Accommodation of foreign genes into the Sendai virus genome: sizes of inserted genes and viral replication

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Received 13 May 1999; received in revised form 5 July 1999

Abstract Sendai virus (SeV) is an enveloped virus with a negative sense genome RNA of about 15.3 kb. We previously established a system to recover an infectious virus entirely from SeV cDNA and illustrated the feasibility of using SeV as a novel expression vector. Here, we have attempted to insert a series of foreign genes into SeV of different lengths to learn how far SeV can accommodate extra genes and how the length of inserted genes affects viral replication in cells cultured in vitro and in the natural host, mice. We show that a gene up to 3.2 kb can be inserted and efficiently expressed and that the replication speed as well as the final virus titers in cell culture are proportionally reduced as the inserted gene length increases. In vivo, such a size-dependent effect was not very clear but a remarkably attenuated replication and pathogenicity were generally seen. Our data further confirmed reinforcement of foreign gene expression in vitro from the V(−) version of SeV in which the accessory V gene had been knocked out. Based on these results, we discuss the utility of SeV vector in terms of both efficiency and safety.

Key words: Sendai virus; Genome; Replication

1. Introduction

Sendai virus (SeV), a member of the genus *Respirovirus* in the family *Paramyxoviridae*, is an enveloped virus with a non-segmented negative sense genome RNA of about 15.3 kb. In the genome RNA of negative sense (−)RNA, the extracistronic 3′ leader and 5′ trailer regions, which are about 50 nucleotides in length and contain *cis*-acting elements essential for replication, flank six genes, which encode the nucleocapsid (N) protein, phospho (P) protein, matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) and large (L) protein in this order from the 3′ terminus. The genome RNA is tightly associated with the structural subunits, N proteins, and further complexed with the RNA polymerase comprising the L and P proteins [1], forming a helical ribonucleoprotein complex, (−)RNP. L represents the catalytic chain and P is an essential modulator in RNA synthesis. The (−)RNP complex, not the naked RNA, is functional as the template for both transcription and replication. There is only a single promoter at the 3′ end for the polymerase. By recognizing the end (E) (termination/polyadenylation) and start (S) signals present at each gene boundary, the polymerase gives rise to each mRNA (reviewed in [2,3]). After translation of these mRNAs and accumulation of the translation products, genome replication begins. Here, the same polymerase copies the same RNP template, but now ignores the successive stop signals and reads through each gene boundary to generate a full length antigenomic (+)RNP. Association of the newly synthesized N subunits with the nascent RNA chain is a precondition for read-through. Thus, encapsidation and replication are tightly coupled and synchronized with each other. The (+)RNP, in turn, serves as the template for the synthesis of (−)RNP. The viral components meet at the plasma membrane and the mature virions are formed and released by budding.

We have established a system to recover SeV entirely from cDNA with a remarkably high efficiency [4]. Thus, the SeV genome can be changed at will and the outcomes can be evaluated in the context of not only viral replication in culture cells but also viral pathogenesis in the natural host, mice ([5–8], for a review see [3]). The technology has also opened the possibility to use SeV as a novel expression vector ([9–11], for a review see [3]). SeV is only moderately pathogenic for cells in culture, reaches an extremely high copy number in cells and has a broad cellular host range. Moreover, the V(−) SeV whose V protein expression was knocked out reached even a higher copy number than the standard SeV [5,6] and is therefore extremely useful to achieve a high level of foreign gene expression in cells of interest. For instance, the expression level of gp120 envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) from the V(−) version in certain cell lines appeared to be the highest available in mammalian cells [10]. Thus, recombinant SeV technology is greatly facilitating biochemical and structural studies of medically important proteins ([12], for a review see [3]). The SeV V protein itself is an accessory protein. Although perfectly competent for tissue culture replication as described above, the V knock out SeV was found to be rapidly cleared from the mouse lung by some innate immunity recruited early in infection and severely impaired in virulence for mice, suggesting that the V protein encodes a luxury function required for in vivo pathogenesis ([5,6], for a review, see [3]).

