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Recombinant Measles Viruses Defective for RNA Editing and V Protein Synthesis Are Viable in Cultured Cells

HENRIETTE SCHNEIDER, KARIN KAELIN,[1] and MARTIN A. BILLETER[2]

Institut für Molekularbiologie Abteilung I, Universität Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

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The measles virus (MV) phosphoprotein (P) gene encodes three proteins, P, C, and V. The V protein is synthesized by pseudo-templated transcription, also designated as RNA editing: during P gene transcription one G residue is inserted at a defined position in about 50% of the mRNAs. To study the importance of sequence elements for the nontemplated G insertion, we generated recombinant MVs in which six different mutations were introduced within the region where editing occurs (3′ UUUUUCCC, template strand). These viruses were then analyzed for their ability to edit their P mRNA and to produce V protein. Single U to C changes within the U stretch abolished editing. Extending the template by three C residues at the site of G insertion resulted in a less precise editing phenotype and overproduction of V. None of these mutants were impaired in their multiplication behavior when analyzed in cultured cells. However, the syncytia of a recombinant MV overproducing V protein were in general smaller and lysed 1 to 2 days later than usual.

INTRODUCTION

Measles virus (MV), a member of the *morbillivirus* genus of the *Paramyxoviridae* family, is an enveloped virus with a single-stranded negative (−) sense RNA genome of 15,894 nucleotides (nt). The RNA is packaged by the nucleocapsid protein (N, 60 kDa) into a helical nucleocapsid (NC). Associated with the NC are the phosphoprotein (P, 70 kDa) serving as a packaging as well as a polymerase cofactor, and the RNA polymerase (L protein, 241 kDa) (for review see Griffin and Bellini, 1996).

The P cistron of all members of the *Paramyxovirinae* subfamily is very peculiar: in addition to the P protein, it encodes in most cases two other proteins from overlapping open reading frames (ORF), accessed by diverse mechanisms. In case of MV, translation of the P mRNA starting at the 5′ proximal AUG gives rise to the P protein, 507 amino acids (aa) long (Bellini *et al.,* 1985). The poorly conserved P proteins have some common characteristics: They are phosphorylated by cellular kinases (Byrappa *et al.,* 1995; Das *et al.,* 1995) and their carboxy-terminal region binds to unassembled N proteins (Curran *et al.,* 1994; 1995; Harty and Palese, 1995; Huber *et al.,* 1991). In addition, for the Sendai virus (SeV) P protein it has been shown that its amino-terminal region is highly acidic and interacts with unassembled N molecules (Curran *et al.,* 1995). Its carboxy-terminal region binds to the L protein (Curran *et al.,* 1994; Smallwood *et al.,* 1994) as well as to NCs (Ryan and Portner, 1990; Ryan *et al.,* 1991, 1993).

Translation of an overlapping ORF initiating downstream of the P/V AUG yields the nonstructural C protein (21 kDa) (Alkhatib *et al.,* 1988; Bellini *et al.,* 1985). The function of the C protein remains unclear. A recombinant MV lacking the C protein has been generated and showed no obvious impairment in multiplication in cultured cells (Radecke and Billeter, 1996). The C protein of SeV, however, has been shown to inhibit transcription (Curran *et al.,* 1992) as well as replication (Cadd *et al.,* 1996).

The V ORF in the P cistron is accessed by RNA editing, a process characterized by the reiterated cotranscriptional insertion of nontemplated G residues at a specific run of Cs in the template by the viral polymerase (Thomas *et al.,* 1988; Cattaneo *et al.,* 1989; Vidal *et al.,* 1990a). In the case of MV, translation of the edited mRNA generates the V protein (43 kDa) whose amino-terminal 231 aa are identical to those of the P protein, but its 68 carboxy-terminal aa are unique and cysteine-rich (Cattaneo *et al.,* 1989). With the exception of human parainfluenzavirus-1 (HPIV-1) and HPIV-3 (Matsuoka *et al.,* 1991; Galinski *et al.,* 1992), all members of the *Paramyxovirinae* subfamily produce (or have the potential to produce) the V protein (Lamb and Kolakofsky, 1996). The highly conserved and cysteine rich carboxy-terminal segment of the V proteins (Thomas *et al.,* 1988) shows similarity to the zinc finger binding motifs of some DNA binding proteins (Klug and Schwabe, 1995) and binds zinc ions (Liston and Briedis, 1994; Paterson *et al.,* 1995). The V protein is phosphorylated (Curran *et al.,* 1991b; Wardrop and Briedis, 1991)

[1] Present address: Laboratoire de Génétique des Virus, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France.

[2] To whom correspondence and reprint requests should be addressed. Fax: 41 1 371 72 05. E-mail: billeter@molbio1.unizh.ch.

