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Replication of Minute Virus of Mice DNA Is Critically Dependent on Accumulated Levels of NS2

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Following transfection of murine fibroblasts, the lymphotropic strain of minute virus of mice (MVMi) does not efficiently produce progeny single-strand DNA (ssDNA). However, changing a single nucleotide in the MVMi 3' splice site to that found in the fibrotropic strain MVMP enabled full DNA replication and production of ssDNA. This change enhanced excision of the large intron and the production of NS2, likely by improving interaction, in fibroblasts with the branch point-binding U2 snRNA. One function of NS2 involves interaction with the nuclear export protein Crm1. The defect in production of MVMi ssDNA in fibroblasts can also be overcome by introducing a mutation in MVMi NS2 that enhances its interaction with Crm1. Although MVMi contains a 3' splice site that performs poorly in fibroblasts, MVMi generated at least as much R2 and NS2 in murine lymphocytes as did MVMP in fibroblasts. Therefore, it appears that MVMP has acquired a mutation that improves the excision of the large intron, as it adapted to fibroblasts to accommodate the need for NS2 for replication in these cells, and that the ratio of NS1 to NS2 may play a larger role in the host range of MVM than previously appreciated.

Two strains of minute virus of mice, fibrotropic strain MVMP and lymphotropic strain MVMi, are reciprocally restricted for growth in murine cells (2, 10, 15, 23, 25, 26). MVMP productively infects murine fibroblasts but not murine lymphocytes, while MVMi does the opposite. The major viral determinant of this host range resides within the capsid. The primary block to restrictive infections is intracellular, following binding to cells but before deposition of viral DNA in the nucleus (1, 3, 4, 11, 12, 20).

Characterization of MVMP and MVMi has also led to the identification of another determinant that affects viral replication in a host cell-dependent manner. Analysis of MVMi virus selected for growth on fibroblasts and analysis of the replication of engineered MVMi and MVMP chimeras on murine lymphocytes have demonstrated that a region encompassing the large-intron 3' splice site and P38 TATA box also plays a role in the efficiency of viral DNA replication in these two cell types (7, 12). The role that this region plays in replication has not been well characterized, but in both cases, differences in the ratio of mRNA R1 to R2, which encode NS1 and NS2, respectively, have been observed (4, 7, 11). The importance of the NS region in cell type-specific replication has also been noted for cells of neuronal origin (22).

Previous studies in our lab have shown that even minor alterations of the MVM large-intron 3' splice site can have significant effects on the steady-state ratio of R1 to R2 and, hence, on the ratio of the viral nonstructural proteins NS1 and NS2 (19, 28). Differences between MVMi and MVMP in this area have been previously noted, and ways in which these differences might affect RNA processing in the two strains have been discussed previously (19). Recently, two reports have

indicated that mutations that increase relative levels of NS2 by enhancing the excision of the large intron lead to increased replication of MVMi in murine fibroblasts (A. D' Abramo, Jr., A. Ali, F. Wang, and P. Tattersall, Abstr. 10th Parvovirus Workshop, abstr. 71, 2004; and E.-Y. Choi, L. Burger, A. E. Newman, and D. J. Pintel, Abstr. 10th Parvovirus Workshop, abstr. 77, 2004).

In this study we have shown that, following transfection of murine A9 fibroblasts, the MVMi genome generated lower relative accumulated levels of R2 and NS2 than did MVMP, and although competent for production of double-stranded monomer replicative form (mRF) DNA, MVMi was deficient for the production of progeny single-strand DNA (ssDNA). Changing a single nucleotide in the MVMi large-intron 3' splice site to that found in MVMP, however, was sufficient to regain full DNA replication. This change did not alter the amino acid sequence of NS1 and was associated with an increase in the excision of the large intron from MVMi P4-generated pre-mRNAs, due to an improved interaction with the branch point-binding U2 snRNA in fibroblasts. This mutation thus led to increased relative accumulated levels of R2 and NS2. A reciprocal mutation, changing the A residue to G in the MVMP background, reduced ssDNA production following transfection into fibroblasts, showing that ssDNA production in the first round following transfection was correlated to accumulated levels of NS2. A mutation previously characterized by the Almendral lab to increase NS2-Crm1 interaction above wild-type levels (14) was found to rescue ssDNA replication in fibroblasts in the MVMi background, suggesting that a threshold level of this function was required for MVM replication in fibroblasts. The necessity of NS2 for replication in murine cells was further underscored by the observation that, although bearing a 3' splice site that performs poorly in fibroblasts, MVMi generated at least as much R2 and NS2 in murine lymphocytes as did MVMP in fibroblasts. These results

