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RNA Replication by a Respiratory Syncytial Virus RNA Analog Does Not Obey the Rule of Six and Retains a Nonviral Trinucleotide Extension at the Leader End

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Genome analogs ("minigenomes") of Sendai and measles viruses replicate efficiently only if their nucleotide length is an even multiple of six, a requirement called the rule of six (P. Calain and L. Roux, J. Virol. 67:4822–4830, 1993; M. S. Sidhu, J. Chan, K. Kaelin, P. Spielhofer, F. Radecke, H. Schneider, M. Masurekar, P. C. Dowling, M. A. Billeter, and S. A. Udem, Virology 208:800–807, 1995). The existence of a comparable requirement was tested for respiratory syncytial virus (RSV), which also is a member of family *Paramyxoviridae* and whose natural genome length also is a multiple of six. An internally truncated analog of RSV positive-sense replicative intermediate RNA (antigenome) bearing the chloramphenicol acetyltransferase gene as a reporter was synthesized from cDNA in vitro. This RNA was transfected into cells which were infected with RSV as a helper. Miniantigenomic RNA was indistinguishable from previously studied negative-sense minigenome RNA in its ability to participate in transcription, RNA replication, and incorporation into transmissible particles. Sixteen miniantigenomes which were of slightly different lengths and which in aggregate represented multiples of a wide range of integers including 1 to 15 were constructed. During transfection and two serial passages, the various miniantigenomes were essentially indistinguishable with regard to the efficiency of transcription, RNA replication, and packaging into transmissible particles. Progeny minigenomes of six different mutants were recovered postpassage, copied into cDNA, cloned, and sequenced completely. The length of each of these RNAs was found to have remained unchanged during replication and passage. Thus, RSV transcription and replication appear to lack the requirement that the template length be an even multiple of an integer such as six, which for Sendai and measles viruses is obligatory for nucleocapsid function. Each of the in vitro-synthesized miniantigenomes used in transfection contained a nonviral extension of three nucleotides, GGG, on the 5′ (leader) end contributed by the T7 promoter. The termini of the recovered minigenomes were examined for five mutants by RNA circularization followed by cDNA synthesis, amplification, cloning, and sequencing. Unexpectedly, each recovered minigenome contained the complement of this nonviral extension on the 3′ (leader) end, showing that it had been faithfully copied and maintained during RNA replication and passage. The nonviral trinucleotide did not appear to affect the activity of the template.

Human respiratory syncytial virus (RSV) is an important agent of pediatric respiratory tract disease and is a representative of the genus *Pneumovirus* of the family *Paramyxoviridae* (9, 22, 28a). Within this virus family, genus *Pneumovirus* was recently segregated into subfamily *Pneumovirinae*, whereas the other three genera, namely, *Paramyxovirus* (represented by Sendai virus [SeV]), *Morbillivirus* (represented by measles virus [MeV]), and *Rubulavirus* (represented by mumps virus), were organized into the separate subfamily *Paramyxovirinae* (28a). The RSV genome is a single nonsegmented negative RNA strand of 15,222 nucleotides (nt). RNA synthesis by RSV has not been studied in detail because of the difficulty of working with this labile, poorly growing virus. It is thought to have general similarities to SeV and vesicular stomatitis virus (VSV [family *Rhabdoviridae*]), which are prototypes for the nonsegmented negative-strand viruses (22, 32).

For these prototypic viruses, genomic RNA is tightly bound by the major nucleocapsid protein N (or NP) and also is associated with the phosphoprotein P and the large polymerase subunit L. This RNA-protein complex comprises the functional nucleocapsid, which is active in template-dependent RNA synthesis and is packaged in the virion. Transcription of the negative-sense genome occurs by entry of the polymerase in the 3′ extragenic leader region followed by the copying of each gene in turn by a sequential stop-start mechanism which is guided by *cis*-acting template signals and yields subgenomic mRNAs. RNA replication occurs when the polymerase switches to a readthrough mode that yields a complete positive-sense replicative intermediate or antigenome which also is tightly encapsidated and serves as the template for the synthesis of progeny genomes.

Methods for direct genetic manipulation of the nonsegmented negative-strand RNA viruses have recently been developed. A number of infectious recombinant viruses have been produced by intracellular coexpression of full-length antigenomes and the proteins of the functional nucleocapsid, all from transfected cDNA. Production of recombinant rabies virus, VSV, SeV, and MeV required coexpression of the N, P, and L proteins, whereas RSV required in addition the M2 protein (6, 17, 23, 28, 29, 34). However, the large genome size is a complicating factor for detailed mutational analysis. Also, many mutations may be unrecoverable in infectious virus because of cumulative effects on replication or transcription and

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thus may be inaccessible to analysis by this method. Such studies can make use of a second type of genetic system, which involves short, helper-dependent "minigenomes" (3, 4, 7, 10–14, 19, 21, 26, 27, 30, 33, 36). These can be versions of naturally occurring defective interfering particle genomes or engineered versions of standard genomes in which the viral genes have been removed and replaced with a marker gene, such as that for chloramphenicol acetyltransferase (CAT), under the control of viral transcription signals. Minigenomes can be complemented by viral proteins supplied by coinfection with standard helper virus (as in this report) or from transfected plasmids.

Studies with RSV minigenomes have confirmed that polymerase entry occurs at the 3′ end and that transcription is sequential and mediated by *cis*-acting gene start (GS) and gene end (GE) sequence signals (21). Also, the N, P, and L proteins were shown to be necessary and sufficient for minigenome RNA replication (19, 36). These observations illustrated aspects of RSV RNA synthesis which are consistent with the model systems described above. On the other hand, RSV transcription was shown to require a processivity factor, the M2 protein, which is without known precedent in the other model systems (7). Expression of a second open reading frame (ORF) of the M2 mRNA was associated with a drastic inhibition of RNA synthesis, suggesting that it encodes an additional, regulatory RSV protein which does not have an obvious counterpart in the other model systems (7). The existence of other features unique to RSV, such as the NS1 and NS2 proteins, is suggestive of additional differences from the model systems.

