Persistent and Stable Gene Expression by a Cytoplasmic RNA Replicon Based on a Noncytopathic Variant Sendai Virus*§

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Persistent and stable expression of foreign genes has been achieved in mammalian cells by integrating the genes into the host chromosomes. However, this approach has several shortcomings in practical applications. For example, large scale production of protein pharmaceutics frequently requires laborious amplification of the inserted genes to optimize the gene expression. The random chromosomal insertion of exogenous DNA also results occasionally in malignant transformation of normal tissue cells, raising safety concerns in medical applications. Here we report a novel cytoplasmic RNA replicon capable of expressing installed genes stably without chromosome insertion. This system is based on the RNA genome of a noncytopathic variant Sendai virus strain, Cl.151. We found that this variant virus establishes stable symbiosis with host cells by escaping from retinoic acid-inducible gene 1-interferon regulatory factor 3-mediated antiviral machinery. Using a cloned genome cDNA of Sendai virus Cl.151, we developed a recombinant RNA installed with exogenous marker genes that was maintained stably in the cytoplasm as a high copy replicon (about 4 × 10⁶ copies/cell) without interfering with normal cellular function. Strong expression of the marker genes persisted for more than 6 months in various types of cultured cells and for at least two months in rat colonic mucosa without any apparent side effects. This stable RNA replicon is a potentially valuable genetic platform for various biological applications.

Delivery and ectopic expression of foreign genes in mammalian cells is now standard in modern biology. Although transient expression of delivered genes is usually sufficient for basic research purposes, their sustained expression is desirable in various medical and industrial applications. For example, persistent expression of therapeutic genes in tissue cells in situ is crucial for gene therapy of congenital metabolic diseases (1). Stable and strong gene expression is also required for large scale production of recombinant proteins in cultured mammalian cells (2). Persistent expression of exogenous genes is achieved routinely by integrating them as a part of the host chromosome, either actively by using integrating viral vectors (1) or transposable elements (3) or passively without any special molecular device. Whereas the former occurs much more efficiently, exogenous DNA elements are inserted into the random location on the host chromosome in either case.

Chromosomal insertion has several drawbacks in practical applications of transgene expression. Because the number of expression cassettes integrated into a cell by a single transfection event is usually limited (2), multiplication of the target genes by laborious drug selection is sometimes required to maximize the gene expression in industrial purposes. In addition, random chromosomal insertion of exogenous genetic elements occasionally induces uncontrollable activation (or suppression) of endogenous genes, and this may lead to fatal side effects in medical applications. For example, in a recent gene therapy trial, the retroviral transfer of therapeutic genes into bone marrow stem cells induced malignant T-cell lymphoma through accidental activation of the LMO2 gene (4). Installing exogenous genes on stable autonomous DNA replicons, such as circular episomal DNA and linear human artificial chromosomes, may avoid these problems (5), although the significance of these replicons in medical and industrial applications is not yet established. Recombinant adeno-associated virus vectors also frequently maintain their genome as an episome in the nucleus, but the mechanism underlying this persistence and the long term stability in the cells have not been established (1).

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**Persistent and Stable Cytoplasmic RNA Replicon**

Sendai virus (SeV) is a nonsegmented negative strand RNA virus belonging to the *Paramyxoviridae* (6). It has a single-strand RNA genome (15,384 nucleotides) encoding eight proteins (NP, P, C, V, M, F, HN, and L). Replication and transcription of SeV occurs in the cytoplasm with extremely low cell specificity and species specificity, although SeV is neither pathogenic nor carcinogenic in human beings. These characteristics make it a candidate for producing harmless viral vectors suitable for medical and industrial applications. Most recombinant SeV vectors developed by reverse genetics have genetic backgrounds of wild-type cytopathic SeV strains (Z and Nagoya) (7, 8). The replication-competent first generation vectors were prepared from the full-length viral genome cDNA with inserted exogenous genes. The replication-defective second generation vectors were prepared from the cDNA by deletion of essential viral genes. These vectors have characteristics reflecting those of the parental SeV strains (efficient gene transduction and strong gene expression, with low cell specificity and species specificity), and a gene therapy trial using a second generation SeV vector has been approved recently in Japan. However, none of these vectors could sustain gene expression for more than 10 days because of the cytopathic nature of the parental SeV strain. Extensive deletion and alteration of the viral genes should reduce the cytotoxicity to some extent (9), but no SeV vector has been reported to express foreign genes stably in a wide variety of cells.

