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The Structurally Diverse Intergenic Regions of Respiratory Syncytial Virus Do Not Modulate Sequential Transcription by a Dicistronic Minigenome

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The first nine genes of respiratory syncytial virus (RSV), a nonsegmented negative-strand RNA virus, are separated by intergenic regions which range in size from 1 to 52 nucleotides for strain A2 and lack obvious consensus elements except that each ends in an A (genome sense). Their significance for gene expression was investigated by using RSV-CAT-LUC RNA, a helper-dependent cDNA-encoded dicistronic analog of RSV genomic RNA in which the viral genes were replaced by a negative-sense copy of the translational open reading frame (ORF) encoding chloramphenicol acetyltransferase (CAT) as the upstream, leader-proximal gene and that encoding luciferase (LUC) as the downstream gene. These foreign ORFs were flanked by the RSV gene-start (GS) and gene-end (GE) transcription signals and separated by the naturally occurring G/F intergenic region. The RSV-CAT-LUC minigenome was synthesized in vitro and transfected into RSV-infected cells, and synthesis of the CAT and LUC mRNAs was monitored by enzyme assay and Northern (RNA) blot hybridization. Surprisingly, substitution of each of the other naturally occurring RSV intergenic regions in turn did not significantly alter the absolute or relative amounts of the two mRNAs. Substitution of a nonnatural 10-nucleotide intergenic region, or elimination of the intergenic region altogether, also had little effect on the level of expression of the two genes. Four of the minigenome variants containing naturally occurring intergenic regions were modified further by replacing part of the LUC ORF with a second copy of the CAT ORF, so that each of the two mRNAs would hybridize equally with a CAT-specific probe and their relative molar amounts could be determined. The level of expression of the downstream gene was 0.30 to 0.36 that of the upstream one. This determined the magnitude of RSV transcriptional polarity across a gene pair and confirmed that this value was very similar among the various intergenic regions. Minigenome transcription also yielded a CAT-LUC readthrough mRNA at a level 0.10 to 0.13 that of the LUC mRNA. In summary, the structurally diverse RSV intergenic regions do not appear to play a role in modulating RSV gene expression.

Human respiratory syncytial virus (RSV), a member of the genus *Pneumovirus* of the family *Paramyxoviridae*, is the leading viral agent of pediatric respiratory tract disease worldwide (7). Its genome is a single negative-sense strand of RNA of 15,222 nucleotides that encodes 10 major subgenomic mRNAs. Characterization of RSV replication and transcription mechanisms has been impeded by the lability of the virus and its poor growth in vitro.

Vesicular stomatitis virus (VSV) and Sendai virus (SeV) are the most extensively characterized nonsegmented negative-strand RNA viruses and serve as prototypes (6, 14, 17, 22, 27, 28). The genomes of these prototypic viruses are tightly encapsidated by the major nucleocapsid N (or NP) protein and are associated with the phosphoprotein P and the polymerase subunit L to form the functional nucleocapsid, which contains the RNA polymerase. The polymerase enters the genome in the 3′ extragenic leader region and transcribes the linear array of genes by a sequential, stop-start mechanism during which the polymerase remains template bound and is guided by short consensus gene-start (GS) and gene-end (GE) signals. This generates a free leader RNA and a series of nonoverlapping subgenomic mRNAs. The abundance of the various mRNAs decreases with increasing gene distance from the promoter, displaying a polarity or attenuation of gene transcription. The genes are separated by short intergenic regions which are not copied into the individual mRNAs and are described further below. RNA replication occurs when the polymerase somehow switches to a readthrough mode in which the transcription signals are ignored. This produces a complete encapsidated positive-sense replicative intermediate, the antigenome, which serves as the template for progeny genomes.

