Chapter 7
Reverse Genetics of Measles Virus and Resulting Multivalent Recombinant Vaccines: Applications of Recombinant Measles Viruses

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Abstract An overview is given on the development of technologies to allow reverse genetics of RNA viruses, i.e., the rescue of viruses from cDNA, with emphasis on nonsegmented negative-strand RNA viruses (*Mononegavirales*), as exemplified for measles virus (MV). Primarily, these technologies allowed site-directed mutagenesis, enabling important insights into a variety of aspects of the biology of these viruses. Concomitantly, foreign coding sequences were inserted to (a) allow localization of virus replication in vivo through marker gene expression, (b) develop candidate multivalent vaccines against measles and other pathogens, and (c) create candidate oncolytic viruses. The vector use of these viruses was experimentally encouraged by the pronounced genetic stability of the recombinants unexpected for RNA viruses, and by the high load

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of insertable genetic material, in excess of 6 kb. The known assets, such as the small genome size of the vector in comparison to DNA viruses proposed as vectors, the extensive clinical experience of attenuated MV as vaccine with a proven record of high safety and efficacy, and the low production cost per vaccination dose are thus favorably complemented.

Development of Techniques Allowing Reverse Genetics of RNA Viruses

Reverse genetics, i.e., the introduction of exactly defined mutations into the genome of organisms (true living organisms and viruses) has become one of the technical cornerstones of molecular biology. Convenient application of this important technique for RNA viruses relies on the possibility to rescue such viruses from cloned DNA that represent sequence variants of their RNA genomes. The underlying DNA segments can be easily obtained, basically by reverse transcription in vitro, manipulated, even conveniently synthesized chemically, and economically amplified in bacteria as segments residing within plasmids.

The primary driving force for establishing reverse genetic systems for RNA viruses was to obtain insights into their biology. Indeed, investigations using this tool are bringing about a wealth of knowledge regarding the propagation of viruses and their interactions with their hosts. In addition, it was recognized early on that reverse genetic technology might also be exploited for practical purposes in medicine, to create new vaccines, and possibly also to combat cancer, taking advantage of the strong and often long-lasting stimulation of immune systems by viruses. Particularly promising was the use of attenuated viruses already widely proven in medical practice to constitute efficient and safe vaccines against their parent wild-type progenitors. Addition to their genome of genetic material encoding proteins or protein fragments of other important pathogens might result in multivalent vaccines mediating protection not only against the virus species used as vector, but also against the pathogens from which the added genetic information is derived.

Over the three decades since the 1970s, great progress has been made to gradually make essentially all RNA virus genera amenable to the application of reverse genetics, and during the last decade the processes to obtain genetically modified viruses by reverse genetics have been refined to facilitate the technical processes, so that practically any desired constructed RNA virus can be made readily available, provided that the changes introduced are compatible with efficient propagation of the construct in cultured cells and packaging into infectious viral particles. As outlined below, manipulated plus-strand RNA viruses were obtained rather easily, and among the more problematic negative-strand RNA viruses, those containing a nonsegmented genome (i.e., the ones grouped in the large order *Mononegavirales*) were more difficult to get hold of than representatives containing segmented genomes.

Positive-Strand RNA Viruses

The bacteriophage Qbeta was the first virus for which this technique could be applied (Taniguchi et al. 1978). In this case, the rescue of virus was particularly easy, as the cloned DNA segment representing the sequence of the viral genome, simply flanked by oligo A and T stretches, was transcribed spontaneously after introduction into *Escherichia coli* cells by the residing RNA polymerase. Since the intracellularly transcribed genomes are first active as messenger RNA to produce all viral proteins and subsequently as template for the viral RNA polymerase/replicase to produce antigenomes, from which progeny genomes are then transcribed, progeny virus is formed automatically. It is still not clear by which mechanisms the primary transcripts containing additional nonviral flanking sequences give rise to progeny RNA containing the precise viral ends.

The next virus made amenable to reverse genetics was poliomyelitis virus (Racaniello and Baltimore 1981). The experimental hurdles to overcome were considerably higher in this case: (a) culturing the host cells was much more demanding, (b) the genome to be cloned was approximately twice as long, and (c) it was necessary to provide a promoter for residing DNA-dependent RNA polymerase. Nevertheless, due to some key properties similar to those outlined for Qbeta, progeny virus could be rescued spontaneously after introduction either of the properly constructed DNA or of in vitro transcribed RNA into host cells. This pioneering work triggered the rescue of a very large variety of plus-strand RNA viruses infecting vertebrates, invertebrates, plants, and microorganisms. Even coronaviruses, the largest RNA viruses with genomes in excess of 30 kb are now amenable to genetic manipulation. Very similar experimental techniques could be employed for all positive-strand RNA viruses, despite their vastly differing propagation mechanisms.

Attenuated poliomyelitis virus was also the first RNA virus to be employed as a vector to create plurivalent vaccine candidates by introduction of foreign genetic material (Andino et al. 1994). Vector use of most positive-strand RNA viruses suffers, however, from two major drawbacks. On one hand, the size of the introduced coding sequences is limited because of the space constraints in icosahedral viral capsids. On the other hand, the additional sequences introduced often survive in the original form only for a few viral generations. This was particularly evident for Qbeta and other RNA bacteriophages, which eliminate and/or modify added or altered sequences at an incredibly fast rate. The underlying reason is the strict requirement of suitable secondary and tertiary RNA structure for optimal RNA replication by the notoriously error-prone RNA-dependent RNA polymerases, usually defined as replicases. RNA structure is of paramount importance in all plus-strand RNA viruses, the genomes of which in the active state inside their host cells are naked or only loosely associated with proteins. In particular, deletions are introduced in their transcripts by recombination due to copy-choice transcription, which comes about by detachment of the replicase associated with the incomplete transcript from the template and reattachment to the template, preferentially at a location complementary to the last nucleotides of the transcript. Since RNA

viruses with deletions of nonessential genome regions replicate more rapidly, such spontaneous mutants soon overgrow the original constructed genomes.

Despite these drawbacks, the genetic stability of some recombinant viruses appears sufficient for practical applications. In particular, alphavirus and flavivirus vectors are promising and have in part advanced to clinical trials. Among the former, Semliki Forest, Sindbis, and Venezuelan Equine Encephalitis viruses can be mentioned (Atkins et al. 1996; Tubulekas et al. 1997; Polo et al. 1999; Pushko et al. 1997). Among the latter, yellow fever virus based on the licensed attenuated vaccine strain (Arroyo et al. 2004; Monath et al. 2003; Brandler et al. 2005) seems particularly promising.

Negative-Strand RNA Viruses

Two distinctive features of all negative-strand RNA viruses render them inherently more difficult to rescue from cloned DNA:

1. Their genomes, which are complementary to sense strands, are not active as messenger RNA to produce the viral proteins, but are merely functional as templates required for the formation initially of mRNAs (transcription step) and subsequently of antigenomic RNA (replication step 1), which then acts as template for the synthesis of genomic RNA (replication step 2). Both transcription and replication are mediated by the viral RNA-dependent RNA polymerase, also defined as transcriptase/replicase. The transcription step is characterized by the formation of less than full-length transcripts, which are typically both capped and polyadenylated, whereas in the replication steps unmodified full-length transcripts are synthesized.
2. The genomic RNA is only biologically active when it is present as ribonucleoprotein (RNP), i.e., associated with at least a nucleocapsid protein (called N or NP) and the viral RNA polymerase. In other words, the minimal infectious unit consists not merely of RNA, but of a specifically associated complex, the RNP. In the case of highly segmented viruses such as Influenza viruses, the polymerase is composed of three polypeptides. In *Mononegavirales*, it consists of only one very large polypeptide (L), frequently associated with a phosphoprotein (P), which fulfills multiple functions in addition to its role as polymerase cofactor, in particular as a chaperone mediating the attachment of N to genome or antigenome RNA as they are being synthesized. Thus, for rescue of virus from cloned DNA, it is necessary to deliver RNP to the host cells by one of two approaches. In vitro-made transcripts must be functionally covered in vitro by the viral proteins mentioned above before introduction into host cells. Alternatively, transcripts, on one hand, and encapsidating proteins (in large quantities) and viral RNA polymerase (in tiny amounts), on the other hand, have to be concomitantly synthesized in the host cells to form biologically active RNPs.

Negative-Strand RNA Viruses with Highly Segmented Genomes

Almost two decades ago, the first partial rescue of a negative-strand RNA virus from cloned DNA was described (Luytjes et al. 1989; Enami et al. 1990). Since the RNP of segmented negative-strand RNA viruses is structured relatively loosely, artificial in vitro transcripts of one cloned segment of an Influenza virus strain X could be encapsidated in vitro by the proteins required for RNP formation (nucleocapsid and the three large polypeptides forming together the viral RNA polymerase). When this in vitro-formed RNP was introduced into cells infected with Influenza virus strain Y used as helper virus, reassortants of Influenza virus could be isolated containing seven segments of the helper virus Y and one segment derived from strain X. However, a method was required to separate the small number of reassortants away from the large excess of nonreassorted helper virus. This was achievable by means of specific antibodies directed against the protein encoded by the segment of strain Y to be replaced.

