Creation of an infectious recombinant Sendai virus expressing the firefly luciferase gene from the 3’ proximal first locus

Mohammad K. Hasan, Atsushi Kato, Tatsuo Shioda, Yuko Sakai, Deshan Yu and Yoshiyuki Nagai

Department of Viral Infection, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

A genetic engineering approach was made to generate a recombinant non-segmented negative-strand RNA virus, Sendai virus (SeV) of the family Paramyxoviridae, that expresses firefly luciferase. The DNA construct containing the entire open reading frame (ORF) of the luciferase gene followed by the SeV transcription stop and restart signals connected with the conserved intergenic three nucleotides was inserted immediately before the ORF of the viral 3’-proximal nucleocapsid (N) protein gene in a full-length SeV cDNA copy. After intracellular expression of full-length antigenomic transcripts from the engineered cDNA and of the viral nucleocapsid protein and RNA polymerase from the respective plasmids, a recombinant SeV expressing luciferase activity at a high level was recovered, although the tendency of this particular reporter gene product to aggregate in cells made it difficult to estimate the maximum level of expression. The increase in genome length brought about by inserting 1728 nucleotides into the 15384 nucleotide parental SeV was associated with reduced plaque size, slightly slower replication kinetics and a severalfold decrease in yield of the virus. The inserted luciferase gene was stably maintained after numerous rounds of replication by serial passages in chick embryos. These results indicate the potential utility of SeV as a novel expression vector.

Introduction

Recent success in recovering infectious virus entirely from cDNA has opened the possibility of engineering non-segmented negative-strand RNA viruses (Schnell et al., 1994; Lawson et al., 1995; Whelan et al., 1995; Radecke et al., 1995; Collins et al., 1995; Garcin et al., 1995; Kato et al., 1996; Baron & Barrett, 1997; for review see Palese et al., 1996 and Rose, 1996). Naked genomic or antigenomic RNA of these viruses cannot be a functional template for either transcription or replication. To be so and to initiate the infectious cycle, the RNA needs to be associated with the viral nucleocapsid (N) protein and RNA polymerase (L and P proteins) to form the ribonucleoprotein complex (RNP). Thus, the basic technique leading to infectious virus recovery involves intracellular expression of full-length viral RNA and N, P and L proteins from the respective plasmids under the control of the bacteriophage T7 promoter. The T7 polymerase is most often provided by a recombinant vaccinia virus (VV). Plasmids which give rise to genome-sense as opposed to antigenome-sense strands have been much less efficient, though not totally ineffective, in initiating virus recovery (Kato et al., 1996), possibly due to their hybridization with miRNAs encoding the trans-acting viral protein and/or to their possession of sequences resembling the T7 polymerase termination sequence (Schnell et al., 1994; Radecke et al., 1995; Whelan et al., 1995).

Our system for a paramyxovirus, Sendai virus (SeV), apparently has the highest recovery rate reported to date for any non-segmented negative-strand virus (reviewed in Rose, 1996). This has come from fine tuning of the system in terms of the concentration of the supporting N, P and L proteins and by constructing plasmids giving rise to transcripts with 5′ and 3′ ends identical to those of the wild-type RNA (Kato et al., 1996; for review see Palese et al., 1996). Optimization of the system also involved the use of cytosine arabinoside (araC) and rifampicin to minimize the cytotoxicity of the VV supplying T7 polymerase. Our system has thus facilitated SeV engineering, and allowed us first to demonstrate that the viral V protein, expressed by a remarkable process known as cotranscriptional editing, is non-essential for virus replication in tissue culture but encodes a luxury function required for in vivo pathogenesis (Kato et al., 1997a), and second to map this pathogenicity determinant to the cysteine-rich carboxy-terminal portion of the V protein (Kato et al., 1997b).
Another important issue to be addressed with this system has been whether SeV is flexible enough to express an additional, foreign gene. There is only a single promoter for RNA polymerase at the 3' end of non-segmented negative-strand viruses. By recognizing the end (E) or stop (termination/polyadenylation) and start (S) signals conserved between the genes, the RNA polymerase gives rise to the leader and to each mRNA. Successful expression of an inserted foreign gene flanked by the new S and E signals has already been reported with other non-segmented negative-strand viruses (Mebatsion et al., 1996; Schnell et al., 1996a, b; Bukreyev et al., 1996). In these cases, the foreign gene was inserted to a genome position remote from the 3' end. Here, we attempted insertion into a more upstream position close to the 3' end of the SeV genome, in view of polar attenuation of transcription toward the 5' end. We have first defined a position in the 3' non-coding region (in negative sense) in the N gene of the SeV genome which tolerated insertion of a small synthetic fragment containing a unique restriction site, and then introduced into this position the firefly luciferase sequence followed by a new set of E and S signals for transcription termination of its own gene and transcription restart of the next N gene, respectively. The created recombinant SeV allowed robust expression of the luciferase gene.

