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Abstract

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A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus

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We have recovered infectious Sendai virus (SeV) from full-length cDNA (FL-3) by transfecting this cDNA and pGEM plasmids expressing the nucleocapsid protein (NP), phosphoprotein and large proteins into cells infected with a vaccinia virus which expresses T7 RNA polymerase. These cells were then injected into chicken eggs, in which SeV grows to very high titers. FL-3 was marked with a *Bgl*III site in the leader region and an *Nsi*I site (ATGTCAT) in the 5' nontranslated region of the NP gene, creating a new, out-of-frame, 5' proximal AUG. All the virus stocks generated eventually removed this impediment to NP expression, by either point mutation or recombination between FL-3 and pGEM-NP. The recovery system was found to be highly recombinogenic. Even in the absence of selective pressure, one in 20 of the recombinant SeV generated had exchanged the NP gene of FL-3 with that of pGEM-NP. When a fifth plasmid containing a new genomic 3' end without the presumably deleterious *Bgl*III site was included as another target for recombination, the new genomic 3' end was found in the recombinant SeV in 12 out of 12 recoveries. Using this approach, a novel copy-back nondefective virus was generated which interferes with wild-type virus replication.

Keywords: copy-back nondefective virus/recombinogenic/Sendai paramyxovirus

Introduction

Paramyxoviruses are enveloped animal viruses containing nonsegmented negative strand [(-)] RNA genomes. Together with rhabdoviruses and filoviruses (such as Ebola virus), paramyxoviruses form the superfamily Mononegaloviridae. Most paramyxovirus genomes contain six genes: M (matrix), F (fusion) and HN (hemagglutinin-neuraminidase), whose products form the viral envelope and are involved in getting the virus into and out of cells, and NP (nucleocapsid protein), P (phosphoprotein) and L (large), which form the viral nucleocapsids and are involved in expressing and replicating the (-) genome. Mononegalovirus genomes are found only as helical nucleocapsids (assembled with NP), which serve as the templates for both mRNA synthesis and genome replication. Upon infection, holonucleocapsids, which for Sendai virus (SeV) contain ~50 L and 300 P proteins, enter the

cytoplasm and carry out mRNA synthesis. On accumulation of the viral translation products, the same nucleocapsids are used as templates for genome replication via the synthesis of antigenome nucleocapsids (reviewed in Lamb and Kolakofsky, 1995).

Unlike (+) RNA viruses, neither the (-) genome nor the (+) antigenome RNA of negative strand RNA viruses is infectious. For mononegaloviruses, neither RNA can be translated by ribosomes to form the critical polymerase L protein. Moreover, even when the viral polymerase is available, it requires an assembled nucleocapsid rather than free RNA as a template. Because of this, the recovery of infectious virus from cDNA has lagged behind that of (+) RNA viruses (Tanaguchi *et al.*, 1978; Racaniello and Baltimore, 1981). Despite these difficulties, the rescue of an infectious (-) RNA virus from cDNA was first achieved for influenza virus which contains eight genome segments, in large part because it was possible to assemble a functional nucleocapsid segment *in vitro*. This could then be transfected into a natural virus-infected cell, and the rare recombinant virus selected via its altered host range (Luytjes *et al.*, 1989; Enami and Palese, 1991). The NP-RNA nucleocapsids of paramyxoviruses (and rhabdoviruses), in contrast, have resisted all attempts so far to be assembled in a functional form *in vitro*, perhaps because they are much more tightly structured. In contrast to influenza virus nucleocapsids, they remain highly active even after banding in CsCl density gradients, and the RNA within this structure remains resistant to RNase attack at any salt concentration (Lynch and Kolakofsky, 1978; Heggeness *et al.*, 1981). A structure for the influenza virus nucleocapsid has been proposed recently based on chemical probing, which has highlighted the differences from the structures of the mononegaloviruses (Baudin *et al.*, 1994).

Another approach to rescuing infectious virus from cDNA has been to synthesize RNA with defined ends *in vivo* via T7 RNA polymerase and the hepatitis delta virus (HDV) ribozyme (Fuerst *et al.*, 1986; Perrotta and Been, 1990) in the presence of vectors which generate the viral NP, P and L proteins required for genome amplification and expression (Curran *et al.*, 1991). This approach is based on the premise that a critical step in this process, the (illegitimate) assembly of the free genome (or antigenome) RNA into a functional nucleocapsid, can be better accomplished inside a living cell. For both the rhabdovirus vesicular stomatitis virus (VSV) and SeV, this approach has been successful for some time now using cDNA copies of natural defective interfering (DI) viral mini-genomes of ~10% the nondefective genome length (Pattnaik and Wertz, 1990; Calain *et al.*, 1992; Calain and Roux, 1993). More recently, this approach has been successful for the full-length genomes (11 kb) of the rhabdoviruses rabies and VSV, and has led to the recovery

of novel rhabdoviruses from cDNA (Schnell *et al.*, 1994; Lawson *et al.*, 1995). Here we report the recovery of infectious Sendai paramyxovirus (15 kb) from cDNA using this approach. We also describe the inherently recombinogenic nature of the recovery system, and the generation of novel viruses with interesting properties.

Results

We have described previously a transfected cell system to study SeV DI genome amplification, in which natural DI genomes (freed of their helper nondefective genomes) are introduced into cells as virus particles and are replicated via the NP, P and L proteins provided from transfected pGEM plasmids (Curran *et al.*, 1991). The T7 RNA polymerase needed to express these SeV genes is provided by infection with a recombinant vaccinia virus (vTF7-3; Fuerst *et al.*, 1986). The natural DI genomes were subsequently replaced by cDNA copies, in which the precise 5' and 3' ends of the mini-genomes are determined by the positioning of a T7 promoter and a HDV ribozyme, respectively. These cDNA-derived DI genomes are presumably first transcribed from the genome plasmids by T7 RNA polymerase, and assembled into nucleocapsids and replicated by the SeV NP, P and L proteins. When the natural DI genomes contained a functional transcription unit, or were engineered to contain one, its protein product could be detected (Calain and Roux, 1995). When this unit contained the mRNA editing region of either the SeV or the bovine parainfluenzavirus type 3 P gene, the ensuing mRNA was edited appropriately (Jacques *et al.*, 1994).