The many other nonsegmented negative-strand RNA viruses probably follow this general scheme, although the existence of differences in genome organization such as in the number and type of encoded proteins suggest that variation may occur. For RSV, numerous features are consistent with the prototypic viruses. The first nine genes, at least, appear to be transcribed by a sequential, polar process (5, 11, 12, 21). Polymerase entry on a dicistronic minigenome was confirmed to occur only at the 3′ end (21). Consensus GS and GE motifs have been identified at the beginning and end of each gene and confirmed to be self-contained transcription signals (2, 21). The N, P, and L proteins are necessary and sufficient for RSV minigenome RNA replication (15, 30). On the other hand, RSV differs from the prototypic viruses in requiring a fourth protein, M2, for processive, sequential transcription (3, 4). Unlike the prototypes, its genome encodes two overlapping mRNAs (10). Also, whereas the intergenic regions of VSV and SeV are consensus di- or trinucleotides, respectively, which are highly conserved

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within and between viruses, the first nine genes of RSV are separated by intergenic regions which vary in length (1 to 52 nucleotides for strain A2), lack obvious consensus elements, and are poorly conserved among divergent RSV strains (2, 16, 19, 22, 25).

The subset of nonsegmented negative-strand viruses which contain consensus-type intergenic regions includes the SeV group (genus *Paramyxovirus*) and measles virus group (genus *Morbillivirus*) of the family *Paramyxoviridae* and the VSV group (genus *Vesiculovirus*) of the family *Rhabdoviridae* (6, 22, 28). Viruses with nonconsensus-type intergenic regions include the RSV group (genus *Pneumovirus*) and mumps virus group (genus *Rubulavirus*) of the family *Paramyxoviridae*; the rabies virus group (genus *Lyssavirus*), bovine ephemeral fever virus group (genus *Ephemerovirus*), and certain plant and insect viruses of the family *Rhabdoviridae*; and Ebola and Marburg viruses of the family *Filoviridae* (2, 6, 7, 22, 24, 26, 28, 29). It seems likely that consensus-type intergenic regions are part of the *cis*-acting sequence elements that guide transcription, as suggested by their conserved nature and close proximity to the GS and GE signals. Also, naturally occurring nucleotide differences in consensus-type intergenic regions are associated with increased accumulation of readthrough transcripts, suggesting an effect on the activity of transcription signals (16, 23). That a large number of nonsegmented negative-strand viruses have variable, nonconsensus intergenic regions suggests that a dimorphism exists in some aspects of gene expression. The nonconsensus intergenic regions might play a variety of roles in gene regulation. For example, intergenic length might control transcriptional attenuation. Nonconsensus intergenic regions might contain *cis*-acting signals which have not been identifiable by sequence analysis alone or which are found in certain intergenic regions but not others.

Here we evaluated the role of intergenic regions in RSV transcription by using a helper virus-dependent minigenome system. In this approach, the negative-sense dicistronic minigenomic RNA is synthesized in vitro from cDNA and transfected into RSV-infected cells, where it participates in transcription, RNA replication, and the production of transmissible particles (8, 21, 25). The results show that the structural diversity of the intergenic regions is not important in modulating sequential transcription.

MATERIALS AND METHODS

cDNAs. cDNA encoding the RSV-CAT-LUC minigenome was constructed previously (21). The various naturally occurring or artificial intergenic regions and flanking GE and GS sequences were constructed by PCR with mutagenic oligonucleotides. Restriction fragment replacements were done by using an *Nco*I site near the end of the chloramphenicol acetyltransferase (CAT) gene and a *Bam*HI site which was immediately adjacent to the luciferase (LUC) GS signal (see Fig. 1). The sequences of DNAs constructed by PCR were confirmed by dideoxynucleotide sequencing. Four RSV-CAT-LUC minigenomes (see Fig. 3) were modified by excising a 511-nucleotide fragment from the LUC ORF with naturally occurring *Spl*I and *Sph*I restriction sites and replacing it with a 631-nucleotide fragment of the CAT gene which was synthesized by PCR with CAT-specific oligonucleotides with flanking *Spl*I and *Sph*I sites. Sticky ends were generated by restriction digestion, and the fragment was inserted by conventional cloning techniques.

Minigenome synthesis, transfection, and passage. Briefly, cultures of human embryonic kidney 293 cells in six-well dishes were infected with RSV at an input multiplicity of approximately 10 PFU per cell, transfected with minigenome RNA 45 min later, and harvested at 30 h when the cytopathic effect was extensive, as described in detail previously (8, 21, 25). For passage, medium supernatants were clarified by centrifugation for 30 s in a microcentrifuge, adsorbed to fresh cells for 2 h, washed, and incubated for an additional 28 h. The cells were harvested as described above.

