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The Minute Virus of Mice P_{39} Transcription Unit Can Encode Both Capsid Proteins

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The right-hand 80% of the genome of minute virus of mice (MVM) was cloned into the bovine papillomavirus type I shuttle vector and used to transfect mouse C127 cells. Transformed lines were isolated that efficiently produce both authentic MVM capsid proteins at a ratio similar to that seen in a normal viral infection, and these proteins assemble into intact empty virions. The only transcription of MVM sequences detected in these lines was representative of the viral P_{39} transcription unit, which therefore contains sufficient information to encode both authentic capsid proteins at the same regulated ratio seen in an infected cell.

Minute virus of mice (MVM) is a member of the autonomous subgroup of the parvovirus family. The prototype strain, designated MVM(p), contains a 5-kilobase (kb) single-stranded genome with terminal palindromic sequences that form hairpin duplex structures. The MVM(p) genome has been completely sequenced (1), and its basic transcriptional organization has been determined (8). The genome is composed of two overlapping transcription units which encode the three major viral transcripts (8). The two largest transcripts, designated R1 and R2, are 4.8 and 3.3 kb in length, respectively, and are initiated at a promoter 4 map units from the left (3′) end of the genome. Transcript R3, which is 3.0 kb in length and the most abundant mRNA produced in vivo, is initiated from a second promoter at 39 map units on the viral DNA and lies within sequences transcribed in both R1 and R2 (Fig. 1A). All three of these mRNAs are polyadenylated near the right-hand terminal hairpin.

MVM and H1, two autonomous rodent parvoviruses that have been studied in great detail, are organized similarly, and each contains two large open reading frames, one in each half of the genome. Hybrid-selected (2) and hybrid-arrested (10) translation studies, as well as direct protein analysis (7, 15) and nucleotide sequence inspection (1, 10), have led to the conclusion that the left open reading frame encodes the larger of two nonstructural proteins, NS-1; the right open reading frame encodes the smaller of the virion proteins, VP2, and participates in coding the larger and less abundant virion protein, VP1 (the amino acid sequence of VP2 is entirely contained within the carboxy-terminal 80% of VP1). Because of the overlapping transcriptional organization of the parvovirus genome, it has been difficult to determine which message(s) encodes the virion proteins and whether information from the left end of the genome is required for the production of VP1. Previous studies have demonstrated that the P_{40} transcription unit of the dependovirus adeno-associated virus type 2 can encode the three adeno-associated virus type 2 capsid proteins (3, 4). In this report, from analysis of the expression of MVM-bovine papillomavirus (BPV) chimeras, we show directly that the right-hand 80% of the MVM genome is sufficient to encode both authentic viral structural proteins, presumably from the viral 3.0-kb message(s).

The MVM-BPV constructs used in these studies are shown in Fig. 1B. An *Eco*RI-*Bam*HI fragment spanning nucleotides 1086 to 5081 from an infectious clone of MVM (6) was first cloned into the plasmid vector pMLd (5, 11). This fragment lacks the MVM promoter at 4 map units, where both R1 and R2 are initiated. The complete BPV genome, removed as a *Bam*HI fragment from the vector p142-6 (11), was then cloned into the MVM-pMLd plasmid in both orientations at the downstream termini (with respect to transcription) of the MVM fragment at the unique *Bam*HI site.

These constructs were used to transfect mouse C127 cells (12) by the calcium phosphate technique (12). Transformed foci were isolated as described previously (9) and screened by an indirect immunofluorescence assay. Cells grown on microscope slides were fixed in methanol for 5 min at 0°C, incubated with a rabbit serum raised against purified empty MVM virions (a gift of P. Tattersall), and subsequently reacted with a fluorescein-conjugated goat anti-rabbit antibody (Cooper Biomedical, Inc.). Constructs in which the transcription of MVM and BPV sequences was codirectional generated transformed cell lines expressing MVM gene products at high efficiency by this assay (data not shown); constructs with opposing orientations generated 25- to 50-fold fewer transformants, and these cell lines did not express detectable MVM gene products.