The above system thus appeared to be suitable for the recovery of infectious virus from full-length cDNA. Moreover, the nucleotide changes needed to mark the recombinant genome to distinguish it from wild-type strains could be examined first in the DI system for possible deleterious effects on replication and expression. The DI used was a modified version of pE307 (Engelhorn *et al.*, 1993), which contains a single deletion of 13 590 nucleotides fusing the beginning of the NP gene to the end of the L gene (Figure 4A) and generates a (+) transcript from the T7 promoter. We found that four base changes creating a *Bgl*III and an *Nsi*I site on either side of the (+) leader-NP mRNA junction (Figure 1) did not affect the efficiency with which pE307 was amplified. These sites were originally introduced to facilitate the mutagenesis of the leader-NP junction in the DI genome, and the *Nsi*I site (AUGCAU) creates a new (out-of-frame) AUG codon at position 12 of the NP mRNA. This start codon is in a reasonable context for initiation (5'-AGUAUGCAU), but its location so close to the 5' end of the mRNA could lower its efficiency. Ribosomes which initiate at this AUG would translate an alternate open reading frame (ORF) which closes well within the NP ORF. However, the presence of this upstream AUG in E307 diminished, but did not eliminate, NP expression (results not shown). As a full-length expression vector could be prepared readily with this backbone, we decided to test its ability to generate infectious virus. This construct also contained a T7 promoter which initiates a transcript with three extra Gs at its 5' end (pppGGGACC... ~25 times more frequently than pppACC...), because this was found to be beneficial for E307 amplification. The extra

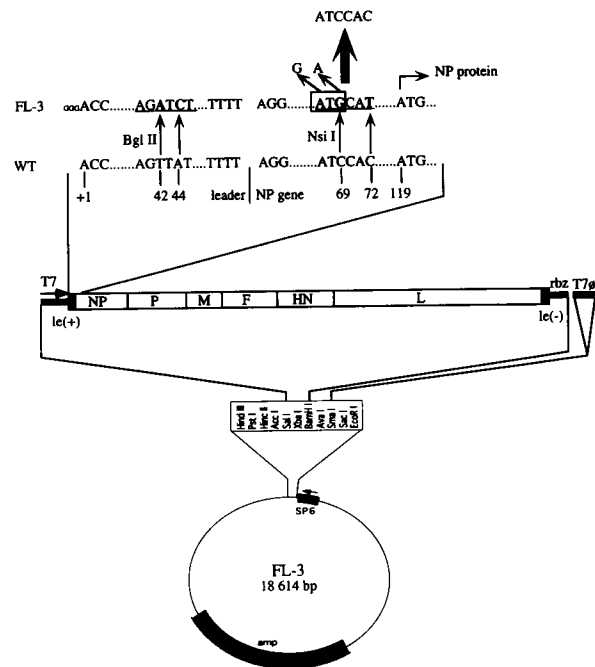


Fig. 1. The full-length infectious clone, FL-3. The 15 384 nucleotide-long genome is shown in the middle as DNA, with the 3' end of the (-) genome on the left, set between the T7 promoter (T7) and the HDV ribozyme (rbz) and a T7 terminator (T7 Φ), all placed within the polylinker region of pSP65, as shown. The 55 nucleotide-long (+) leader region (black box on the left) and the start of the NP gene are expanded above, showing the base changes made to create the *Bgl*III and *Nsi*I sites. The ATG of the latter site is boxed, and its position relative to that which starts the NP protein is shown. The arrows above the *Nsi*I site show the changes found in the recovered viruses (see text). The three smaller G residues at the 5' end of the FL-3 sequence are derived from the T7 promoter, and have been removed in the recovered viruses.

5' G residues were quickly eliminated from the amplified DI genomes (results not shown), as found in other systems (Tanaguchi *et al.*, 1978; Pattnaik and Wertz, 1990; Schnell *et al.*, 1994). There is also a T7 terminator downstream of the HDV ribozyme, whose presence was found to be neutral for E307 amplification. The missing 13 590 nucleotides were then reinserted (see Materials and methods), generating FL-3, a full-length (15 384 nucleotides) cDNA with four base changes creating a *Bgl*III and an *Nsi*I site on either side of the (+) leader-NP junction (Figure 1).

Virus recovery

Infectious virus was recovered from the full-length cDNA as follows. First, HeLa or BHK cells (in 9 cm dishes) were infected with vTF7-3 [multiplicity of infection (MOI) ~3] for 1 h (all operations were carried out at 33°C). Then the infecting medium was removed and replaced by 2.5 ml medium containing FL-3 and the NP, P and L plasmids (whose ratios were optimized using the DI parent) and home-made TransfectAce (Rose *et al.*, 1991). At 16–20 h post-infection, the cells were washed and the medium replaced with modified Eagle's medium (MEM) containing 100 μ g/ml AraC to prevent further DNA synthesis. At 36–48 h post-infection, the cells (showing clear cytopathic effects) were scraped from the dishes, pelleted and resuspended in 2 ml/dish MEM. Up to 500 μ l of this suspension were injected into the allantoic cavity of four 9 day old

