Rescue of Rinderpest Virus from Cloned cDNA

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Rinderpest virus is a morbillivirus and is the causative agent of a widespread and important disease of cattle. The viral genome is a single strand of RNA in the negative sense. We have constructed plasmids containing cDNA copies of the 5' and 3' termini of the virus separated by a reporter gene and have shown that antigenome-sense RNA transcripts of these model genomes can be replicated, transcribed, and packaged by helper virus, both rinderpest virus and the related measles virus. Further, these genome analogs can be replicated and transcribed by viral proteins expressed from cDNA clones by using a recombinant vaccinia virus expressing T7 RNA polymerase (MVA-T7). Using this latter system, we have rescued live rinderpest virus from a full-length cDNA copy of the genome of the RBOK vaccine strain. The recombinant virus appears to grow in tissue culture identically to the original virus.

Rinderpest is an economically highly important disease affecting cattle and wild bovids. Widespread in sub-Saharan Africa in the mid-1980s, it has now been restricted to areas of Kenya and Ethiopia by the efforts of the Pan African Rinderpest Campaign. The disease remains enzootic in parts of the Indian subcontinent, as well as in several countries in the Near and Middle East, with recent outbreaks reported in Pakistan, Afghanistan, Iran, Yemen, and Turkey. The causative agent, rinderpest virus (RPV), belongs to the morbilliviruses, a genus of the family *Paramyxoviridae*, and is thus related to measles virus (MV), canine and phocid (seal) distemper viruses, the dolphin morbillivirus, and peste des petits ruminants virus. Like all the morbilliviruses, there is only one serotype of RPV, yet enormous variation can be found in the pathogenicity of field isolates, from those causing essentially 100% mortality to others causing barely detectable clinical signs (35, 37). The molecular basis of this variation is unknown.

The most commonly used vaccine strain of RPV is the RBOK vaccine (RPV-R), which was derived from the virulent Kabete O strain (RPV-K) in the late 1950s by multiple passages in primary calf kidney cell cultures (26). The latter strain, unadapted to tissue culture, is available for comparative studies since it has been kept by limited animal-to-animal passage. As part of our investigations into virulence factors in RPV, we have cloned and sequenced the entire genome of the vaccine strain (1a–3, 5, 8, 13) and completed the sequencing of the virulent parent (4). The advantage of this pair of strains is that differences in the sequences will have arisen purely due to the tissue culture adaptation and not to geographical or temporal drift. A total of 87 differing bases (of a total of 15,882) were found between the two sequences, and we now wish to test the functional significance of these differences; this requires the synthesis of recombinant RPV containing defined genetic elements from both strains.

Although rescuing positive-strand RNA viruses from cDNA copies of their genomes has been possible for a number of years (for a recent review, see reference 6), there was no way of doing the same with negative-strand RNA viruses. The main difficulty has been that the isolated genomic RNA of such

viruses is not infectious, the minimum infectious unit being the encapsidated genome (whether segmented or nonsegmented) plus the viral RNA polymerase proteins. The problem is particularly acute for the nonsegmented negative-strand viruses, a group which includes the families *Paramyxoviridae* and the *Rhabdoviridae*, where genomes of 16 to 18 kb must be manipulated. Recent developments (excellently reviewed by Conzelmann [10]) have overcome these problems and allowed the rescue of a number of rhabdo- and paramyxoviruses, including rabies virus (31), vesicular stomatitis virus (VSV) (22, 38), Sendai virus (15), measles virus (28), and respiratory syncytial virus (RSV) (9). The basic technique has been to clone a DNA copy of the viral genome between a T7 RNA polymerase promoter and a ribozyme sequence in such a way that viral antigenomes with exact, or nearly exact, 5' and 3' termini are produced when the plasmid is transcribed by T7 polymerase. Plasmids encoding the viral nucleocapsid (N), polymerase (L), and polymerase-associated (P/NS) proteins are cotransfected with the genome plasmid. For the rescue of the first three of these viruses, the T7 polymerase was produced by the recombinant vaccinia virus vTF7-3 (14). This system, however, requires that the rescued virus be able to be separated from the vaccinia virus by physical (31) or biochemical (38) means or by repeated passage in cells or tissues that are not good hosts for the poxvirus (15). For MV, these problems were avoided by creating a cell line that expressed T7 polymerase as well as the viral N and P proteins. Rescue was achieved by transfecting the genome plasmid and the L gene plasmid into this helper cell line. The reported efficiency of this method was, however, no higher than that achieved with the vaccinia virus system and required the creation of the helper cell line. The advantages of the vaccinia virus system can be obtained without the problems of concurrent poxvirus replication by using the recently described recombinant form of the host range-restricted vaccinia virus MVA-T7 (34), which expresses the T7 RNA polymerase, but does not replicate, in mammalian cells. This was the method used in the rescue of RSV (9). We report here the construction and rescue of recombinant RPV (rRPV) entirely from cloned cDNA.

