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Rescue of measles viruses from cloned DNA

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A system has been established allowing the rescue of replicating measles viruses (MVs) from cloned DNA. On one hand, plasmids were constructed from which MV antigenomic RNAs with the correct termini are transcribed by phage T7 RNA polymerase. On the other hand, helper cells derived from the human embryonic kidney 293 cell line were generated constitutively expressing T7 RNA polymerase together with MV nucleocapsid protein and phosphoprotein. Simultaneous transfection of the helper cells with the MV antigenomic plasmid and with a plasmid encoding the MV polymerase under direction of a T7 promoter led to formation of syncytia from which MVs were easily recovered. A genetic tag comprising three nucleotide changes was present in the progeny virus. As a first application of reverse genetics, a segment of 504 nucleotides from the 5′ non-coding region of the fusion gene was deleted, leading to an MV variant whose replication behaviour in Vero cells was indistinguishable from that of the laboratory Edmonston B strain. Since no helper virus is involved, this system, in principle, should be applicable to the rescue of any member of the large virus order *Mononegavirales*, i.e. viruses with a non-segmented negative-strand RNA genome.

Keywords: encapsidation/helper cell line/live vaccines/measles virus reverse genetics/paramyxovirus rescue

Introduction

The rescue of negative-strand RNA viruses from cloned DNA and hence the application of reverse genetics has lagged behind the use of these techniques for most positive-strand RNA viruses (Taniguchi *et al.*, 1978; Racaniello and Baltimore, 1981). In the latter case, in general, the introduction of the genomes into host cells is sufficient to trigger virus production, due to the dual function of these genomes both as mRNA and as templates for replication. In contrast, in negative-strand RNA viruses, neither the genomic nor the antigenomic RNAs can act as mRNA or as templates. To be biologically active, they must be encapsidated with viral nucleocapsid (N) protein in the form of flexible rod-like ribonucleoprotein complexes (RNPs). Genomic RNPs are the templates both for transcription, to produce naked mRNAs, and for replication, to form concomitantly encapsidated antigenomic RNPs which in most of these viruses are templates exclusively for replication.

Rescue from cloned DNA has been possible for several years in the case of influenza virus, a negative-strand RNA virus containing eight genome segments. Their RNPs, which are small in size and loosely structured, as revealed by the susceptibility of their RNA component to RNase, can be assembled *in vitro* from RNA and the required viral proteins, N and the polymerase components. Initially, an artificial RNA was used carrying the coding sequence for the reporter enzyme chloramphenicol acetyltransferase (CAT) embedded in the non-coding terminal segments of an influenza virus genome subunit (Luytjes *et al.*, 1989). Later, single authentic or altered genome subunit RNAs transcribed *in vitro* from cloned DNA were used (Enami and Palese, 1991). The assembled RNPs replicated and transcribed upon transfection into influenza-infected cells, as monitored by CAT production and by rescue of a reassorted influenza virus, respectively. Purification of virus containing the introduced subunit from the vast excess of non-reassorted virus in some cases can be accomplished by selection, e.g. using a specific neutralizing antibody directed against the protein encoded by the cognate subunit of the helper virus.

In contrast, for the viruses with a non-segmented negative-strand RNA genome, grouped together in the order *Mononegavirales* (Murphy *et al.*, 1995), the much more tightly structured and longer RNPs containing, in addition to the N protein, the assembly and polymerase cofactor phosphoprotein (P) and the viral RNA polymerase (large protein, L), have been refractory to functional association *in vitro*. [For schematic representations of genomic measles virus (MV) RNP see Figure 3B, and Figure 8A top; for descriptions of genomic RNP function see figure legends.] To facilitate the artificial assembly of RNPs in exploring feasible intracellular assembly systems, many laboratories approached the rescue of representatives of the *Mononegavirales* starting out with small subgenomic RNAs containing only essential sections of the viral genomes. First, naturally arising subgenomic RNAs, competing with the viral replication and thus known as defective interfering particle (DI) RNAs (Re, 1991), were used, being substituted later by artificial DI RNAs containing reporter genes, transcribed from plasmid constructs. A common type of these mini-replicons, first devised by the group of M.Krystal (Park *et al.*, 1991) according to the replicon in the initial influenza rescue model (Luytjes *et al.*, 1989), carries a CAT coding sequence inserted into viral non-coding terminal regions of Sendai virus (SeV) and has been used successfully also

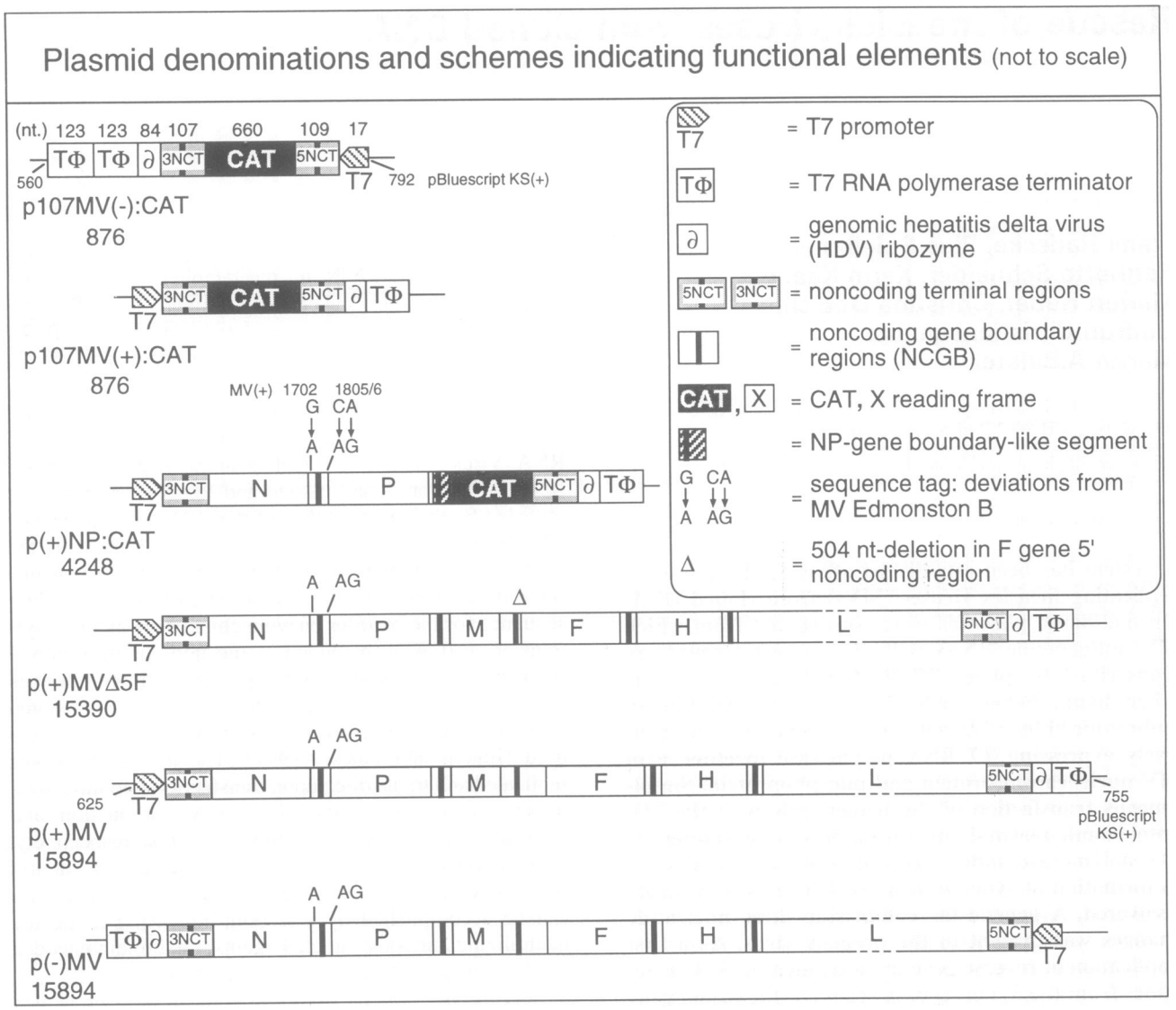

Fig. 1. Plasmid vectors specifying RNAs with correct MV-specific termini. The numbers below the plasmid names indicate the length in nucleotides of the RNAs generated after ribozyme self-cleavage. X open reading frame indicates any of the regions encoding N, P, M, F, H and L. The genomic or antigenomic sense of the specified RNAs are indicated by (–) and (+), respectively. Note that the MV nucleotide sequences present in these plasmids are according to EMBL accession No. Z66517, deviating in 30 positions from accession No. K01711, most notably by a deletion of an A residue at position 30, compensated by insertion of an A at position 3402. For an overview of an MV consensus sequence see Radecke and Billeter (1995).

for respiratory syncytial virus (Collins *et al.*, 1991, 1993), human parainfluenza virus 3 (Dimock and Collins, 1993), rabies virus (RV) (Conzelmann and Schnell, 1994) and MV (Sidhu *et al.*, 1995).

In all these systems, the required proteins mediating encapsidation, transcription and replication, designated here as helper proteins, were provided either by the homologous viruses or by the vaccinia vector vTF7-3 encoding phage T7 RNA polymerase (Fuerst *et al.*, 1986) to drive T7-specific transcription of transfected plasmids encoding the required proteins N, P and L, as pioneered for vesicular stomatitis virus (VSV) by Pattnaik and Wertz (1990). These investigations using mini-replicons have allowed important insights into the non-coding regulatory regions of the corresponding viral genomes and anti-genomes (for a recent discussion, see Wertz *et al.*, 1994). Finally, the substitution of mini- by full length viral RNAs and thus the rescue of viruses to allow the application of reverse genetics has been accomplished for RV by Schnell *et al.* (1994). Adopting the same experimental set-up, the rescue of VSV, like RV a member of the *Rhabdoviridae*, has now also been reported (Lawson *et al.*, 1995).