Studies with SeV minigenomes of both the copy-back and internally deleted varieties led to the remarkable finding that minigenome RNA replication was efficient only when the RNA's nucleotide length was evenly divisible by six, a requirement called the rule of six (3, 4). This finding was confirmed for MeV, representing a second genus of the paramyxovirus family (30), and also appears to hold for human parainfluenza virus type 3, a member of the same genus as SeV (14). The rule of six is thought to reflect a requirement that each NP monomer associate with exactly six nucleotides. The natural genome of RSV, like those of SeV and MeV, is evenly divisible by six. It was of interest to determine whether this rule extended to RSV, representing another subfamily of *Paramyxoviridae*.

Here, we tested the existence for RSV of a rule based on 6 or on any other integer from 1 to 15 by using a series of antigenomic RNA analogs designed to be of incrementally different lengths. The RNAs were synthesized in vitro and complemented by transfection into RSV-infected cells, a strategy which offers several advantages for this particular study over the alternative method of complementation by plasmid-encoded RSV proteins (19, 36). RSV infection of permissive cells should provide the most authentic context available for examination of RSV-specific RNA synthesis, since all of the viral proteins would be represented and each would be supplied in the correct amount and location and with the correct kinetics. RSV complementation also directs highly efficient incorporation of progeny minigenome RNA into transmissible particles, making it possible to monitor levels of RNA replication and transcription during serial passage (10, 11, 21). Unlike the plasmid-based system, complementation by RSV does not involve recombinant vaccinia virus, precluding potential artifacts due to vaccinia virus-specific nucleic acid-modifying enzymes or perturbation of cellular or RSV activities. Under these conditions, it was shown that RSV appears to be free of any requirement comparable to the rule of six. Surprisingly, three nonviral nucleotides contributed to the 5′ end of the miniantigenome were faithfully copied into the minigenome and retained during passage.

MATERIALS AND METHODS

cDNAs. In previous work, cDNA was constructed to encode the negative-sense 934-nt minigenome H15 which contained the 3′-terminal 86 and 5′-terminal 179 nt of authentic genomic RNA (21). These RSV-specific terminal segments flank a negative-sense copy of the CAT ORF, placing it under the control of RSV GS and GE signals (21). The cDNA was flanked by the T7 promoter and an *Hga*I site such that transcription from a linearized plasmid yielded a negative-sense minigenome containing exactly correct 5′ and 3′ ends. The H15 cDNA was modified such that the locations of the T7 promoter and the *Hga*I linearization site were interchanged. The result was cDNA KSG, which encodes the positive-sense complement, or antigenome, of H15. The mutagenesis was performed by PCR using synthetic oligonucleotide primers containing the desired sequence changes, followed by restriction fragment replacement. The two fragments involved were (refer to Fig. 1, which shows KSPX, a closely related derivative of KSG) the left-hand (leader) end of the minigenome from a *Kpn*I site in the vector to the *Xba*I site at the upstream end of CAT and the right-hand (trailer) end from the *Pst*I site at the downstream end of CAT to an *Hin*dIII site in the vector. This mutagenesis involved one additional change: three transcribed G residues were added to the T7 promoter such that they formed a trinucleotide nonviral extension on the 5′ (leader) end of the KSG RNA. The sequence of each PCR product was confirmed by dideoxynucleotide sequence analysis.

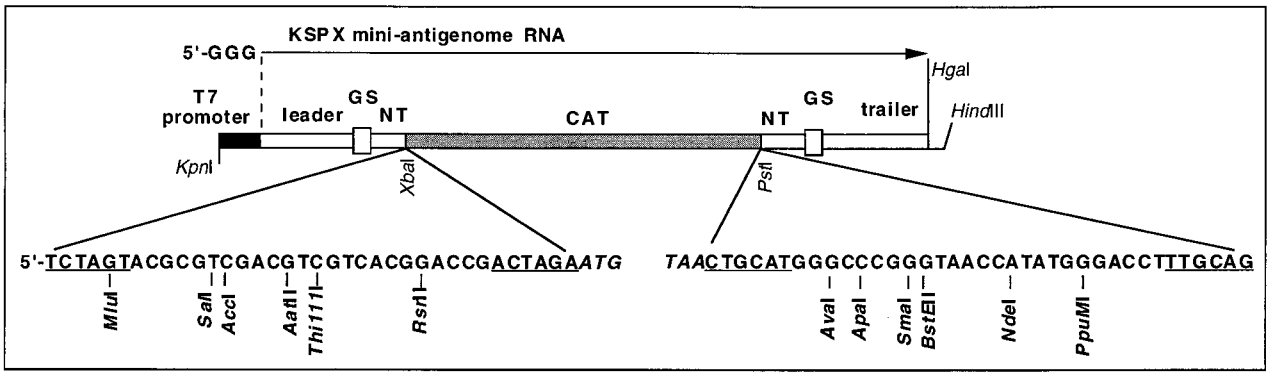
The KSG cDNA was modified (Fig. 1) by the insertion of one short DNA duplex made from synthetic oligonucleotides into the *Xba*I site to generate KSX (Fig. 1), by the insertion of a second such duplex into the *Pst*I site to generate KSP, or by the insertion of both duplexes into the two sites to generate KSPX. Both single-insertion mutants were made to determine whether either insertion altered minigenome activity, and it was found that neither did. The DNA inserted into the *Xba*I site was made by hybridization of the synthetic oligonucleotides 5′-CTAGTACGCGTCGACGTCGTCACGGACCGA (positive strand) and 5′-CTAGTCGGTCCGTGACGACGTCGACGCGTA (negative strand), and that inserted into the *Pst*I site consisted of 5′-TGGGCCCGGGTAACCAT ATGGGACCTTTGCA (positive strand) and 5′-AAGGTCCCATATGGTTAC CCGGGCCCATGCA (negative strand). The *Xba*I and *Pst*I sites were destroyed by the insertions. These short DNA inserts each contained several unique restriction enzyme sites (Fig. 1) which were modified to increase or decrease the cDNA length. Increases in length were made by restriction enzyme digestion, treatment with T4 DNA polymerase in the presence of 0.3 mM each deoxynucleoside triphosphate, and religation. Decreases in length were made by restriction digestion, treatment with exonuclease 7, and religation. Twelve such modifications were made and confirmed by sequence analysis, and the resulting plasmids were designated KSPX-1 to -12 (Fig. 1).