Enzyme assays. CAT activity in cell lysates was assayed (8, 21, 25) by acetylation of [^{14}C]chloramphenicol as measured by thin-layer chromatography. Quantitation was performed by liquid scintillation of excised spots or by phosphorimager analysis, and the CAT activity was expressed as percent acetylation. A typical assay contained 1/100 or 1/1,000 of the cell lysate from a single well of a six-well dish. Alternatively, the CAT protein was measured by a commercial enzyme-linked immunoadsorption assay (CAT-ELISA; Boehringer Mannheim) and expressed as picograms of protein per 1/200 of one well of a six-well dish. LUC activity was measured with a commercial buffer system (luciferase assay system; Promega) and a Turner 20-TD luminometer; a typical assay involved lysate representing 1/250 of a single well of a six-well dish.

Northern (RNA) blot hybridization. Total cellular RNA was purified with Trizol reagent (Life Technologies) (15). Oligo(dT) chromatography was performed with oligo(dT)-cellulose by a minibatch method involving differential centrifugation in a microcentrifuge tube (15). RNA was denatured with glyoxal, electrophoresed through a 1.5% agarose gel, and blotted to nylon membrane (see Fig. 2) (21). Alternatively, electrophoresis was performed through a 1.5% agarose gel in the presence of formaldehyde with blotting onto nitrocellulose (see Fig. 4) (15). Hybridization was performed with ^{32}P-labeled riboprobe synthesized in vitro by runoff transcription. CAT-specific negative- and positive-sense probes were synthesized from *Xba*I-digested RSV-CAT C2 or *Nco*I-digested C4 cDNA (15), respectively; negative-sense LUC probe was synthesized from pGem-LUC plasmid (Promega); and negative- and positive-sense riboprobes specific to the RSV phosphoprotein (P) gene were synthesized from a P cDNA contained in pGem3 or pTM1, respectively (15, 21).

RESULTS

Comparison of various intergenic regions inserted into the RSV-CAT-LUC dicistronic minigenome. cDNA was constructed to encode RSV-CAT-LUC RNA, a dicistronic, negative-sense analog of RSV genomic RNA in which the RSV genes were removed and replaced with negative-sense copies of the open reading frames (ORFs) encoding the CAT and LUC marker enzymes (Fig. 1). The CAT and LUC ORFs were each placed under the control of separate sets of RSV GS and GE signals, with CAT as the upstream, leader-proximal gene and LUC as the downstream one. The cDNA was engineered so that the intergenic region between the two genes could be easily modified. A series of mutants representing all eight naturally occurring intergenic regions of strain A2 was made. These vary in length from 1 to 52 nucleotides and lack obvious consensus elements except that they are rich in A and U, as is the RSV genome in general, and each ends with an A residue in genome sense (Fig. 1). The SH/G intergenic region sustained a single nucleotide deletion during cDNA construction (Fig. 1) and thus was 1 nucleotide shorter than the authentic one and was designated SH/G(−1). The GS signal used in the mutants was that which was conserved exactly among the nine smaller genes of RSV strain A2 (Fig. 1) (2, 7). The GE signal has some variability between genes of the authentic genome, but we chose to keep this feature constant in the mutants and used a single signal, that of the N and M genes. This particular GE signal has a single nucleotide difference from the naturally occurring ones of the NS1, SH, and L genes and two nucleotide differences from the other GE signals (2, 7). All of these nucleotide differences (except for one in NS2) occur in a divergent domain between the 3′-UCAAU and oligo(U) motifs. When evaluated in the RSV-CAT-LUC minigenome, the various GE signals of strain A2 were similar in activity, except that those of the NS1 and NS2 genes were 60% as active (20a). This suggested that the few nucleotide differences were inconsequential except those for the NS genes.