To avoid helper viruses which have to be separated from the rescued virus, cell lines were developed providing as helper functions T7 RNA polymerase and MV N and P protein. Transfection of plasmids specifying antigenomic RNA and MV L mRNA allows the rescue of MV, as directly monitored by syncytia formation in the transfected cell culture. Using this system, recombinant MVs were generated carrying either a sequence tag or a large deletion. In principle, this approach should be applicable for all *Mononegavirales*.

Results

Genomic and antigenomic plasmids specifying mini-, midi- and full-length replicons

The plasmid constructs used in this study are shown in Figure 1. p107MV(–):CAT and p107MV(+):CAT specify genome- and antigenome-sense RNAs, respectively, in which all MV coding regions are replaced precisely by the CAT coding region. In MV-infected cells or in helper cells (see below), they give rise to mini-replicons and to capped and polyadenylated CAT mRNA comprising the 5′ N and the 3′ L non-coding region. p(+)NP:CAT, containing in addition also the MV N and P coding regions in their ordinary MV sequence context, gives rise to midi-replicons. Full-length or partially deleted antigenomic or genomic RNAs are specified by p(+)MVΔ5F, p(+)MV and p(–)MV. For all these plasmids, transcription with T7 RNA polymerase yields RNAs bearing the authentic nucleotides of the viral genomic and antigenomic termini, respectively (Sidhu *et al.*, 1995). Correct initiation was accomplished by direct fusion of the T7 promoter (devoid of its transcribed part) to the genomic and antigenomic sequence. Starting all transcripts with the MV-specific nucleotides ACC rather than the T7-specific GGG reduces the RNA yield by about one order of magnitude, as revealed by *in vitro* transcription studies using precursor plasmid constructs (unpublished data). To mediate formation of the correct MV 3′ termini, the hepatitis delta virus genomic ribozyme sequence (Perrotta and Been, 1990) was cloned immediately adjacent to the MV 3′-terminal nucleotides; the introduction of T7 terminators increased the efficiency of self-cleavage (K.Kaelin *et al.*, in preparation).

Helper cells stably expressing MV N and P proteins as well as T7 RNA polymerase

The human embryonic kidney cell line 293 was chosen because it is highly permissive for MV. In addition, these cells can be transfected efficiently by the calcium phosphate coprecipitation method; 30–60% of the cells stained blue 24 h after transfection with a plasmid encoding β-galactosidase.

Following cotransfection of 293 cells with pSC6-N, pSC6-P and pSC6-T7-NEO, ~100 colonies were expanded under drug selection, as described in Materials and methods. The expression of N and P was screened by Western blotting, and the activity of T7 RNA polymerase was evaluated by transfection with a reporter plasmid containing the firefly luciferase coding region under the control of a T7 promoter. Many clones expressed high levels of P, but only few co-expressed N efficiently. Figure 2 shows N and P expression of two selected cell lines at levels comparable with that of MV-infected 293 cells; T7 RNA polymerase activity detected in clone 293-3-46 was among the highest of all clones, whereas it was ~100 times lower in clone 293-3-64 which turned out not to rescue MV. A third cell line, 293-3-43, expressing the three proteins at levels comparable with 293-3-46 was also active in rescue (data not shown).

The expression of the introduced genes did not reduce the susceptibility for MV infection (results not shown). The helper cell line 293-3-46 principally used for MV rescue, although growing at a rate 2–3 times slower than the parental 293 line, proved to be very stable and fully functional after >80 cell splittings at dilutions of 1:4 –1:8.

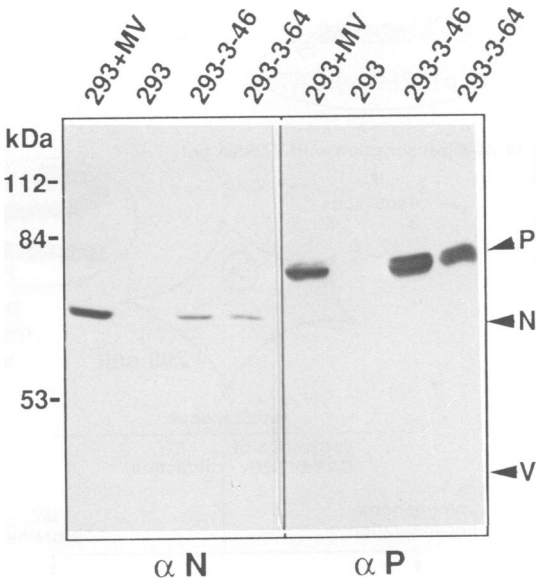

Fig. 2. Western blot showing the expression of MV N and P proteins in MV-infected 293 cells, uninfected 293 cells and in cell line clones 293-3-46 and 293-3-64, respectively. Arrows indicate the positions of the structural MV N and P proteins as well as the non-structural V protein arising from MV P gene transcript editing. αN and αP below the panels indicate the antibodies used for probing the blots as specified in Materials and methods.

From MV mini-replicon rescue using helper MV to MV rescue using helper cells 293-3-46

The MV rescue system was developed stepwise, enabling all components to be tested functionally. MV-dependent rescue of mini- and, later, successively longer midi-replicons was ascertained by CAT reporter assays. Similarly, the functionality of the 293-3-46 cells was compared with the MV-based help described before (Sidhu *et al.*, 1995).

The mini-replicon rescue test is shown schematically in Figure 3A. Small transcripts from p107MV(–):CAT, p107MV(+):CAT (Sidhu *et al.*, 1995) and later longer transcripts, e.g. generated from p(+)NP:CAT (Figure 1), behaved like mini- and midi-replicons, respectively. They were encapsidated, transcribed to produce CAT, replicated and packaged into infectious virion particles. During the first 2–4 infection cycles, they amplified massively, whereas in later cycles replication of both MV and the mini-replicons was curtailed, as observed for naturally occurring DI RNAs (Re, 1991). Analyses of the amplified RNAs showed that the encapsidated replicons and the CAT transcripts contained the respective different MV-specific terminal regions (Sidhu *et al.*, 1995). Most importantly, it turned out that, for efficient function, the total number of nucleotides of the replicons had to be a multiple of six (K.Kaelin *et al.*, in preparation), a requirement—termed the rule of six—previously found essential for natural and slightly modified SeV DI RNAs of the copy-back type (Calain and Roux, 1993). Adherence to this rule was crucial for the construction of plasmids specifying a variety of mini- and midi-replicons such as those shown in Figure 1 (K.Kaelin *et al.*, in preparation; P.Spielhofer,

