inability of the P4 activator sequences to confer detectable levels of basal expression to P38 from its native position approximately 2 kb upstream is not a consequence of the intervening functional P4 promoter; rather, this region functions in a position-dependent manner. Thus, at the earliest times postinfection, when NS1 has not yet reached a critical concentration, P38 activity remains low due not only to a lack of proximal enhancer elements but also to additional positional constraints within the context of the viral genome which can be overcome by NS1.

MATERIALS AND METHODS

Cells and transfections. Murine A92L cells were propagated as described previously (36). All transient transfections were performed with DEAE-dextran as described previously (33). Where indicated, NS1 was supplied by plasmid pKONS1m (23), from which NS1 expression is driven by the SV40 early promoter. Within this construct, the nucleotides between positions 2016 and 2047 were altered such that degenerate changes were introduced into the overlapping NS1 reading frame. The nucleotide alterations within MVM mRNAs generated

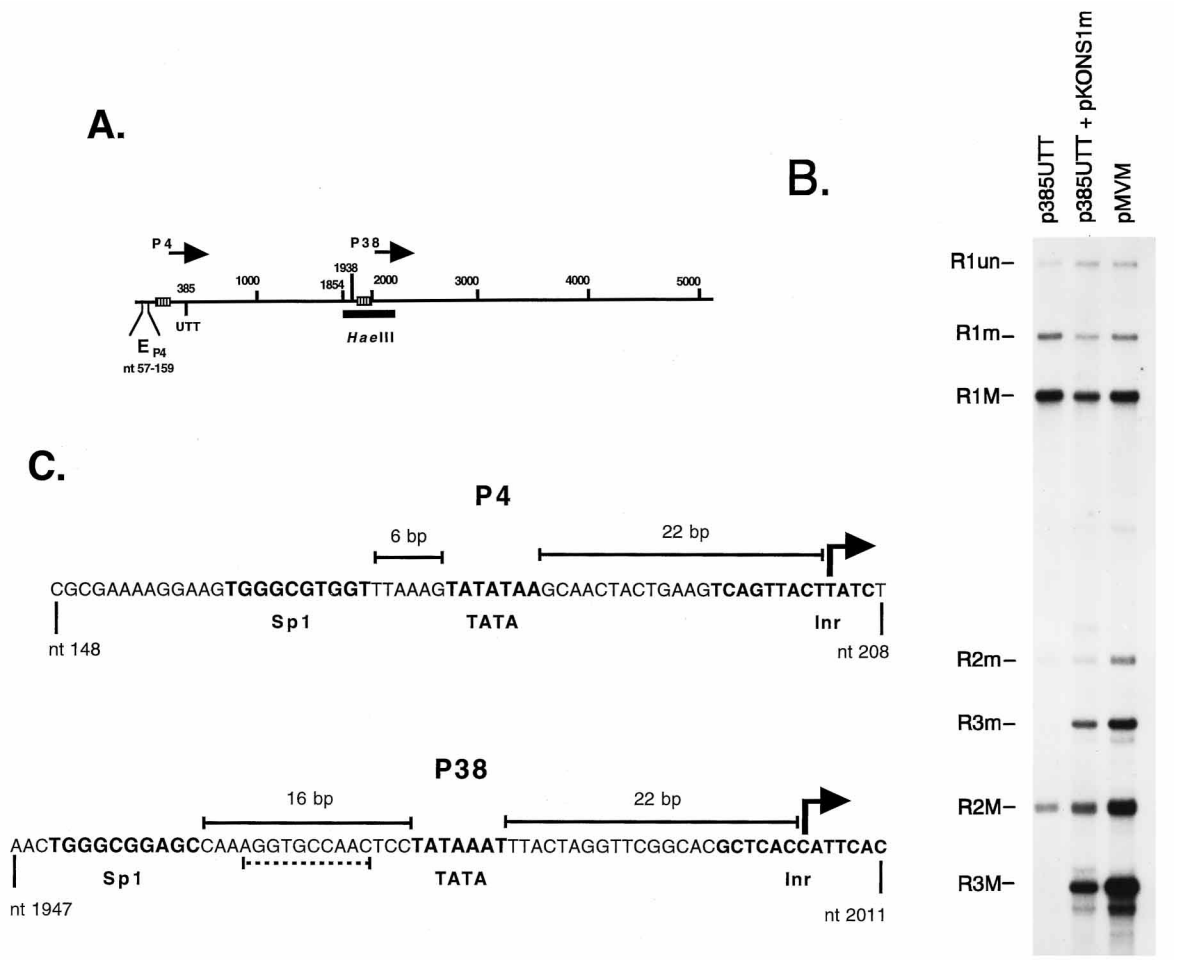


FIG. 1. (A) MVM genome. Locations of the P4 and P38 promoters, as well as the translation termination cassette (UTT) at nt 385, are shown. The thick bar shows the area of the *Hae*III RNase protection probe (nt 1854 to 2378). The hatched areas denote the regions of the P4 and P38 promoters that are shown in detail in panel C. Nucleotides 1938 and 1854, the sites of some of the insertions described in the text and shown below, are also indicated. The region of the genome designated E_{P4}, MVM nt 57 to 159, containing the P4 enhancer region is identified. (B) P38 expression is undetectable in the absence of NS1. 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 after transfection with 20 μg of p385UTT (lane p385UTT), 20 μg of p385UTT plus 10 μg of pKONS1m (lane p385UTT + pKONS1m), or 20 μg of pMVM, a genomic clone of MVM (lane pMVM), 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 (25). The p385UTT P38/P4 ratio is consistently between 0.8 and 1.2 after cotransfection with pKNOS1m. The ratio of basal (untransactivated) P4 activity to NS1-transactivated P4 activity is consistently approximately 0.50 to 0.60. The various MVM RNA species are indicated to the left (see Materials and Methods for explanation). (C) Sequence comparison of core promoter elements from the MVM P4 and P38 promoters. The sequence of the wild-type P4 and P38 core promoter regions are shown, and Sp1, TATA, and putative initiator (Inr) elements are shown in boldface. Modest sequences differences can be seen between the P4 and P38 elements; in addition, there are 6 nt between the P4 Sp1 and TATA motifs and 16 nt between the P38 Sp1 and TATA motifs. Sequences deleted in p385UTTΔ1964-1973 are underlined with dashes (see text for explanation).

from this mutant construct allowed us to differentiate between mRNAs from test plasmids and the NS1-supplying cotransfected plasmid.