In this article, we describe a novel RNA replicon based on a noncytopathic persistent variant SeV strain. We found that this unique virus strain established stable symbiosis with host cells by escaping from the antiviral reaction mediated by interferon regulatory factor 3 (IRF-3). This remarkable characteristic was maintained even after exogenous marker genes had been inserted into the virus genome, thus enabling us to express the marker genes stably for more than 6 months. We discuss the potential of this novel cytoplasmic RNA replicon in medical and industrial applications.

**Experimental Procedures**

**Viruses and Cells**—Sendai virus was grown in 10-day-old fertilized chicken eggs at 35.5 °C for 3 days (Z and Nagoya strains and the derivatives) or at 32 °C for 5 days (Cl.151 strain, GenBank accession number AB275417) or at 32 °C for 5 days (Cl.151 strain, GenBankTM accession number AB275416) and was purified by sucrose step gradient centrifugation as described (10). Vaccinia virus MVAGKT7 (provided by Dr. G. R. Kovacs) was propagated using chicken embryo fibroblasts as described (11). LLCMK2 and CV-1 cells were cultured in Eagle’s minimum essential medium, COS-1 and NIH/3T3 cells were cultured in Dulbecco’s modified Eagle’s medium, Chinese hamster ovary (CHO)-K1 cells were cultured in Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (1:1), and U937 cells were cultured in RPMI 1640 medium at 37 °C in an atmosphere containing 5% CO₂. Each medium was supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

**cDNA Cloning**—All of the recombinant DNA experiments were performed according to the guidelines of the Institutional Recombinant DNA Experiment Committee of the National Institute of Advanced Industrial Science and Technology. Genomic RNA of SeV was isolated from the purified virion as described (12). First strand cDNA corresponding to the SeV genome RNA was synthesized using the Superscript III first strand synthesis system (Invitrogen) according to the manufacturer’s protocol. Double-stranded genome cDNA was cloned into pBluescript II SK (+) (Stratagene, La Jolla, CA) as three overlapping clones (nucleotide numbers 1–2875, 2870–10484, and 10479–15384) after PCR amplification using Pfu Ultra high fidelity DNA polymerase (Stratagene). The nucleotide sequences of four independent SeV cDNA clones were determined by Takara Bio Inc. (Otsu, Japan). For constructing full-length genome cDNA of the Z strain, pSeV (+) (provided by Dr. A. Kato) was used as a template for PCR. A full-length SeV genome cDNA was finally assembled on λ DASH II (Stratagene). The primers used in this article are summarized in supplemental Table S2, and the detailed procedure of the cloning and assembly of the full-length SeV genome cDNA is described in supplemental Fig. S1. To construct the SeV vector, marker genes were inserted into a unique NotI site created in SeV genomic cDNA as described (13). cDNAs encoding enhanced green fluorescent protein (EGFP), *Cypridina noctiluca* luciferase (CLuc), and fibroblast growth factor 1-proteoglycan fusion protein (PG-FGF-1) were amplified by PCR to add the transcription initiation signal (S) and termination signal (T), using pEGFP-1 (Clontech, Mountain View, CA), pCLm (14), and pMex-RyU1 (15) as templates, respectively. The detailed procedure for the construction of the vector cDNA with marker genes is described in supplemental Fig. S2.

**Rescue of Recombinant SeV by Reverse Genetics**—Recombinant SeV and the derivatives were rescued essentially as described (11). LLCMK₂ cells (1 × 10⁶ cells/well) in 6-well plates were infected with MVAGKT7 at a multiplicity of infection (MOI) of 10 plaque-forming units/cell and incubated at 37 °C for 1 h. The culture medium was removed, and the cells were supplemented with 2 ml of fresh medium and transfected with SeV genome cDNA. A complex of Lipofectamine 2000 (Invitrogen) (10 μl), λ phage DNA encoding SeV genomic cDNA (5 μg), pGEM-NP (2 μg), pGEM-P (1 μg), and pGEM-L (2 μg) (pGEM-NP, -P, and -L were provided by Dr. D. Kolakofsky) was prepared according to the protocol provided by the supplier, added to each well of cell culture, and incubated for 4 h at 37 °C. The complex was removed, and the cells were incubated in fresh medium for 20 h at 32 or 37 °C. A cell lysate was prepared and inoculated into 10-day-old fertilized chicken eggs as described (13). The eggs were incubated at 35.5 °C for 3 days (Z and Nagoya strains and the derivatives) or at 32 °C for 5 days (Cl.151 strain and the derivatives), after which the viruses were recovered from the allantoic fluid. The titer of SeV in allantoic fluid was determined by measuring hemagglutinating activity (10). The recombinant virus seeds were propagated in fertilized chicken eggs once and then purified using sucrose.
step density centrifugation as described (10). The presence of defective-interfering (DI) genomic RNA in the virus preparation was determined as described (16).

