The Plowright vaccine strain of Rinderpest virus has attenuating mutations in most genes

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The currently used vaccine strain of Rinderpest virus was derived by serial passage of the highly virulent Kabete ‘O’ strain (KO). A full-length cDNA copy of the KO strain was made from which a virus identical in pathogenicity to the wild-type virus was rescued. A series of chimeric viruses was prepared in which the coding sequences for the N, P, F, H or L proteins were replaced with the corresponding sequences from the vaccine strain. The KO-based virus with the vaccine strain H gene and that with the carboxy-terminal half of the L gene replaced with the corresponding sequence from the vaccine strain retained all or almost all of the virulence of the original KO virus. Animals infected with the KO-based virus containing the vaccine strain N, P or F gene, or the amino-terminal half of the L gene, developed high and prolonged pyrexia and leukopenia, but with reduced or absent lesions and other clinical signs; although partially attenuated, none was nearly as attenuated as the vaccine strain itself. These data indicate that the high attenuation and stability of the current vaccine are due to the accumulation of a number of separate mutations, none of which is itself so sufficiently debilitating that there is strong selective pressure in favour of the revertant.

INTRODUCTION

Viral vaccines can crudely be divided into three categories: attenuated strains of the virus, which elicit a full immune response yet do not cause disease, inactivated (killed) viruses, and subunit vaccines, in which a part of the virus is injected into or expressed in the host using one of a variety of systems. Of these vaccine types, the second is usually the quickest to develop for use, but frequently produces limited protection or protection of short duration (as is the case for the foot-and-mouth disease virus vaccines currently in use). It is a matter of record that the vast majority of viral vaccines that have been used successfully to control and/or suppress human or animal disease are of the first type, the attenuated live viruses. Where these can be produced and are stable (i.e. do not revert to the virulent phenotype), they are the vaccine of choice. However, the derivation of such attenuated strains by classical methods takes considerable time, since it depends on suitable changes occurring in the viral genome by chance and being selected for by whatever unnatural means of growing the virus (e.g. abnormal host, tissue culture, tissue culture at abnormal temperature) has been chosen. Pains must also be taken to check for possible reversion and other safety concerns. Targeted engineering of viruses to create attenuated strains would be of great help; the development of a reverse genetics technique for viruses of the order Mononegavirales (Schnell et al., 1994) has made such genetic engineering possible for this group of viruses and various strategies are being developed in a number of laboratories (Bukreyev et al., 1999; Skiadopoulos et al., 1999; Whitehead et al., 1999a, b, c; Tao et al., 2000; Flanagan et al., 2001).

One of the most successful vaccines for this group of viruses has been the Plowright vaccine strain (RBOK) of Rinderpest virus (RPV) (Plowright & Ferris, 1962). RPV is a paramyxovirus of the genus Morbillivirus that causes a severe and often fatal disease of cattle and buffalo, as well as several wild species of the order Artiodactyla. It is most closely related to Measles virus (MeV) of humans. The virus is enveloped, pleiomorphic and has a single-stranded RNA genome of negative-sense with six genes (or transcription units). These encode the nucleocapsid (N) protein (co-transcriptionally complexed with the viral genome RNA to form the RNase-resistant ribonucleoprotein), the phospho-(P) and large (L) proteins (which together make up the viral polymerase), the attachment (H) and fusion (F) proteins (glycoproteins found in the viral envelope that respectively are responsible for the binding of the virus to, and fusion of the virus membrane with, the host cell) and the matrix (M) protein (thought to link the ribonucleoprotein to the viral glycoproteins to enable budding of new virions). The P gene also encodes two so-called non-structural proteins (V and C), which seem to play a variety of roles in virus replication and possibly pathogenicity (Garcin et al., 1997; Kato et al., 1997; Tober et al., 1998; Escofier et al., 1999; Baron & Barrett, 2000; Mebatsion et al., 2001; Sweetman...
et al., 2001; Shaffer et al., 2003). The Plowright vaccine was derived by 90 serial passages in tissue culture and has been used throughout Africa, the Middle East, parts of Europe and the Indian subcontinent in a global campaign to eradicate rinderpest, once the most feared of all cattle diseases. So successful has this campaign been that the virus is now restricted to a small area of east Africa (Mariner & Roeder, 2003) and may be fully eliminated by or soon after the target date of 2010 (FAO, 2003). We have previously compared the sequence of this vaccine strain with that of the virulent Kabete ‘O’ (KO) strain, from which it was derived (Baron et al., 1996), and found sequence differences throughout the genome, although concentrated in one or two areas; none of the changes was so great as to indicate that this was the primary attenuating mutation. Since we have developed methods of rescuing RPV from cDNA copies of the viral genome (Baron & Barrett, 1997; Das et al., 2000a), we used chimeric constructs containing genes from both virulent and avirulent strains to investigate which of the genetic differences observed were important in determining the phenotype of the RBOK strain. Our results indicate that the complete attenuation of the vaccine strain is due to a combination of less-attenuating mutations distributed throughout the genome.

