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# The NS2 Polypeptide of Parvovirus MVM Is Required for Capsid Assembly in Murine Cells

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Mutants of minute virus of mice (MVM) which express truncated forms of the NS2 polypeptide are known to exhibit a host range defect, replicating productively in transformed human cells but not in cells from their normal murine host. To explore this deficiency we generated viruses with translation termination codons at various positions in the second exon of NS2. In human cells these mutants were viable, but showed a late defect in progeny virion release which put them at a selective disadvantage compared to the wildtype. In murine cells, however, duplex viral DNA amplification was reduced to 5% of wildtype levels and single-strand DNA synthesis was undetectable. These deficiencies could not be attributed to a failure to initiate infection or to a generalized defect in viral gene expression, since the viral replicator protein NS1 was expressed to normal or elevated levels early in infection. In contrast, truncated NS2 gene products failed to accumulate, so that each mutant exhibited a similar NS2-null phenotype. Expression of the capsid polypeptides VP1 and VP2 and their subsequent assembly into intact particles were examined in detail. Synchronized infected cell populations labeled under pulse–chase conditions were analyzed by differential immunoprecipitation of native or denatured extracts using antibodies which discriminated between intact particles and isolated polypeptide chains. These analyses showed that at early times in infection, capsid protein synthesis and stability were normal, but particle assembly was impaired. Unassembled VP proteins were retained in the cell for several hours, but as the unprocessed material accumulated, capsid protein synthesis progressively diminished, so that at later times relatively few VP molecules were synthesized. Thus in NS2-null infections of mouse cells there is a major primary defect in the folding or assembly processes required for effective capsid production.



## INTRODUCTION

Parvoviruses encode two separate gene complexes, with transcripts from one half of their small (5-kb) linear DNA genome programming the synthesis of an overlapping set of capsid polypeptides, while the other half gives rise to nonstructural proteins involved in DNA replication. The murine virus MVM encodes two types of nonstructural proteins; an 83-kDa nuclear phosphoprotein, NS1, which is a site-specific DNA-binding protein essential for many aspects of viral DNA replication, transcriptional regulation, and modulation of the cellular environment, and three distinct forms of a 25-kDa NS2 polypeptide (Cotmore and Tattersall, 1987, 1995). NS2 subtypes all share a central exon of 98 amino acids and have an 84-amino-acid N-terminal domain in common with NS1, but they differ from each other in having unique carboxy-terminal peptides, 6–13 amino acids long (Cotmore and Tattersall, 1986, 1990; Jongeneel *et al.,* 1986; Morgan and Ward, 1986). Each NS2 subtype exists in approximately equal numbers of phosphorylated and nonphosphorylated forms and while the former are totally excluded from the nucleus, the nonphosphorylated forms show a more uniform distribution (Clemens *et al.,* 1990; Cotmore and Tattersall, 1990). Although both NS1 and NS2 are synthesized from the same promoter (P4) early in S-phase, NS2 polypeptides accumulate approximately three times as fast as NS1, making them the predominant virally coded protein in the cell at this time. Since the half-life of all subtypes is relatively short (~1 hr), NS2 accumulation peaks early in infection but rapidly diminishes thereafter as the activity of the P4 promoter declines (Clemens and Pintel, 1988; Cotmore and Tattersall, 1990).

Although not essential for productive infection in a variety of nonmurine cell lines in culture, NS2 is absolutely required in cells from its natural host species both in culture and in the whole animal (Naeger *et al.,* 1990; Brownstein *et al.,* 1992; Cater and Pintel, 1992; Naeger *et al.,* 1993). MVM mutants which do not encode full-length NS2 polypeptides have been reported to exhibit multiple, diverse DNA replication defects in mouse cells and to accumulate reduced levels of the viral capsid polypeptides (Naeger *et al.,* 1990, 1993). Although biochemical analyses have so far failed to yield any information concerning the specific function(s) of NS2 molecules, their high-level expression early in infection suggests that they are unlikely to be purely catalytic or regulatory and may well perform multiple roles in the viral life cycle. A similar species-specific host range defect in viral DNA

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replication has been reported for NS2$^-$ mutants of the closely related H1 parvovirus (Li and Rhode, 1991), but in these mutants the expression of all viral proteins was reduced, and experiments with reporter-gene constructs suggested that a sequence present in the 3′ untranslated region of all viral mRNAs might render them susceptible to translational modulation by NS2 (Li and Rhode, 1993). Similar widespread defects in viral protein accumulation were not apparent in the MVM NS2 mutants studied previously (Naeger *et al.,* 1990, 1993), and in this report we analyze the phenotypes of a new set of MVM NS2 mutants and similarly fail to find any evidence for a generalized lesion in protein synthesis at early times in infection. We have essentially confirmed and redefined the defects in DNA replication reported earlier and have used pulse–chase labeling conditions and differential immunoprecipitation to demonstrate that even the reduction in capsid accumulation associated with this phenotype at early times in infection is not primarily due to a reduction in virion protein synthesis per se, but rather reflects an underlying defect in capsid assembly.

## MATERIALS AND METHODS

### Construction of mutant MVM genomes

Four NS2$^-$ mutant genomes, summarized in Fig. 1, were constructed in the infectious cloned form of the prototype virus MVMp by site-directed mutagenesis using chimeric polymerase chain reaction (PCR) as previously described (Nuesch and Tattersall, 1993).

*NS2-F86am.* The serine codon at residue 578 of NS1 was changed from TCG to AGC by chimeric PCR. The final PCR product was digested with *Bst*EII and *Xho*I and used to replace the *Bst*EII (1884) to *Xho*I (2070) fragment of plasmid pMVMp-D1 (Gardiner and Tattersall, 1988). While this change conserves the NS1 sequence, it replaces the first codon of the second intron of NS2, which normally specifies phenylalanine (TTC) at residue 86, with the amber termination codon TAG.

*NS2-SA86am.* The leucine codon at residue 576 in the NS1 gene encoded by pNS2-F86am was changed from CTA to CTC using chimeric PCR and by replacement of the *Bst*EII to *Xho*I fragment of plasmid pNS2-F86am. This mutation destroys the consensus splice acceptor site used in all NS2-encoding transcripts (Naeger *et al.,* 1990; Li and Rhode, 1991).

*NS2-Q100am.* The glutamine codon CAG at residue 100 in NS2 was replaced with the amber termination codon TAG by chimeric PCR. pMVMp-D1 was used as template, and the mutation was transferred back into pMVMp-D1 by replacement of the *Bst*EII to *Xho*I fragment as described above.

*NS2-R132op.* The internal MVM *Eco*RI fragment (MVM nts 1084 through 3522) was transferred from plasmid pULB3224 (Brandenburger *et al.,* 1990) into *Eco*RI-digested pMVMp-D1. pULB3224 contains an A to T point mutation at nucleotide 2129, which changes an arginine (AGA) codon at residue 132 in NS2 to the opal termination codon TGA.

### Cells and viral stocks

The prototype strain MVMp and its mutant derivatives were propagated in the SV40-transformed newborn human kidney cell line 324K (Tattersall and Bratton, 1983), in Dulbecco's modified Eagle's minimal medium containing 5% fetal calf serum. Viruses were initially recovered from infectious plasmids by Lipofectin (Gibco/BRL) - mediated transfection of 324K monolayers with 5 $\mu$g plasmid DNA. First-passage viral stocks were titered on 324K cell monolayers essentially as described previously (Tattersall and Bratton, 1983), except that the time allowed for plaque formation was increased to allow for any possible reduction in the efficiency with which mutant stocks spread through the culture. These stocks were used to infect asynchronous cultures of 324K cells or the mouse L-cell derivative, A9 ouab$^r$11, at 3 PFU per cell unless otherwise stated in the text. Some experiments involved the use of A9 cell populations synchronized by a double block schedule described elsewhere (Cotmore and Tattersall, 1987). Briefly, cells were allowed to accumulate in G0 by depriving them of isoleucine for 48 hr, and they were then allowed to progress to the G1/S boundary by incubating the cultures in medium containing isoleucine and 10 $\mu$g/ml of the DNA polymerase $\alpha$ and $\delta$ inhibitor aphidicolin, for 20 hr. Viruses were added to the cells along with the aphidicolin and were able to penetrate the cells and accumulate at an immediately pre-DNA synthetic step in the viral life cycle prior to removal of the inhibitor. All infection times referred to here initiate at the point when the aphidicolin was removed and the cells became free to transit the S-phase.