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and with the exception of the *rubulaviruses* (Paterson *et al.,* 1995; Samson *et al.,* 1991; Takeuchi *et al.,* 1990), the V protein has been detected only in infected cells and not within the virion itself (Curran *et al.,* 1991b; Wardrop and Briedis, 1991). The biological role of V has not been clarified yet. Recently it has been demonstrated for SeV that V binds to unassembled N molecules and thus inhibits its genome replication (Curran *et al.,* 1991a; Horikami *et al.,* 1996).

Two different P cistron expression strategies have been observed among the members of the subfamily *Paramyxovirinae* (Cattaneo, 1991). The *morbilliviruses* and the *paramyxovirus* SeV express the P and the C proteins from ORFs on the unaltered P mRNA, and the V reading frame is generated by insertion of one G residue within the transcript. (Cattaneo *et al.,* 1989; Blixenkrone-Møller *et al.,* 1992; Vidal *et al.,* 1990a). The *paramyxovirus* bovine parainfluenzavirus-3 (BPIV-3) deviates slightly from this scheme in that one to six Gs are found introduced in its P transcript at equal frequency (Pelet *et al.,* 1991). The genomes of *rubulaviruses,* on the other hand, contain short P cistrons without coding potential for a C protein. SV-5, mumps virus, and HPIV-2 and HPIV-4 encode their P protein by two overlapping ORFs (Thomas *et al.,* 1988; Paterson *et al.,* 1995; Southern *et al.,* 1990; Kondo *et al.,* 1990). Faithful transcription yields V mRNA, whereas the P mRNA is produced by insertion of 2G residues. The rubulavirus Newcastle disease virus (NDV) is exceptional in that its P expression stategy is like that of the former group of viruses. P is translated from unedited mRNA and the V reading frame becomes accessible after the insertion of one G (Steward *et al.,* 1993). The sequence at the editing sites are highly conserved and can be distinguished according to the respective editing phenotype. For the *morbilli-* and *paramyxoviruses,* the template sequence at the editing site is 3′ UUUUUCCC, except for BPIV-3 whose sequence is 3′ UUUUUCCCC. Excluding NDV, the editing template of *rubulaviruses* is 3′ UUCUCCCCCC (except for SV-5: UUCUCCCCGU). Based on this characteristic sequence ambiguity of the editing templates, a model for the specific cotranscriptional insertion of Gs has been proposed by Vidal *et al.* (1990b). The viral polymerase pauses at the run of Cs in the template allowing for separation of the newly synthesized transcript from the template. According to the model, a shift by either 1 or 2 nt during the subsequent realignment of the mRNA with the template is determined by the upstream sequence which differs between the two virus groups. In case of the *morbilli-* and *paramyxoviruses,* slippage upstream by one nt creates a permissible G:U base pair (bp) and hence these viruses insert preferentially a single G residue. In contrast, slippage by 1 nt in case of the *rubulaviruses,* would demand an unlikely A:C bp, and slippage by 2 nt creates permissible G:U bp. These viruses, therefore, preferably insert 2Gs. This model has been tested, but could not be confirmed for SeV using synthetic minigenomes (Jacques *et al.,* 1994).

In order to test the editing model in the authentic viral context and to evaluate the function of the V protein we constructed recombinant MVs (rMVs) into which mutations were introduced within the conserved editing region. The editing phenotype of these mutants was analyzed directly on P mRNAs isolated from infected cells, and thus, the influence of mutations on RNA editing was for the first time studied under natural conditions. It was found that the short conserved site is a major cis-acting determinant of editing. However, it remains unclear how the precise insertion of either 1 or 2 G residues is controlled. Furthermore, the consequence of the altered editing phenotypes on V protein expression were analyzed.

MATERIALS AND METHODS

Plasmid constructions

Plasmids carrying mutations within the editing regions were constructed as follows (for an overview see Fig. 1). The nucleotide numbering is according to EMBL Accession No. Z66517; (−) and (+) refers to genome or antigenome, respectively. Mutations are marked in bold. For construct #3, the *Hin*dIII (MV 2419)–*Bam*HI (MV 2943) fragment of pePMF-2 (Bluescript vector containing the MV*Sac*II (MV 2043)–*Apa*I (MV 5059) fragment; Spielhofer *et al.,* in preparation) was replaced by that of pmfPM13 carrying already three additional C residues in the editing template. The same fragment was exchanged with that of peV1 which contains an extra C for construct #4. The *Sac*II (MV 2043)–*Nar*I (MV 4923) fragment of these pePMF-2-derivatives was then transferred into peMV(−) (Ballart *et al.,* 1990). Mutations contained within constructs #1, #2, and #5 were introduced by PCR on pePMF-2 using the external primers #66 (5′-TCCAGAGGCAACAACTTTCC-3′, MV(+) 2398–2417) and #67 (5′-ATTTCGACATCTGCAGTGGG-3′, MV(−) 2975–2956) and two internal mutation primers each. For construct #1, the primer pair #68 (5′-TCTGTGCCCTCTTTAATGGG-3′, MV(−) 2504–2485) and #69 (5′-CCCATTAAAGAGGGCACAGA-3′, MV(+) 2485–2504), for construct #2, #72 (5′-CTGTGCCCTTCTTAATGGGT-3′, MV(−) 2503–2484) and #73 (5′-ACCCATTAAGAAGGGCACAG-3′, MV(+) 2484–2503), and for construct #5, primers #70 (5′ TCTGTGCCCCTCTTTAATGG 3′, MV(−) 2504–2486) and #71 (5′ CCATTAAAGAGGGGCACAGA 3′, MV(+) 2486–2504) were used. A C residue has been erroneously exchanged by T at position 2625 [MV(−)] by the Taq-polymerase during PCR amplification with primer pair #70 and #71. This transition leaves the P ORF intact yet introduces a stop codon in the V reading frame which is eliminating the carboxy-terminal 25 aa of V. This mutated fragment served for the synthesis of construct #6. The PCR fragments were subcloned into pePMF-2 by cleaving with *Hin*dIII (MV 2419) and *Bam*HI (MV 2943). The *Sac*II (MV