In previous studies, transfection of in vitro-synthesized monocistronic RSV-CAT or dicistronic RSV-CAT-LUC RNA into RSV-infected cells was shown to result in abundant RNA replication, yielding mini-antigenome and progeny minigenome, and abundant transcription, yielding subgenomic CAT and LUC mRNAs and their encoded enzymes (references 21 and 25, and unpublished data). Also, the minigenome is incorporated into particles released into the medium (unpublished data), and passage of posttransfection medium supernatants to fresh cells results in high levels of RNA replication and transcription (8, 21, 25). Figure 2 shows the positive-sense RNAs synthesized in response to the panel of RSV-CAT-LUC mini-

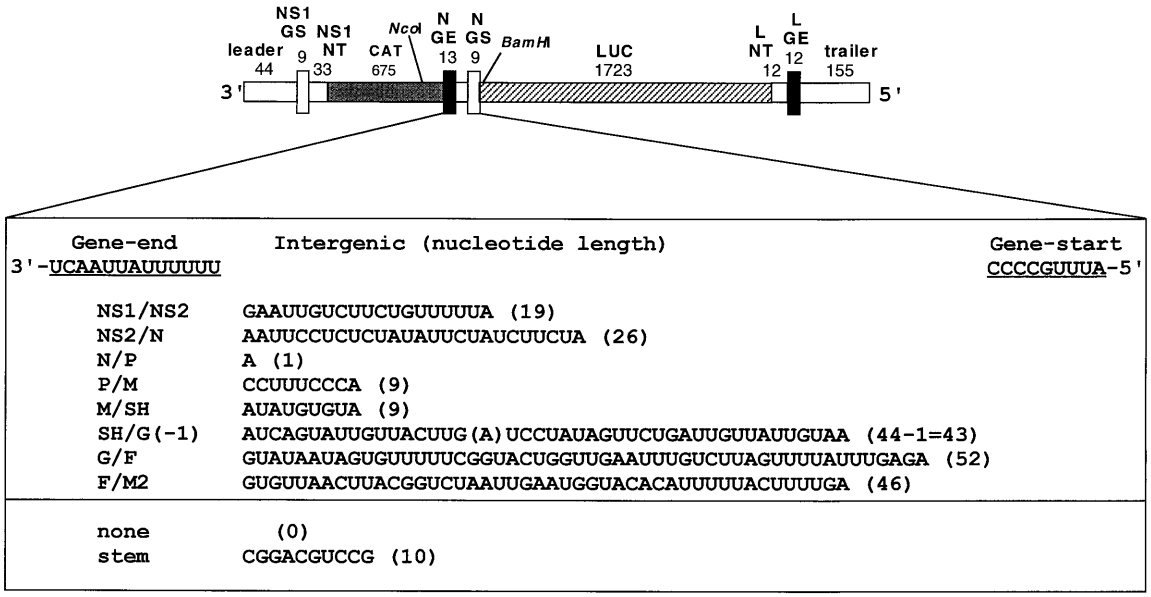

FIG. 1. Diagram (3′ to 5′, not to scale) of RSV-CAT-LUC RNA, a negative-sense, dicistronic minigenome synthesized from cDNA. RSV-specific sequences are shown as open boxes except for the GE signals, which are solid. CAT and LUC sequences are shaded and hatched, respectively. The 3′ (left-hand) end of the minigenome contained the 3′-terminal 86 nucleotides of the authentic RSV genome, consisting of the 44-nucleotide extragenic leader region, the 9-nucleotide GS signal, and first 33 nontranslated (NT) nucleotides of the nonstructural protein NS1 gene. This was followed, from 3′ to 5′, by a negative-sense copy of the CAT ORF, the N GE signal, the intergenic region, the N GS signal, and a negative-sense copy of the LUC ORF. The 5′ (right-hand) end of the minigenome contained the 5′-terminal 179 nucleotides of the authentic genome, consisting of the last 12 NT nucleotides and adjoining GE signal of the L gene and the extragenic 155-nucleotide trailer region. The box below the diagram contains the sequences (negative-sense, with each nucleotide length in parentheses) of the various naturally occurring and artificial intergenic regions which were inserted between the CAT and LUC genes. During construction, the reconstituted SH/G intergenic sustained a deletion of a single A residue (parentheses), and the region was designated SH/G(−1). The *Nco*I and *Bam*HI sites used for restriction fragment replacement (see Materials and Methods) are shown.