In this study, we attempted to insert into SeV and express from SeV foreign genes of various lengths to know how far SeV can tolerate additional length and how viral replication both in vitro and in vivo is affected by the length. The results indicated that SeV can stably express genes up to 3.2 kb in length. The viral replication in cell culture was retarded conversely as the inserted gene length was increased. Viral multi-

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0014-5793/99/$20.00
PII: S0014-5793(99)00960-6

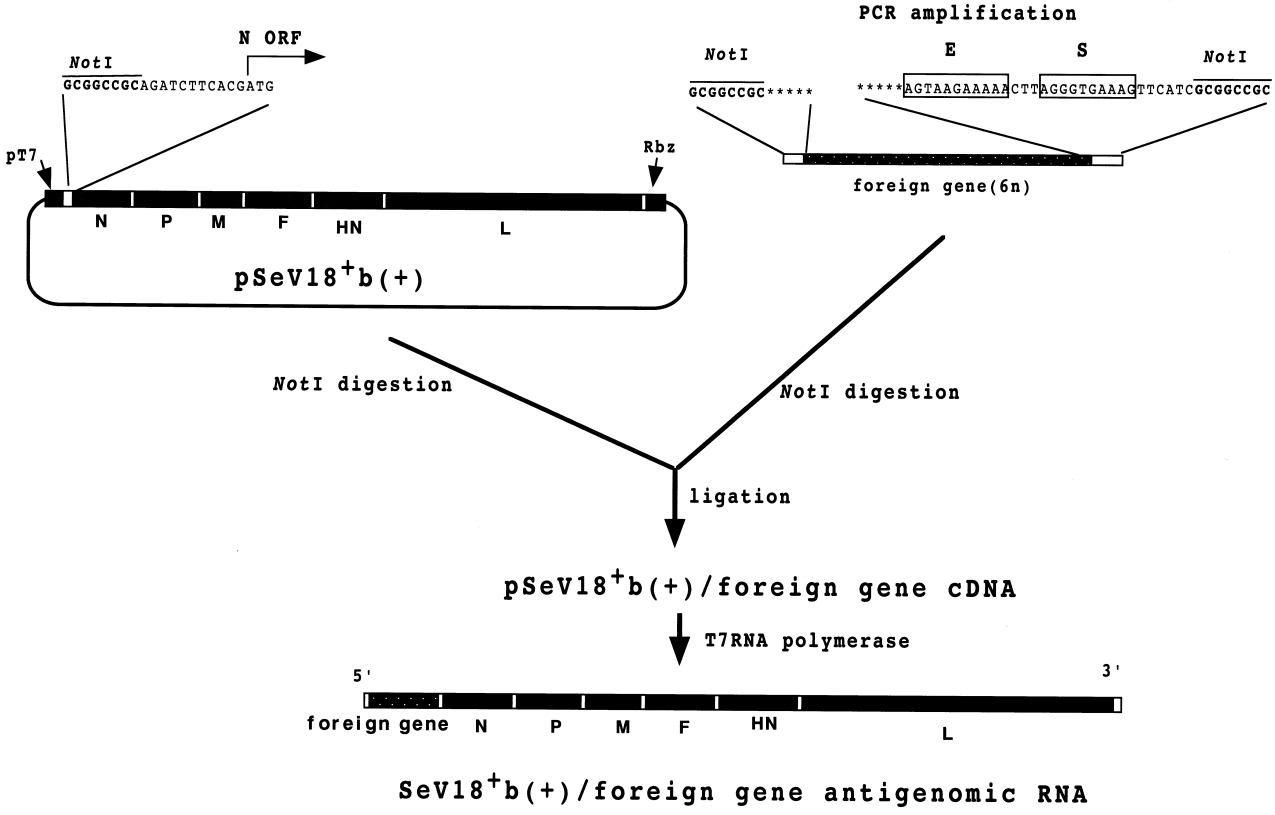

Fig. 1. Insertion of a foreign gene into the SeV cDNA plasmid. The SeV cDNA plasmid, named pSeV18$^+$b(+), contains an 18 nucleotide insert with a unique *Not*I site just upstream of the N ORF within the 3′ proximal N gene and generates antigenomic positive sense RNA. This construct allowed for a straightforward insertion of a gene PCR-amplified with *Not*I-tagged primers in a cassette-like fashion. SeV specific transcription termination (E) and restart (S) signals connected with a trinucleotide intergenic sequence were included in the antisense primer for the PCR reaction. Not only the standard SeV but also the V(−) version were used (see the text).

plication and pathogenicity in mice were also attenuated. The results confirmed the superiority of the V(−) version for a higher expression as well as convenience of the cassette-like insertion developed previously [9].