2043)–*Nar*I (MV 4923) fragment of peMV(−) was subsequently replaced by the corresponding mutagenized fragments of pePMF-2. The *Sac*II (MV 2043)–*Pac*I (MV 7242) fragment of p(+)MV (Radecke *et al.,* 1995) was then exchanged with that of the peMV(−) derivatives #1 or #2. For constructs #4, #5, and #6 the corresponding fragment of p(+)MVΔ*Sal*I-1 (see below) and for construct #3 the one of p(+)MVΔ*Sal*I-3 (see below) was used for the exchange. Plasmid p(+)MVΔ*Sal*I was generated by cleaving p(+)MV with *Sal*I (MV 3364, 6271), followed by religation of the plasmid backbone. Deletion of 1 or 3 nucleotides within the intercistronic region of the hemagglutinin (H) and L protein genes of p(+)MVΔ*Sal*I resulted in the production of p(+)MVΔ*Sal*I-1 or -3. This was done by PCR using two primers carrying either a 1 or 3 nucleotide deletion when compared to the genomic sequence. PCR was carried out on p(+)MV using either primer #308 (5′-CATACCCACTAGTTGAAATAGACATCAG-3′, MV(+) 9168–9196) or primer #309 (5′-CATACCCACTAGTAAATAGACATCAGAA-3′, MV(+) 9168–9198) together with primer #310 (5′-GGAGCCTAGGCCAAGCCGTGAG-3′, MV(−) 9650–9629). The generated PCR fragments were then digested with *Spe*I and *Eco*RI and replaced this segment of p(+)MVΔ*Sal*I.

Cells

Cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) for Vero (African green monkey kidney) and HeLa (human cervix epitheloid carcinoma), with 10% FCS for 293 (human embryonic kidney) and with 10% FCS and 1.2 mg/ml G418 for the stably transfected 293-3-46 cells.

Rescue of recombinant MVs

Rescues of MV tag-Edmonston B and mutant MV using the 293-3-46 cell line were carried out as in Radecke *et al.* (1995). Briefly, 293-3-46 cells were seeded into a 9-cm^2 well to reach ~50–70% confluency when being transfected. Five micrograms of each p(+)MV plasmid containing the mutations within the editing region together with 100 ng of pEMC-La were transfected. Three days after transfection cells were transferred to a 75-cm^2 dish; 1 to 2 days later syncytia developed. Cells were then scraped into the medium and subjected once to freezing and thawing. After clearance of the supernatants from cell debris, the crude virus stocks were kept at −80°.

The rescue of MV using the MVA-T7 based system was achieved as described in Schneider *et al.* (1996). In brief, HeLa cells seeded in 9-cm^2 wells were infected at ~50% confluency with MVA-T7 at a multiplicity of infection of 3–5. The cells were transfected 45 min later with pEMC-Na (5 μg), pEMC-Pa (2.5 μg), pEMC-L (2.5 μg) together with the different p(+)MV derivates (5 μg). Three days after transfection, the cells were scraped off the plate and the cleared supernatants were added for 2 hr onto HeLa, Vero, or 293 cells. Crude virus stocks were prepared when syncytia were visible, usually after 2 days.

Plaque purification and titration of rMV

In order to plaque purify recombinant MV strains #1, #2, #3 and the MV tag-Edmonston B strain, Vero cells were inoculated for 2 hr with appropriate diluted crude virus stocks obtained from the rescue experiments and overlaid with 2 ml DMEM containing 5% FCS and 1% SeaPlaque agarose. When syncytia were visible, single ones were transferred to a 9-cm^2 Vero cell culture each and later expanded to one or more 175-cm^2 dishes. Viral stocks were then prepared by scraping the cells into the medium when syncytia formation was pronounced. Subsequently, one round of freezing and thawing was followed by clearance of the supernatant. Virus titrations were performed on 9-cm^2 Vero cell cultures. Four to five days after agarose-overlay, cells were fixed with 1 ml 10% trichloroacetic acid for 1 hr followed by 30 min of UV treatment. The agarose was removed and the cells stained with crystal violet in 4% ethanol.