MATERIALS AND METHODS

Cells and virus. Vero cells were maintained in Dulbecco's modified Eagle's medium–10 mM HEPES (pH 7.3)–5% fetal calf serum. 293 cells were maintained in the same medium without HEPES. B95a cells were grown in RPMI 1640 containing 5% fetal calf serum. Chicken fibroblasts were grown in Eagle's

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minimal essential medium containing 5% fetal calf serum, 2% chicken serum, and 10% tryptose phosphate broth. RPV (RBOK vaccine or Saudi 81/1) stocks were grown in Vero cells. MVA-T7 was the generous gift of G. Sutter, GSF Environment and Health Research Centre, Oberschleissheim, Germany. Stocks were grown in primary chicken fibroblasts or in the chicken fibroblast line OÜ2 (the gift of J. McCauley, Institute for Animal Health, Compton, United Kingdom). Tissue culture media were either prepared in-house or purchased from Life Technologies.

Molecular biology and assays. Except where indicated, all DNA and RNA manipulation was performed by standard methods. Plasmids were cloned and grown in *E. coli* JM109 or DH5 α and purified on CsCl gradients or Qiagen columns. Catecholamine acetyltransferase (CAT) was assayed by the CAT enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim); transfected cells were lysed in 0.5% Nonidet P-40–150 mM NaCl–50 mM Tris/HCl (pH 7.5), and 5 to 100 μ l was used to measure CAT activity. RNA from RPV-infected cells was prepared with Trizol (Life Technologies), and reverse transcription PCR (RT-PCR) was performed as previously described (3). RNA transcription in vitro from plasmids was performed with the Ribomax kit (Promega) as specified by the manufacturer. Routinely, 25- μ l reaction mixtures containing 2.5 μ g of plasmid were used. The reaction mixtures were treated with RNase-free DNase, made 0.3 M with sodium acetate (pH 4.0), extracted successively with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), and precipitated with ethanol. The purified RNA was redissolved in diethylpyrocabonate-treated water. Phosphatidylethanolamine-dimethyldioctylammonium bromide transfection reagent (homemade LipofectACE [HML]) was prepared as described previously (29).

Construction of pMDB1. To produce either model antigenomes or the full-length antigenome of RPV, the viral sequences would have to be cloned between a T7 RNA polymerase promoter (to provide the exact 5' end to the transcript) and a hepatitis delta virus ribozyme sequence (to provide the exact 3' end to the transcript). A suitable vector (pMDB1) was constructed to allow this. Plasmid pBTVE3 (36) contains a *BsmI* site immediately downstream of a T7 promoter, positioned in such a way that cleavage with *BsmI* and polishing of the ends with T4 DNA polymerase allows the cloning of a blunt-ended DNA fragment exactly at the start-transcription site of the promoter. This plasmid was further modified by inserting two tandem copies of the T7 RNA polymerase terminator at the *HindIII* site at the other end of the multiple-cloning site, giving pBTVE3.2. The ribozyme sequence was amplified by PCR from an MV-based minigenome (the kind gift of M. Billeter), incorporating a *PstI* site and an *EagI* site at the 5' end and an *NsiI* site at the 3' end. After digestion with *PstI* and *NsiI*, this fragment was inserted into the *PstI* site in pBTVE3.2 to give pBTVE3.3. The sequence of the ribozyme and terminators was confirmed. Unfortunately, since the backbone of pBTVE3 contains an *EagI* site, that next to the ribozyme was not unique. The backbone of the plasmid was therefore exchanged for that of pGEM5Zf(+) (Promega) by a three-fragment ligation of the *PvuII/AsnI* fragment of pBTVE3 with the *AsnI/AlwNI* and *AlwNI/PvuII* fragments from pGEM5Zf(+). The resultant vector (pMDB1) contains, in addition to the *BsmI* and *EagI* sites, a short multiple-cloning region with unique *SacI*, *SnaBI*, *EcoRI*, *BamHI*, *XbaI*, *Sall*, and *PstI* sites. Digestion with *EagI* and polishing with T4 DNA polymerase allows the cloning of DNA exactly at the cleavage point of the ribozyme.

