In Vivo Model for Pseudo-Templated Transcription in Sendai Virus

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A synthetic Sendai virus-like recombinant RNA was used to develop a model system for pseudo-templated transcription of the *P/C* gene. The synthetic RNA molecule contains a 42-base stretch of nucleotide sequence derived from the RNA editing site of the *P/C* gene embedded into the chloramphenicol acetyltransferase gene. When this construct was rescued into Sendai virus, it was found that this 42-base sequence was sufficient to allow the Sendai virus polymerase to transcribe mRNAs with G-nucleotide insertions. Edited mRNA species containing a single nontemplated G insertion were found at a frequency of 6.5%, while rare messages had two G residues inserted. Edited viral RNA was not apparent, suggesting that this event is appropriately excluded during replication of the model genome. By progressively deleting from the 3' end, we found that a 24-nucleotide sequence spanning the G-insertion site was sufficient for pseudo-templated transcription in our system.

Sendai virus, a paramyxovirus, is an enveloped virus that has a nonsegmented negative-sense RNA genome of 15.3 kb. The genome (viral RNA [vRNA]) is transcribed from the 3' end by the viral polymerase, generating an extracistronic leader RNA followed by six transcription units (*NP*, *P/C*, *M*, *F*, *HN*, and *L*, in 3'-to-5' order) (for reviews, see references 1, 5, and 6). The viral polymerase complex, which consists of the *L* (large) and *P* (phospho) proteins, is thought to terminate and reinitiate at each gene junction, capping and polyadenylating all mRNAs except the leader RNA. The vRNA also serves as a template for the synthesis of a full-length positive-sense complementary RNA, which is the replication intermediate. In contrast to mRNA, both the vRNA and complementary RNA are unmodified and always found encapsidated by the nucleoprotein into nuclease-resistant helical nucleocapsids.

Most paramyxoviruses have the unique property of promoting the transcription of mRNAs containing nontemplated insertions at one locus in the *P/C* gene. Thus, the paramyxovirus *P/C* gene is transcribed into mRNAs that usually differ by one (e.g., measles virus and Sendai virus) or two (e.g., simian virus 5 and mumps virus) nontemplated G-nucleotide insertions (2, 14, 20, 21). There is a short consensus sequence among these viruses (3'-UUYUCCC-5', where Y is either U or C) where the viral polymerase is suggested to stutter and cotranscriptionally insert a G residue (21). A model for how the consensus and its neighboring sequences may promote one or two G insertions for various paramyxoviruses has been proposed (22). First, the polymerase pauses at or near the insertion site because of an intrinsic property of the template. When the pause time is sufficiently long, slippage of the nascent mRNA occurs by one base (Sendai virus and measles virus) or two bases (mumps virus and simian virus 5). In this way, the polymerase reiteratively adds one or two G's at the slippage site. The insertion(s) creates a frameshift in the ribosomal reading frame, resulting

in the synthesis of polypeptides with common N-terminal but alternate C-terminal sequences. For simian virus 5 and mumps virus, the edited mRNA is translated into P protein (14, 20), whereas for Sendai and measles virus, it usually gives rise to a cysteine-rich V protein (2, 21). For Sendai virus, and likely for other viruses, one outcome of this type of gene expression appears to be a down-regulation of RNA synthesis by proteins sharing domains for a protein-protein interaction(s) but with alternate functional modules (4).

A number of questions about the nature of RNA editing in paramyxoviruses remain unanswered. For example, what is the *cis*-acting sequence element(s) required for pausing and stuttering of the polymerase and slippage of the template? Why is it not detected at other regions of the genome? What are the proteins involved in initiating and regulating the process of nontemplated transcription that is excluded during replication? To begin to answer these questions via reverse genetics, a model system which accurately mimics the vRNA editing process and allows mutational analysis at both the RNA and protein levels would be fundamental.