METHODS

Cell culture. B95a cells (Epstein–Barr virus-transformed tamarin lymphoblastoid cells; Kobune et al., 1991) were grown in RPMI 1640 (Invitrogen) containing 10% fetal calf serum (FCS). Vero cells were grown in Dulbecco’s modified Eagle’s medium containing 5% FCS. Transfections were performed with TransFast (Promega) following the manufacturer’s directions using 6 µl TransFast (µg DNA) \(^{-1}\).

Recovery of virus from full-length genomic cDNA. Virus rescue was performed by two methods: (i) Vero cells were plated in six-well plates (2 x 10^5 per well). The following day, cells were infected with recombinant fowlpox expressing the T7 RNA polymerase (FP-T7; Britton et al., 1996) and transfected with plasmids encoding the RBOK strain N, P and L proteins plus plasmid containing full-length virus genome cDNA as previously described (Das et al., 2000b). Three days post-infection (p.i.), cells were frozen and thawed once to release virions, clarified by microcentrifugation at 5000 r.p.m. for 5 mins, and 25% of the supernatant was added to B95a cells in a six-well plate. (ii) B95a cells were plated at a concentration of approximately 2 x 10^6 cells per well in a six-well plate 2 days before transfection. Cells were infected with recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3; Fuerst et al., 1986) (m.o.i. of ~0-2) for 1 h before transfecting with the genome and helper plasmids as above. In each case, cells were observed daily for cytopathic effect (CPE). Recombinant virions were released by a single freeze–thaw cycle and clarified as above. Clarified supernatants were stored at ~70 °C. Recombinant virions were further amplified by growth in B95a cells through two or three passages before use. All viruses were titrated by determine of TCID50 (Reed & Muench, 1938) on B95a cells.

Molecular biology techniques. RNA was purified from infected cells using Trizol (Invitrogen); cDNA was prepared as previously described (Baron & Barrett, 2000). All preparative amplification by PCR used Pfu polymerase (Stratagene) according to the manufacturer’s instructions. Diagnostic RT-PCR to detect viral RNA in peripheral blood leukocytes (PBLs) was performed essentially as previously described (Forsey & Barrett, 1995), except that HotStarTaq (Qiagen) was used. Other DNA manipulations were performed by standard techniques. Plasmids were transfomed in Escherichia coli strains DH5x or JM109; large-scale preparation and purification of plasmids was performed using CsCl gradients or Qiagen Midiprep Plasmid Purification kits.