Construction of RPV minigenomes. Both model genome and the full-length genome cDNAs were constructed so that the RNA transcribed would be of antigenome sense, since this had given the best results in recovering infectious rabies virus (31). Cloning of the leader (5' end of the antigenome) and trailer (5' end of the genome) regions of RPV has been described previously (2). These clones contained additional non-RPV sequence as a consequence of the method used to isolate them. We therefore used PCR with the proofreading Vent polymerase (New England Biolabs) to isolate exact fragments and checked each cloned PCR product by sequencing. The last 109 bases of the antigenome were amplified by PCR and cloned into *EagI*-cut, blunt-ended pMDB1 to give pMDB4. This region contains part of the 3' untranslated region, including gene termination and polyadenylation signals, from the L gene of RPV, followed by the extragenic terminal sequence of the genome. The first 107 bases of the antigenome were amplified and cloned into *BsmI*-cut, blunt pMDB4. These 107 bases contained the extragenic terminal sequences, followed by the gene start signal and 5' untranslated region of the N gene. PCR products were made with and without two extra G residues at the beginning of the sequence, since T7 RNA polymerase transcribes RNA much more efficiently if it incorporates G residues at the start (23), and we were concerned about whether the transcription from the normal 3' end of the genome (TGGT) would be sufficient. The resultant plasmids (pMDB5 [plus GG] and pMDB6) therefore contained the start and end of the antigenome, with sufficient viral gene sequence to direct the transcription and processing of a single gene. We cloned the CAT open reading frame (ORF) between the two sections of viral sequence so that it was in the same orientation as a viral ORF (pMDB7A and pMDB8A); as controls, constructs with the CAT ORF in the reverse orientation were also isolated (pMDB7B and pMDB8B). The predicted transcripts from pMDB8A and pMDB8B would be 900 bases, an exact multiple of 6, which appeared to be necessary for MV-based replicons (33). Those from pMDB7A and pMDB7B would have an extra 2 bases, but earlier work on VSV-based replicons suggested that minor 5' extensions would be tolerated and eliminated (24).

Construction of the full-length cDNA copy of the RPV genome. The full-length genome of RPV was assembled from previously existing clones in a 10-step procedure. During this process, overlap PCR mutagenesis (17) was used to insert two new restriction enzyme sites into nonconserved, untranslated regions of the genome (see below). These acted as genetic tags to identify recombinant virus.

The first 201 bases of the antigenome were amplified by overlap PCR, introducing a unique *Clal* site immediately 5' to the initial ATG of the N protein ORF (⁹⁹TTCTTTAAAATGG¹¹¹ to ⁹⁹TTCATCGATATGG¹¹¹). This fragment was cloned into the *BsmI* site of pMDB1, and the sequence was checked. This plasmid was digested with *EcoRI* and *SapI* (partial) and ligated to the *SapI/SapI* fragment from the N gene and the *SapI/EcoRI* fragment containing the rest of the N gene, the N/P intergenic region, and the whole P gene taken from clone P14 (5). As this clone was from a V protein-encoding mRNA, the editing region of the P gene was swapped as a *KpnI/ApaI* fragment with the corresponding region of a P protein-encoding clone (but one which lacked the N/P intergenic region), giving pMDBNP. We had previously isolated a clone containing most of the M gene and the whole of the F gene [MF(L)] (3, 13); the M gene was completed with a PCR fragment which extended into the P gene (3); the PCR product was cloned into pGEM5Zf, and the sequence was checked. The required part was then cloned into MF(L2), giving pKSMF. The M and F genes were then added to pMDBNP by using the *SphI* site that lies almost at the end of the P gene, giving pMDBN2F. The other end of the genome was amplified, again by overlap PCR, and a *Sall* site was inserted 70 bases from the end (¹⁵⁸⁰³CTGGTCAATAAAA¹⁵⁸¹⁵ to ¹⁵⁸⁰³CTGGTGCACAAA¹⁵⁸¹⁵). This fragment was cloned into the *EagI* site of pMDB1 (pMDBT), and the sequence was checked. As the *Sall* site was needed to transfer the L gene clone from pGEM (where it had been assembled), the PCR product was also cloned into pGEM5Zf, the sequence was checked, and the genomic terminal sequences were inserted correctly downstream of the L gene in another three-fragment ligation. Finally, we ligated the *HpaI/SalI* fragment containing most of the L gene, the small *SalI/NarI* fragment from pMDBT containing the end of the genome and part of the ribozyme, the *NarI/SunI* fragment containing almost all of pMDBN2F, and the *SunI/HpaI* fragment from L9, a previously isolated genomic clone that ran from the F gene through the H gene and into the L gene (2). The resultant plasmid (pMDBRPV) was checked to ensure that all restriction sites used in the cloning were intact.

