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Expression of Minute Virus of Mice Structural Proteins in Murine Cell Lines Transformed by Bovine Papillomavirus-Minute Virus of Mice Plasmid Chimera

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Recombinant plasmids containing the genomes of both bovine papillomavirus type I and minute virus of mice (MVM) were constructed and used to transform mouse C127 cells. Transformed lines that express MVM gene products with high efficiency were isolated and characterized. These transformants synthesize large amounts of MVM structural polypeptides and spontaneously assemble them into empty virion particles that are released into the culture medium. These lines were, however, genetically unstable; they slowly generated subpopulations that failed to express MVM-specific proteins, and they possessed episomal DNA in which both MVM and bovine papillomavirus sequences were deleted or rearranged, or both. Clonal isolates of these transformants were also superinfectible by infectious MVM virus. Therefore, in spite of their instability, they should be useful host cell lines for transcomplementing mutations introduced into the MVM genome and for growing defective viruses as virions.

Minute Virus of Mice (MVM) is a member of the autonomous parvovirus family. The prototype stain of this virus, designated MVM(p), contains a single-stranded DNA genome 5,081 nucleotides long with palindromic sequences at each terminus which form hairpin duplex structures. The MVM(p) genome has been completely sequenced (1) and cloned into plasmid pBR322 in a double-stranded form that is infectious when transfected into mammalian cells (22). The emerging picture of the MVM chromosome is that it contains two overlapping transcription units which produce four known species of mRNA (23). The two largest viral transcripts, designated R1 and R2, are 4.8 and 3.3 kilobases (kb) in length, respectively, and are initiated from a promoter at 4.0 map units (mu), near the left (3′) end of the genome. Transcript R3, which is 3.0 kb in length and the most abundant mRNA seen in vivo, is initiated from a second promoter at ca. 39 mu on the DNA. The fourth viral transcript, 1.8 kb in length, is present in such low abundance that its genomic origin has not yet been defined precisely. These four mRNAs encode the VP1 and VP2 structural proteins of the virion and two recently identified nonstructural polypeptides of 85,000 and 25,000 daltons, designated NS-1 and NS-2, respectively (5). Although the R1 transcript has been shown unambiguously to code for the NS-1 protein and the R3 transcript to encode VP2, the assignment of the other viral gene products to specific mRNA species has not been established unequivocally because of complexities associated with the organization of the overlapping transcription units.

To further characterize the gene products of MVM(p) and the RNA species which encode them, we have constructed a series of defined, site-specific, mutations within the MVM genome cloned into plasmid pBR322 (M. Merchlinsky and D. C. Ward, unpublished data). Since the infectivity of both wild-type MVM DNA and MVM-plasmid clones is low (ca. 100 PFU/μg), it has been difficult to be solely dependent on DNA transfection assays for analyzing the phenotype of such mutants. To circumvent these problems, we set out to establish superinfectible cell lines which constitutively express MVM(p) gene products. These lines could supply complementing proteins in *trans* and permit the growth of defective genomes as viable virions which could then be used for mass infection of permissive host cells. Such cell lines have already proven extremely useful in studies of adenovirus (10) and simian virus 40 (9) tumor antigens.

The bovine papillomavirus (BPV)-based shuttle-vector system (13, 26, 27) provided an opportunity to develop a complementing cell system for MVM. BPV DNA can transform mouse cells to malignancy and can be maintained as an episome in the nuclei of transformed cells (7, 17). When genes from a wide variety of organisms are inserted into these vectors, cell lines stably expressing these products can be generated (7, 13, 17, 26). Because mouse cells are the natural permissive host for MVM infection, the utility of MVM-BPV chimeric vectors in generating cell lines which both stably express MVM gene products and remain superinfective to MVM virions has been examined.

MATERIALS AND METHODS

Virus and cell methods. The growth and purification of MVM(p), fluorescent antibody techniques, hemagglutination assays, and CsCl and sucrose gradients for sizing assembled capsids have been previously described (31–33).

Antibodies against MVM capsid polypeptides (a gift of P. Tattersall) were used for both fluorescent antibody assays and immunoprecipitation experiments. The antiserum was raised in rabbits by repeated subcutaneous injection of purified empty (noninfectious) virions.

All cell lines were grown in Autopow monolayer medium (Flow Laboratories, Inc.) supplemented with either 5 or 10% heat-inactivated fetal calf serum.

Construction of MVM-BPV recombinants. The plasmid p142-6, which contains the complete BPV genome inserted into the *Bam*HI site of the bacterial plasmid pML, was

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originally constructed in the laboratory of P. Howley and is described elsewhere (26). Plasmid p142-6 was partially digested with *Bam*HI, and the resultant linear molecules were doubly purified from neutral agarose gels. The linear duplex form of MVM DNA was released from MVM genomic plasmid clones (22; M. Merchlinsky et al., in preparation) by *Bam*HI digestion, isolated from the agarose gels, and ligated into the purified *Bam*HI-linearized p142-6 DNA. This ligation mixture, containing DNA in which the MVM and BPV sequences had four orientations with respect to each other, was used to transform the competent *Escherichia coli* strain LE392. Clones containing all four sequence orientations were obtained and verified by the alkaline minilysis procedure of Birnboim and Doly (2). The DNA from each clone was then assayed for transformation of C127 cells.