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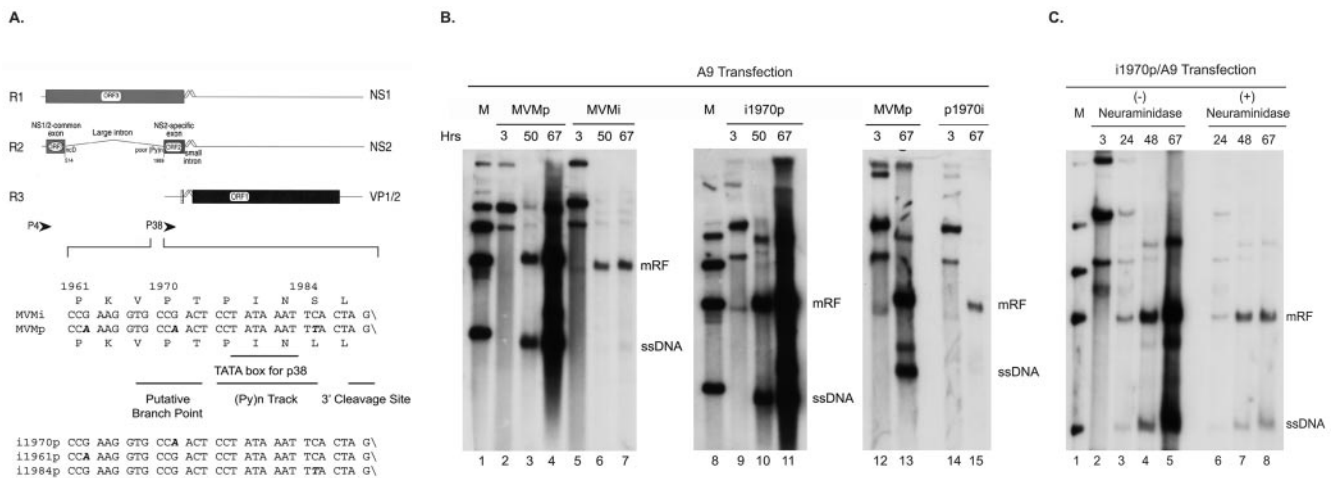


FIG. 1. MVMi generates little progeny ssDNA following transfection of murine fibroblasts, which could be overcome by the exchange of MVMi nt 1970 within the 3' splice site of the large intron to that present in MVMp. (A) A genetic map of MVM is shown. The region of MVM required to generate ssDNA is expanded, showing RNA processing landmarks in the region and the nucleotide sequences of i1970p, i1961p, and i1984p. (B) Southern analysis of cell-associated DNA forms, generated at the hours indicated above the blots, following transfection of A9 cells by either wild-type MVMp or MVMi or the mutant i1970p or p1970i. (C) Southern analysis of cell-associated DNA forms, generated at the hours indicated above the blots, following transfection of A9 cells by mutant i1970p, either in the presence (+) or absence (-) of neuraminidase. M, marker lanes, containing double-stranded DNA fragments of (from bottom) 2.5, 5.0, and 7.5 kb. Bands above those are partial digestion products.

suggested that MVMp has acquired by mutation the ability to improve the excision of the large intron as it adapted to fibroblasts, in order to accommodate the need for NS2 for replication in these cells. The MVMi construct containing only an improved 3' splice site spread well following transfection of fibroblasts in culture, suggesting that the accumulated level of NS2 may play a larger role in the host ranges of MVMp and MVMi than previously appreciated.

MATERIALS AND METHODS

Cells and virus. Murine A9 and EL4 cells and human NB324K cells were propagated as previously described (17). All virus was made directly from transfection of NB324K cells without further propagation, and the titers of the virus were determined by plaque assay on NB324K (6).

Plasmids. The MVMp and MVMi parent plasmids used in this study were the original infectious clones derived by Merchinsky et al. (16) and Gardiner and Tattersall (11), and all subsequent derivatives were constructed from these plasmids. Mutations were made using standard PCR techniques, as described previously (21), and were sequenced to ensure that they were as expected. The mutation which increased NS2 interaction with Crm1 was K96E/L103P, as described by Lopez-Bueno et al. (14). The U2 snRNA mutations were made in the wild-type human U2 snRNA gene, obtained from Alan Weiner (29), by using standard techniques. The branch point-binding 5' ends of the human and mouse U2 snRNA are identical.