Replication of model genomes with helper virus. 293 cells in six-well plates were infected with RPV (Saudi 81/1) at a multiplicity of infection (MOI) of approximately 0.1 for 3 h before transfection. The cells were then washed once with Optimum I (Life Technologies) and transfected with 6 μ g of RNA per well, using 10 μ l of Lipofectin (Life Technologies) in a final volume of 1 ml. The transfection was allowed to proceed for 5 h or overnight; the cells were then washed once, incubated in normal medium, and harvested on day 2 or 3 postinfection. The cells were lysed, and CAT was assayed as indicated above. Where indicated, medium from transfected and infected cells was harvested and used to reinfect 293 cells or Vero cells for 2 h. The inoculum was removed, and the cells were cultured for a further 3 days before the cell lysate was harvested for the CAT assay.

Replication of model genomes and rescue of the full-length genome from plasmids with MVA-T7. 293 cells were washed with Optimum I and then infected with MVA-T7 (MOI, 0.1 to 0.4) in the same medium for 30 to 40 min. HML (12.5 μ l/10 cm²) was diluted in Optimum I (625 μ l per 10 cm²), allowed to stand at room temperature for 30 min, and mixed with an equal volume of Optimum I containing the plasmids. The plasmids used were pKS-N1 (the N protein ORF in pBluescriptKS+) (2), pGEM-L (the L protein ORF plus viral trailer in pGEM5Zf+) (2), and pKS-P (the P protein ORF in pBluescriptKS+) (5). The virus inoculum was removed from the cells and replaced with the transfecting mixture. The cells were transfected for 5 to 6 h, and an equal volume of normal medium containing 10% serum was added. Cell lysates were harvested for the CAT assay after a further 18 to 20 h; for virus rescue, the cells were incubated for 3 days and then frozen and thawed once, cell debris was removed by centrifugation (10 min at 1,280 $\times$ g), and 0.5 ml of the supernatant was used to infect Vero or B95a cells.

Virus growth. Vero cells (5 $\times$ 10⁵ per 35-mm well) were infected with rRPV or normal RPV-R at an MOI of 0.1 for 2 h. The inoculum was removed and the cells washed twice with medium and 1.5 ml of medium added to each well. Samples were taken immediately and at various times thereafter and stored at -70°C. After about 60 h, cell layers were harvested in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for Western blot assays. The 50% tissue culture infective dose (TCID₅₀) of released virus was quantitated by standard methods.

SDS-PAGE and Western blot analysis. Samples of infected or uninfected Vero cells were analyzed by SDS-PAGE (21) and transferred to Hybond-C (Amersham, Little Chalfont, United Kingdom). Filters were blocked with 3% (wt/vol) dried milk and 2% (vol/vol) Tween 20 in 20 mM Tris (pH 7.5)–150 mM NaCl (TBS), washed with 0.1% Tween 20, and incubated with antibody overnight at 4°C. After being washed, the filters were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Sigma) for 1 h at room temperature, washed again, and developed with 3,3'-diaminobenzidine-NiCl₂ (16). The anti-sera used were a serum from a rabbit hyperimmunized with RPV (RHIS), an anti-MV V protein antiserum (the kind gift of D. Bredis) and a specific anti-RPV P protein antiserum raised against a bacterially expressed glutathione-S-

FIG. 1. Replication and transcription of RPV- and MV-based minigenomes by helper virus. 293 cells were infected with RPV or MV or left uninfected; they were then transfected with in vitro-transcribed RNA or plasmid as shown. The CAT activity was determined by ELISA.

transferase GST fusion protein containing the carboxy-terminal half of the P protein (1).