Revertant viruses were obtained by plaquing 324K-derived stocks on A9 monolayers and amplifying the revertant plaques in 324K cells. The NS2 exon 2 region of each genome was then amplified using polymerase chain reaction techniques with viral stocks as template and sequenced by the chain termination method using Sequenase as directed by the supplier (U.S. Biochemicals, Cleveland, Ohio), essentially as described previously (Ball-Goodrich and Tattersall, 1992).

### Detection and analysis of viral DNA

To quantitate viral DNA synthesis, asynchronous monolayers of A9 and 324K cells were labeled with $^{32}PO_4$ at 0.5 mCi/ml for 20 hr in Dulbecco's modified Eagle's minimal medium containing $\frac{1}{10}$ of the normal concentration of unlabeled phosphate, 5% dialyzed fetal calf serum, and 100 $\mu$g/ml neuraminidase (TypeV, from *Clostridium perfringens,* obtained from Sigma, St. Louis, MO), in order to limit replication to a single round by preventing readsorption of virus to cell receptors. Labeling was started

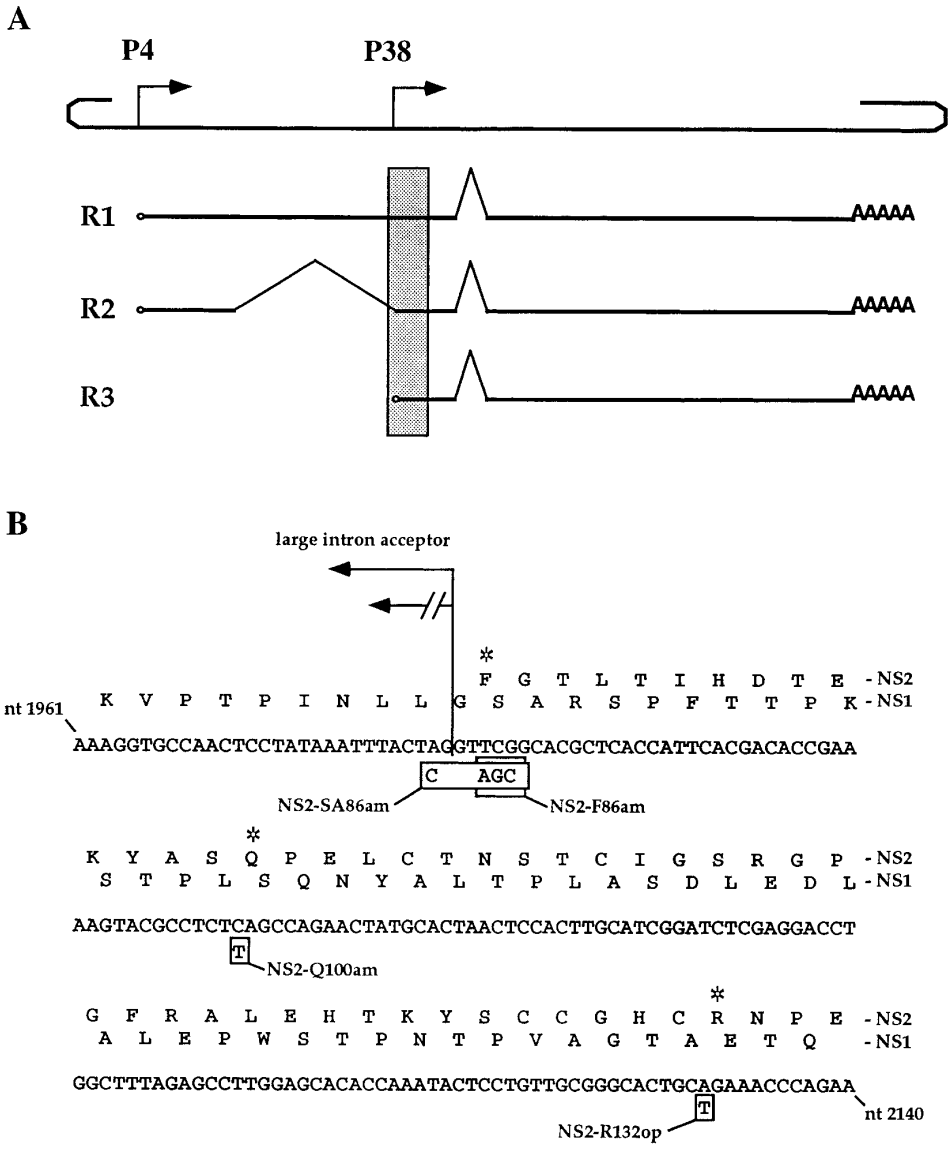


FIG. 1. Mutagenesis strategy in the second intron of NS2. (A) The line diagram of the single-stranded MVM genome shows the position of the nonstructural gene promoter (P4) and the capsid gene promoter (P38). Aligned below this are the three size classes of mRNAs encoded by MVM, denoted R1 through R3. The stippled box indicates the region which is shown in greater detail in B. (B) DNA sequence between MVMp nucleotides 1961 and 2140, showing the predicted protein coding for this region in open reading frames 2 and 3, used to encode the second exon of NS2 and part of the body of NS1, respectively. Exon 2 of NS2 initiates at the indicated large intron splice acceptor site. Mutations introduced into this sequence to create the various $NS2^{-}$ genomes are shown in boxes below the wildtype sequence, and the resulting mutants were named as described under Materials and Methods.

at 10 hr after infection and terminated with the separate collection of cells and medium. Total DNA was extracted from each sample and analyzed by electrophoresis through agarose gels as previously described (Cotmore and Tattersall, 1992).

## Production of specific antisera

*Anti-allopeptide serum.* A peptide, $NH_2$-QGSRHGTTQ-MGVNWVSK-COOH, which corresponds to the sequence of VP2 between residues 311 and 327 in MVMp, was synthesized. This spans a mutable genetic element, designated the allotropic determinant, which controls switching between lymphotropic and fibrotropic phenotypes (Gardiner and Tattersall, 1988; Ball-Goodrich and Tattersall, 1992). An additional cysteine residue was added at the carboxy-terminus of the peptide to allow efficient conjugation to keyhole limpet hemocyanin, and the conjugate was used to raise polyclonal antibodies in rabbits according to standard procedures.

*Anti-VP1-specific serum.* Rabbit anti-VP1-specific serum was raised against a gel-purified, bacterially expressed polypeptide encoding the entire VP1-specific region of MVM together with a carboxy-terminal $(His)_6$-tag.

This antigen will be described in detail elsewhere (S. F. Cotmore, A. M. D'Abramo, and P. Tattersall, manuscript in preparation).

*Anti-capsid sera.* Anti-capsid sera PN1 and PN2 were derived by repeated immunization of rabbits with 100 hemagglutination units per injection of cesium chloride equilibrium gradient-purified empty MVMp particles. These sera recognize and neutralize all MVM strains (Tattersall and Bratton, 1983) and have been used as a source of anti-MVM capsid antibodies in studies published by our laboratory since 1978.

### Analysis of viral protein synthesis

*Western transfer.* For Western transfer, cells were harvested by scraping into the medium. After centrifugation the cell pellet was briefly drained and flash-frozen on dry ice. For analysis, pellets were solubilized from the frozen state by rapid boiling in SDS sample buffer and fractionated by electrophoresis on linear 11–23% gradient gels (Phorcast, Amersham), and blots were probed with rabbit antiserum directed against the amino-terminal peptide common to both NS1 and NS2 (Cotmore and Tattersall, 1986) and detected with $^{125}$I-labeled protein A (Amersham).

*Radiolabeling of viral proteins.* For long-term labeling of asynchronous cultures, cells were exposed to a [$^{35}$S]-methionine/cysteine labeling mix (Tran$^{35}$S-label, ICN Radiochemicals, Irvine, CA) at 20 $\mu$Ci/ml in Dulbecco's modified Eagle's minimal medium containing $\frac{1}{20}$ of the normal concentration of unlabeled methionine and cysteine and 5% dialyzed fetal calf serum and neuraminidase, starting 10 hr after infection, and the cells were harvested 20 hr later.