RNA synthesis, transfection, and passages. Plasmid-borne cDNAs were digested with *Hga*I and transcribed (2 μg per 100-μl reaction mixture) in vitro with T7 RNA polymerase under standard conditions. Infection, transfection, passage, and harvesting of monolayers of adenovirus E1-transformed human embryonic kidney (293) cells in six-well dishes were carried out as described previously (11, 21). CAT enzyme was measured by acetylation of [^{14}C]chloramphenicol as measured by thin-layer chromatography (18) and quantified by phosphorimagery (Molecular Dynamics).

Northern (RNA) blot hybridization using strand-specific probes. Harvested cell pellets were resuspended in a drop of water and dissolved in 1 ml of Trizol reagent (Life Technologies) according to the supplier's protocol except that the RNAs were extracted twice with phenol-chloroform following the isopropanol precipitation (19). RNA samples (each representing one-fifth of one well of cells, approximately 15 μg) were analyzed by electrophoresis on 1.5% agarose gels containing 0.44 M formaldehyde, transferred to nitrocellulose by using alkaline high-salt buffer (5, 19), and probed with a strand-specific riboprobe synthesized in vitro by runoff transcription of RSV-CAT C2 or C4 cDNA, as described in detail elsewhere (19). Quantitation was done by phosphorimagery.

RNA circularization, cDNA cloning, and nucleotide sequencing of minigenome RNA. Cells were infected with RSV and transfected with miniantigenome RNA which had been treated with DNase to remove residual template. Transfection was followed by extensive washing to remove residual input nucleic acid. Posttransfection medium supernatants were clarified and passaged to fresh cells as described above, and this was followed again by extensive washing to remove residual inoculum. The medium was harvested at 30 h postpassage and clarified, and extracellular particles were collected, concentrated with polyethylene glycol (19), and purified with Trizol. The cell monolayers were harvested, and intracellular RNAs were purified with Trizol.

To sequence the minigenome termini, RNA isolated from extracellular particles was circularized with RNA ligase (24, 25, 30, 35). Briefly, RNA from particles obtained from a single well of cells was denatured at 75°C for 2 min, treated with 4 U of tobacco acid pyrophosphatase (Epicenter Technologies) for 1 h at 37°C, and then subjected to phenol extraction and ethanol precipitation. The RNA was denatured again in the same way, assembled into an 80-μl ligation reaction mixture containing 60 U of T4 RNA ligase and 10% dimethyl sulfoxide in a standard buffer, and subjected to phenol extraction and ethanol precipitation. Ligation was carried out overnight at 16°C. cDNA was synthesized from the ligated RNA by reverse transcription (RT) using as a primer a negative-sense oligonucleotide corresponding to nt 179 to 197 of the CAT ORF, which made the reaction specific to progeny minigenomes. PCR using the above-described neg-



RSV-CAT RNA ([+] sense except as noted)	Restriction sites used for modification	length change at XbaI site	length change at PstI site	nucleotide length (including three nonviral G residues)	divisible evenly by the indicated integer(s)
KSG				937	
KSX				967	
KSP				968	4, 8
KSPX				998	
KSPX-1	Tth111I	+1	0	999	3, 9
KSPX-2	AccI	+2	0	1000	4, 5, 8, 10, 20
KSPX-3	RsrII	+3	0	1001	7, 11, 13
KSPX-4	MluI	+4	0	1002	3, 6
KSPX-5	MluI+Tth111I	+5	0	1003	17
KSPX-6	MluI+AccI	+6	0	1004	4
KSPX-7	MluI+RsrII	+7	0	1005	3, 5, 15
KSPX-8	MluI+SalI	+8	0	1006	
KSPX-9	NdeI	0	+2	1000	4, 5, 8, 10, 20
KSPX-10	BstEII	0	+5	1003	17
KSPX-11	AccI	-2	0	996	3, 4, 6, 12
KSPX-12	MluI	-4	0	994	7, 14