RESULTS

Transcription and rescue of model genomes by helper virus.

The CAT ORF in the minigenomes allowed us to examine the function of our constructs in a quantitative manner, which was important given the low efficiency of rescue reported for most members of the *Mononegavirales* (9, 15, 22, 28, 31) and therefore the need to optimize the system as much as possible. The nature of the construct is such that only viral polymerase transcripts of the pseudogene should give translatable CAT mRNA, since the in vitro-synthesized T7 polymerase transcripts (i) have no 5' cap and (ii) have an upstream, out-of-frame ATG codon, albeit in a poor context (³¹TCAATGA³⁷). Transcription of the CAT pseudogene requires the copying of the antigenome RNA into an encapsidated genome which can act as a template for the viral polymerase. The production of CAT therefore reflects both replication and transcription of the model genome.

Examination of the in vitro transcripts from pMDB1-based constructs by formaldehyde gel showed that about 60% of the RNAs terminated at the ribozyme cleavage point, most of the rest terminating at one of the two T7 terminator sequences (data not shown). Primer extension analysis showed that the 5' ends of the transcripts began at the predicted position. When RNA from RPV-based model genomes was transfected into RPV-infected 293 cells, CAT was produced in a virus-dependent manner (Fig. 1); no CAT was produced when the ORF was in the reverse orientation, showing that, as expected, mRNA was not transcribed from the antigenome (Fig. 1). MV could also function as a helper virus for the RPV-based model genome, although to a lesser extent; this seems to be a consequence of a difference in the types of strains of RPV and MV used rather than a major difference in the abilities of the two viruses to replicate and transcribe the RPV-based model genomes (see below). We also included in these experiments an MV-based minigenome plasmid pMVCAT (33) (the generous gift of M. Billeter). It is notable that although the MV-based minigenome was transcribed by RPV proteins, it consistently gave a much lower yield of CAT than did the RPV-based equivalents. This was also true even when MV was used as the helper virus, to the extent that CAT was not detectable by

ELISA in our hands from a combination of MV and pMVCAT RNA (Fig. 1). The most probable explanation for this observation is that the MV-CAT construct produces genome-sense transcripts. Plasmids which give rise to genome-sense as opposed to antigenome-sense transcripts appear to be ineffective or very inefficient in initiating viral rescue (18, 31), possibly due to hybridization between the primary transcripts and the mRNAs transcribed from them (31) and/or to the occurrence of sequences resembling the T7 RNA polymerase termination sequence in the viral genome (28, 38). The T7 polymerase transcripts from pMVCAT appeared to be full length by formaldehyde gel, but the results obtained with the two model genomes underline the importance of using antigenome-sense primary transcripts. No CAT was produced if untranscribed plasmids were transfected into RPV-infected cells (data not shown), and similar results were obtained from pMDB7A (two extra G nucleotides at the 5' end of the transcript) and pMDB8A (normal antigenome start) (data not shown), suggesting that a small 5' extension was tolerated by the viral packaging and replication system.

To demonstrate that the resultant pseudogenomes were also

FIG. 2. Packaging of minigenomes by helper virus. (a) 293 cells were infected with RPV or MV or left uninfected; they were then transfected with in vitro-transcribed RNA as shown. After 2 days, the cells were lysed and the CAT activity was assayed (P0). The medium from these cells was used to infect 293 cells (b) or Vero cells (c). After a further 3 days, the infected cells were lysed and the CAT activity was assayed (P1).