Recently, we developed an artificial minigenome that could be expressed, replicated, and packaged efficiently by Sendai virus (13). The RNA minigenome consists of the antisense chloramphenicol acetyltransferase (CAT) coding region flanked by 145 and 119 5'- and 3'-terminal sequences derived from the Sendai virus genome. Since the minigenome is derived from *in vitro* transcription of appropriately constructed recombinant DNA (pSend-CAT), mutations or sequence manipulations introduced at the DNA level would in turn alter the RNA minigenome. A similar system developed for influenza virus (12, 23) has enabled the development of new approaches to molecular analyses of negative-strand virus replication (8, 9, 11). An analogous method was also applied to respiratory syncytial virus (3), leading to the mutational analysis of the transcription/replication signal(s). In this study, we have developed an *in vivo* model for RNA editing in Sendai virus. By inserting a short nucleotide stretch spanning the editing site of the *P/C* gene into the minigenome, we have used the minigenome as a template to reconstitute the pseudo-templated transcription process.

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MATERIALS AND METHODS

Viruses and cells. Sendai virus (Sendai/52) was grown in 10-day-old embryonated chicken eggs as described previously (13). Madin-Darby bovine kidney (MDBK) cells were used for all transfection experiments. Cytoplasmic vRNAs were prepared from virus-infected baby hamster kidney (BHK) cells.

Construction of plasmids. The original plasmid construct was made via polymerase chain reaction (PCR) mutagenesis, using the original pSend-CAT transcription vector (13). Briefly, two separate PCR fragments were synthesized and then joined via a common *Bam*HI site. One PCR product was constructed from pSend-CAT via an oligonucleotide which contains, in 5'-to-3' order, a *Bam*HI site, the *P/C* sequence, and the CAT sequence (5'-GCGCCGGATCCCG ACTCAACAAAAAGGGCATAGGAGAGAACACATCA TCTATGAGAAAAAATCACTGGG-3'; primer A) and an oligonucleotide spanning the *Hind*III site in pSend-CAT (primer B) (Fig. 1A). The second PCR product was constructed from pSend-CAT by using an oligonucleotide containing a *Bam*HI site followed by 3' untranslated sequences from pSend-CAT (5'-CCCCCGGATCCATCGTGAAC TTTGGCAGC-3'; primer C) and an oligonucleotide encompassing the *Eco*RI and *Eae*I sites and the 3' end of the Sendai virus genome (primer D). The PCR products were joined at the engineered *Bam*HI site and cloned into the *Eco*RI-*Hind*III sites of pUC19 (pPC-CAT). All subsequent constructs were made via PCR mutagenesis by cloning into the unique *Bam*HI-*Acc*I sites surrounding the *P/C* sequence (Fig. 1A).

In vitro transcription and transfections. T7 RNA polymerase runoff transcription of *Eae*I-digested plasmids was performed as instructed by the manufacturer (GIBCO-BRL, Gaithersburg, Md.). The transcript was purified by multiple phenol-chloroform extractions, and the yield was quantitated on a 1.2% agarose gel. Approximately 1 mg of RNA was used to transfect a 35-mm-diameter dish of MDBK cells (about 10^6 cells) by the DEAE-dextran method as described previously (12).

CAT assays and passaging of transfectant viruses. CAT assays were performed 18 h after transfection as detailed previously (7). Viruses were grown in serum-free minimal essential medium containing 0.2% bovine serum albumin (BSA) and trypsin (5 mg/ml) for 48 h after transfection. Undiluted supernatant (100 ml) was injected into 10-day-old embryonated eggs for high-titer transfectant virus preparations. Allantoic fluid was harvested after 48 h and tested for rescued PC-CAT virus through infection of monolayer and CAT assays.

Cytoplasmic RNA preparation. Approximately 10^7 BHK monolayer cells were infected with egg-grown transfectant viruses at a multiplicity of infection of 10 for 20 h at 33°C. The infected cells were disrupted in a hypertonic buffer containing 0.6% Nonidet P-40, and the viral nucleocapsids and the infected cell cytoplasmic RNA were separated on a CsCl gradient (20 to 40%) as described previously (10). The nucleocapsid band was pooled and dialyzed against TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) for 1 h and then treated with 500 U of micrococcal nuclease for 30 min at 37°C. The RNA pellet and nucleocapsid band were extracted with phenol-chloroform, precipitated in ethanol, and used for PCR and limited primer extension analysis.