Construction of pMDB-KO. The KO strain genome was amplified in sections by RT-PCR. Individual PCR products were treated with T4 polynucleotide kinase and ligated into pT7Blue/blunt (Novagen). Amplified sections of the KO virus genome were sequenced. We found two differences between the H gene in our KO isolate and the sequence previously published (Yamanaka et al., 1988), leading to an Arg at position 556 instead of Lys, and a Glu instead of Lys at position 603. Both of these residues were Lys (like the RBOK sequence) in the originally published KO strain H sequence and may be due to differing passage histories of the two KO isolates (ours had only been grown in cattle or B95a cells). In addition, we found a number of places in the previously published KO strain N, P, F and L genes (Hsu et al., 1988; Yamanaka et al., 1992; Ismail et al., 1994; Baron et al., 1996) where predicted differences between the two viruses did not exist. Our fully corrected sequence for the KO genome has been deposited in GenBank with accession number X98291. We previously engineered restriction sites into the ends of each gene or open reading frame (ORF) of the RBOK strain in plasmids pMDBRPV2B and pMDBRPV2C (Baron & Barrett, 2000; Das et al., 2000a). These sites are: a ClaI site at nt 102 in the antigenome, immediately upstream of the N ORF; a Pad site at nt 1711 in the antigenome, 25 nt downstream of the end of the N ORF; an SbfI site at nt 3354 in the antigenome, 23 nt downstream of the end of the P ORF; a Swal site at nt 4456 in the antigenome, 11 nt downstream of the M protein ORF and 401 nt upstream of the intergenic trinucleotide; an SgfI site at nt 5435 in the antigenome, 566 nt after the M–F intergenic trinucleotide and 15 nt upstream of the F protein ORF; an AscI site at nt 7195 in the antigenome, 97 nt downstream of the F protein ORF; a PmlI site at nt 9092 in the antigenome, 3 nt downstream of the H protein ORF; and a SalI site at nt 15807 in the antigenome, 33 nt downstream of the L protein ORF (and outside the conserved hexamer motif forming part of the promoter; Mioulet et al., 2001). Identical sites were introduced into the KO sequence during construction of the full-length genome clone, with the exception of the PmlI site at the end of the H gene, which was replaced with a Pml site in the KO strain plasmid (there is a PmlI site in the KO virus L gene); both of these enzymes leave blunt ends and the Pml site was positioned to cleave at the same point in the H gene 3′ untranslated region (UTR) as the PmlI site in pMDBPV2C.

The various full-length plasmid constructs described in this paper were made by standard DNA manipulation techniques (full details are available from the authors on request, along with sequences of all primers used for RT and PCR, if required).

The promoter regions of RPV KO were amplified from the plasmids containing the constructs sequenced in Baron & Barrett (1995) and ligated into pMDBI to give pMDBKOGEN. This plasmid contained the genome 3′ 101 nt and the 5′ 70 nt of the virus plus the engineered ClaI and SalI sites to attach the rest of the genome. The rest of the KO genome was then built into this construct in several stages to give pMDB-KO. In this plasmid, all of the sequence was derived from the KO strain apart from nt 4464–5434, comprising 401 nt of the M gene 3′ UTR, the M–F intergenic trinucleotide and 566 nt of the F gene 5′ UTR.

Construction of chimeric replicons. In plasmids pRPV-KON, pRPV-KOP and pRPV-KOH, the ClaI–PciI, PciI–SbfI and AscI–PmlI segments, respectively, of RBOK were replaced by the equivalent KO sequences. Plasmids pMDBKO-NR, pMDBKO-PR and

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pMDBKO-MR were made similarly by digesting pMDBKO and pRPV2C with the appropriate restriction enzymes bracketing the sequence to be exchanged, isolating the large and small fragments, respectively, from the two digests and ligating them together. Plasmids pMDBKO-LRa and -LRb were made using the natural Ncol site at the start of the L protein ORF and the AatII site at nt 3634 in the L gene. In pMDBKO-LRa, therefore, the first 3601 nt of the L ORF were from RBOK, while in pMDBKO-LRb the remainder of the L ORF (2935 nt) plus 33 nt of the L gene 3' UTR were from RBOK. For plasmid pMDBKO-HR, the region from the AscI site engineered in the F gene 3' UTR to the same Ncol site in the L gene was replaced with RBOK sequence. Plasmid pMDBKO-FR, made during assembly of the full KO strain replicon and in which the whole of the F gene and the first 878 nt of the H gene were derived from RBOK, was used as the F gene exchange plasmid.