Although this work not only constituted the first breakthrough in this area, but also permitted the generation of some interesting designed Influenza virus mutants, experimentation was limited, since the necessity of a counter-selection step restricted the range of rescuable manipulated constructs. One decade of intensive work by several groups was required to elaborate a method enabling the rescue of Influenza virus entirely from DNA, i.e., without a helper virus (Neumann et al. 1999; Fodor et al. 1999). This method, using a large array of simultaneously cotransfected plasmids, specifying the eight antigenomic RNAs as well as the mRNAs for production of the proteins required for RNP formation, allows rescue of practically any desired Influenza virus compatible with the required biological properties of this class of viruses.

Negative-Strand RNA Viruses with Nonsegmented Genomes

Preparatory Work Using Minireplicons

Numerous research groups attempted, but invariably failed, to assemble in vitro RNPs of *Mononegavirales*, following or modifying the lessons provided in the initial accounts describing the rescue of Influenza virus. There are three main reasons for this failure: first and foremost, the RNPs of *Mononegavirales* are much tighter and more rigid than those of segmented Influenza virus (indeed, in contrast to the latter they are completely resistant to RNase digestion). Second, the RNA genomes to be covered are much longer than the one Influenza segment being encapsidated by the original Influenza virus rescue procedure. Third, the extremely large and multidomain RNA polymerase (L) of *Mononegavirales* is more prone to nonfunctional aggregation than the three subunits of the Influenza enzyme. Consequently, many investigators reasoned that genomes or antigenomes of *Mononegavirales* should be encapsidated inside cultured cells in statu nascendi, i.e., while they are

being synthesized artificially from a cDNA template by a foreign DNA-dependent RNA polymerase expressed in the host cells. Indeed, soon after the successful rescue of Influenza viruses, the first steps toward rescue of *Mononegavirales* were achieved: RNA minireplicons of certain members of this viral order triggered from cloned DNA were propagated in various cell lines. The DNAs underlying these minireplicons were initially derived by reverse transcription from natural defective interfering particles (DIs) generated spontaneously when *Mononegavirales* are propagated in serial succession at high infection rates. Later, minireplicon DNAs were constructed that minimally contained the noncoding 5′- and 3′-terminal signal sequences essential for virus-specific RNA replication; the viral genes were either partially or completely deleted and replaced by reporter genes for convenient detection of virus-specific transcription and replication (Collins et al. 1991, 1993; Park et al. 1991; Conzelmann and Schnell 1994; Sidhu et al. 1995). Investigators used two kinds of helper viruses to provide the proteins to intracellularly encapsidate and replicate these minireplicons. In a simple approach, in vitro transcripts from the small cloned DNA segments were introduced into cells infected with the cognant parent nondefective virus. In a more advanced strategy, the DNA specifying the minireplicons were transfected, along with DNA encoding the encapsidating proteins nucleocapsid (N), phosphoprotein (P), and the large polymerase (L) required for formation of biologically active RNP, into cells infected with a Vaccinia virus construct (vTF7–3), engineered to express T7 RNA polymerase at high levels. All DNAs contained T7 promoters; thus, this strategy allowed efficient transcription of all virus-specific cloned DNA segments in the transfected cells.

First Rescues Employing Helper Viruses

Obviously, the goal of these experiments was to find optimal conditions to achieve replication of the much longer genomes of the full-length nondefective viruses, and then to isolate spurious amounts of assembled rescued viruses away from the helper viruses present in large quantities. This was first achieved for rabies virus (RV) (Schnell et al. 1994). Using almost identical technical approaches, approximately 1 year later, two groups were able to rescue vesicular stomatitis virus (VSV) (Lawson et al. 1995; Whelan et al. 1995). Both RV and VSV belong to the family of *Rhabdoviridae*, the members of which are characterized by a sturdy bullet-shaped virion structure, and by highly efficient, vigorous replication achieving high viral titers. Furthermore, their replication/propagation is only scarcely inhibited by the vaccinia-T7 helper virus. Thus, the tiny amounts of rescued virus could be isolated by a series of elaborate purification steps, including passage through filters.

Rescues Relying Exclusively on Transfected DNA

Although the pioneering work described in the previous section established that functional RNPs of entire genomes of *Mononegavirales* can indeed be formed

intracellularly, this approach necessarily had to fail in the rescue of measles virus (MV), a representative of the family *Paramyxoviridae*, which contains larger genomes, replicates to titer orders of a magnitude lower than RV and VSV, and forms large polyploid virions that are easily inactivated by chemical and physical stress such as filtration. Therefore, it appeared desirable to develop a rescue method avoiding helper viruses altogether, thus obviating the need for a purification step. This was achieved by relying only on transfected plasmids encoding N and P proteins as well as T7 RNA polymerase; transcription of these artificial mRNAs was governed by strong CMV promoters recognized by cellular RNA polymerase II residing in nuclei, rather than by T7 promoters. An intron and a polyadenylation sequence in the transcribed region of the plasmids was inserted to ensure capping, splicing, and polyadenylation, thus enabling efficient export to the cytoplasm as well as a long half-life of the mRNAs. It was clear that, despite these precautions, expression from a plasmid would allow a much weaker expression of T7 RNA polymerase than that achievable with vaccinia-T7. However, in the strategy chosen, the plasmids encoding the viral proteins N and P, which are both required in large quantities, did not rely on T7 promoters, as in the procedure enabling rescue of *Rhabdoviridae*. Thus, a relatively low level of T7 RNA polymerase was thought to be sufficient for the synthesis of antigenomic RNA and the mRNA encoding the large viral polymerase, which is required only in tiny amounts. It should be mentioned that, to maximize the chances of success, initially an approach involving two separate steps was employed. First, helper cells were stably transfected with the three plasmids endowed with CMV promoters encoding all three proteins: N, P, and T7 RNA polymerase. The plasmid encoding T7 RNA polymerase contained a neomycin (Neo) resistance marker, and among approximately 100 neoresistant colonies, two were chosen to propagate lines of helper cells, which, in addition to T7 RNA polymerase, express both N and P at appreciable levels. (These helper cell lines are still being used today by several research groups). Second, the helper cells were transiently transfected with two plasmids containing T7 promoters. One specified precisely initiated and terminated minireplicon (and later full-length antigenomic) transcripts; the other, used in very small amounts, encoded the large viral RNA polymerase L.

This approach triggered high levels of chloramphenicol acetyl transferase (CAT) in cells transfected with artificial minireplicon DNA bearing a CAT reporter gene, similar to the level that had been obtained by introducing the in vitro transcripts of CAT minireplicon DNA into MV-infected cells. In further experiments, successively longer viral antigenomic regions were added to the minireplicon sequence, giving rise to what is termed midireplicons. As can be expected, the CAT signal elicited became weaker with increasing midireplicon size. Finally, all of the missing antigenomic sequence was added, leading, at the end of 1994, to the first rescued MVs, which were clonally derived from single syncytia, thus constituting an additional benefit in comparison to rescues using a helper virus that yields mixtures of different clones (see Fig. 1A). This virus was denominated MV Edtag, as it had been tagged by three nucleotide exchanges in a nontranslated region, to distinguish it from the parent attenuated vaccine-derived MV Edmonston B type strain passaged extensively in many laboratories. However, it took more than 6 months before