**Methods**

**Plasmid construction.** The plasmid pSeV(+) was constructed to generate the exact SeV full-length antigenic plus-sense RNA of 15384 nucleotides (Kato et al., 1996) and used as the starting material for plasmid construction. In pSeV(+), SeV cDNA was placed in the antigenic orientation between the T7 promoter (pT7) and a cDNA copy of the self-cleaving ribosome (Rbs) from the antigenic strand of hepatitis delta virus. The plasmid pSeVASK(+) is a derivative of pSeV(+) which was made by cleavage of pSeV(+) at unique SplI and Kpnl sites to remove a large portion (~14670 nucleotides) of the virus-specific sequence, followed by circularization with a linker (Fig. 1A). This small derivative containing only the viral terminal regions was the background on which manipulations to insert an additional short sequence were made. This short sequence consisted of 18 nucleotides and was designed to contain a unique NolI site for introducing a foreign gene of interest. Insertion of the 18 nucleotide sequence was attempted at two different positions, after residues 79 and 119 of the 3' non-coding region of the N gene, in pSeVASK(+) (Fig. 1A, B) [numbering is on the negative-sense genomic strand (Shioda et al., 1983)]. For these insertions, we used site-directed mutagenesis by a PCR-mediated overlap primer extension method (Ho et al., 1989). Briefly, two DNA fragments with overlapping ends were generated by using the complementary primers (CPs) with overlapping complementary 18 nucleotides containing a NolI site and the outer primers (OPs) (Fig. 1A). The two fragments were combined in the subsequent fusion reaction in which the overlapping ends anneal, allowing the 3' overlap of each strand to serve as a primer for 3' extension of the complementary strand. The following are the sequences of primers used.

- OP1, 5' TCTGACACATGCAGCTCCCG 3';
- OP2, 5' CATGCCAGCACCCAGCAACAA 3';
- CP1a, 5' cgtgagctggcgcagtCACGAGTTCCAGACCTTTTGG 3';
- CP2a, 5' aattgcggccgctagACCACTCCAGACCTTTTGG 3';
- CP1b, 5' cgtgagctggcgcagtCACGAGTTCCAGACCTTTTGG 3';
- CP2b, 5' ggagctgacagcttacagATGGCCGGTGTTGAGCAAC 3'.

The complementary NolI site-containing sequences are shown in lowercase letters. The two different amplified products were individually digested with ApaI and SplI and cloned into pSeVAVSK(+). Into these plasmids, the above described SplI–Kpnl fragment of pSeV(+) spanning the 14670 nucleotide viral sequence, was replaced, thereby generating plasmids with an extra 18 nucleotides after position 79 (pSeV18'+(+) or 119 (pSeV18'+(+) in full-length SeV cDNA. The luciferase gene from the firefly *Photinus pyralis* was derived from the plasmid pHLucRT4(−) (Kato et al., 1996) by PCR amplification of the open reading frame (ORF) of 1653 nucleotides with a pair of NolI-tagged primers [5' AAgccggccgagCTCTCCCG 3' (sense) and 5' TggagctgacagcttacagATGGCCGGTGTTGAGCAAC 3']. Underlined are a new set of SeV E and S signals connected with the conserved intergenic 3' GAA 5' sequence and the lower case letters represent the NolI tag. After digestion with NolI, the amplified fragments were directly introduced into the NolI site of pSeV18'+(+) (Fig. 1C). This final construct was named pSeVLuc(+). All PCR were performed with ExTaq polymerase (Takara, Tokyo). All PCR products were verified for their sequence authenticity.