All restriction enzymes and DNA ligase were obtained from New England Biolabs.

DNA transformation and isolation of transformed lines. DNA transformations were carried out exactly as described by Sarver et al. (27). After ca. 3 weeks, transformed foci were isolated with cloning cylinders; not more than one colony was isolated per dish. To reclone transformed lines, cells were plated at ca. 100 cells per dish and foci were again isolated; these lines were then cloned by single cell endpoint dilution in 96-well plates. Cells were diluted so that ca. 30 cells were distributed among the 96 wells. Typically, colonies formed in 12 to 15 of these wells.

DNA and RNA isolation and Northern and Southern blotting procedures. Low-molecular-weight DNA was isolated either by the method of Hirt (12) or by the alkaline minilysis technique of Birnboim and Doly (2). Total DNA was isolated by the method of Wigler et al. (36). RNA was isolated by the guanidinum hydrochloride method of Cox (6) as modified by Strohman et al. (30). DNA was run on neutral agarose gels, and RNA was run on agarose gels containing 2.2 M formaldehyde (24) as described by Heilman et al. (11). DNA and RNA transfers and hybridization were done essentially as described by Thomas (34). Probes were nick-translated with ^{32}P-labeled deoxynucleotide triphosphates by the method of Rigby et al. (25).

Immunoprecipitation and protein gel electrophoresis. Immunoprecipitation was performed essentially as described by Kessler (14) with Formalin-fixed, heat-killed *Staphylococcus aureus* (Cowan 1 strain). Samples to be precipitated were pretreated once with normal rabbit serum and three times with the immunoadsorbent and incubated with antiserum overnight at 4°C.

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (15) with 10% acrylamide resolving gels. Samples were reduced with dithiothreitol and alkylated with iodoacetamide before application. Gels were equilibrated with 1 M sodium salicylate (pH 7.0), and the [^{35}S]methionine-labeled proteins were detected by sodium salicylate fluorography (4) on presensitized Kodak XAR-5 film after exposure at −70°C (16).

RESULTS

Susceptibility of mouse cell lines C127 and ID13 to infection with MVM(p). The C127 mouse cell line is a well characterized host for BPV transfection (8, 13, 17). However, it was first necessary to see whether this line, as well as BPV virion-transformed derivatives of C127 such as ID13 (8, 17), would support the growth of MVM(p), the prototype strain of MVM. Since it had been shown previously that expression of the MVM(p) genome is dependent on the interaction between a differentiation-specific host cell factor(s) and a *cis*-acting viral gene sequence(s) (29, 32), it was important to establish that these new cell lines were MVM-replication competent. Both cell lines did indeed support the growth of MVM(p), although the yields of infectious virus are somewhat lower than those normally obtained from standard MVM-permissive A-9 (18) or Ehrlich ascites (35) cell lines (Table 1). Nevertheless, both cell lines yielded higher titers of MVM(p) than NB324K cells, a simian virus 40-transformed human newborn kidney line (28), which supports plaque formation by both MVM(p) and MVM(i), an immunosuppressive strain of MVM (32). Additional experiments (data not shown) also showed that both the C127 and ID13 cell lines were susceptible to infection by transfected MVM DNA. These results indicated that transformants of C127 obtained after transfection with BPV-MVM chimeric plasmids should be fully competent for MVM gene expression.

Construction of chimeric vectors and their transformation efficiencies. MVM-BPV chimeras were first constructed by ligating various fragments of the MVM genome into a plasmid, pBPV$_{69T}$, which contains the transforming fragment of BPV DNA (19, 27). Numerous transformed lines were established after transfection with these constructs; however, none of those analyzed was found to produce MVM-specific RNAs. Furthermore, when the episomal DNA present in these lines was examined, both the BPV and MVM sequences were found to be extensively rearranged. The general instability of some chimeric vectors constructed with the transforming 69% fragment of BPV DNA has been reported by others (7, 13).