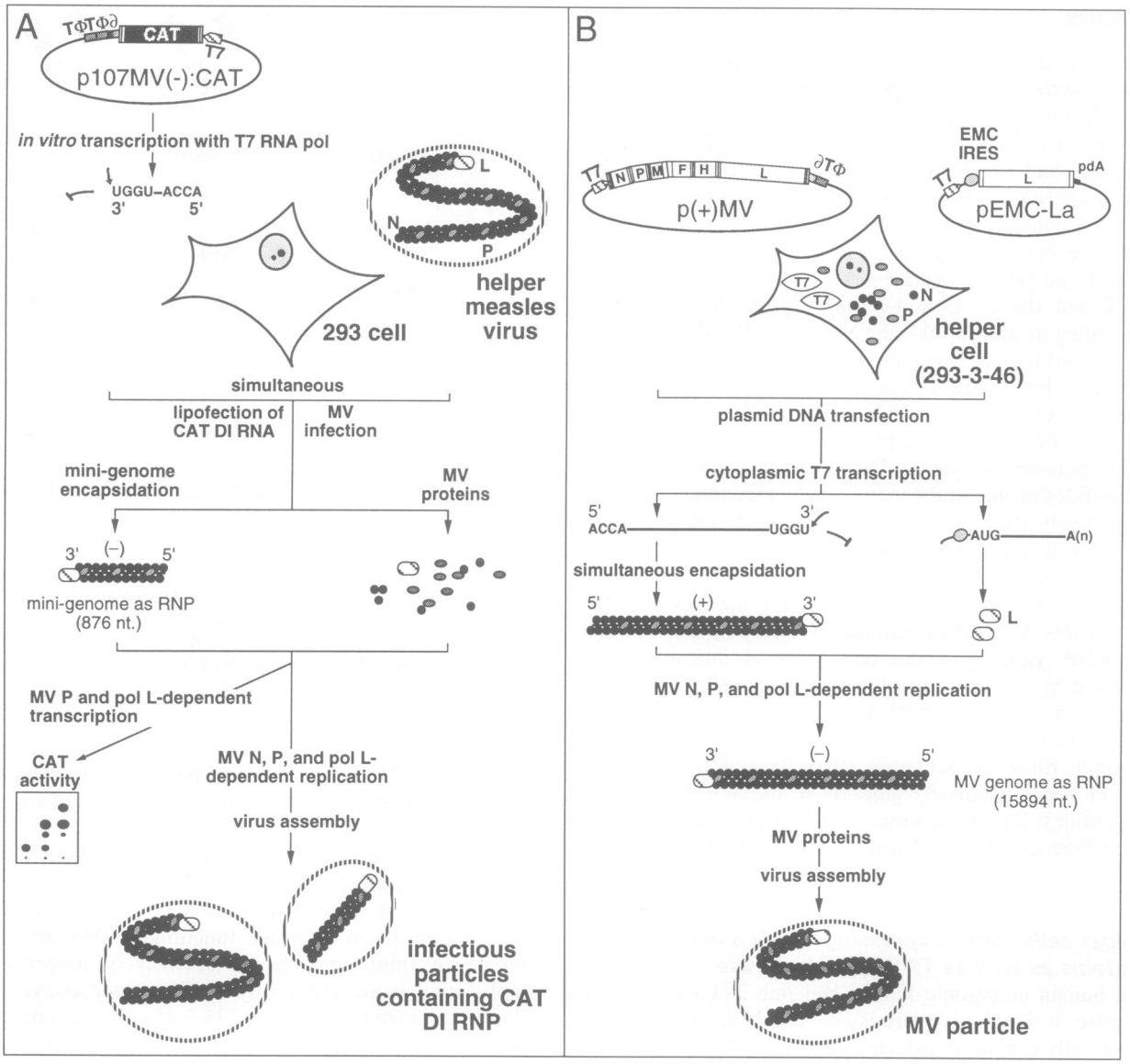

Fig. 3. Overview of experimental components and procedures for rescue. (**A**) Mini-replicon rescue, implicating transfection of *in vitro* transcribed RNA and coinfection with MV, supplying helper proteins N, P and L (and for later stages also M, F and H, as well as non-structural proteins C and V). (**B**) MV rescue, implicating transfection of plasmid DNAs into helper cells mediating both artificial T7 transcription and N and P functions. For explanation of most symbols see Figure 1. The L-encoding plasmid pEMC-La contains an internal ribosome entry site derived from encephalomyocarditis virus (stippled oval, EMC IRES), fused to the L-coding region such that the initiator AUG of EMCV and L coincide; a poly(dA) tract downstream (~40 dAs) is indicated as pdA. These two devices ensure transcript stability as well as efficient translation from the transcripts generated in the cytoplasm. Note that in natural infections MV genome RNPs (B, lower third) are delivered from the virions to the cytoplasm, where they are transcriptionally active, to form naked mRNAs for the production of viral proteins, whereupon they replicate to produce first antigenomic RNPs (B, middle) and then progeny RNPs.

unpublished data). This was also the case for full-length clones.

The helper function of stably transfected cell clones was tested with the set-up represented in Figure 3B, using, however, either plasmid p107MV(–):CAT, p107MV(+):CAT or p(+)NP:CAT (Figure 1) instead of p(+)MV. As shown in Figure 4, CAT activity arose in the transfected cells, although at levels considerably lower than in 293 cells infected with MV and cotransfected directly with mini-or midi-replicon RNA (results not shown). The cotransfection of plasmid pEMC-La encoding the MV L protein was an absolute requirement. As expected, low background CAT activity was detected when the plus-sense mini-replicon construct was used. The two constructs containing only the CAT reading frame in the plus and minus sense elicited about equal amounts of CAT activity; the midi-replicon construct gave rise to ~100 times less CAT activity than the mini-replicon. The observation that the efficiency of rescue shows a very strong inverse correlation with the size of the replicon has been made for all viruses implicated in studies using artificial replicons (Collins *et al.*, 1993; Conzelmann and Schnell, 1994; Lawson *et al.*, 1995).

The transfection protocol was optimized in terms of maximal achievable CAT activity, using mini- and midi-replicon plasmids. Then, the full-length constructs p(+)MV and p(–)MV were tested. About 10^6 cells contained in each 35 mm well were transfected; it is estimated that about one tenth of these actually received full-length as well as the L-encoding plasmids. Usually, following

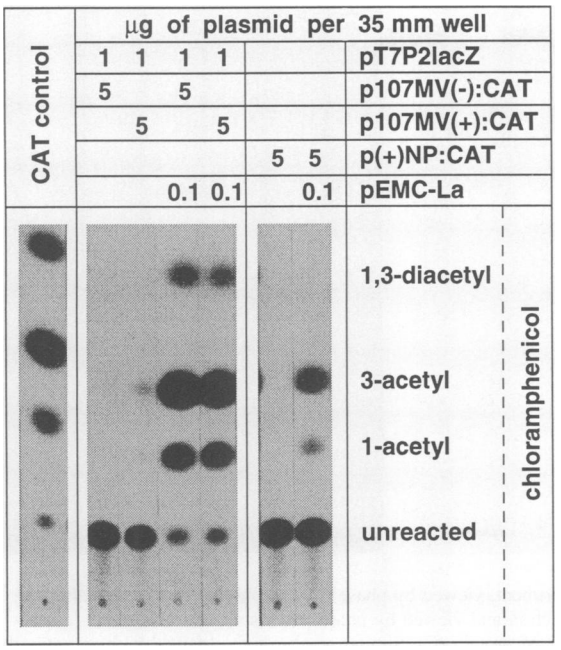

Fig. 4. Assay of CAT activity elicited in 293-3-46 helper cells by transfection of the plasmid constructs p107MV(+):CAT and p107MV(−):CAT, specifying mini-replicons, and construct p(+)NP:CAT, specifying a midi-replicon. The plasmid pT7P2lacZ, similar to constructs described for CAT by Pelletier and Sonenberg (1988), was used to set the total amount of input DNA to 6 μg.

cotransfection of p(+)MV and pEMC-La, 1–6 syncytia developed after 2–3 days in each well. No syncytia were found when the latter was omitted or when the p(−)MV plasmid was used. The rescue experiments were reproducible; using different DNA preparations, the efficiency was slightly variable, but at least 30% of the transfected wells revealed rescue. Figure 5 shows typical syncytia formed in these experiments, viewed either directly (phase contrast, Figure 5A) or after fixation of cells grown on cover slips (phase contrast, Figure 5B, or immunofluorescence of the same area, Figure 5C).

Characterization of rescued MV

First, it had to be ascertained that the rescued MVs contained the genetic tag which had been introduced into the MV full length plasmid clones. The 3 nt tag indicated in Figure 1 originated from a variant 176 nt N/P non-coding gene boundary region (NCGB) recovered from a persistent MV derived from a case of subacute sclerosing panencephalitis (SSPE; for a recent review, see Billeter *et al.*, 1994), replicating in IP-3-Ca cells (Ballart *et al.*, 1990). Rescued viruses were amplified in Vero cells, either directly from the transfected cells or after plaque purification; the products recovered by reverse transcription followed by PCR (RT-PCR) were analysed by cycle sequencing. Figure 6 shows an example of these analyses, revealing the AG tag instead of CA in the Edmonston B strain passaged in our laboratory.

We did not analyse the entire sequence of rescued MVs to exclude any error introduced either during the assembly of the antigenomic plasmid clones or during T7 RNA polymerase transcription in the rescue step. However, major deleterious changes could be ruled out by analysing the replication behaviour of the rescued virus in comparison with that of the Edmonston B strain. Figure 7 shows that both the speed of replication and the final titres reached in repeated experiments were indistinguishable between single plaque-purified normal (MV EdB) and rescued (MV tag EdB) viruses. The apparent difference at day 1 after infection was not a consistent observation. Non-plaque-purified virus stocks gave similar results (data not shown).

MV missing 504 nucleotides in the F gene 5′ non-coding region

As a first application of the reverse genetics system, most of the 5′ non-coding region of the F gene was eliminated by deleting 504 nucleotides encompassing nucleotides 4926–5429 (Radecke and Billeter, 1995), thus generating a shortened genome compatible with the rule of six mentioned above. The long enigmatic non-coding M/F NCGB is typical for MV and the other morbilliviruses, whereas the representatives of the other two genera of the subfamily *Paramyxovirinae*, paramyxovirus and rubulavirus, contain only a short NCGB. Remarkably, the mutant was viable and moreover replicated in Vero cell culture at a rate indistinguishable from that of the Edmonston B and the rescued non-deleted MV strain (Figure 7, MVΔ5F EdB). To determine the size of the F gene-derived RNAs, the MV-specific mRNA induced by these plaque-purified viruses was analysed, using probes specific for the F and for the M and H genes situated up- and downstream of F, respectively. Indeed, as shown in Figure 8, the F mRNA as well as the MF and FH bicistronic RNAs are consistently shorter in cells infected with the MVΔ5F EdB variant.

Discussion

Different methods for the rescue of Mononegavirales

The experiments reported in this article show that genetically tagged MV variants can be rescued reproducibly from cloned DNA specifying complete antigenomic RNA in helper cells constitutively expressing T7 RNA polymerase and the MV N and P proteins and transiently MV L. This method relies on a similar basic principle to the method described for the rescue of RV (Schnell *et al.*, 1994), where the required proteins are transiently expressed using the vTF7-3-based expression system. In both cases, the antigenomic viral RNA is produced directly in cells in which the proteins encapsidating the RNA to form biologically active RNPs are available.