Cytotoxic Assay—Because the ratio of plaque-forming units and viral particles differed significantly between different SeV strains and because Cl.151 did not make plaques at 37 °C, we used the 50% tissue culture infectivity dose (TCID₅₀) as an index of viral infectivity. This was determined from the ratio of the cells expressing NP protein in the culture examined with an indirect immunofluorescent assay using a mouse anti-NP monoclonal antibody as described (17). One TCID₅₀ unit of SeV suspension of the Cl.151 and Z strains corresponds to 1.25 and 0.125 pg of viral protein/cell, respectively. Cytotoxicity was determined using a cytotoxicity detection kit (Roche Applied Science) that measures lactose dehydrogenase activity as described (9).

Detection of EGFP—To measure EGFP production in cultured cells, the cells were fixed with 2% paraformaldehyde at room temperature for 10 min and mounted in VECTASHIELD with 4’,6’-diamino-2-phenylindole HCl (Vector Laboratories, Burlingame, CA). The EGFP signal was then examined by fluorescence microscopy as described (18).

Animal Use—All of the animal experiments were performed according to the regulations of the Institutional Animal Care and Use Committee of the Advanced Industrial Science and Technology with permission of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Eight-week-old male Wistar rats were fasted for 24 h and then anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Darinppon Sumitomo Pharma, Osaka, Japan). The animals were restrained in a supine position on a thermocontrolled sheet to maintain body temperature during the experiment. Three hundred micrograms (protein) of SeV vector suspended in 0.3 ml of buffered salt solution (150 mM NaCl, 10 mM Tris-HCl, 1 mM CaCl₂, pH 7.4) was administered directly through a 3-cm-long tube through the anus. The animals were euthanized at the times described in Fig. 2B, and tissue specimens were prepared and observed as described (19), except that the thickness of the transverse section was 20 μm.

Determination of Secreted Marker Proteins—Cluc activity was determined as described (14) with modification: 50 μl of filtrated culture medium was mixed with 50 μl of C. noctiluca luciferin (ATTO, Tokyo, Japan; 216 nm in 10 mM Tris-HCl, pH 7.4) at room temperature, and the emission was determined with a luminometer (Aloka, Tokyo, Japan). The amount of C. noctiluca luciferase was quantified using purified enzyme (ATTO) as a standard. To express PG-FGF-1 from the conventional DNA-based vector, the full-length PG-FGF-1 cDNA was cloned into the EcoRI/NotI site of pMKIT-neo (provided by Dr. K. Maruyama) to create pMKIT-neo–PG-FGF-1, in which the cDNA was driven by the strong SRα promoter (20). CHO cells (6.5 × 10⁵ cells/well with 0.8 ml medium) were cultured in 12-well plates for 8 h. The cells were transfected with the complex of 2 μg of pMKIT-neo–PG-FGF-1 and 4 μl of Lipofectamine 2000 prepared in 200 μl of Opti-MEM according to the protocol provided by the supplier. The cells were incubated for 14 h, the medium was replaced with 1 ml of Opti-MEM, and the cells were incubated for a further 48 h for transient expression. Under this condition, about 80% of the cells expressed the marker gene. To ensure stable expression, transfected cells were selected further with G418 (800 μg/ml), and a clonal cell line expressing the highest level of PG-FGF-1 was cultured for 48 h as described above. PG-FGF-1 was determined using an enzyme-linked immunosorbent assay as described (15).

Interferon β Promoter Activation Assay—A 172-bp KpnI/HindIII fragment containing the 140-bp human interferon β (IFNβ) promoter (from −119 bp to +21 bp, +1 = transcription start site) was excised from the pGL3-IFNβ-promoter-luc (provided by Dr. A. Matsuda) and inserted into a KpnI/HindIII site of pGL4.12 (Promega, Madison, WI) to create pIV3. Nineteen micrograms of pIV3 and 1 μg of pRSVHyg were cotransfected into LLCMK₂ cells with DOTAP transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. One of the hygromycin-resistant clones (LLCMK₂/pIV3/clone 16) containing the complete IFNβ promoter-luciferase transcription unit was used in this study. Luciferase activity was determined using a luciferase assay system (Promega), and protein concentration was measured using a BCA assay kit (Pierce). The IFNβ promoter was activated either by viral infection or by transfecting with a 147-nt 5’-triphosphate single-stranded RNA synthesized in vitro using a RibomAX Large Scale RNA production system-T7 (Promega) (21, 22) with the aid of Lipofectamine 2000 (Invitrogen) or by adding poly(rI):poly(rC) (Sigma-Aldrich) to the culture medium (23) as described in the figure legend. LLCMK₂/pIV3/16/RIG-IC/clone 20 was the cell line derived from LLCMK₂/pIV3/clone 16 and expressed RIG-IC (dominant-negative form of RIG-I) (23) stably. This cell line was established by cotransfecting a RIG-IC expression cassette pHN63-8 (Flag-RIG-IC cDNA driven by CAG promoter) pRSV-Neo and selected by G-418 (1600 μg/ml) as described above.