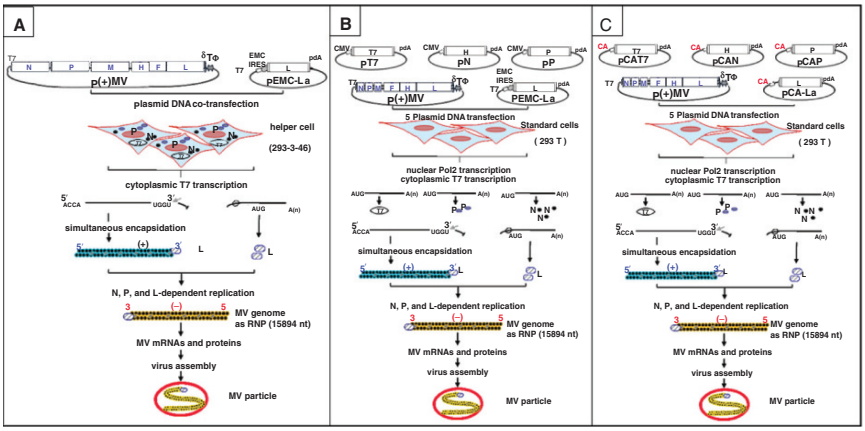

Fig. 1 **A–C** Rescue methods for MV strains and recombinant MVs. **A** Rescue as first described (Radecke et al. 1995), using as helper cells a clone of 293 cells (293–3-46) stably transfected with expression plasmids encoding T7 RNA polymerase, N and P proteins. Note that the intracellular T7 antigenomic transcripts specified by the p(+)MV type plasmids start precisely at the antigenomic 5' end and are cleaved automatically by the hepatitis ' ribozyme sequence after termination at the T7 termination signal T'. **B** Slightly modified rescue protocol: simultaneous transfection of 293 cells or any other cell line supporting replication of MV, using exactly the same five plasmids used in the original method, where the plasmids pT7, pN, and pP had been utilized to generate the 293–3-46 cell clone. **C** Similar method as in **B**, but using the highly efficient chicken actin (CA) promoter rather than the cytomegalovirus (CMV) promoter (Martin et al. 2006)

a patent application was filed, and almost 1 year for the first original research paper to be published (Radecke et al. 1995), since all experimental procedures had to be verified by different collaborators, and the genetic stability of rescued viruses had to be ascertained by adding marker genes. Even chimera were constructed where the envelope proteins of MV were genetically substituted by the unique envelope protein of another virus, VSV (Spielhofer 1995; Spielhofer et al. 1998).

A comment regarding all rescue procedures in general is necessary here. It might appear odd to artificially express antigenomic rather than genomic RNA, since this requires a replication step to yield genomic RNP, from which transcription to produce all viral mRNAs and subsequently all viral proteins can take place. However, several research groups independently adopted this strategy, which circumvents hybridization and thus inactivation of artificially expressed antigenomes with the large amounts of artificially expressed mRNA encoding N and P.

Methodological Refinements Allowing Experimentally Straightforward Rescue of Virtually All *Mononegavirales*

Soon after the publication of RV rescue, the so-called MVA-T7 virus was constructed, based on the modified virus Ankara (MVA) strain of vaccinia virus. This

recombinant expresses T7 RNA polymerase at levels similar to those produced by the previously available helper virus vTF7-3. Importantly, however, the MVA variant replicates only up to a certain point in mammalian cells, and forms progeny virus only in avian cells. Thus, use of this defective helper virus, circumventing the elimination of progeny helper virus, was a second means for the rescue of the large majority of constructed *Mononegavirales* that are not obtainable by the original procedure enabling the rescue of *Rhabdoviridae*. Indeed, shortly after publication of the independent methodology described above, the rescue of human respiratory syncytial virus (RSV) employing MVA-T7 was reported (Collins et al. 1995). Numerous publications describing the rescue of different genera of *Mononegavirales* with this method, which is actually a variant of the rhabdovirus rescue procedure, appeared after this account. It should be mentioned that a further complex method only loosely similar to the RV and VSV rescue procedure, employing vTF7-3, but a completely different purification approach, applicable only for rescue of Sendai virus (SeV) and a few particular representatives of *Paramyxoviridae*, was published (Garcin et al. 1995) concurrently with the first rescue of RSV (Collins et al. 1995).

It is worth noting however, that all methods based on vaccinia-derived helper virus, regardless of whether these form progeny during the rescue procedure, suffer from several important drawbacks, the most important being the high rate of recombination induced by vaccinia virus among the transfected plasmids, which leads to frequent incorporation of sequences residing in the plasmids encoding N and P protein into the antigenomic plasmid and thus into the rescued virus. Therefore, several of the most proficient investigators in the field later switched to procedures similar to the one described above, exclusively using expression from plasmids, thus circumventing the need for a helper virus. The first published account in this line was used for the rescue of bovine RSV (Buchholz et al. 1999).

In this context, it should also be mentioned that, curiously even in 2001, Yusuke Yanagi's group described a protocol for rescuing wild-type MV strains (Tatsuo et al. 2001), which is based on the replication proficient vaccinia-T7 strain vTF7-3 also used originally for the rescue of RV. However, it should be noted that the Yanagi group used a very particular cell line shown earlier to be suitable for isolation of wild-type MV strains from acutely infected individuals, the marmoset B cell derived line B95a expressing SLAM, the receptor exclusively used by most wild-type MV strains. In this cell line, the helper virus vTF7-3 replicates exceedingly slowly and forms only a small number of progeny. Thus, the experimental condition closely resembles that using the replication-deficient MVA-T7 helper virus as discussed in the preceding paragraph.

Advantageous modifications of the original method for MV rescue involved a gradual shift from the use of persistently transfected helper cells to only transiently transfected cell lines. The modifications are particularly advantageous when new viral species are to be rescued from cloned cDNA, since they circumvent the tedious selection of cellular clones expressing, in addition to T7 RNA, the N and P proteins of the new viral species at suitable ratios. Standard cell lines able to support replication of the new virus are transfected simultaneously with all five required plasmids. (See Fig. 1B. Note that for MV rescue, the exact original transient transfection

procedure is utilized, varied only by inclusion of the three plasmids expressing T7 RNA polymerase and the viral proteins N and P, which in the original set-up are already present in the persistently transfected helper cells.) The rescue efficiency using only one-step transient transfection is higher than in the original method and is enhanced further when instead of CMV promoters chicken actin promoters (Martin et al. 2006) are used (compare Fig. 1B and C). This principle was first adopted for the rescue of mumps virus (Clarke et al. 2000) and later for many manipulated representatives of *Mononegavirales*, as described in great experimental detail in a recent international patent application (WO 2004/113517 A2).

An additional simplification of MV rescue has recently been described utilizing a CMV promoter for the antigenomic expression plasmid, recognized by the nuclear polymerase II, rather than the traditional T7 promoter (Martin et al. 2006). The reason for trying this strategy was actually dictated by Borna virus (BV) rescue. BV replicates in the nucleus; therefore, it was reasonable to test pol I and pol II promoters, and the latter proved to be optimal. The finding that this strategy was also successful for MV suggests that MV RNP can be formed in the nucleus concomitantly with the synthesis of antigenomic RNA by pol II, thus impeding splicing at fortuitous splice sites, and that RNPs then reach the cytoplasm rather efficiently. RNP formation in the nucleus appears likely attributable to the fact that N protein, when artificially expressed alone, migrates efficiently to the nucleus (Huber et al. 1991) and likely also carries along some associated chaperone P when both proteins are simultaneously expressed.

Genomic Modifications of Existing Measles Virus Strains

As mentioned above, there was one major driving force behind the development of systems enabling reverse genetics for *Mononegavirales*: to gain better insight into the biology of the representatives of this viral order, as more comprehensively illustrated for MV in other chapters of this book. Since this chapter mainly deals with the possible practical applications enabled by these techniques, only some of the first designed artificial mutants of MV are mentioned here.

The first of these modifications consisted in a large deletion of 504 nucleotides, almost the entire 5′ untranslated region (UTR) of the F gene (Radecke et al. 1995), which is very GC-rich. In cell cultures, this mutant did not show any deterioration in propagation. No experiments in the animal models described in other chapters (ferrets, cotton rats, transgenic mice, and macaques) have been conducted to date, which might demonstrate that the deleted region is indeed dispensable for viability in infected model animals, and thus likely also in humans. (It should be noted, however, that in human thymus/liver implants engrafted in SCID mice, this mutant replicated somewhat more slowly and the virus titer reached was roughly ten times lower than that of the parent MV Edtag; Valsamakis et al. 1998.) Also, in the F gene of other representatives of the genus morbillivirus, relatively long 5′ UTRs exist, although none with a comparable length. Recently, this region as well as the preceding long 3′ UTR of the M gene have been reexamined in different cell lines, and

it has been concluded that the former decreases F protein production, thus inhibiting MV replication and reducing cytopathogenicity, whereas the latter increases M production and promotes virus replication (Takeda et al. 2005). One can speculate that the F protein in predecessors of MV might have been much longer; when a shorter functional version of F protein arose, the remaining untranslated region, rather than being eliminated as would be dictated by the maximal compaction of genetic material in RNA viruses, became the target of biased G/I hypermutation (Cattaneo et al. 1988; Bass et al. 1989), resulting in replacement of U by C residues in the genome. (This type of massive mutational alteration has been observed frequently in defective M genes of MV triggering subacute sclerosing panencephalitis SSPE; Cattaneo et al. 1988, 1989b; Cattaneo and Billeter 1992.) Maintenance of superfluous sequences in MV might only be a somewhat realistic hypothesis in view of the virtual absence of recombination by copy choice and by the obligatory adherence to the rule of six, hallmarks of all representatives of the subfamily *Paramyxovirinae*. (For a discussion of this rule, see the following chapter.)