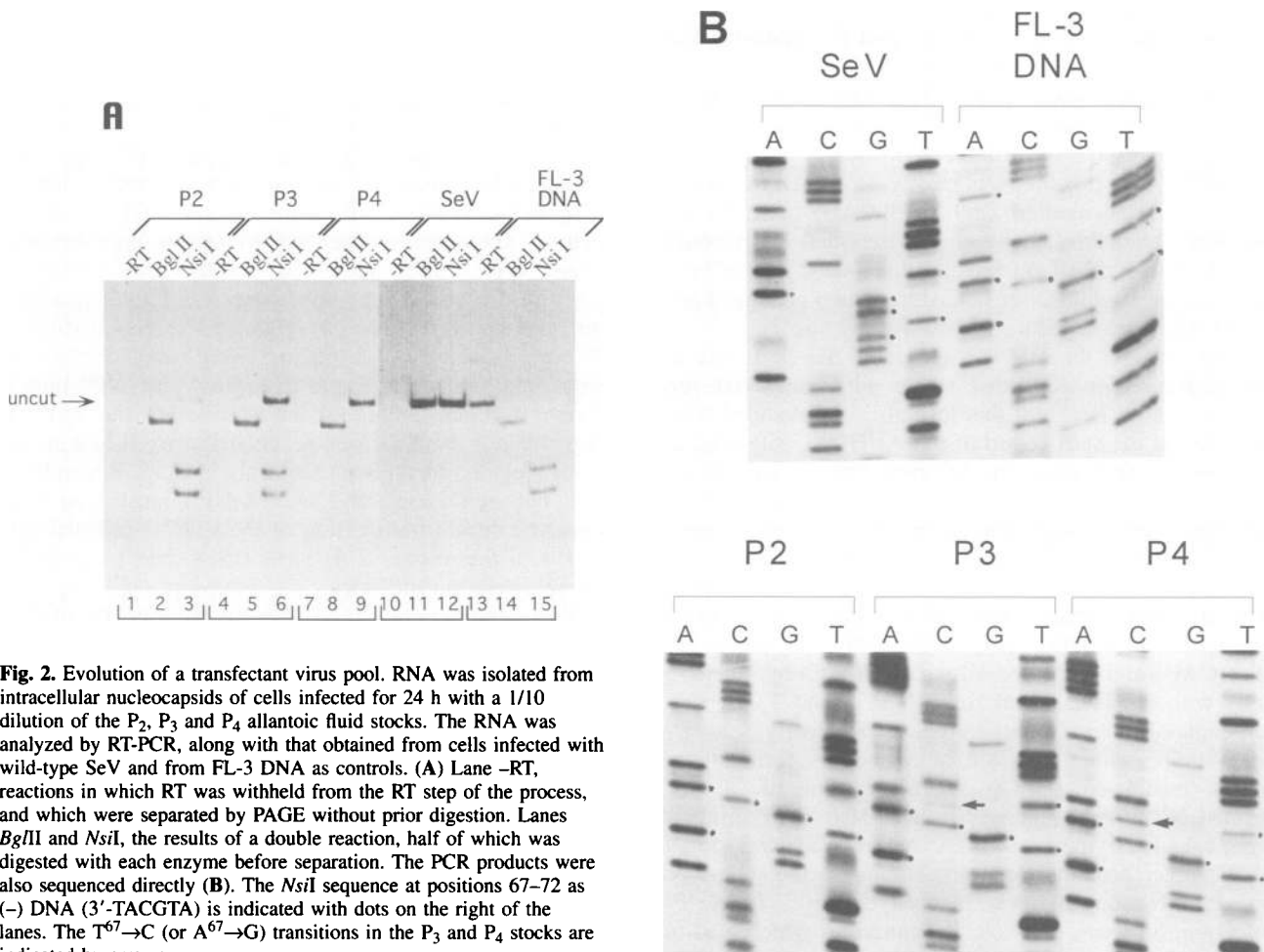


Fig. 2. Evolution of a transfectant virus pool. RNA was isolated from intracellular nucleocapsids of cells infected for 24 h with a 1/10 dilution of the P₂, P₃ and P₄ allantoic fluid stocks. The RNA was analyzed by RT-PCR, along with that obtained from cells infected with wild-type SeV and from FL-3 DNA as controls. (A) Lane -RT, reactions in which RT was withheld from the RT step of the process, and which were separated by PAGE without prior digestion. Lanes *Bgl*II and *Nsi*I, the results of a double reaction, half of which was digested with each enzyme before separation. The PCR products were also sequenced directly (B). The *Nsi*I sequence at positions 67–72 as (-) DNA (3'-TACGTA) is indicated with dots on the right of the lanes. The T⁶⁷→C (or A⁶⁷→G) transitions in the P₃ and P₄ stocks are indicated by arrows.

embryonated chicken eggs which were kept at 33°C for 2 days. The allantoic fluid from all the eggs was harvested and combined, and 100 µl of this P₁ (passage 1) fluid were injected into each of four eggs whose allantoic fluids were combined to yield the P₂ virus stock. Further passage stocks were obtained after dilutions of 1/1000 in a similar manner. The presence of virus in these fluids was determined by infecting BHK or HeLa cells with a 1/10 dilution of each stock. Cytoplasmic extracts were prepared and centrifuged on CsCl density gradients, where viral nucleocapsids form a sharp band at 1.31 g/ml and the vast majority of other (unencapsidated) nucleic acids pellet. These gradients are not only useful for purifying the nucleocapsids; they are also diagnostic of SeV infections because relatively small amounts (e.g. containing 1 µg genome RNA) are visible to the naked eye with indirect light at their characteristic buoyant density (they scatter blue light). The gradients are also diagnostic for vaccinia virus, where the presence of other (white) bands at ~1.25 g/ml indicates this infection. vTF7-3 appears to be eliminated from the stock by P₃, according to this test.