FIG. 1. Diagram of cDNA encoding the positive-sense KSPX miniantigenomic RNA (upper panel) and length variants derived therefrom (lower panel). (Upper panel) The left-hand end of KSPX abuts the promoter for T7 RNA polymerase (filled box) and contains the RSV leader (44 nt), the GS transcription signal (9 nt), and the adjoining nontranslated (NT) region of the NS1 gene (33 nt). The T7 promoter contributes three nonviral G residues to the 5′ end of the RNA. The right-hand end of KSPX contains part of the nontranslated region of the L gene (9 nt), the GE signal (13 nt), and the trailer region (144 nt) and is followed by an *Hga*I site for linearization. RSV-specific sequences are shown by open boxes, and the CAT gene is shaded. The CAT gene is flanked on each side by a short insert (expanded sequences; positive sense) containing unique restriction sites for manipulation of miniantigenome length. The underlined sequences show the *Xba*I (left-hand sequence) or *Pst*I (right-hand sequence) sites which were each destroyed by a single nucleotide change resulting from insertion of the synthetic DNA. (Lower panel) The various RNA analogs are shown. To generate KSPX-1 to -12, the KSPX cDNA was linearized at the indicated site(s) with the indicated restriction enzyme(s), made blunt by filling in or removal of sticky ends, and religated to yield the indicated change in length. Total nucleotide lengths include the three nonviral 5′-terminal G residues. Those integers (from 3 to 20) which can be divided evenly into each nucleotide length are indicated. KSG and KSPX-1 to -4 are outlined with dotted boxes to indicate that these were sequenced in their entirety from particles isolated from postpassage medium supernatants (Fig. 4 and 5).

ative-sense primer together with a positive-sense one corresponding to nt 438 to 457 of the CAT ORF was then employed to amplify the ligated ends. The resulting ~700-bp product was cloned by using the TA system (Invitrogen). Sequence analysis was performed on several clones per sample.

To determine the sequence of the remainder of the minigenome RNA, intracellular RNA was reverse transcribed with a positive-sense oligonucleotide corresponding to the first 20 nt of the leader region. One-fiftieth of the first-strand cDNA was made double stranded and amplified by PCR (1 min at 94°C, 1 min at 37°C, and 2 min at 72°C for 35 cycles) using the same positive-sense primer together with a negative-sense one corresponding to the last 30 nt of the trailer region. The ~1,000-bp product was electrophoresed on an agarose gel, recovered by the Gene Clean procedure (BIO101) and cloned and sequenced as described above.

RESULTS

cDNAs encoding positive-sense miniantigenomes. cDNA was constructed to encode RNA KSPX, an analog of RSV antigenomic RNA in which the viral genes had been deleted and replaced with the CAT ORF flanked by the RSV GS and GE transcription signals (Fig. 1). The KSPX cDNA was bordered on the leader and trailer ends by a T7 promoter and an *Hga*I site for linearization, respectively. The RNA transcribed from an *Hga*I-linearized plasmid was positive sense and contained the correct 5′ and 3′ ends of the authentic antigenome except that the former contained an extension of three nonviral G residues contributed by the T7 promoter, which are included in the nucleotide length of 998. These three transcribed G residues are part of the optimal configuration of this particular promoter, although it is well known that the promoter is functional without them. Indeed, the amount of transcription in vitro was increased by only two- or threefold by their presence (data not shown), but T7 polymerase is added in great excess in vitro and the efficiency of initiation might not be a limiting factor in that situation. We and others (12, 27) found that the

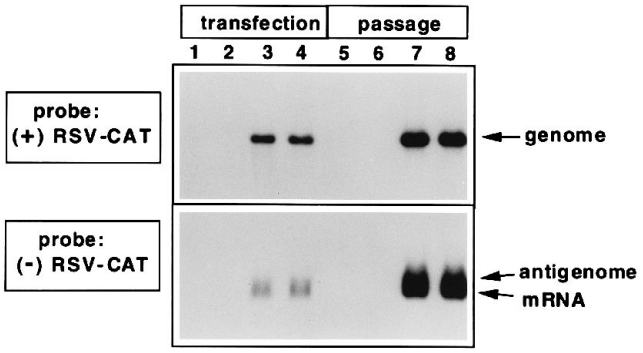


FIG. 2. Northern blot analysis of intracellular RSV-CAT RNAs synthesized during transfection and a subsequent passage in response to in vitro-synthesized negative-sense C2 minigenomes (lanes 3 and 7) or positive-sense C4 miniantigenomes (lanes 4 and 8). Duplicate Northern blots were prepared from total intracellular RNA harvested at 30 h posttransfection or postpassage. The blots were hybridized with a positive-sense (top panel) or negative-sense (bottom panel) RSV-CAT probe. Lanes 1 to 4 represent transfected cells. Lane 1, uninfected cells transfected with C2 RNA; lane 2, uninfected cells transfected with C4 RNA; lane 3, RSV-infected cells transfected with C2 RNA; lane 4, RSV-infected cells transfected with C4 RNA. Lanes 5 to 8 represent passage cells which received the medium supernatants of lanes 1 to 4, respectively.

presence of two or three transcribed G residues was necessary for synthesis of sufficient plasmid-encoded minigenome material in vivo, a situation in which T7 polymerase is synthesized in situ and the efficiency of initiation probably is much more important. The predicted sequences of the ends of KSPX RNA synthesized in vitro were confirmed by RNA circularization with RNA ligase followed by RT-PCR, cloning and sequence analysis (data not shown). The KSPX cDNA contained two short DNA inserts at the *Xba*I and *Pst*I sites which contained several closely spaced unique restriction sites for the purpose of making small changes in length (Fig. 1).

The present study compared the replication efficiencies of RSV RNA analogs that differed slightly in length. Thus, it was important to be able to recover replicated RNAs for sequence analysis to determine whether their lengths had changed. It is more feasible to recover minigenomes than it is to recover miniantigenomes, since the genome is the major product of RNA replication. The choice of miniantigenomic RNA for transfection would preclude the possibility that recovered minigenome material represented residual input material. However, it was necessary to confirm that in vitro-synthesized miniantigenome material, like the previously studied minigenome material (10, 11, 21), could be complemented by RSV and participate in the full viral replicative cycle. This was tested with two previously constructed plasmids which encode minigenome C2 and its cognate miniantigenome C4 (19), the latter being very similar to KSPX.