Further attempts to construct MVM-BPV chimeras that would be maintained as stable episomal elements in C127 cells used the BPV vector p142-6 (26). This construct contains the complete BPV genome cloned into the bacterial plasmid pML, a pBR322 derivative which lacks sequences that interfere with its replication in eucaryotic cells (21). This vector offered the additional advantage that it could be shuttled readily between eucaryotic and procaryotic host cells, and it has been noted that some heterologous DNAs are more stable in p142-6 than in pBPV$_{69T}$ (P. Howley, personal communication). In addition, to maximize the stability of the MVM DNA insert, the viral genome was modified in two ways. First, DNA containing a small deletion within the 5′-terminal hairpin sequence (between 97 and 99 mu) was chosen for cloning. This deletion, which lay outside the known coding regions in the DNA, is spontaneously generated when the complete MVM genome, cloned in duplex form into pBR322, is passaged in *E. coli* (M. Merchlinsky, C. Astell, and D. C. Ward, submitted for publica-

TABLE 1. Susceptibility of mouse cell lines to infection by MVM(p)[a]

Cell line	Infectious titer (PFU per 10^5 cells)
A-9	1×10^9
C127	4×10^7
ID13	1×10^7
NB324K	5×10^6
1A-57	2×10^4
4C-52	2×10^4

[a] Cell lines were seeded at a density of 10^5 per 60 mm^2 and infected with 1×10^5 to 2×10^5 PFU of purified MVM(p). After 7 days the cells were harvested, frozen and thawed three times in 1 ml of 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.7), and centrifuged to remove cellular debris, and the supernatant plaque was assayed on NB324K cells (32).

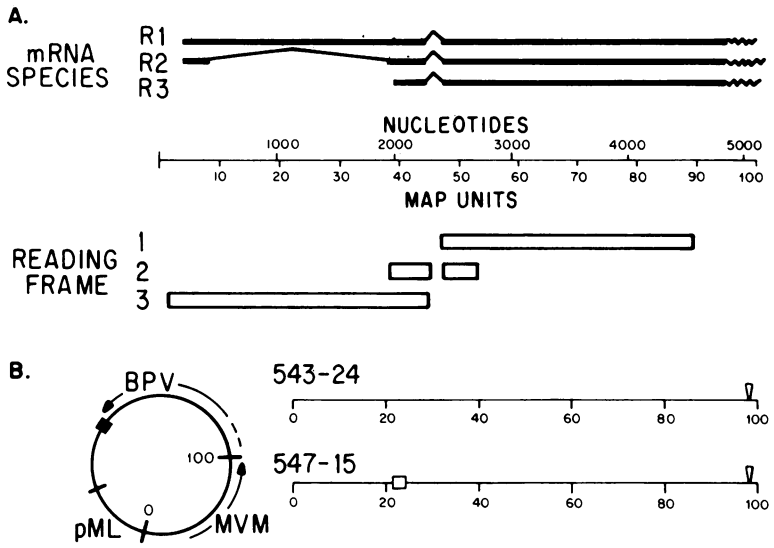

FIG. 1. (A) Schematic diagram of the regions of the MVM genome from which the three major transcripts (R1, R2, and R3) are derived and of the distribution of sequences free of translational stop codons in all three possible reading frames. (B) Sequence organization of the two BPV-MVM chimeric plasmids used to produce the 1A-57 and 4C-52 transformed cell lines. Arrows, Direction of transcription from the MVM and BPV components of the plasmids; ■, location of the BPV enhancer sequence at the distal end of the early transcription unit (20).

tion). Plasmid clones containing this deletion do not have the MVM DNA insert excised from the vector as efficiently as clones containing the complete, wild-type genome. By using the deletion mutation we hoped to lower the possibility of generating infectious DNA molecules that could lyse the desired transformed lines. The chimeric vector, clone 543-24, contained this deletion (Fig. 1). A second construct, clone 547-15, also contained a 10-nucleotide insertion at nucleotide 1061. This mutation causes a reading frame shift in the R1 mRNA which results in premature translational termination of the major nonstructural gene product, NS-1 (M. Merchlinsky and D. C. Ward, unpublished data). This mutation also prevents the excision of MVM sequences from recombinant plasmids and abolishes the ability of cloned viral DNA to induce plaques when transfected into cultured cell lines. Thus, one construct, clone 543-24, would be expected to express all MVM gene products, whereas the other, 547-15, would be expected to express all but the NS-1 gene product.

BPV-MVM chimeric plasmids were constructed as described above, such that the MVM DNA sequences were inserted in all four possible orientations with respect to the BPV sequences. Although some of the clones failed to generate transformed lines when transfected into C127 cells, transformants were obtained with each type of plasmid construct with roughly equal efficiencies. Transformed C127 cell lines obtained after transfection with clones 543-24 and 547-15, in which the MVM and BPV transcription units were aligned in the same direction (Fig. 1), were selected for further study. The efficiency of transformation by these vectors was 10- to 17-fold less than that by the parent vector, p142-6, alone (Table 2). Although the reason for the lower transformation efficiency is unclear, it is interesting to note that MVM-BPV chimeras which lacked the left 20% of the MVM genome (and therefore contained neither the 3'-end hairpin sequence nor the left promoter at 4.0 mu) transformed C127 cells at the same efficiency as did p142-6 (data not shown).