genomes following transfection (Fig. 2A, top portion) and passage (Fig. 2B, top portion), as visualized by Northern blot hybridization of total intracellular RNA. These RNAs include subgenomic CAT mRNA, subgenomic LUC mRNA, and a minigenome-sized band containing mini-antigenome and a small amount of CAT-LUC readthrough mRNA. Side-by-side comparison of the panel of minigenomes showed that they were very similar with regard to the pattern of encoded intra-

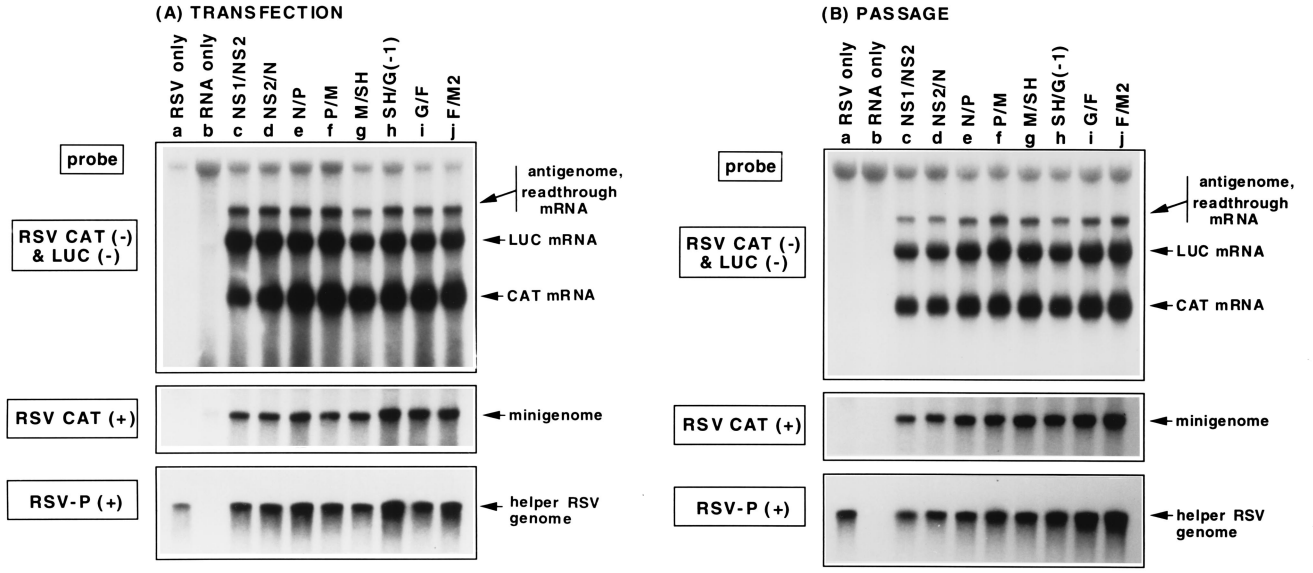

FIG. 2. Northern blot analysis of RNAs synthesized in RSV-infected cells in response to transfection with RSV-CAT-LUC minigenomes that each contain one of the naturally occurring intergenic regions of RSV strain A2 (as indicated, lanes c to j). Control cells received RSV alone (lane a) or RSV-CAT-LUC-N/P minigenome alone (lane b). Intracellular RNAs were isolated 30 h posttransfection (A) or 30 h following the passage of posttransfection medium supernatants to fresh cells (B). Replicate Northern blots were analyzed by hybridization with an equal mixture of negative-sense RSV-CAT and LUC riboprobes to detect subgenomic mRNAs and antigenome (top portions), positive-sense RSV-CAT riboprobe to detect minigenome (middle portions), or positive-sense P riboprobe to detect helper virus genome (bottom portions).