**Cattle experiments.** All animal experiments were carried out in accordance with national legislation governing the use of animals for research and with the approval of the local ethical review body. Outbred Freisan Holstein bullock calves (approx. 6 months old) were subcutaneously infected with $10^4$ TCID₅₀ virus. Rectal temperatures were monitored daily and blood was sampled before infection and at 2–3-day intervals thereafter. The white cell count was determined on heparinized blood by lysing the erythrocytes in 0.9% (v/v) acetic acid and counting surviving cells in a haemocytometer. PBLs were purified from heparinized blood as previously described (Ohishi *et al.*, 1999). For virus isolation, cells from approximately 10 ml blood were cultured with B95a cells in a 25 cm² flask to detect cell-associated virus in the blood (viraemia). Cultures were considered positive if virus was detected within 1 week, without further passage of the cells. For viral RNA detection by RT-PCR, a similar number of cells were lysed in Trizol for RNA purification. Trizol lysates were stored at −20 °C until RNA extraction and purification. In some experiments, ocular secretions were sampled on sterile cotton buds at the same time as blood sampling and the cotton buds extracted with Trizol to isolate RNA from any secreted virus, or washed with PBS, which was then added to B95a cells and cultured as above to look for secreted virus by isolation. The presence of viral RNA in PBLs and ocular secretions was determined by RT-PCR using primers F3b and F4d as previously described (Forsyth & Barrett, 1995).

**RESULTS**

The first three chimeric viruses we prepared were based on the RBOK strain previously rescued (Baron & Barrett, 1997). We selected three genes that seemed most likely to have mutations that would affect virus infectivity (H gene) or replication (N and P genes) and replaced these genes individually in the RBOK cDNA clone with the equivalent gene from the virulent KO strain (see Fig. 2). Each of these viruses behaved indistinguishably from the vaccine when tested in animals. As an example, Fig. 1(a) and (b) compares the results obtained with RBOK-KOH with those obtained with rescued RBOK. Reasoning that the vaccine

![Fig. 1. In vivo studies of recombinant RPV.](http://vir.sgmjournals.org)
might have multiple defects and that it might therefore be impossible to detect correction of any one defect in the vaccine, it was decided to create a full clone of the KO strain and change individual genes in that strain to the equivalent from the vaccine strain. In this way, we hoped to determine which genes of the RBOK vaccine contained attenuating mutations.

We therefore prepared a full-length cDNA copy of the KO genome, as described in Methods, using KO-infected B95a cells as the source of viral RNA. We had previously observed that virus from wild-type RPV-infected animals grew without apparent adaptation in B95a cells, giving rise to noticeable CPE after no more than 2 days (M. D. Baron, unpublished observations) and that virus grown in these cells caused severe disease in cattle. The individual genes of the KO strain cDNA had unique restriction sites engineered in non-conserved UTRs at the 5' and/or 3' end of the transcription units to facilitate gene swapping with the RBOK clone. These cDNAs were assembled into the plasmid pMDB1 (Baron & Barrett, 1997) as described in Methods to give pMDB-KO, in which all of the viral sequence was derived from the KO strain, apart from the RBOK clone. These cDNAs were assembled into the plasmid pMDB1 (Baron & Barrett, 1997) as described in Methods to give pMDB-KO, in which all of the viral sequence was derived from the KO strain, apart from the long GC-rich region between the M and F ORFs consisting of the M gene 3' UTR, the intergenic trinucleotide and the F gene 5' UTR. This region was taken from the RBOK plasmid, as we were unable to amplify it from KO cDNA in such a way that we could incorporate the sequence into the KO plasmid.