PCR. Approximately 2 mg of purified cytoplasmic RNA was added in a first-strand cDNA synthesis reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM

MgCl₂, 0.1% (wt/vol) gelatin, 1 mM MgCl₂, 2.5 mM each deoxynucleoside triphosphate (dNTP), 1 U of RNasin (Promega, Madison Wis.), 2.5 U of mouse mammary leukemia virus reverse transcriptase (GIBCO-BRL), and 1 mg of downstream primer specific for the CAT gene. Either 5'-TGAAAACGTTTCAGTTTGCTCATGGAAAAC-3' for analyzing mRNA (primer 1) or 5'-GACCCCTTTGCTTTGCTGC CAAAGTTCACGATGGATC-3' for analyzing vRNA (primer 2) was used. The mixture was incubated at 42°C for 30 min, and the enzyme was inactivated at 99°C for 15 min. PCR was performed by diluting the mix to 100 µl in the same salt buffer with 0.4 mM MgCl₂ and adding 1 mg of upstream primer specific for the *P/C* gene (primer 2 for analyzing mRNA or primer 1 for analyzing vRNA) and 2.5 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.). The reaction was carried out at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min for 30 cycles.

Limited primer extension analysis. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by electrophoresis on a 7 M urea-12% polyacrylamide gel. Primer extension analysis of pseudo-template transcription was based on previous studies (17). Primers were end labeled with T4 polynucleotide kinase and [γ -³²P]ATP. Approximately 25 to 50 ng of PCR products was mixed with 5×10^6 cpm of labelled primer in 40 mM Tris-HCl (pH 7.5)-20 mM MgCl₂-50 mM NaCl, boiled for 3 min, and allowed to anneal at 37°C for 20 min. Either ddA or ddT and appropriate mixes of three dNTPs were added to a final concentration of 150 mM, and the mixture was incubated at 37°C for 30 min after addition of 2 ml of Sequenase (U.S. Biochemical) diluted 1:8 in 10 mM Tris-HCl (pH 7.5)-5 mM dithiothreitol-0.5 mg of BSA per ml. For the PC-CAT PCR product, primer 3 (5'-CTCATAGATGATGTGTTCTC TCCTA-3') was used with ddATP (see Fig. 4B); for Para 45, 44, and 46 PCR products, primer 4 (5'-CAGCATGGATCCCGACT-3') was used with ddTTP (Fig. 2A).

Differential oligonucleotide hybridization. PCR products were digested with *Bam*HI and *Eco*RI (see Fig. 4A) and cloned into an appropriately digested pUC19 vector. Colonies were replica plated to nitrocellulose filters and screened by oligonucleotides as detailed elsewhere (21). Primers 5'-CTCCTATGCCCTTTTTTGT-3', 5'-CTCCTATGCCCTTTT TTTG-3', and 5'-TCCTATGCCCCCTTTTTTG-3' were end-labeled and used in screening for 3G, 4G, and 5G colonies, respectively. Probes were prehybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.5% nonfat dry milk-0.05% PP_i overnight at 37°C and washed in 1× SSC-0.1% sodium dodecyl sulfate (SDS)-0.05% PP_i three times at room temperature for 5 min each time and then at 50°C for 20 min. After the filters were air dried, autoradiography was performed as described elsewhere (18), and appropriate colonies were picked and grown overnight in 3 ml of TB containing ampicillin. Plasmid DNAs were prepared as described elsewhere (18) and sequenced by the dideoxynucleotide chain termination method (19), using Sequenase.

RESULTS

RNA editing detected by CAT expression. Previously we showed that synthetic RNAs containing the 5'- and 3'-terminal sequences of the Sendai virus genome could be expressed, replicated, and packaged after transfection in Sendai virus-infected cells (13). In this system, the specific expression of the artificial genome, Send-CAT, was conveniently assayed by expression of the CAT gene inserted