Another site-directed mutation was the inactivation of the C reading frame in the P gene (Radecke and Billeter 1996). This mutant propagated normally in Vero cells, most likely due to the absence of interferon type I response in these cells. This view is supported by later studies with Sendai virus, showing that ablation of C-type proteins leads to heavily compromised viruses, since Sendai C-type proteins interfere with interferon responses. In addition, these proteins are involved in transcription/replication (Delenda et al. 1998; Garcin et al. 1999, 2000, 2001, 2002). The MV C-mutant replicated poorly in human peripheral blood cells (PBMCs) (Escoffier et al. 1999) as well as in human thymus/liver implants in SCID mice (Valsamakis et al. 1998), suggesting a role of C protein also in the transcription/replication of MV.

Ablation in MV Edtag of the V protein, which arises in all naturally occurring and attenuated MV strains by co-transcriptional editing (Cattaneo et al. 1989a), or overexpression of V showed no impairment in the interferon-deficient Vero cell line (Schneider et al. 1997), nor in PBMCs (Escoffier et al. 1999), suggesting that V is not essentially required for transcription/replication. Interestingly, the V^- mutant multiplied more slowly and the V overexpressing mutant more rapidly than the parent virus in human implants in SCID mice (Valsamakis et al. 1998). Furthermore, C^- and V^- mutants appear to be overattenuated, since they multiplied poorly in $Ifnar^{ko}$/CD46Ge transgenic mice, which express CD46 and are devoid of the interferon type I response (Mrkic et al. 1998). However, all these results must be interpreted with caution; in the MV Edtag strain the C-terminal portion of V protein deviating from the P protein is already defective to begin with, by replacement of important amino acids (Cys272Arg and Tyr291His) conserved in all approved MV vaccine strains (Combredet et al. 2003). Very recent studies with wild-type MV and derivatives lacking V and or C in macaques indicated that the defective MVs replicated less extensively, because they were more restricted by the interferon and inflammatory responses, whereas antibody and MV-specific T cell responses were equal for wild-type and variant viruses (Devaux et al. 2008).

The function of the matrix protein M was addressed by either eliminating most of the M reading frame (Naim et al. 2000) or by replacing the M frame in MV

Edtag by a hypermutated M reading frame recovered from the brain of a deceased SSPE patient (Patterson et al. 2001). These mutant viruses were able to replicate, albeit very slowly, in cultures of polarized cell lines derived from lung epithelia, as well as in the brain of transgenic mice expressing CD46, and in primary brain cell cultures. The experiments also showed that migration and co-localization of N and the envelope proteins F and H to particular cell compartments depend on M (Naim et al. 2000), directly demonstrating the interaction of M with nucleocapsids on one hand and with the cytoplasmic tails of the envelope proteins on the other.

Also, replacements in the MV envelope proteins were studied by directed mutations of MV strains. Replacement of single amino acids in the H protein of wild-type MV able to use only SLAM for cell entry also gained the ability to utilize CD46, the generic second receptor recognized only by attenuated vaccine strains (Erlenhofer et al. 2002). For the F protein, it has been shown that the single replacement of Leu at position 278 in the AIK-C vaccine strain by Phe, which is typical for the Schwarz/Moraten strain, resulted in enhanced syncytium formation, a distinguishing feature between the two vaccine strains (Nakayama et al. 2001).

Measles Viruses with Inserted Foreign Coding Sequences

Technical Aspects of Foreign Gene Expression

Additional transcription units (ATUs) can be inserted rather easily into the MV antigenomic sequence by placing short sequence stretches embracing an intergenic region into the 3′ nontranslated region of a resident MV gene. Addition of a cassette of unique restriction sites not present in plasmids bearing antigenomic MV sequences then allows insertion of foreign reading frames (see Fig. 2 for an overview).

Care has to be taken that the final constructs obey the rule of six, which states that the number of nucleotides from the genomic 5′ to the 3′ terminus has to be an integral multimer of six, i.e., that the genome has to be precisely covered by N molecules, each contacting six nucleotides. This rule was first found to be valid for SeV copy-back minireplicons (Calain and Roux 1993) and then for constructed internal deletion/replacement MV minireplicons (Sidhu et al. 1995). Later, adherence to this rule was shown to be a strict requirement for full-length recombinant MV as well (Rager et al. 2002) and parainfluenzavirus type 2 (Skiadopoulos et al. 2003b) and is likely imperative for all representatives of the viral subfamily *Paramyxovirinae*. (Whenever an antigenomic cDNA construct deviates by mutation from this rule, viruses can only be rescued if they bear a second mutation preferably close to the first one, correcting the deviation.) Additional ATUs have been inserted first after the P and the H genes (Singh et al. 1999), then also before the N gene (Hangartner 1997; Zuniga et al. 2007), and recently also after the L gene (del Valle et al. 2007).

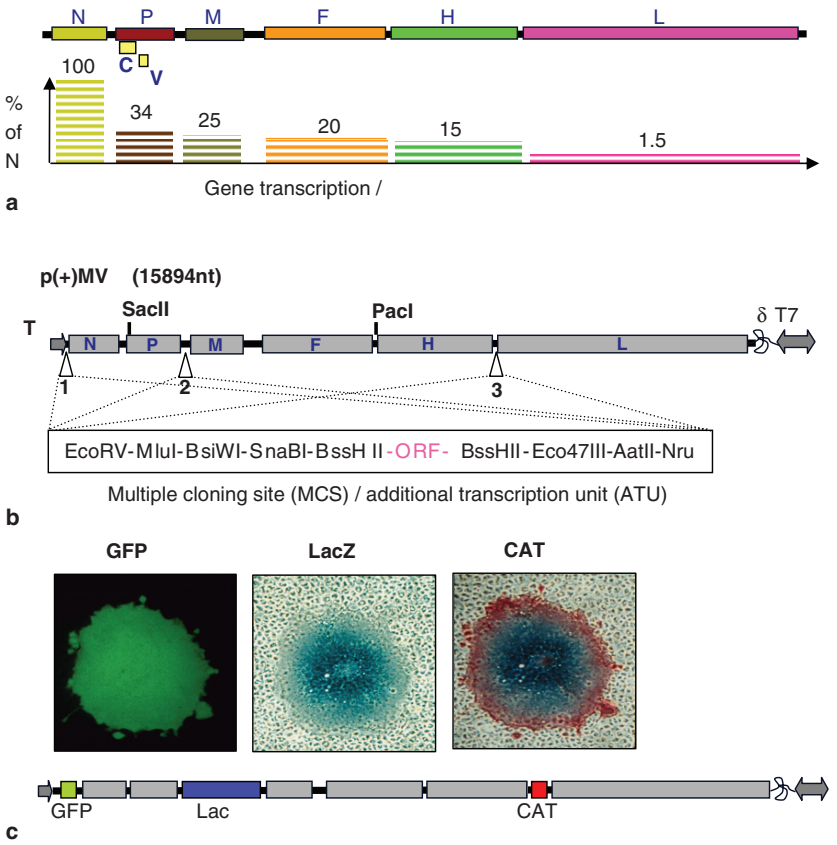

Fig. 2 Strategy for inserting transgenes into the MV backbone. **A** The MV antigenome is schematically shown, and the relative transcript levels of the six MV genes are indicated. **B** In three positions, 1 (upstream of the N gene), 2 (downstream of the P gene), and 3 (downstream of the H gene), additional transcription units (ATUs) have been inserted into antigenomic plasmids, each containing a transcription start signal, a multicloning site (MCS) embracing an open reading frame (ORF), and a termination/polyadenylation signal (Hangartner 1997; Wang et al. 2001). **C** Three different staining patterns of one syncytium is shown, induced by a triporter MV containing an eGFP ORF in position1, a beta-galactosidase (LacZ) ORF in position 2, and a CAT ORF in position 3 (Zuniga et al. 2007)

Two other approaches to express additional foreign coding sequences were also explored with the aim of ensuring that the inserted reading frames remain functionally intact, i.e., cannot be mutated without inactivating the recombinant virus. In particular, generation of viruses containing premature stop codons by the error-prone viral RNA polymerase during repeated viral passages were to be avoided. In one approach, the foreign reading frame was linked to a downstream resident MV reading frame by a stop/restart signal, by overlapping a stop codon with an adjacent initiation codon [UGAUG], as featured by influenza B viruses for the expression of the downstream NS reading frame. In the second approach, the protein self-cleavage featured by foot and mouth disease virus was adopted by

linking the upstream foreign coding region with a downstream MV coding region through a stretch of nucleotides generating a very short polypeptide able to cleave almost exactly upstream of the MV protein (Ryan and Drew 1994). By this design, the transgenic protein should bear a C-terminal elongation of 17 amino acids, whereas the MV protein should bear a single foreign amino acid at its N-terminus. In both approaches, the inserts were placed upstream of the L gene, which has to be expressed only at low levels. The first method did not lead to rescuable viruses, whereas the second approach did work and might actually be employed in particular cases (L. Martin, unpublished results). However, since in a large variety of recombinant MVs mutational formation of premature stop codons was not found to actually be a problem, this approach has not been further explored in practice, at least for the time being.

