Mutations within Noncoding Terminal Sequences of Model RNAs of Sendai Virus: Influence on Reporter Gene Expression

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Received 17 June 1994/Accepted 22 December 1994

A reverse-genetics system employing the chloramphenicol acetyltransferase (CAT) reporter gene has been established previously for Sendai virus. We utilized PCR-directed mutagenesis to introduce nucleotide additions, deletions, and/or substitutions within terminal Sendai virus RNA sequences. The influence of these mutations on replication-transcription of these model Sendai-CAT RNAs was analyzed by assaying CAT activity. Results from these experiments indicate that (i) Sendai-CAT RNAs expressing wild-type levels of CAT activity conform to the Sendai virus rule of six, (ii) apparent exceptions to the rule of six exist in that the 5' terminus of the Sendai-CAT RNA is more tolerant than the 3' terminus of nucleotide additions or deletions, and (iii) the 3' leader region of Sendai-CAT RNA appears to be sensitive not only to mutagenesis (single-nucleotide addition or deletion) but also to changes in its total nucleotide length.

Sendai virus, a member of the *Paramyxoviridae* family, possesses a nonsegmented, negative-sense RNA genome of approximately 15.0 kb (1, 13, 22). The close relationship of Sendai virus with other members of the *Paramyxoviridae* family (measles virus, parainfluenza virus type 3, respiratory syncytial virus, and mumps virus) that infect humans and the ability of the virus to replicate to high titers in tissue culture and embryonated eggs have resulted in Sendai virus serving as a model paramyxovirus for examining molecular aspects of viral replication. Sendai virus utilizes a typical negative-sense RNA replicative strategy whereby the minus-sense genomic RNA (virion RNA [vRNA]) is transcribed by the viral polymerase complex into a short, plus-sense noncoding leader RNA (14), as well as into subgenomic-length mRNAs which are capped and polyadenylated. The mechanism by which the viral polymerase complex switches from a transcriptional mode to a replicative mode characterized by the synthesis of a plus-sense, genomic-length RNA (cRNA) template is also not fully understood (21).

Because of the negative polarity of the genome of negative-strand RNA viruses, standard genetic approaches, until recently, were ineffective for molecular analyses of viral replication. However, a reverse-genetics approach, developed with influenza A viruses, has allowed for direct genetic manipulation of specific viral genes (9, 10, 16). This general approach has been adapted and used successfully to establish similar transfection systems employing other negative-sense RNA viruses including Sendai virus (17, 18), respiratory syncytial virus (4, 5), human parainfluenza virus type 3 (HPIV3) (7, 8), and rabies virus (6). In addition to transfection systems using reporter genes, transfection systems employing defective interfering (DI) RNAs to assess RNA replication have also been established (2, 3, 19), and most recently, the rescue of infectious rabies virus from cloned DNA was achieved (20).

Park et al. (17) first developed the transfection system for Sendai virus by constructing a plasmid (pSendCAT) containing the chloramphenicol acetyltransferase (CAT) open reading frame flanked by Sendai virus terminal sequences. The in vitro-

transcribed Sendai virus-CAT RNA was shown to be replicated, transcribed, and rescued into viral particles following transfection into cells which were infected with Sendai virus (17). These results indicated that the viral sequences within this synthetic RNA contained the necessary *cis*-acting elements for transcription and replication (17). In this report, we have mutated both 5'- and 3'-terminal sequences of Sendai virus-CAT RNA and sequences within the 3' leader region. The effect of these mutations on the ability of the Sendai virus-CAT RNAs to be expressed in this system was assessed by standard CAT assay.