Transfections. Transfection of A9 cells was as previously described (17). Transfections of EL4 cells were performed with an Amaxa nucleofector device (Amaxa, Inc.) by using program B24.

Southern and Western analysis and RNase protection assays. Southern analysis and Western blot analysis were performed exactly as previously described (17, 24). The complete homologous genomic clones were used as probes for Southern analysis, and the antibody for NS1/NS2 Western analysis was a polyclonal rabbit antibody raised to the NH₂-terminal common exon of NS1 and NS2. Where indicated, 0.5 U of neuraminidase/ml of medium was added to cultures 3 h postinfection and replenished every 12 h. RNase protection assays were performed with homologous probes spanning MVM nucleotides (nt) 1858 to 2377, as previously described (24). Quantifications were performed as described previously (24) by using a Bio-Rad phosphorimager and are the averages, with standard deviations, of multiple experiments, as indicated in the figure legends.

RESULTS

The inability of MVMi to generate progeny ssDNA following transfection of murine fibroblasts could be overcome by the exchange of MVMi nt 1970 within the 3' splice site of the large intron to that present in MVMp. In contrast to the extensive replication of an infectious plasmid clone of MVMp, transfection of murine A9 fibroblasts with an infectious replicating clone of MVMi resulted in the production of very little ssDNA either associated with cells (Fig. 1B, compare lanes 2 to 4 with lanes 5 to 7) or in the medium (data not shown). MVMi mRF DNA was generated at levels consistent with the lack of spread to a second round of infection, which was not prevented in these assays.

Analysis of a series of hybrids between MVMp and MVMi initially mapped the determinant required for efficient ssDNA production to a region that included the large-intron 3' splice site and the P38 TATA box (data not shown). This region contains three nucleotide differences between MVMp and MVMi (Fig. 1A). Replacement of the G at nt 1970 of MVMi with an A residue, as found in MVMp (i1970p), but neither of the other two changes (i1961p or i1984p) allowed full replication of the MVMi genome following transfection of A9 cells (mutant i1970p [Fig. 1B, lanes 9 to 11]). The reciprocal mutant, in which the A residue at nt 1970 of MVMp was changed to G, as found in MVMi, resulted in a significant loss of MVMp ssDNA production following transfection of A9 cells (mutant p1970i [Fig. 1B, lanes 14 to 15]), demonstrating that ssDNA production could be controlled by this nucleotide alone.

The extent of i1970p replication suggested that even though this mutant bore an MVMi coat, it was able to spread through the fibroblast culture following transfection. The presence of neuraminidase in the culture, which destroys the MVM receptor and prevents reinfection, prevented the accumulation seen in its absence (Fig. 1C, compare lanes in the absence and the

presence of neuraminidase). This effect was also seen in the presence of anti-MVM antibody (data not shown). Thus, the generation of higher levels of NS2 allowed the extracellular spread of virus with an MVMi capsid through a murine fibroblast culture. i1970p mutant stocks remained impaired for initiating infection in fibroblasts de novo, however. Like MVMi (data not shown), they formed plaques on A9 cells at least 10,000-fold less efficiently than on permissive NB324K cells, and DNA replication could not be detected following infection of either untreated or mock-transfected A9 cells, even at multiplicities of infection of 1,000 (data not shown).

The G to A substitution at MVMi nt 1970 allowed greater excision of the MVMi large intron and production of increased levels of NS2 in murine fibroblasts. Following transfection of murine A9 fibroblasts with the infectious clone of MVMi, the relative accumulated levels of both R2 and its protein product NS2 were significantly less than was seen following transfection of the infectious clone of MVMp (Fig. 2A and B, compare lanes 1 and 2). The magnitude of the differences in accumulated relative levels of R2 is not as great as that seen for differences in the accumulated levels of NS2, which may reflect the short half-life of the NS2 protein. In contrast to their parent constructs, however, the i1970p mutant generated levels of both R2 and NS2 similar to those generated by MVMp (Fig. 2A and B, lanes 3), and the p1970i mutant generated levels of R2 and NS2 similar to those of MVMi following transfection of A9 cells (Fig. 2A and B, lanes 4). Thus, the ability to generate ssDNA following transfection of murine fibroblasts correlated with high levels of NS2 production.