Seven clones of cells transformed by each of the two vectors were selected, and total RNA was rapidly extracted and applied to nitrocellulose in the presence of NaI as described by Bresser et al. (3). These samples were screened for the presence of MVM-specific RNA by hybridization with ^{32}P-labeled MVM DNA probes. Two transformants that were found to contain the highest abundance of MVM-specific RNA (line 1A, transformed by clone 543-24, and line 4C, transformed by clone 547-15) were chosen for further analysis.

Biochemical analysis of cell lines 1A and 4C. Lines 1A and 4C were first examined for the production of MVM virion proteins by the indirect immunofluorescence technique with antibodies raised against purified MVM virions as the primary detector reagent. Both lines expressed MVM polypeptides that were immunoreactive; however, ca. 60% of the cells in each line showed no immunofluorescence at all, whereas the remaining 40% showed a wide variation with respect to both the intracellular localization and the relative intensity of the fluorescent signal. Since both lines 1A and 4C were established directly from primary transformed foci, these results suggested that neither line was a true clonal isolate derived from a single cell. Both lines were therefore recloned several times, first as individual transformed foci and then by endpoint dilution. At each recloning step, numerous subclones were screened by immunofluorescence for the expression of MVM-specific proteins. With each recloning, colonies that were totally negative by immunofluorescence were observed, as well as colonies that were immunopositive. However, none of the positive clones had

TABLE 2. Transformation of C127 cells by BPV and MVM-BPV vectors

Vector	No. of foci per μg of transfecting DNA in experiment:	
	1	2
p543-24	4	20
p547-15	6	28
p142-6	70	250
Mock	<1	<1

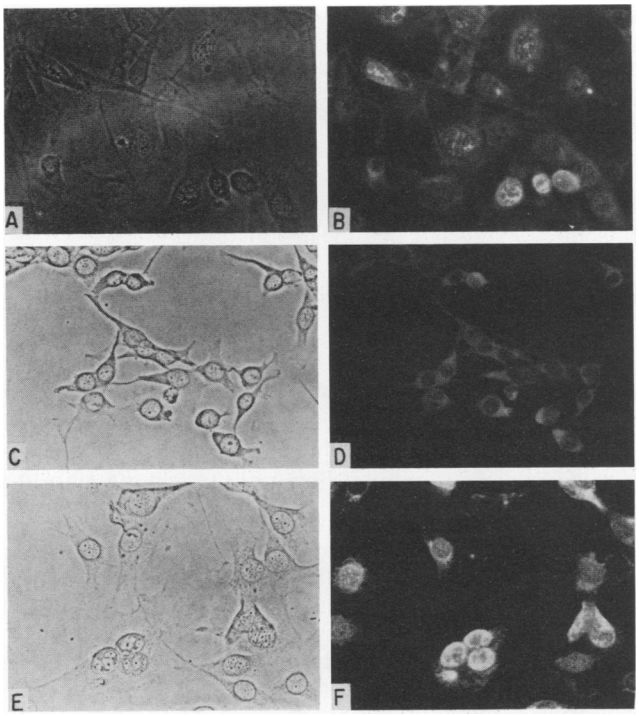

FIG. 2. Phase-contrast and immunofluorescence micrographs of 1A-57 cells (A and B), ID13 cells (C and D), and 4C-52 cells (E and F) after incubation with rabbit anti-MVM capsid protein antisera. Immunopositive cells were visualized after a 1-h incubation with fluorescein-labeled goat anti-rabbit immunoglobulin.

more than 80% of the cells immunopositive. Two of the most immunopositive subclones, designated 1A-57 and 4C-52, were analyzed further. Surprisingly, both lines, even after being doubly recloned, showed the variable pattern of immunofluorescence initially observed in the parent lines. In addition to the 10 to 20% of those cells in the population that were devoid of immunofluorescence, cells were observed in which the viral antigens were localized exclusively in either the nucleus or the cytoplasm, in both the nucleus and the cytoplasm, or exclusively in the perinuclear region (Fig. 2). Control experiments with uninfected or MVM-infected C127 cells clearly showed that these observations were not due to nonspecific antibody interactions; the uninfected C127 cells showed no reactivity at all, whereas the MVM-infected C127 cells, harvested 24 h postinfection, showed the expected nuclear pattern of immunofluorescence seen in typical MVM infections.

The appearance of immunonegative cells within the clonal lines can be most readily explained by a random loss of MVM DNA sequences resulting from the segregation of the episomal plasmid DNA; this is currently being tested directly by using in situ hybridization. The unexpected variation in the intracellular distribution of the MVM polypeptides in the transformed lines is, however, much more difficult to rationalize. MVM replication is known to occur only in the S phase of the cell cycle (31). Therefore, we initially thought that the unusual immunofluorescence results might indicate that MVM gene expression from the plasmid chimeras was also restricted to a specific stage of the cell cycle. If so, the differences in the intracellular distribution of MVM proteins may be a reflection of the cell-cycle distribution within the cell population. To test this hypothesis, randomly growing populations of 1A-57 and 4C-52 cells were separated into G1-, S- and G2/M-phase subpopulations by a fluorescence activated cell sorter, and equal numbers of each cell subpopulation were analyzed by antibody immunofluorescence and RNA dot-blot hybridization. These studies indicated that all cell-cycle stages showed the same relative abundance of MVM mRNA and the variable intracellular distribution of MVM proteins (data not shown).