Since the rescue system now developed, in contrast to the one used for rescue of RV (Schnell *et al.*, 1994), VSV (Lawson *et al.*, 1995) and, very recently, also for SeV (D.Garcin and D.Kolakofsky, personal communication), does not rely on any helper virus; there is no need to separate the rescued virus from the vast excess of the vaccinia virus used as helper. Elimination of vaccinia virus from rescued virus is accomplished by a simple filtration step in the case of the rigidly structured virions of *Rhabdoviridae* but would involve more complex purification schemes in the case of pleomorphic *Paramyxoviridae*, particularly those not replicating to high titres, such as MV. Furthermore, for viruses impaired in replication and/or budding by the vaccinia virus, rescue might fail altogether. The helper cell system circumvents these prob-

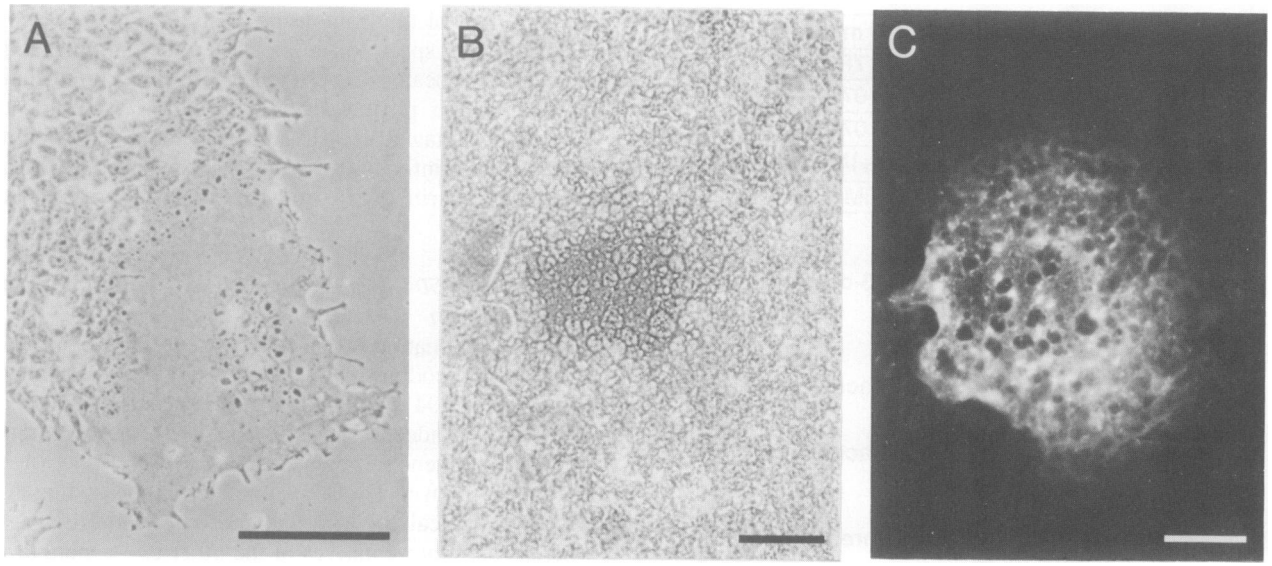

Fig. 5. Visualization of syncytia formed in 293-3-46 helper cells. (**A**) Rescue experiment, viewed by phase contrast microscopy 4 days after transfection. (**B** and **C**) Cells grown on glass cover slips, fixed 3 days after transfection and viewed by phase contrast (B) or indirect immunofluorescence microscopy using a monoclonal antibody directed against MV M protein (C). Similar results were obtained with an antibody against H. The bar length represents 100 μm.

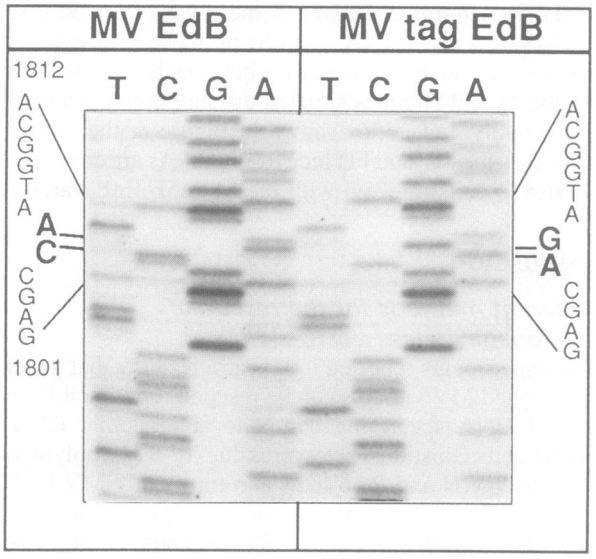

Fig. 6. Sequence determination of plaque-purified viruses, carried out by RT-PCR followed by cycle sequencing as described in Materials and methods. The left lanes of the relevant area reproduced from a sequencing gel relate to our laboratory Edmonston B strain, the right lanes to the rescued virus. Nucleotide positions indicated correspond to those in the MV consensus sequence as defined in Figure 1.

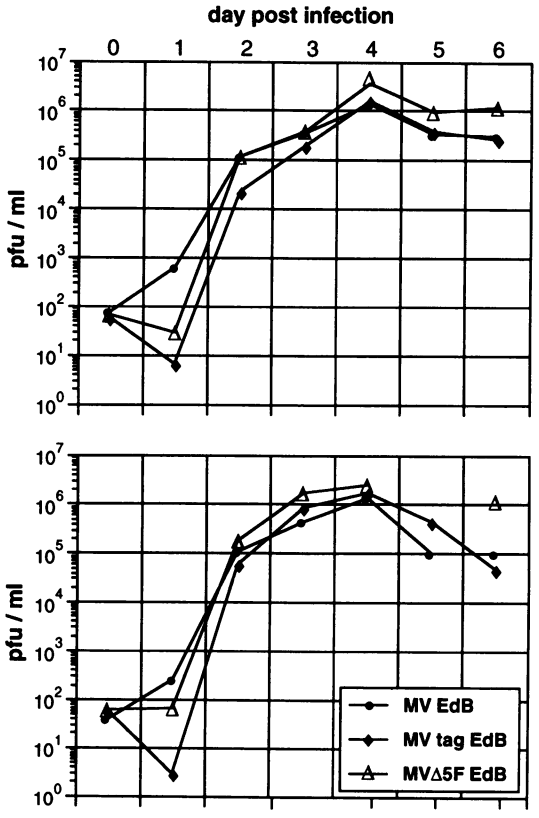

Fig. 7. Replication behaviour of plaque-purified viruses, evaluated by an overlay technique as described in Materials and methods. The derivatives of rescue experiments, the standard MV tag EdB and the 504 nucleotide deletion mutant MV Δ5F EdB are compared with a clone from our laboratory Edmonston B virus strain. The results of two independent experiments using representative clones of each virus species are shown.

lems and should, in principle, be applicable to the rescue of any of the *Mononegavirales* replicating in vertebrate cells.

It may not be necessary for the rescue of any single representative of *Mononegavirales* to establish a helper cell line expressing the cognate N and P proteins. The non-coding terminal regions (NCTs) of canine distemper virus (CDV, like MV a morbillivirus) differ by 35%. Nevertheless, mini-replicon constructs containing the CDV NCTs replicate in the MV-specific helper cells at an efficiency approaching that of the analogous MV mini-replicon (K.Kaelin *et al.*, in preparation; F.Radecke, unpublished data). Thus, possibly, CDV could be rescued with the 293-3-46 cells and, more generally, any helper cell line might be able to rescue a number of not too distantly related *Mononegavirales*. This will probably depend on the compatibility of the proteins elicited by the related

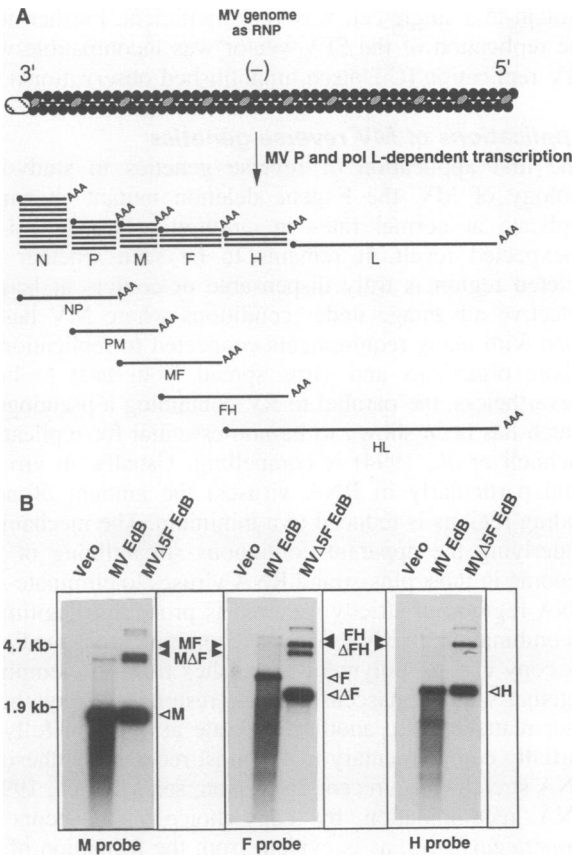

Fig. 8. (**A**) Schematic representation of capped and polyadenylated products of transcriptionally active antigenomic MV RNP; this pattern is similar for all *Mononegavirales*, although the number of genes varies from 5 to 10. The relative quantities of the different transcripts are indicated, showing the transcriptional gradient resulting in the highest amounts of monocistronic N mRNA and progressively lower levels of mRNAs encoding P, M, F, H and L. Bicistronic transcripts (NP, PM, MF, FH and HL) arise at a 10–20 times lower level, roughly following the gradient of the monocistronic transcripts (not indicated; for a review, see Cattaneo *et al.*, 1987). Since only the upstream coding sequence of the bicistronic (and of the very scarce tri- and tetracistronic) transcripts is translated, it remains unclear whether these large transcripts are biologically relevant or represent only erroneous products, resulting from occasional readthrough at gene boundaries rather than polyadenylation by slippage on oligo(U) stretches of the template, followed by termination and partial reinitiation. (**B**) Northern blots revealing mRNAs of the rescued MV derived from p(+)MV, and the MV deletion mutant derived from p(+)MVΔ5F (Figure 1). The monocistronic F, M and H mRNA species (open triangles) and the bicistronic MF and FH mRNAs (black triangles) are revealed by M-, F- and H-specific probes. The F-specific mono- and bicistronic RNAs induced by the deletion mutant are clearly smaller than the corresponding RNAs induced by the rescued standard MV [ΔF, 1869 rather than 2373 nt calculated, without considering poly(A) tails; MΔF, 3338 rather than 3842 nt, and ΔFH, 3830 rather than 4334 nt]. The bicistronic RNAs migrating at 4–5 kb which do not differ between the standard MV and the deletion mutant are not marked. The slowest migrating band visible only in the RNA derived from the mutant but not in the partially degraded RNA from the standard MV probably represents some MV genomic RNA not eliminated by oligo(dT) selection. The marker RNAs corresponding to 1.9 and 4.7 kb, respectively, were 18 and 28S rRNAs.

viruses, which was shown not to be the case for SeV-specific N and P and parainfluenza virus type 3 (PIV3)-specific L (Curran and Kolakofsky, 1991).