Determination of Viral RNA—Total cellular RNA was purified with ISOGEN (Nippon Gene, Tokyo, Japan) according to the supplier’s protocol. Copy numbers of Cluc mRNA and SeV genome RNA in the cells were determined by a quantitative S1 nuclease assay using a double-strand DNA probe, which was ³²P-labeled at the 5’ end with [γ-³²P]ATP (185 Tbq/mmol), as described (24). Details of the S1 assay, including the structure of the probes against Cluc mRNA, SeV genomic RNA, and monkey β-actin mRNA, are described in supplemental Fig. S3. Five or 10 μg of total RNA was hybridized with 2 fmol each of the Cluc probe and monkey β-actin probe or with 2 fmol each of SeV genome probe and monkey β-actin probe in 10 μl of hybridization buffer (3 mM sodium trichloroacetate, 50 mM PIPES-NaOH, 5 mM EDTA, pH 7.0) at 45 °C for 16 h. The mixture was digested with S1 nuclease (400 units, in 200 μl of 250 mM NaCl, 40 mM sodium acetate, 1 mM ZnCl₂, pH 5.5) at 37 °C for 60 min. Digested materials were recovered with ethanol precipitation and analyzed on a prewarmed, denatured 5% polyacrylamide gel containing 8 mM urea. Signals corresponding to the protected probes were quantified using the STORM 830 image analyzer (Molecular Dynamics, Sunnyvale, CA). The results were normalized against monkey β-actin mRNA, and the copy numbers were estimated from the signal of predetermined single-strand RNA synthesized in vitro using a Ribol...
MAX large scale RNA production system-T7 (Promega) according to the supplier’s protocol.

RESULTS

Characterization of the Genome Structure of SeV Clone 151 Strain—A noncytopathic persistent variant SeV strain Cl.151 (25) was isolated originally from the parental cytopathic Nagoya strain (26, 27) as a variant that caused stable persistent infection in baby hamster kidney cells at a nonpermissive temperature (38 °C). Cl.151 can establish persistent infection even in sensitive CV-1 cells without cytopathic effects (Fig. 1C). We showed previously that amino acid substitutions in the M protein of Cl.151 lead to temperature-sensitive virion production (12). Defective virion production might be responsible for the reduced cytotoxicity because accumulation of unassembled NP protein affects the relative levels of transcription and replication (6, 28). However, the recombinant M-deleted SeV is still highly cytopathic, although it is defective in virion production, as is Cl.151 (29). Therefore, SeV injures the infected cells by a mechanism that is independent of virus morphogenesis.

To uncover the mechanism underlying the noncytopathic persistent nature of Cl.151, we cloned a cDNA corresponding to the full-length genome RNA of Cl.151 and characterized it in comparison with that of the parental Nagoya strain. Although no deletion or insertion was identified, we found 50 nucleotide substitutions throughout the viral genome, 35 of which led to the amino acid substitutions (Fig. 1A and supplemental Table S1). Because the characteristics of different SeV strains are affected largely by external factors such as the presence of DI genome RNA, we then characterized the recombinant virus recovered from the cloned cDNA by reverse genetics to examine whether these substitutions alone were responsible and sufficient for exhibiting the phenotype of Cl.151.
Recombinant SeV was recovered from the full-length genome cDNA by converting it to the antigenome RNA in the cytoplasm with the aid of T7 RNA polymerase. Simultaneous expression of NP, P, and L proteins in the cell encapsulates the de novo synthesized antigenome RNA and initiates the virus replication in the cytoplasm (30). One of the drawbacks of the original procedure is that full-length SeV genome cDNA (15384 bp) cloned into plasmid vectors was unstable in bacterial cells and frequently caused partial deletion during amplification (data not shown). To overcome this problem, we reconstructed the full-length SeV genome cDNAs on a nonlysogenic lambda vector, which maintained the long SeV cDNA more stably in Escherichia coli than did plasmid vectors (Fig. 1B and supplemental Fig. S1). Antigenome RNA is transcribed from this cDNA under the control of the T7 promoter and is trimmed precisely by hairpin ribozyme derived from the tobacco ringspot virus (31). We recovered virus at 32 °C, but not at 37 °C (the standard temperature for recovering wild-type SeV) from Cl.151 genomic cDNA; this is consistent with the temperature-sensitive replication of Cl.151. In addition, this recombinant SeV had characteristics indistinguishable from the original Cl.151: it infected various cultured cells persistently at nonpermissive temperature (≥37 °C) without cytopathic effects (Fig. 1C), and virion production occurred only at the permissive temperature (32 °C) (data not shown). We conclude that this cloned cDNA contains all the genetic information necessary and sufficient for reproducing the characteristics of Cl.151.