**Transfection of cDNAs and virus recovery.** Viruses were recovered from cDNAs essentially according to the previously described procedures (Kato et al., 1996). Briefly, 1.2 × 10⁷ LLCMK2 cells were infected with VV vTF7-3, expressing T7 polymerase (Fuerst et al., 1986), at a multiplicity of 2 p.f.u. per cell. Then, 0.0 µg of pSeV(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or pSeV18'+(+) or...
Fig. 1. Construction of the plasmid pSeVluc(+) encoding the SeV antigenome with an insertion of the luciferase gene in the non-coding region of viral N gene. (A) pSeVΔSK was generated by digestion of pSeV(+), encoding the full-length SeV antigenome, with SphI and KpnI followed by circularization. An 18 nucleotide fragment designed to contain a NotI site was inserted after position 79 or 119 by site-directed mutagenesis (PCR-mediated overlap primer extension method; for details, see Methods). CP, complementary primers; OP, outer primers. The larger SphI–KpnI fragment of SeV spanning the 14670 nucleotide SeV sequence was then returned back into pSeVΔSK, now containing an extra 18 nucleotides, thereby generating plasmids encoding the SeV antigenome with an additional 18 nucleotide sequence. The plasmid with the insertion after position 119 was named pSeV18b(+). (B) The nucleotide sequence (in positive sense) around the site of insertion of the 18 nucleotide fragments [18a(+)] and [18b(+)] in lower-case letters. BB3 is assumed to have critical functions in replication and/or transcription. S represents the start signal for N gene transcription. For details, see the text. (C) The ORF of the luciferase gene was PCR-amplified with NotI-tagged primers from plasmid pHvLuc-RT4, digested with NotI and introduced into pSeV18b(+). A set of new E and S signals connected with GAA was invented in the antisense primer to be placed at the end of the amplified luciferase gene. The resulting plasmid gives rise to an antigenomic SeV RNA of 17112 nucleotides which contains the luciferase-expressing unit in its first locus.
The longer non-coding region of N gene (Fig. 1A, B). Insertion was between residues 79 and 80 or between 119 and 120 in the mutagenized plasmids, creating pSeV18 virus infection, these cells were maintained in MEM without serum. After + with α hybridized with [α-32P]dCTP labelled cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific riboprobe synthesized in vitro using SP6 polymerase (Epicient Technologies).

**Western blotting.** Infected cell lysates were electrophoresed in 12.5% SDS–polyacrylamide gels (Laemmli, 1970). The proteins in the gels were electrotransferred onto PVDF membranes (Millipore) and probed with anti-luciferase antibody (Promega) or anti-SeV rabbit serum as described previously (Kato et al., 1996). The virus probes that had been labelled with [α-32P]dCTP using the Multiprime DNA Labelling System (Amersham). The same filters were also probed with anti-luciferase antibody (Promega) or anti-SeV rabbit serum as described above.

**Cell cultures.** The monkey kidney cell lines LLCMK2 and CV1 were grown in MEM supplemented with 10% foetal bovine serum. After virus infection, these cells were maintained in MEM without serum.