FIG. 1. (A) Diagrammatic representation of relevant regions of pPC-CAT. The CAT gene is in an antisense orientation (as indicated by upside-down letters) relative to the T7-derived RNA transcript (Tx RNA). After the initiating methionine codon, the gene is interrupted by a *Bam*HI linker and the insertion of a 42-nucleotide sequence derived from the *P/C* gene. The CAT gene is flanked by 145 and 119 noncoding nucleotides of the Sendai virus genome 5' and 3' termini, adjacent to the T7 phage promoter sequence and the restriction enzyme *Ear*I site, respectively. The primers shown below the gene represent the original PCR oligonucleotides which were used to construct pPC-CAT from the parental pSend-CAT (13). (B) Amino-terminal sequence of the PC-CAT gene. The faithful transcript translates into a protein which is out of frame with the subsequent CAT protein. However, if pseudo-templated transcription acts to insert a single G nucleotide, the correct reading frame is restored and CAT protein should be expressed. The *P/C* gene sequences are underlined.

between the viral 5' and 3' sequences. Using this system, we attempted to reconstitute the RNA editing event that occurs during transcription of the *P/C* gene of Sendai virus.

Initially, a 42-nucleotide sequence derived from the Sendai virus *P/C* gene spanning the G-insertion site was placed two codons downstream of the initiating methionine residue of the CAT gene (Fig. 1A). The resulting construct, PC-CAT, is identical to its parent, Send-CAT, except for the insertion at the initiation codon. The clone was constructed such that the downstream CAT coding region was shifted out of reading frame by one base, so that PC-CAT should not express CAT. If the Sendai virus polymerase recognizes the *P/C* sequence, it may insert one or more G residues. If a single guanine nucleotide were inserted by the viral polymerase, the CAT gene would be returned to the correct reading frame, and CAT activity should be restored, assuming that the 16-amino-acid insertion at the amino terminus of the CAT gene is a tolerated insertion (Fig. 1B).

Initially, the PC-CAT RNA was transcribed *in vitro* by T7 bacteriophage RNA polymerase from *Ear*I-digested pPC-CAT. To ensure that this vRNA-like transcript was faithful and did not contain any insertions, this transcript was subjected to a limited primer extension analysis. RNA was hybridized with an end-labeled primer (Fig. 2A) and subjected to extension with reverse transcriptase in the presence of dATP, dGTP, dCTP, and ddTTP (Fig. 2A). The product will extend through the presumptive editing region and terminate to produce a 32-nucleotide product. The resulting 32-nucleotide single band (and a lack of 33-nucleotide product) indicated that the transcript did not incorporate any extra nucleotides during the T7 transcription reaction (Fig. 2B). This result is consistent with the previous finding that pseudo-templated transcription was not detected when the *P/C* mRNA was expressed by an alternate RNA polymerase via a vaccinia virus recombinant (21).

The vRNA-like transcripts are then transfected into cells which are subsequently infected with Sendai virus (Fig. 3A).

FIG. 2. (A) Schematic of the limited primer extension analysis. The sequence of the region of the PC-CAT transcript (Tx) where the primer hybridizes is shown. The predicted G-insertion site is indicated below as the editing region. The termination site of the extension reaction by reverse transcriptase (R.T.) after incorporation of ddT is 15 bases from the start site, thus generating either a 32-nucleotide (no editing) or a 33-nucleotide (one G nucleotide inserted) product. (B) Limited primer extension analyzed on a polyacrylamide-urea gel. A sequencing reaction of pPC-CAT with the same primer was used to show the proper termination at the first thymidine residue. A 33-nucleotide (nt.) extension product was used as a molecular weight marker.

The helper virus will express, replicate, and package the synthetic RNA transcript. Assays for CAT activity can be used to monitor the transfection. As shown previously (13) and used here as a positive control (Fig. 3B, lane 1), the Send-CAT RNA gives rise to a relatively high level of CAT expression after transfection and Sendai virus infection. When the PC-CAT RNA was transfected, a relatively small but reproducible amount of CAT expression was detected (Fig. 3B, lane 2). This activity could be the result of pseudo-templated transcription of the PC-CAT mRNA through G insertion by the viral polymerase. Since the edited version of *P/C* mRNA during a normal virus infection has been estimated to be only about 20 to 31% of the total *P/C* mRNA produced (17, 21), the reduced level of CAT activity would be expected. On the other hand, the CAT activity could also result from unedited mRNA by mechanisms such as an alternate transcription start or a translation frameshift (i.e., pseudoknot). To further address this possibility, the PC-CAT RNA was rescued into progeny viruses and grown to a high titer by undiluted passage through embryonated chicken eggs (Fig. 3A). We have previously suggested that