In a recent report, Calain and Roux (3) demonstrated that DI RNAs of Sendai virus replicate most efficiently when the total nucleotide length of the RNA is a multiple of six. As noted by the authors, this observation is compatible with the notion that one molecule of the Sendai virus nucleocapsid protein covers 6 nucleotides of vRNA. Although the rule of six was shown to be important for a Sendai virus DI RNA capable of only replication, this concept had yet to be proven equally important for the Sendai virus-CAT RNA, an RNA capable of both replication (cRNA synthesis) and transcription (mRNA synthesis) (17, 18). To address this question, mutations were introduced by PCR techniques into the pSendCAT construct to determine whether the rule of six applies to this model RNA (Fig. 1). Deletions of 1, 3, or 6 nucleotides were engineered into the pSendCAT construct within the 5' Sendai virus sequences adjacent to the stop codon for the CAT open reading frame, generating plasmids pSendCAT-1, pSendCAT-3, and pSendCAT-6, respectively (Fig. 1). Also, one additional nucleotide was introduced into the same region of pSendCAT to generate construct pSendCAT+1 (Fig. 1). This region of pSendCAT was chosen for mutagenesis since it was unlikely to contain any essential *cis*-acting elements. Interestingly, following in vitro transcription and subsequent transfection of the resultant RNAs into Sendai virus-infected cells, CAT activity was detected exclusively from the two RNAs whose lengths were multiples of six, pSendCAT and pSendCAT-6 (Fig. 1). CAT expression from pSendCAT-1, pSendCAT-3, and pSendCAT+1 RNAs was less than 1% of that derived from pSendCAT and pSendCAT-6 RNAs (Fig. 1). To demonstrate that the loss of CAT expression was indeed due to the introduced mutation and not due to some other defect in the plasmid

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FIG. 1. Application of the rule of six to Sendai virus-CAT RNA. A schematic diagram of the wild-type pSendCAT plasmid is shown at the top depicting key restriction endonuclease sites used for generating mutant constructs. Abbreviations are as follows: SV, Sendai virus; CAT, the CAT open reading frame cloned in the antisense orientation; T7, bacteriophage T7 promoter. A portion (16 nucleotides) of the T7-derived RNA sequence of the region spanning the junction of the CAT open reading frame and the 5'-terminal sequences of Sendai virus RNA is depicted below. The UUA triplet (lowercase) represents the stop codon of the CAT open reading frame. pSendCAT-1 has a 1-nucleotide deletion (U), pSendCAT-3 has a deletion of 3 nucleotides (UUA), pSendCAT-6 has a deletion of 6 nucleotides (UUAUUA), and pSendCAT+1 has a 1-nucleotide addition (lowercase c). The total length of each RNA is indicated along with the percent relative CAT activity obtained following transfection of these RNAs. One hundred percent CAT activity corresponds to the level measured with wild-type Sendai virus-CAT RNA. All PCR products containing the appropriate mutation were inserted into the *SacII*-*NcoI* window of pSendCAT. nt, nucleotides.

itself, the *SacII*-*NcoI* fragment of both pSendCAT+1 and pSendCAT-1 was repaired. Sendai virus-CAT RNAs synthesized from these plasmids expressed levels of CAT activity indistinguishable from that of wild-type Sendai virus-CAT RNA (data not shown). Thus, these data support the concept of the rule of six and confirm its relevance to the Sendai virus-CAT RNA transfection system. It should be noted that the total nucleotide lengths of derivatives of the pSendCAT RNA used to study RNA editing were also divisible by six (18).

Because of the conserved nature of the 3'- and 5'-terminal sequences of the genomes of negative-sense RNA viruses, it was of interest to ascertain the effects of mutations within these sequences on CAT expression from the Sendai virus-CAT model RNA. To determine whether precise ends of the Sendai virus-CAT RNA are required for efficient CAT expression, nucleotide additions or deletions were introduced at either the 5' or the 3' terminus of the Sendai virus-CAT RNA. Sendai virus-CAT RNAs containing 1 (pSendCAT5'G) or 2 (pSendCAT5'GG) extra nucleotides at the 5' terminus expressed levels of CAT activity virtually identical to that of the wild-type Sendai virus-CAT RNA (Fig. 2). Results from similar experiments using the HPIV3-CAT system demonstrated that the addition of 1 or 2 nucleotides to the 5' terminus of an HPIV3-CAT RNA decreased CAT activity by 50 and 90%, respectively (7). In contrast to the results with HPIV3-CAT, a vesicular stomatitis virus (VSV) DI RNA was shown to be tolerant of up to 4 or 5 extra nucleotides at its 5' terminus, and extra 5'-terminal nucleotides on VSV DI RNAs were also reported to be repaired by the VSV polymerase (19). Thus, the possibility exists that the extra nucleotides added at the 5' terminus of the Sendai virus-CAT RNA may be repaired; however, no attempt was made to confirm this possibility.