Pulse–chase and pulse-only analyses employed synchronized A9 populations. In pulse–chase experiments cells were labeled with 100 $\mu$Ci/ml Tran$^{35}$S-label in medium lacking methionine or cysteine for 20 min, starting 8 hr after release from aphidicolin, and were either harvested 20 min later or transferred into unlabeled medium containing normal levels of methionine and cysteine, and harvested, as described above, after indicated chase periods. In pulse-only experiments cells were labeled with 100 $\mu$Ci/ml Tran$^{35}$S-label in medium containing $\frac{1}{20}$ of the normal methionine and cysteine concentrations for periods of 3 hr starting 6, 9, or 12 hr after release from aphidicolin, and were harvested, as described above, at the end of the labeling period.

The labeled wildtype virus used to demonstrate antibody specificity was harvested from the medium 30 hr after the release from aphidicolin of MVMp-infected, synchronized A9 cells. These infected cells were labeled from 6 to 13 hr into S-phase with Tran$^{35}$S-label, as described above for the 3-hr pulses, and then incubated from 13 to 30 hr in normal medium containing 100 $\mu$g/ml neuraminidase, in order to prevent readsorption of virus to cell receptors.

*Immunoprecipitation.* For immunoprecipitation of total cell extracts, pellets of approximately $1 \times 10^6$ cells were extracted into 0.45 ml of buffer A (0.01 *M* Tris–HCl, pH 8, 0.15 *M* NaCl, 0.002 *M* EDTA, 1% Nonidet P-40) containing 0.35% sodium dodecyl sulfate (SDS), and either kept at room temperature (native samples) or fully denatured by heating to 85° for 30 min prior to dilution with buffer A to a final SDS concentration of 0.25%, and immunoprecipitated as previously described (Cotmore and Tattersall, 1986).

*Immunofluorescence.* A9 cells were grown, synchronized, and infected with virus on glass spots on Teflon-coated slides (Cel-line Associates, Inc., Newfield, NJ). Cells were released into S-phase in medium containing neuraminidase, to ensure the removal of all noninternalized virus, and fixed 10 hr later. Cells were permeabilized and stained as previously described (Cotmore and Tattersall, 1990), and primary antibodies were detected with Texas-red conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

## RESULTS

### NS2$^-$ mutant virus stocks can be generated by transfection into 324K cells

Full-length infectious plasmid clones of the various wildtype and NS2$^-$ mutant genomes were transfected into A9 and 324K cells, and the cultures were followed for cytopathic degeneration and production of viral hemagglutinin. As reported in previous studies by Naeger and colleagues (Naeger *et al.,* 1990, 1993), no virus production was observed from repeated transfections of mutant plasmids into A9 cells, whereas the wildtype virus could be recovered readily by transfection into these cells. However, mutant virus could be recovered routinely from transfected 324K cells, although the mutant viruses clearly amplified more slowly than those recovered from parallel wildtype MVMp transfections. Initial stocks of mutant virus recovered in this way consistently yielded between 1 and 10% of the infectious virus, as measured by the 324K plaque assay, of a wildtype stock prepared in parallel. We have measured the particle:infectivity ratios of these wildtype and NS2$^-$ mutant virus stocks by digesting samples with micrococcal nuclease and quantitating the protected DNA by Southern transfer following alkaline agarose gel electrophoresis. We have generally found them to contain equal numbers of viral genomes per 324K PFU, indicating that the low titers of the mutants are probably due to inefficient growth rather than a low intrinsic infectivity. Mutant viruses do require longer to produce visible plaques, and these tend to be much smaller than those produced by wildtype virus. Occasionally discrepancies were observed between numbers of genomes and 324K PFU, and we believe that this reflects the diminished ability of mutant stocks to generate visible plaques efficiently. Because of this potential variability,

we have used packaged DNA content, measured by nuclease treatment combined with alkaline gels as described above, to normalize the NS2$^-$ mutant stocks relative to titers determined for the wildtype virus for analyses of infection at matched multiplicities.

Consistent with their inability to produce virus when transfected into A9 cells, NS2$^-$ mutant virus stocks exhibited about $10^{-5}$-fold less infectivity in an A9 plaque assay than in a similar assay carried out in 324K cells. Expanded stocks, however, exhibited intermediate phenotypes, and we assessed whether this was due to reversion of the original mutation. PCR-mediated direct sequencing of several plaques formed on A9 monolayers infected with NS2-Q100am viral stocks confirmed that these plaques were produced by viral genomes in which the amber codon had reverted to a coding triplet. In two independent revertants the amino acid encoded at position 100 in NS2 was tyrosine and in another two it was glutamic acid. While none of these had reverted the NS2 sequence to wildtype, the glutamic acid change did conserve the collinear sequence of NS1, whereas the tyrosine change induced a concomitant conservative change in NS1 from serine to threonine. The revertant viruses appeared to have a considerable growth advantage over the mutant, even in 324K cells, and rapidly came to predominate in the population upon multiple serial passage. Because of this reversion and selection problem, only early passage stocks of transfection-derived virus were used in subsequent experiments.

The reversion rate of NS2-F86am appeared to be much lower than that of the other single amino acid substitution mutants, presumably because it is an NS1 serine codon replacement which cannot revert to wildtype sequence for both polypeptides in a single step. The low reversion frequency of NS2-F86am made it the mutant of choice for many subsequent analyses. It was also chosen as a starting point for the generation of NS2-SA86am, a more complex mutant which, lacking a functional splice acceptor sequence as well as carrying the F86am mutation, cannot make the spliced mRNAs needed to encode isolated copies of the amino-terminal peptide domain common to both NS1 and NS2. As its design would predict, NS2-SA86am has proven to be the most stable of all of these NS2$^-$ mutants. However, in those analyses where it was included, it showed exactly the same phenotype as each of the other second exon NS2$^-$ mutants studied.

## Truncated NS2 molecules do not accumulate in mutant virus infections of murine A9 cells

NS2 synthesis peaks early in infection and rapidly diminishes thereafter (Cotmore and Tattersall, 1990; Schoborg and Pintel, 1991). Since viral infections in A9 cells can be efficiently synchronized using a coupled isoleucine-deprivation/aphidicolin block protocol (Cotmore and Tattersall, 1987), we analyzed expression of the viral nonstructural proteins in these cells 8 hr after their release into S-phase, at a time when both NS1 and NS2 are normally abundant. At this time cells infected with wildtype virus contained approximately equimolar accumulations of the two nonstructural proteins, as determined from Western blots developed using an antibody directed against the amino-terminal peptide common to both molecules (Fig. 2, lane 2). In contrast, neither full-length nor truncated forms of NS2 were seen in cells infected with NS2 translation-termination mutants (Fig. 2, lanes 3–5). Since the mutant viruses would be expected to synthesize NS2 molecules of between 85 and 131 amino acids, stable forms of such molecules should be resolved on the 11–23% gradient gel used in this analysis. A small immunoreactive species does appear in all lanes containing infected cells, but its apparent molecular weight is identical in all viruses, and hence we consider that it is more likely to be a breakdown product of the highly abundant NS1 protein than a truncated form of NS2. Thus the abbreviated NS2 polypeptides encoded by these translation-termination mutants do not accumulate in A9 cells, at least to within one or two orders of magnitude of wildtype levels, suggesting that they must be much less stable and/or synthesized less efficiently. Since all of these mutants appear to exhibit the same NS2-null phenotype they cannot, as was originally hoped, provide information about possible different functions for the N- and C-terminal domains of NS2.

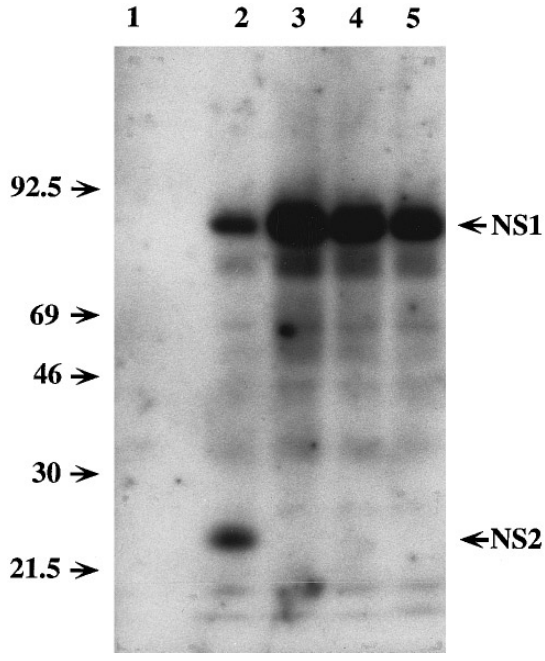


FIG. 2. Western blot analysis of nonstructural protein expression in the highly synchronized infection of murine A9 cells. Synchronized uninfected A9 cells (lane 1) and similar cultures infected at 3 PFU/cell with wildtype virus (lane 2), NS-F86am (lane 3), NS2-Q100am (lane 4), or NS2-R132op were harvested 8 hr after release into S-phase. Blots were developed with an antiserum directed against the amino-terminal peptide common to both NS1 and NS2.