Plasmid constructs. (i) Swapping mutants. Plasmid p385UTT, previously described (25), contains a 16-nucleotide (nt) addition containing ochre TAA signals in all three reading frames (5′-GCTTAATTAATTAAGC-3′) at the *Eco*RV site at nt 385 and a small deletion within the right-hand hairpin generated in *Escherichia coli*. Site-specific mutations were introduced into p385UTT by oligonucleotide-mediated M13 mutagenesis. To create p385UTTP38S_{P4}, the native P38 Sp1 element (5′-TGGGCGGAGC-3′) in p385UTT was mutated to that of the P4 Sp1 element (5′-TGGGCGTGGT-3′) (Fig. 1C). p385UTTΔ1964-1973 was created by deleting 10 nt (nt 1964 to 1973 [Fig. 1C]) between the P38 Sp1 and TATA elements. p385UTTP38T_{SV} was constructed by changing the native P38 TATA sequence, 5′-TATAAATTT-3′, to that of the SV40 early TATA sequence, 5′-TATTTATTT-3′ (mutated sequences are underlined).

(ii) pMVMΔ133-1886, p385UTT1938E_{P4}, p385UTTSV21, p385UTTSV21LS11, and p385UTTΔ1-60. The previously described full-length clone of MVM, pMVM (23), was restricted with *Pme*I and *Bst*EII (deleting MVM nt 133 to 1886) to create pMVMΔ133-1886. p385UTT1938E_{P4} was created by cloning MVM sequences 57 to 159 (termed E_{P4}) between the *Bst*EII site (MVM nt 1886) and a *Bgl*II linker at MVM nt 1938 into the previously described parent plasmid, p385UTT-LS9 (23). p385UTTSV21 and p385UTTLS11SV21 were created by cloning a double-stranded oligonucleotide containing the SV40 21-bp repeats (comprised of six Sp1 sites; referred to as SV21 repeats) into the *Bst*EII site (MVM nt 1886) of the parent plasmids, p385UTT and p385UTT-LS11, respectively. LS11 disrupts the native P38 Sp1 element (2, 23). p385UTTΔ1-60 was constructed by deleting the MVM nt 1 to 60 within p385UTT.

Transfection efficiencies of these constructs in experiments shown in Fig. 4B (using plasmids pMVMΔ133-1886 and p385UTT), 4C (using plasmids p385UTTΔ1-60 and p385UTT), and 5 were standardized by using the following procedure. Test plasmids were cotransfected in parallel with 0.25 μg of a control c-*fos*(Luc) vector (gift from M. Hannink) into two additional 60-mm-diameter plates, which were separately used in luciferase activity analysis, as described below. Total P4 and P38 expression levels from constructs shown in Fig. 4B, 4C, and 5, as determined by RNase protection assays, were then standardized relative to luciferase expression from the cotransfect c-*fos*(Luc) vector as determined by luciferase expression.

(iii) P4 promoter disruptions within p385UTT. Mutations which disrupted or deleted critical P4 transcription regulatory motifs were introduced into p385UTT to generate the following constructs: p385UTTLM3 (contains a linker insertion between nt 171 to 180 [disrupting the P4 TATA element]); p385UTTLM4 (contains a linker insertion between nt 159 to 168 [disrupting the P4 Sp1 element]); and p385UTTID1 (contains a linker insertion between 142 to 180 [de-

leting both the P4 Sp1 and TATA elements]). The original P4 linker mutations were created in D. Ward's laboratory, Yale University (1).

(iv) Luc and bGH constructs. P4 and P38 constructs were cloned into either the pGL2-basic plasmid polylinker sequence (Promega, Madison, Wis.) driving expression of the luciferase gene, or into the polylinker of a derivative of pGL2-basic, pGL2-bGH, in which we replaced the luciferase gene with the genomic sequence of bovine growth hormone (bGH) (18). The suffixes (Luc) and (bGH) denote derivation from expression plasmids pGL2-basic and pGL2-bGH, respectively. As described below, Luc constructs were used in luciferase assays, and bGH constructs were used in RNase protection assays. All pGL2(bGH) and pGL2(Luc) derivative constructs contain an additional poly(A) signal immediately upstream of the promoter constructs described in the text, which serves to reduce the level of nonspecific background readthrough.

Isolated core promoter constructs. pP4S/T was created by annealing the following complementary single-stranded oligonucleotides: 5′-CTAGCTGGGCGTGGTCCATGGTATATAATC-3′ (top) and 5′-TCGAGATTATATACCATGGACCACGCCCA-3′ (bottom). When annealed, the double-stranded substrate contains compatible cohesive 5′ and 3′ ends, which allows this fragment to be directionally cloned between the *Nhe*I and *Xho*I sites within the pGL2-bGH polylinker. pP38S/T was created by annealing the following complementary single-stranded oligonucleotides: 5′-CTAGCTGGGCGGAGCATCGTCCATGGAGCGATATAAATTC-3′ (top) and 5′-TCGAGAATTTATATCGCTCCATGGATGGACGATGCTCCGCCCAGG-3′ (bottom). This double-stranded substrate also contains 5′ and 3′ ends compatible with *Nhe*I- and *Xho*I-generated ends and was cloned between the *Nhe*I and *Xho*I sites within the pGL2-bGH polylinker. These oligonucleotides contain the respective wild-type Sp1 and TATA sequences (underlined) of either P4 or P38. The flanking and intervening sequences are heterologous, but the nucleotide spacing between the Sp1 and TATA elements is identical to the native promoter spacing (6 nt at P4 and 16 nt at P38). The putative initiator elements were introduced by using complementary single-stranded oligonucleotides containing 13 MVM nt spanning the authentic RNA initiation site of either P4 or P38. To generate pP4S/T/I, oligonucleotides 5′-TCGAGTTCGCGTTCAGTTACTTATCA-3′ (top) and 5′-GATCTGATAAGTAACTGAACGCGAAC-3′ (bottom) were annealed and ligated into pP4S/T at the *Xho*I and *Bgl*II sites in the polylinker. To generate pP38S/T/I, oligonucleotides 5′-TCGAGTTCGCGTGTCCGCTCACCATTCACA-3′ (top) and 5′-GATCTGTGAATGGTGAGCGGACACGCGAAC-3′ (bottom) were annealed and ligated between the *Xho*I and *Bgl*II sites of pP38S/T. The P4 and P38 RNA initiation site within the initiator elements was positioned such that the distance from the TATA element within pP4S/T/I and pP38S/T/I was the same as in the native promoter contexts; however, the intervening sequence is heterologous. Sequence inspection revealed that the intervening sequences did not introduce any known binding elements for additional factors.