Eight of the above cell lines that were positive by the immunofluorescence assay were then examined for the production of viral VP1 and VP2 by immunoprecipitation as previously described (9), with the same rabbit anti-MVM capsid antiserum as above. All of the cell lines tested produced immunoreactive proteins the same size as authentic viral VP1 (83 kilodaltons) and VP2 (64 kilodaltons) and furthermore in the same ratio as that seen in a normal viral infection (14) (Fig. 2).

To examine whether these proteins were capable of assembling into intact (empty) virions, cleared lysates from the transformed cell lines were first tested and shown to agglutinate guinea pig erythrocytes (data not shown), a property characteristic of MVM virions (14). To confirm the presence of intact capsids, two transformed cell lines, 127-4-1A and 127-9-1B, were chosen for further examination by electron microscopy. Cell pellets were fixed in 3% glutaraldehyde followed by 1% OsO_4, embedded in LX-112 resin (a generic replacement for Epon 812; Ladd Research Industries, Inc.), and stained in 2% uranyl acetate followed by lead citrate. These cell lines do in fact contain empty capsids, proving

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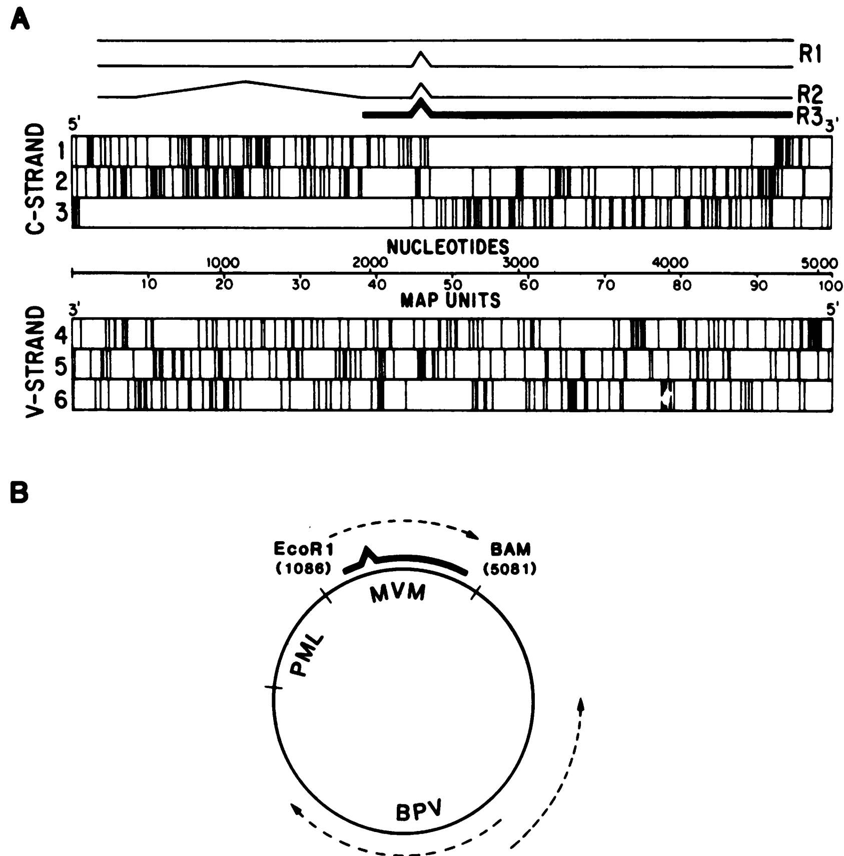
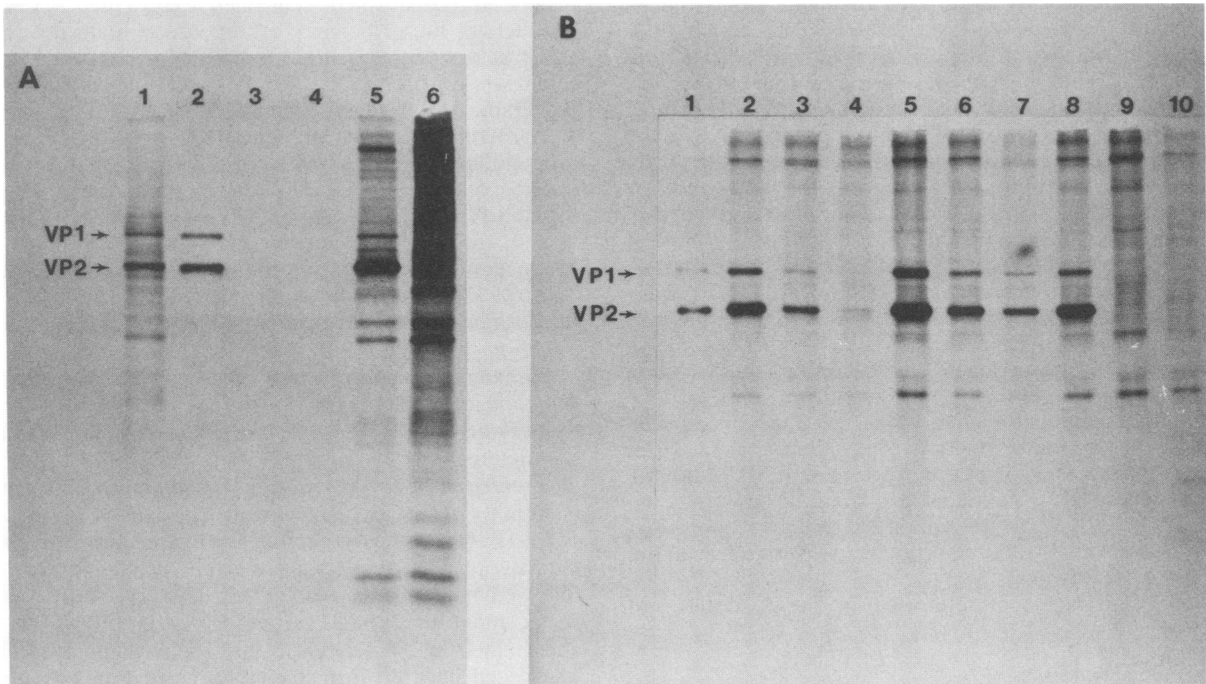