Primer extension analysis of P cistron transcripts

For analysis of the P cistron mRNAs, either Vero cells were infected with the plaque-purified viral stocks of rMV #1, #2, and #3, or in case of rMV #4, #5, and #6, 293-3-46 cells were used (note that in these cases rMV stocks were directly prepared from infected cells, since 293 cells and its derivatives are not suitable for plaque purification). Total RNA was isolated according to Chomczynski and Sacchi (1987). Subsequently, poly(A)+ RNA was prepared from 250 μg of total RNA using Oligotex resin (Qiagen). One-fourth of the mRNA was hybridized with 0.5 pmol of the 5′ end-labeled primer #90 (5′-CTAATCTCGCGTCTGTG-3′, MV(−) 2515–2499) by heating to 95° for 2 min and then cooling on ice. Primer extension was carried out with 200 U of mouse mammary tumor virus reverse transcriptase [GIBCO BRL] in a buffer containing 20 m*M* Tris–HCl, pH 8.4, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, and 0.1 mg/ml bovine serum albumin and in the presence of 0.5 m*M* dCTP, 0.5 m*M* dTTP, and 0.32 m*M* ddATP. As control, 1 μg of RNAs synthesized *in vitro* from P and V expression vectors (pAeV, pAeP, Huber, 1993) were used as templates. The 20-μl reactions were kept at 20° for 10 min, incubated at 42° for 60 min, and finally heated at 95° for 5 min. One-sixth of the reaction mix was resolved on a 12% sequencing gel. Quantification was performed using the Molecular Dynamics PhosphorImager and ImageQuant 3.3 software.

Western blots

To analyze the synthesis of V protein, 9-cm^2 monolayers of 293 cells were infected with the rMV #1, #2, or

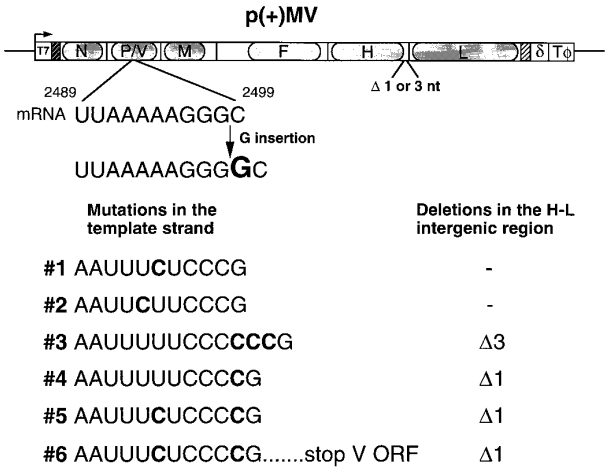

FIG. 1 Mutations introduced into the plasmid specifying the full-length antigenomic MV RNA [p(+)MV (Radecke *et al.,* 1995)]. The MV full-length cDNA was cloned between the T7 promoter (T7) and the genomic hepatitis delta virus ribozyme (δ) followed by the T7 RNA polymerase terminator (TΦ). MV 3′ noncoding terminal region (NCT; dark hatched box) is directly adjacent to the T7 promoter, the 5′ NCT (light hatched box) adjacent to the ribozyme. Below the cDNA the relevant portion of the P transcript is shown. Note that the precise site of G insertion (either after the 3 templated Gs, as indicated, or after the A stretch) is not known. The bottom of the figure shows the mutations introduced in the genomes template strand. Alterations are as indicated in bold. The nucleotide numbering is according to EMBL Accession No. Z66517.

#3; 293-3-46 cells were infected with the rMV #4, #5, or #6. When syncytia formation was pronounced, the cells were washed with PBS (137 m*M* NaCl, 2.7 m*M* KCl, 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4) and lysed by addition of 300 μl lysis buffer (50 m*M* Tris–HCl, pH 8, 62.5 m*M* EDTA, 1% NP-40, 0.4% deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin). About 1/30 of the total proteins were separarted on 10% SDS–PAGE and transferred onto Immobilon-P membranes (Millipore). The filters were incubated with a 5000-fold dilution of the primary antibodies (rabbit polyclonal anti-P #171, anti-N antisera #179 (kindly provided by Dr. C. Oervell) or anti-peptide-H) in TBST (10 m*M* Tris–HCl, pH 8, 150 m*M* NaCl, 0.05% Tween 20). The antiserum against the amino-terminal region of H (Buchholz *et al.,* 1996) was kindly provided by R. Cattaneo. Bound antibodies were detected with swine anti-rabbit antibody coupled to horseradish peroxidase (DAKO) followed by enhanced chemiluminescence (ECL Amersham Life Science). For final quantification, the blots were scanned using the Molecular Dynamics Personal Densitometer and the Image QuaNT 4.1 software.