To generate the pSV21P4S/T and pSV21P38S/T constructs, the SV21 repeat was first cloned into the *Sma*I site in the pGL2-bGH polylinker (a generous gift from M. Martin). P4 and P38 Sp1 and TATA elements from p4S/T and p38S/T constructs were then cloned approximately 60 nt downstream of the SV21 repeats in the polylinker of the pGL2-SV21/bGH construct.

To generate the pE_{P4}P4S/T and PE_{P4}P38S/T constructs, the P4 enhancer factor-binding sequence (MVM nt 57 to 159), designated E_{P4}, was first excised as a *Bss*HII-*Bgl*II fragment from an MVM-containing plasmid in which the P4 Sp1 element was disrupted by a *Bgl*II-containing linker. This fragment was then cloned into the pP4S/T(bGH) and pP38S/T(bGH) constructs immediately upstream of the Sp1 elements within pP4S/T(bGH) and pP38S/T(bGH).

Expression values generated from the Luc constructs are shown relative to values for pE_{P4}P38S/T(Luc), transfected at the same time, and are the averages of four individual experiments in which each test plasmid was transfected in duplicate. We find that analysis of the persistence of plasmid DNA in transfected cells is not an appropriate tool for standardizing values between such transfections; in our hands, the great majority of this DNA remains extranuclear.

E_{P4} series. pE_{P4}1854-2072, pE_{P4}1854-5000, and pE_{P4}1938-2072 contain MVM nt 1854 to 2072, 1854 to 5000, and 1938 to 2072, respectively, which were cloned between the *Sac*I and *Xho*I sites in the pGL2-bGH polylinker. P4 enhancer factor-binding sequences, nt 57 to 159 (E_{P4}), were introduced immediately upstream of MVM nt 1854 (pE_{P4}1854-2072 and pE_{P4}1854-5000) or 1938 (pE_{P4}1938-2072).

Transfection efficiencies of these constructs were controlled for in the experiments presented in Fig. 3C and 4A by the following procedure. pKONS1mΔ1248-2072 (2 μg/100-mm-diameter plate), a derivative of the SV40-driven NS1-supplying plasmid, pKONS1m (23), in which MVM nt 1248 to 2072 have been deleted (including all previously identified P38 regulatory motifs), was cotransfected with the experimental P38 plasmids shown in Fig. 3C and 4A. The cotransfecting pKONS1m-deleted plasmid produced an mRNA transcript, driven by the SV40 early promoter, which was specifically detected by using an MVM RNase protection assay probe spanning nt 650 to 385. Expression from this cotransfecting plasmid was quantitated by RNase protection assay analysis. The levels of P38 expression from constructs shown in Fig. 3C and 4A were then standardized relative to the amount of expression from the cotransfected pKONS1mΔ1248-2072 plasmid.

RNA isolation and characterization. Total RNA was isolated, and RNase protection assays were performed as described previously (23, 25). mRNAs designated R1M to R3M use the small intron donor at nt 2280, those designated R1m to R3m use the small intron donor at nt 2317, and that designated R1un is unspliced in the small intron region. Quantitations were performed on a Molecular Dynamics PhosphorImager. Expression levels were analyzed by using an antisense probe of the bGH cDNA, generated from a *Pst*I-*Pst*I fragment (bGH genomic nt 718 to 1757), cloned into pGEM3Z, that produces a protected species of approximately 410 bp (23).

Luciferase assays. Briefly, 10^6 A92L cells, in a 60-mm-diameter dish, were transfected with 1 μg of template DNA. At 48 h posttransfection, whole-cell extracts were prepared by the Triton X-100 basic protocol and assayed as described previously (4). Values shown represent four individual transfection experiments. When indicated, the NS1-supplying plasmid, pKONS1m (23), was cotransfected at 0.25 μg/60-mm-diameter dish. A titration experiment indicated that this amount was the minimal concentration which induced the maximal level of p1854-2072(Luc) expression.

RESULTS

Comparative analysis of the MVM P38 promoter. As previously determined, P38 transcriptional regulatory elements consist of an NS1-binding motif, an Sp1 motif, a TATA element, and a putative initiator element (2, 16, 20, 22, 23). Following transfection, in the absence of NS1, although the basal activity of the P4 promoter is substantial, transcription from P38 occurs at levels which are undetectable by RNase protection assays (Fig. 1B; note the absence of R3M and R3m in lane p385UTT) (23, 25, 40). Upon the addition of NS1 via a cotransfected plasmid (pKONS1m), however, P38 is transactivated highly; R3 transcripts accumulate to approximately twice the levels of R1 and R2 transcripts generated by the P4 promoter (Fig. 1B; compare p385UTT with and without pKONS1m) (23, 33). P4 transcription is also activated modestly (approximately 0.5-fold) upon cotransfection of wild-type NS1 (Fig. 1B); therefore, steady-state basal P4 activity reaches approximately 25% of NS1-transactivated P38 expression. Although within the context of the MVM chromosome, P4 and P38 mediate dramatically different levels of basal (in the absence of NS1) expression, the sequences of the core regulatory elements (Sp1 and TATA) of these promoters are remarkably similar (Fig. 1C).