Virus rescue was initially performed by a minor modification of our previous protocols (Baron & Barrett, 1997; Das et al., 2000b), in which transfections were performed in Vero cells that had been infected with modified fowlpox expressing the T7 RNA polymerase (FP-T7) and virus that formed in the Vero cells was immediately (48 h post-transfection) passed to B95a cells. Recombinant RPV KO strain (rKO) was rescued by this technique and was fully virulent in a bullock infected with the virus (Fig. 1e and f; rKO 3), when compared with the clinical signs of pathogenic RPV (Fig. 1c and d). These have been fully described in the past (Anderson et al., 1996); briefly, the pathogenic virus causes a pyrexia, starting on day 3 or 4; this can remain high for 7–8 days, during which more severe symptoms (mouth lesions, diarrhoea, anorexia, etc.) develop. There is loss of approximately 80% of the PBLs, which slowly recover over the following 2–3 weeks, if the animal survives. In some animals, the temperature may fall after 4 or 5 days as a prelude to collapse and death, often due to dehydration. The severe dehydration leads to a rise in the blood packed-cell volume and an apparent rise in the white cell count. This could be seen in one animal given the wild-type non-recombinant virus in Fig. 1 (wild-type RPV 3). The exact outcome depended to some extent on the individual animal, as these were all outbred stock. For the animal given rKO, in addition to the fever and severe leukopenia, the infected animal developed diarrhoea and became apathetic and anorexic. Due to severe dehydration and the consequent increase in packed-cell volume, the white cell count was not recorded in this animal on day 7 or 9. It was killed at day 9 and post-mortem examination showed the typical internal lesions of rinderpest.

We also rescued virus by a modification of the method for rescuing wild-type MeV (Takeda et al., 2000), in order to eliminate any possibility that the initial replication in Vero cells would lead to selection for a mutation arising during replication in these cells; wild-type RPV has to be blind passed several times in Vero cells in order to 'adapt' the virus (i.e. select a mutant that grows better in those cells). In this method, the genome and helper plasmids were transfected directly into B95a cells that had been infected with recombinant vaccinia as a source of T7 RNA polymerase (vTF7-3). This method proved to be highly effective, and virus was rescued from essentially all wells containing transfected cells, as judged by the development of typical syncytia by 2 or 3 days post-transfection. We did not observe virus rescue if FP-T7 was used instead of vTF7-3. As also observed by Takeda et al. (2000), the recombinant vaccinia did not appear to replicate productively in B95a cells, and infected but non-transfected cells showed no CPE. In addition, we examined recombinant virus stocks after two rounds of amplification in B95a cells for any contamination with vaccinia. No CPE was seen in BHK cells (a host for vaccinia, but not RPV) exposed to undiluted virus, nor could we find any trace of vaccinia by immunofluorescent staining of RPV-infected Vero cells with an antirabbit-pox antiserum (a kind gift of Professor R. Moyer, University of Florida, USA) that readily stained vTF7-3-infected cells. Recombinant KO virus rescued by this technique was also fully virulent, with the animals developing fever and leukopenia (Fig. 1e and f; rKO 1 and rKO 2) as well as oral and nasal discharges, gum lesions and erosions in the oral papillae. The clinical signs observed in these animals were again indistinguishable from those observed in animals infected with non-recombinant virulent RPV. Virus could be detected in PBLs by RT-PCR from day 2 until euthanasia.

We then prepared a set of chimeric viruses based on rKO in which the N, P, M, F, H or L coding regions were replaced with the equivalent sequence from RBOK. Because of its large size and the consequent large number of differences between the RBOK and KO L genes, we exchanged only half of the coding sequence of the L protein in any one chimera, leading to a total of seven chimeric KO-based viruses (Fig. 2). These viruses were rescued as described above, using both techniques. All the viruses were rescued at a similar efficiency (virus being found in three to five wells out of five transfected in each case). All grew to titres similar to those observed for rKO (10^4–10^5 TCID50 ml^-1) in preparative tissue culture. Single- or multi-step growth curves were not performed with these viruses, since our sole interest was the level of virulence of the recombinant viruses. As has recently been shown for recombinant vesicular stomatitis virus (Novella et al., 2004) and was
also found with a series of recombinant chimeric rinderpest viruses with altered promoter sequences (Banyard et al., 2005; accompanying paper), virus growth in tissue culture does not correlate with virulence in vivo.