Organization of MVM DNA and RNA sequences in cell lines 1A-57 and 4C-52. Another possible explanation for the results described above is that the MVM insert had been subjected to a deletion or rearrangement event resulting in the production of a modified or truncated protein(s) that was immunoreactive but had an atypical intracellular distribution. To examine the expression of the chimeric episome in more detail, the organization of MVM DNA sequences within the transformed cells was compared with that of the original plasmid used in the transfection of Southern blotting, and the sizes and quantities of viral transcripts synthesized in the transformants, were analyzed by the Northern blotting method, as described above.

Line 1A-57 generated two transcripts of ca. 4.6 and 3.0 kb in length, respectively, which hybridized with cloned MVM DNA; the 4.6-kb species was more abundant than the 3.0-kb message (Fig. 3, lane 4). Line 4C-52 generated three major transcripts that hybridized to clones MVM DNA, a predominant transcript of 3.0 kb and two less abundant transcripts of

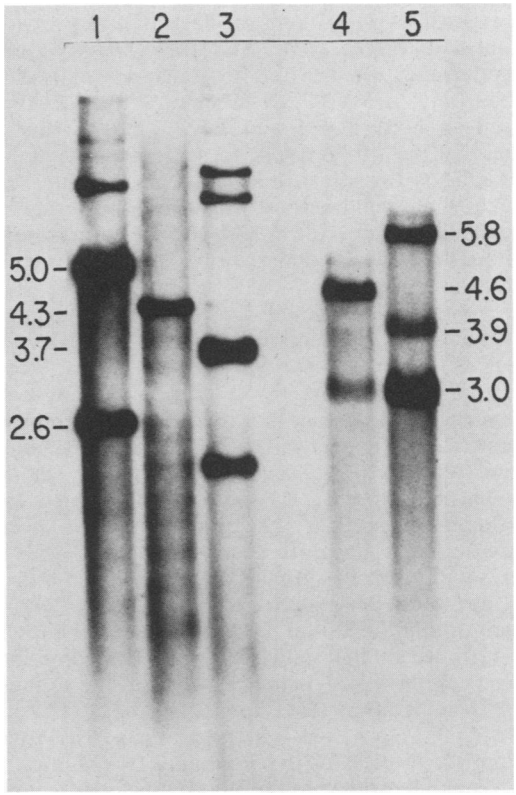

FIG. 3. Hybridization of ^{32}P-labeled MVM DNA prepared by nick-translation of the MVM plasmid clone pMM984 (22) to 4 μg of total RNA extracted from cell lines 1A-57 (lane 4) and 4C-52 (lane 5). Lanes 1 to 3 each contain 1 ng of a *Bam*HI-digested plasmid DNA molecular weight marker. Lane 1, pMM984, a genomic clone of MVM DNA (22); lane 2, pBR322 DNA; lane 3, pAT153 DNA.

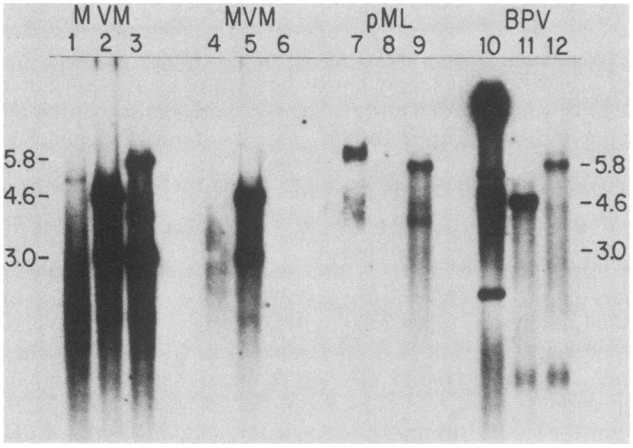

FIG. 4. Hybridization of 700 ng of total RNA extracted from cell line 1A-57 (lanes 2, 5, 8, and 11) or 4C-52 (lanes 3, 6, 9, and 12) with ^{32}P-labeled purified MVM DNA (lanes 1 to 6), ^{32}P-labeled purified pML DNA (lanes 7 to 9), or ^{32}P-labeled purified BPV DNA (lanes 10 to 12). The sample in lane 6 was treated with pancreatic RNase (25 μg/ml) before electrophoresis. Lanes 1, 4, 7, and 10 contain MVM, pML, or BPV DNA fragments as size reference markers.

ca. 3.9 and 5.8 kb, respectively (Fig. 3, lane 5). Since only the 3.0-kb transcripts corresponded in size to an mRNA species seen in MVM-infected cells (23), additional experiments were done to further characterize these RNAs.