Initially, to determine whether the subtle differences between P4 and P38 core promoter elements could account for their dramatically different levels of basal transcription, we constructed the following series of swapping mutants. Core promoter elements of P38 were replaced with the analogous motifs from either P4 or the constitutively expressing SV40 early promoter, in an attempt to confer detectable levels of basal expression to P38. In the first P38 construct, p385UTTΔ1964-1973, the number of nucleotides between the Sp1 and TATA elements was reduced from 16, as in the native P38 promoter, to 6, as in the native P4 promoter (Fig. 1C). In a separate construct, $p385UTTP38S_{P4}$, the P38 Sp1 site was replaced with the P4 Sp1 sequence. Finally, we exchanged the P38 TATA element, 5′-TATAAAT-3′, for the more divergent TATA element from the SV40 early promoter, 5′-TATTTAT-3′ ($p385UTTP38T_{SV}$). Basal P38 activity remained undetectable by RNase protection assays for all modified P38 promoter constructs tested (Fig. 2, R3M and R3m in −NS1 lanes). Following cotransfection of NS1, however, all of the P38 chimeric promoters were highly transactivated (Fig. 2, +NS1 lanes). These results suggested that differences in expression of P4 relative to P38 might be independent of the nature of the core sequences themselves.

To further characterize and compare the relative activities of the individual core regulatory elements of P4 and P38, the Sp1 and TATA motifs of each promoter were inserted into an otherwise heterologous plasmid backbone (the polylinker sequence of pGL2 [Promega]) in the same orientation and spacing as they exist in their natural contexts, upstream of the bGH gene, which served as the reporter gene for RNase protection

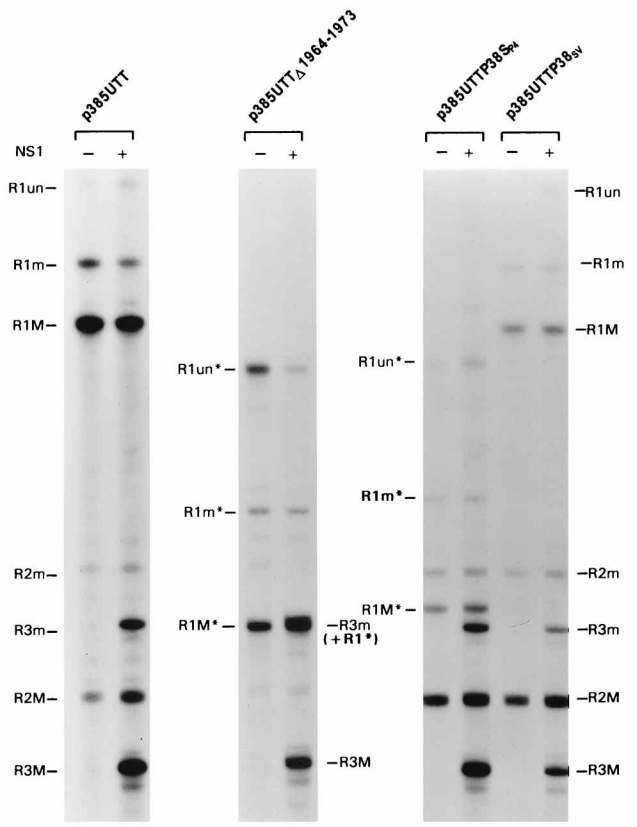


FIG. 2. Significant levels of basal expression cannot be conferred to chimeric P38 promoters by replacing core P38 regulatory elements with those from constitutively expressing promoters. Shown are RNase protection assays, using the wild-type *Hae*III probe shown in Fig. 1A (p385UTT, p385UTTΔ1964-1973, and p385UTTP38S$_{P4}$) or a homologous *Hae*III-like probe (p385UTTP38T$_{SV}$), of 20 μg of total RNA taken 48 h after transfection of A9 cells with 20 μg of each experimental plasmid, with or without 10 μg of the NS1-supplying pKONS1m as indicated. P4 R1 mRNAs produced from plasmids p385UTTΔ1964-1973 (indicated by *) and p385UTTP38S$_{P4}$ (indicated by **) protect less than full-length fragments because the engineered mutations are within the same region protected by the wild-type *Hae*III probe; however, the protected R2 and R3 mRNAs species are not altered. The R1M* species of p385UTTΔ1964-1973 migrates at approximately the same position as wild-type R3m. Analysis with a homologous *Hae*III-like probe demonstrates that p385UTTΔ1964-1973 generates no significant levels of either R3M or R3m in the absence of NS1 (data not shown).

assay analysis (see Materials and Methods for details). Neither the core elements from P38 nor those from the constitutively expressing P4 promoter generated detectable transcript levels by RNase protection assays (data not shown). When the bGH gene was replaced with a luciferase reporter gene, however, allowing comparison of P4 and P38 at a greater level of sensitivity, the isolated P38 Sp1 and TATA elements displayed activity slightly greater than that of the analogous Sp1 and TATA elements from the constitutively expressing P4 promoter [Fig. 3A; compare pP38S/T(Luc) and pP4S/T(Luc)]. The introduction of the respective putative initiator elements had little effect with either a P4 or P38 construct [Fig. 3A, pP38S/T/I(Luc) and pP4S/T/I(Luc)].

Thus, direct comparisons of the isolated promoter elements of P4 and P38 demonstrate that the Sp1 and TATA elements of P38 are not intrinsically deficient and in fact mediate modestly higher levels of expression than the analogous elements from the constitutively expressing P4 promoter. We conclude that the core promoter elements of P38 are themselves relatively strong, and their lack of activity within the wild-type MVM genome is likely due to additional constraints related to their position within the viral chromosome.