2. Materials and methods

2.1. Insertion of foreign genes into SeV cDNA plasmid

Fig. 1 illustrates the basic strategies for insertion of a foreign gene into the SeV genome. The SeV genome length appears to have to be a multiple of six nucleotides for efficient replication [13]. Without violating this 'rule of six', the SeV cDNA plasmid, named pSeV18b(+), was constructed, which contains an 18 nucleotide insert with a unique *Not*I site just upstream of the N open reading frame (ORF) within the 3′ proximal N gene (in genomic negative sense) and generates antigenomic positive sense RNA [9] (Fig. 1). This construct allowed for a straightforward insertion of a gene PCR-amplified with *Not*I-tagged primers (again without violating the rule of six) in a cassette-like fashion [9]. SeV specific transcription termination (E) and restart (S) signals connected with a trinucleotide intergenic sequence were included in the antisense primer for the PCR reaction. The recovered virus was named SeV18b+. Using the synthetic E and S signals, the inserted gene expression is terminated and the downstream neighbor N gene expression is initiated, respectively (Fig. 1). The transcription start of the inserted gene itself is driven by the S signal originally used for the N gene start of the parental SeV. In SeV transcription, restart at each gene boundary is efficient but not perfect, causing polar attenuation of gene expression toward the 5′ end (reviewed in [2]). All insertions were therefore made in the upstream region of the 3′ proximal N gene ORF. Not only the standard SeV but also the V(−) version were used for a higher level of expression [10]. The foreign genes, which we attempted to insert, included those encoding stromal cell-derived factor 1α (SDF-1α) [11], green fluorescent protein of *Aequore victoria* (GFP), HIV-1 gp120 [10], firefly luciferase (luci) [9], β-glucuronidase (β-glu) and *lacZ* encoding β-galactosidase (β-gal). The sizes of these foreign genes ranged from 0.4 to 3.2 kb (Table 1). β-Glu is relevant to mucopolysaccharidosis VII.

2.2. Transfection of cDNAs and infectious virus recovery

Viruses were recovered from cDNAs essentially according to the previously described procedure [4]. Briefly, 1.2×10^7 LLCMK2 cells, a monkey kidney line, were infected with a recombinant vaccinia virus (VV), vTF7-3, expressing T7 polymerase [14] at a multiplicity of two plaque forming units (PFU) per cell. Then, 60 μg of a SeV plasmid with a given insert and the plasmids encoding *trans*-acting proteins, pGEM-N (24 μg), pGEM-P (12 μg) and pGEM-L (24 μg), were transfected simultaneously with aid of the lipofection reagent DOTAP (Boehringer-Mannheim). The cells were maintained in serum free minimal essential medium in the presence of 40 μg/ml cytosine arabinofuranoside and 100 μg/ml rifampicin to minimize VV cytopathogenic-

Table 1
Sizes of inserted genes and the final titers of recovered recombinant SeVs after propagation in embryonated chicken eggs

Inserted gene	Size (kb)	Titer (10^9 PFU/ml)	
		SeV18$^+$b	V(−) SeV18$^+$b
18$^+$b		7.93	4.33
SDF-1α	0.4	9.74	4.79
GFP	0.7	1.14	4.47
HIV gp120	1.6	0.39	2.45
Luci	1.7	3.03	–
β-Glu	2.1	0.74	–
β-Gal	3.2	2.79	1.62

–, not done.

ity and thereby maximize the recovery rate [4]. 40 h after transfection, cells were harvested, disrupted by three cycles of freezing and thawing and injected into the allantoic cavity of 10 days old embryonated chicken eggs. After 3 days of incubation, the allantoic fluid was harvested. The titers of recovered viruses were expressed in hemagglutination units (HAU) and PFU per ml as described previously [4]. The allantoic fluid of the eggs contained 10^8–10^9 PFU per ml of the recovered virus along with the helper VV in an amount of 10^3–10^4 PFU per ml. The latter was completely eliminated by the second propagation in eggs at a dilution of 10^{-6} [4]. The second passages were used as the stock viruses for all the experiments.