Mechanism Underlying the Noncytopathic Phenotype of SeV Cl.151 Strain—Wild-type SeV induces apoptotic death in infected cells. This phenomenon is triggered by caspases 3, 8, and 9 (32, 33), which are induced as one of the IRF-3-mediated antiviral responses, which include IFNβ induction (34). To clarify the mechanism underlying the noncytopathic phenotype of Cl.151, we examined the IRF-3-dependent induction of IFNβ using the activation of the IFNβ promoter as an indicator (23). To avoid artificial activation of the IFN system by transcription of plasmid DNA, we established a stable cell line (LLCMK/pIV3/clone 16) carrying the reporter gene (destabilized firefly luciferase cDNA driven by the human IFNβ promoter; see “Experimental Procedures”). Because the background luciferase activity in this cell line was extremely low, we were able to detect the activation state of the IFNβ promoter with high sensitivity. The wild-type SeV (Z and Nagoya strains) activated the IFNβ promoter by 18 times, whereas SeV Cl.151 achieved only a 1.8-fold activation (Fig. 1D, open bars), indicating that IRF-3 was not activated significantly in the cells infected with Cl.151.

Several viral factors, such as DI genome RNA, viral C/Y/V proteins, and the 3′-terminal structure of genome RNA, affect IRF-3-mediated antiviral response differently in SeV-infected cells. DI genome RNA contaminating the virus preparation super-induces IFNβ (16). C/Y proteins and V proteins interfere with IRF-3-mediated IFNβ induction and apoptotic cell death (35–37). Alteration of the 3′-terminal structure of genome RNA also affects IFNβ induction (37) and apoptotic cell death (38, 39). Because no DI genome RNA was detected in any of the SeV preparations used in this study (data not shown), we examined the phenotype of chimeric SeV between Cl.151 and the parental Nagoya strain to clarify the genetic element(s) responsible for the noncytopathic phenotype.

We separated the entire SeV genome into three regions: a 3′-terminal region (1-2870 nt) containing the 3′-leader region, the entire NP and C genes, and a part of the P gene, which included a frameshift site to produce V mRNA by nucleotide insertion (40); a central region (2871–9593 nt) containing the entire M, F, and HN genes; and a 5′-terminal region (9594–15384 nt) containing most of the L gene and a 5′-trailer region. We recovered four recombinant SeVs (N/N/C, N/C/N, N/C/C, and C/C/N) from chimeric cDNAs constructed on the phage vector (Fig. 2A) and characterized them with regard to the cytotoxicity and to IFNβ induction.

First, we found that the 3′-terminal region was not responsible for the noncytopathic phenotype of Cl.151; the N/C/C chimera with the 3′-terminal region identical to that of Nagoya strain did not induce IFNβ and was not cytopathic (Fig. 2).
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To obtain further insight into the differential activation of the IFNβ promoter, we then examined whether exogenous RNA ligands could induce IFNβ in Cl.151-infected cells. SeV-induced IFNβ production is absolutely dependent on RIG-I helicase (41), which recognizes 5'-triphosphate single-stranded RNA (21, 22). We reasoned that if some virus component interferes with the RIG-I/IRF-3-mediated pathway, the IFNβ promoter should not be activated by exogenous RNA ligands. We found that transfection of 5'-triphosphate single-stranded RNA activated the IFNβ promoter by 10 times in both Cl.151-infected cells and mock-infected cells (Fig. 1D). Activation of the IFNβ promoter by 5'-triphosphate single-stranded RNA was absolutely dependent on RIG-I because it was blocked specifically by a dominant-negative RIG-I variant (RIG-IC) (23) (Fig. 1D). These data indicate that the RIG-I/IRF-3-mediated signal transduction pathway is not interfered with in Cl.151-infected cells. We also found that the toll-like receptor 3/IRF-3-mediated signal transduction pathway (23) functions normally in Cl.151-infected cells because the addition of RNA duplex poly(rI):poly(rC) into the culture medium induced IFNβ (data not shown). These data strongly suggest that viral RNA(s) recognized by RIG-I are missing in the Cl.151-infected cells, possibly because of the alteration of L protein, putative RNA-dependent RNA polymerase. The role of L protein in IFNβ induction has not been established, and this finding clearly indicates that Cl.151 escapes from the IRF-3-mediated antivirus response by a mechanism not yet described. Further details of this phenomenon are now under investigation.