RNA from lines 1A-57 and 4C-52 was first probed with highly purified MVM, pML, and BPV DNA sequences, respectively (Fig. 4). The 3.0-kb transcript of line 1A-57 hybridized only to MVM DNA, whereas the 4.6-kb RNA hybridized to both MVM and BPV probes. This result suggested that the 3.0-kb transcript was an authentic MVM-encoded mRNA (i.e., R3), initiated from the internal promoter (at 39 mu) and terminating properly within MVM DNA sequences. Since the MVM and BPV transcriptional units within the original vector were in the same orientation, we hypothesize that the 4.6-kb RNA in the 1A-57 cell line is initiated from the same MVM promoter (at 39 mu) but that the RNA continues into, and terminates within, BPV-specific sequences.

The 3.0-kb RNA of line 4C-52 hybridized only to MVM DNA sequences, indicating that this RNA also represented an authentic MVM R3 transcript. However, the 3.9-kb RNA hybridized also to pML sequences, whereas the 5.8-kb species hybridized to pML, MVM, and BPV sequences (Fig. 4). We therefore conclude that these latter two species of RNA are initiated in pML sequences; the 3.9-kb RNA terminates within the MVM insert, whereas the 5.8-kb RNA extends into the BPV sequence as well.

To confirm that the 3.0-kb RNAs found in cell lines 1A-57 and 4C-52 were in fact initiated from the major internal promoter of the MVM genome, RNA from these lines was probed with cloned fragments from either the left (3′) or right (5′) end of MVM DNA. The left-end probe, a *Pvu*II fragment cloned in plasmid pBR322 that contained MVM nucleotides 1 to 762, did not hybridize to the 3.0-kb transcript from either cell line (Fig. 5, lanes 3 and 4). However, this probe did hybridize to the 5.8- and 3.9-kb RNAs from line 4C-52, (Fig. 5, lane 4), consistent with the observation that both of these transcripts were initiated within pML sequences and transcribed into the MVM DNA insert. In contrast, the right-end probe, a *Hin*dIII fragment containing MVM DNA sequences between nucleotides 3996 and 5081, hybridized to all MVM-positive transcripts from both cell lines (Fig. 5, lanes 7 and 8). These observations suggested that the left end of the genome was altered or deleted during the intracellular propagation of the original plasmid.

Southern blot analysis of the episomal DNA extracted from these lines by alkaline minilysis (2) has in fact confirmed that a majority of the MVM sequences from the left 20% of the genome are missing in both transformed cell lines (data not shown). In addition, BPV and pML sequences are also missing or rearranged in the residual plasmids present in both lines. We are currently in the process of determining the extent and nature of the structural alterations in these plasmids. Although we cannot rigorously rule out the presence of less than one copy of viral DNA within the host genomes of the 1A-57 and 4C-52 cell lines, Southern analysis of total cellular DNA has failed to detect MVM or BPV DNA sequences present in an integrated form (data not shown). Thus, all MVM gene products appear to be transcribed from the rearranged episomal DNAs.

Transformants 1A-57 and 4C-52 make full-size and functional capsid polypeptides. Cell lines 1A-57 and 4C-52 were next examined for their ability to produce authentic and functional MVM structural proteins. The cells were grown in the presence of [^{35}S]methionine for 24 h, and cell extracts were incubated with anti-MVM virion antibodies and Formalin-fixed *S. aureus* as described above. The immunoprecipitated proteins were analyzed by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and compared with

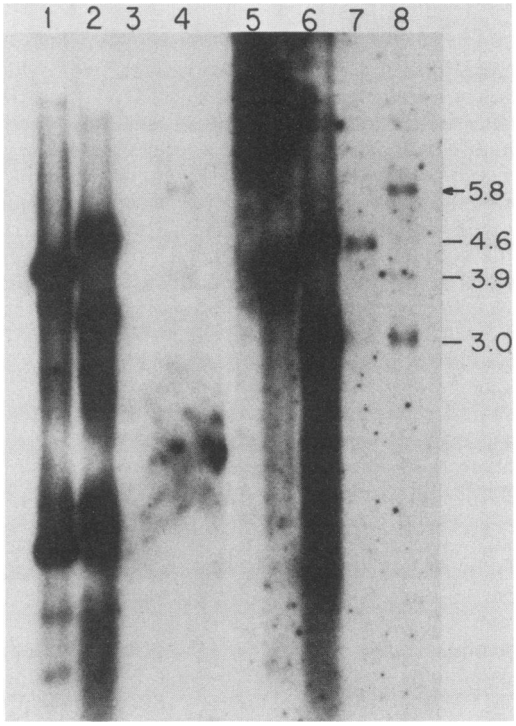

FIG. 5. Hybridization of 350 ng of total RNA from cell line 1A-57 (lanes 3 and 7) or 4C-52 (lanes 4 and 8) with ^{32}P-labeled cloned MVM DNA sequences representing the left end (nucleotides 0 to 762) of the genome (lanes 3 and 4) or the right end (nucleotides 3996 to 5081) of the genome (lanes 7 and 8). Lanes 1 and 5 contain 500 pg of pMM 984 DNA cut with *Eco*RI; lanes 2 and 6 contain 5 μg of total RNA extracted from MVM-infected A9 cells 24 h postinfection.