The C2 and C4 RNAs were synthesized in vitro and transfected in parallel into RSV-infected cells. Thirty hours later, when cytopathic effect was extensive, the medium supernatants were passaged to fresh cells. Intracellular RNAs from the transfection and passage were isolated and analyzed by Northern blot hybridization using strand-specific riboprobes (Fig. 2). C2 and C4 were found to be indistinguishable on the basis of the pattern of intracellular RNAs synthesized during transfection and passage. For example, hybridization with the positive-sense riboprobe revealed the abundant synthesis of minigenome material (Fig. 2, upper panel, lanes 3, 4, 7, and 8). Hybridization with the negative-sense probe revealed a doublet of positive-sense RNA (Fig. 2, lower panel, lanes 3, 4, 7, and 8). Previous work showed that the upper band consists of antigenome RNA and the lower, more diffuse band consists of polyadenylated mRNA. Only trace amounts of RSV CAT RNA, representing residual input material, were detected in uninfected cells which had been transfected in parallel with equivalent amounts of input RNA (Fig. 2, lanes 1 and 2).

The short inserts on either side of the CAT ORF in cDNA KSPX (Fig. 1) were manipulated to make small changes in length by digestion, blunt-ending, and religation. This created a panel of twelve variants, KSPX-1 to -12, which differed slightly in length (Fig. 1). Together with the KSG, KSX, and KSP RNAs, which were intermediates in construction (see Materials and Methods), 16 miniantigenomes ranging in size from 937 to 1,006 nt were tested. These miniantigenomes included ones evenly divisible by various integers such that in aggregate every integer from 1 to 15 was represented. The three nonviral 5′-terminal G residues were included in the length calculations because, as described later, they were found to be retained during transfection and passage.

Efficiencies of replication and CAT expression by miniantigenomes of different lengths. The various miniantigenome RNAs were synthesized individually in vitro and compared by transfection into RSV-infected cells followed by two serial passages. The passages were performed with the idea that modest differences in replication efficiency might be missed in the original transfection but, if significant, should become apparent during serial passage. The amounts of minigenome material synthesized by the 16 variants were very similar during transfection (data not shown), the first passage (data not shown), and the second passage (Fig. 3). Also, the levels of CAT enzyme expression, a marker for transcription, among the various mutants also were very similar following transfection and each passage (data not shown).

Detailed data for the six mutants KSPX-1 to -6 are shown in Table 1, which compares levels of minigenome accumulation, and Table 2, which compares levels of CAT enzyme accumulation. The levels of minigenome material increased by approximately 22-fold from the transfection to the first passage but fell during the second passage to be approximately 6.5-fold higher than they were after the original transfection. The considerable increase in levels of minigenome material (and CAT) during passage is typical and appears to reflect a replicative

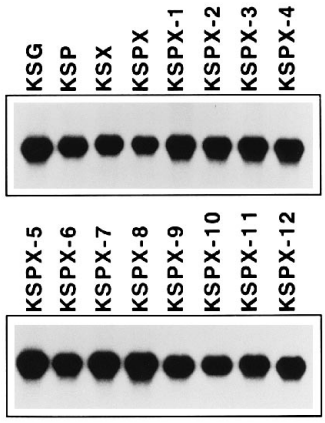


FIG. 3. Northern blot analysis of minigenome RNA synthesized in response to the indicated miniantigenomes. RSV-infected cells were transfected with the indicated miniantigenomes, and after two serial passages, intracellular RNA was isolated and analyzed by hybridization with a positive-sense RSV-CAT probe. Lanes representing cells which were infected with RNA but did not receive a miniantigenome or uninfected cells which received a miniantigenome were blank and are not shown.

TABLE 1. Intracellular accumulation of minigenome material during transfection and two serial passages initiated with miniantigenomes KSPX-1 to -6 measured by Northern blot hybridization[a]

RNA	Amt of minigenome accumulation (mean ± 1 SD)		
	Transfection	First passage	Second passage
KSPX-1	59.2 ± 1.2	1,239.3 ± 250	271.1 ± 30.7
KSPX-2	54.0 ± 11.0	2,000.7 ± 99.4	316.2 ± 35.1
KSPX-3	55.1 ± 4.8	1591.3 ± 834	369.9 ± 15.6
KSPX-4	73.6 ± 12.5	2,391.8 ± 1011.8	779.5 ± 179.8
KSPX-5	78.2 ± 4.8	1,072.2 ± 322.3	492.8 ± 70.8
KSPX-6	88.5 ± 11.5	612.2 ± 151.1	473.7 ± 101.3

[a] Triplicate cultures of RSV-infected cells were transfected with the indicated in vitro-synthesized miniantigenome RNAs, and intracellular RNAs were isolated 30 h later (primary transfection). Medium supernatants were passaged to fresh cells, and intracellular RNAs were isolated 30 h later (first passage). A second passage was performed, and intracellular RNAs were isolated 30 h later (second passage). RNAs were electrophoresed on formaldehyde gels, transferred to nitrocellulose, and hybridized with a positive-sense ^{32}P-labeled RSV-CAT probe. Quantitation was done by phosphorimagery, and data are expressed in arbitrary units. Negative controls were RSV-infected cells which did not receive RNA or uninfected cells which were subjected to RNA transfection; in either case the levels of hybridization were negligible (see Fig. 2).

advantage for the minigenome versus the helper virus (5a). Decreases such as those seen in the second passage also are common and seem to be due to the inconsistency of RSV helper virus infection during serial single-step growth cycles as well as to interference from high levels of minigenome replication (5a). Because these experiments involve many steps between the starting plasmid and the final PhosphorImager images which might introduce sample-to-sample variability, we considered a consistent three- to fourfold difference to be the threshold of significance. By this measure, the differences among the triplicate samples of each mutant, and between the various mutants, were insignificant.