FIG. 3. Replication and transcription of RPV-based minigenomes without helper virus. 293 cells were infected with MVA-T7 as described in Materials and Methods and then transfected with the indicated amounts of various plasmids. After overnight incubation, the cells were lysed and the amount of CAT was assayed. (These experiments were performed in 11-mm wells, but amounts of plasmids and yield have been calculated per 35-mm well to be comparable with previous figures.)

packaged and enveloped by viral proteins, the medium from infected or transfected cells was harvested and used to infect either further 293 cells or Vero cells. As shown in Fig. 2, cells infected with the cell-free supernatants from CAT-expressing cells also expressed CAT. In addition, the expression levels were greatly increased when the cell line used was more permissive for viral growth, as is the case for Vero cells (Fig. 2c: note the difference in the x axes). The latter cell line showed extensive cytopathic effect (CPE), especially in the case of the MV-infected cells, while much less CPE was apparent in the 293 cells. Hence, the poor initial expression of CAT from RPV- or MV-based genomes seen when using MV may be due to our use of a highly attenuated form of the Edmonston strain that simply grows more poorly in 293 cells than does the virulent Saudi strain of RPV. Although 293 cells are not ideal hosts for RPV, we continued to use them because of the much greater efficiency of transfection we have been able to achieve, which was important when we came to rescue the full-length genome.

Transcription of model genomes without helper virus. To rescue rRPV, it was necessary to provide the viral N, P, and L proteins from cDNA clones. These plasmids would be transfected into cells along with the plasmid containing the cDNA copy of the RPV genome, and appropriate transcripts would be made by the T7 polymerase produced by MVA-T7. Experiments were carried out to investigate the optimal amounts of N, P, and L plasmids to be used. Since satisfactory amounts of transcription were obtained from the model genomes with the natural viral RNA sequence start, and since we wished to work with as near to the native sequence as possible, we built the full-length genome without the extra G nucleotides present in pMDB7A and used pMDB8A in the optimization studies. As shown in Fig. 3, not only was high-level expression of CAT dependent on the presence of N, P, and L proteins, but also the levels of expression were much higher than those achieved with helper virus, presumably because there were no native RPV genomes to compete for the viral proteins. As expected, the optimal conditions were found with a high ratio (about 20:1 in molar terms) of N or P to L. Higher ratios than those shown in Fig. 3 were tried in other experiments without improving the yield of CAT. Ratios of N to P greater or less than 1 did not improve the expression of CAT at the optimal level of L plasmid, although at higher levels of L, a ratio less than 1 was

better (data not shown). Increasing the amount of pMDB8A had very little effect, although a further doubling of expression could be achieved by increasing the amount of MVA-T7 beyond that used here. A small amount of background expression of CAT was seen in the absence of the full set of RPV proteins, probably due to capping of the pMDB8A transcript by the normal MVA capping enzyme and skipping of the first (weak) ATG in the transcript. In the data set shown, above-background production of CAT was found in cells transfected with L plasmid, or N plus P plasmids; this was not reproduced in other studies.

Rescue of rRPV from cDNA. Based on the preceding results, we infected 293 cells with MVA-T7 and transfected them with 1 μ g each of N and P plasmids, 100 ng of L plasmid, and 1 to 2 μ g of pMDBRPV per 35-mm well (six-well plate), as described in Materials and Methods. Unlike the case of MV, we have never observed syncytia in RPV-infected 293 cells, so that direct observation of viral replication in the transfected cells was not possible. After 3 days, the cells were freeze-thawed and the medium was clarified. Vero cells infected with the medium from infected or transfected cells for 36 h showed a small number of foci of RPV infection by immunofluorescence, equivalent to <10 PFU/ml in the medium from the 293 cells. However, more prolonged culture of the supernatants with Vero cells showed CPE characteristic of RPV in all the cases where the original cells had received all the plasmids plus MVA-T7. In other experiments, we have extended the incubation of the infected and transfected 293 cells to 5 days and also cocultivated them from days 5 to 10 with B95a cells in attempts to improve the recovery of rRPV, without success. During these studies, no difference could be seen between MVA-infected and noninfected cells. Prolonged incubation of cells with supernatants from negative controls that had been infected with MVA-T7 failed to show any CPE.

Vero cells infected with rRPV were passaged once to increase the stock of virus, and total cellular RNA was isolated. RT-PCR was used to amplify the expected fragments from the genomic termini of both native RPV-R and the rescued virus (Fig. 4a), and those from the rRPV contained the restriction sites that had been engineered into the cDNA copy of the genome (Fig. 4c). If reverse transcriptase was omitted from the first stage of the RT-PCR, no amplified products were found (Fig. 4b), emphasizing that the DNA amplified was derived from RNA and not from any possibly contaminating plasmids. The virus grew to similar final titers (10^6 to 10^7 TCID₅₀ units per ml) to those found for RPV-R, and the growth curves of the recombinants and normal RPV-R were similar (Fig. 5). Western blots showed the presence of the same viral proteins, in the same relative amounts, as in cells infected with normal RPV-R (Fig. 6). The presence of the V protein, produced from an alternate reading frame by the introduction of an extra nontemplated nucleotide by the viral polymerase during about half the transcriptions of the P gene mRNA (5), confirms the presence of active RPV replication in these cells.