Initially, four recovery experiments were carried out, and a nucleocapsid band, whose identity was confirmed by immunoblotting with anti-NP, was visible by P₂ in all cases. RNA was extracted from these nucleocapsids and the pertinent region (positions 21–126, Figures 1 and 4A) was amplified by RT-PCR. A -RT control was always included to ensure that RNA was being amplified, and the

same region was also simultaneously amplified from natural virus RNA and FL-3 DNA. All four recoveries gave similar results, and one is shown in Figure 2. An amplified product which depended on the RT step of the RT-PCR (Figure 2A, lanes -RT) was present in all the recombinant virus (rSeV) pools and, in this case, was digested with both *Bgl*II and *Nsi*I to yield fragments of the expected mobility (Figure 2A, lanes 2 and 3). The product amplified from the natural virus was not digested by either enzyme (Figure 2A, lanes 11 and 12). However, while the P₂–P₄ products were completely cut by *Bgl*II, a small number of the P₂ products were not digested with *Nsi*I under conditions where that amplified from FL-3 DNA was digested completely (Figure 2A, lane 15). This fraction also increased from P₂ to P₄. To investigate this progressive loss of the *Nsi*I site, and to confirm the presence of the markers in the recombinant genomes, the PCR products from each infection were also sequenced directly. The P₂ products were found to contain the same sequence as FL-3 at the four different positions (Figure 1, bold letters), whereas the product from the natural virus (SeV) infection contained none of these changes, as expected [Figure 2B; positions 67–72, as (-) DNA, are marked with dots on the right-hand sides of the lanes]. However, the sequence from P₃ contains a C as well as the expected T at nucleotide 67 in the (-) DNA (Figure 2B, arrow). This converts to 5'-(A/G)UGCAU as (+) RNA, and G becomes the predominant base at this position

in the P₄ sequence. No other changes from the FL-3 sequence were detected in the P₃ and P₄ products. The progressive loss of digestion with *Nsi*I can thus be explained by the progressive replacement of A67 with G. Virus recovered from the second experiment was similar, with a progressive loss of the *Nsi*I site from P₂ to P₄, except that here this loss was mostly accounted for by the progressive transition of G69 (AUGCAU) to A. In the two other recoveries, none of the amplified DNA could be cut at all by *Nsi*I by P₂, and this was found to be a result of the simultaneous reversion of two changes back to the wild-type sequence (AUCCAC; Figure 1).

Given that in these four rSeV pools the *Bgl*II site in the leader region remained stable while the *Nsi*I site was invariably lost, and this loss always coincided with mutation of the start codon of the AUGCAU site (and in three out of four cases by different events), viruses are clearly being selected which have removed the block on NP expression, as might have been expected. To determine the extent of this block that SeV can tolerate and remain viable in the recovery test, we compared the rate of viral protein synthesis in cells infected with the P₂ stock shown in Figure 2A (~90% AUGCAU) with a P₆ stock (all GUGCAU) and wild-type virus (AUCCAC) by labelling cells with [³⁵S]Translabel for 1 h at 8 and 13 h post-infection. The viral proteins could be clearly seen by SDS-PAGE without the aid of antibodies during these pulses (results not shown). When the ratios of the ³⁵S-labeled NP protein relative to the P, HN and F proteins were calculated, the level of NP synthesis relative to that of the other proteins was very similar in the wild-type and P₆ infections but was reduced by ~50% in the P₂ infection. SeV can thus tolerate mutations which lead to a 2-fold decrease in the level of NP synthesis and remain viable in this recovery system.

Recombination during virus recovery

There appear to be two ways in which the SeV genome mutates during virus recovery to eliminate the upstream AUG. One involves base transitions (A67→G and G69→A, presumably due to an error-prone SeV replicase; reviewed in Holland *et al.*, 1992) where the selection of the AUG⁻ virus is relatively slow; these stocks become mostly AUG⁻ only by P₄. The other is the simultaneous reversion to the wild-type sequence (G69→C and U72→C), where all the viruses are AUG⁻ by P₂. The more rapid selection of these latter viruses may be a result of their increased fitness because they contain the wild-type sequence at nucleotides 67–72. However the question arises as to how this simultaneous reversion took place. The region containing the *Nsi*I site also occurs in pGEM-NP which generates NP during the initial stages of recovery, so homologous recombination is possible. However, the pGEM-NP gene is slightly shorter at its 5' end, beginning at nucleotide 61, leaving only eight nucleotides between this end and the first base change (G69→C). One of the putative recombinational crossover events would thus have to occur within this remarkably short interval.

Therefore we sought further evidence that recombination was occurring at a significant frequency during virus recovery. Our full-length clone is from strain Z and pGEM-NP is from strain H. Although there are few differences between these NP genes, a substitution in the

C-terminal 100 amino acids of the protein leads to an increased mobility of NP^Z during SDS-PAGE. These mobility differences are seen in immunoblots of the initial transfection (Figure 3, lane 2), which also show that by 24 h of incubation as much NP^Z is being generated as NP^H. This unexpectedly high level of NP^Z expression is probably caused, at least in part, by the reciprocal recombination of the NP^Z sequences into pGEM-NP (see below). Therefore we searched among our recombinant Z strain stocks for those which, on infection, also made NP^H. Of 20 stocks examined, one was found in which, remarkably, most of the NP migrated like that of strain H (Figure 3, lane 6). Two plaques were isolated from this stock, and both were found to express only NP^H (one is shown in Figure 3, lane 7) and to have lost the *Nsi*I site. Nevertheless both viruses retained the *Bgl*II site in the leader region, which is present only in FL-3. Recombination between FL-3^Z and pGEM-NP^H must then have occurred during transfection of the vTF7-3-infected cell, and both the mosaic Z-H virus (rSeV-NP^H) and rSeV^Z are recovered in the stock.