Sequence analysis of recovered minigenomic RNA. In order to draw conclusions concerning the importance of minigenome length, it was necessary to determine whether the lengths of the various input KSPX RNAs remained unaltered during RNA replication. RNAs KSPX-1 to -6 were individually transfected into RSV-infected cells, and the posttransfection medium supernatants were passaged to fresh cells. Intracellular RNA was isolated at 30 h postpassage, converted into cDNA by RT using a primer containing the complement of the 3′ end of the minigenome, amplified by PCR using in addition a second primer containing the 5′ end of the minigenome, cloned, and sequenced. Several nucleotide substitutions were noted, but none of the minigenomes contained changes in nucleotide length.

Because the primers used for RT and PCR contained the ends of the KSPX derivatives, they precluded determination of these sequences. Therefore, terminal sequencing based on RNA circularization was performed for KSG and KSPX-1 to -4. Particles were precipitated from postpassage medium supernatants, and the RNA, which is predominantly minigenomic (5a), was extracted and circularized by end-to-end ligation. This RNA was copied by RT into cDNA by using a positive-sense oligonucleotide primer, which would make the reaction specific to minigenome material. cDNAs were amplified by PCR, cloned, and analyzed by sequencing.

A total of 22 cDNA clones representing the five different analogs (Table 3 and Fig. 4) were analyzed. Remarkably, 20 of the cDNAs contained three nonviral C residues (negative sense) between the RSV-specific 5′ and 3′ minigenome ends, and the other two cDNAs had one or two C's. Thus, the three G residues at the 5′ end of the input miniantigenome had been faithfully copied into minigenomic RNA. All 22 cDNAs contained the correct, complete virus-specific 3′ (leader) minigenome end. The 5′ (trailer) end was contained in full in 21 DNAs, whereas the remaining one lacked nt 934, the last nucleotide at the 5′ end. Nucleolytic degradation in the cell or during manipulation might account for the loss of nucleotides from the two ends. Because the minigenome ends had been ligated together prior to cDNA synthesis, the sequence alone did not establish whether the nonviral C residues had been contained on the leader end or the trailer end. However, the former situation (as represented in Fig. 4) would be expected from direct copying from the miniantigenome. Also, the loss of a virus-specific terminal nucleotide from the trailer in one cDNA and of one or two C residues from the nonviral extension in two other cDNAs would be consistent with these ends being free and the leader terminus being the one attached to the nonviral triplet.

The finding that the nonviral triplet contributed by the T7 promoter was maintained in progeny minigenomes raised the question of whether it might nonetheless reduce the efficiency of transcription and RNA replication. Figure 5 shows a comparison of CAT expression in response to transfected miniantigenome KSG and that in response to transfected miniantigenome KS, the latter being identical to KSG except that it lacks the nonviral triplet. Since the transfected RNA was miniantigenomic, its encapsidation into a functional nucleocapsid would not be sufficient alone to make template capable of being transcribed into CAT mRNA. Rather, RNA replication to yield minigenome nucleocapsid material would be required, and thus the expression of CAT depended on both RNA replication and transcription. The two miniantigenomes produced similar high levels of CAT activity following transfection into RSV-infected cells, and CAT was not detected when miniantigenome material was transfected into uninfected cells.

DISCUSSION

RSV RNA replication and transcription were studied with a system in which short, internally truncated analogs of antigenomic RNA were produced by transcription in vitro from linearized cDNA and transfected into RSV-infected cells. This should represent the most authentic method of complementation available, since it involves a permissive homologous infec-

TABLE 2. Intracellular CAT enzyme accumulation during transfection and two serial passages initiated with miniantigenomes KSPX-1 to -6[a]

RNA	Intracellular CAT activity (mean ± 1 SD)		
	Transfection	First passage	Second passage
KSPX-1	40.9 ± 12.3	74.4 ± 3.6	70.7 ± 12.9
KSPX-2	63.5 ± 20.8	78.1 ± 2.5	42.6 ± 2.9
KSPX-3	59.4 ± 7.5	73.9 ± 5.3	53.8 ± 4.3
KSPX-4	43.0 ± 18.2	70.2 ± 3.7	66.7 ± 10.8
KSPX-5	33.7 ± 16.5	71.7 ± 1.6	46.8 ± 3.1
KSPX-6	43.8 ± 20.8	69.2 ± 3.1	55.1 ± 7.4

[a] As part of the experiment described in Table 1, aliquots of cells harvested posttransfection or postpassage were analyzed for intracellular CAT activity. Cell lysate samples, each representing of 3.33×10^{-3} (transfection) or 8.33×10^{-5} (passages) of a single well of a six-well dish, were incubated with [^{14}C]chloramphenicol and then subjected to thin-layer chromatography and quantitation by phosphorimagery. Data for KSPX-1, -2, and -3 represent triplicate transfections performed in parallel. Values are percents acetylation. CAT activity of negative controls described in Table 1 was <0.04%.

TABLE 3. Sequences at the 3′ and 5′ ends of minigenomes isolated from extracellular particles following passage

Minigenome RNA	No. of cDNAs sequenced	3′ leader end (no. of clones)	5′ trailer end (no. of clones)
KSG	4	3′-CCC. . .(3), 3′-_CC. . .(1)[a]	. . .GCA-5′(3), . . .GC_-5′(1)[a]
KSPX-1	3	3′-CCC. . .(3)	. . .GCA-5′(3)
KSPX-2	3	3′-CCC. . .(3)	. . .GCA-5′(3)
KSPX-3	4	3′-CCC. . .(3), 3′-__C. . .(1)	. . .GCA-5′(4)
KSPX-4	8	3′-CCC. . .(8)	. . .GCA-5′(8)

[a] These two deletions were from different cDNAs.

tion in which the only extraneous factors were the in vitro-synthesized RNA and the transfection reagent.