For the establishment of new helper cell lines for other viruses, the following considerations might be helpful. The constitutive expression of the T7 RNA polymerase and the MV proteins N and P did not impair the long-term stability of the 293-3-46 cell line, as mentioned in the Results section. Thus, inducible expression of these proteins, e.g. by the approaches described by the group of Bujard (for a review, see Gossen *et al.*, 1993) will probably not be necessary, although it cannot be excluded that the N and P proteins of other viruses are more deleterious for cell growth than those of MV. Titration of the plasmids used for transfection proved to be essential, showing that a ratio of ~1:1000 of L-encoding and antigenome-producing plasmid, respectively, was optimal, in agreement with the deleterious effect of high VSV L expression for VSV replication noted by Schubert *et al.* (1985). An alternative mode of transiently supplying L, using a plasmid containing a Rous sarcoma virus promoter/enhancer and an intron upstream rather than downstream of the L-coding region to facilitate the export of some long L mRNA from the nucleus, was also successful in rescue, but the efficiency was not better than with the standard method of cytoplasmic T7-dependent L expression (G.Christiansen, unpublished data). In view of these experiences, the decision not to include an L-encoding plasmid for the generation of helper cells, thus allowing expression of L at adjustable ratios, was probably advantageous. Nevertheless, it should be mentioned that a cell line stably expressing SeV-derived N, P and L which mediates long-term replication of natural SeV DIs has been described (Willenbrink and Neubert, 1994).

Evaluation of various rescue methods

The rescue efficiency, in comparison with rescue of positive-strand RNA viruses (for a recent review, see Boyer and Haenni, 1994), is low because only 1–6 out of 10^6 transfected cells, each exposed to an average of 2.5×10^5 molecules of antigenomic and 80–800 molecules of L-encoding plasmid, trigger the formation of syncytia. The MV rescue compares well with the rescue method described for RV and VSV, where $\sim2\times10^7$ cells are transfected to obtain one rescue event (Schnell *et al.*, 1994; Lawson *et al.*, 1995), particularly in view of the fact that the MV genome size is ~4.5 kb larger and thus, in principle, more difficult to rescue. Importantly, the low efficiency should not constitute a difficulty for the rescue of MV variants replicating only to titre levels even orders of magnitude lower than the Edmonston B strains, since the bottle-neck of rescue most probably is constituted by an early event. Note that on cells fixed at various times after transfection, immunofluorescence indicating H or M gene expression was monitored exclusively in syncytia, and there was no indication that rescue was confined to single cells (data not shown). When the rescue is visible directly by syncytia formation, already thousands of progeny MV genomes have arisen. Impaired and thus slowly replicating virus variants might not form visible syncytia initially, but these should be revealed after splitting of the transfected cell culture or upon seeding onto Vero cells.

Generally, the critical event for rescue appears to be the efficiency of RNA transcription by the T7 RNA polymerase and/or the encapsidation step. With regard to T7-specific transcription, the template activity of the MV plasmid constructs, as judged from *in vitro* transcription experiments, is probably about an order of magnitude

lower than that of the constructs described for RV and VSV rescue, due to the omission of three G residues preceding the viral sequences which are optimal for T7 transcription initiation. However, this advantage is presumably more than compensated for by the necessity for the extra nucleotides to somehow disappear later. With regard to encapsidation, both systems must overcome the same problem. Encapsidation normally seems to initiate at or very near to the 5′ terminus of the nascent transcript and then to progress, tightly coupled with the RNA elongation by the MV polymerase in the replication mode. In contrast, under rescue conditions, encapsidation is presumably not coupled to transcript elongation by the T7 RNA polymerase which progresses at a rate of >200 nucleotides/s (Chamberlin and Ryan, 1982), i.e. ~50 times faster than the polymerases of *Mononegavirales* (Iverson and Rose, 1981). Thus, the antigenomic RNA formed is initially naked over most of its length, constituting a target for RNase attack as well as erroneous, non-functional encapsidation. Non-specific encapsidation of completely heterologous RNA has been shown in the case of several *Mononegavirales*, and in particular for MV, by the group of R.Drillien who demonstrated extensive formation of heterogeneous RNPs in cells infected with recombinant vaccinia virus expressing MV N protein (Spehner *et al.*, 1991).

Our consistent failure to rescue MV from plasmids specifying genomic RNA is noteworthy, since assembled antigenomic RNPs have first to be replicated to genomic RNPs before mRNA synthesis can occur (see Figure 3B). Most likely, the underlying cause is annealing of the vast quantity of N and P mRNA present in the helper cells to newly synthesized genome sense RNA. Other explanations cannot be ruled out altogether. A mistake in the genomic plasmid seems very unlikely. Nevertheless, it might be relevant that T7 RNA polymerase produces significantly lower amounts of full-length transcripts *in vitro* from plasmids specifying genomes, due to an adventitious strong stop of T7-dependent minus-strand transcription in the L sequence.

Recently, an alternative MV rescue approach has been developed in our laboratory, using the overall methodology reported for RV and VSV rescue, but adopting a T7 RNA polymerase-expressing vaccinia vector (G.Sutter, in preparation) derived from the chicken cell-adapted vaccinia virus variant MVA (Sutter and Moss, 1992) which does not form progeny virus in mammalian cells. The efficiency of MV rescue obtained by this approach so far seems roughly comparable (H.Schneider and G.Sutter, unpublished data). Since it is easy to express a large variety of variant plasmids, this modified vaccinia–T7 system might be used for studying artificially altered MV proteins in terms of encapsidation or transcription/replication function. In addition, the ability of any mammalian cell line devoid of the measles receptor CD46 (Naniche *et al.*, 1993) to support the later steps of MV replication can be tested.

Finally, it should be mentioned that the expression system based on Semliki forest virus (SFV; Sjoberg *et al.*, 1994), which, like MVA–T7, does not give rise to progeny vector virus, proved not to be applicable for the purpose of MV rescue. Although single proteins were produced efficiently, the simultaneous expression of more than one protein in a single cell was very inefficient. Furthermore, the replication of the SFV vector was incompatible with MV replication (C.Dötsch, unpublished observations).

Applications of MV reverse genetics

The first application of reverse genetics to study the biology of MV, the F gene deletion mutant shown to replicate at normal rates in cultured cells, yielded an unexpected result. It remains to be seen whether the deleted region is truly dispensable or confers at least a selective advantage under conditions where MV has to cope with many requirements connected to replication in whole organisms and virus spread from host to host. Nevertheless, the parallel to RV containing a pseudogene which has been shown to be non-essential for replication (Schnell *et al.*, 1994) is compelling. Usually, in viruses (and particularly in RNA viruses) the amount of non-coding regions is reduced to a minimum. The mechanism underlying the apparent continuous streamlining of the genome in most plus-strand RNA viruses to eliminate any RNA region not strictly required is probably illegitimate recombination. In RNA viruses, recombination is mediated by copy choice; polymerase detaches from the template, together with the nascent RNA, to resume RNA synthesis after reattaching to another template at a region fully or partially complementary to the most recently synthesized RNA stretch (for a recent discussion, see Mindich, 1995). RNA recombination by copy-choice also occurs in *Mononegavirales*, as is evident from the formation of DI RNA. However, such recombination events must be several orders of magnitude less frequent than in most positive-strand RNA viruses, as also expected theoretically from the fact that both template and product RNA are tightly encapsidated as RNPs, thus impeding base pairing and attachment of nascent RNA–polymerase complexes to new templates.

Some study objects accessible by MV reverse genetics are listed here: the definition of the RNA signals (*cis*-acting elements) operative in nucleation of encapsidation, replication, transcription including stop–restart at gene boundaries and editing of P gene transcripts (Cattaneo *et al.*, 1989); the role of non-structural proteins V (arising through editing) and C (arising also from P gene transcripts by initiation at a downstream initiation codon) studied by interruption of their coding regions; functional appraisal of mutations found typically in MV variants responsible for fatal SSPE (for a review on SSPE, see Billeter *et al.*, 1994); and identification of the mutations responsible for attenuation of MV vaccine strains. The last item should also be of practical value, because the recognition of the mutations converting wild-type to attenuated MVs should allow the updating of the existing vaccine strains (derived from MV isolates 40 years ago) to more closely resemble currently circulating MV strains without interfering with attenuation traits (Norrby, 1995).