In contrast to nucleotide additions, the deletion of 1 nucleotide (pSendCAT5'-1) from the 5' end resulted in a twofold reduction in CAT activity relative to that of wild type, while a 2-nucleotide (pSendCAT5'-2) deletion reduced CAT activity greater than 100-fold relative to that of the wild-type Sendai

Relative CAT Activity			
Wild Type Seq.	5'	ACCAGACAAGAG.....	100%
pSendCAT5'G	5'	GACCAGACAAGAG.....	116%
pSendCAT5'GG	5'	GGACCAGACAAGAG.....	100%
pSendCAT5'-1	5'	CCAGACAAGAG.....	52%
pSendCAT5'-2	5'	CAGACAAGAG.....	<1%

FIG. 2. Mutational analysis of the extreme 5' terminus of Sendai virus-CAT RNA. The 5'-terminal sequences of the Sendai virus-CAT RNAs pSendCAT5'G (1 G nucleotide added), pSendCAT5'GG (2 G nucleotides added), pSendCAT5'-1 (5'-terminal A deleted), and pSendCAT5'-2 (5'-terminal AC dinucleotide deleted) are shown. The relative CAT activity obtained following transfection of these RNAs is indicated. PCR products containing the appropriate mutation were inserted into the *HindIII*-*NcoI* window of pSendCAT-6 (Fig. 1).

virus-CAT RNA (Fig. 2). These results demonstrate that nucleotide deletions at the 5' terminus were more deleterious to CAT expression than were nucleotide additions at the 5' terminus. If indeed Sendai virus possesses the capability to excise extra nucleotides at its 5' terminus, a similar mechanism to repair its 5' terminus by adding back nucleotides is not apparent from these results.

Results differing from those described above were observed when similar mutations were made at the 3' terminus of the Sendai virus-CAT RNA. Either 1 or 2 nucleotides were added (plasmids pSendCAT3'A and pSendCAT3'AA, respectively) or deleted (plasmids pSendCAT3'-1 and pSendCAT3'-2, respectively) from the 3' terminus (Fig. 3). The addition of 1 nucleotide at the 3' terminus resulted in a threefold decrease in CAT activity, while the addition of 2 nucleotides resulted in a 12-fold decrease in CAT activity relative to that observed with wild-type Sendai virus-CAT RNA (Fig. 3). An even greater decrease in CAT expression was observed when either 1 or 2 nucleotides were deleted from the 3' terminus (Fig. 3). CAT expression from both pSendCAT3'-1 and pSendCAT3'-2 RNAs was diminished greater than 100-fold compared with that of wild-type Sendai virus-CAT RNA. These results indicate that mutations at the 3' terminus are less tolerated than corresponding mutations at the 5' terminus. Similar observations have been reported for the HPIV3-CAT RNA (7) and VSV DI RNAs (19). This difference in tolerance between the 5' and 3' termini may reflect the fact that 3'-terminal sequences of the negative-sense RNA are responsible for the synthesis of both cRNA and mRNA, whereas 5'-terminal sequences are required for vRNA synthesis only. In addition, transcription of the positive-sense leader RNA of Sendai virus is also initiated at the extreme 3' terminus (14).