2.3. Infection and pathogenicity studies

CV1 cells, a monkey kidney cell line, were used for studies of tissue culture replication as described previously [5,6]. For in vivo replication and pathogenicity studies, specific pathogen free, 3 weeks old mice of the strain ICR/Crj (CD-1) (Charles River, Tokyo, Japan) were used. Virus titers in the lungs were determined by cell infectious units per lung and pathogenicity by consolidation scores of the lungs and disturbance of body weight gain as described [5,6,8].

3. Results

3.1. Recovery of recombinant viruses

We inserted various foreign genes into the cDNAs encoding the standard or V(−) SeV antigenome RNA or both and attempted virus recovery according to the protocol described in Section 2 and illustrated in Fig. 2. The *Not*I-based cassette insertion has been generally convenient as no *Not*I site was present in the genes to be inserted except *lacZ*. One *Not*I site was present in the *lacZ*. This one was disrupted prior to PCR amplification. Virus recovery was successful in most attempts. However, there were some but rare unsuccessful cases despite repeated trials. The reason for this was initially unclear but careful inspection of the sequences to be inserted revealed that these genes contained a region resembling the SeV E signal (AUUCUUUUU). Most likely, such an E-like sequence induced premature transcription termination, eventually leading to failure of virus recovery. In these cases, E-like sequences were disrupted and viruses were successfully recovered. The final titers of recovered recombinants after propagation in embryonated chicken eggs are shown in Table 1.

We previously showed that the recombinant SeV18b+ with an 18 nucleotide insert and the original wild-type SeV were very similar in replication in cultured cells and multiplication and pathogenicity in mice. SeV18b+ but not wild-type cDNA was the starting material for insertion of foreign genes (see Section 2). Thus, we used SeV18b+ or its V(−) version as a control for all subsequent experiments.

Fig. 2 compares replication kinetics of various recombinant viruses derived from the standard V(+) version under the conditions of both a single round and multiple rounds of replication in CV1 cells. Replication kinetics were found to become slower as the inserted gene became longer. The peak titers also became lower, but the reductions were only by several folds at most under single cycle growth conditions (cf. β-gal and control in Fig. 2). As expected, under multiple cycle conditions which involved numerous rounds of replication, more profound differences were seen between the control and each recombinant except SeV/SDF-1α in replication kinetics and the final yield (Fig. 2). Thus, size-dependent replication retardation and titer reduction were both quite clear. These results suggest that the products from inserted genes are so far neutral without giving a particular advantage or disadvantage to the viral replication capability and argues for that the speed of genome replication is inversely correlated simply with the length of inserted genes. A very similar tendency was found with recombinant viruses based upon the V(−) version (Fig. 2). If a pair of recombinant viruses expressing a given gene were compared, the V(−) version always displayed a faster replication than the V(+) version, confirming our previous results [5,6,10].

3.2. Expression of foreign genes from recombinant viruses and its reinforcement with the V(−) version

Fig. 3A illustrates the expression of GFP and β-gal from the respective V(−) recombinant viruses in CV1 cells. Their