The MVMi 3' splice site was inefficiently utilized by the murine fibroblast U2 snRNP. Nucleotide 1970 is in a position that could function in the branch point for excision of the large intron. The pre-mRNA branch point engages in an essential base pairing interaction with the 5' end of the U2 snRNA molecule. This interaction results in the bulging out of an A residue, which is required for the first step of intron excision. The A residue at nt 1970 in the putative MVMp branch point is predicted to form a bulged A structure with the endogenous U2 snRNA (Fig. 3B, diagram 1). However, the presence of the G residue at that position, as found in MVMi, allows for two possible structures (Fig. 3B, diagram 2). If the G residue base pairs with the uridine residue of the endogenous snRNA, the adjacent A could bulge out (Fig. 3B, diagram 2, left). However, the A residue may also base pair with the U residue, leaving the G residue at nt 1970 mismatched and the A residue unfavorably duplexed (Fig. 3B, diagram 2, right). Although the i1970p G to A mutation is predicted to allow a bulged A in the MVMi duplex, substitution of either a T or a C at this position (Fig. 3A) would not (Fig. 3B, shown for i1970 T in diagram 4), and in contrast to the A substitution in i1970p, neither T nor C substitution increased excision of the MVMi large intron (Fig. 3C, compare lane 3 [i1970p] with lanes 4 and 9 [i1970T and i1970C, respectively]). Cotransfection of a mutant U2 snRNA gene, however, which had been mutated to potentially allow the A residue at nt 1971 of the i1970T mutant to bulge out from the duplex (Fig. 3B, U2 snRNA-T, compare diagrams 4 and 5), increased splicing of the i1970T, but not the MVMi, large intron (Fig. 3C, compare lane 4 with lane 5 and lane 2 with lane 6). Full levels of excision were likely not reached due to the remaining presence of the endogenous U2 snRNA.

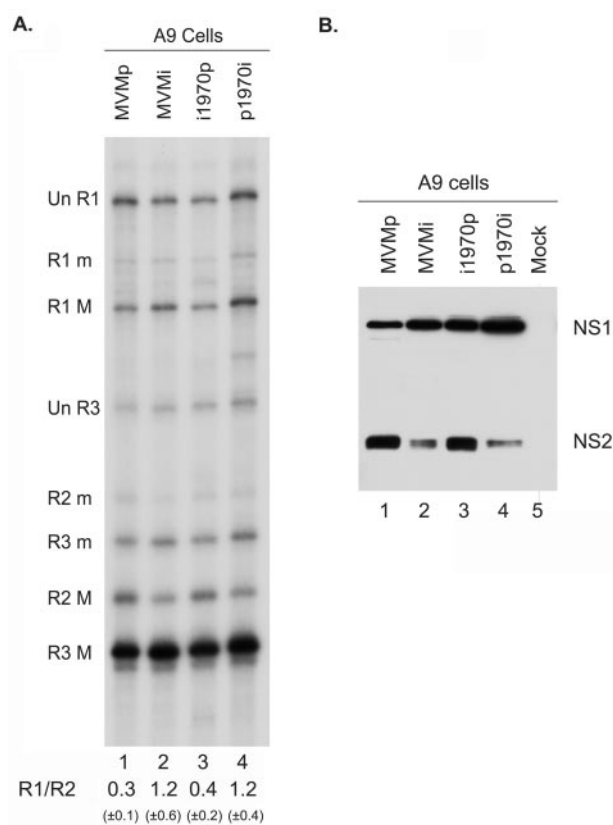


FIG. 2. Replacement of G by A at MVMi nt 1970 allows greater excision of the MVMi large intron and production of increased levels of NS2 in murine fibroblasts. (A) RNase protection analysis, using homologous antisense probes spanning nt 1858 to 2377, of RNA present 48 h following transfection of A9 cells by the infectious viral clones indicated. A representative experiment is shown, below which are quantifications with standard deviations of 25 experiments for MVMp, 25 experiments for MVMi, 16 experiments for i1970p, and 4 experiments for p1970i. The identities of the RNA species are given on the left: M refers to usage of small-intron donor D1 at nt 2280, m refers to usage of small-intron donor D2 at nt 2317, and Un refers to RNA remaining unspliced through the probe region. (B) Western analysis, using antibody reacting with the NH₂-terminal region common to both NS1 and NS2, of protein present 24 h following transfection of A9 cells with the infectious viral clones, as indicated.