Relative CAT Activity			
Wild Type Seq.	CUCUUGUUUGGU	3'	100%
pSendCAT3'A	CUCUUGUUUGGUA	3'	32%
pSendCAT3'AA	CUCUUGUUUGGUA	3'	8%
pSendCAT3'-1	CUCUUGUUUGG	3'	<1%
pSendCAT3'-2	CUCUUGUUUG	3'	<1%

FIG. 3. Mutational analysis of the extreme 3' terminus of Sendai virus-CAT RNA. The 3'-terminal sequences of Sendai virus-CAT RNAs pSendCAT3'A (1 A nucleotide added), pSendCAT3'AA (2 A nucleotides added), pSendCAT3'-1 (3'-terminal U deleted), and pSendCAT3'-2 (3'-terminal UG dinucleotide deleted) are shown. The relative CAT activity obtained following transfection of these RNAs is indicated. PCR products containing the appropriate mutation were inserted into the *AccI*-*SacI* window of pSendCAT (Fig. 1).

FIG. 4. Effect of mutations involving the 3' leader region. (Top) A diagram of the Sendai virus-CAT plasmid is shown. The numbers represent nucleotide positions beginning at the 3' terminus. The names of the mutant constructs are shown on the left. The addition (nucleotide position 13, 33, or 780) or deletion (nucleotide position 18, 114, or 784) of 1 nucleotide is illustrated by a +1 or -1 adjacent to the name of the plasmid. The relative percent CAT activity obtained following transfection of these RNAs is shown at the right. Nucleotides 13, 18, and 33 are located within the region of the 3' leader. Nucleotide 114 lies within the noncoding 3' sequence of Sendai virus RNA, while nucleotides 780 and 784 are within the 5' noncoding sequence of Sendai virus RNA. PCR products containing mutations within the 5' sequences (plasmids p+1/SVL-1, p5'+1-1, and p+1/-1) were inserted into the *SacII-NcoI* window of pSendCAT-6, while PCR products containing mutations within the 3' sequences (plasmids pSVL-1, pSVL+1, pSVL+1*, pSVL-0, pSVL-0', p+1/SVL-1, p+1/-1, p-1/SVL+1, and p-1) were inserted into the *SacI-AccI* window of pSendCAT-6. (Bottom) One representative CAT assay of extracts of cells transfected with the above RNAs. The lane designations are as follows: lane 1, wild-type pSendCAT-6 (100-fold dilution); lane 2, pSVL+1* (undiluted); lane 3, pSVL-0' (10-fold dilution); lane 4, pSVL-0 (10-fold dilution); lane 5, pSVL-1 (10-fold dilution); lane 6, pSVL+1 (undiluted); lane 7, p-1/SVL+1 (undiluted); lane 8, p+1/-1 (100-fold dilution); lane 9, p-1 (undiluted); lane 10, p5'+1-1 (100-fold dilution); and lane 11, p+1/SVL-1 (undiluted).

To assess the importance of sequences within the noncoding 3' leader domain on CAT expression, a series of mutants were constructed that contained mutations within the 3' leader region of the Sendai virus-CAT RNA (Fig. 4). The 3' leader region was altered by the addition of 1 nucleotide (adenine) at position 13 (plasmid pSVL+1*) or 33 (plasmid pSVL+1) or by the deletion of 1 nucleotide at position 18 (uridine; plasmid pSVL-1) (Fig. 4). As expected (on the basis of the rule of six), Sendai virus-CAT RNAs containing these single-nucleotide mutations were unable to direct CAT expression above 1% of the wild-type level (Fig. 4). Interestingly, compensatory mutations outside of the 3' leader region which were designed to restore the length of the RNA to a number divisible by six failed to restore CAT activity (Fig. 4, constructs p+1/SVL-1 and p-1/SVL+1). Surprisingly, constructs pSVL-0 and pSVL-0', which contained both the first and second compensatory mutation inside the 3' leader domain, did indeed exhibit a 10- and 20-fold increase in CAT activity, respectively, relative to that of pSVL-1 (Fig. 4). Furthermore, Sendai virus-CAT RNAs synthesized from constructs p+1/-1 and p5'+1-1 expressed CAT levels that were comparable (40 and 80%, respectively) to that of wild-type Sendai virus-CAT RNA (Fig. 4). Thus, the 1-nucleotide deletion at position 114 (plasmid p-1; adenine) and the 1-nucleotide addition at position 780