Recombination during virus recovery was also evident when additional targets for this event were provided. When pE307, which contains the wild-type leader and beginning of the NP gene (Figure 4A), was included as an additional plasmid during the transfection, virus which had lost both sites was recovered in 12 out of 12 recoveries. Moreover, P₁ stocks of these viruses were of sufficiently high titer that their infection of cell cultures led to visible levels of intracellular nucleocapsids. Although these viruses had lost all four markers distinguishing them from wild-type sequences, parallel recoveries without pE307 led exclusively to viruses which had retained the *Bgl*II site.

Generation of a copy-back nondefective interfering SeV via recombination

Assuming that rSeV-NP^H has little selective advantage (or disadvantage) over rSeV^Z in our mixed infections, this suggests that FL-3 cDNAs recombined over virtually the entire NP gene, with pGEM-NP in at least 5% of the recoveries. The remarkable frequency of recovery (100%) of revertant virus with wild-type leader sequences when pE307 is included during transfection is presumably a result of both the high rate at which recombination occurs and the competitive advantage of viral genomes containing the wild-type (+) leader region. The (+) leader region of mononegaloviruses is the short sequence between the 3' end of the genome and the start of the first (NP) gene (55 nucleotides for SeV; Figure 4A, hatched box). There is an analogous region between the 3' end of the antigenome and the end of the last (L) gene: the (-) leader (or trailer) sequence (also 55 nucleotides for SeV; Figure 4A, black box). The (+) and (-) leader regions are thought to control antigenome and genome synthesis, respectively, because the 3' ends of the templates contain at least part of the replicase promoters. Their complements (the 5' ends of the nascent chains) contain the site for the initiation of nucleocapsid assembly. As genome replication is coupled to the concurrent assembly of the nascent chain, these 5' ends can also be considered as part of the replication promoters. Because the reversion of both of the *Bgl*II and *Nsi*I sites to the wild-type sequences appears to confer a strong advantage on progeny genomes, we examined

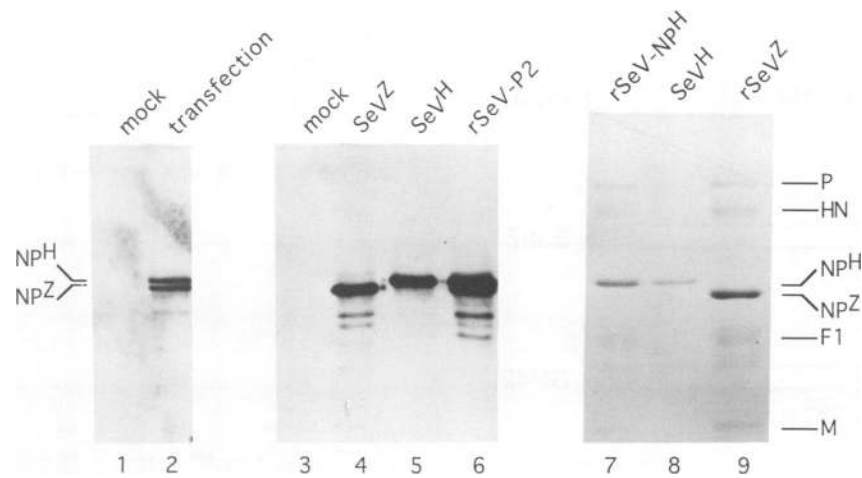


Fig. 3. Isolation of a virus with a variant NP^H protein. Lanes 2 and 1 show an immunoblot of vTF7-3-infected cells transfected with pFL-3 and pGEMs NP, P and L for 24 h, and a mock-transfected control, respectively. Lane 6 shows an immunoblot of cells infected with the P₂ stock of one particular pool (rSeV-P₂), along with those infected with strains H and Z (SeV^{H/Z}). Lane 7 shows a Coomassie Blue-stained gel of 20 μl allantoic fluid which was pelleted, along with that of natural strain H (SeV^H, lane 8) and a rSeV^Z (lane 9).

whether the (+) leader region of FL-3 could be replaced by other sequences which might be functional, e.g. those found on copy-back DI genomes.

DI genomes are deletion mutants which are thought to be generated by a copy-choice mechanism in which the viral replicase, carrying its nascent chain, either jumps forward on its template, creating an internal deletion (e.g. E307; see Figure 4A), or jumps backwards onto the nascent chain itself, creating a terminal complementary repeat upon finishing the chain. These latter DIs are called copy-backs, and all natural examples have replaced their (+) leader regions of their genomes with (-) leader or trailer regions (Figure 4A, black boxes at both ends). Copy-backs tend to have a strong competitive advantage in replication over the nondefective genome, presumably because they have replaced the genomic promoter with the stronger antigenomic promoter. These notions of promoter strength have been given more substance recently by Calain and Roux (1995), who compared the amplification levels of different DI constructs in the transfected cell system. They found that the relative levels of amplification between different DI genomes in this system are determined primarily by whether the genomic or antigenomic sequences are present at the 3' end of the genome. One of the E307 constructs created for this study, GP42 (Figure 4A), in which the first 42 nucleotides of its genomic promoter were replaced by those of the antigenomic promoter (creating a copy-back of 42 nucleotides), was of particular interest. This DI, with a chimeric antigenomic/genomic promoter at the 3' end of its genome, was found to amplify as strongly as a natural copy-back DI, yet its ability to transcribe an mRNA from its single transcription unit was reduced only ~2-fold relative to that of E307.