In marked contrast to NS2 levels, NS1 synthesis was elevated at this point early in S-phase in the NS2$^-$ mutant infections. NS1 was consistently expressed to higher levels than are seen in the wildtype infection, and this overexpression correlated inversely with the position of the termination codon in the NS2 gene. This result was not surprising since others have shown that the introduction of termination codons into the second exon of NS2 impedes the splicing required to convert R1 transcripts, encoding NS1, to R2 transcripts encoding NS2 (Naeger *et al.,* 1992). Moreover, the level to which splicing was inhibited in the previous study correlated in a polar fashion with the inverse of the codon position, so that the earlier the exon was interrupted, the fewer R2 transcripts were formed and the more NS1-encoding R1 transcripts remained. While the failure to process R1 transcripts contributes in some part to our inability to detect NS2 gene products, as seen in Fig. 2, it is probably the major factor responsible for the relative overexpression of NS1 in NS2$^-$ mutant infections.

## Infection of human 324K cells by NS2$^-$ mutants is productive but exhibits a late defect in progeny virion release

Initially we monitored viral DNA synthesis in 324K cells by Southern transfer of total cellular and viral DNA, but the presence of input viral genomes made progeny single-stranded synthesis difficult to assess. In order to obtain precise quantitative data on progeny genome production and distribution, we used the *in vivo* $^{32}$P-labeling approach illustrated in Figs. 3A and 3B. Asynchronous 324K cells were infected at 3 PFU per cell and labeled with [$^{32}$P]orthophosphate between 10 and 30 hr postinfection in the presence of neuraminidase, which served to restrict infection to a single round. Cells and medium were then harvested and analyzed separately. Cellular DNA replication and viral monomer and dimer duplex replicative-form (mRF and dRF, respectively) DNA synthesis appeared similar in cells infected with wildtype and mutant viruses (Fig. 3A), although single-stranded progeny DNA synthesis appeared somewhat disparate. Cells infected with wildtype virus retained relatively little single-stranded DNA at this late time in infection. However, significant amounts of progeny single-strands were found in all mutant-infected cells. This progeny DNA migrated rather diffusely in the gel shown in Fig. 3A, for reasons which are not currently understood. However, when extracted under nondisruptive conditions these genomes could be immunoprecipitated with anti-capsid sera and were unit-length and single-stranded when subsequently analyzed by electrophoresis under denaturing conditions (data not shown). A band of fragmented, partially duplex DNA was also seen in some NS2 mutant-infected cells, migrating between mRF and single-stranded DNA, but the significance of this product is currently unknown.

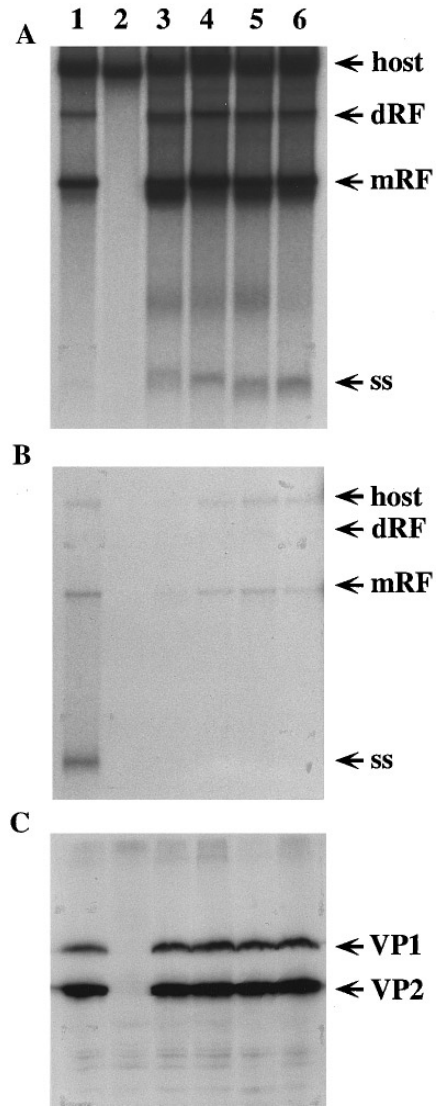


FIG. 3. DNA replication and capsid gene expression in infected human 324K cells. (A) Autoradiograph of a neutral agarose gel showing $^{32}$P-labeled DNA replication products present in cell pellets harvested from uninfected 324K cells (lane 2) or infected cells 30 hr after the addition of wildtype MVMp (lane 1) or the NS2 mutant viruses NS2-Q100am (lane 3), NS2-F86am (lane 4), NS2-R132op (lane 5), or NS2-SA86am (lane 6). Monomeric (mRF) and dimeric (dRF) duplex viral replication intermediates and progeny single-stranded (ss) DNA are indicated. (B) Autoradiograph of a neutral agarose gel showing $^{32}$P-labeled DNA replication products present in culture supernatants from the 324K monolayers indicated in A, using the same lane designations and labeling abbreviations. (C) Autoradiograph of a discontinuous SDS–PAGE gel showing $^{35}$S-labeled capsid proteins (VP1 and VP2) immunoprecipitated with rabbit anti-capsid serum from combined cell pellet and culture supernatant samples from uninfected or infected 324K cells harvested 30 hr after addition of virus. Lane designations are as for A.

The retention of single-stranded progeny DNA at this late time in cells infected with NS2$^-$ mutant viruses was in marked contrast to the situation in wildtype infections (compare Figs. 3A and 3B). Although cell degeneration was still limited at this stage in the infection, as shown

by the small amount of cellular or RF DNA in the culture medium (Fig. 3B) relative to that in the cell (Fig. 3A), most of the progeny wildtype genomes had already been transported or otherwise released into the medium by 30 hr postinfection (Fig. 3B, lane 1). At the same time, only 1 to 2% of the NS2$^-$ mutant genomes had been similarly translocated (Fig. 3B, lanes 3–6). While there is no direct evidence, at present, for a nonlytic mechanism for progeny virus release, these results clearly show that it is the release phase, rather than progeny synthesis per se, which is impaired in NS2$^-$ mutant infection of 324K cells.

In parallel we examined the synthesis of capsid proteins in single-round infections of 324K cells with wildtype and mutant virus under culture conditions comparable to those used in the DNA replication analysis presented above but using [$^{35}$S]methionine/cysteine as the isotopic label. Since single-strand synthesis is concomitant with and dependent upon packaging (reviewed in Cotmore and Tattersall, 1995), a major reduction in the availability of capsid proteins at this time could theoretically reduce and/or retard progeny synthesis. However, when assessed by immunoprecipitation of pooled cells and medium under nondenaturing conditions with nonlimiting amounts of the anti-capsid serum PN1, capsid synthesis appeared essentially identical in 324K cells infected with wildtype and mutant viruses (Fig. 3C).