Insertion of Genes Encoding Markers and Immunomodulators

It was convenient to insert reading frames encoding various marker proteins for monitoring the location of MV replication in cells (various primary cell types from infected model animals and cell lines) and in the organs of infected animals. Furthermore, the functionality of inserted ATUs can be readily ascertained, and the reading frames encoding markers can then conveniently be replaced by coding sequences of medical relevance. The most frequently used markers, apart from luciferase, are shown in Fig. 2, which also illustrates the insertion sites and the levels of expression. The most frequently used marker is green fluorescent protein (GFP) and its various derivatives (Spielhofer 1995; Duprex et al. 1999a, 1999b; Naim et al. 2000). In numerous publications not mentioned here, MV recombinants expressing markers have been used for a large variety of purposes.

Since GFP readily diffuses away from the site of its production, several attempts have been made to incorporate GFP into viral proteins, to directly visualize their location and viral assembly. Insertion into strongly expressed MV proteins has not yielded reasonably viable viruses, but rather surprisingly, one insertion into the large polymerase L was successful (Duprex et al. 2002).

Expression of cytokines, mainly to act as adjuvants to enhance the immunogenicity of vaccines, have been used in various systems. With MV, so far only IL-12 has been employed, in an attempt to completely eliminate the immunosuppression, which is the hallmark of wild-type MV infection and also characterizes vaccine strains to some extent. Functional IL-12 was expressed at high levels from inserts containing the coding regions for the two components p35 and p40 separated by an internal ribosome entry site (Singh and Billeter 1999). However, in macaques this recombinant virus failed to eliminate immunosuppression, although both humoral and cellular immune responses were altered (Hoffman et al. 2003).

Insertion of Genes from Other Pathogens: Candidate Multivalent Vaccines

Reading frames encoding proteins from other pathogens have usually been inserted into the MV context by replacement of the marker genes described above. MVs with either single or multiple insertions have been constructed, as shown only for a few examples in Fig. 3 (Zuniga et al. 2007). Most of the recombinants show replication properties in cell lines very similar to the parent empty MVs; usually the speed of propagation is only slightly delayed and the same end titers are reached. For the purposes of vaccine production, the slight delays are not counterproductive, but in the vaccinated host, a delay in propagation might result in a considerably reduced immunogenicity of the vaccine, which, however, could be counterbalanced

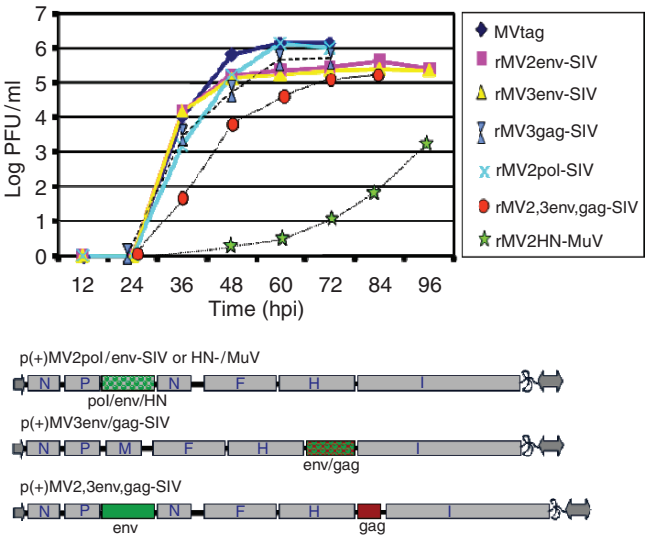

Fig. 3 Growth kinetics of selected recombinant MVs with one or two ATUs containing ORFs derived from other pathogens. Recombinant MVs expressing one or two ORFs of SIV or mumps virus were grown in comparison to the parental MV under the same physiological conditions. Growth is plotted as a function of infectious viruses released (y-axis) and time (x-axis). Experimental conditions were as described previously (Wang et al. 2001; Zuniga et al. 2007). The cloning position of all antigens is illustrated in the *lower panel*. Abbreviations: *tag* Parent empty tagged MV (without ATU), *rMV2env* or *rMV2pol* MV with a single ATU containing SIV full-length (anchored) envelope ORF or polymerase in position 2, *rMV3env* or *rMV3gag* ATU of SIV env or group-specific antigen (gag: capsid proteins) ORF in position 3, *rMV2,3env, gag* MV with 2 ATUs, containing a SIV envelope ORF in position 2 and a SIV capsid ORF in position 3, *rMV2HN-MuV*MV with a single ATU containing mumps virus hemagglutinin/neuraminidase ORF in position 2

by using higher viral doses. Given that the approved MV vaccines are administered in low doses ranging between 10^3 and 10^4 plaque forming units, this would be perfectly feasible in practice. Multiple inserts do not generally lead to more slowly growing recombinants than single inserts; the position of the inserts and the length and quality of the expressed additional proteins are much more important. Inserts at position 1 upstream of the N gene are most delaying, particularly if they are long. Glycoproteins targeted to the endoplasmic reticulum (ER) are usually more problematic than proteins remaining in the cytoplasm; furthermore, membrane-anchored glycoproteins are more deleterious than excreted ones, probably because they tend to compete with MV envelope proteins. Nevertheless, only in one instance, when MV with inserts derived from mumps virus (MuV) have been tested, was the replication delay not tolerable for practical application. This was most likely due to competition between MV and MuV glycoproteins in the assembly of viral particles (Wang et al. 2001). As evident from Fig. 3, the initial slow propagation of the recombinant expressing MuV H later resumed a speed similar to empty MV; however, the more rapidly growing virus turned out to have lost MuV H expression.

Obviously, the quality of the MV backbone is of paramount importance for the viability and thus the immunogenicity of the recombinants in vivo. As mentioned above, the MV Edtag is mutationally altered and thus exposed to the interferon type I response. (Other mutations than those in V as mentioned above, mainly in the P/C/V and the L gene, might also cripple this virus.) When tested in Ifnar^{ko}/CD46Ge mice (Mrkic et al. 1998), where the IFN-α/β receptor is inactivated, the recombinants based on this laboratory strain replicate efficiently and are reasonably immunogenic. However, in macaques, even high doses of this strain are poorly immunogenic, in contrast to the licensed Schwarz vaccine strain, which elicits strong humoral and cell-mediated immune responses (Combredet et al. 2003). It should be kept in mind that prior to 2004 in none of the published vaccination studies cited below were MV recombinants based on licensed vaccine strains used. Today, two groups (see Lorin et al. 2004; del Valle et al. 2007) use recombinants based on the Schwarz/Moraten strain obtained from different sources. (It must be recalled that the sequences of Schwarz and Moraten strains are identical, despite the reported different attenuation regimen; Parks et al. 2001a, 2001b.) Conversely, vectors based on licensed Edmonston Zagreb (EZ) vaccine strains have been developed and are being employed in several studies (H.Y. Naim et al., unpublished results).

Below, some of the pathogens addressed by recombinant MVs as described in published reports are addressed individually. In addition, a variety of projects are underway, addressing among others malaria, human papilloma virus (HPV), and avian influenza virus.

Hepatitis B Virus

Hepatitis B virus (HBV) was the first pathogen against which candidate MV-based vaccines were developed (Singh et al. 1999). The recombinant expressing the small

surface antigen (HBsAg) proved to be genetically stable for at least ten serial viral transfers, a criterion subsequently checked routinely for all recombinant candidate vaccines, and induced strong humoral immune responses in Ifnar^{ko}/CD46Ge mice. A recombinant with inserts encoding both the surface and the core antigen from different cassettes in a single MV vector was also shown to express both proteins. However, this project was discontinued since development of a new HBV vaccine was deemed not to be commercially promising given that efficient vaccines against HBV already exist and that attenuated MV vaccine is not efficient in infants below the age of 6 months, whereas in developing countries chronically HBV-infected mothers usually transfer the virus to their newborns. Nevertheless, in a different laboratory, recently MV-vectored HBV vaccine candidates expressing the HBsAg have again been constructed (del Valle et al. 2007). The same insert as used previously was placed in a MV Schwarz-derived vector after the P, H, and L genes. In Ifnar^{ko}/CD46 mice, the recombinants induced antibodies; as expected, the vectors with the P and L cassettes elicited the highest and the lowest immunogenicity, respectively. In macaques, only two of the four animals inoculated with the P cassette vector generated a humoral anti-S response. It was concluded that for optimal immune responses against the insert antigens, it is important to choose the strongest expression compatible with reasonably high replication speed of the MV recombinant, or that repeat vaccinations with the same recombinant, or prime-boost regimens using either protein with adjuvant or other viral vectors should be adopted. It is noteworthy that the recombinants protected the macaques as efficiently against challenge by wild-type MV as the commercial vaccine.