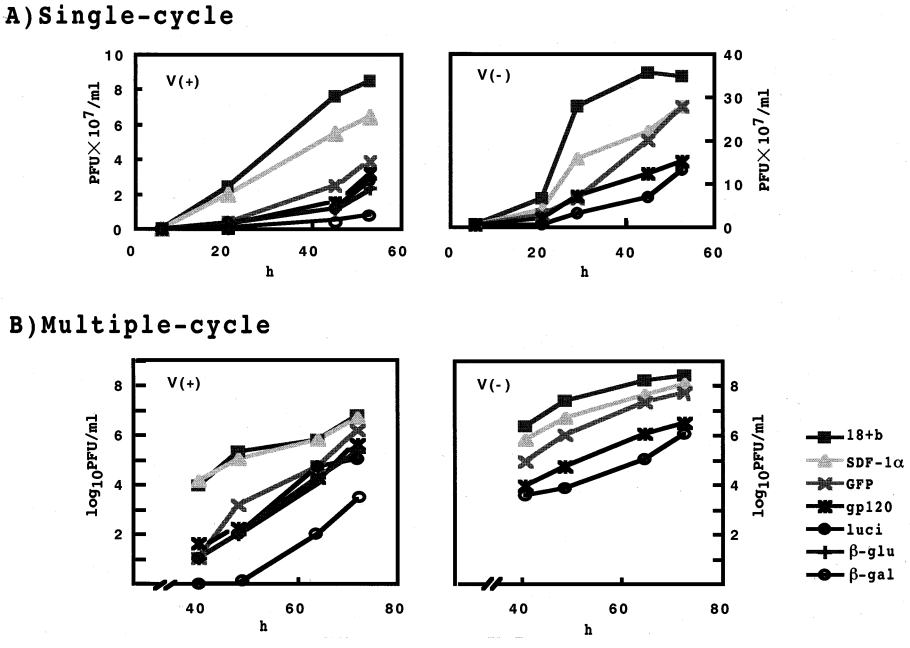

Fig. 2. Replication of various recombinant SeVs in CV1 cells. Growth kinetics of V(+) (A) and V(−) (B) recombinant viruses under the conditions of both single round (multiplicity of infection (moi) is 10 PFU/cell) and multiple rounds (moi 0.01 PFU/cell) of replication are compared.