(plasmid pSendCAT+1; cytosine) could be complemented (as judged by CAT activity) by a second mutation mapping outside of the 3' leader region but could not be complemented by a second mutation mapping within the 3' leader region (Fig. 4). These data suggest that maintaining not only the wild-type nucleotide sequence but also the proper nucleotide length of the 3' leader domain may be important for expression from this model Sendai virus-CAT RNA. Indeed, the potential importance of the length of the 3' leader domain is implied by the observation that the distance from the 3'-terminal nucleotide to the putative mRNA initiation motif of several paramyxoviruses (Sendai virus, measles virus, Newcastle disease virus, HPIV3, bovine parainfluenza virus type 3, and mumps virus) is conserved (1, 13). Moreover, studies with VSV DI RNAs suggested that potential structural elements within the 3' VSV leader domain, and not simply the nucleotide sequence itself, may be important for binding and/or initiation of transcription by the viral polymerase (11, 12). It should also be noted that the 3' leader domain of Sendai virus-CAT RNA was highly sensitive to more extensive mutations including the addition or deletion of large stretches of nucleotides, even though these alterations abided by the rule of six (data not shown).

A more thorough understanding of the results described above would be possible with direct biochemical analysis of each of the mutated Sendai virus-CAT RNAs with respect to replication (Sendai virus-CAT cRNA synthesis) versus transcription (Sendai virus-CAT mRNA synthesis). However, all attempts to directly and simultaneously quantitate the levels of both Sendai virus-CAT cRNA and mRNA in the transfected cells by using RNase protection assays with a Sendai virus-CAT vRNA probe (minus sense) have been unsuccessful. To our knowledge, the ability to quantitate and compare cRNA and mRNA levels for any of the paramyxovirus RNA transfection systems employing a CAT reporter gene and wild-type helper virus has yet to be achieved. One possible explanation is that vRNA from the required helper virus is diluting out or competing with the Sendai virus-CAT vRNA probe for binding to Sendai virus-CAT cRNA and mRNA. This does not appear to be the case for similar experiments reported for influenza virus (15), possibly because the lengths of the 5' and 3' influenza virus sequences flanking CAT are significantly shorter than those of Sendai virus. Thus, in the influenza virus system, the vRNA molecules do not appear to hybridize to a significant extent with the mRNA and cRNA of the reporter gene. In an attempt to avoid the problem of the RNase protection assay in Sendai virus-infected cells, primer extension analysis was performed. A radiolabeled (10^8 cpm/ μ g) primer specific for the CAT open reading frame was used; however, once again, no signal was detected. Although the interference of the terminal Sendai virus sequences is avoided with primer extension, this method may not be sensitive enough to detect Sendai virus-CAT cRNA and mRNA. Despite our failure to measure specific cRNA and mRNA levels, the ability to measure reporter gene expression from this model RNA has provided valuable insights into viral sequences important for RNA synthesis.

Specifically, it has been demonstrated that the rule of six applies in general to a Sendai virus system capable of replication as well as transcription but also that there are potential exceptions to this rule. Although results presented here for Sendai virus-CAT RNAs and earlier reports for HPIV3-CAT and respiratory syncytial virus-CAT RNAs have contributed to our knowledge, future progress in these systems will depend heavily on the ability to generate infectious virus from cloned cDNA as has been achieved for rabies virus (5-8, 17, 18, 20).

We thank M. Krystal for kindly providing the pSendCAT plasmid. We also thank S. Kerns and R. Lin for technical support and R. O'Neill, W. Barclay, A. Garcia-Sastre, and T. Zürcher for helpful discussions.

Support for this work was provided by grants from the NIH (P.P.), by a Charles H. Revson Fellowship (R.N.H.), and by National Research Service Award AI09045-02 (R.N.H.).