It might be speculated that optimal MV-vectored HBV vaccine could even be used therapeutically for the very large number of chronically HBV-infected patients present mainly in Southeast Asia.

SIV/HIV

The target of most studies using MV vectors has been AIDS, which is arguably the most important infectious disease against which no vaccine exists as yet. Due to the very favorable record of attenuated MVs in terms of both efficacy and safety, as discussed elsewhere in this book and also in the next principal chapter, MV-vectored vaccines are among the strongest candidates for final success against AIDS. The first MV-based attempts against AIDS involved SIV antigens (Wang et al. 2001). In two laboratories, a variety of different reading frames encoding Gag, Pol, Env, and Nef derived from SIV and HIV have since been inserted, either isolated directly or manipulated in various ways, e.g., as resynthesized consensus sequences or with deletions and/or fusions. In view of the high variability of HIV, particularly env has been manipulated in different ways, to get rid of highly variable regions and thus to expose mainly conserved epitopes. In an interesting early study, anchored env appeared to be preferable to excreted forms to induce neutralizing antibodies against several heterologous HIV primary isolates and to elicit cell-mediated

immune responses (Lorin et al. 2004). It is noteworthy that, as observed for HBV vaccine, MV preimmunization did not prevent these anti-HIV immune responses, as revealed in Fig. 4. In several recent reviews, the potential of MV vectored vaccines in general, and in particular against AIDS, is discussed (Tangy and Naim 2005; Zuniga et al. 2007; Brandler and Tangy 2007).

Flaviviruses: West Nile Virus, Dengue Virus

Among the human diseases caused by members of the family *Flaviviridae*, yellow fever, Japanese encephalitis, Dengue fever, and West Nile (WN) fever are the most important. Efficient vaccines exist against the first two, but morbidity and lethality caused by these diseases is still high in Western Africa/South America and in the Asia-Pacific region, respectively. In contrast, no vaccines are available against the latter two. Thus, as recently reviewed, WN and Dengue fever have been addressed using the MV-Schwarz strain (MVSchw) vector (Brandler and Tangy 2007).

The exodomains of all Flaviviruses have similar structures and are highly immunogenic; thus, rather than expressing the anchored glycoprotein as in the case of candidate AIDS vaccines, only the regions encoding the exodomains of Flaviviruses have been inserted into MV.

First, Western Nile virus (WNV) was addressed, which can efficiently infect mice by peripheral inoculation, causing a lethal neuroinvasive disease similar to encephalitis in humans. The secreted form of the glycoprotein E (sE) was expressed, and low doses of MVSchw-sEWNV protected mice against lethal challenges by WNV at least for a period ranging from 8 days to 6 months after vaccination (Despres et al. 2005). In currently unpublished experiments, the efficacy of the recombinant was also demonstrated in a primate model: squirrel monkeys (*Saimiri sciureus*) were completely protected against WNV by a single-dose vaccination.

Dengue virus (DV) poses a greater problem, as four serotypes exist. The exposed portion of gpE folds into three globular ectodomains ED I, II, and III; the outermost immunoglobulin-like ED III, which can fold independently through a single disulfide bond, determines the serotype specificity and contains the major neutralizing epitopes. Thus, as announced by the Tangy group (Brandler et al. 2007), a construct encoding all four EDIII domains of DV 1, 2, 3, and 4 has been inserted into the MVSchw vector, and immunogenicity studies are ongoing.

SARS Corona Virus

Immediately following the SARS epidemic, and because of the fear that SARS Corona virus (CoV) might be used for bioterrorism, both the nucleocapsid protein N and the codon-optimized spike glycoprotein S of SARS-CoV were inserted independently into position 2 between the P and M gene of MV Edtag, yielding p(+)MV-SARS-N and p(+)-SARS-S. In MV-susceptible Ifnar^{ko}/CD46Ge mice,

the recombinants induced high antibody titers against N and S. The antibodies generated against the S-expressing MV strongly neutralized both SARS-CoV and MV. N-expressing MV elicited a cellular immune response measured by IFN-γ ELISPOT. Also in this case, preimmunization with empty MV did not impair the immune responses against SARS CoV, and a 1:1 mixture of the two recombinants induced responses of a similar magnitude to that of the single vaccines (Liniger et al. 2008).

Creation of Targeted MVs with Oncolytic Properties

The projects to develop recombinant MVs against cancer are extensively discussed elsewhere in this book (see the chapter by S. Russel et al., this Volume). Here it should only be mentioned that these oncolytic viruses are constructed and rescued basically as the recombinants discussed above. Oncolytic properties of MV have been recognized by the early finding that MV infection resolved Burkitt's lymphoma (Bluming and Ziegler 1971). To enhance these properties, numerous alterations have been introduced into the MV derived from vaccines, with the intent of retargeting the recombinants specifically to cancer cells and ablating interaction with the known MV receptors SLAM and CD46. Thus, primarily the H reading frame has been manipulated to create slightly shortened H proteins carrying at their C-termini ligands fitting onto different receptors expressed abundantly by diverse types of cancer cells. The first promising recombinants have been created by the authors of a recent review (von Messling and Cattaneo 2004; for later original publications see Springfeld et al. 2006; Ungerechts et al. 2007a, 2007b).

Development of Segmented MVs Carrying Foreign Genes

One of the most interesting developments concerning manipulated MVs is the recent creation of MVs containing bi- or even tri-partite genomes, denominated 2 seg-MV and 3 seg-MV, respectively (Takeda et al. 2006). The DNA constructs underlying these segmented viruses were designed to bear precisely the 3′ and 5′ terminal untranslated regions present in the parent MV. In addition to the MV genes, the 3 seg-MV could accommodate up to six additional transcription units, and efficient expression of five of the encoded proteins was demonstrated. Importantly, expression of a long insert encoding beta-galactosidase placed upstream of the N gene in a segment comprising only the MV N and P genes impaired the replication of the recombinant much less than beta-galactosidase residing in the same position of a monopartite MV. This suggests that long, multiple inserts could be expressed more efficiently when segmented MVs are used as vectors. The surprisingly efficient propagation can be explained by the fact that the MV

particles grown in cell lines are largely polyploid (Rager et al. 2002), containing far more than one genome. In addition, when relatively short genomes are replicated, the probability of the replicase to quit the template prematurely is lower than on long templates.

Until such segmented MVs can be used for evaluation in practice, much additional experimentation is needed. First, an attenuated licensed MV vaccine strain has to be used rather that the wild-type isolate from which the constructs were derived. Second, a rescue method has to be used employing exclusively transfected plasmids rather than vaccinia-T7 helper virus. Third, for the construction of tripartite virus, each segment should contain one of the genes required for replication, N, P, or L. (Ideally, to fully exploit the potential of segmented MV vectors, one segment should bear only one short transgene upstream of the measles genes encoding M, F, H, and L, whereas two segments encoding N and P, respectively, could bear multiple and relatively long inserts up- and downstream of the MV genes.) Fourth, propagation and immunogenicity in animal models have to be checked in comparison to monopartite MV vectors. These provisos by no means reduce the importance of this pioneering work.

Considerations for Practical Applications of Recombinant Multivalent MV Vaccines

Stability of Recombinant MVs

RNA polymerases are known to be much more error-prone than DNA polymerases due to the lack of proofreading; thus, the error rate in RNA viruses is on average approximately three orders of magnitude higher than in DNA viruses. The so-called quasispecies concept for RNA viruses was explored in an early study of bacteriophage Qbeta (Domingo et al. 1978). Therefore, it came as a great surprise to find that in the MV recombinants the transgenes are rather stably expressed, although they are nonessential and in some cases deleterious for virus propagation and expected to be even more rapidly mutated than the MV genes conserved by positive selection. The expression of the first transgene studied in MV encoding chloramphenicol acetyl transferase (CAT), inserted after the P and H gene, was completely maintained in all analyzed progeny clones derived from single original rescued clones after ten serial virus passages, which represent an amplification factor of 10^{33} (Spielhofer 1995). However, in that experiment, 35% and 100% of the clones derived from second rescued clones serially transferred in parallel had lost CAT expression. It seems likely that the errors already occurred in these second original rescued clones during transcription by the particularly error-prone T7 RNA polymerase. In almost all later rescued recombinants, the transgene expression was maintained. Only the expression of MuV-derived transgenes encoding MuV F and HN was lost, resulting in viruses propagating as fast as empty MV, whereas the freshly rescued recombinants were slow, as discussed above and shown in Fig. 3.