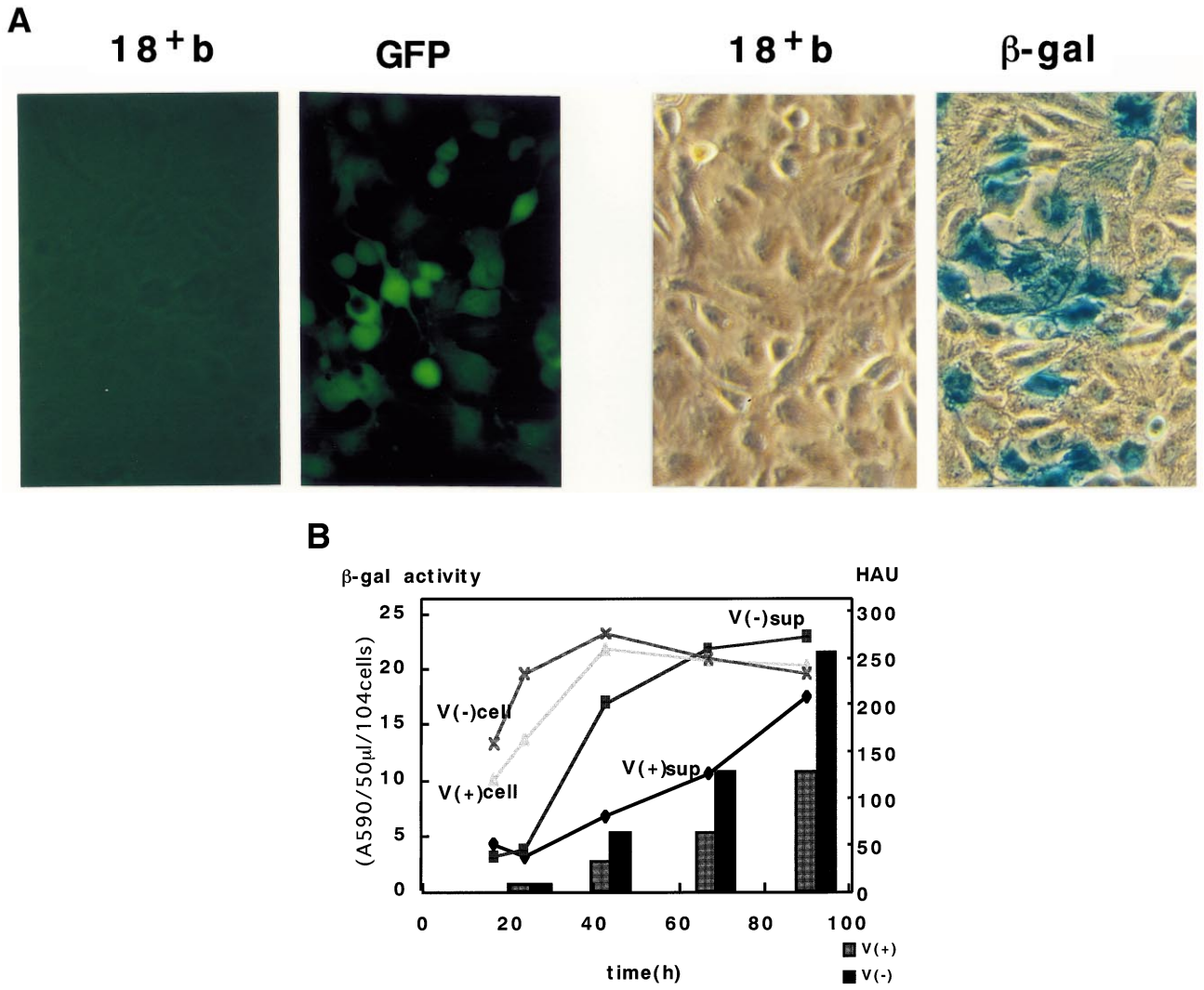

Fig. 3. Expression of foreign genes from recombinant viruses and its reinforcement with the V(−) version. (A) Expression of GFP and β-gal from the respective V(−) recombinant viruses in CV1 cells at 24 h post-infection at a moi of 0.1 PFU/cell. (B) Comparison of intracellular and extracellular β-gal activities expressed from the standard V(+) and V(−) versions at various times post-infection. Virus titers at each time point are shown by HAU.

expression from the V(−) version was already extensive at 24 h post-infection under single cycle growth conditions. Reinforced expression by the V(−) version was confirmed by comparing to intracellular and extracellular β-gal activities expressed from the standard V(+) and V(−) versions (Fig. 3B). Similar vigorous expression from the V(−) SeV in CV1 and other cell lines as well as primary cells of human and avian origins were previously demonstrated for SDF-1α [11], luci [9] and HIV-1 gp120 [10]. These results clearly argue for the utility of SeV, particularly its V(−) version, as a novel expression vector to produce foreign proteins of interest in cultured cells of interest.

3.3. In vivo pathogenicity of recombinant viruses

Fig. 4A shows the replication and pathogenicity of various V(+) recombinant viruses. The virus expressing the shortest gene, SDF-1α, appears to have retained a certain degree of pathogenicity, as body weight gain was greatly disturbed for two of the three mice. Consistent with this, the virus replicated remarkably well and produced high consolidation scores in the lungs. In contrast, the viruses expressing GFP and others longer than the GFP were greatly attenuated, allowing a body weight gain almost comparable to those of the mock-infected mice. This attenuation appeared to be paralleled with lower consolidation scores and less efficient viral replication in the lungs, compared with the control infection. Thus, the extra genes also appeared to affect in vivo viral replication in a length-dependent manner. However, while insertion of a relatively short gene, GFP, resulted in a reduction of the virus titer in the lungs by as much as 100-fold, no further attenuation of replication was seen with longer genes, gp120, luci, β-glu and β-gal. Therefore, in vivo virus growth was not so finely graded by the inserted gene length as in vitro growth. These results suggest the presence of much more complex conditions in vivo where replication would not only be limited by extra gene length but also affected somehow by the biological nature of the gene products and/or differences in host response to the products. The V(−) SeV was previously found to be greatly attenuated in multiplication and pathogenicity in vivo [5,6]. This was confirmed here (Fig. 4B). The V protein appeared to encode a luxury function for the virus to cope with some early host response [5,6]. The V(−) virus is thus