RESULTS

Rescue of recombinant MV containing mutations within the editing region

To test whether the sequence at the editing site determines the mode of editing and to analyze whether the MV editing phenotype can be converted to that of other members of the *Paramyxovirinae* subfamily by exchanging the editing sequence of MV with that of BPIV-3 (mutant #4) or the *rubulaviruses* (mutant #1, #5 or #6), six recombinant MVs carrying mutations in the editing site were reconstituted in cell culture. Two different reverse genetics systems were established for the rescue of recombinant MV. In both systems, a transcript corresponding to the entire MV antigenomic RNA is generated by T7 RNA polymerase. This RNA is then encapsidated, transcribed, and replicated by coexpressed viral N, P, and L proteins. Either a helper cell line constitutively expressing T7 RNA polymerase and the viral N and P proteins was transfected with the plasmid specifying the MV antigenomic RNA (p(+)MV) and a plasmid encoding for the L protein (pEMC-La) (Radecke *et al.,* 1995). Alternatively, HeLa cells were transfected with p(+)MV together with the plasmids encoding the viral N, P, and L proteins (pEMC-Na, pEMC-Pa, pEMC-L). Generation of antigenomic MV RNA and expression of N, P, and L proteins was accomplished by the recombinant vaccinia virus MVA-T7 (Schneider *et al.,* 1996). The different mutations introduced in the editing region of p(+)MV are shown in Fig. 1. Insertions of nucleotides within the editing region were compensated by deletion of 1 nt at position 9181 (p(+)MV-1 for #4, #5, #6) or 3 nt at positions

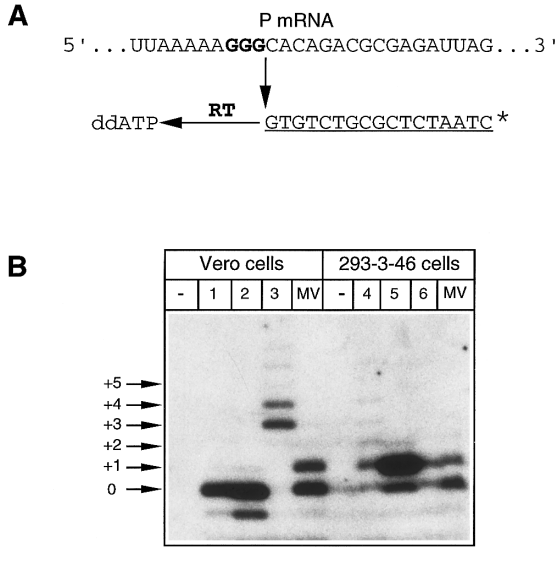

FIG. 2. Limited primer extension analysis of P cistron transcripts of rMVs. The principle of the method is depicted in A. The site of nt insertion in the mRNA is depicted with 3 bold Gs (top lane), and the [γ-^{32}P]dATP-labeled oligodeoxynucleotide hybridizing to the message just downstream of the insertion site is depicted with an asterisk. Primer extension was performed using dCTP and dTTP, and ddATP. Analysis of extension products by 12% PAGE is shown in B. The primer was extended on the mRNAs of each rMV #1 to #6 (lane 1 to 6). MV refers to RNA of cells infected with the rMV tag-Edmonston B strain and (–) to RNA of uninfected cells. The number of residues present at the editing site in addition to the 3Gs of the wild-type message are indicated with arrows. *In vitro*-synthesized P and V RNAs served as controls for the extension analysis (not shown).

9181–9183 (p(+)MV-3 for #3) within the H-L intercistronic region. This was to insure that the MV antigenomic RNA follows the rule of six, a requirement for efficient replication of SeV and MV viral RNAs (Calain and Roux, 1993; Kaelin, 1995). Generation of most rMVs can be performed by using both the helper-cell line system and the MVA-T7 based expression system. However, mutants #4, #5, and #6 were rescued only using the helper cell line system. If the editing phenotypes were +1G for all of these mutants, rMV #1, #2, and #3 would produce P and V proteins, whereas mutants #4 and #5 (and #6) would only produce V protein: These mutants already carry a 1G insertion in the genome and thus produce V mRNA and would generate P mRNA only by insertion of 2Gs. Moreover, if the editing model is correct, mutants #1 and #5 (and #6) would edit by insertion of 2Gs and thus mutant #1 should produce P protein, but no V protein. Conversely, mutant #5 (and #6) should produce V (in case of #6 a truncated V protein) as well as P proteins. The fact that generation and passage of rMV #4 and #5 (and #6) was always dependent on the 293-3-46 cell line constitutively expressing P protein suggested that the predictions of the model were not realized.