Application of SeV Cl.151 Strain as a Gene Expression Vector—
These characteristics of Cl.151 are clearly advantageous for developing a virus vector capable of persistent and stable gene expression without interfering with normal cellular functions. Therefore, we constructed a chimeric SeV Cl.151 vector by installing marker genes upstream of the NP gene (Fig. 1B and supplemental Fig. S3) and examined their phenotype in comparison with those based on the cytopathic Z strain. SeV Cl.151 carrying the enhanced EGFP gene, termed rCl.151-EGFP, was not cytopathic (Fig. 1C), nor did it activate the IFNβ promoter (Fig. 1E), as did the parental Cl.151. In contrast, the Z strain-based recombinant SeV carrying EGFP (rZ-EGFP) was cytopathic (Fig. 1C) and activated the IFNβ promoter (Fig. 1E), even though the insertion of the marker gene diminished the cytotoxicity slightly. We also

Because the structure of C/Y and V proteins of the N/C/C chimera were identical to those of the Nagoya strain, we conclude that alterations of these proteins are not related to the reduced cytopathogenicity and IFNβ inducivity of Cl.151.

In contrast, the central region and the 5'-terminal region contributed differently to the phenotype of Cl.151. The central region was closely linked to acute cytotoxicity, as revealed by the LDH assay (Fig. 2B) and the morphology of the infected cells on day 2 (Fig. 2B). Replacement of the central region of the genome of the Nagoya strain with that of Cl.151 (N/C/N chimera) reduced the cytotoxicity by 85%. This phenomenon may correspond with the previous finding that the deletion of all M, F, and HN genes diminishes the cytotoxicity of second generation SeV vectors (9).

On the other hand, the 5'-terminal region was closely linked to IFNβ induction (Fig. 2A) and to long term persistency (Fig. 2B). Comparison of the SeV with the altered 5'-terminal region derived from Cl.151 indicated reduced activation of the IFNβ promoter by 78% for Nagoya versus N/N/C, 82% for N/C/N versus N/C/C, and 82% for C/C/N versus Cl.151 (Fig. 2A). Because the Nagoya strain and Cl.151 have identical 48-nt 5'-trailer regions (39), this indicates that the L gene with four missense mutations and one mutation in the noncoding region was responsible for this phenomenon (Fig. 1A and supplemental Table S1).

FIGURE 2. Characterization of recombinant chimeric SeV in the Nagoya strain and the Cl.151 strain. A, genome structures (left) and biological activity (right) of chimeric SeV. Chimeric genome cDNA was reconstructed from XhoI-EcoRI 3'-terminal cDNA (1–2870 nt), from EcoRI-NcoI central cDNA (2871–9593 nt), and from NcoI-Sall 5'-terminal cDNA (9594–15384 nt) on the λ phage as described in supplemental Fig. S1. Four recombinant SeVs (N/N/C, N/C/N, N/C/C, and C/C/N) were then recovered from these cDNAs. The shaded parts indicate the regions derived from the Cl.151 genome. Cytotoxicity in CV-1 cells and induction of IFNβ using the activation of IFNβ promoter were determined as shown in Fig. 1 (C and D, respectively). B, morphology of CV-1 cells infected by the recombinant SeV. CV-1 cells (5 × 10^5 cells/2 ml of culture medium/well) were seeded in 6-well plates on Day 0. On Day 1, the cells were infected with the SeV described above at an MOI of 10 TCID50 units/cell and incubated for 1 day (Day 2) and 3 days (Day 4). The cells were then examined by phase contrast microscopy (Nikon Eclipse TE300) equipped with a 10 × objective. Bar, 100 μm.