These results suggested that nt 1970 was part of the large-intron branch point and that the A residue at nt 1970 found in MVMp and i1970p provided functional improvement for the excision of their large introns. Suppression of the wild-type MVMi branch point was not successful. Mutations of the U2 snRNA, which are predicted to enhance the generation of a bulged A residue in MVMi pre-mRNA, by necessity also introduced an additional G-C base pair flanking the A residue (Fig. 3B, U2 snRNA-i, diagram 3). Such a change may have hindered subsequent rearrangements in the spliceosome required for intron excision.

The inability of MVMi to generate ssDNA in murine fibroblasts could also be overcome by increasing the strength of the NS2-Crm1 interaction. Recently, Lopez-Bueno et al. isolated a group of MVMi mutants that were selected for the ability to escape passive immunotherapy in immunosuppressed mice (14). Interestingly, a number of these viruses contained muta-

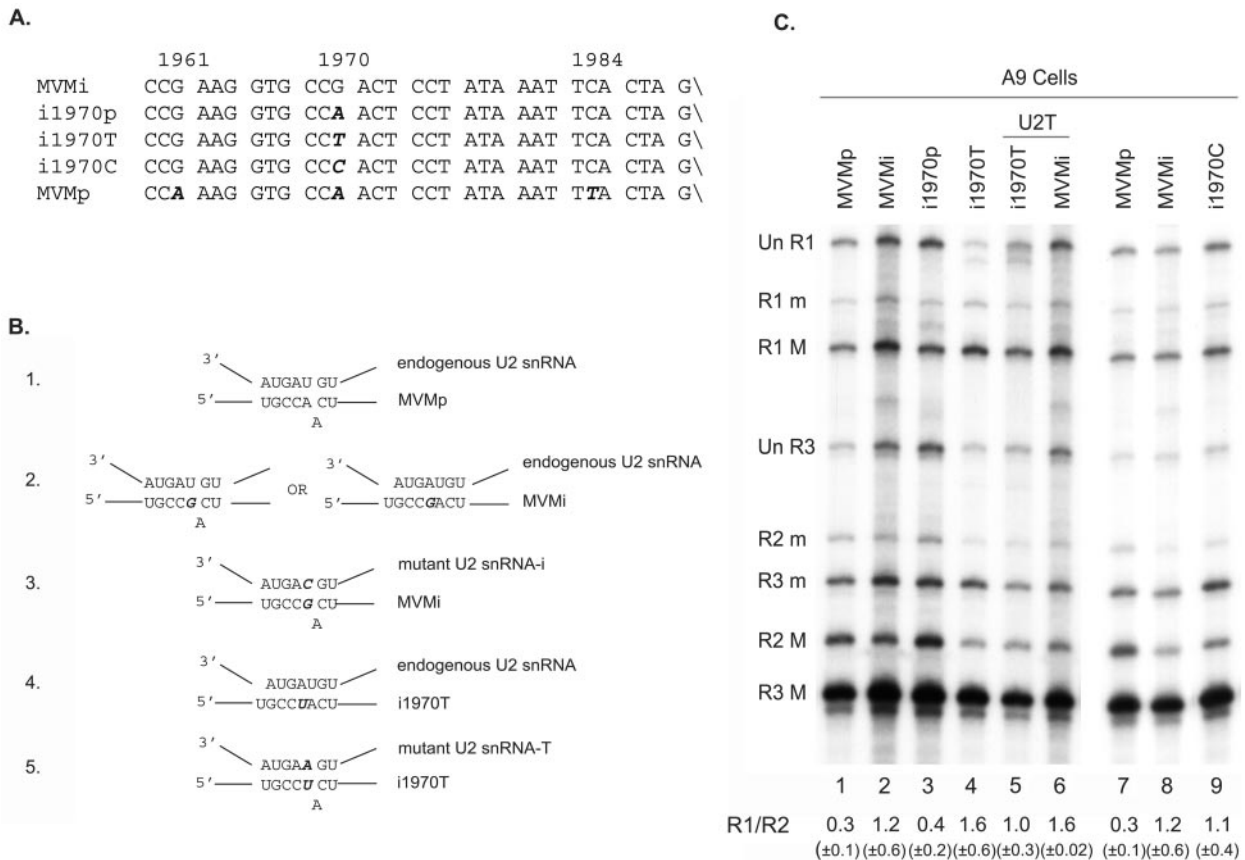


FIG. 3. The MVMi 3' splice site is inefficiently utilized by the murine fibroblast U2 snRNP. (A) Nucleotide sequences of the changes made (denoted by bold, italic letters) in i1970p, i1970T, and i1970C. (B) Potential duplex structures between the 5' end of the endogenous U2 snRNA and the putative large-intron branch point for MVMp (diagram 1), MVMi (diagram 2), and i1970T (diagram 4), between the 5' end of mutant U2 snRNA-T (as described in the text) and i1970T (diagram 5), and between the 5' end of mutant U2 snRNA-i and MVMi (diagram 3). (C) RNase protection assay performed, as described in legend for Fig. 2, with RNA present 48 h following transfection of A9 cells with MVM infectious clones indicated and including cotransfection of mutant U2 snRNA in the samples assayed in lanes 5 and 6. The identities of the RNA species are described in the legend for Fig. 2. A representative experiment is shown, below which are quantifications with standard deviations of 25 experiments for MVMp, 25 experiments for MVMi, 16 experiments for i1970p, 16 experiments for i1970T, 7 experiments for i1970C, 8 experiments for i1970T with mutant U2 snRNA-T, and 3 experiments for MVMi with mutant U2 snRNA-i.