authentic virion proteins prepared from purified virus particles. The results clearly show that both cell lines synthesize proteins which are immunoprecipitable by anti-virion antibody and possess the same molecular weights as the authentic viral capsid proteins VP1 (A) and VP2 (B) (Fig. 6). It is also interesting to note that in both transformed lines the MVM virion proteins are produced in approximately the same molar ratio as is normally produced in a lytic viral infection. Since the primary rabbit antiserum was raised against virion particles, this serum does not recognize the nonstructural proteins NS-1 and NS-2. Hamster antiserum, which has been shown to have anti-NS-1 activity (5), does not immunoprecipitate an NS-1-like polypeptide from either cell line. This result is not unexpected, however, in the light of the observations described above, which indicate that neither cell line contains the MVM left-end promoter in a functional form and that NS-1 is encoded by the R1 transcript initiated from this promoter.

The VP1 and VP2 proteins are the only two polypeptide species found in empty MVM virions. However, neither capsid protein has any known enzymatic activity which could be assayed to assess the functionality of the polypeptides synthesized in the transformants. We therefore asked whether the proteins could interact with sufficient fidelity to assemble into empty virion particles. Cultures of both cell lines were lysed in 10 mM Tris hydrochloride–1 mM EDTA (pH 8.7) and frozen and thawed three times to release virus from cellular debris, and the lysate was then cleared by low-speed centrifugation. The supernatants were then assayed for their ability to hemagglutinate guinea pig erythrocytes, a characteristic trait of MVM virions but not of individual viral proteins. The results indicated that extracts from both transformed cell lines agglutinated the guinea pig cells, whereas extracts of the parent cell line, C127, did not. A quantitative study of the amount of hemagglutinating activity indicated that both cell lines were producing ca. 10^4 MVM virions per transformed cell, which is similar to that seen in a typical lytic infection of the permissive A9 cell line.

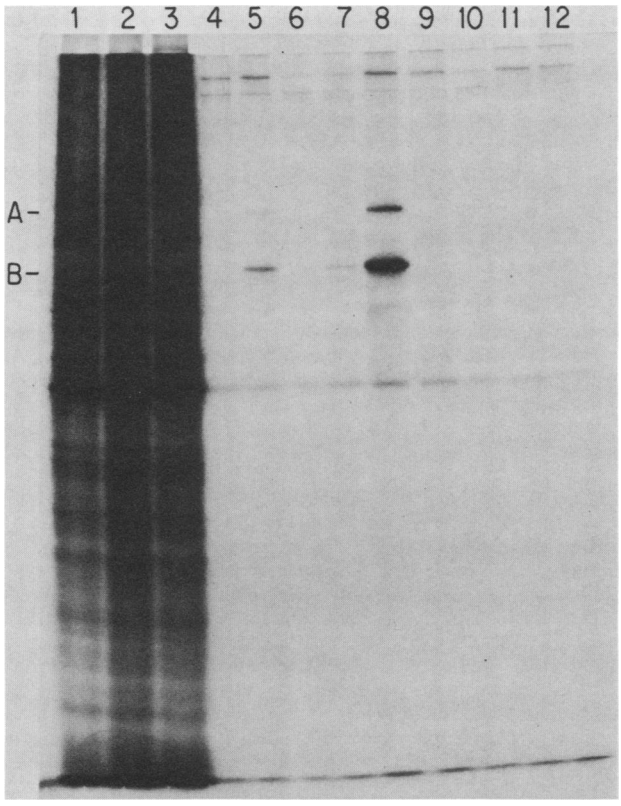

FIG. 6. Immunoprecipitation of [^{35}S]methionine-labeled extracts from cell line 1A-57 (lanes 4, 5, and 6), 4C-52 (lanes 7, 8, and 9) or ID13 (lanes 10, 11, and 12) with nonimmune control serum (lanes 4, 9, and 10), rabbit anti-MVM capsid serum (lanes 5, 8, and 11), or hamster anti-H1 virus serum (lanes 6, 7, and 12). Lanes 1, 2, and 3 show the pattern of [^{35}S]methionine-labeled proteins in cell lines 1A-57, 4C-52, and ID13, respectively, before immunoprecipitation. A and B, Positions of MVM capsid proteins VP1 and VP2, respectively.

The majority of these virion particles are excreted into the culture medium and bound to virus receptors on the outer surface of the plasma membrane. These virions can be released from the receptors by treatment with isotonic buffer at pH 9.0, conditions which do not alter the integrity or viability of the cells. Cells depleted of surface-bound virions contain only 1/30th the hemagglutinating activity of untreated cells. Furthermore, since 1 U of hemagglutinating activity under standard conditions is equal to 10^8 physical particles (31), we could calculate that both cell lines have virtually all virus receptors (ca. 2×10^5 per cell) saturated with empty virions at all times.