Although we constructed and rescued KO-MR as part of this series, it was not used in vivo as the only differences between the M proteins of RBOK and KO were conservative substitutions (Baron et al., 1996) and it was felt to be unethical to infect animals with what was almost certainly going to be a lethal disease. We infected two or three cattle with each of the other chimeric viruses. Due to the outbred nature of the experimental animals, some variability in the responses to infection was always observed (notably in the temperature responses of the two animals infected with KO-FR and the leukopenia of the two animals given KO-LRa). Nevertheless, clear conclusions could be drawn. As shown in Figs 3 and 4, all the viruses showed at least limited pathogenicity as shown by the rapid onset of pyrexia (defined as rectal temperature in excess of 39.5 °C) and concomitant leukopenia. Virus was detectable by RT-PCR in PBLs at day 2 or 3 p.i. in all cases and could be detected up to day 13 in some animals (Fig. 4). Virus isolation by co-cultivation of PBLs with B95a cells was less sensitive; in experiments where this was attempted, virus was detected sometimes at day 2 and always at days 5, 7 and 9, if it was also detected by RT-PCR. Ocular swabs showed virus at days 5–7 only, by either detection method.

Viruses KO-NR, KO-PR, KO-FR and KO-LRa all showed...
some attenuation, with none of the animals becoming distressed or anorexic. In addition to the fever and leukopenia, slight ocular congestion was seen in most cases, in addition to slight nasal discharge in a few animals and diarrhoea for 1 day in one animal given KO-PR. All these animals recovered from the fever and their white cell counts returned to normal by the end of the experiment (21 days p.i.).

In contrast, animals infected with KO-HR or KO-LRb showed prolonged pyrexia (Fig. 3) and more severe nasal and ocular discharge. The apparently rapid ‘recovery’ in white cell count in these cases (Fig. 4) was due to dehydration with the concomitant increase in packed-cell volume and is regularly seen in severe rinderpest infection. In several cases, animals infected with these viruses had to be killed before the end of the experiment due to the severity of the symptoms. While not as consistently lethal as the original KO virus, these chimeras were clearly still highly pathogenic.

**DISCUSSION**

The ability to manipulate the genomes of negative-strand RNA viruses has led to the development of several strategies to engineer attenuated viruses for use as vaccines. Replacement of viral genes with equivalent genes of related viruses that use different hosts has been tried with some success (Bailly *et al*., 2000; Haller *et al*., 2000; Tao *et al*., 2000; Schmidt *et al*., 2002), although this amounts to some extent in simultaneously inserting a large number of mutations of unknown effect. The elegant idea of Wertz *et al*. (1998) to attenuate a virus by changing the natural order of, and therefore the relative expression levels of, the viral genes has also proved effective (Flanagan *et al*., 2001). Although it remains to be proved whether such attenuation is stable in practice, the general replication strategy of this group of viruses means that it is hard to envisage mutations that could restore the normal expression levels of each gene. A third way to produce a fully attenuated vaccine candidate is to combine mutations from several partially attenuated strains. This type of manipulation has already been applied to vaccine strains of parainfluenza and respiratory syncytial viruses (Skiadopoulos *et al*., 1999; Whitehead *et al*., 1999b; Newman *et al*., 2004). It is possible that combinations of these strategies could lead to multivalent, attenuated vaccines.