## Infection of murine A9 cells by NS2$^-$ mutants is abortive for progeny genome synthesis

In order to identify steps in the viral life cycle affected by the NS2$^-$ lesion in nonpermissive host cells, we performed a series of *in vivo* labeling studies in asynchronous A9 cells, exactly comparable to those described above for 324K cells. In marked contrast to the results from the human cells, in murine A9 cells incorporation of [$^{32}$P]orthophosphate into duplex viral RF DNA in the mutants was reduced to approximately 5% of the wildtype level, and single-stranded progeny DNA was undetectable in either cells or medium even after prolonged autoradiographic exposure (Figs. 4A and 4B). Moreover, analysis of capsid protein production, assessed by the same PN1 anti-capsid serum immunoprecipitation procedure used previously, suggested that capsid protein accumulation was severely depressed in mutant-infected cells (Fig. 4C). This apparent failure in expression from the P38 promoter has been observed previously (Naeger *et al.,* 1993), but is surprising since NS1 accumulation during the same 20-hr labeling period appeared relatively normal (data not shown), and transactivation of a capsid-gene promoter-construct was shown to be dependent on NS1 expression but completely independent of NS2 (Doerig *et al.,* 1988). We have extended these studies using constructs containing a reporter CAT gene initiating

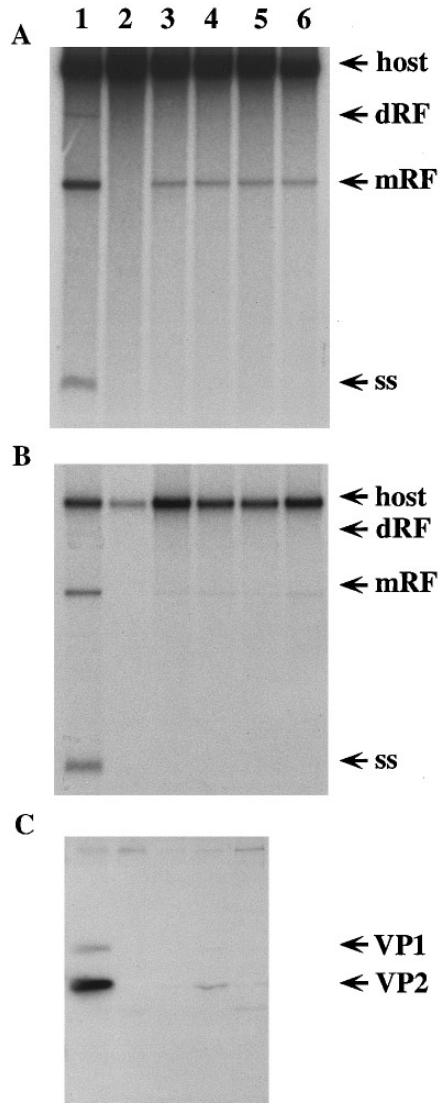


FIG. 4. DNA replication and capsid gene expression in infected murine A9 cells. (A) Autoradiograph of a neutral agarose gel showing $^{32}$P-labeled DNA replication products present in cell pellets harvested from uninfected A9 cells (lane 2) or infected cells 30 hr after the addition of wildtype MVMp (lane 1) or the NS2 mutant viruses NS2-Q100am (lane 3), NS2-F86am (lane 4), NS2-R132op (lane 5), or NS2-SA86am (lane 6). Monomeric (mRF) and dimeric (dRF) duplex viral replication intermediates and progeny single-stranded (ss) DNA are indicated. (B) Autoradiograph of a neutral agarose gel showing $^{32}$P-labeled DNA replication products present in culture supernatants from the A9 monolayers indicated in A, using the same lane designations and labeling abbreviations. (C) Autoradiograph of a discontinuous SDS–PAGE gel showing $^{35}$S-labeled capsid proteins (VP1 and VP2) immunoprecipitated with rabbit anti-capsid serum from combined cell pellet and culture supernatant samples from uninfected or infected A9 cells harvested 30 hr after addition of virus. Lane designations are as for A.

close to the normal VP2 start codon in otherwise unaltered P38 transcripts and have shown that, even within this context, reporter gene expression was similarly NS2-independent but dependent on NS1 (L. F. Carbonell and P. Tattersall, unpublished observations). Thus it seemed improbable that VP2 expression per se

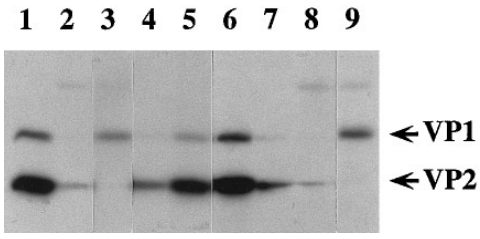


FIG. 5. Immunoprecipitation of polypeptides from purified virus with anti-capsid and anti-allopeptide sera. $^{35}$S-labeled wildtype MVMp virions were either left intact (lanes 1, 2, 4, 6, and 8) or denatured and dissociated into monomer polypeptides by heating to 85° for 30 min in 0.375% SDS (lanes 3, 5, 7, and 9) before being immunoprecipitated with anti-capsid serum (lanes 1, 6, and 7), with anti-VP1-specific serum (lanes 2 and 3), or with anti-allopeptide serum (lanes 4 and 5). Unbound material from the precipitation of native capsids with anti-capsid serum shown in lane 6 is shown reprecipitated with anti-VP1-specific serum in lane 8. Unbound material from the precipitation of denatured capsids with anti-capsid serum shown in lane 7 is shown reprecipitated with anti-VP1-specific serum in lane 9.

would be severely impaired in NS2$^-$ mutant infections, prompting us to undertake the more detailed analysis of capsid protein expression described below.

## Anti-capsid and anti-peptide antibodies differentiate between mature empty particles and unassembled VP2 polypeptides

The polyclonal antisera PN1 and PN2, raised by repeated immunization of rabbits with small quantities of intact capsids, reacted very efficiently with intact particles (Fig. 5, lanes 1 and 6), but not with capsids that have been fully disrupted by incubation to 80° or above (Fig. 5, lane 7). When the unbound material from the latter immunoprecipitation was reprecipitated with anti-VP1 serum, the remaining VP1 proteins were recovered, showing that the conditions used had fully dissociated them from complexes with VP2 (Fig. 5, lane 9). These two anti-capsid sera, PN1 and PN2, thus react predominantly with secondary or higher-order structural determinants on the surface of the capsid shell, but poorly with any of the predominantly linear epitopes presented by fully denatured capsid proteins. This finding accords with our previous observations that these particular sera give very poor signals when used to probe Western transfers of viral capsid proteins (data not shown).

In contrast to the anti-capsid sera described above, anti-allopeptide- and anti-VP1-specific sera react efficiently with isolated VP polypeptides, but very poorly with intact particles. These sera thus allow discrimination between capsid protein synthesis per se and the processes required for authentic capsid assembly in cells infected with MVMp wildtype and NS2$^-$ mutant viruses. A more detailed characterization of the sera will be presented in a separate publication (S. F. Cotmore, A. D'Abramo, and P. Tattersall, manuscript in preparation). The two anti-peptide sera react efficiently and specifically with the expected viral capsid proteins when analyzed by Western transfer (data not shown) or by immunoprecipitation of [$^{35}$S]methionine/cysteine-labeled capsid proteins which have been thoroughly denatured by heating to 85° in 0.375% SDS prior to immunoprecipitation (Fig. 5A, lanes 3 and 5). Under these conditions the anti-allopeptide sera react with both VP1 and VP2 (Fig. 5, lane 5), while the anti-VP1 sera precipitate only isolated VP1 molecules (Fig. 5, lane 3). However, these sera were only able to precipitate a small proportion of $^{35}$S-labeled intact viral particles when such samples were prepared for precipitation without prior heating (Fig. 5A, lanes 2 and 4). In the experiment shown here the anti-allopeptide serum precipitated approximately 16% of the native $^{35}$S-labeled capsids present in this population of newly released virus harvested from the medium of synchronized MVMp-infected A9 cells, while the anti-VP1 specific serum reacted with approximately 7% of such particles. Since VP2 molecules account for approximately 85% of capsid polypeptides, five to six VP2 molecules are coprecipitated with each VP1 molecule in intact particles.

## The primary capsid defect in NS2$^-$ infection operates at the level of particle assembly, not capsid protein expression or trafficking

A9 cultures synchronized using the standard double-block procedure were simultaneously infected with either wildtype MVMp or NS2-F86am, a particularly stable NS2$^-$ mutant virus. Under this regimen cells are released into S-phase 20 hr after infection, so that when the polymerase block is removed, viral genomes are already present in the cell nucleus and are able to initiate viral replication and gene expression rapidly. Eight hours after release into S-phase, at the normal peak of NS2 synthesis, cells were pulsed with [$^{35}$S]methionine/cysteine for 20 min and then harvested immediately or transferred back into normal medium for various chase periods. Immunoprecipitation under native conditions with antiserum PN2, directed against intact capsids, confirmed the capsid accumulation defect reported earlier, with wildtype infections yielding approximately four- to fivefold more precipitable material than mutant virus infections in both pulse and pulse–chase samples (Fig. 6A). However, in both wildtype and NS2-F86am infections, samples harvested after a chase period, rather than immediately after the pulse, showed a two- to threefold increase in material reacting with this antibody (Fig. 6A, compare lanes 2 and 5 or lanes 3 and 6). Since we have shown, in the previous section, that this anti-capsid serum reacts preferentially with structural determinants on the viral particle, but very poorly with isolated viral polypeptides, it seemed possible that the increased levels of labeled protein precipitated with PN2 after the first chase period might reflect the process of particle assembly, rather than further synthesis.