### Results

**Insertion of an 18 nucleotide fragment into SeV cDNA and recovery of recombinant viruses**

SeV genome length appears to have to be a multiple of six nucleotides (Calain & Roux, 1993). Taking this rule of six into consideration, we synthesized an 18 nucleotide fragment and recovery of infectious virus. In repeated trials, virus recovery was un-

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Passage in eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st passage</td>
</tr>
<tr>
<td></td>
<td>LLCKM2 cell no. per egg</td>
</tr>
<tr>
<td>SeV(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>SeV18&lt;sup&gt;a&lt;/sup&gt;(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SeV18&lt;sup&gt;b&lt;/sup&gt;(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SeVluc(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup> Not tested.

wild-type SeV recovered from pSeV(+) were once again passaged in eggs at a dilution of 10<sup>–8</sup> and were the same sequence difference or chance mutations possibly caused during manipulation of the plasmids was responsible for the failure of virus recovery from pSeV18<sup>a</sup>(+). The failure could also be due to disruption of the BB3-equivalent sequence from positions 75 to 92 (Fig. 1B), which has been supposed to

<table>
<thead>
<tr>
<th>1st passage</th>
<th>2nd passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLCKM2 cell no. per egg</td>
<td>HAU</td>
</tr>
<tr>
<td>SeV(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>SeV18&lt;sup&gt;a&lt;/sup&gt;(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SeV18&lt;sup&gt;b&lt;/sup&gt;(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SeVluc(+)</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup> Not tested.
Table 2. Luciferase expression in CV1 cells from SeV/luc which had been serially passaged in chick embryo

Each passage was initiated with an input dose of 10 p.f.u. per egg and ended with the final virus yield expressed by HAU and p.f.u./ml. The viruses were then inoculated to CV1 cells at a multiplicity of 20 p.f.u. per cell, and the luciferase activity and p.f.u. were determined at 14 h p.i.

<table>
<thead>
<tr>
<th>Passage in eggs</th>
<th>Infection in CV1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>HAU</td>
</tr>
<tr>
<td>1</td>
<td>1024</td>
</tr>
<tr>
<td>2</td>
<td>2048</td>
</tr>
<tr>
<td>3</td>
<td>2048</td>
</tr>
<tr>
<td>4</td>
<td>2048</td>
</tr>
<tr>
<td>5</td>
<td>1024</td>
</tr>
<tr>
<td>6</td>
<td>1024</td>
</tr>
<tr>
<td>7</td>
<td>1024</td>
</tr>
<tr>
<td>8</td>
<td>1024</td>
</tr>
<tr>
<td>9</td>
<td>1024</td>
</tr>
</tbody>
</table>

be important for guiding and facilitating polymerase attachment (Blumberg et al., 1991).

Insertion of the luciferase gene into SeV cDNA and recovery of a recombinant virus

The luciferase gene was PCR amplified from pHvLuc-RT4 with NotI-tagged specific primers as described in Methods. The antisense primers contained a set of E and S signals connected with the conserved intergenic trinucleotide which were derived from those between the N and P genes. The amplified fragment was introduced to the NotI site of the 18 nucleotide insert in pSeV18+b(+) generating pSeVluc(+) (Fig. 1C). If this construct could generate infectious virus, the inserted luciferase gene would be expressed as a monocistronic mRNA under the control of the S signal originally used for starting N gene expression in the wild-type SeV and the newly introduced E signal. The following S signal would operate to initiate transcription of the downstream neighboring N gene. The sequence inserted into the 15,384 nucleotide parental wild type SeV genome was quite long (1728 nucleotides). Nevertheless, we were able to recover infectious progeny from pSeVluc(+) (Table 1). The recovery rate appeared to be about 10 times lower, compared with that of the wild-type SeV or SeV18+, as ten times more transfected cells were required for the former (Table 1). However, the comparably high titres (HAU) after the first passage in eggs strongly suggested that the recovered recombinant virus was fully infectious and contained no deleterious chance mutations. The lower rate of recovery thus appeared to be due to enlarged genome size. After the second passage in eggs to eliminate vTF7-3, the titre was also satisfactorily high, being comparable to those of SeV18+ and the wild-type SeV (Table 1). This stock virus, named SeV/luc, was confirmed to have the exact sequence insert of 1728 nucleotides by reverse transcription, PCR amplification and sequencing of the corresponding region of the viral genome.