FIG. 3. (A) Protocol for obtaining high-titer transfectant viruses. SEN, wild-type Sendai virus infection 1 h after transfection; Tx, transcript; 1°C, primary. (B) Comparative CAT assays after transfection of RNA or infection with the transfectant viruses grown in eggs. Cells were harvested either 18 h after transfection (lanes 1 and 2) or 12 h after infection (lanes 3 and 4). Equivalent amounts of RNA and allantoic fluids were used for both Send-CAT and PC-CAT.

the Send-CAT RNA may be analogous to a fusion defective interfering RNA, and we were able to partially test and confirm one characteristic of a defective interfering RNA by serial passaging of undiluted transfectant viruses (Fig. 3B, lanes 3 and 4).

Identification of PC-CAT mRNA with G insertions at the correct position. To determine whether the observed CAT activity was due to PC-CAT mRNA which had undergone pseudo-templated transcription, the rescued transfectant viruses were used to infect large numbers of BHK cells ($\sim 10^8$ cells). These cells were permeabilized with lysocleithin and cytoplasmic RNA, and nucleocapsids were isolated via CsCl gradient centrifugation (10). The PC-CAT-infected cytoplasmic RNA was amplified by reverse transcription-PCR, using primers specific for the CAT gene (primer 1) and the inserted *P/C* gene sequence (primer 2) (Fig. 4A). These primers extend only on the synthetic CAT construct, not on viral *P/C* gene mRNA. To control for any possible artifacts which may arise during the reverse transcription-PCR process, the T7-transcribed PC-CAT vRNA was also amplified at the same time. These PCR products were then analyzed by limited primer extension analysis with T7 DNA polymerase (Sequenase) and *P/C* gene-specific primers in the presence of dGTP, dTTP, dCTP, and ddATP (Fig. 4B). The extension product would terminate at the first adenosine, which is 16

nucleotides from the end of the primer and would read through the proposed editing site (Fig. 4B). Therefore, faithful transcripts should align with the first adenosine in the sequence (Fig. 4B, lane 2), while mRNAs with additional G's inserted would align with bands of increased size. By this analysis, an edited 4G product was clearly detected when the PCR sample from PC-CAT mRNA was used, whereas with PC-CAT transcript PCR, only the unedited 3G product was evident (Fig. 4B, lanes 5 and 7). PCR fragments corresponding to mRNAs with more than one insertion could not be definitely identified. However, when the PC-CAT mRNA PCR sample was cloned and screened by a differential oligonucleotide hybridization method that could distinguish three G's from four or five G's at the putative editing site (21), 6.5% of the clones contained four G's while less than 1% had five G's (Table 1). Again, no four- or five-G insertions were identified when the control PC-CAT transcript PCR sample was screened (data not shown). Finally, sequencing analysis of the positive 4G and 5G clones confirmed that the clones contained one and two nontemplated G insertions, respectively, at the precise position (Fig. 5). In addition to examining mRNA, we examined the PC-CAT vRNA purified from nucleocapsids isolated from a CsCl gradient. To further purify the ribonucleoprotein, the banded nucleocapsid was additionally treated with micrococcal nuclease. Since ribonucleoproteins of Sendai virus are nuclease resistant, this treatment would remove any traces of cytoplasmic RNA which may have copurified with the nucleocapsid. The limited primer extension analysis showed no evidence of an edited product (Fig. 4B, lane 2), and screening by oligonucleotide hybridization exclusively detected the genomic-like three-G sequence (data not shown). This finding strongly suggests that the vRNA in our model system undergoes RNA editing with constraints similar to those affecting the intact viral genome, as it occurs during transcription but not replication (21).