tions in NS2 that increased the affinity of NS2 with Crm1. We reasoned that if the failure of MVMi to generate ssDNA following transfection of A9 cells is due to loss of a required NS2-Crm1 interaction, then perhaps an NS2 mutation which increases NS2-Crm1 interaction above wild-type levels [K96E/L103P (14)] can rescue ssDNA replication in fibroblasts in the MVMi background, in spite of the reduced levels of NS2 in these cells. Introduction of this mutation into the infectious clone of MVMi restores its ability to replicate ssDNA following transfection of A9 cells (Fig. 4A, lane 4), even though low MVMi-like levels of NS2 are present (Fig. 4B, lane 4). This suggested that the NS2-Crm1 interaction is essential for progeny ssDNA production in these cells.

MVMi generated the same high levels of NS2, relative to levels of NS1, in murine lymphocytes as did MVMp in murine fibroblasts. Although MVMi has a poor branch point, the large intron is removed efficiently following infection of EL4 cells, and as much R2 and NS2 was produced in these cells as was seen with an MVMp infection of A9 cells (Fig. 5B, lanes 1 and 3, compare to Fig. 2). i1970p virus bearing the MVMp-like

branch point generated levels of R2 and NS2 similar to those of MVMi following infection of EL4 cells (Fig. 5B, lanes 2 and 4) and efficiently generated all replicative DNA forms following transfection of EL4 cells (Fig. 5A, lanes 3 and 4). These results suggest that, rather than less NS2 being required for efficient replication in EL4 cells, the same high levels are required, and the MVMi 3' splice site is competent for large-intron excision in these cells. MVMp and p1970i were able to generate all replicative DNA forms in the first round of replication following transfection of EL4 cells; however, the ability of p1970i infections to spread in these cells, compared to that of MVMi, could not be accurately ascertained (data not shown).

DISCUSSION

In this study, we have shown that differences in the relative accumulated amounts of NS2 can affect the ability of MVM to generate progeny ssDNA following transfection of murine fibroblasts. MVMi replicated vigorously in murine lymphocytes