Analysis of editing of P cistron transcripts

Transcripts of the P cistrons of the mutant rMVs were directly analyzed by the limited primer extension method (Fig. 2; Pelet *et al.,* 1991). For this, mRNA isolated from Vero cells infected with rMV #1, #2, or #3 or from 293-3-46 cells producing rMV #4, #5, or #6 was used, and the editing phenotypes were compared to that of MV tag-Edmonston B strain (Fig. 2). No edited mRNA species carrying an insertion of one G residue was found for the rMV #1 and #2. The genomes of these viruses contain a single U to C exchange at different positions (3′ UUUCUCCC, 3′ UUCUUCCC) within the U stretch upstream of the G insertion site. Only a product corresponding to unedited P mRNA (position 0) was detected. The band migrating at position -1, in case of rMV #2, was not found in further analyses. Mutant #3 (3′ UUUUUCCCCCC) which carried a 3C residue insertion on the template strand within the editing site, showed two major bands at positions +3 and +4 and a ladder of minor bands at the positions +5 to +8. About 55% of the mRNA molecules (+3 position) represent faithful, nonedited copies of the genome. The other mRNA molecules [+4 (~35%) to +8 (~2–5%)] contain insertions of 1 to 5 nucleotides, respectively. Insertions of one or four G residues (+4 or +7 position) generated mRNAs competent to code for the V protein. The ratio of +1 edited to the unedited mRNA was comparable to that of MV tag-Edmonston B (MV) (~50% uninserted mRNA, ~40% edited mRNA with one inserted G).

Mutant rMVs #4, #5, and #6 all carry 4C instead of 3C residues on the template strand. In addition, mutant rMV

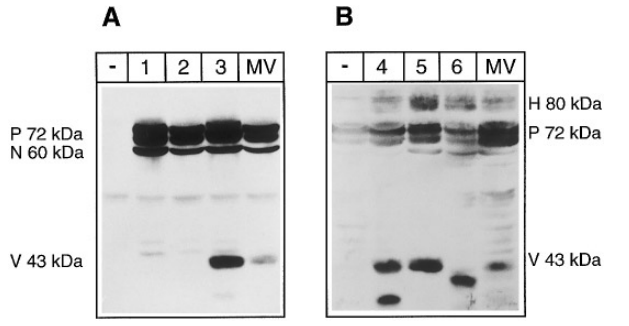

FIG. 3. Western blot analysis of proteins isolated from 293 cells infected with the rMVs #1, #2, #3 or rMV tag-Edmonston B (MV) (A), and proteins isolated from 293-3-46 cells infected with the rMVs #4, #5, #6 or rMV tag-Edmonston B (MV) (B). Cells not infected are shown in lane (−). P, V, and N proteins were detected using polyclonal anti-P and anti-N antisera in A and P, V, and H proteins were detected using polyclonal anti-P and anti-peptide-H antisera in B. The position of the P, V, N, and H proteins is indicated.

#5 and #6 have a U to C mutation within the U stretch (3′ UUUCUCCCC) and they are, according to the editing model, expected to edit by insertion of 2Gs. These mutants were grown on 293-3-46 cells, which constitutively produce P mRNA and thus give rise to a band at position 0, as shown in the uninfected 293-3-46 cells (Fig. 2). All three mutants showed the two major P (0 position) and V (+1 position) specific mRNA bands. Mutant rMV #4 (3′ UUUUUCCCC) carrying the BPIV-3 editing site was able to generate a ladder of minor bands ranging from insertions of 1 to 5 nucleotides (+2 to +6 position; 3–13%). With the exception of rMV #4, these mutants were therefore unable to edit their genomically encoded V mRNAs. Although mutant #4 generated a small amount of mRNAs with 2 inserted G residues (+3 position) which code for the P protein, the amount of P synthesized was insufficient to allow growth in cell lines not expressing P protein.

In summary, a single U to C exchange on the template strand within the U stretch (3′ UUUCUCCC (#1), 3′ UUCUUCCC (#2), 3′ UUUCUCCCC (#5, #6)) eliminated insertions entirely, suggesting that an uninterrupted U stretch upstream of the insertion site is necessary for MV RNA editing. Therefore, the editing model relying entirely on the RNA and not on interacting polymerase structure was not confirmed in these analysis. Furthermore, increasing the length of the C stretch from 3 to 4 (#4) or 6 (#3) C residues resulted in a different but less precise editing phenotype giving rise to a high proportion of multiple G insertions.

Analysis of V protein expression

To determine whether the nondefective rMVs #1, #2, and #3 produced V protein during infection, plaque purified viral stocks were used to infect 293 cells. Proteins were analyzed by Western-blotting using polyclonal anti-P antibodies recognizing the common amino-terminal