To determine whether this chimeric promoter could replace the natural genomic promoter sufficiently for virus to be recovered in our system, GP42 was similarly included in the transfection and putative virus was passaged in eggs. Two independent transfections were carried out and gave identical results (one transfection is shown in Figure 4). When these stocks were used to infect cell cultures to assay for the presence of infectivity, they gave rise to a

much reduced but clearly visible nucleocapsid band in CsCl density gradients by P₂. RT-PCR analysis of the nucleocapsid RNA was carried out using four primer sets, as depicted in Figure 4A. Primer set a is specific for either FL-3 or E307, because primer le21 can anneal only to the genomic promoter. Primer sets b, c and d are all specific for antigenomic sequences at the 3' end of the genome because primer tr-15 can only act as a left to right primer here. Primer set b will detect both GP42 and the possible full-length infectious recombinant (rSeV-GP42). Set c is specific for rSeV-GP42 because primer NP643 cannot anneal to GP42, and set d is specific for GP42 because the PCR product from rSeV-GP42 would be too long (14 253 bp) to be made efficiently in our protocol. As shown in Figure 4B, only primer set a amplifies the expected product (105 bp) from natural SeV (lanes 5–8), whereas primer set d but not set c amplifies the expected product (659/661 bp) from a natural virus stock also containing DI-GP42 (lanes 19 and 20). The PCR products of the P₂ stock with primer sets a and b are shown in Figure 4C and show that genomes with both the genomic (set a, lane 3) and antigenomic sequences (set b, lane 4) at their 3' ends are present in roughly equal amounts at this passage level. Further passage of this stock led to the disappearance of the wild-type genomes (lane 5) but not those with the copy-back 3' end by P₄ (lane 6), and no defective GP42 genomes were found at P₆ (Figure 4B, lanes 2–4).

An unexpected property of all the infections with these progeny was that the level of intracellular nucleocapsids did not improve upon further passage of the stock. The progeny also produced mild cytopathic effects only by 48 h post-infection, whereas all the other stocks produced strong effects by 24 h. These stocks also produced very small plaques: ~0.1–0.2 mm, when the other stocks gave plaques of 1.0–2.0 mm, with a titer of ~10⁸/ml, ~2.0 logs less than that of the other stocks. However, this titer appears to be grossly underestimated because both stocks have similar amounts of NP protein and genome RNA (results not shown). Two of these pin plaques from the P₅ stock were expanded in eggs, and a PCR analysis of these

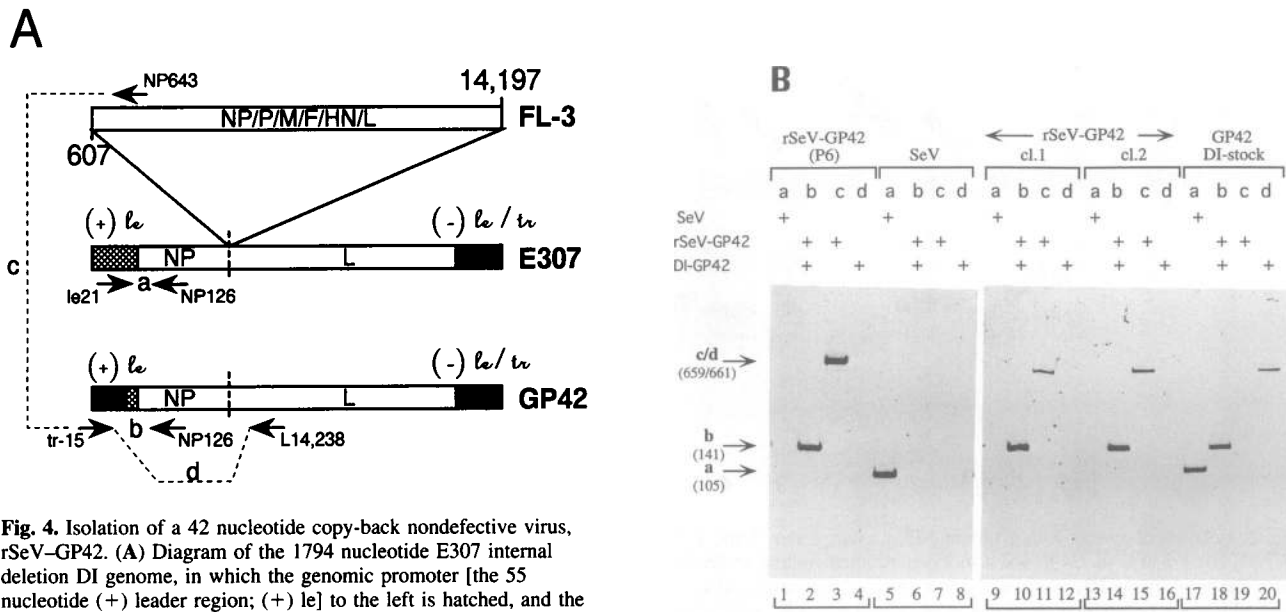


Fig. 4. Isolation of a 42 nucleotide copy-back nondefective virus, rSeV-GP42. (A) Diagram of the 1794 nucleotide E307 internal deletion DI genome, in which the genomic promoter [the 55 nucleotide (+) leader region; (+) le] to the left is hatched, and the antigenomic promoter [the 55 nucleotide (-) leader or trailer region; (-) le/tr] to the right is in black. The sequences deleted from the nondefective genome in creating E307 are shown above. FL-3 was obtained from a modified E307 and is represented by the sum of the top two boxes, as shown. GP42 is a derivative of E307, in which the first 42 nucleotides of the genomic promoter (hatched) were replaced with the same region of the antigenomic promoter (black). The horizontal arrows show the positions and orientations of the primers used in the RT-PCR analysis, and a–d indicate the four primer sets used. (B) Nucleocapsid RNA was isolated from cells infected with the P₆ allantoic fluid of a transfection which included pGP42 [rSeV-GP42 (P6)], wild-type SeV, two clones isolated from the rSeV-GP P₅ fluid, and a stock of wild-type SeV containing DI-GP42, as shown above. RT-PCR was carried out with the primer sets a–d, as indicated, and the products were separated by PAGE. The plus signs above each lane indicate which viral genomes can be detected by each primer set, as detailed in (A) and in the text. The sizes of the products expected for the different primer sets are indicated to the left. (C) The same analysis carried out with the P₂ and P₄ allantoic fluids of rSeV-GP42, and the P₁ and P₂ fluids of a 1:1 mixed infection of wild-type SeV and the P₆ stock of rSeV-GP42, as shown above. Only primer sets a and b were used.