Furthermore, the MV rescue system might also lead to the use of MV for vector purposes. As a logical extension of the construction of midi-replicon plasmids such as p(+)NP:CAT (Figure 1), MV antigenomic midi-replicons have been developed containing, in addition, parts or the entire L gene downstream of the CAT gene. This demonstrates that a foreign gene can be expressed in an MV-dependent fashion by embedding it in MV-derived up-

and downstream gene boundary sequences (P.Spielhofer, unpublished data). Thus, chimeric vaccine MVs might be constructed expressing heterologous antigens, conferring immunity not only against MV but against other pathogens as well. In addition, vector applications of MV or other *Paramyxoviridae* for specific purposes implicating somatic gene therapy can also be envisaged. Obviously, any vector use of MV critically depends on the stability of the foreign genetic material during the replication of the virus which is generally problematic in RNA viruses due to their lack of proofreading inherent in RNA polymerases. Nevertheless, in comparison with vaccine vectors based on various positive-strand RNA viruses, e.g. as recently proposed for poliovirus (Andino *et al.*, 1994), MV appears *a priori* more suitable due both to the lack of extensive recombination as discussed above, which should effectively preclude large deletions, and to the rule of six, which should impede propagation of recombinant MV genomes containing small deletions or insertions introduced during the replication by error-prone RNA polymerase.

Materials and methods

Cells and viruses

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

To grow MV virus stocks reaching titres of ~10^7 p.f.u./ml, recombinant viruses were propagated in Vero cells, and the vaccine strain Edmonston B was grown in Vero or 293 cells. One round of plaque purification was carried out by transferring a syncytium to a 35 mm Vero cell culture which was expanded to a 175 cm^2 dish. Virus stocks were made from 175 cm^2 cultures when syncytia formation was pronounced. Cells were scraped into 3 ml of OptiMEM I (GIBCO BRL) followed by one round of freezing and thawing. The virus titrations were carried out on 35 mm Vero cell cultures. After 2–3 h of virus adsorption, the inoculum was removed and the cells were overlaid with 2 ml of DMEM containing 5% FCS and 1% SeaPlaque agarose. After 4–5 days, cultures were fixed with 1 ml of 10% trichloroacetic acid for 1 h, then UV cross-linked for 30 min. After removal of the agarose overlay, cell monolayers were stained with crystal violet dissolved in 4% ethanol, and the plaques were counted.

Generation of cell line 293-3-46

Before the transfection, all plasmids were linearized by digestion with *Sfi*I and sterilized by ethanol precipitation. Cells were seeded into one 35 mm well for transfection during 13 h as described below. The transfection mix contained 5 μg of pSC6-N, 4 μg of pSC6-P and 1 μg of pSC6-T7-NEO. Then, cells were washed once with 2 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4), and DMEM containing 10% FCS was added. After 2 days in culture, the cells of the 35 mm well were split into two 75 cm^2 dishes, and selection under 1.2 mg/ml G418 was started, changing the medium every second day. After ~2 weeks, the first clones of a total of ~100 clones were transferred to 5 mm wells. When a clone had expanded to a 21 mm or 35 mm well, cells were seeded for screening. The expression of the MV N and P proteins was analysed by Western blotting (see also below) using ~1/3 to 1/10 of the total lysate of a confluent 21 mm well. To monitor the functionality of the T7 RNA polymerase, a 35 mm cell culture was transfected with 4 μg of pEMC-Luc (Deng *et al.*, 1991), and the luciferase activity in 1/125 of the cleared total lysate (Promega protocol; harvest 1 day after transfection) was measured in a luminometer. Clones expressing the MV N and P proteins comparable with the same number of 293 cells infected with MV and showing a T7 RNA polymerase activity as high as possible were chosen to test their performance in allowing MV DI RNAs to express CAT. Here, 5 μg of the plasmids p107MV(+):CAT, p107MV(–):CAT or p(+)NP:CAT with or without 100 ng of pEMC-La were transfected. After 1 day, cells were lysed, and 1/4 of the cleared lysates was tested for CAT activity.

Plasmid constructions

All cloning procedures were basically as described in Sambrook *et al.* (1989). PCR amplifications were carried out using the proofreading *Pfu* DNA polymerase (Stratagene) and primers with a 3′-terminal phosphorothioate bond instead of a phosphodiester bond (Skerra, 1992). DNA sequences of the synthetic oligonucleotides are given in lower case for non-MV nucleotides and in upper case for the MV nucleotides; sequences of relevant restriction endonuclease recognition sites are underlined. The construction of the plasmid p107MV(–):CAT can be found in Sidhu *et al.* (1995). Plasmid p107MV(+):CAT is the analogue of the plasmid p107MV(–):CAT. The additional intercistronic region of p(+)NP:CAT that is similar to the N–P intergenic boundary was constructed by inserting 5′-ctaGCCTACCCTCCATCATTGTTATAAA-AAACTTAGGAACCAGGTCCACACAGCCGCCAGCCCATCAACgc-gtatcgcgata-3′, MV(+) 1717–1782 and the internally complementary oligonucleotide into the *Spe*I site of the P gene. The initially PCR-amplified and subcloned CAT coding region was inserted as depicted in Figure 1.

The description of the assembly of the first MV full-length DNA, the source of MV nucleotides 2044–14937 in later versions of full-length clones such as peuT7MV(–) (see below), is given in Ballart *et al.* (1990). The main features of the plasmid p(+)MV (Figure 1) are as follows. The T7 promoter allows the synthesis of the MV antigenomic RNA precisely starting with the first nucleotide. The genomic hepatitis delta virus (δ) ribozyme liberates the correct MV 3′-terminal nucleotide upon self-cleavage. Directly downstream of the δ ribozyme, the T7 RNA polymerase terminator TΦ stops most of the transcribing polymerases. This ensures that adjacent sequences derived from the vector backbone will not interfere with the cleavage activity. The cloning of p(+)MV started by annealing two internally complementary oligonucleotides #191 (5′-ggggaaccatcgatggataagaatgcggccgcaggtac-3′) and #192 (5′-ctgcggccgcattcttatccatcgatggttcccgc-3′), yielding a short polylinker that carries the restriction sites for *Sac*II, *Cla*I, *Not*I and *Kpn*I. This new polylinker replaced the *Sac*II–*Kpn*I fragment in pBloT7 derived from pBluescript KS(+) (Stratagene) containing the T7 promoter fused to a *Nsi*I site (Kaelin, 1989), thus forming the plasmid pBloT7NSCNK. To clone in the 5′-terminal 2041 bp of the MV antigenome (up to the *Sac*II site), a *Nsi*I digestion was followed by treatment with Klenow polymerase in the presence of all four dNTPs. This created a blunt-end cloning site flush to the non-transcribed part of the T7 promoter sequence. An MV fragment comprising the nucleotides 1–2078 was generated from the 3351 bp *Pvu*I fragment of peuMV(–) by PCR amplification using primers #182 [5′-ACCAAACAAAGTTGGGTAAGGATAG-3′, MV(+) 1–25] and #183 [5′-CAGCGTCGTCATCGCTCTCTCC-3′, MV(–) 2077–2056]. Note that the additional A residue at position MV(+) 30 (Sidhu *et al.*, 1995) derived from the MV sequence of peuMV(–) was later deleted by mutational PCR. Upon *Sac*II treatment, the MV fragment was ligated into the vector to yield pT7MV(+)5′. Next, the 3′ terminus of the antigenome was linked to the sequence of δ followed downstream by TΦ. The MV 3′ fragment (nucleotides 14907–15894) was generated from the 14 046 bp *Pvu*I fragment of peuMV(–) by PCR amplification using the primers #186 [5′-GAGAAGCTAGAGGAATTGGCAGCC-3′; MV(+) 14 907–14 930] and #187 [5′-ttctgaagactcACCAGACAAAGC-TGGG-3′, MV(–) 15 894–15 879]. Another PCR amplification on the plasmid peu3aδTΦ with the primers #184 (5′-ataagaatgcggccgcatccggata-tagttcctcc-3′) and #FR4 [5′-ttctgaagactcTGGTggccggcatggtcccag-3′, MV(+) 15 891–15 894] yielded the genomic δ ribozyme linked to the TΦ. Both primers #FR4 and #187 contain, close to their 5′ ends, the recognition sequence for *Bbs*I which creates a sticky end on both fragments comprising the four 3′-terminal MV nucleotides [MV(+) TGGT]. After digestion of the MV 3′ fragment with *Cla*I and *Bbs*I, of the δ/TΦ fragment with *Bbs*I and *Not*I and of pT7MV(+)5′ with *Cla*I and *Not*I, a three-way ligation yielded the plasmid pT7MV(+)5′3′δTΦ. The final step to generate p(+)MV was to fill in the remaining antigenomic MV nucleotides 2044–14 937 by a three-way ligation. The *Sac*II–*Pac*I fragment [MV(+) nucleotides 2044–7242] and the *Pac*I–*Cla*I fragment (MV nucleotides 7243–14 937) were released from plasmid peuT7MV(–). These two fragments were ligated into pT7MV(+)5′3′δTΦ from which the remaining polylinker (*Sac*II–*Cla*I) had been removed. The plasmid p(–)MV (Figure 1) was constructed similarly. The self-cleavage activity of δ was demonstrated by detecting the expected small 3′ fragments of *in vitro* made RNAs on a 5% polyacrylamide/7 M urea gel. To generate p(+)MVΔ5F carrying a 504 nt deletion [MV(+) 4926–5429] in the 5′ non-coding region of the F gene, first a PCR was carried out on plasmid pAeF1 (Huber, 1993) using primers #88 [5′-CcGAATCAAGACTCATCCAATGTCCATCATGG-3′, MV(+) 5430–5461] and #89 [5′-AGAGAGATTGCCCCAATGGATTTGACCG-3′,

MV(–) 5550–5523]. The PCR fragment digested with *Hpa*I replaced the *Nar*I–*Hpa*I fragment in pAeF1. The *Nar*I–*Pac*I fragment of this vector then replaced the corresponding fragment in p(+)MV.