Expression of luciferase gene from the recombinant SeV/luc

Fig. 2(A) shows the kinetics of luciferase activity expression in CV1 cells infected with SeV/luc under single-cycle growth conditions with an input multiplicity of 20 p.f.u. per cell. The enzyme activity increased exponentially up to 14 h p.i.
reaching a titre as high as about $3 \times 10^5$ c.p.s. Note that this titre is about 20-fold higher than that routinely obtained by transfection of the same cells with a luciferase-encoding pGEM plasmid driven by T7 polymerase from vTF7-3. However, little further increase occurred, in contrast to the viral HAU and p.f.u. titres which continued to increase beyond 14 h p.i. and peaked at 20 to 26 h. This was probably caused by inactivation of luciferase due to extensive intracellular aggregation occurring late in infection (see below).

Fig. 2(A) further shows a detailed comparison of virus replication between SeV/luc and wild-type SeV. The replication of the former was slightly but significantly retarded and the yields were consistently reduced by severalfold in p.f.u. and by two- to fourfold in HAU. In accordance with this, the plaque sizes of the former were significantly smaller than those of the latter (Fig. 2B). These results suggest that SeV/luc is slightly but significantly attenuated in replication capacity in CV1 cells.

Under the same single-cycle growth conditions, expression of the luciferase gene and of viral genes was studied by Northern hybridization and Western blotting. The luciferase-specific RNA was clearly identified at the 18S region and in the genomic 50S RNA at 14 or 20 h p.i. in the total RNA extracted from SeV/luc-infected CV1 cells (Fig. 3A). The bands migrating between the 18S and 50S regions could be readthrough products. The luciferase-specific RNA at the 18S, but not that at 50S, region was also clearly detected for oligo(dT)-selected RNA (data not shown). These results indicated that the luciferase gene was expressed in a monocistronic manner from SeV/luc and also replicated as a part of the genome. The luciferase gene product (62 kDa) became detectable at 6 h p.i., and then increased in amount (Fig. 3B). Its production kinetics appeared to correlate well with that of luciferase activity in cells as shown in Fig. 2A. Later in infection, this band did not increase in intensity. Instead, high molecular mass material appeared between the stacking and separating gels, and the amount increased with time (data not shown). This material most likely represented the intracellular aggregation of expressed and accumulated luciferase molecules. These aggregates could be enzymatically inactive and their preferential accumulation would account for the fact that luciferase activity did not increase beyond 14 h p.i. (Fig. 2A).

The virus-specific products were also examined. As represented by the data for N-specific RNAs and P and N proteins (Fig. 3A, B), SeV/luc displayed slightly slower production kinetics and lower levels at each time-point. These observations were also in good agreement with the attenuated phenotype characteristic of the recombinant virus shown in Fig. 2A, B.

Genetic stability of the inserted luciferase gene

Expression of a foreign gene from the 3’ proximal first locus has not been described for other non-segmented negative-strand RNA viruses. To learn whether such a foreign gene would be stably maintained, SeV/luc was serially passaged in chick embryos. Because each passage was initiated with an input dose of as low as 10 p.f.u. per egg and ended with a final virus yield as high as about $10^6$ p.f.u. per egg, assuming each egg contains 10 ml of allantoic fluid, the virus underwent numerous rounds of replication even within a single passage. After each passage, the virus was propagated in CV1 cells under single-cycle replication conditions exactly as described above and its capability to generate luciferase activity at 14 h p.i. was determined. The stock at each passage gave a similar luciferase activity up to the 9th passage (Table 2). Thus, the inserted luciferase gene appeared to be faithfully maintained in a functional form.