3' deletions of the *P/C* gene editing sequence. To determine whether sequences 3' to the G-insertion site were essential for pseudo-templated transcription, three new constructs which contained progressive deletions of six nucleotides were made (Fig. 6A). Thus, Para 45, 44, and 46 contained 36, 30, and 24 bases, respectively, of the *P/C* gene. All of these constructs still retained the conserved 3'-UUUCCCC-5' consensus sequence. The three new derivatives were then transfected, rescued, and used to prepare cytoplasmic RNA. After reverse transcription and PCR, the limited primer extension analysis of the DNA showed that all three constructs underwent editing during transcription by the viral polymerase and now contained an extra nucleotide (Fig. 6B). Cloning, screening, and sequencing of the PCR product of the shortest clone (Para 46) confirmed the presence of pseudo-template transcription products. Although a smaller sample size was screened, 2 of 35 clones of Para 46 PCR products contained a single G insertion. Thus, a sequence as short as 24 nucleotides could induce editing by the viral polymerase. It should be noted that an additional band corresponding to a one-base-shorter extension fragment is also evident in Fig. 6B (lanes 5 to 8). The intensity of this band is highly variable between experiments and is always present in control lanes (Fig. 4B, lane 7). As PCR products shorter by one base have never been isolated, we presume that this band is an artifact due to premature termination prior to the dideoxy base addition.

Finally, one other construct was tested for its ability to induce pseudo-templated transcription. Para 54 is identical to PC-CAT except that the three C's in the consensus

FIG. 4. (A) Procedure for detecting edited RNA by PCR amplification and limited primer extension analysis. For analysis of mRNA, primer 1 was used in the first-strand synthesis reaction and

TABLE 1. Frequency of G-nucleotide insertions in PC-CAT mRNA

Expt	No. (%) of PC-CAT cDNA clones with the indicated number of G's inserted		
	0	1	2
I	168 (94)	10 (5.6) ^a	1 (0.6) ^a
II	178 (94)	11 (6)	0 (0)
III	125 (91)	12 (8.7)	1 (0.7)
Total (%)	471 (93.1)	33 (6.5)	2 (0.4)

^a These clones were picked and sequenced. All those sequenced showed the correct number of inserted G nucleotides.

sequence were changed to three G's (Fig. 6A). When this construct was rescued after transfection and analyzed by limited primer extension, no visible edited product was evident (Fig. 6B). This result strongly suggests that the Sendai virus editing mechanism specifically requires the stretch of C's in the vRNA for the insertion to occur, and the substitution by G residues reduces the efficiency of editing below the detectable level.

DISCUSSION

The recent success in developing artificial genomes for negative-strand RNA viruses has greatly increased our ability to apply reverse genetics and understand the structure-function requirements of viral elements (3, 8, 9, 11-13, 15, 16, 23). In particular, the analyses of various *cis*-acting signals such as those directing transcription, replication, and packaging of the viral genome have benefited immensely, since mutations are introduced into the model genome with ease and quantitations of the effects of the modifications are accomplished by simple assays of reporter gene expression. Combined with the ability to express viral proteins *in vivo* by T7- or vaccinia virus-based vectors (4, 8, 9, 15, 16), the artificial genome system provides a powerful tool with which to dissect and understand the functions of individual *cis*- and *trans*-acting elements as well as their interactions.

In this report, we describe the successful reconstitution of pseudo-templated transcription by the Sendai virus polymerase by placing a 42-nucleotide sequence derived from the P/C gene into the model genome. The initial evidence was provided by CAT expression, which by design could conceivably result from a nontemplated G-nucleotide insertion. This conclusion was proven by limited primer extension analysis and subsequent cloning and sequencing studies. By our assay procedure, which entails rescuing the recombinant genome into transfectant viruses and using these viruses to infect and prepare cytoplasmic RNA samples, we found that

primer 2 was added for PCR amplification. For analysis of transcript or rescued vRNA, primer 2 was used in the first-strand synthesis and primer 1 was used for amplification. R.T., reverse transcriptase; PAGE, polyacrylamide gel electrophoresis. (B) Limited primer extension analysis on PCR products. Sequences of the end-labelled primer and template are shown at the top. The three PCR products indicated as Cyto, NC, and Tx are those corresponding to the cytoplasmic RNA, nucleocapsid, and transcript, respectively. A sequence reaction of pPC-CAT with use of the same labelled primer was used as a size marker and proper termination site indicator. The unedited and edited (one-G insertion) products are indicated by 3G and 4G, respectively.