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Because the structure of C/Y and V proteins of the N/C/C chimera were identical to those of the Nagoya strain, we conclude that alterations of these proteins are not related to the reduced cytopathogenicity and IFNβ inducivity of Cl.151.

In contrast, the central region and the 5'-terminal region contributed differently to the phenotype of Cl.151. The central region was closely linked to acute cytotoxicity, as revealed by the LDH assay (Fig. 2A) and the morphology of the infected cells on day 2 (Fig. 2B). Replacement of the central region of the genome of the Nagoya strain with that of Cl.151 (N/C/N chimera) reduced the cytotoxicity by 85%. This phenomenon may correspond with the previous finding that the deletion of all M, F, and HN genes diminishes the cytotoxicity of second generation SeV vectors (9).

On the other hand, the 5'-terminal region was closely linked to IFNβ induction (Fig. 2A) and to long term persistency (Fig. 2B). Comparison of the SeV with the altered 5'-terminal region derived from Cl.151 indicated reduced activation of the IFNβ promoter by 78% for Nagoya versus N/N/C, 82% for N/C/N versus N/C/C, and 82% for C/C/N versus Cl.151 (Fig. 2A). Because the Nagoya strain and Cl.151 have identical 48-nt 5'-trailer regions (39), this indicates that the L gene with four missense mutations and one mutation in the noncoding region was responsible for this phenomenon (Fig. 1A and supplemental Table S1).
found that rCl.151-EGFP did not affect cell proliferation (Fig. 1F). Thus, inserting an exogenous genetic element did not affect the phenotype of Cl.151.

We then characterized the expression of marker genes, focusing on their persistence. Gene expression induced by SeV Cl.151-based vectors was stable in cultured cells; LLCMK₂ cells infected with rCl.151-EGFP sustained EGFP expression for up to 6 months (Fig. 3A). The infected cells neither produced infectious virions nor delivered the nucleocapsid to adjacent uninfected cells by cell-to-cell fusion (data not shown), indicating that this persistent EGFP expression was not maintained by horizontal transmission of the rCl.151-EGFP genome. We estimated the loss rate of the rCl.151-EGFP genome from LLCMK₂ cells to be less than 0.04%/cell division (39.1% retention after 180 population doublings), indicating that the Cl.151-based RNA replicon was much more stable than are circular Epstein-Barr virus-derived DNA replicons in permissive human cells (1.8–4.4% loss/cell division) (42). This remarkable stability was observed in various cultured cells derived from mouse, hamster, monkey, and human tissues (data not shown).

We also examined EGFP expression induced by rCl.151-EGFP in rat tissue cells. Airway epithelium is a natural site of infection by SeV, but this virus can also infect many other tissue cells (8), including colon epithelium (19). EGFP expression induced in the colon epithelium by rZ-EGFP was very strong on day 3 after transanal administration but was undetectable on day 14 (Fig. 3B). In contrast, EGFP expression induced in the colon epithelium by rZ-EGFP was very strong on day 3 after transanal administration but was undetectable on day 14 (Fig. 3B). In both cases, EGFP expression was detectable only in the epithelium, not in the lamina propria or muscularis mucosae (Fig. 3B). Considering the rapid renewal rate (5 days) of colon epithelial cells, these observations suggest that rCl.151-EGFP infected the tissue stem cells located at the

**FIGURE 3.** Expression of EGFP in the cells infected with recombinant SeV vectors. A, EGFP expression in cultured cells. On Day 0, LLCMK₂ cells (2 × 10⁵/well) in six-well plates were infected with recombinant SeV vector (rCl.151-EGFP or rZ-EGFP) at an MOI of 10 TCID₅₀ units/cell for 24 h. On Day 2, the cells were propagated into a new six-well plates and subcultured as usual. One day before EGFP observation, the cells were subcultured into eight-well chambers (LAB-TEK II chamber slide; Nalge) at 2 × 10⁴/chamber. On the day indicated, the cells were fixed and examined under fluorescent microscopy as described under “Experimental Procedures.” N.D., not detectable because no cells survived. Bar, 40 μm. B, EGFP expression in rat colon epithelium. On Day 0, 300 μg (protein) of recombinant SeV vector (rCl.151-EGFP or rZ-EGFP) was administered transanally. The animals were euthanized at the times indicated, and the specimens were prepared and examined as described under “Experimental Procedures.” Bar, 200 μm. DAPI, 4’,6’-diamino-2-phenylindole.
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FIGURE 4. Levels of secreted marker proteins in the cells infected with recombinant SeV vectors. A, quantification of C. noiluca luciferase in the culture supernatant. On Day 0, LLCMK2 cells (5 \times 10^5/dish) in a 100-mm culture dish were infected with recombinant SeV vector (rCl.151-Cluc or rZ-Cluc) at an MOI of 10 TCID_{50} units/cell for 24 h. One day before harvest at the times indicated, the cells were subcultured into six-well culture plates (1 \times 10^5 cells/well in 0.8 ml medium) in 12-well plates were transfected with DNA vector strategy: CHO cells carrying SeV Cl.151 vector produced four times more PG-FGF-1 than did a cloned CHO cell line carrying multiple expression cassettes in the chromosome (Fig. 4D).