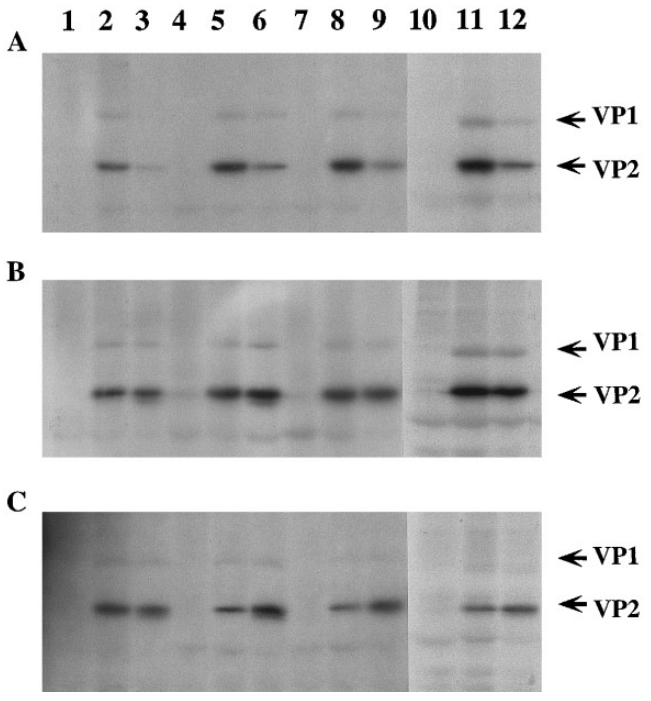


FIG. 6. Immunoprecipitation of pulse–chase-labeled viral structural polypeptides. Autoradiographs of discontinuous SDS–PAGE gels showing $^{35}$S-labeled capsid proteins (VP1 and VP2) immunoprecipitated from synchronized A9 cells harvested immediately after a 20-min pulse with Tran$^{35}$S-label initiated 8 hr after release into S-phase (lanes 1–3 in all panels), or after an additional 40-min chase period in unlabeled medium (lanes 4–6 in all panels), after an 80-min chase period (lanes 7–9), or after a 2-hr chase period (lanes 10–12). Samples in B were denatured by heating to 85° for 20 min in 0.375% SDS prior to immunoprecipitation, while samples in A and C were not heated. Samples in A were immunoprecipitated with rabbit anti-capsid serum, and those in B and C were precipitated with anti-allopeptide serum. Samples were harvested from uninfected synchronized A9 cells (lanes 1, 4, 7, and 10) or from synchronized cultures infected with either wildtype MVMp (lanes 2, 5, 8, and 11) or NS2-F86am (lanes 3, 6, 9, and 12).

To explore this possibility, we first denatured the samples by incubating them at 85° for 30 min in 0.375% SDS and then precipitated them quantitatively with anti-allopeptide serum, which reacts preferentially with isolated proteins. In contrast to the previous result all samples harvested within 80 min of the pulse were found to contain approximately equivalent amounts of capsid protein, when analyzed in this way, irrespective of whether the infecting virus was wildtype or NS2-F86am (Fig. 6B). Thus in each infection newly synthesized capsid proteins require a finite time to assemble into particles and become precipitable with anti-capsid serum, but in the wildtype infection this process is essentially complete by the end of the first chase period (i.e., within 40 min). In NS2-F86am-infected cells, however, relatively little folding or assembly occurred during this or subsequent chase periods, so that most of the labeled protein remained unprecipitable by anti-capsid serum. Thus at 8 hr postinfection the defect in capsid synthesis exhibited by NS2$^-$ mutant viruses appeared to reflect a defect in particle assembly rather than a defect in the synthesis of capsid proteins per se.

This finding was confirmed by immunoprecipitating the extracts with the same anti-allopeptide serum, but now under native conditions (Fig. 6C). As expected, in both wildtype and NS2-F86am infections most of the labeled capsid protein present at the end of the pulse period could be precipitated with this antibody, indicating that at this time few capsid proteins were assembled into normal capsid structures. At later times, however, most of the capsid proteins from NS2-F86am infections remained recognizable by the anti-allopeptide serum, whereas those synthesized during wildtype infection chased into a form which was not recognized. The NS2-F86am capsid proteins which were not assembled into particles remained in the cell in their unassembled state for at least 80 min after synthesis (Figs. 6B and 6C, lanes 6 and 9). However, by 2 hr after synthesis the level of unassembled protein had begun to decline (Fig. 6C, lane 12), matched by a concomitant drop in the total capsid protein level (Fig. 6B, lane 12), without any sign of its gradual conversion into assembled particles (Fig. 6A, lane 12). Thus in NS2$^-$ mutant virus infections any capsid proteins which did not assemble into particles soon after synthesis were retained in the cell for protracted periods in their unfolded state. That they did not assemble at a later time suggests either that these proteins became and remained incompetent for assembly or that a limiting cellular function was exhausted.

Immunofluorescence analysis of synchronized cells fixed 8 hr after infection and stained with anti-allopeptide serum revealed that a similar proportion of cells in both cultures synthesized capsid polypeptides (Figs. 7A and 7B). When stained with the anti-peptide antibody, infected cells showed intense cytoplasmic fluorescence, as expected for newly synthesized, nonassembled capsid proteins. In both populations of infected cells there was equivalent accumulation of signal in the nucleus, suggesting that capsid polypeptides can be transported into the nucleus prior to assembly. This observation supports the recent finding that the structural proteins contain a C-terminal nuclear localization signal, located between residues 528 and 538 of VP2 (Lombardo *et al.,* 1995). The recently determined atomic structure of the MVM particle (Llamas-Saiz *et al.,* 1997) indicates that these residues would be substantially buried in the intact capsid, and therefore this signal can probably function only prior to assembly.

In marked contrast to the anti-peptide antibody result, staining with PN2 anti-capsid serum revealed correctly assembled particles concentrated in the nuclei of cells infected with wildtype virus, but a greatly diminished level of nuclear signal in cells infected with the NS2-F86am mutant virus (compare Figs. 7C and 7D). These observations are entirely consistent with the metabolic labeling and immunoprecipitation studies reported above. The