Basal activity of minimal P38 constructs can be stimulated by proximal enhancer elements. Although the core transcription regulatory elements of P4 and P38 are quite similar, the native 5′ upstream region of the P4 promoter contains, in addition to the left-end hairpin, numerous binding elements for cellular transcription activators which have been shown to affect P4 transcription (1, 15, 17, 27). The introduction of the P4 enhancer sequences (E_{P4}) upstream of the Sp1 motif in both the pGL2 P4 and P38 Sp1/TATA minimal promoter constructs (described above), in a position relative to the Sp1 element similar to the relative position of the E_{P4} sequence within the native P4 promoter, resulted in substantial and similar levels of basal expression from both synthetic promoters [Fig. 3B; compare pE_{P4}P38S/T(Luc) and pE_{P4}P4S/T(Luc)]. The sequences introduced into these constructs excluded the majority of the left-hand hairpin structure and extended to immediately 5′ of the P4 Sp1 site (Fig. 1A). The introduction of the SV21 repeats (which contain six Sp1-binding sites) upstream of the minimal P4 and P38 S/T constructs also elevated expression to similar levels (Fig. 3B). E_{P4} sequences stimulated expression of both promoter constructs to approximately 12% (and the SV21 repeats to approximately 6%) of fully NS1-transactivated P38 expression, as determined by RNase protection assays using the bGH reporter gene for direct comparison (Fig. 3C). These results demonstrated that substantial levels of basal expression can be achieved from the core P38 promoter elements by addition of either enhancer-factor binding sites present at the native P4 promoter or the SV21 repeats.

E_{P4} can also confer modest basal activity to a small P38 cassette (nt 1938 to 2072) taken from the viral chromosome. In this construct [pE_{P4}1938-2072(bGH)], the upstream enhancer sequences are positioned 12 nt upstream of the P38 Sp1 element, similar to their position in the pS/T constructs. P38 activity of this construct was similar to that seen for the E_{P4}pS/T construct [approximately 12% of NS1-activated P38 levels; compare pE_{P4}P38S/T(bGH) with pE_{P4}1938-2072(bGH) in Fig. 4A], which suggested that proximally positioned enhancer factor-binding sequences can also confer substantial levels of basal activity to a P38 promoter in a more natural context. When E_{P4} was positioned approximately 96 nt upstream from the P38 Sp1 site at nt 1854, however, only approximately 3% of NS1-transactivated levels was achieved (Fig. 4A, pE_{P4}1854-2072 and pE_{P4}1854-5000), suggesting that the ability of E_{P4} to stimulate P38 maybe subject to a positional effect.

E_{P4} elements alone stimulated levels of P38 basal transcription in these abbreviated constructs to approximately 12% of NS1-transactivated levels, which is equivalent to approximately half of the basal transcription achieved by the native P4 promoter. When the full-length left-hand end of MVM, including both the enhancer factor-binding sites and hairpin sequences (MVM nt 1 to 133), were positioned upstream proximal to P38 at nt 1886, however, basal expression from P38 was stimulated an additional twofold, to approximately 25% of NS1-transactivated levels (Fig. 4B, pMVMΔ133-1886, R3). These results demonstrated that the hairpin sequences of P4, which contain an additional NF-Y and ATF binding site, can contribute to P38 basal activation and, together with E_{P4} sequences, stimulate P38 basal activity to levels comparable to P4 basal activity (approximately 25% that of NS1-transactivated P38 activity). Consistent with this observation, a deletion of the left-hand hairpin sequences (nt 1 to 60) from an otherwise full-length viral genome decreased P4 basal transcription approximately

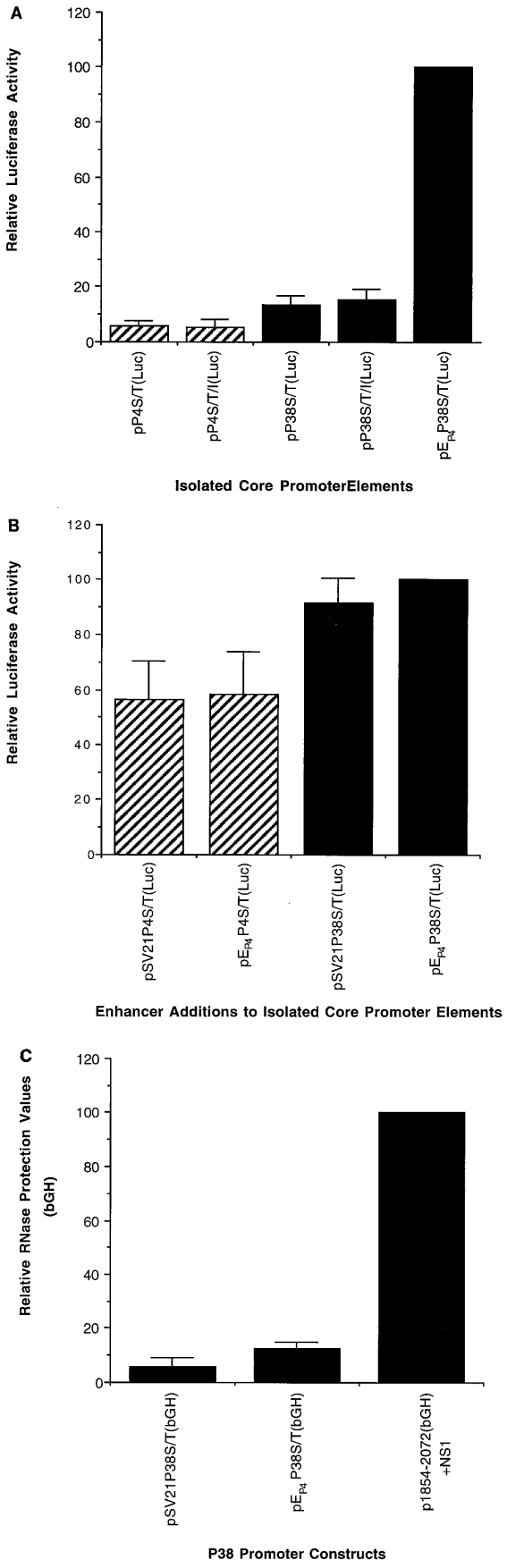
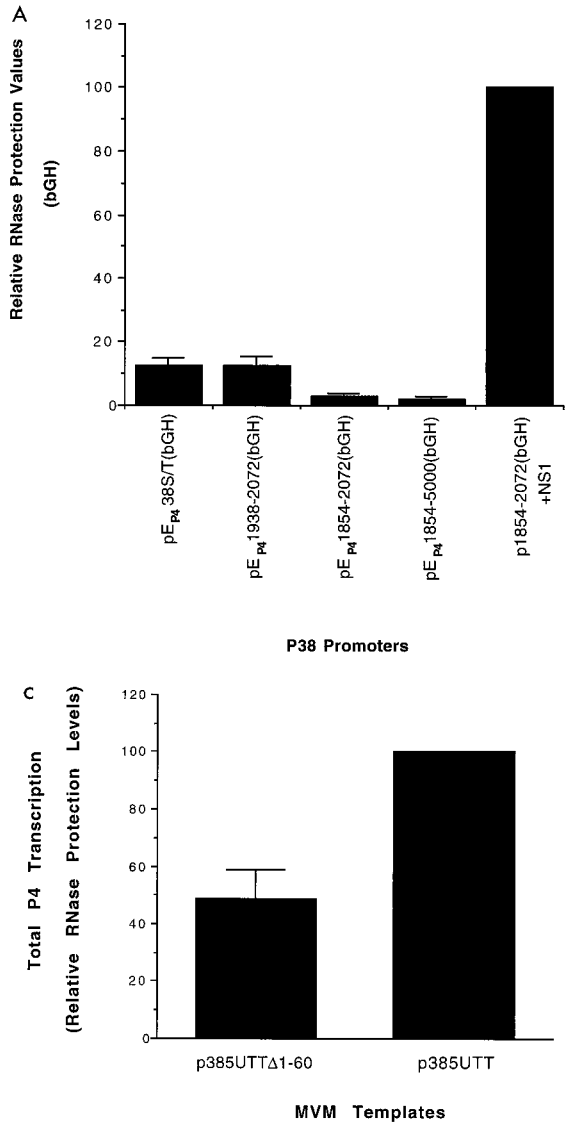