FIG. 1. (A) Map displaying the organization of the three major transcripts of MVM in relation to the open reading frames deduced from sequence analysis. (B) MVM-BPV chimeric constructs used in this study. These constructs contain MVM sequences spanning nucleotides 1086 to 5081 and include only the P_{39} promoter. The direction of transcription is indicated by arrows.



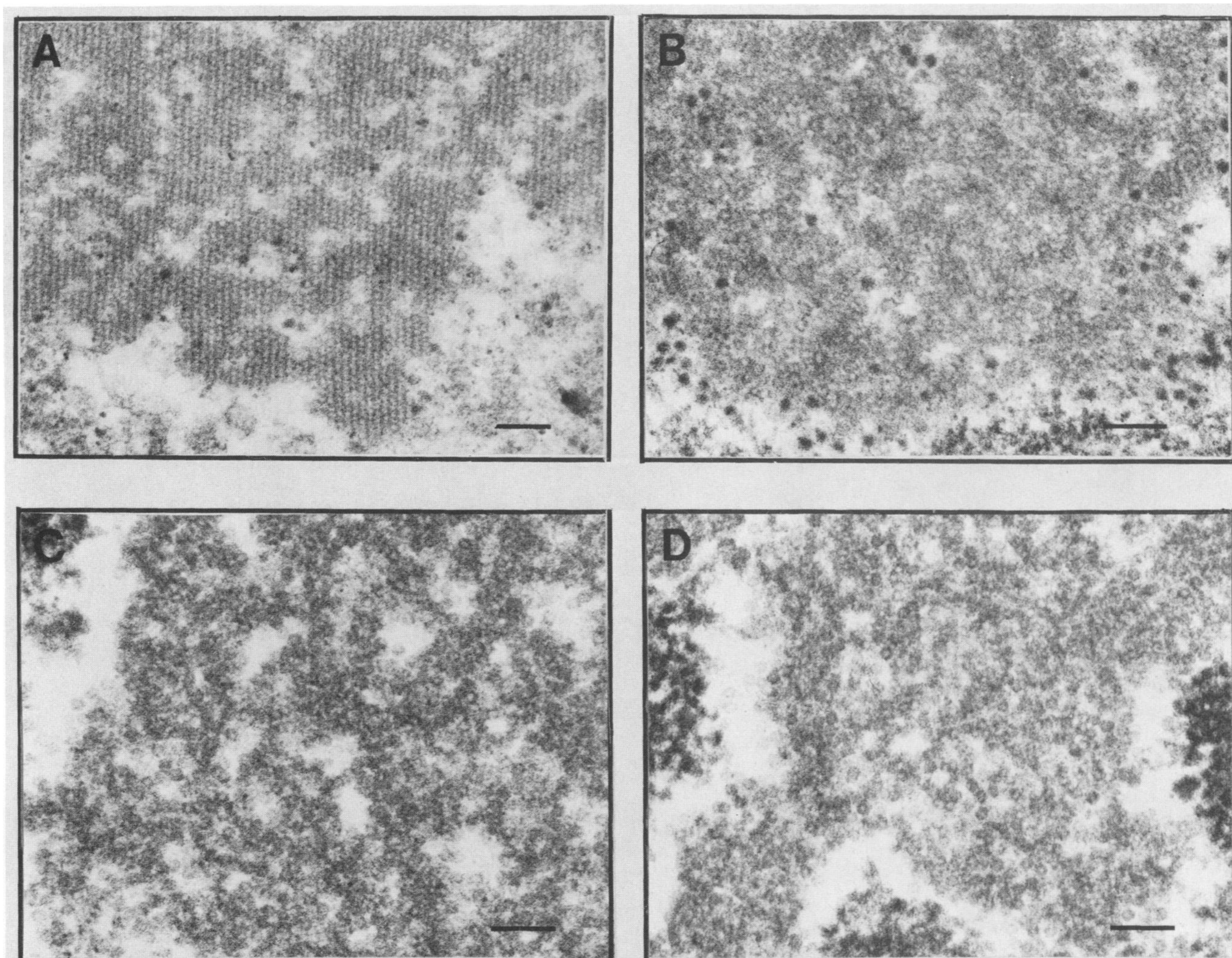


FIG. 3. Electron microscopy of cell lines transformed by MVM-BPV chimeras. Noncrystalline array of empty capsids found in nuclei from MVM-BPV-transformed cell lines 127-4-1A (C) and 127-9-1B (D) (each ×90,000 magnification). MVM-infected NB324K cells showing nuclei containing crystalline (A; ×80,000 magnification) and noncrystalline (B; ×90,000 magnification) arrays of full (electron dense) and empty capsids. Bar, 0.1 μm.

that the right-hand 80% of the MVM genome, which lacks the viral P_4 promoter, contains sufficient information to encode both authentic virion proteins (Fig. 3).

Northern analysis was performed as described previously (9) on RNA extracted from the capsid-producing transformed cell lines to determine whether they were producing the 3.0-kb R3 message. Hybridization with a double-stranded M13 probe containing the MVM *Bgl*II-*Hpa*II fragment spanning nucleotides 4212 to 4976 demonstrates that both cell lines contain an RNA species of approximately 3.0 kb which comigrates with the authentic MVM R3 message (Fig. 4). Although at least one larger band and several smaller bands are also seen by this analysis, hybridization with a double-stranded M13 probe containing MVM sequences immediately upstream of the known R3 initiation site detected no transcription from MVM sequences prior to the P_{39} promoter (data not shown). Previous analysis has detected readthrough of MVM transcription into BPV sequences in similarly constructed vectors (9). S1 hybridization analysis and primer extension analysis are currently in progress to determine the exact initiation site of the mRNAs detected in these lines.