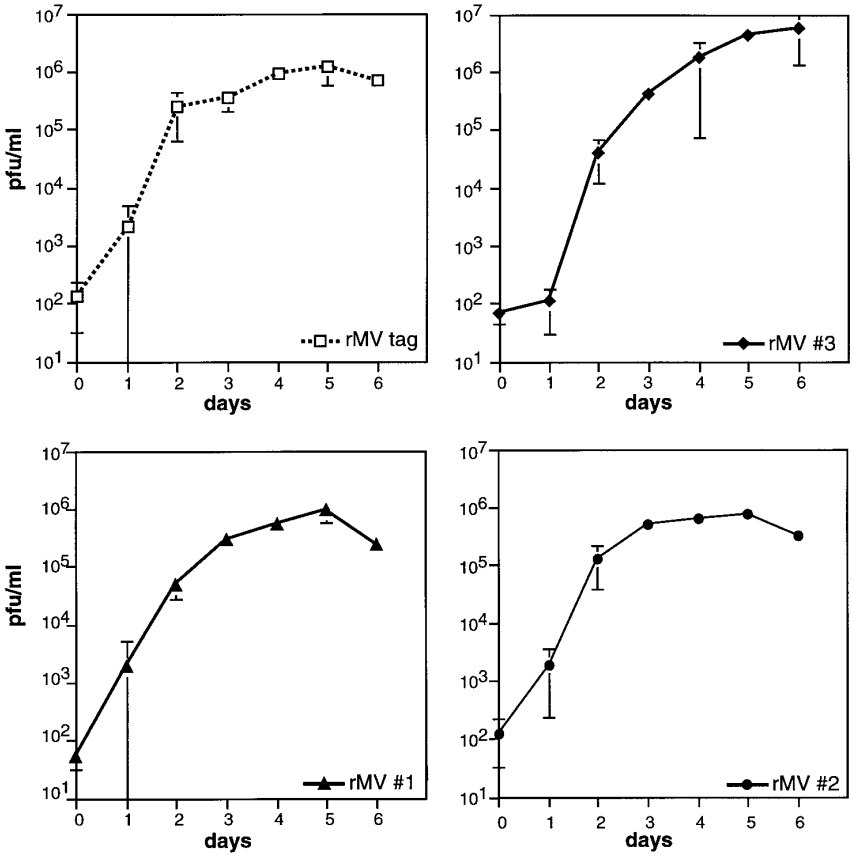

FIG. 4. Analysis of the multiplication behavior of plaque purified rMVs #1, #2, #3 and rMV tag-Edmonston B was performed on Vero cells over a period of 6 days as described under Materials and Methods.

231 aa of P and V. The two mutants #1 and #2 which both contain a single U to C nucleotide exchange, failed to produce V protein (Fig. 3A). The inability to produce V protein is a consequence of the altered editing function. In contrast, rMV #3 which carries an insertion of three C nucleotides on the template strand produced on average 4 times more V protein when compared to the MV tag-Edmonston B strain (MV) (Fig. 3A). The degree of infection of the cell cultures from which proteins were isolated was measured by using anti-N antibodies in case of rMV #1, #2, and #3 (Fig. 3A). Since rMV #4, #5, and #6 were grown on the 293-3-46 cell line expressing P and N protein the degree of viral infection was standardized with anti-H antibodies in these cases. These three rMVs contain an insertion of one C residue on the template strand and thus they derive the V protein from a nonedited transcript (Fig. 3B). Due to the stop codon introduced in the V reading frame, a truncated V protein was produced by mutant #6 (Fig. 3B). In the case of rMV #4 an additional smaller protein was detectable. This mutant inserts multiple G residues and thus accesses all three reading frames downstream of the editing site. Insertion of 1G residue into the genomically encoded V mRNA could therefore lead to the synthesis of W protein, i.e., a P protein truncated shortly after the editing site.

In general, these data show a correlation of the level of V protein with the level of the corresponding mRNAs. Only in case of rMV #3 the level of V appears higher than expected from the proportion of edited transcripts. This might be due to a slightly increased stability of the V protein containing an additional glycine residue.

Multiplication rate in cultured cells of rMVs #1, #2, and #3

In order to determine whether the absence or overproduction of V protein had any effect on the multiplication behavior of the rMVs #1, #2, and #3, we analyzed their growth rate in Vero cells over a period of 6 days. None of these viruses showed a significant difference in growth rate. The final titers were similar to those of MV tag-Edmonston B (Fig. 4). However, the syncytia produced by rMV #3 were in general smaller and lysed 1–2 days later than usual (Fig. 5).

DISCUSSION

We report the study of RNA editing phenotypes of rMVs carrying mutations within the editing region of their genome. The test systems used allow the MV polymerase to edit on nondefective templates. The transcripts were