FIG. 3. Isolated P38 core regulatory elements mediate significant levels of basal activity and can be further elevated by proximal positioning of E_{P4} or the SV21 repeats. (A) A9 cells were transfected (2 μg per 60-mm-diameter plate) with experimental plasmids containing core regulatory motifs from either P4 (hatched bars) or P38 (solid bars) (S, T, and I correspond to the appropriate Sp1, TATA, and initiator elements from each promoter, as described in Materials and Methods). Luciferase values are presented relative to those for pE$_{P4}$P38S/T(Luc) (described below); they represent four independent experiments and are as follows (percentages including 95% confidence limits): pP4S/T(Luc), 5.59 ± 1.98; pP4S/T/I(Luc), 5.53 ± 2.60; pP38S/T(Luc), 13.33 ± 3.77; and pP38S/T/I(Luc), 15.64 ± 3.69. (B) P38 basal activity can be stimulated by proximal enhancer elements. The SV21 repeats or E_{P4} was positioned approximately 65 or 12 nt upstream, respectively, of the Sp1 element within p4S/T (hatched bars) or p38S/T (solid bars) constructs. Transfections and luciferase assays were performed as for panel A; values are relative to those for pE$_{P4}$P38S/T and represent four independent experiments (percentages including 95% confidence limits): pSV21P4S/T(Luc), 56.23 ± 13.98; pE$_{P4}$P4S/T(Luc), 58.48 ± 15.28; and pSV21P38S/T(Luc), 91.38 ± 9.42. (C) Proximal enhancer elements conferred significant levels of expression to P38 relative to NS1-transactivated levels. pSV21P38S/T(bGH), pE$_{P4}$P38S/T(bGH), and p854-2072(bGH) drive expression of the bGH gene, which served as a reporter gene for RNase protection assay analysis, by using a bGH cDNA-generated probe as described in Materials and Methods. A9 cells were transfected (20 μg per 100-mm-diameter plate) with each experimental plasmid indicated. p1854-2072(bGH), which is able to be transactivated to wild-type levels (23), was cotransfected with 10 μg of pKONS1m. Quantitations and standardizations using a cotransfected reported plasmid were performed as described in Materials and Methods. Values are presented relative to those for p1854-2072 plus pKONS1m; they represent four independent experiments and are as follows (percentages including 95% confidence limits): pSV21P38S/T(bGH), 5.9 ± 3.1; and pE$_{P4}$P38S/T, 12.5 ± 2.5.

50% (Fig. 4C, pUTT385Δ1-60), further demonstrating that extreme left-end sequences are required for full P4 expression.

Enhancer factor-binding sequences do not stimulate P38 in the context of the full viral genome. Enhancer sequences can often function over long distances; however, since P38 transcription levels, in the absence of NS1, are extremely low in the context of the viral genome, left-hand hairpin and P4 enhancer factor-binding sequences do not significantly effect P38 basal activity from their native position 2 kb upstream of P38. To investigate whether the intervening P4 promoter might interfere with the left-hand end elements interacting with P38, P38 activity was assayed from mutants in which critical P4 basal transcription elements were disrupted in otherwise full-length MVM genomes. Three such P4 mutants, which had linker disruptions in either the P4 Sp1 site or the P4 TATA element or had a deletion of the nucleotides between positions 142 and 180 (which includes the P4 Sp1 and TATA elements) (see Materials and Methods), exhibited dramatically decreased or completely abolished P4 transcription as determined by RNase protection assays (data not shown). In none of these constructs, however, was basal P38 transcription activated, suggesting that neither a functional P4 promoter nor the core P4 promoter elements themselves had inhibitory effects on E_{P4}-P38 interactions (data not shown). It is most likely, therefore, that the inability of left-end sequences to stimulate basal levels of the strong P38 core elements within the wild-type genome in the absence of NS1 is due to a positional effect, either because of extreme distance or because of effects of intervening sequences.