FIG. 2. Immunoprecipitation of cell extracts from individual cell lines transformed by MVM-BPV chimeras. (A) MVM-BPV-transformed cell line 127-4-2B labeled with [^{35}S]methionine and immunoprecipitated with rabbit anti-MVM antiserum (lane 5) and preimmune rabbit serum (lane 6). MVM-infected NB324K cells (a simian virus 40-transformed human kidney cell line routinely used to plaque MVM [13]) labeled with [^{3}H]lysine and immunoprecipitated with rabbit anti-MVM antiserum (lane 2), preimmune rabbit serum (lane 3), and an ethanol precipitate of total protein (lane 1). BPV-transformed cell line 74-2-1B labeled with [^{3}H]lysine and immunoprecipitated with rabbit anti-MVM antiserum (lane 4). (B) All cell lines were labeled with [^{35}S]methionine. MVM-BPV-transformed cell lines 127-9-1C, 4-1A, 4-2A, 9-1A, 9-1B, 9-2B, and 4-2E immunoprecipitated with rabbit anti-MVM antiserum (lanes 1 through 7, respectively). MVM-BPV-transformed cell line 127-4-2B immunoprecipitated with rabbit anti-MVM antiserum (lane 8) and preimmune rabbit serum (lane 9). BPV-transformed cell line 123-2-4A immunoprecipitated with rabbit anti-MVM antiserum (lane 10).

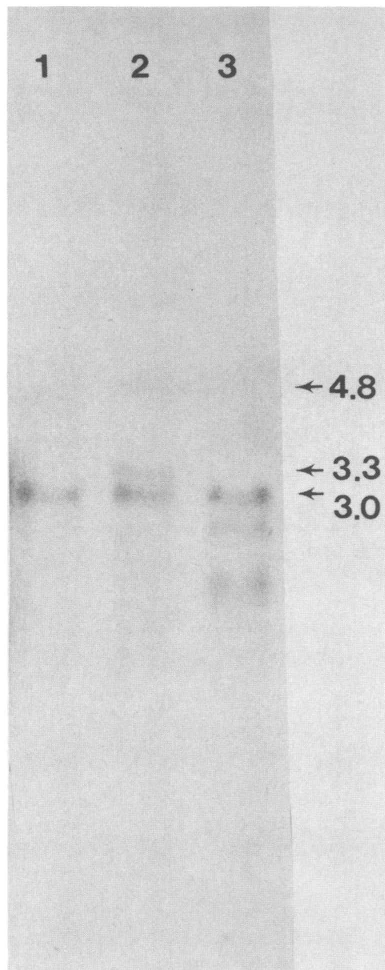


FIG. 4. Northern analysis of RNA extracts from MVM-BPV-transformed cell lines probed with a double-stranded M13 clone containing MVM sequences spanning nucleotides 4212 to 4976. Total RNA (4 μg) from MVM-BPV-transformed lines 127-4-1A (lane 1) and 127-9-1B (lane 3). Total RNA (2 μg) from MVM-infected mouse A9 cells showing the three major viral transcripts of 3.0, 3.3, and 4.8 kb (lane 2).

To confirm that VP1 and VP2 are independent translation products of the P_{39} transcription unit and that the translation initiation site of VP1 precedes that of VP2, we constructed the following mutation of the MVM-BPV chimeras analyzed above. A 15-nucleotide palindrome containing translation termination signals in all three reading frames (Pharmacia) was inserted at the *Bss*HII site at nucleotide 2636 prior to the initiating AUG codon of VP2 (7) but beyond the presumptive initiating AUG codon of VP1 (Fig. 5A). As can be seen by the immunoprecipitation analysis as described above, C127 cell lines transformed by these chimeras generate only authentic VP2 (Fig. 5B). Presumably the translation of VP1 is truncated by this substitution; however, we have not yet detected a truncated polypeptide corresponding to the amino terminus of VP1 by immunoprecipitation with antibody raised to either intact or reduced and alkylated capsid preparations (a gift of P. Tattersall). This experiment suggests that VP1 and VP2 are independent translation products of the P_{39} transcription unit and confirms that the translation initiation site of VP1 precedes that of VP2.

The R3 message of MVM had previously been mapped as a single species by S1 hybridization analysis (8); therefore it was somewhat surprising that this region of the genome could encode both viral proteins thought to be independent translation products (2, 14). Either a single mRNA species encodes the two overlapping proteins, or two different transcripts of similar size but spliced differently originate from the P_{39} promoter. Sequence inspection of the area around the small splice of the R3 message shows that multiple-splice donor and acceptor consensus sequences indeed exist which would permit differential splicing by almost identically sized messages into the large open reading frame in the right-hand end of the molecule. We are currently examining whether the product of the P_{39} promoter is more than one message or whether a single molecular species can generate both virion proteins.