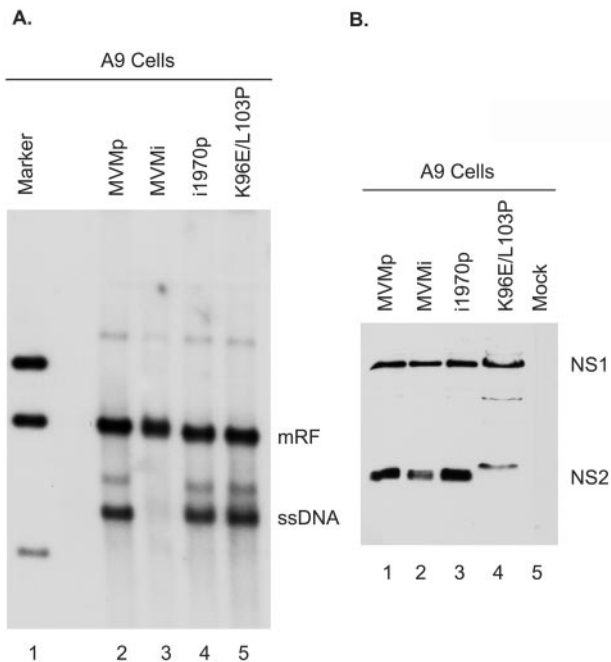


FIG. 4. The inability of MVMi to generate ssDNA in murine fibroblasts can also be overcome by an increase in the strength of the NS2-Crm1 interaction. (A) Southern analysis of cell-associated DNA forms generated 48 h following transfection of A9 cells by either wild-type MVMp or MVMi or the mutant i1970p or K96E/L103P. Marker lane contains double-stranded DNA fragments of (from bottom) 2.5, 5.0, and 7.5 kb. (B) Western analysis, using antibody reacting with the NH₂-terminal region common to both NS1 and NS2, of protein present 24 h following transfection of A9 cells with the infectious viral clones, as indicated. Why the NS2 protein generated by K96E/L103P migrates more slowly than the other NS2 proteins is not known, but this result was also given in the original report of its isolation (14).

and generated substantial amounts of NS2 in these cells; however, MVMi did not generate progeny ssDNA following transfection of murine fibroblasts, due to less-efficient splicing of the large intron from P4-generated pre-mRNAs and a consequent reduction in the accumulated levels of NS2. The differences in the final accumulated levels of NS2 generated in murine fibroblasts by the two variants are somewhat greater than the differences in the steady-state levels of the encoding R2 RNA. This may be a reflection of the differences in the relative half-lives of the nonstructural proteins, although other possibilities that might account for this difference cannot be ruled out. Changing MVMi nt 1970 in the large-intron 3' splice site from G to A, as found in MVMp, improved the interaction of MVMi pre-mRNA with U2 snRNA and thus excision of the large intron in fibroblasts enough to generate sufficient NS2 to recover production of ssDNA. Reducing the efficiency of large-intron excision and NS2 production in MVMp by the reciprocal A to G mutation in the 3' splice site led to reduced production of progeny MVMp ssDNA, demonstrating that the generation of ssDNA could be controlled by this element alone.

It is not clear why a reduced amount of NS2 resulted in inefficient progeny ssDNA production in fibroblasts. It has been known for some time that, while the production of mRF

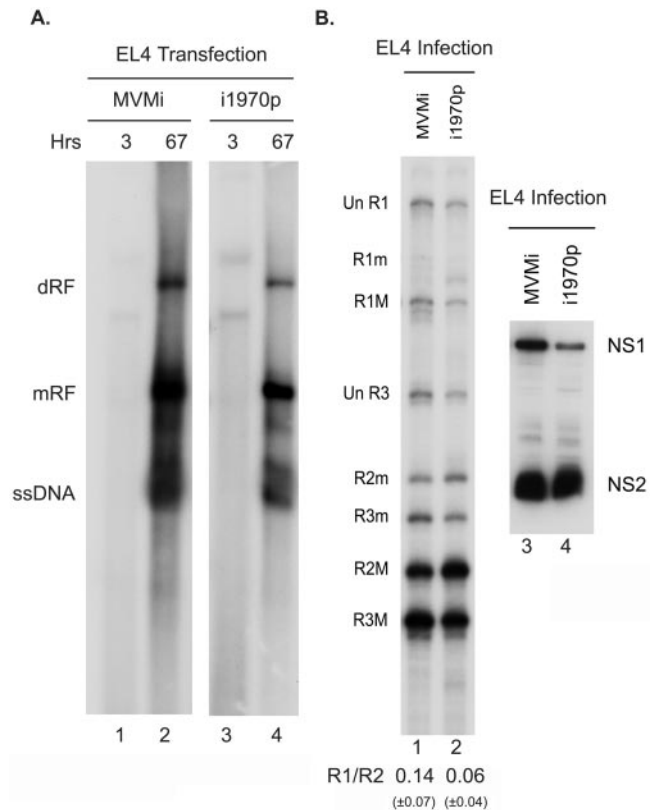


FIG. 5. MVMi generates high levels of NS2, relative to NS1, in murine lymphocytes. (A) Southern analysis of cell-associated DNA forms, generated at the hours indicated, following transfection of EL4 cells by either wild-type MVMi or i1970p. dRF, dimer replicative form. (B) RNase protection assay performed, as described in legend for Fig. 2, with RNA present 24 h following infection of EL4 cells with either MVMi or i1970p. The identities of the RNA species are described in the legend for Fig. 2. A representative experiment is shown, below which are quantifications with standard deviations of three experiments for MVMi and three experiments for i1970p. Far right: Western analysis, using antibody reacting with the NH₂-terminal region common to both NS1 and NS2, of protein present 24 h following infection of EL4 cells with either MVMi or i1970p.