To our surprise, however, neither E_{P4} nor the SV21 repeats could stimulate significant basal expression when placed proximal to P38 in the context of the full-length viral genome. In one construct, p385UTT served as the parent plasmid into which the E_{P4} sequences were inserted 12 nt upstream of the P38 Sp1 site at MVM nt 1938 (p385UTT1938E$_{P4}$). In two additional constructs, the SV21 repeats were positioned approximately 65 nt upstream of the native P38 Sp1 site at MVM nt 1886 within either p385UTT (p385UTTP38SV21) or a p385UTT derivative in which the native P38 Sp1 element has



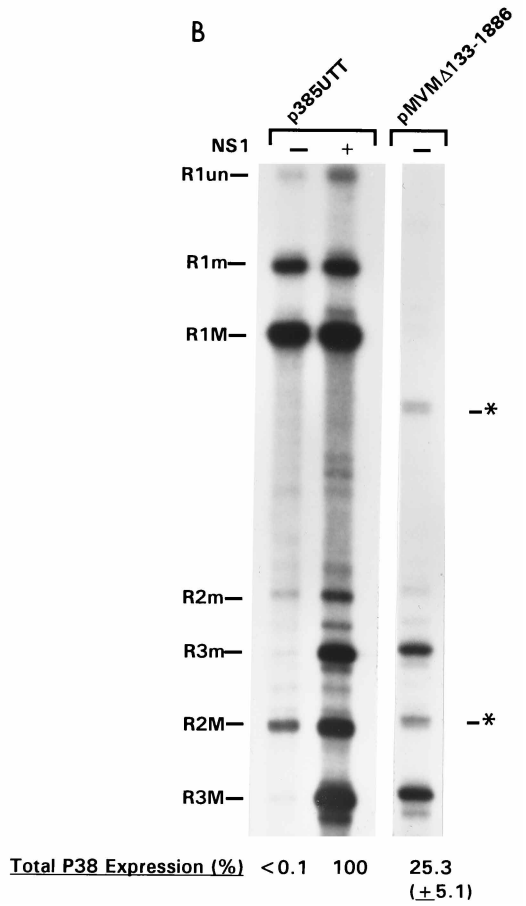


FIG. 4. (A) E_{P4} stimulates P38 expression in a position-dependent manner. Shown are P38 expression levels from various E_{P4}-P38(bGH) experimental templates, analyzed by RNAse protection assays, using a bGH-specific probe. Values cotransfected with the NS1-supplying plasmid, pKONS1m (23). E_{P4} was positioned either 12 nt (pE_{P4}P38S/T and pE_{P4}1938-2072) or 96 nt (pE_{P4} 1854-2072 and pE_{P4}1854-5000) upstream of the P38 Sp1 element. Quantitations and standardizations using a cotransfected reporter plasmid were performed as described in Materials and Methods. Values from four separate experiments, determined by RNase protections of 20 μg of total RNA isolated from A9 cells 48 h posttransfection as described for Fig. 1A, are as follows (percentages including 95% confidence limits): pE_{P4}P38S/T, 12.5 ± 2.5; pE_{P4}1938-2072, 12.5 ± 3.1; pE_{P4}1854-2072, 2.7 ± 0.4; and pE_{P4}1854-5000, 2.1 ± 1. (B) Proximally positioned MVM left-hand sequences confer basal expression levels to P38 equivalent to basal levels of wild-type P4. Shown are RNase protection assays, using the wild-type *Hae*III probe of 20 μg of total RNA taken 48 h after transfection of A9 cells, as described for Fig. 1. Specifically initiated P38 transcription from pMVMΔ132-1886 (R3m and R3M) is expressed relative to NS1-transactivated P38 levels from p385UTT from three separate experiments and is as follows (percentages including 95% confidence limits): pmVMΔ132-1886, 25.3 ± 5.10; and p385UTT, 100. Quantitations and standardizations using a cotransfected plasmid were performed as described in Materials and Methods. Low levels of nonspecific, spliced read-through products are indicated by asterisks. The low basal P38 levels from p385UTT seen in this experiment are likely due to spillover from the adjacent lane; P38 basal levels are reproducibly undetectable by RNase protection assays. (C) Extreme left-end sequences are required for maximal P4 basal expression. RNase protection assay values of P4 basal expression, relative to p385UTT P4 basal expression, for p385UTT and p385UTTΔ1-60 were obtained by using the wild-type *Hae*III probe, with quantitations and standardizations using a cotransfected plasmid performed as described in Materials and Methods. The level for p385UTTΔ1-60 relative to p385UTT (including 95% confidence limits) is 48.5% ± 10.5%.

been disrupted (p385UTTSV21LS11). In the latter two constructs, the SV21 repeats were placed in a position similar to their positions in the P4 and P38 SV21S/T constructs, from which expression levels were approximately 6% of NS1-transactivated levels (Fig. 3C). In none of these constructs did the introduction of enhancer sequences proximal to P38, within the context of full-length viral genome, result in significant basal P38 expression levels (R3M and R3m) in the absence of NS1 (Fig. 5). All three mutant constructs can be transactivated fully by NS1, indicating that the introduction of the enhancer elements did not functionally inhibit the P38 core promoter elements. Additionally, P38 expression was not enhanced when these templates were linearized prior to transfection (data not shown).

DISCUSSION

While the MVM P4 promoter is highly active in the absence of viral proteins (1, 14, 15, 17, 27), the MVM capsid gene promoter, P38, has been shown to be dependent on NS1 for transcriptional activation (12, 13, 16, 20, 22, 23, 30, 35). In the absence of NS1, P38 expression is undetectable by sensitive RNase protection assays, whereas cotransfection of an NS1-supplying plasmid results in high levels of P38 expression (20, 22, 23, 25, 30–32, 40). The discrepancy between the ability of P38 to mediate very high levels of activated transcription yet