Previous results analyzing cell lines transformed by the

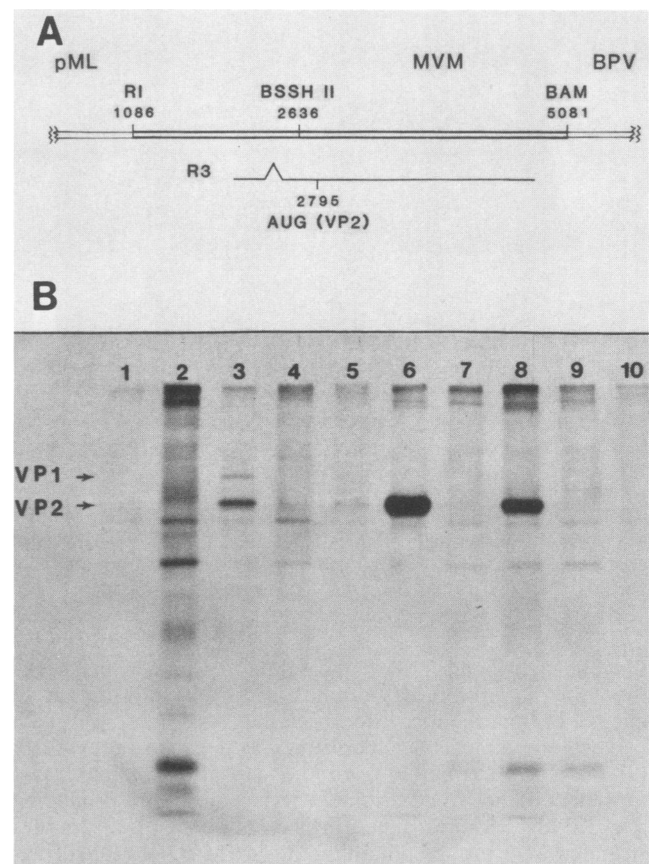


FIG. 5. (A) Schematic diagram of that portion of the MVM genome inserted into MVM-BPV chimeric constructs showing the position of the 3.0-kb R3 message, the presumptive initiation of VP2 and the *Bss*HII site where a 15-nucleotide translation termination signal was inserted. (B) Immunoprecipitation of [^{35}S]methionine-labeled cell extracts of cell lines transformed by the substituted MVM-BPV construct. Substituted MVM-BPV-transformed cell lines 1-2-5A and 89-2-6C immunoprecipitated with rabbit anti-MVM antiserum (lanes 6 and 8, respectively) and preimmune rabbit serum (lanes 7 and 9, respectively). MVM-BPV-transformed cell lines 123-4-4D and 127-9-2B immunoprecipitated with rabbit anti-MVM antiserum (lanes 3 and 5, respectively) and preimmune rabbit serum (lanes 4 and 10, respectively). BPV-transformed cell line 123-2-4A immunoprecipitated with rabbit anti-MVM antiserum (lane 1) and preimmune rabbit serum (lane 2).

MVM-BPV chimeras in which the whole MVM genome was utilized (9) alluded to the results presented here; however, rearrangements of plasmids in those cell lines prevented a definitive assignment of the coding sequences for VP1 and VP2. Southern analysis of cell lines transformed by the MVM-BPV chimeras described above has also indicated that a slow, complicated rearrangement takes place as the lines are being passaged (data not shown). However, since our original constructs contained only the right-hand 80% of the MVM genome, we can unambiguously assign the coding region of VP1 and VP2 to this fragment.

In summary, we have shown that the P_{39} transcription unit of the MVM genome contains sufficient information to encode both authentic virion proteins, VP1 and VP2, presumably from the viral 3.0-kb R3 message(s). These proteins are produced in ratios similar to those seen in infected cells and can assemble into empty virions. Whether the viral 3.3-kb R2 message has a role to play in the generation of the virion proteins during viral infection has yet to be determined.

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