FIG. 5. DNA sequencing of the cDNA clones of PC-CAT mRNA. Clones positive for the 3G (unedited), 4G (+1G), and 5G (+2G) oligonucleotides were sequenced by the ddNTP method. Circles and arrowheads indicate the unedited 3G products and inserted G's, respectively.

about 6% of the mRNA contained a G insertion at the precise position and less than 1% contained a two-G insertion. During normal paramyxovirus infection, insertion of non-templated nucleotides occurs only during transcription, not during replication (1). Our model PC-CAT template behaved accordingly, as we did not detect any edited PC-CAT replication products by limited primer extension and oligonucleotide screening. We did, however, find one edited (one-G insertion) clone derived from vRNA upon screening of the shorter Para 46 construct (data not shown); we believe that this clone is probably due to contamination but may also have arisen from an error introduced during our cloning procedure. In this regard, Paterson and Lamb (14) also found one mumps virus vRNA cDNA clone, after sequencing 70 clones, which was considered an error introduced by reverse transcriptase or *Taq* DNA polymerase. Since no edited PCR product was found when the T7-expressed transcript was used as a starting material for first-strand synthesis and PCR amplification, we feel that errors due to our cloning procedure would be introduced at an extremely low rate.

Previous studies found that during normal Sendai virus infection, 20 to 31% of viral *P/C* mRNA had a single G insertion while 5 to 7% had multiple G insertions (17, 21). This variation in frequencies was considered to be a result of the actual method of quantitation (17). In our studies, reverse transcription, PCR amplification, and cloning were used, and levels of approximately 6 and 0.5% of single and double G insertions, respectively, were found. Further stud-

ies are needed to examine whether this lower value for pseudo-template transcription is an inherent property of the multiple steps in our quantitation system or whether the PC-CAT RNA or its derivatives are lacking a regulatory element which may promote more efficient editing. However, it is clear that transposition of a sequence as short as 24 nucleotides is sufficient to promote this polymerase-induced event.

From studies of the known and predicted editing sites among paramyxoviruses, it has been suggested that the linear sequence element plays only a minor role in directing the precise G-nucleotide insertions (21), since the consensus site is only weakly preserved among different paramyxoviruses, with a strict consensus of only seven nucleotides present among all paramyxoviruses. In addition, this consensus site is present multiple times throughout the Sendai virus genome and even in other regions of the *P/C* gene itself without promoting editing. However, the data presented here suggest that the primary sequence does play a major role in editing. In this respect, the 3' sequence deletion

FIG. 6. (A) Sequences of 3' deletion mutants. Para 45, 44, and 46 contain progressive deletions of six nucleotides from the 3' end, whereas Para 54 contains three C's (shaded) instead of the G's in the consensus sequence. The predicted insertion site is indicated by an arrow. (B) Limited primer extension analysis on PCR products of cytoplasmic RNA derived after infection by rescued viruses of the modified clones. The primer for extension is shown in Fig. 2A. The sequence of pPC-CAT, obtained by using the same primer, is shown to indicate the proper termination site at the first thymidine residue. The unedited and edited products are indicated by 3G and 4G, respectively.

analysis strongly suggests that a sequence element as short as 24 nucleotides (Para 46 construct) can by itself confer the ability to undergo RNA editing at the precise position. This property is context independent, since we have removed these bases from all other *P/C* gene sequences and placed it within a foreign gene. In addition, a substitution of the three G residues by C's in the consensus sequence appears to abrogate editing in our system, indicating that there is a very specific sequence requirement. Clearly, a more extensive mutational analysis of the sequence element should allow a better understanding of the sequence requirements.

In our artificial genome model, the basic rules and mechanisms of pseudo-template transcription appear to be preserved. For example, the number of G's inserted heavily favors a single G residue, rather than two or more, and the insertion occurs at the correct predicted position. Furthermore, the editing is detected only as a Sendai virus-encoded activity and is not seen when other polymerases, such as T7 RNA polymerase, T7 DNA polymerase (Sequenase), mouse mammary leukemia virus reverse transcriptase, and *Taq* polymerase, are used. Thus, the results obtained with the PC-CAT genome or its derivatives should provide valuable insights into the mechanism of pseudo-templated transcription by the Sendai virus polymerase.

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