DISCUSSION

We have shown in these studies that we can recover infectious RPV from a cDNA clone. The recovered virus appears to replicate normally in tissue culture; as a clone of the vaccine strain of RPV, it should give rise to a subclinical infection in cattle, and we are beginning those investigations.

The efficiency of recovery of recombinant virus in our hands is similar to or better than that reported for most other systems, about 1 rescue event per 10^6 cells. About 10% of cells were transfected, and, although not determined in these stud-

FIG. 4. Identification of genetic tags in rRPV. Total RNA from infected cells was reverse transcribed, and the cDNA was amplified with primer pair GEN8A and RPVN6 (amplifies the first 201 bases of the antigenome) or RPVL6 and GEN10 (amplifies the last 297 bases of the antigenome). (a) PCR products from RPV-R and from two independent isolates of rRPV (I and II); (b) as panel (a) but with reverse transcriptase enzyme omitted from the RT step; (c) the PCR products from panel (a) digested with *ClaI* or *SalI* as indicated. In this and Fig. 6, original gel pictures or blots were photographed with a Kodak DCS420 digital camera, transferred to a PowerMac 7500, converted to grey scale and clipped in Adobe Photoshop, laid out and labelled in Quark Express, and printed on a Kodak XLS 8600 printer.

ies, the percentage of transfected cells that receive all four plasmids in similar systems appears to be about 40% (25). Since we used a relatively low MOI of MVA-T7 (very high doses had seemed to be toxic to the cells), only ~25% of cells would have been expressing T7 RNA polymerase. Taking these factors into account, roughly 1 in 100 cells would have received all four plasmids and MVA-T7. This could probably be improved slightly by using the highest nontoxic dose of MVA-T7 or by extensively optimizing the transfection conditions; however, it seems as if viral replication is successfully initiated in only 1 in 10^4 cells in which all necessary viral proteins and the viral genome are being made. The limiting step in all the systems used is probably the encapsidation of the primary full-length antigenome transcript to generate a replication-competent nucleocapsid. During normal viral replication, (anti) genome synthesis is coupled to encapsidation; in virus rescue, the T7 polymerase-transcribed RNA must be encapsidated by the viral N protein before it folds on itself irreversibly. Simply expressing high levels of the antigenome transcript does not seem to be the most important step: T7 RNA polymerase

FIG. 5. Growth of RPV-R and rRPV. Single-step growth curves plotting the growth of rRPV and rRPVII and normal RPV-R after infection of Vero cells at low MOI are shown.

initiates more efficiently at multiple G residues, and the rabies virus, VSV, Sendai virus, and RSV rescues have all used constructs in which transcription begins with 2 or 3 G nucleotides. The MV and RPV constructs, on the other hand, have used the normal viral sequence (beginning with ACCA) without loss of rescue efficiency, and Kato et al. (18) claim that the use of the normal viral sequence is essential for maximum efficiency of rescue of Sendai virus. Interestingly, two groups have reported the rescue of VSV from cDNA clones (22, 38); the latter group reported the recovery of similar amounts of virus (50 PFU/9-cm dish) to that seen here, while the former group reported up to 8×10^4 PFU, the main difference seeming to be that the high recovery came from cells transfected with linearized genome plasmid. Further work will need to be done to see if this is repeatable in other viruses.