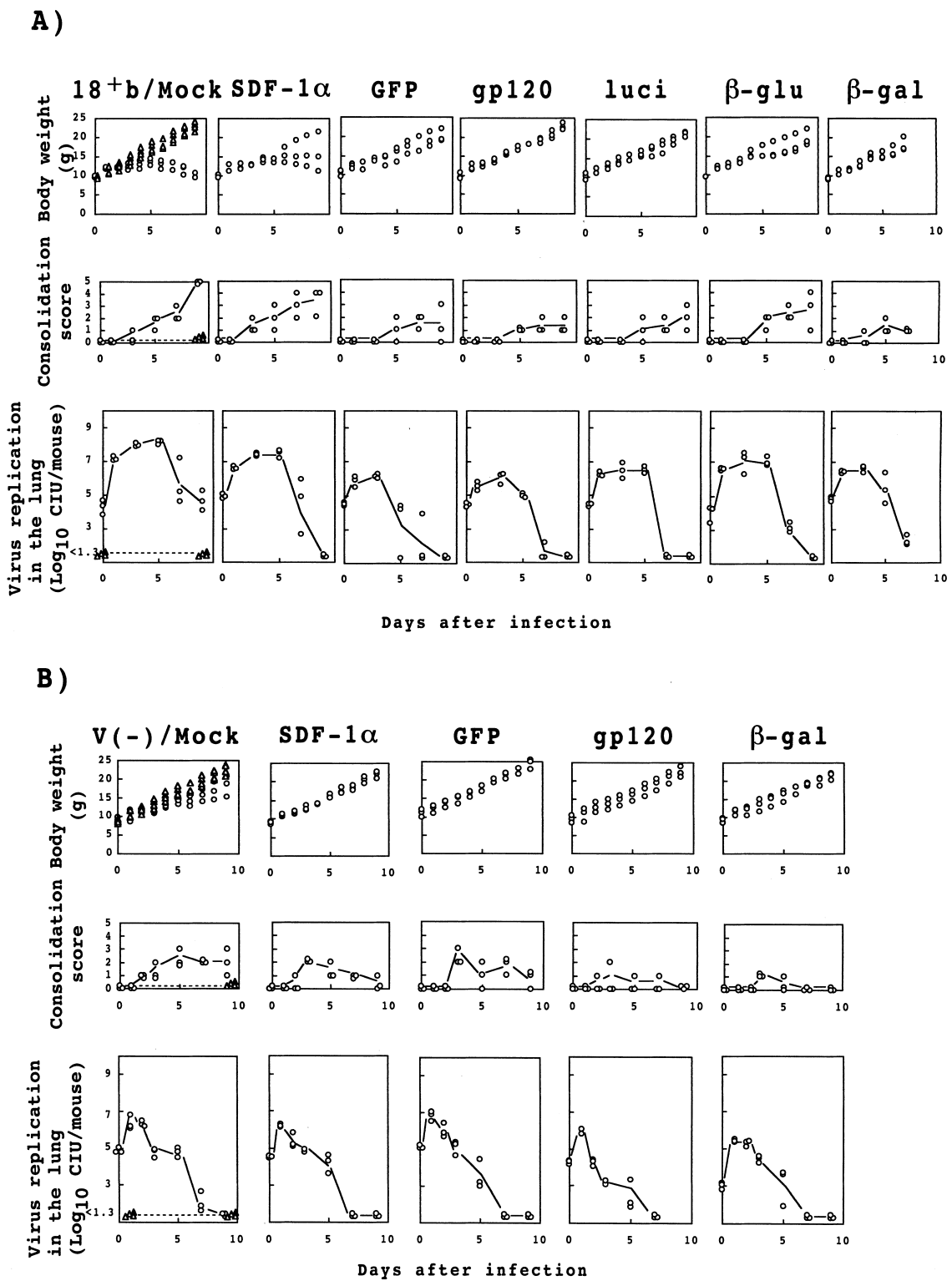

Fig. 4. Replication and pathogenicity of various recombinant viruses in mice. Virus titers in the lungs were determined by cell infectious units per lung and pathogenicity by consolidation scores of the lungs and disturbance of body weight gain as described [5,6]. △, mock-infected.

characterized by an apparently normal rapid replication at the initial day of infection followed by a rapid clearance from the body (Fig. 4B). Its overall in vivo replication pattern was simpler than that of the V(+) counterpart which was featured by maintenance of high virus titers for a long period (Fig. 4A). It may be interesting to note that extra gene length-dependent growth restriction has been seen more clearly in V(−) infections than in V(+) infections (cf. Fig. 4A and B).