In none of these few cases where transgene expression was lost could any deletion be detected as monitored by PCR, suggesting that recombination by copy-choice must be an extremely rare event, in contrast to plus-strand RNA viruses, which readily delete inserted foreign sequences. Copy-choice events must occur sometimes in MV, as the generation of defective interfering particles (DIs) cannot arise in any other fashion. However, most of the DIs are the copy-back type, where the polymerase, after leaving the original template together with the unfinished transcript, attaches to this transcript rather than to the original template. Virtual absence of deletion events must be due to the extremely tight RNP structure, which renders reattachment of the replicase to its template in another position almost impossible.

Misincorporation of single nucleotides certainly does occur in MV to an appreciable extent, as documented by nucleotide changes in characteristic positions during the attenuation process to obtain vaccine strains (Parks et al. 2001a, 2001b) and by loss of virulence upon passage of wild-type virus isolates in nonlymphoid cells (Kobune et al. 1990; Tatsuo et al. 2000). However, in these cases, the mutational changes resulted in a propagation advantage in the cells used for attenuation and were thus selected for against the original virus. In contrast, loss of expression of a protein which does not interfere with viral propagation is neutral. Nevertheless, it is surprising that ablation of protein expression, which could easily arise by nucleotide exchanges, leading to premature stop codons and thus interruption of the coding region, has not been documented. Single nucleotide deletions and insertions, which could also lead to truncation of the expressed protein, are virtually excluded due to the strict adherence of MV and all other *Paramyxovirinae* to the rule of six discussed above. Missense mutation in transgenes are obviously more difficult to detect than nonsense mutations, and to our knowledge for none of the inserts in MV have such events been excluded by sequencing after ten standard serial viral transfers. However, as long as the antigenic properties of the expressed protein are not severely hampered, in most cases such changes are expected to deteriorate the recombinant vaccine only very slightly.

Efficacy and Safety

The efficacy of vaccination using attenuated MVs is quite remarkable: most vaccinees seroconvert and are then protected for life, at least against clinically manifest MV infection. At present, it is only a hope that MV-expressed single antigens of other pathogens will elicit a comparable strong and durable protection. Nevertheless, it is encouraging that in the first instance where protection of an animal model close to humans has been tested, in the case of MV expressing the exodomain of WNV gpE, a complete protection up to 6 months has been documented in monkeys, as mentioned above (Brandler et al. 2007). Clinical trials with any recombinant vaccine candidate based on MV are only in the planning stage; thus, practical success is still to be awaited for some years to come in view of the severe and costly hurdles imposed by regulatory agencies.

Nevertheless, the potential of MV recombinants as efficient multivalent vaccines has started to be appreciated by increasing numbers of vaccination experts. Even if in particularly difficult cases such as malaria, elicited by several different and variable strains of *Plasmodium*, complete protection cannot be expected, MV recombinants might elicit immune responses comparable to that triggered by the first *Plasmodium* infection that every infant in sub-Saharan Africa contracts. This would already be an important success, since the first infection is responsible for more than 80% of deaths caused by malaria, whereas further *Plasmodium* infections involve much less morbidity. The impact of MV recombinants on AIDS might be considered less optimistically, since a partially protecting vaccine would most likely result in increased negligence of other protective measures.

How does MV compare with other vaccination regimens based on delivery of genetic material? In comparison with plasmid DNA, the efficacy is expected to be much higher, and in terms of safety it is certainly favorable that the danger of integration of genetic material into the human genome is negligible in comparison to delivered DNA or to DNA viruses. This expectation appears valid despite reported integration of cDNA derived from lymphocytic choriomeningitis virus (LCMV) into mouse genomes (Klenerman et al. 1997). An additional advantage is the relatively small size of MV in comparison with DNA viruses used as delivery vehicles; only a few proteins of the vector are expressed in addition to those expressed from the artificial inserts, thus minimizing superfluous immune responses. In fact, the immune responses against the vector must also be considered beneficial, at least as long as MV is not eradicated, a goal of the WHO that will take many years to achieve and might never be reached. (Here it should be mentioned that in the opinion of some medical experts of MV vaccination in Africa, attenuated MV vaccination is generally beneficial for the development of the immune system in infants and therefore should be maintained even if measles is eradicated one day.) In comparison with RNA virus vectors, the high genetic stability of MV is noteworthy, which is shared with the representatives of the viral subfamily *Paramyxovirinae*, all representatives of which adhere to the rule of six, whereas the other *Mononegavirales* are somewhat less stable. In addition, the high possible payload of inserts, unrestricted by viral capsid structure, should be noted.

What are the consequences of MV vaccines being replication-competent? Replication-deficient vectors such as most DNA viruses in use and RNA virus vectors based on alpha-viruses are broadly considered to be generally safer. However, replication-deficiency entails two major drawbacks. On one hand, doses higher by many orders of magnitude in comparison with replicating vehicles have to be delivered. Even more importantly, the range of action is very limited, as only one-step infection of cells bearing suitable virus receptors at the place of delivery is possible. This contrasts significantly with attenuated MV vaccines, which owe their high efficacy to systemic spread and preferential infection of professional antigen-presenting cells and lymphoid tissues. In the case of MV replication, competence appears only as an advantage in terms of safety and efficacy, since MV vaccines

have an extremely good safety record (MV vaccination is recommended even for immunocompromised patients, e.g., HIV), and severe sequelae of wild-type MV infection such as SSPE have been stopped by vaccination. Importantly, cell targeting of MV recombinants is not altered by transgenes (no envelope proteins, except MV-related glycoproteins as those of the MuV envelope have been shown to be incorporated into MV particles; Wang et al. 2001).

Quite generally, it is noteworthy that killed vaccines or subunit vaccines are not necessarily safer than replication-competent vaccines, as shown for killed MV vaccine, which proved not only to lack efficacy, but even exacerbated the effects of subsequent wild-type MV infection. Thus, there is no general increasing gradient of safety when one compares replication-competent versus replication-restricted versus dead vaccines. Any component delivered to humans must be planned, developed, and evaluated with equal care, without a priori bias based on theoretical general and illusive classifications of danger to human health.

Preimmunity: Prime-Boost Regimens

Preimmunity is a concern for the use of recombinant MV vaccines given that (a) almost all adolescent and adult individuals are immune against measles due to prior wild-type MV infection or MV vaccination and (b) maternal antibodies against measles in young infants below 6–9 months of age prevent immunization by attenuated MV vaccines. Therefore, vaccination with recombinant MVs was initially considered exclusively for young infants at an age when MV vaccination is recommended, thus replacing the standard vaccine with a bi- or multivalent vaccine protecting not only against measles, but also against other important diseases such as malaria. Such a regimen is certainly still a preferred option, and care has been taken to ascertain that in animal models various transgenes in MV do not hamper the immunogenicity against measles (Brandler et al. 2007; Brandler and Tangy 2007; del Valle et al. 2007). However, measles immunity can be boosted in children who are preimmune after a first vaccination, particularly when the boost is given as an aerosol (Bennett et al. 2002). Thus, it was probably wrong to assume that the immune reactions against measles in young infants, where the immune system is still immature, is necessarily similar in older children and adults. Since, for example, MV-based AIDS vaccines should be given much later, before sexual activity begins, it was important to ascertain, for the time being in animal models, that preimmunity from vaccination with standard MV vaccine does not prevent a boost with recombinant MV. In fact, boosts resulted in enhancement of immune responses against MV and induction of immune responses against the transgenes (del Valle et al. 2007; Brandler and Tangy 2007; see Fig. 4).

Nevertheless, efficiency of boosting with MV recombinants will probably wane with increasing numbers of boosts. Thus, prime-boost regimens using either subunit

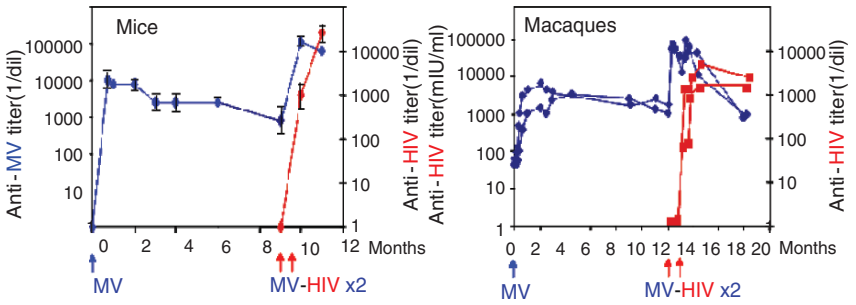

Fig. 4 Induction of immune responses against antigen ORFs in recombinant MV in presence of anti-MV antibodies. Antibody titers against MV (*blue*) and against HIV (*red*) in mice and macaques. *Left panel* A group of six transgenic CD46^{ko}/IfnarGe mice (Mrkic et al. 1998) were preimmunized with 10^5 $TCID_{50}$ of MV Edtag and 9 months later twice with 5×10^6 $TCID_{50}$ of recombinant MV containing the HIV envelope glycoprotein $gp140_{HIV89.6}$ in position 2. *Right panel* Two cynomolgus macaques were preimmunized with a standard human vaccination dose (10^4 $TCID_{50}$) of commercial Rouvax MV, and immunized at months 12 and 13 with the same recombinant utilized for the mice, using 5×10^6 $TCID_{50}$ (Lorin et al. 2004; Tangy and Naim 2005). (Modified with permission from Brandler and Tangy 2007)

vaccines with adjuvants or delivery with other viral vectors may be advantageous in practice. Adenovirus vectors should induce immune responses in newborns already and might close the gap until MV vaccines can be used. A particularly attractive regimen might entail vectors based on attenuated MuV, which, similar to MV, is being used widely for human vaccination. Furthermore, other vectors based on *Mononegavirales* and among those preferentially belonging to the *Paramyxovirinae* should also be considered. Representatives of all genera belonging to this subfamily and thus far unclassified viruses might be explored, as no cross-reactivity between these genera exists. However, attenuated MV and MuV are the only viruses for which extensive experience in humans exists, and are therefore preferred options for vector use, particularly since they are able, at least in principle, to induce life-long immunity.