progeny (Figure 4B, lanes 9–12 and 13–16) showed that, like the uncloned P₆ stock (Figure 4B, lanes 1–4), they produced the expected products only with primer sets b and c. A direct sequence analysis of these cloned viruses showed that they contained the expected sequence at the pertinent region (results not shown). Finally, when the rSeV-GP42 P₆ stock (Figure 4B, lanes 1–4) was mixed 1:1 with that of SeV (Figure 4B, lanes 5–8) and passed in eggs, the progeny produced only small amounts of intracellular nucleocapsids, similar to infection with rSeV-GP42 alone. A RT-PCR analysis showed that there were roughly equal amounts of both genomes at P₁ (Figure 4C, lanes 7 and 8), but the wild-type genomes had largely been excluded by P₂ (Figure 4C, lanes 9 and 10). Taken together, these results indicate that a 42 nucleotide copy-back nondefective virus (rSeV-GP42) with the property of interfering with the growth of standard virus (similar to classic copy-back DI genomes) has been generated by recombination between pFL-3 and pGP42 during transfection of the vTF7-3-infected cells.

Discussion

The infection of cells with vTF7-3 not only provides cytoplasmic T7 RNA polymerase to generate transcripts

from T7-promoted plasmids, but the vaccinia virus infection also amplifies these DNAs (presumably via rolling circles) and recombines them at a high frequency (and fidelity) if they contain homologies (Evans *et al.*, 1988; Parks and Evans, 1991a,b; Zhang and Evans, 1993; Ball, 1995). In other experiments, in which two pGEM plasmids expressing different forms of the SeV P protein were transfected into these cells, we found that a single crossover event (as monitored by immunoblotting) occurred with a frequency as high as 50% in some cases (when there are >100 nucleotides of homology). Double crossovers (such as that which presumably generated rSeV-NP^H) were found at a frequency of ~10%; even evidence of triple crossovers could be clearly seen, all in the absence of selection. Given that our full-length construct (FL-3) contains four base changes in the first 72 nucleotides of the viral genome which may all be deleterious, this promotes the selection of the recombined genome. Therefore it is not so surprising, in retrospect, that homologous recombination has played such a large role in this study.

We have exploited the highly recombinogenic nature of this system to generate two modified rSeV. The first, rSeV-NP^H, in which most of the NP^Z gene of FL-3 was replaced by that of pGEM-NP^H, is of interest (so far)

primarily because it shows what can be done in this system without selective pressure. The other modified virus, rSeV-GP42, containing a chimeric promoter at the 3' end of the genome chain, has interesting properties. All the other viruses generated in the recoveries yield roughly the same level of viral nucleocapsids intracellularly and infectivity titers as wild-type SeV ($\sim 10^{10}$ /ml of allantoic fluid). Nevertheless, all these rSeV are lost completely in a single round of mixed infections in eggs in competition with an equal amount of SeV. In contrast, rSeV-GP42 predominates in these mixed infections of eggs and, in fact, eliminates the wild-type virus by the second passage (Figure 4C). In this respect, rSeV-GP42 is an interfering nondefective virus. However, despite its advantage in mixed infections, rSeV-GP42 amplifies intracellularly to levels far lower than any of the other viruses. Thus, unlike classic copy-back DI viruses, its interfering ability does not appear to be linked to an amplification advantage. Further experiments will hopefully determine why this chimeric promoter does not confer the same amplification advantage to the nondefective virus as it does to the DI genome (Calain and Roux, 1995). We also note that DI-GP42, which was used to generate rSeV-GP42, cannot compete with rSeV-GP42 which contains the same constellation of ends, further indicating that small size is apparently not the determining factor in the competition for a limited supply of packaging proteins. It will be of interest to determine whether rSeV-GP42 competes as well against SeV in infections of mice, the natural host. rSeV-GP42 can also be considered as a dominant negative mutant, similar to viruses isolated from persistent infections of VSV (Youngner *et al.*, 1986; Jordan and Youngner, 1987). The copy-choice mechanism thought to generate copy-back DI genomes could also conceivably generate viruses similar to rSeV-GP42 in cell culture if the crossover back onto the nascent chain were carried out conservatively with respect to the genome chain length.

It was indeed fortunate that the *Bgl*III and *Nsi*I sites introduced as markers had such negative effects on the recovered virus, so that viruses altered by point mutation or recombination became evident so quickly. One of the difficulties in working with full-length paramyxovirus cDNAs is the paucity of convenient restriction sites and the unexpected effects of introducing such sites (much data not shown). The homologous recombination which operates so efficiently in the vaccinia virus-infected cytoplasm can, of course, be used to circumvent this problem. It should also be possible to create positive selection in this system by constructing almost full-length cDNAs missing a particular region or gene, such that virus can only be recovered after its replacement from a cotransfected plasmid. In this case, the sequence alterations can be carried out more reasonably on a much smaller cDNA. For alterations of the NP and P genes, their pGEM expression plasmids can be replaced by those carrying the human parainfluenzavirus type 1 gene to avoid recombination between the almost full-length cDNA and the expression plasmid (data not shown). Our recovery system is also more robust than expected; it has yet to fail in >40 recoveries, and it can accommodate negative elements such as an upstream out-of-frame AUG and still yield virus (in the absence of other virus competition). Thus it

should also be possible to mutate interesting regions, e.g. the ACG which acts as a start codon for the C' protein of the P gene. If virus is recovered, it can be further passaged to see whether C' expression will eventually be restored, and by what route. In a similar vein, we are currently passaging the rSeV-GP42 stock looking for spontaneous large plaque variants which may have circumvented the limitation of genome amplification. Because the solution of these problems will be provided by nature, this adds a true genetic component to the reverse genetic system.