Clearly, one factor affecting the amount of virus recovered is the release of the virus from the transfected cell. For most tissue culture-adapted laboratory strains of virus, this will not be a problem. In the studies reported here, both RPV and the packaged genome analogs were found to replicate in 293 cells, although viral release was not efficient and required a freeze-thaw step to allow us to collect the small amount of virus made in the rescue experiments. Adaptation of morbilliviruses to growth in common tissue culture lines has been shown in most cases to result in significant changes in the sequence (see, e.g., reference 32), so that future attempts to rescue wild-type vi-

FIG. 6. Synthesis of viral proteins by rRPV. Vero cells were infected with RPV-R, rRPV, or rRPVII or left uninfected. Proteins from infected and uninfected cells were analyzed by SDS-PAGE and transferred to cellulose filters. Replicate filters were probed with rabbit anti-RPV (reacts primarily with N, P, and H proteins), rabbit anti-P protein, or rabbit anti-V protein antiserum.

ruses, such as the RPV-K virulent parent of the RPV-R vaccine strain or even chimeras of the two viruses, will require rescue to occur in a cell line which supports wild-type virus growth without adaptation. Fortunately, both MV and RPV isolates grow without apparent adaptation in the marmoset lymphoblastoid cell line B95a (19, 20), and we are attempting to develop a rescue system involving this cell line. One of the advantages of the MVA-T7-based system over the helper cell line is that one does not have to develop a new stable cell line when one changes the host cell in which rescue is to occur. It also allows greater control over the relative amounts of the viral proteins in the system; the helper cell line used by Raddecke et al. expressed a large excess of P protein over N protein (28), whereas most studies have suggested that the normal virus expresses significantly more N mRNA than P (see, e.g., references 7 and 12). Rescue of VSV defective interfering particles was optimal with an excess of N plasmid over NS (P) plasmid, and rescue of rabies and Sendai viruses was accomplished with an excess (11) or equal amount (15) of N. The lower reported efficiency of rescue in the MV helper cell system (about 30% of transfected wells) may be accounted for by this lack of the optimal ratio of N and P. The importance of finding the optimum amounts of the various component plasmids is emphasized by a recent optimization of the rescue of Sendai virus (18). Under these conditions, rescue of virus from a negative (genome)-sense initial transcript was achieved, although at about 200-fold-lower efficiency than from the equivalent positive sense transcript.

On the other hand, as the one system that does not involve a poxvirus, the helper cell line is free of concerns about homologous recombination, which appears to occur in significant amounts between the genome plasmid and those from which the helper proteins are expressed, catalyzed by vaccinia virus proteins (15). This would have had no impact on the work reported here, since the genome plasmid was constructed from either the same cDNAs as were used to make the helper plasmids or cDNAs with identical sequence, so that all recombination events would generate the same genome. However, future attempts to recover virus with mutations in the N, P, or L genes may recover primarily vaccinia virus-catalyzed recombination revertants, especially if the mutation confers any replicative disadvantage on the virus. Our observation that the MV N, P, and L proteins appear to be capable of utilizing the RPV transcription and packaging signals in the RPV model genomes raises the possibility that we can use these proteins as helpers in rescuing rRPV. There is sufficient sequence dissimilarity between the two viruses to prevent homologous recombination, although it remains to be seen whether the MV proteins would interfere with the functioning of the RPV analogs by forming inactive N-P or P-L complexes.

Although of great importance to the health of humans and both domestic and wild animals, the replication and assembly of the morbilliviruses remains poorly understood. The development of systems to replicate and transcribe simple model genomes (replicons), either with (33) or without (the work reported here) helper virus, will allow us to investigate the *cis*- and *trans*-acting signals in the terminal and intergenic regions of these viruses. The replication of these viruses is already being dissected, with the demonstration that the C protein, a nonstructural protein translated from an alternate reading frame in the P gene, is not necessary for MV replication in tissue culture (27). The recovery of infectious RPV from cloned cDNA shown in this work will allow us directly to address questions of the molecular basis of virulence in this virus, since the recovered virus can be tested in the natural host animal, where the pattern of disease and the histopathology

are already well described (39, 40). In addition, it has already proved possible to introduce an entire new coding unit into VSV (30). The immunity conferred by the RPV vaccine appears to be lifelong after a single dose, raising the possibility of using RPV to express epitopes of other viruses, thereby immunizing cattle against diseases for which no vaccine, or no long-lasting vaccine, exists. We are currently constructing a variant of RPV-R which expresses epitopes of foot-and-mouth disease virus nonstructural proteins; there are currently only killed-virus, serotype-specific vaccines for foot-and-mouth disease. If such a recombinant-virus double vaccine proves functional, there is no *a priori* reason why triple or higher-order vaccines could not be created.

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