In seeking to understand the genetic basis for the avirulent phenotype of the RBOK vaccine strain of RPV, one possibility was that a significant change had occurred in one protein or the expression of a protein that resulted in loss of virulence in cattle. Such an effect of a relatively simple change in viral sequence has been observed in related viruses (Garcin *et al*., 1997; Itoh *et al*., 1997; Juhasz *et al*., 1997; Whitehead *et al*., 1998) and it may have been that such a change had effects on viral growth that were particularly favoured in tissue culture. Another possibility was that a number of changes, possibly in different genes, had accumulated during virus passage in culture, each of which was partly attenuating, and that the sum of these changes was such as to render the virus avirulent. This latter possibility also offered an explanation for the noted stability of the Plowright vaccine. Single base changes are unlikely to be stable in an RNA virus, all of which show high levels of spontaneous mutation (Steinhauer & Holland, 1986; Ward *et al*., 1988) and indeed RPV isolated after 41 passes in tissue culture reverted to partial virulence after two rounds of needle passage in cattle (Plowright & Ferris, 1962); a further 49 passes produced a virus that did not revert to pathogenicity. A virus with a large accumulation of mutations, of which perhaps any two or three would be enough for full attenuation, would be unlikely to undergo sufficient spontaneous mutation during infection to restore any detectable degree of pathogenicity. Since viruses of the order Mononegavirales do not show recombination (apart from very particular, rare circumstances;
Spann et al., 2003), there is no mechanism to revert several mutations other than a sequential series of stochastic events, with some selective pressure for maintenance of intermediate stages.

The conclusions from the studies reported here, coupled with those in Banyard et al., (2005; accompanying paper) showing that there are further, even more strongly, attenuating mutations in the leader and/or trailer regions of the RBOK strain, is that a vaccine that has been shown to be nearly ideal in use in the field has a large number of attenuating mutations distributed throughout the genome. Attempts to engineer an attenuated RNA virus for use as a vaccine should therefore attempt to insert attenuating mutations into several places in the genome. These mutations could be naturally occurring, selected by, for example, growth at low temperature or deduced by comparison with other viruses. Our results with the RBOK vaccine also indicate that the individual mutations do not have to be fully attenuating by themselves, as long as the sum of their effects is full attenuation.

In the course of these studies we have confirmed and extended the utility of the combination of vTF7-3 with B95a cells (Takeda et al., 2000) for morbillivirus rescue. Although our initial attempts to transfect B95a cells were inefficient, we have found that they seem to transfect more readily when infected with vaccinia, at least enough to allow rescue to occur. Our observations were that multiple rescue events took place in each well; typically CPE was seen within 2 or 3 days of transfection. The vaccinia infection appears to be abortive in B95a cells, a lymphoblastoid line, as progeny vaccinia were not observed in the rescued RPV stocks. Removal of vaccinia by centrifugation or filtration, as has been necessary in other protocols for rescue of negative-stand RNA viruses (Lawson et al., 1995; Whelan et al., 1995), was not required. This method allowed us to place more confidence in our rescues of various chimeric viruses (the work reported here and in Banyard et al., 2005; accompanying paper), as growth of RPV in Vero cells has been shown to lead to attenuation (Kobune et al., 1991). In fact, in the studies reported here, the combined Vero transfection/B95a amplification strategy first applied to the rescue of the KO strain and KO-based chimeric viruses produced viruses that had identical pathogenicity to viruses rescued by the B95a-only method. This may be because the primary defect in KO replication in fibroblast cells (e.g. see Banyard et al., 2005; accompanying paper) is in virus binding to the host, as it can be localized to the KO H protein (M. D. Baron, unpublished data). The first round of virus replication in the transfected Vero cells, depending only on the transfected plasmids, bypasses this defect; released virus could then infect the B95a cells in the amplification stage. Interestingly, although the H protein of the RBOK strain has a greater concentration of differences compared with the virulent parent than any of the other viral proteins (Baron et al., 1996), exchanging this protein had only a slight effect on pathogenesis, suggesting that mere concentration of sequence changes is no guide to in vivo effects.

ACKNOWLEDGEMENTS

We thank J. Garrett for technical assistance. This work was supported by the BBSRC.

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of either the NS2 or SH gene is attenuated in chimpanzees. *J Virol* 73, 3438–3442.