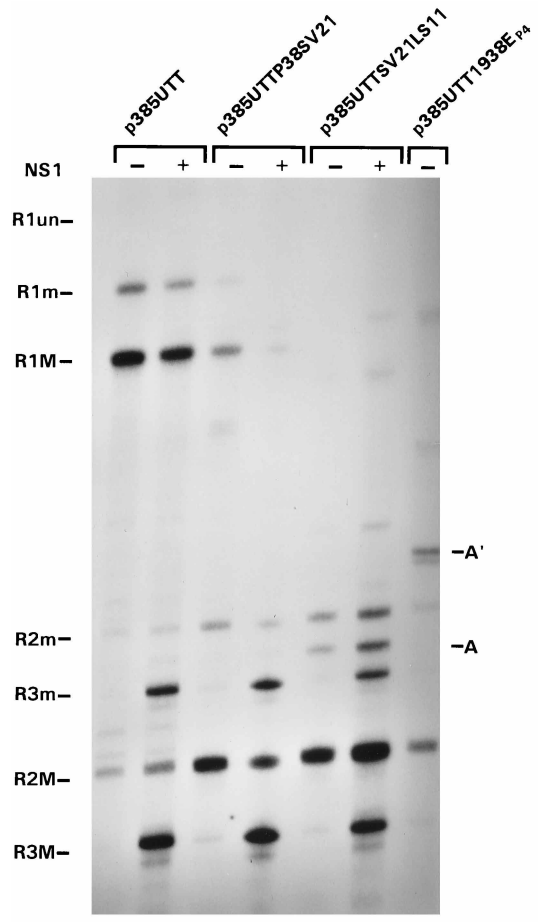


FIG. 5. Within the context of the full-length genome, proximally positioned enhancer elements are not sufficient to confer significant levels of P38 basal expression. Shown are RNase protection assays, using the wild-type *Hae*III probe, of RNA generated from full-length MVM constructs which have either E_{P4} positioned 12 nt upstream of the P38 Sp1 element (p385UTT1938E_{P4}) or the SV21 repeats positioned 65 nt upstream of the P38 Sp1 site (p385UTTSv21 and p385UTTSV21LS11). The native P38 Sp1 element within p385UTTSV21LS11 has been disrupted by a 12-nt linker substitution (LS11) (23). R1 transcripts generated from the P38-enhancer constructs result in wild-type *Hae*III protected fragments (A and A′) which are smaller than p385UTT-generated R1 protected fragments because the SV21 repeats, E_{P4}, and the P38 Sp1 disruption (LS11) are within the *Hae*III-protected region. A corresponds to p385UTTSV21LS11-generated R1m; A′ corresponds to p385UTT1938E_{P4}-generated R1M. Low levels of wild-type-length R1-protected fragments are detectable from p385UTTSV21 even though the SV21 repeats are present in the protected region; however, these bands result from incomplete RNase digestion of probe sequences. Analysis with a homologous *Hae*III-like probe confirms the presence of the SV21 repeats; such probes protect slightly larger than wild-type R1 species (data not shown). P38 basal expression from p385UTT1938E_{P4}, p385UTTSV21, and p385UTTSV21LS11 was quantitatively less than 1% of NS1-transactivated levels (as described in Materials and Methods). When indicated, NS1 was supplied in *trans*. As expected, NS1 transactivated the P38 promoters of p385UTTSV21 and p385UTTSV21LS11, demonstrating that the insertion of the SV21 repeats did not functionally inhibit the P38 core promoter elements. Additionally, p385UTT1938E_{P4}, containing the large substitution mutation upstream of P38 (see Materials and Methods), can also be transactivated to near wild-type levels by NS1 (data not shown).

only low levels of basal expression led us to investigate the determinants that govern P38 basal expression and compare them to the constitutively expressing P4 promoter.

Using a more sensitive reporter system, we could show that the core promoter signals of P38 (the Sp1 site and TATA box) are at least as competent for basal expression as those of P4, suggesting that the fundamental difference between the constitutive activities of the two promoters might be due to the enhancer factor-binding sites upstream of P4. We could find no role for specific sequences at the RNA initiation site of P38 or P4 even though these regions are both similar to previously identified consensus initiator sequences (34). The conclusion that the P38 core promoter elements are indeed strong is supported by in vitro transcription experiments: the P4 and P38 promoters exhibit similar high levels of activity in the absence of NS1 in both murine FM3a and HeLa cell nuclear extracts, using purified templates (24, 29).

The isolated P38 core promoter signals (pP38S/T) and the natural P38 promoter in abbreviated constructs (containing nt 1938 to 2072) could be stimulated to the same levels as the core P4 elements both by P4 enhancer factor-binding sequences (E_{P4}) and by the SV21 repeat Sp1-binding sites, placed proximal to P38. These results again suggested that a fundamental difference between the basal activities of P4 and P38 is the presence of the enhancer factor-binding region upstream of P4. Although the elements within E_{P4} responsible for mediating transcriptional activation have not been characterized, given the similarities between P4 and P38, it is reasonable to assume that the elements which govern P4 expression, specifically the NF-Y, ATF, and USF sites (1, 14, 17, 27), also contribute to P38 expression. It is interesting that both the P4 enhancer factor-binding sequences and the SV21 Sp1-binding sites can stimulate equivalent levels of expression from either P4 or P38 constructs, since it is likely that P4 enhancer binding factors and Sp1 act very differently. These results do suggest, however, that both sets of core promoter elements are capable of being activated through similar mechanisms. Furthermore, full activation of either set of core elements required proximal positioning of the full left hand-end, including the hairpin sequences. Consistent with the later observation was the demonstration that full basal activity of the native P4 promoter in the full-length genome required the full left-hand end, which contains additional NF-Y and ATF sites.

The P4 enhancer factor-binding sequences do not stimulate P38, in the absence of NS1, from their natural position 2 kb upstream. One reason may be that the P4 enhancer factor-binding region is ineffective at a distance. The NF-Y sites within E_{P4}, although imperfect, contribute substantially to native P4 basal expression (17). The major histocompatibility complex class II-associated invariant chain promoter, which also has an imperfect NF-Y site, requires the presence of an Sp1-binding element adjacent to this site for full activation by NF-Y (39). Therefore, it could easily be envisioned that activation by NF-Y in the MVM genome is distance or position dependent.

However, neither the P4 enhancer factor-binding sequences nor the SV21 repeats could stimulate P38 in the context of the complete viral genome when placed proximal to P38. These results suggest that perhaps its local context renders P38 recalcitrant to enhancer activation (at least by the factors that bind to the P4 enhancer or by multiple Sp1-binding sites), leaving P38 under the more temporally regulatable control of NS1. Such nonresponsiveness to enhancer-binding factors could easily be due to local chromatin constraints and may be related to the mechanism of NS1 transactivation.

Our results have also permitted an evaluation of the relative strengths of P4 and P38 under various conditions. As shown by both RNase protection analysis (likely to be valid for these evaluations since MVM RNA is quite stable [33]) and previous nuclear run-off experiments (33), the transactivated level of P38 activity is approximately twice that of transactivated P4. P4 activity in the presence of NS1 is approximately twice that of

basal P4, and therefore P4 basal activity is approximately 25% of NS1-transactivated P38 activity, while basal P38 activity (which relies only on core promoter sequences) is less than 1% of the activity of transactivated P38.

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