To further characterize the virions assembled in the transformants, cleared cell lysates from uncloned 1A and 4C lines were centrifuged to equilibrium in CsCl gradients, fractions were collected by bottom puncture, and hemagglutination assays were performed on each fraction. A single peak of hemagglutinating activity was found at a density of 1.31 g/cm^3, the buoyant density of authentic empty virions. Cell lysate material was also analyzed in a similar way after velocity sedimentation in a 5 to 20% sucrose gradient. Again, a single peak of hemagglutination activity was observed, and this activity comigrated with an authentic sample of ^{125}I-labeled empty MVM virions at 70S (data not shown). Cleared lysates from the recloned lines, 1A-57 and 4C-52, were also analyzed by velocity sedimentation in 5 to 20% sucrose gradients, and each showed a single peak of hemagglutinating activity at 70S. Collectively, these results indicate that both 1A-57 and 4C-52 cell lines synthesize MVM proteins in relatively high concentrations and that these polypeptides can spontaneously assemble into virion particles indistinguishable by the above criteria from those produced in normal lytically infected cells.

Susceptibility of cell lines 1A-57 and 4C-52 to superinfection by MVM. The transformed cell lines 1A-57 and 4C-52 do support the growth of a superinfecting MVM(p); however, they do so less well than the parent C127 cell line or C127 derivatives transformed with the BPV clone (Table 1). One possible reason for this is the relatively slow growth rate of these cells (unpublished observation). Since MVM requires a cellular function transiently present during the S phase of the cell cycle for viral growth, cell lines with a long generation time are generally less efficient hosts for lytic infections. In addition, it may be more difficult to initiate a superinfection cycle since the surface receptors of both lines are virtually always saturated with empty virions, a result of the efficient expression of MVM structural gene products. The combination of these two factors most likely leads to the low infectivity. We are currently examining the infectibility of these cells by input virus after stripping the virions from their cell membrane receptors, and we are currently developing transfection assays for DNA-mediated infections.

DISCUSSION

We have isolated mouse cell lines after transformation by chimeric MVM-BPV vectors that constitutively express MVM capsid proteins and assemble them into empty virions. Since these cells are superinfectible by MVM virus, they are good candidates as host cells in which to grow defective mutants of MVM by complementation, analogous to 293 cells in the adenovirus system (10) and COS cells in the simian virus 40 system (9). It should be noted, however, that the lines 1A-57 and 4C-52 described here, which were selected as strong producers of MVM gene products, are not stable and do not propogate virus well when superinfected. The selection of clones which produce the largest abundance of MVM polypeptides may not, in retrospect, have been the wisest decision. These clonal lines have a longer generation time than the parent C127 cells, and because of their efficient expression of MVM structural proteins, the virus-binding receptors on the plasma membranes of these lines are constantly being occupied by empty virion particles that are released by the transformed cells. The slow growth rate and the high receptor occupancy are likely to contribute to the low superinfection efficiency observed. Another possible contributing factor to the low level of superinfectibility is that the cellular protein(s) which is required to interact with the MVM genome to establish efficient transcription (29, 32) might be saturated by the episomal BPV-MVM chimeric DNA and thus suppress expression of incoming genomes. Indeed, it has been observed previously that the yield of infectious virus as a function of input virus multiplicity follows a bell-shaped rather than a linear profile and that infection multiplicities above 100 PFU per cell decrease the yield of progeny virus per infected cell quite markedly. Experiments are in progress to determine which factors are most responsible for the low superinfectibility. However, cell lines that express MVM gene products to lesser extents or selectively express individual polypeptides encoded by specific subgenomic segments of the MVM genome should yield transformed lines that are highly susceptible to MVM superinfection. We are currently screening the numerous cell lines generated to date to identify clonal isolates which possess these characteristics.

It is also interesting to note that all the MVM-BPV chimeric plasmids examined were genetically unstable upon propagation in the transformed C127 cells, irrespective of whether the subgenomic ($pBPV_{69T}$) or whole genomic (p142-6) BPV plasmids were used to construct the chimeras. In the case of the 1A-57 and 4C-52 lines, it was apparent that the left (3′) end of the MVM DNA insert was selectively lost. The observation that both VP1 and VP2 structural polypeptides were produced in both cell lines in the same molar ratio as seen in an MVM lytic infection—even though the left-end promoter sequences at 4.0 mu were extensively deleted—implies that the 3.0-kb MVM transcript (R3) can be used efficiently for the translation of both structural proteins.

In summary, we have generated cell lines which express MVM gene products and remain superinfectible to MVM. These and related lines should yield interesting results regarding MVM gene expression and hopefully serve as tools with which to grow defective mutants of MVM for further analysis on permissive cells.

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