4. Discussion

We have previously shown that foreign genes, such as those

encoding SDF-1α, SDF-1β, luci and HIV-1 gp120, can be inserted into and expressed from the SeV genome [9–11]. In this study, we investigated how far SeV can tolerate insertion of foreign genes in terms of their lengths and found that a gene up to 3.2 kb can be readily inserted and expressed. This size accounts for as much as about 1/5 of the original 15 384 bases of the SeV genome. The non-segmented, linear and helical RNP is capable of growing longer and thereby can accommodate such a long extra gene. However, as insertion of a gene longer than 3.2 kb was not attempted, our present study has been unable to define the upper limit.

Viral replication speed must be inversely correlated with the entire genome length. This concept has been used to explain the superiority of the shorter defective interfering viral genome to the full length wild-type genome. However, this has not been illustrated experimentally for any virus. Here, we were able to show for the first time such a genome size-dependent growth retardation because all products from the genes inserted happened, most likely, to be neutral for SeV replication at least in in vitro cells. Probably because of much more complex conditions existing in vivo, the principle could not be very clearly seen in mice but still appeared to be applicable. The fact that recombinant viruses were significantly attenuated as long as the inserted gene was sufficiently long suggests the utility of an even replication competent SeV vector in terms of safety.

Despite the error-prone nature of RNA polymerase in general, maintenance of inserted genes was satisfactory [9]. SeV transcription undergoes polar attenuation toward the 5′ end of thegenome, because the re-initiation at the restart signals of the downstream genes is efficient but not perfect. Consequently, the highest level of foreign gene expression is expected when the gene is placed in the first locus [9]. Furthermore, because of the augmented gene expression of the SeV V(−) mutant (see above), the use of the V(−) version and insertion of a gene upstream of its N ORF guaranteed an extremely high level of expression as shown typically here for β-gal and previously for HIV-1 gp120. The V(−)-based expression level of the latter was the highest currently available in mammalian cells [10]. SeV reaches quite a high copy number in infected cells, is only moderately cytopathic and possesses a broad cellular host range. Hence, replication competent SeV vector, particularly the V(−) version, is extremely useful in producing large quantities of medically important proteins in cells of interest, thereby greatly contributing to functional and structural studies (reviewed in [3]).

Large DNA viruses such as VV have most often been used for production of proteins of interest in mammalian cells. Because of their large genome sizes, e.g. about 190 kb for VV, it has been difficult to create a unique restriction site. Therefore, the step of homologous recombination is necessary for foreign gene insertion. In contrast, because of its short size, a unique *Not*I site has been created in SeV cDNA. This has greatly facilitated insertion of foreign genes in a cassette-like fashion.

The viral vectors most frequently used for gene therapy have derived from retro- and adenoviruses. With the exception of lentiviruses, retroviruses cannot infect non-dividing cells and adenoviruses hardly infect cells of blood origin. Our recombinant SeVs can infect non-dividing cells such as neurons and replicate there and express foreign genes vigorously (in preparation). They also replicate and express foreign genes in the primary human peripheral mononuclear cells and macrophages in culture [10]. SeV replication is independent of nuclear functions and does not have a DNA phase. Thus, it does not transform cells by integrating its genetic information into the cellular genome. Furthermore, homologous recombination has not been observed. Thus, replication incompetent viral constructs grown in complementing cells should be free of a contaminating virus generated by a recombination event. These properties weigh heavily in favor of SeV and related non-segmented negative strand RNA viruses in terms of both utility and safety (reviewed in [3]). Therefore, vectors of the second generation are awaited, which will no longer be replication competent but replication incompetent. They will not encode endogenous envelope proteins but instead incorporate them expressed in *trans* or are pseudotyped with foreign viral envelope proteins.

Acknowledgements: We thank W.S. Sly, Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, for providing the cloned β-glu gene. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, the Ministry of Health and Welfare, the Organization for Pharmaceutical Safety and Research and the Bio-oriented Technology Research Advancement Institution.

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