The vector backbone of pEMC-La is based on pTM1 (Moss *et al.*, 1990) in which a *Nco*I site overlaps with an ATG trinucleotide. Using this ATG as the start codon, an open reading frame inserted into this *Nco*I site is translationally controlled by the encephalomyocarditis (EMC) virus internal ribosome entry site (IRES). The MV L coding sequence linked to an artificial poly(dA) tract was taken from vector pAeL (Huber, 1993) in two steps: first, a 405 bp fragment containing the MV nucleotides 9234–9630 was generated by PCR using primers #194 [5′-gtggatccATGGACTCGCTATCTGTCAACC-3′, MV(+) 9234–9255] and #195 [5′-AGTTAGTGTCCCTTAAGCATTGGAAAACC-3′, MV(–) 9630–9602]; second, a 6265 bp fragment comprising nucleotides 9572–15 835 of the MV L gene sequence joined to the poly(dA) tract was excised with *Eco*RI. After removing the *Nco*I–*Eco*RI part of the polylinker in pTM1 and digesting the PCR fragment also with *Nco*I and *Eco*RI, a three-way ligation including the 6265 bp *Eco*RI fragment yielded pEMC-La.

To eliminate the T7 promoter located 5′ of the cytomegalovirus promoter/enhancer in the vectors pSC-N and pSC-P (Huber *et al.*, 1991), pSC6-N and pSC6-P were constructed by replacing a *Pvu*I–*Eco*RI fragment with the corresponding fragment of pC GR N-795 (kindly provided by S.Rusconi). pSC6-T7 was generated by exchanging the N gene insert of pSC6-N by the fragment carrying the T7 RNA polymerase gene of pAR 1173 (Davanloo *et al.*, 1984). pSC6-T7-NEO was constructed by ligation of the phosphoglycerol kinase promoter–neomycin resistance cassette (Soriano *et al.*, 1991) into the unique *Avr*II site of pSC6-T7 using appropriate linker oligodeoxyribonucleotides. All cloning sites were verified by sequencing.

Transfection of plasmids and harvest of the reporter gene products

Cells were seeded into a 35 mm well to reach ~50–70% confluence when being transfected. At 3–8 h before transfection, the medium was replaced with 3 ml of DMEM containing 10% FCS. G418 was omitted subsequently because of its toxic effect during transfection. All plasmids were prepared according to the QIAGEN plasmid preparation kit. The protocol for the Ca^{2+} phosphate coprecipitation of the DNA was adapted from Rozenblatt *et al.* (1979). The plasmids (2–10 μg per 35 mm well) were diluted with 300 μl of 1× transfection buffer (137 mM NaCl, 4.96 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM dextrose, 21 mM HEPES pH 7.03). $CaCl_2$ solution (1 M) was added to a final Ca^{2+} concentration of 125 mM, and the mix was incubated at 20°C for 30–120 min. The coprecipitates were added dropwise to the culture and the transfection was carried out at 37°C and 5% CO_2 for ~15 h. Then, the transfection medium was replaced with 3 ml of DMEM containing 10% FCS. The products of the reporter genes were harvested 24–37 h after transfection. Cells were washed and lysed with Reporter lysis buffer (Promega), and CAT and luciferase assays were done following the supplier's protocol.

Experimental set-up to rescue MV

293-3-46 cells prepared for transfection as described above were transfected with 5 μg of the plasmid harbouring the MV antigenomic DNA in the presence or absence of 1–100 ng of the plasmid specifying the MV L mRNA. The first syncytia appeared ~2–3 days after transfection when the cells were still subconfluent. To allow syncytia formation to progress more easily, almost confluent cell monolayers of each 35 mm well were then transferred to a 75 cm^2 dish. When these cultures reached confluence, cells were scraped into the medium and subjected once to freezing and thawing. Cleared supernatants were used to infect monolayers of Vero cells either to grow virus stocks or to harvest total RNA for analysis.

RT-PCR, cycle sequencing, Northern blot, Western blot and immunofluorescence

For RT-PCR followed by cycle sequencing, Vero cells were infected with cleared virus suspensions either harvested from rescue cultures or from later passages, and total RNA was isolated according to Chomczynski and Sacchi (1987). Total RNAs (2 μg) were first hybridized with 10 pmol or 1 nmol of random hexamer primers by heating to 80°C for 1 min and then quick-cooled on ice. Reverse transcriptions were carried out with 200 U of mouse mammary tumour virus reverse transcriptase (GIBCO BRL) in the presence of 1 mM dNTPs in a buffer containing 20 mM Tris–HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, and 1 U RNAsin (Promega). The 20 μl mixes were kept at 20°C for 10 min, incubated at 42°C for 1 h, and terminated by heating at 95°C for 10 min. One tenth of the reaction volumes was used as templates for the PCR amplification with the primers #59 [5′-ACTCGGTATCACTGCCGAGGATGCAAGGC-3′, MV(+) 1256–1284] and #183 [5′-CAGCGTCGTCATCGCTCTCTCC-3′, MV(–) 2077–2056]. After 40 cycles, the 822 bp fragments were isolated using the QIAquick gel extraction kit (QIAGEN). The sequencing reactions were done according to the linear amplification protocol (Adams and Blakesley, 1991). Primer #76 [5′-ctaGCCTACCCTCCATCATTGTTATAAAAAACTTAG-3′, MV(+) 1717–1749] was used for the tag in the 5′ non-coding region of the P gene and primer #6 [5′-ccggTTATAACAATGATGGAGGG-3′, MV(–) 1740–1722] for the tag in the 3′ non-coding region of the N gene.

Total cellular RNA for Northern blot analysis was isolated from Vero cells using the TRI REAGENT® (Molecular Research Center, Inc.) and poly(A) RNA was purified using oligo$(dT)_{25}$-coated paramagnetic polystyrene beads (Dynal) and a magnetic particle concentrator. The RNA was electrophoresed through a 1% agarose gel in 6% formaldehyde-containing running buffer and transferred to a Hybond-N^+ membrane (Amersham) by capillary elution in 20× SSC. Filters were prehybridized at 42°C for 4 h. Hybridization was performed overnight at 42°C in 50% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulfate, 1% SDS, yeast tRNA (0.1 mg/ml) containing 2×10^6 c.p.m./ml of an [α-^{32}P]dATP-labelled DNA probe prepared with Prime-It II (Stratagene). The following DNA fragments were used for random priming: the 1.4 kb *Sal*I–*Bam*HI fragment from pSC-M (Huber *et al.*, 1991), the 1.7 kb *Hpa*I–*Pac*I fragment from pCG-F (Cathomen *et al.*, in preparation); and the 1.6 kb *Sma*I–*Xba*I fragment from pSC-H (Huber *et al.*, 1991). Filters were washed in 2× SSC at 20°C for 10 min and twice in 2× SSC, 1% SDS at 65°C for 30 min. Bands were visualized by autoradiography.

To analyse the expression of the MV N and P proteins by Western blotting, cells were washed with PBS and cytoplasmic extracts were prepared using 300 μl of lysis buffer (50 mM Tris–HCl pH 8, 62.5 mM EDTA, 1% NP-40, 0.4% deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin). About 1/60 of the total lysates was run on SDS–8%PAGE and blotted onto Immobilon-P membranes. As first antibodies, either the rabbit polyclonal anti-N antibody #179 (kindly provided by C.Oervell) in a 6000-fold dilution in TBST (10 mM Tris–HCl pH 7.2–8, 150 mM NaCl, 0.05% Tween 20) or the rabbit polyclonal anti-P antibody #178 (Oervell and Norrby, 1980) in a 3000-fold dilution in TBST was used. The second antibody was a swine anti-rabbit antibody coupled to horseradish peroxidase, allowing the visualization of the bands by the enhanced chemiluminescence kit (ECL™ Amersham Life Science, RPN 2106).

For immunofluorescence microscopy, 293-3-46 cells were seeded for a rescue experiment on 24×24 mm glass cover slips in 35 mm wells, cultured overnight and transfected as described above. Three days after transfection, cells were permeabilized with acetone:methanol (1:1) and indirect immunofluorescence was performed essentially as described (Oervell and Norrby, 1980; Hancock *et al.*, 1990), except that PBS was supplemented with 1 mM $MgCl_2$ and 0.8 mM $CaCl_2$ and that *p*-phenylendiamine was omitted from the mountant. Viral M and H proteins were detected using mouse monoclonal anti-M-16BB2 and anti-H-I29 antibodies (Sheshberadaran *et al.*, 1983) and rabbit anti-mouse IgG [F(ab′)$_2$] antibodies coupled to rhodamine (Pierce, 31666).

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References

Adams,S.M. and Blakesley,R. (1991) Linear amplification DNA sequencing. *Focus*, **13**, 56–58.

Andino,R., Silvera,D., Suggett,S.D., Achacoso,P.L., Miller,C.J., Baltimore,D. and Feinberg,M.B. (1994) Engineering poliovirus as a vaccine vector for the expression of diverse antigens. *Science*, **265**, 1448–1451.