Upon completing this work, we learned that the M. Billeter laboratory in Zurich has successfully recovered measles morbillivirus from cDNA (Radecke *et al.*, 1995), and the P. Collins laboratory at NIH has recovered respiratory syncytial pneumovirus from cDNA (personal communication).

Materials and methods

Construction of the full-length infectious SeV cDNA

cDNAs spanning the entire SeV Z strain genome (Shioda *et al.*, 1983, 1986; Hidaka *et al.*, 1984) were assembled into a single chain by ligation of the restriction fragments (kindly provided by Y. Sakai and T. Shioda, manuscript in preparation). For the final assembly step, a pE307 minigenome (Engelhorn *et al.*, 1993) contained in plasmid pSP65 was used. pE307 (Figure 4A) contains nucleotides 1–607 fused to nucleotides 14 197–15 384, inserted between a T7 RNA polymerase promoter which generates three extra G residues at the 5' end, and the HDV antigenome ribozyme sequence (Perrotta and Been, 1991) which generates a precise 3' end; a T7 terminator sequence is also present downstream of the ribozyme (Figure 1). The pE307 used was modified to contain two genetic tags in the SeV sequence: two base changes in the (+) leader sequence, T42→A and A44→C, creating a *Bgl*III site (Figure 1), and two base changes in the NP untranslated region, C69→G and C72→T, creating an *Nsi*I site and a new (out of frame) AUG (Figure 1). The SeV sequence was inserted in two pieces [a *Pf*MI (nucleotide 507)–*Pf*MI (nucleotide 13 831) fragment and a *Pf*MI (nucleotide 13 831)–*Kpn*I (nucleotide 15 279) fragment] into the two unique sites *Pf*MI (nucleotide 507) and *Kpn*I (nucleotide 15 279) of the modified pE307 (tpE307) (Figure 1).

Transfections

Transfection experiments were carried out as described previously (Curran *et al.*, 1991). BHK or HeLa cells were grown overnight in 9 cm diameter dishes in modified Eagle's medium (MEM) supplemented with 5% fetal calf serum to ~80% confluency, and infected with the recombinant vaccinia virus vTF7-3 (Fuerst *et al.*, 1986) at a MOI of 3. At 1 h post-infection, cells were washed twice with MEM lacking serum and transfected with a plasmid mixture containing 1.5 µg pGEM-L, 4.5 µg pGEM-NP, 4.5 µg pGEM-P, 15–30 µg pFL-3 and 20 µl home-made transfectase (Rose *et al.*, 1991) in 2.5 ml MEM. At 16–20 h post-infection, the cells were washed and the medium replaced with MEM containing 100 µg/ml AraC.

Analysis of rSeV stocks

The presence of virus in the allantoic fluids of the egg was determined by infecting BHK or HeLa cells with a 1/10 dilution of each stock. At 24 h post-infection cells were harvested, lysed with 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, plus 0.5% NP40; the cytoplasmic extracts were centrifuged on 20–40% (w/w) CsCl density gradients to purify the viral nucleocapsids (Lynch and Kolakofsky, 1978).

Titration and plaquing of transfectant viruses (rSeV) were carried out in LLC-MK2 cells covered with a 0.3% agarose overlay containing 1.2 µg/ml acetyl-trypsin for 3–4 days at 33°C.

RT-PCR

RNA from viral nucleocapsids purified on CsCl density gradients (from one-quarter of a 9 cm Petri dish) was used for reverse transcription and PCR with the following primers (Figure 4A): NP126 (5'-CGGCC-ATCGTGAACCTTTGGC-3'), nucleotides 126–107; le21 (5'-GTATGGA-ATATATAATGAAG-3'), nucleotides 21–40; NP643 (5'-CCAGACTTG-

GACAATTATTGCTCC-3'), nucleotides 643-620; L14,238 (5'-CGAGCTATTCTGTAATTCATTC-3'), nucleotides 14 238-14 216; and tr-15 (5'-ATTGGATCCGAATGCTACCAGACAAGAGTTTAAGAGATATGTATC-3'). The sequence in italic is a T7 promoter; that in bold type is nucleotides 15 384-15 356, i.e. from the right-hand extremity.

Reverse transcription reactions were carried out with the M-MLV enzyme, as described by Gibco/BRL. One-fifth of the reaction was then used directly for DNA amplification with 1 U *Taq* DNA polymerase in 50 µl reactions. After 17 cycles (denaturation 30 s, 92°C; annealing 30 s, 50°C; elongation 20-60 s, 72°C), the PCR products were digested with restriction enzymes or separated directly on 10% polyacrylamide gels and visualized with ethidium bromide. The PCR products were purified using the QIAquick PCR purification Kit (Qiagen) and sequenced with T7 DNA polymerase.

Protein analysis

Proteins contained in the NP-40 cytoplasmic extract were analyzed by immunoblotting, as described in Curran et al. (1991), except that the signal was visualized using the chemiluminescent substrate AMPPD (Boehringer). For virion proteins, 1 ml allantoic fluid was centrifuged on 500 µl 25% glycerol in 10 mM Tris-HCl pH 7.4, 100 mM NaCl and 1 mM EDTA at 12 000 r.p.m. for 30 min in an Eppendorf tube. The pellet was resuspended in protein sample buffer and 1/50 was separated by SDS-PAGE and stained with Coomassie Blue.

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