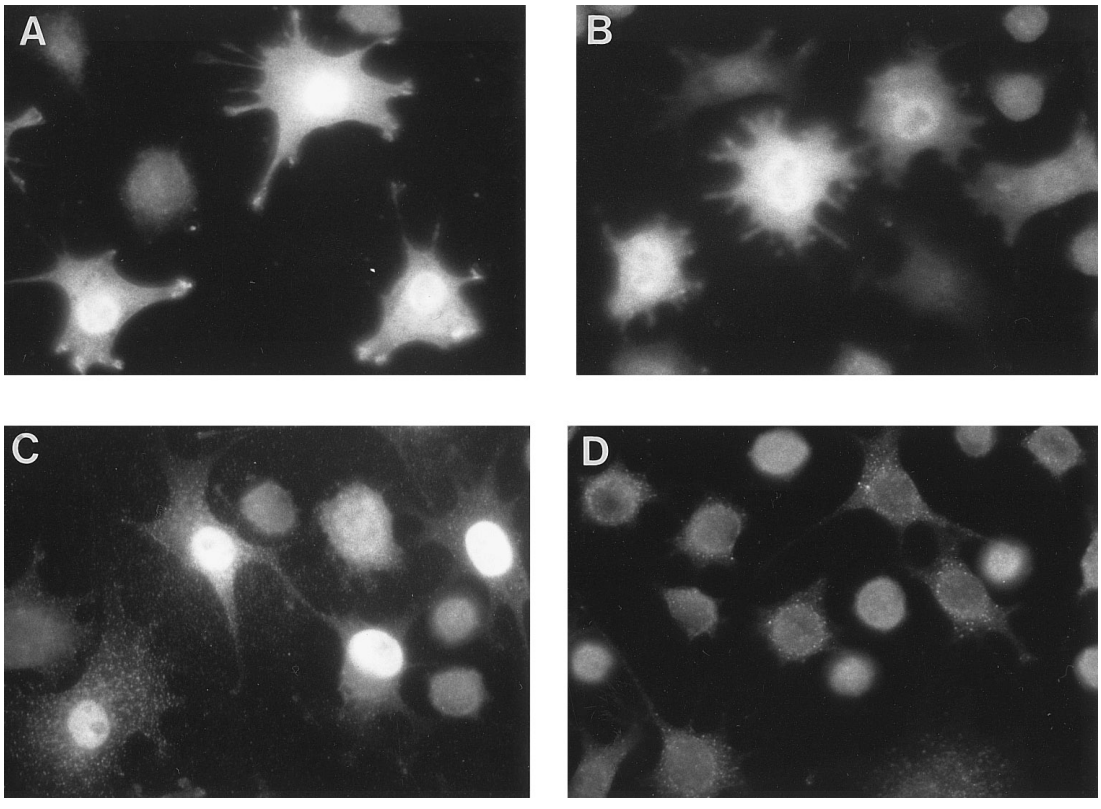


FIG. 7. Immunofluorescence staining of viral structural polypeptides. Synchronized A9 cells infected with wildtype MVMp (A and C) or with NS2-F86am (B and D) were fixed 10 hr after release into S-phase and stained with either anti-allopeptide serum (A and B) or anti-capsid serum (C and D).

anti-capsid antibody also stains viral antigen within endosomal vesicles in the cytoplasm in both populations of infected cells with equivalent intensity. This type of staining, which diminishes with time as infection proceeds, can also be detected in cells which have not been released from aphidicolin (data not shown), indicating that it represents residual input virus.

## Total capsid synthesis progressively declines in NS2$^-$ mutant-infected cells

Synchronized cells, infected with either wildtype or NS2-F86am virus, were labeled with [$^{35}$S]methionine/cysteine for 3-hr periods between 6 and 9, 9 and 12, or 12 and 15 hr after release into S-phase. Immunoprecipitation of fully denatured extracts with anti-allopeptide serum showed that in the wildtype infection the rate of capsid synthesis and accumulation increased as the infection progressed (Fig. 8, lanes 1–3), while in cells infected with the NS2$^-$ mutant capsid synthesis was efficient at early times in infection, but became progressively impaired at later times (Fig. 8, lanes 4–6). Since unassembled capsid proteins had a half-life of approximately 2 hr in the previous pulse–chase analysis, it seems probable that this progressive diminution reflects both markedly reduced synthesis at later times in infection and slightly elevated rates of protein degradation. When extracted and analyzed under nondenaturing conditions, the diminishing amounts of NS2-F86am capsid protein which was synthesized during each of the three pulse periods continued to be precipitable far more efficiently by anti-peptide serum than by anti-capsid serum, while the reverse was true for the increasingly abundant wildtype proteins (data not shown).

## DISCUSSION

In this report we detail specific host range-specific defects in viral replication that result from the premature translational termination or ablation of the viral NS2 polypeptides. As reported previously by Naeger and colleagues (Naeger *et al.*, 1990), cloned MVMp genomes encoding truncated forms of the NS2 gene could not be recovered in, and were unable to grow productively in, their normal murine host cell, but were able to give rise to progeny virus in the SV40-transformed human fibroblast line 324K, albeit with reduced efficiency. The defects in mutant virus growth in these two cell types are significantly different. In murine cells major lesions in both viral DNA replication and capsid assembly were observed, while the only growth defect found in human cells, impaired progeny virus release, was manifested much later in the life cycle. From this we conclude that many of the roles played by NS2 in the early replicative

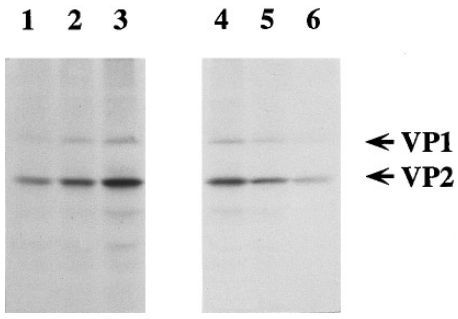


FIG. 8. Extended pulse-labeling of viral structural polypeptides. Autoradiograph of a discontinuous SDS–PAGE gel showing metabolically $^{35}$S-labeled capsid proteins (VP1 and VP2) immunoprecipitated from denatured extracts of synchronized A9 cells which had been infected with wildtype MVMp (lanes 1–3) or NS2-F86am (lanes 4–6) and harvested immediately after a 3-hr pulse with Tran$^{35}$S-label spanning the period from 6 to 9 hr after release into S-phase (lanes 1 and 4), from 9 to 12 hr after release (lanes 2 and 5), or from 12 to 15 hr after release (lanes 3 and 6). Each sample was immunoprecipitated with anti-allopeptide serum.

processes of MVM are likely to be indirect, since they can be complemented by an appropriate cellular environment. While in agreement with previous analyses (Naeger *et al.,* 1990, 1993), this finding is remarkable given the high-level expression of NS2 early in wildtype infections, which originally suggested a more direct role for NS2 in progeny production, perhaps structural or at least noncatalytic. Possibly such high-level expression is required simply because the virus is unable to express its genes prior to S-phase and so must reorient the cellular replication machinery for viral replication as rapidly as possible to ensure productive takeover of the cell cycle.

Although NS2$^-$ mutant viral stocks could be generated in 324K cells, the growth of these viruses was still substantially impaired relative to that of wildtype MVMp. In consequence, stocks of all NS2$^-$ mutant viruses which relied upon a single nonsense mutation to inactivate NS2 could be rapidly compromised by the appearance of wildtype revertants or forward mutants with restored NS2 expression. In contrast, mutant NS2-F86am, which carries a complex termination mutation, was unable to revert with appreciable frequency and so became the virus of choice in those experiments requiring high-titer pools of NS2$^-$ virus. The selective pressure which drives reversion to an NS2$^+$ genotype in 324K cells appears to act late in the viral life cycle. While even at late times in a single-round infection, viral RF DNA replication and capsid protein synthesis appeared relatively normal, and single-stranded progeny synthesis was within twofold of normal, packaged virions were not efficiently released. Whether or not there is a nonlytic mechanism for progeny virus release from 324K cells remains uncertain, but such an exocytotic mechanism has been proposed for the early phase of SV40 release from infected cells (Clayson *et al.,* 1989). While the molecular basis for this late defect in progeny export is at present uncertain, it clearly restricts the effective spread of virus through the culture and provides a rationale for the rapid selection and preferential growth of wildtype revertants that we have observed during stock expansion.

Although we introduced nonsense codons at three different positions in the second exon of NS2, and coupled one of these with a splice acceptor mutation designed to prevent the expression of the isolated first exon domain, all mutant viruses had the same host range phenotype and all failed to show any detectable accumulation of truncated NS2 products. Thus the phenotype we report appears to reflect the absence of NS2, rather than the stable expression of any particular peptide domain. Brockhaus *et al.* (1996) were similarly unable to express stable forms of NS2 in murine cells infected with two mutant MVM viruses which carried mutations near the extreme carboxy-terminus of exon 2 (NS2-2268 and Df18), although detectable levels of these modified proteins could be produced in a c-Ha-ras-transformed Fisher rat cell, FREJ4. In a previous study these two particular mutant viruses had also been shown to express detectable levels of NS2 in 324K cells (Naeger *et al.,* 1990), suggesting that the differential stability of mutant NS2 proteins in different cell types might influence host range. However, the viruses used in the present study, as well as the series of NS2 mutant viruses generated by Naeger *et al.* (1990) which had termination codons more centrally located in the body of exon 2, all failed to stably express truncated forms of NS2 in 324K cells (Naeger *et al.,* 1990 and data not shown), and thus it is unlikely that protein stability per se is of primary importance. In part, the failure to accumulate truncated NS2 species likely reflects a polar shift in the splicing pattern of P4-driven transcripts reported previously (Naeger *et al.,* 1992), as also suggested by the polar shift in NS1 expression seen in the present study. However, the magnitude of the lesion in NS2 synthesis greatly exceeds the reported splicing shift, suggesting that transcripts encoding the affected gene might be poorly translated or that truncated NS2 polypeptides are extremely unstable *in vivo.*