REFERENCES

1. Banerjee, A. K., S. Barik, and B. P. De. 1991. Gene expression of nonsegmented negative strand RNA viruses. *Pharmacol. Ther.* **51**:47–70.
2. Calain, P., J. Curran, D. Kolakofsky, and L. Roux. 1992. Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. *Virology* **191**:62–71.
3. Calain, P., and L. Roux. 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J. Virol.* **67**:4822–4830.
4. Collins, P. L., L. Kuo, K. Dimock, M. Mink, D. Stec, and H. Grosfeld. 1993. Rescue of synthetic analogs of respiratory syncytial virus genomic RNA, p. 42–45. Workshop on Reverse Genetics of Negative Stranded RNA Viruses, Madrid, Spain.
5. Collins, P. L., M. A. Mink, and D. S. Stec. 1991. Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. *Proc. Natl. Acad. Sci. USA* **88**:9663–9667.
6. Conzelmann, K.-K., and M. Schnell. 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. *J. Virol.* **68**:713–719.
7. De, B. P., and A. K. Banerjee. 1993. Rescue of synthetic analogs of genome RNA of human parainfluenza virus type 3. *Virology* **196**:344–348.
8. Dimock, K., and P. L. Collins. 1993. Rescue of synthetic analogs of genomic RNA and replicative-intermediate RNA of human parainfluenza virus type 3. *J. Virol.* **67**:2772–2778.
9. Enami, M., W. Luytjes, M. Krystal, and P. Palese. 1990. Introduction of site-specific mutations into the genome of influenza virus. *Proc. Natl. Acad. Sci. USA* **87**:3802–3805.
10. Enami, M., and P. Palese. 1991. High-efficiency formation of influenza virus transfectants. *J. Virol.* **65**:2711–2713.
11. Keene, J. D., I. M. Chien, and R. A. Lazzarini. 1981. Vesicular stomatitis virus defective interfering particle containing a muted internal leader RNA gene. *Proc. Natl. Acad. Sci. USA* **78**:2090–2094.
12. Keene, J. D., B. J. Thornton, and S. U. Emerson. 1981. Sequence-specific contacts between the RNA polymerase of vesicular stomatitis virus and the leader RNA gene. *Proc. Natl. Acad. Sci. USA* **78**:6191–6195.
13. Kingsbury, D. W. 1990. Paramyxoviridae and their replication, p. 945–962. *In* B. N. Fields and D. M. Knipe (ed.), *Virology*. Raven Press Ltd., New York.
14. Leppert, M., L. Rittenhouse, J. Perrault, D. F. Summers, and D. Kolakofsky. 1979. Plus and minus strand leader RNAs in negative strand virus-infected cells. *Cell* **18**:735–747.
15. Li, X., and P. Palese. 1994. Characterization of the polyadenylation signal of influenza virus RNA. *J. Virol.* **68**:1245–1249.
16. Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese. 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. *Cell* **59**:1107–1113.
17. Park, K. H., T. Huang, F. F. Correia, and M. Krystal. 1991. Rescue of a foreign gene by Sendai virus. *Proc. Natl. Acad. Sci. USA* **88**:5537–5541.
18. Park, K. H., and M. Krystal. 1992. In vivo model for pseudo-templated transcription in Sendai virus. *J. Virol.* **66**:7033–7039.
19. Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz. 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**:1011–1020.
20. Schnell, M. J., T. Mebatslon, and K.-K. Conzelmann. 1994. Infectious rabies virus from cloned cDNA. *EMBO J.* **13**:4195–4203.
21. Vidal, S., and D. Kolakofsky. 1989. Modified model for the switch from Sendai virus transcription to replication. *J. Virol.* **63**:1951–1958.
22. Wertz, G. W., and J. A. Melero. 1993. Workshop on reverse genetics of negative stranded RNA viruses sponsored by the Juan March Institute, Madrid, Spain. *Virus Res.* **30**:215–219.