Following transfection into RSV-infected cells, positive-sense antigenome material was indistinguishable from the previously studied negative-sense minigenome material in its ability to participate in transcription, RNA replication, and the production of transmissible virus. Minigenome material and miniantigenome material were equally active on the basis of the amount of transfected RNA. Since antigenome material is at least 10-fold less abundant than genome material in RSV-infected cells, it might have been expected that its analog would initiate a correspondingly higher level of RNA replication relative to an equal amount of minigenome material, but this was not observed.

The small variations in length among the different antigenome analogs were without significant consequence for transcription, RNA replication, or packaging into transmissible particles. This is in contrast to the case with SeV and MeV, for which RNA replication was efficient only if the genome length was an even multiple of six (the rule of six) (3, 4, 17, 28, 30). This rule of six seems to be a fundamental aspect of nucleocapsid structure and function for those viruses, but RSV appears to be independent of such a requirement. Consistent with this idea, we recently showed that infectious virus could be produced from a cDNA-encoded antigenome RNA whose nucleotide length was not a multiple of six (6). However, this last observation is made with the caveat that the nucleotide length of the recovered recombinant virus was not confirmed.

The detailed evaluation described here for RSV shows that the rule of six is not an obligatory characteristic of the nonsegmented negative-strand RNA replicative strategy. Among the four genera of the family *Paramyxoviridae*, the rule of six has been demonstrated for *Paramyxovirus* (SeV and human parainfluenza virus type 3) and *Morbillivirus* (MeV), does not hold for *Pneumovirus* (RSV), and remains to be tested for *Rubulavirus* (mumps, Newcastle disease, and simian type 5 viruses), whose members currently lack minigenome or recombinant virus experimental systems. For rabies virus and VSV, which represent two genera of the family *Rhabdoviridae*, minigenome replication and the recovery of recombinant virus did not appear to have a requirement comparable to the rule of six, although detailed evaluations have not been described (12, 23, 27, 29, 33, 34). (A rule of nine rather than six might have been predicted for VSV, since each N monomer associates with 9 rather than 6 nt [31]). The viruses which adhere to the rule have three differences from the ones which do not (with the assumption that rabies virus and VSV represent the latter): (i) they possess larger N (or NP) and P proteins (examples of the lengths in amino acids of N protein and P protein are as follows: 524 and 568 [SeV], 525 and 507 [MeV], 422 and 265 [VSV], 450 and 297 [rabies virus], and 391 and 241 [RSV], respectively); (ii) they possess the C and V proteins (although VSV and rabies virus do have a protein which might be a counterpart to C); and (iii) they engage in RNA editing, which for these two genera involves editing the P ORF to shift the register into the V ORF (8, 9, 22, 32). *Rubulavirus*, whose

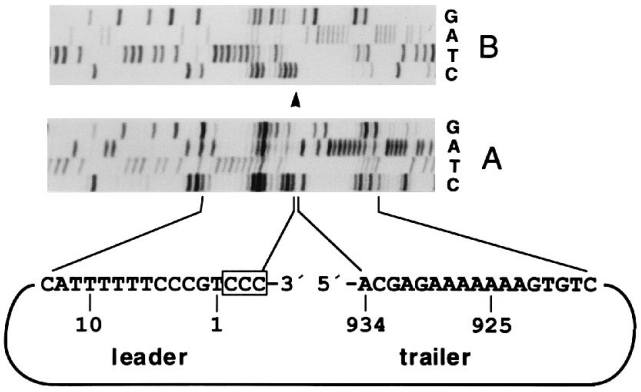


FIG. 4. Sequence determination by RNA circularization and RT-PCR of the ends of two minigenome isolates derived from the KSG miniantigenome, illustrated by typical sequencing gels. KSG RNA was transfected into RSV-infected cells, posttransfection medium supernatants were passaged to fresh cells, and extracellular particles were precipitated from the postpassage medium supernatants. RNA was isolated, ligated end-to-end, copied into cDNA by using a primer specific for minigenome material, amplified by PCR, cloned, and sequenced. The sequences and sequence ladders are in minigenome-sense and are numbered from 1 (leader end) to 934 (trailer end); the three terminal C residues of nonviral origin are boxed but not numbered. Sequence ladder A represents a minigenome in which both termini were intact. Ladder B represents a second minigenome which lacks nt 934, with the new 5′ trailer end (C at nt 933) indicated with an arrow. The diagram at the bottom illustrates the 3′ and 5′ ends of the minigenome prior to ligation, with the positions of nt 1, 13, 918, and 934 indicated relative to the gel.

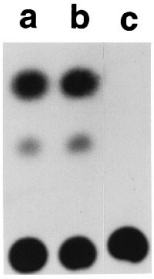


FIG. 5. Effect on CAT expression of three nonviral G residues on the 5′ end of the miniantigenome. RSV-infected cells were transfected with KS RNA (lane a), a miniantigenome which is identical to KSG except that it lacks the 5′-terminal nonviral G triplet extension, or KSG RNA (lane b), which contains the extension. As a control, uninfected cells (lane c) were transfected with KSG RNA. At 30 h posttransfection cells were harvested and an aliquot representing 10^{-4} (lanes a and b) or 10^{-2} (lane c) of a single well of a six-well dish was assayed for acetylation of [^{14}C]chloramphenicol. Nonacetylated chloramphenicol remained at the origin (as in lane c), whereas acetylated forms moved in the vertical dimension.

members have small N and P proteins, lack the C protein, and edit the V ORF to yield P, will be an interesting subject for comparison once methods for genetic manipulation have been developed. Another interesting subject for comparison will be human parainfluenza virus type one, which is a close relative of SeV but appears to lack editing capability (reference 28b and references therein).