In both MVM and the closely related parvovirus H1 the inability of NS2$^-$ mutants to replicate productively in rodent cells is associated with a complex series of defects in viral DNA replication (Naeger *et al.,* 1990; Li and Rhode, 1991). Under the conditions used in the present study duplex DNA replication was reduced to around 5% of wildtype levels and there was absolutely no evidence of any single-stranded DNA synthesis. Why NS2 has such a critical role in replication remains unknown. Although these observations might suggest that in murine cells it plays a direct role in viral duplex DNA replication, there is no published *in vitro* evidence to support this premise and we have been unable to substantially influence NS1-driven *in vitro* MVM DNA replication in A9 extracts by adding wildtype NS2 expressed from recom-

binant vaccinia constructs (J. P. F. Nuesch, S. F. Cotmore, and P. Tattersall, unpublished results). Moreover, an indirect effect mediated through NS1 is also unlikely since expression of NS1 in NS2$^-$ mutant infections is somewhat elevated rather than depressed, and replication-competent NS1 can be generated in the absence of NS2 in mammalian and insect cells infected with recombinant vaccinia or baculovirus vectors (Nuesch *et al.,* 1992; Christensen *et al.,* 1997). Whether or not NS2 directly influences progeny single-strand DNA synthesis is more difficult to evaluate. All single-stranded viral DNA found in infected cells is encapsidated, and its synthesis likely involves direct sequestration of displaced strands into preformed or partially formed capsids (Cotmore and Tattersall, 1996). Thus the defect in capsid assembly seen in NS2$^-$ mutant infections would be expected to impinge severely on single-strand synthesis in its own right, making it impossible to ascertain whether or not NS2 plays a direct role in the synthesis of progeny strands and/or their encapsidation.

Although our preliminary anti-capsid immunoprecipitations did suggest that capsid protein synthesis might be defective, this seemed unlikely since NS1 expression appeared relatively normal and the NS1-encoding R1 transcripts contain all of the sequences present in the capsid mRNAs. Moreover, transactivation of both a simple P38 promoter-construct (Doerig *et al.,* 1988) and a construct in which a CAT-reporter gene initiated at the VP2 start of otherwise unaltered P38 transcripts (L. F. Carbonell and P. Tattersall, unpublished observations) had been shown to be dependent on NS1 expression but completely independent of NS2. In a previous study capsid protein synthesis in NS2 mutant infections was measured by immunoprecipitation of $^{35}$S-labeled cell extracts with anti-capsid serum and related to the level of P38-driven transcripts in the same infection (Naeger *et al.,* 1993). Using cell extracts prepared under nondenaturing conditions, these authors showed a 10-fold defect in the accumulation of immunoprecipitable VP2 relative to transcript accumulation following a 1-hr pulse 9, 12, or 14 hr after entry into S-phase. Although we initially obtained somewhat similar data with our series of mutants, it was clear that the magnitude of the observed defect depended on the timing and duration of the labeling period, so that long pulses late in infection suggested a vastly more profound defect than short pulses instituted early in infection (compare Fig. 8 with Fig. 6A). This led us to conclude that the capsid defect, whatever its basis, developed progressively as infection proceeded.

Since MVM capsid particles are known to be exceptionally rugged, we first used immunoprecipitation with anti-VP1-specific serum to explore conditions required to fully dissociate and denature such particles and thus render the VP1 proteins fully susceptible to immunoprecipitation without coprecipitating VP2. Such dissociation could only be achieved after heating (with or without SDS) to temperatures in excess of 80°, conditions which we considered so stringent that we were prompted to reassess whether or not our various anti-intact-capsid sera could react with similarly dissociated material. The anti-allopeptide serum used here is particularly useful because it reacts quantitatively with monomeric VP1 and VP2 molecules released by heating capsids to 80°, but is unable to gain access to the relevant epitopes in the great majority of intact, native particles (S. F. Cotmore, A. D'Abramo, and P. Tattersall, manuscript in preparation). Using these two types of sera with cell extracts prepared under native and denaturing conditions at different times in infection thus allowed us to differentiate between the processes of capsid protein synthesis and particle assembly in cells infected with mutant MVM genomes.

The observations presented here make it clear that cells infected with wildtype virus assemble capsid particles far more efficiently than mutant-infected cells and thus suggest that the virus is able to manipulate the cellular environment to optimize this process. Nonetheless, in NS2 mutant-infected A9 cells a small number of the VP proteins synthesized throughout the course of the infection do assemble into capsid-like structures, although we do not know if they assemble into packaging-competent intermediates. Similarly discrete particles can be produced in cells stably expressing parvoviral capsid genes but lacking the nonstructural gene sequences (Pintel *et al.,* 1984; Kajigaya *et al.,* 1989) or when these proteins are expressed in insect cells from recombinant baculoviruses (Brown *et al.,* 1991; Kajigaya *et al.,* 1991; Lopez de Turiso *et al.,* 1992; Martinez *et al.,* 1992; Saliki *et al.,* 1992; Christensen *et al.,* 1994) and in mammalian cells from recombinant vaccinia viruses (Clemens *et al.,* 1992). Some of the VP proteins expressed under these conditions do assemble into 70S particles carrying a variety of neutralizing, structure-related antigenic epitopes, but most of the expressed proteins do not. Moreover there is crystallographic evidence to suggest that B19 particles assembled in this way may be somewhat disordered (Agbandje *et al.,* 1994). The crystal structure of the MVM virion core has recently been determined to atomic resolution (Llamas-Saiz *et al.,* 1997), but the disposition of the amino-termini of the VP polypeptides remains uncertain, and as yet we have few reagents available to monitor precise assembly or capsid function.

Since some capsid particles are assembled in mutant-infected cells, it is unlikely that this process normally involves a direct interaction between NS2 and the VP polypeptides. Thus, NS2 once again appears to mediate its effects indirectly by modulating cellular function(s). A subset of phosphorylated NS2 molecules was recently shown to bind directly to two members of the highly conserved 14-3-3 family of eukaryotic proteins (Brockhaus *et al.,* 1996). 14-3-3 molecules have previously been shown to bind to a broad range of cellular and viral proteins, apparently acting as modulators or "adaptors"

in many critical biochemical activities including signal transduction, exocytosis, protein transport, and cell cycle regulation (Freed *et al.,* 1994; Reuther *et al.,* 1994), but at present the biological consequences of this interaction for MVM are unclear. The middle T antigen of polyoma virus also binds 14-3-3 proteins (Pallas *et al.,* 1994), and middle T is known to induce the activity of so-called "permissivity factors" in the host cell (Garcea and Benjamin, 1983; Garcea *et al.,* 1985, 1989). These allow stable phosphorylation of threonine residues in the capsid protein VP1 and assembly of mature 240S virions from a 75S nucleocapsid intermediate (Garcea *et al.,* 1989), but whether there is a causal link between these two activities is unproven. MVM capsid proteins also show complex patterns of posttranslational modification (Peterson *et al.,* 1978; Santaren *et al.,* 1993), which might well influence the assembly or ultimate structure of the viral particle, but at present the significance of such processing remains unclear.

We conclude that in mouse cells *in vivo* the absence of NS2 down-regulates capsid assembly without initially down-regulating VP synthesis and that, while a lesion in VP polypeptide expression does arise late in infection, this may well be a secondary effect resulting from product accumulation. A similar progressive drop in capsid synthesis at late times in infection was seen in cells infected with a group of SV40 host range mutants which carry small deletions in the extreme carboxy-terminal region of Large T antigen (Spence and Pipas, 1994). Like the MVMp NS2 mutants described here, at early times in infection these SV40 host range mutants showed a defect in virion assembly, but not in capsid protein synthesis per se. Exactly why this progressive deterioration occurred remains uncertain. In the case of MVM it may simply reflect a progressive drop in the level of accumulated transcripts, as evidenced by the studies of Naeger and colleagues (Naeger *et al.,* 1993), but there may also be direct inhibition of the synthetic pathway due to product-mediated negative feedback or the depletion of critical cellular components such as chaperone polypeptides. Such progressive defects may thus be symptoms of NS2 gene dysfunction rather than its primary effect.

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