Impediments Imposed by Regulatory and Commercial Issues

It is sad that the progress toward new vaccines and the improvement of existing vaccines is blocked or severely slowed down by issues other than those based on scientific and technical considerations. This is true in general: just one example is the failure to improve the Sabin vaccine against polio by planned mutations, which would impede reversion to wild-type viruses that repeatedly led to small epidemics. Such impediments are highly regrettable in the case of MV and similar viruses for vector use, which appear promising in the battle against important diseases. Vaccines developed on the basis of such vectors may particularly enhance public

health in developing countries, considering the reasons outlined above and the low costs of production per vaccination dose.

Regulatory Hurdles

The great importance for human health and the high level of responsibility expected from regulatory agencies such as the FDA in the United States is fully recognized. Nevertheless, it is difficult to understand why, in cases such as the polio vaccine mentioned in the preceding section, phase III clinical trials costing hundreds of millions of dollars would be imposed even for applying minor changes that would render the vaccine much safer; one cannot blame manufacturers for refraining from improving their product in view of the enormous costs entailed. Similarly, imposing such extensive and costly clinical trials might not be warranted for recombinants based on the widely used MV vaccines, provided that it can be rigorously demonstrated that any of the additionally expressed antigen is not toxic, the cell targeting of the recombinant virus is not altered by inserted transgenes, the immune response generated against measles is the same as in the traditional MV vaccines, and the manufacturing process is practically the same as that used for the traditional vaccine.

It might also be asked whether society should not generally accept the prevailing principle of ethical behavior, i.e., maximizing the average benefits for the largest possible number of individuals. Risks accompany every human invention, and in many cases, such as traffic and sport activities, risks are tacitly accepted. Why is this not the case with vaccines? Rare adverse effects of medically active compounds, as routinely explained in leaflets accompanying drugs, are usually tolerated. Admittedly, vaccines must be considered differently, as they are administered to healthy individuals, particularly to children. Nevertheless, why should even highly adverse effects of vaccines not be acceptable, as long as such effects remain very rare, when severe morbidity and death can be prevented for large populations? Fact-based, nonemotional dialogue between scientists and society is clearly necessary. It should be possible to find acceptable legal and procedural solutions to this and similar problems even though at present in general the rights of the individual prevail over the rights of the community.

Patent Conflicts and Disruption of Scientific Collaborations

Unfortunately, patents are unavoidable to actually produce, based on scientific endeavors either in academic or corporate research laboratories, medically valuable commercial products. Competition between companies and scientists is an important driving force and may be considered more beneficial than detrimental in the long run. However, for the development of *Mononegavirales* as vaccine vectors, many researchers in different laboratories have invented tools that are indispensable to the success in establishing reverse genetics and foreign gene expression. In this

instance, competition and financial avidity not only among companies, but also among scientists, has severely hampered progress. Patent offices must necessarily adhere to legal considerations, but research institutions should be amenable to compromises based on the recognition of scientific merit. Patent conflicts are costly and demotivating in general. They become intolerable when, for example, scientific collaborations that have arisen over many years and are subsidized by public grant money are unilaterally discontinued by decree of an institutional director, forcing his researchers, for the financial benefit of his institution, to discontinue the flow of materials and information.

Other Recombinant *Mononegavirales* as Vaccines

As discussed in various sections above, a variety of *Mononegavirales* have been rescued from DNA. The first reported rescues of a number of these viruses are addressed in an excellent general review on *Paramyxoviridae* and their interactions with the host (Lamb and Parks 2007). The list includes RABV (Schnell et al. 1994), VSV (Lawson et al. 1995; Whelan et al. 1995), MV (Radecke et al. 1995), human respiratory virus hRSV (Collins et al. 1995), SeV (Garcin et al. 1995; Kato et al. 1996), rinderpest virus (Baron and Barrett 1997), hPIV3 (Durbin et al. 1997; Hoffman and Banerjee 1997), SV5/PIV5 (He et al. 1997), NDV (Peeters et al. 1999), and bRSV (Buchholz et al. 1999).

Some examples of foreign gene insertions in *Paramyxoviridae* are reported in the review on Parainfluenza viruses, which comprise the genera *Respirovirus*, *Rubulavirus*, and *Avulavirus* (Karron and Collins 2007). The bPIV3 backbone bearing the hPIV3 F and HN genes rather than their bovine counterparts (Schmidt et al. 2000), and hPIV in which only a few internal protein genes such as N and P are replaced by their bovine counterparts (Bailly et al. 2000; Skiadopoulos et al. 2003a) are candidate vaccines against human parainfluenza. Additional foreign genes derived from other viruses such as hRSV and human metapneumovirus (hMPV) have also been inserted into hPIV3 (Schmidt et al. 2002; Skiadopoulos et al. 2002), as well as in hPIV1, hPIV2, and SeV. Vectors based on a representative of the genus *Avulavirus*, Newcastle disease virus (NDV, now officially called AMPV1) appear promising as vaccines and oncolytic agents for veterinarian and human use (Ge et al. 2007; Lorence et al. 2007; DiNapoli et al. 2007; Estevez et al. 2007; Vigil et al. 2007; Janke et al. 2007). A drawback for respiroviruses as vaccination vectors is that even a full infection with these nonattenuated viruses does not protect from reinfection by the same agent. Thus, protection by attenuated respirovirus vectors cannot be expected to be long-lasting.

Of the two main genera *Lyssavirus* and *Vesiculovirus* of the recently reviewed *Rhabdoviridae* (Lyles and Rupprecht 2007), rabies virus (RABV) and the two main subtypes of VSV (Indiana and New Jersey) have been proposed both as vaccination

vectors, mainly against AIDS, and as therapeutic agents (Roberts et al. 1999; Roberts and Rose 1999; Rose et al. 2001; Schnell et al. 2000; Palin et al. 2007; Simon et al. 2007; Clarke et al. 2006; Bergman et al. 2007; Diaz et al. 2007; Brandsma et al. 2007; Schwartz et al. 2007; Tani et al. 2007; Cooper et al. 2008). RABV, and particularly VSV and derived recombinants containing inserts, have the advantage of replicating to extremely high titers. Since the natural hosts of VSV are cattle, horses, swine, mosquitoes, and sandflies, human use is somewhat problematic, particularly since VSV shows some neurovirulence in primates (Johnson et al. 2007). However, VSV can be greatly attenuated by shuffling its genes (Wertz et al. 1998).

Conclusions

In comparison with other *Mononegavirales*, in particular VSV and NDV/AMPV1, MV vectors have lagged behind, as is evident from the recently published reports cited in the preceding section. In part, this may stem from the relatively low titers obtained for MVs, to preexisting immunity against MV in the adolescent and adult human population, as discussed above, and suboptimal and/or expensive animal models. Clinical studies involving MV vectors for vaccine applications are only in the planning phase and are presently ongoing only with oncolytic MV recombinants requiring less strict previous testing. Thus, the promise of recombinant MV vaccines obtained in studies with animal models has yet to be corroborated in humans, the unique MV host. Nevertheless, for a variety of reasons discussed in this chapter, it can be predicted that the search for medical applications with recombinant MVs will rapidly gain momentum, as suggested by recent reviews comparing different viral vectors for vaccination purposes (Li et al. 2007; Brave et al. 2007).

In comparison with other vectors based on RNA viruses, MV and generally *Mononegavirales* appear clearly superior. Will the hope presently attached to these vectors gradually wane, as has occurred for many DNA virus applications and delivered plasmid DNA? Comparative studies are clearly needed, as are studies comparing replication restricted to replication-competent vectors, which are thought to be superior at least in case of MV and MuV.

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