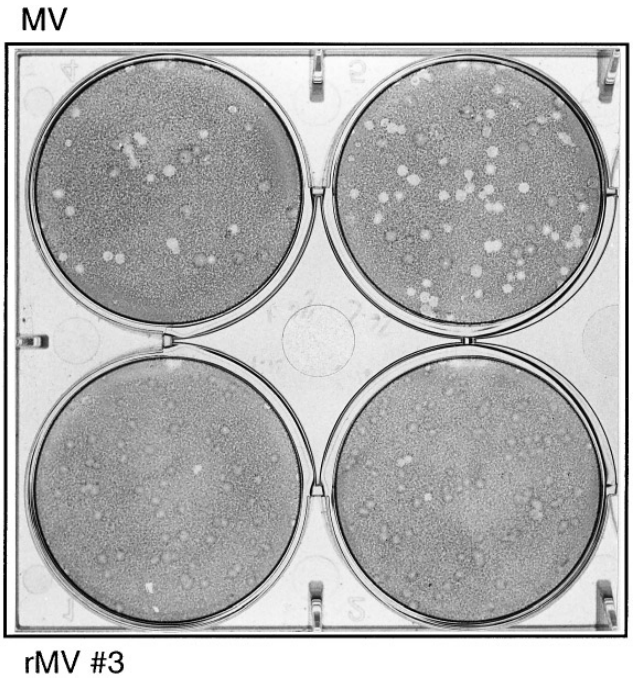

FIG. 5. Plaque morphology of rMV #3 and rMeV tag-Edmonston B (MV) on Vero cells. Staining of plaques was performed 5 days after infection.

directly analyzed, and the consequence of altered editing on synthesis of V protein was studied. Since production of rMV requires its genome to comply with the rule of six (Calain and Roux, 1993; Kaelin, 1995; Radecke *et al.,* 1995; and unpublished observations), the introduction of residues in the editing templates had to be compensated for by deletion of the corresponding number of nt in the H-L intergenic region. For the analysis of RNA editing on Sendai virus minigenomes (Jacques *et al.,* 1994), the rule of six was not taken into account since the cotranscriptional insertion of Gs was considered a process that is not affected by the replication efficiency.

Six different mutant rMVs were produced to obtain information on the sequence elements determining the nontemplated G insertion. We found that the short U rich sequence on the template strand upstream of the G insertion site is critical for editing, since U to C exchanges at two different positions (3′ UUUCUCCC, 3′ UUCUUCCC) completely abolished insertion of any G residues. Similar results were obtained from editing studies on Sendai virus minigenome templates (Jacques *et al.,* 1994). Neither of these findings agree with a stuttering model based on the minimal sequence difference between the *paramyxo-/morbillivirus*- (3′ UUUUCCC) and the *rubulavirus* (3′ UUCUCCC) groups (Vidal *et al.,* 1990b). However, it remains to be analyzed whether changing the MV editing site into the conserved site of the *rubulaviruses* (3′ UUCUCCCCCC, except for SV-5: 3′ UUCUCCCCGU) would result in insertion of 2G residues. On the other hand, a pattern close to that of BPIV-3 editing (Pelet *et al.,* 1991) was obtained by introducing its editing site (3′ UUUUUCCCC) into the MV genome. Using Sendai virus minigenomes, this phenomenon had not been observed (Jacques *et al.,* 1994). Furthermore, the length of the C stretch has been shown to be critical in both systems. However, with none of our mutants have we reproducibly detected deletions as reported by Jacques *et al.* (1994). To conclude, major determinants for editing appear to be the length of the C stretch as well as the upstream U-rich sequence.

The fact that the same alterations within the short editing site cause differing editing phenotypes in MV and defective Sendai viruses could either be ascribed to the different systems used or that additional factors are involved. In particular, sequences further upstream as well as slightly distinct intrinsic editing functions of different viral polymerases could be involved. Interestingly, it has been reported that editing in the neurotropic hamster MV strain shows a shift away from single toward multiple G insertions. The only sequence difference found is a C to U exchange 19 nucleotides upstream of the insertion site (Vanchiere *et al.,* 1995).

Two mutant rMVs did not synthesize and one overproduced V protein. The overproduction of V can be partly explained by the less precise editing mechanism that generates different mRNA species at overall higher levels encoding the V protein; additionally, the added glycine residue might increase the stability of V. None of the mutant rMVs showed an altered growth behavior in Vero cells. This suggests that absence or overproduction of V protein does not interfere with MV propagation in tissue culture. For Sendai virus, it has been shown that V interferes with viral RNA replication, probably because it binds to N and thus inhibits encapsidation (Curran *et al.,* 1991a; Horikami *et al.,* 1996). The role of the highly conserved and cysteine-rich carboxy-terminal region of the V protein is still unclear. It has been suggested that the cysteine-rich domain sharing homologies to zinc-finger motifs confers a nucleic acid binding property to V. By binding to DNA or to cellular proteins, V could conceivably be involved in delaying MV-induced apoptosis (Esolen *et al.,* 1995). The retarded lysis of syncytia and the smaller plaques of the rMV overproducing V would be consistent with such processes. However, other mechanisms to restrict virus replication (e.g., interacting with the interferon system) are equally feasible. It remains puzzling why essentially no change in phenotype was evident for the mutant rMVs producing no V protein. One possible explanation is that the fifth of seven cysteine residues is changed to an arginine in our attenuated MV Edmonston B strain, which could alter the structure and thus the function of the V protein. The effect of V-deletion or V-overproducing MV in animal models has not been tested yet. Such experiments might reveal a role of V in persistent infections, as it has been hypothesized by Gombart *et al.* (1992), or in neurovirulence (Van-

chiere *et al.,* 1995). The rMVs which we generated will help to clarify the possible involvement of V protein in these processes.

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