DNA can proceed in the absence of assembled viral capsids, the production of progeny ssDNA in their absence is aborted (27). Furthermore, NS2 is required in murine cells for the production of properly assembled viral capsids (8). Exactly how capsids function during replication to allow production of progeny ssDNA is not fully understood, and the relationship between NS2, assembled capsids, and the production of progeny ssDNA is not clear. There is little evidence to suggest that NS2 is directly involved in the replication process, and interaction between NS2 and capsid proteins has not been reported.

However, our experiments do suggest that at least one NS2 function lacking for replication in fibroblasts for virus that produces low levels of NS2 is its interaction with Crm1. We found that the previously characterized NS2 mutation (14) which increases NS2-Crm1 interaction above wild-type levels could rescue ssDNA replication in fibroblasts in the MVMi background, in spite of the reduced levels of NS2 in these cells. NS2 mutants that generate wild-type levels of an NS2 protein that no longer interacts with Crm1 have multiple restrictions

during infection, including a block to the egress of assembled capsids from the nucleus and a deficiency in the accumulation of progeny ssDNA (9, 17). How the NS2-CRM1 interaction might affect viral replication is not known; however, it may be that this interaction somehow influences the status of assembled capsids required for ssDNA production.

The levels of NS2 apparently play a larger role in MVM tissue tropism than previously thought. Unlike MVMi, the i1970p mutant, which still has the parental MVMi coat, spread readily through a fibroblast culture. Spread following transfection was inhibited by both neuraminidase and anticapsid antibody, suggesting that transmission included an extracellular step. However, like the parent MVMi, the i1970 mutant remained extremely inefficient at initiating infection of either untreated or mock-transfected A9 cells (data not shown). The explanation for this apparent discrepancy is unclear. Perhaps the high local concentration of virus produced by an infected cell allows spread to adjacent cells, if the relative amount of NS2 is high.

Splicing of the large intron of MVM is controlled both by sequences at the 3' end of the intron and within the upstream NS2-specific exon itself. Therefore, mutations in either of these regions may have effects on the levels of NS2 and hence ssDNA production. The 5' end of the NS2-specific exon, adjacent to the large-intron 3' splice site, contains both an amino acid motif important for the NS2 interaction with Crm1 (5) and a nucleotide motif that functions as an exon splicing enhancer that plays a critical role in upstream large-intron excision (13). The six-amino-acid NS2-CRM1 interaction mutant we previously characterized, which is deficient in ssDNA production (17), shows no decrease in excision of the large intron and makes at least wild-type levels of mutant NS2 (data not shown), and so the phenotype of this mutant is related to the loss of NS2 function rather than a decrease in protein abundance. However, as might be predicted, a single nucleotide mutation in the exon splicing enhancer immediately upstream in this region that inhibits excision of the upstream MVMp large intron in fibroblasts shows, similarly to MVMi, decreased ability to make ssDNA (18).

The inefficiency of large-intron excision in MVMi is likely due to a poor interaction between the MVMi pre-mRNA branch point and U2 snRNA. Excision of the large intron from i1970T-generated pre-mRNA could be enhanced by the addition in *trans* of a mutant U2 snRNA that is predicted to more efficiently regenerate a bulged A residue in the duplex between the two. Suppression of the MVMi branch point was not successful; U2 mutations which are predicted to enhance the generation of a bulged A residue in MVMi pre-mRNA necessitated the introduction of an additional G-C base pair flanking the A residue, which may have hindered subsequent essential rearrangements in the spliceosome.

Surprisingly, the poor-consensus MVMi branch point is used efficiently in murine lymphocytes. Thus, it is likely that there are either cellular factors in lymphocytes, absent in fibroblasts, that allow efficient excision of the MVMi large intron or, alternatively, that there is repression of excision of the large intron in fibroblasts, which needs to be overcome by a stronger branch point-U2 snRNA interaction. (The levels of U2AF are similar in both cell types [E.-Y. Choi and D. Pintel, unpublished data].) That MVMi generates abundant amounts of R2

and NS2 in lymphocytes suggests that a high level of NS2 is not merely required for MVM replication in fibroblasts but is likely to be required by MVM in general. Interestingly, both H1 virus and canine parvovirus have poor-consensus 3' splice sites that are similar to MVMi rather than MVMp. Perhaps in adapting to murine fibroblasts, MVMp has acquired the nt 1970 G to A transition to allow the greater production of NS2 needed for its replication in fibroblasts.

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