Ballart,I., Eschle,D., Cattaneo,R., Schmid,A., Metzler,M., Chan,J., Pifko-Hirst,S., Udem,S.A. and Billeter,M.A. (1990) Infectious measles virus from cloned cDNA. *EMBO J.*, **9**, 379–384. (Retracted by Eschle,D., Cattaneo,R., Schmid,A., Metzler,M., Chan,J., Pifko-Hirst,S., Udem,S.A. and Billeter,M.A. in *EMBO J.*, **10**, 3558; 1991).

Billeter,M.A., Cattaneo,R., Spielhofer,P., Kaelin,K., Huber,M., Schmid,A., Baczko,K. and ter Meulen,V. (1994) Generation and properties of measles virus mutations typically associated with subacute sclerosing panencephalitis. *Ann. N.Y. Acad. Sci.*, **724**, 367–377.

Boyer,J.C. and Haenni,A.L. (1994) Infectious transcripts and cDNA clones of RNA viruses. *Virology*, **198**, 415–426.

Calain,P. and Roux,L. (1993) The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J. Virol.*, **67**, 4822–4830.

Cattaneo,R., Rebmann,G., Schmid,A., Baczko,K., ter Meulen,V. and Billeter,M.A. (1987) Altered transcription of a defective measles virus genome derived from a diseased human brain. *EMBO J.*, **6**, 681–688.

Cattaneo,R., Kaelin,K., Baczko,K. and Billeter,M.A. (1989) Measles virus editing provides an additional cysteine-rich protein. *Cell*, **56**, 759–764.

Chamberlin,M. and Ryan,T. (1982) Bacteriophage DNA-dependent RNA polymerases. In Boyer,P.D. (ed.), *The Enzymes*. 3rd edn., Academic Press, New York/London, Vol. 15, pp. 87–108.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Collins,P.L., Mink,M.A. and Stec,D.S. (1991) Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. *Proc. Natl Acad. Sci. USA*, **88**, 9663–9667.

Collins,P.L., Mink,M.A., Hill,M.G., Camargo,E., Grosfeld,H. and Stec,D.S. (1993) Rescue of a 7502-nucleotide (49.3% of full-length) synthetic analog of respiratory syncytial virus genomic RNA. *Virology*, **195**, 252–256.

Conzelmann,K.K. and Schnell,M. (1994) Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. *J. Virol.*, **68**, 713–719.

Curran,J.A. and Kolakofsky,D. (1991) Rescue of a Sendai virus DI genome by other parainfluenza viruses: implications for genome replication. *Virology*, **182**, 168–176.

Davanloo,P., Rosenberg,A.H., Dunn,J.J. and Studier,F.W. (1984) Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proc. Natl Acad. Sci. USA*, **81**, 2035–2039.

Deng,H., Wang,C., Acsadi,G. and Wolff,J.A. (1991) High-efficiency protein synthesis from T7 RNA polymerase transcripts in 3T3 fibroblasts. *Gene*, **109**, 193–201.

Dimock,K. and Collins,P.L. (1993) Rescue of synthetic analogs of genomic RNA and replicative-intermediate RNA of human parainfluenza virus type 3. *J. Virol.*, **67**, 2772–2778.

Enami,M. and Palese,P. (1991) High-efficiency formation of influenza virus transfectants. *J. Virol.*, **65**, 2711–2713.

Fuerst,T.R., Niles,E.G., Studier,F.W. and Moss,B. (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl Acad. Sci. USA*, **83**, 8122–8126.

Gossen,M., Bonin,A.L. and Bujard,H. (1993) Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem. Sci.*, **18**, 471–475.

Hancock,J.F., Paterson,H. and Marshall,C.J. (1990) A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell*, **63**, 133–139.

Huber,M. (1993) Expression of measles virus genes: analysis of interactions between nucleocapsid protein and phosphoprotein. Ph.D. Thesis, University of Zürich, Switzerland.

Huber,M., Cattaneo,R., Spielhofer,P., Oervell,C., Norrby,E., Messerli,M., Perriard,J.C. and Billeter,M.A. (1991) Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. *Virology*, **185**, 299–308.

Iverson,L.E. and Rose,J.K. (1981) Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. *Cell*, **23**, 477–484.

Kaelin,K. (1989) RNA editing in the measles virus phosphoprotein gene provides an additional protein. Diploma Thesis, University of Zürich, Switzerland.

Lawson,N., Stillman,E.A., Whitt,M.A. and Rose,J.K. (1995) Recombinant vesicular stomatitis viruses from DNA. *Proc. Natl Acad. Sci. USA*, **92**, 4477–4481.

Luytjes,W., Krystal,M., Enami,M., Parvin,J.D. and Palese,P. (1989) Amplification, expression, and packaging of a foreign gene by influenza virus. *Cell*, **59**, 1107–1113.

Mindich,L. (1995) Heterologous recombination in the segmented dsRNA genome of bacteriophage ϕ6. *Semin. Virol.*, **6**, 75–83.

Moss,B., Elroy-Stein,O., Mizukami,T., Alexander,W.A. and Fuerst,T.R. (1990) Product review. New mammalian expression vectors. *Nature*, **348**, 91–92.

Murphy,F.A., Fauquet,C.M., Bishop,D.H.L., Ghabrial,S.A., Jarvis,A.W., Martelli,G.P., Mayo,M.A. and Summers,M.D. (1995) *Virus Taxonomy, Sixth Report of the International Committee on Taxonomy of Viruses*. Springer-Verlag, Wien.

Naniche,D., Varior-Krishnan,G., Cervoni,F., Wild,T.F., Rossi,B., Rabourdin-Combe,C. and Gerlier,D. (1993) Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *J. Virol.*, **67**, 6025–6032.

Norrby,E. (1995) The paradigms of measles vaccinology. *Curr. Top. Microbiol. Immunol.*, **191**, 167–180.

Oervell,C. and Norrby,E. (1980) Immunological relationships between homologous structural polypeptides of measles and canine distemper virus. *J. Gen. Virol.*, **50**, 231–245.

Park,K.H., Huang,T., Correia,F.F. and Krystal,M. (1991) Rescue of a foreign gene by Sendai virus. *Proc. Natl Acad. Sci. USA*, **88**, 5537–5541.

Pattnaik,A.K. and Wertz,G.W. (1990) Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs. *J. Virol.*, **64**, 2948–2957.

Pelletier,J. and Sonenberg,N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature*, **334**, 320–325.

Perrotta,A.T. and Been,M.D. (1990) The self-cleaving domain from the genomic RNA of hepatitis delta virus: sequence requirements and the effects of denaturants. *Nucleic Acids Res.*, **18**, 6821–6827.

Racaniello,V.R. and Baltimore,D. (1981) Cloned poliovirus cDNA is infectious in mammalian cells. *Science*, **214**, 916–919.

Radecke,F. and Billeter,M.A. (1995) Appendix: measles virus antigenome and protein consensus sequences. *Curr. Top. Microbiol. Immunol.*, **191**, 181–192.

Re,G.G. (1991) Deletion mutants of paramyxoviruses. In Kingsbury,D.W. (ed.), *The Paramyxoviruses*. Plenum Press, New York, pp. 275–298.

Rozenblatt,S., Koch,T., Pinhasi,O. and Bratosin,S. (1979) Infective substructures of measles virus from acutely and persistently infected cells. *J. Virol.*, **32**, 329–333.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schnell,M.J., Mebatsion,T. and Conzelmann,K.K. (1994) Infectious rabies viruses from cloned cDNA. *EMBO J.*, **13**, 4195–4203.

Schubert,M., Harmison,G.G., Richardson,C.D. and Meier,E. (1985) Expression of a cDNA encoding a functional 241 kDa VSV RNA polymerase. *Proc. Natl Acad. Sci. USA*, **82**, 7984–7988.

Sheshberadaran,H., Chen,S.N. and Norrby,E. (1983) Monoclonal antibodies against five structural components of measles virus. *Virology*, **128**, 341–353.

Sidhu,M.S. *et al.* (1995) Rescue of synthetic measles virus minireplicons: measles genomic termini direct efficient expression and propagation of a reporter gene. *Virology*, **208**, 800–807.

Sjoberg,E.M., Suomalainen,M. and Garoff,H. (1994) A significantly improved Semliki forest virus expression system based on translation enhancer segments from the viral capsid gene. *Bio-Technology*, **12**, 1127–1131.

Skerra,A. (1992) Phosphorothioate primers improve the amplification of DNA sequences by DNA polymerases with proofreading activity. *Nucleic Acids Res.*, **20**, 3551–3554.

Soriano,P., Montgomery,C., Geske,R. and Bradley,A. (1991) Targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice. *Cell*, **64**, 693–702.

Spehner,D., Kirn,A. and Drillien,R. (1991) Assembly of nucleocapsid like structures in animal cells infected with a vaccinia virus recombinant encoding the measles virus nucleoprotein. *J. Virol.*, **65**, 6296–6300.

Sutter,G. and Moss,B. (1992) Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl Acad. Sci. USA*, **89**, 10847–10851.

Taniguchi,T., Palmieri,M. and Weissmann,C. (1978) Qβ DNA-containing

hybrid plasmids giving rise to Qβ phage formation in the bacterial host. *Nature*, **274**, 2293–2298.

Wertz,G.W., Whelan,S., LeGrone,A. and Ball,L.A. (1994) Extent of terminal complementarity modulates the balance between transcription and replication of vesicular stomatitis virus RNA. *Proc. Natl Acad. Sci. USA*, **91**, 8587–8591.

Willenbrink,W. and Neubert,W.N. (1994) Long-term replication of Sendai virus defective interfering particle nucleocapsids in stable helper cell lines. *J. Virol.*, **68**, 8413–8417.

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