DISCUSSION

In this article, we describe a unique prototype RNA replicon capable of expressing inserted genes persistently from its stable RNA genome in the cytoplasm. In addition to the retrovirus vectors and lentivirus vectors already established as a practical tool for gene transfer, RNA virus vectors based on alphaviruses, rhabdoviruses, coronaviruses, picornaviruses, and paramyxoviruses have been developed (43, 44). However, except for the vectors belonging to Retroviridae that stabilize their genome cDNA by chromosomal insertion, most RNA virus vectors have not been considered as tools for stable and persistent gene expression because of their potent cytotoxicity. This is partly because viral RNAs synthesized explosively by RNA-dependent RNA polymerase trigger antiviral responses, including IFN\(\beta\) induction, in host cells. IFN\(\beta\) is a critical molecule for initiating pleiotropic antiviral responses, and the detailed mechanism by which viral RNAs induce IFN\(\beta\) has been uncovered recently (21–23, 41). Our finding that the noncytopathic persistent variant SeV Cl.151 escapes from the RIG-1/IRF-3-mediated induction of IFN\(\beta\) allowed us to use this unique variant virus as a platform for stable gene expression. The characteristics of this gene expression platform will be particularly advantageous in future medical applications because it can evade the undesired side effects caused by cytokine burst or insertional mutagenesis.

Strong and steady gene expression from the cytoplasmic RNA replicon is also advantageous for industrial protein production. Although a high producer cell line is a key for success, the chance of obtaining cell lines that integrate a sufficient number of exogenous gene cassettes is quite low, and laborious gene amplification procedures may be required (2). In contrast, the RNA replicon described in this article readily established a cell line carrying multiple expression cassettes in the chromosome (Fig. 4D), which can be useful in various applications.

We next used a secretary marker (Cluc) (14) and an engineered fibroblast growth factor 1 (PG-FGF-1) to characterize the gene expression more quantitatively (15). Secretion of Cluc to the culture medium was efficient and constant at 50 pg/cell/day over 4 weeks, fulfilling the standard level for industrial protein production (2) (Fig. 4A). This phenomenon reflected the steady level of Cluc RNAs in the cell: \(\sim 1 \times 10^6\) copies/cell of Cluc mRNA and \(\sim 4 \times 10^6\) copies/cell of vector genome RNA (Fig. 4B) were present constantly during the assay period. This vector system was also applicable for producing PG-FGF-1, a protein difficult to manufacture on a large scale because of extensive glycosylation (15). This vector system was applicable to many cultured cells derived from various species (Fig. 4C), including CHO cells, a popular cell line for large scale protein production. Productivity was much more efficient in CHO cells cultured in serum-free media than the conventional DNA vector strategy: CHO cells carrying SeV Cl.151 vector produced four times more PG-FGF-1 than did a cloned CHO cell line carrying multiple expression cassettes in the chromosome (Fig. 4D).
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Our finding will also contribute to identifying the RNA molecule(s) that trigger IFNβ induction in infection by wild-type SeV. Recent biochemical and genetic analysis has identified the major cellular players in IFNβ induction. In the case of SeV, RIG-I helicase was identified as a molecular sensor that recognizes viral RNA (41), but the viral RNA that actually triggers RIG-I has not been characterized. Furthermore, all the variant SeV strains reported previously induce IFNβ more strongly than do the parental wild-type SeV strains, and no SeV strain defective in IFNβ production has been reported. RIG-I directly recognizes uncapped 5'‐triphosphate single‐strand RNA (21, 22), and transfection of 5'‐triphosphate single‐strand RNA induces IFNβ in both Cl.151‐infected cells and control cells (Fig. 1D). We conclude that the 5'‐triphosphate uncapped viral RNAs needed to trigger RIG‐I were missing in Cl.151‐infected cells and control cells; and Dr. Y. Kato for useful advice.

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