Each in vitro-synthesized miniantigenome contained a 5′-terminal (leader end) extension of three G residues contributed by the T7 promoter. Surprisingly, minigenomes recovered from postpassage extracellular particles contained the complement of this extension, three 3′-terminal C residues. This indicates that the nonviral extension was faithfully copied during RNA replication. Two minigenome isolates lacked 1 or 2 nt of the nonviral extension, and a third isolate lacked the last nucleotide from the trailer end. These deletions identified these two sequences as constituting the free ends, which confirmed the expectation that the nonviral extension was attached to the leader end as shown in Fig. 4.

The intracellular levels of minigenome material increased by approximately 22-fold during the first passage, indicating that RNA replication was robust. In other experiments performed under similar conditions, transcription resulting from comparable levels of input minigenomes or miniantigenomes exceeded that of the helper virus by the second or third passage, illustrating that the extent of minigenome amplification was massive (5a). This implies that the process was authentic and shows that both the miniantigenomes and the minigenomes were very active as templates. The efficiencies of amplification and expression were identical for a miniantigenome that contained the nonviral extension and one that did not. This indicates that the nonviral extension was well tolerated during multiple rounds of replication and also did not interfere with transcription.

As is the case with nonsegmented negative-strand RNA viruses in general, the RSV genome has circumscribed terminal complementarity. Ten of the 11 terminal nucleotides and 21 of the terminal 24 to 26 nt are complementary, with complementarity being insignificant thereafter. It is generally thought that these conserved ends contain *cis*-acting sequence elements, namely, that the 3′ ends of the genome and the antigenome each contain a promoter and the 5′ end of each contains an encapsidation signal (22). It also has been suggested that terminal complementarity is important in its own right in RNA replication (33). Given the presumption that the structures of these highly conserved 5′ and 3′ ends are critical, it was somewhat surprising that a nonviral extension would be tolerated and maintained. For example, in the VSV model system an extension of two G residues at the 3′ (leader) end of a negative-sense minigenome rendered this minigenome inactive, while an extension of two G residues on the 5′ (trailer) end was not copied during RNA replication (27). Nonviral G residues added to the 5′ ends of positive-sense RNA viral genomes also were not maintained (1).

When present at the 5′ end of the RSV miniantigenome, the nonviral trinucleotide extension had the potential to interfere with the encapsidation signal thought to be located at that end. The lack of an inhibitory effect implies that this postulated signal does not require a particular nucleotide at the 5′ end or a precise spacing relative to the end. The presence of the complementary nonviral extension on the 3′ end of the minigenome had the potential to interfere with the genomic promoter. The lack of an inhibitory effect implies that (i) the promoter can tolerate being displaced by three positions from the free 3′ end, (ii) the first 3 nt of that end can be 3′-CCC instead of 3′-UGC, and (iii) RNA synthesis can be initiated efficiently on a C residue in place of the native U residue (a purine might have been less well tolerated, given the strong preference of polymerases to initiate new strands at a pyrimidine [discussed in reference 16]). This situation would be inconsistent with a model wherein the polymerase initiates synthesis at the first nucleotide of a terminally situated promoter sequence. Rather, it suggests a model in which the polymerase contacts the genome at the viral promoter (which remains to be defined but is contained within the 3′-terminal 23 nt [28c]) and reaches back to begin synthesis at the free 3′ end.

For a number of RNA viruses, RNA replication is more complicated than direct correspondence between genomes and antigenomes that are exactly colinear. Some positive-strand RNA viruses, such as tobacco mosaic virus and bacteriophage Qβ, synthesize virion and replicative intermediate RNAs which contain a single 3′ A residue which is nontemplated and is not copied (references 1 and 2 and references therein). Genomic and antigenomic RNAs of the Tacaribe arenavirus, a segmented negative-strand RNA virus, have overhanging 5′ G residues which were proposed to arise from an unusual "prime and realign" mechanism (15). Nontemplated single nucleotides also were found at the ends of antigenomic and genomic RNAs of the lymphocytic choriomeningitis arenavirus and were suggested to have been added to 3′ ends during RNA replication (25). The initiation of RNA replication by the Hantaan bunyavirus, another segmented negative-strand RNA virus, has been hypothesized to involve a prime and realign mechanism which might also involve nucleolytic trimming (16). Other RNA viruses have unexpectedly been found to have abundant naturally occurring terminal deletions (25, 35) or to repair artificially deleted terminal nucleotides (20), and these characteristics also are suggestive of unexpected complexity of RNA replication. It is generally assumed that RNA replication by nonsegmented negative-strand viruses such as RSV occurs by a less complicated mechanism, involving initiation at the 3′-terminal nucleotide and copying without priming, slippage, nuclease action, or nontemplated addition. The finding here that a nonviral extension was tolerated and faithfully maintained would be consistent with this description while revealing an unexpected degree of tolerance regarding end structure and spacing. It will be important to further investigate the terminal structures of RSV RNAs during RNA replication.

We wonder whether the ability of RSV to tolerate a short template extension and its apparent independence from the rule of six are interrelated. One interpretation of the rule is that nucleotides additional to 6*n*, where *n* represents any integer, interfere with RNA replication by causing unencapsidated nucleotides to protrude beyond the 3′ end of the nucleocapsid, perhaps altering a ribonucleoprotein end structure that is critical for subsequent polymerase entry. Perhaps RSV does not have such a strict requirement for the structure at the 3′ end of the nucleocapsid and thus can tolerate extensions, deletions, and nonviral terminal nucleotides.

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