Discussion

We have constructed a recombinant, helper-independent SeV expressing a foreign gene, the firefly luciferase gene. Insertion of the gene and expression strategies involved creation of a NotI insertion site and attachment of a new set of E and S signals to the end of the gene to be inserted. The new E and S signals had been incorporated within the antisense
primer used for amplification of the gene to be inserted. Luciferase gene expression from the recombinant SeV/luc in CV1 cells appeared to be highly efficient. Efficient expression was not only due to generally robust SeV gene expression in CV1 and many other cell lines, but might also come from placing the gene in the first locus of the recombinant virus in view of the polar attenuation characteristic of paramyxovirus transcription. However, we have been unable to assess the maximum capability of SeV to express a foreign functional protein by this particular reporter gene because of inactivation of the gene products, probably due to extensive aggregation in cells.

The presence of the additional gene slightly but significantly retarded virus replication and resulted in a severalfold decrease in virus yield during single-cycle growth. This attenuation should be regarded as the minimal cost paid for SeV to tolerate an increase in genome length of 1728 nucleotides to a total of 15384 nucleotides. The transcriptional attenuation at the border between the luc and N genes could also be responsible for reduced virus replication. Similar or greater attenuation may also be inevitable when the foreign gene has been inserted downstream (Bukreyev et al., 1996). Insertion as a NotI-tagged cassette into the first locus should be the principal method for construction of a recombinant SeV expressing other foreign genes. Indeed, we have also recovered SeV recombinants with the gene encoding the gp120 envelope protein by this particular reporter gene because of inactivation of the polar attenuation characteristic of paramyxovirus. The foreign gene insert was not always stable (Mattion et al., 1994; Bredenbeek et al., 1993). In non-segmented negative-strand RNA viruses, a foreign gene can be expressed from a separate transcription unit and maintained stably. Such a relatively simple mode of gene expression and high stability seem to be of great technical merit and may further allow insertion of multiple transcription units. Influenza A virus, a segmented negative-strand RNA virus, is also an excellent tool for foreign gene expression. It can express foreign peptides incorporated into viral proteins, and complete foreign proteins by fusing a foreign ORF to a viral ORF or to a second ORF in a bicistronic gene segment (Garcia-Sastre et al., 1994; Percy et al., 1994). The segmented nature of the viral genome, however, could strictly limit the size of insertion.

Fidelity of the RNA polymerase as well as its ability to copy the entire genome and to express all genes with sufficient efficiency would also be important factors determining the size of insert sequence. It will be interesting to learn the real limit of insertion into paramyxovirus genomes and how this limit is determined.

Various positive-strand RNA viruses have been used as helper-dependent replicons or infectious chimeric viruses to express foreign genes (Bredenbeek et al., 1993; Frolov et al., 1996; Mattion et al., 1994; Pletnev et al., 1992; Porter et al., 1995; Pugachev et al., 1995; Sjoberg et al., 1994). In these cases their expression must involve a complex process of polypeptide cleavage. The foreign gene insert was not always stable (Mattion et al., 1994; Bredenbeek et al., 1993). In non-segmented negative-strand RNA viruses, a foreign gene can be expressed from a separate transcription unit and maintained stably. Such a relatively simple mode of gene expression and high stability seem to be of great technical merit and may further allow insertion of multiple transcription units. Influenza A virus, a segmented negative-strand RNA virus, is also an excellent tool for foreign gene expression. It can express foreign peptides incorporated into viral proteins, and complete foreign proteins by fusing a foreign ORF to a viral ORF or to a second ORF in a bicistronic gene segment (Garcia-Sastre et al., 1994; Percy et al., 1994). The segmented nature of the viral genome, however, could strictly limit the size of insertion.

We thank Y. Yogo, M. Asakawa, E. Nakayama, C. Moriya, M. Takeda, and X. Xin for technical advice and valuable discussion. We also thank K. Kakuta and A. Mitsuzawa for their help in preparing the manuscript. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, the Ministry of Health and Welfare, and the Science and Technology Agency of the Japanese Government and from the Human Science Promotion Foundation and the Organization for Drug Relief, RTD Promotion and Product Review of Japan.

References
transcription elongation factor from the 5’ proximal open reading frame of the M2 mRNA and provides a capability for vaccine development.


Received 25 April 1997; Accepted 2 July 1997