TABLE 1. Ratio of LUC enzyme versus CAT enzyme expressed by RSV-CAT-LUC minigenomes representing the eight naturally occurring intergenic regions of RSV strain A2[a]

Intergenic region (nucleotide length)	LUC/CAT ratio (mean ± 1 SD)[b] in:				Normalized ratio[c] in:				Mean ± 1 SD[d]
	Expt 1 (n = 6)	Expt 2 (n = 3)	Expt 3 (n = 2)	Expt 4 (n = 2)	Expt 1	Expt 2	Expt 3	Expt 4	
NS1/NS2 (19)	22.72 ± 0.96	23.48 ± 1.89	22.35	22.68	1.12	0.77	0.97	1.20	1.01 ± 0.19
NS2/N (26)	21.72 ± 2.17	28.39 ± 2.39	22.83	21.30	1.07	0.93	0.99	1.13	1.03 ± 0.09
N/P (1)	20.36 ± 2.83	30.48 ± 4.38	23.04	18.87	1.00	1.00	1.00	1.00	1.00 ± 0.00
P/M (9)	24.89 ± 2.57	26.77 ± 5.88	26.28	24.74	1.22	0.88	1.14	1.31	1.14 ± 0.19
M/SH (9)	20.10 ± 1.26	23.68 ± 2.98	20.47	17.79	0.99	0.78	0.89	0.94	0.90 ± 0.09
SH/G(−1) (43)	21.06 ± 1.26	29.53 ± 2.00	18.55	19.68	1.03	0.97	0.81	1.04	0.96 ± 0.11
G/F (52)	19.86 ± 3.33	32.40 ± 3.66	19.20	21.48	0.98	1.06	0.83	1.14	1.00 ± 0.13
F/M2 (46)	17.23 ± 1.41	25.57 ± 2.68	19.99	19.57	0.85	0.84	0.87	1.04	0.90 ± 0.09

[a] Four independent experiments are represented, with the number of replicate samples in each indicated as n.
[b] CAT activity was measured as the percent acetylation of [^{14}C]chloramphenicol determined by thin-layer chromatography, and LUC activity was measured as luminometer light units. Taking these values as arbitrary units, the latter was divided by the former to yield a LUC/CAT ratio (see the text for rationale). Standard deviations (SD) are given when n = 3 or 6. The expression of CAT or LUC in control cells, which consisted of uninfected cells transfected with minigenome and nontransfected infected cells, was negligible.
[c] The LUC/CAT ratios were normalized within each experiment relative to that of the N/P minigenome as 1.00.
[d] Mean of the four experiments. Mean for the eight regions, 0.99.

cellular positive-sense RNAs. Because the blots were hybridized with a mixture of two different probes, one specific for CAT and the other specific for LUC, the relative molar amounts of CAT and LUC mRNAs could not be determined from this experiment. A second set of duplicate blots was analyzed to detect the accumulation of negative-sense minigenome (Fig. 2, middle portions), and a third set was analyzed to monitor the accumulation of helper virus genome (Fig. 2, bottom portions).

The Northern blots exhibited sample-to-sample differences in RNA quantity which appeared to be due to experimental variability, which might occur at any one or more of the many steps involved including RNA isolation, gel loading, or blot transfer (1). For example, within a single experiment, the amount of minigenome or helper virus genome varied somewhat among the different samples (Fig. 2, middle and lower portions, respectively). However, comparison of several independent experiments (results not shown) showed that the differences were not consistent and that, on average, the level of minigenome or helper virus genome was uniform. Thus, the transfection and passage of the various minigenomes did not appear to affect helper virus replication and the various intergenic substitutions did not appear to affect minigenome RNA replication. The various intergenic substitutions also did not alter the expression level of the upstream CAT gene (Fig. 2, upper portions). Somewhat surprisingly, these substitutions also had little or no effect on the expression level of the downstream LUC gene.

Because the lack of effect on transcription of the downstream gene was somewhat unexpected, it was of interest to examine this further in case small but consistent differences might exist. Therefore, gene expression by the various RSV-CAT-LUC minigenomes was examined by measuring CAT and LUC activities in four independent experiments that each involved two to six replicate samples (Table 1). Assay of enzyme activities simplified the measurement of multiple samples, involved many fewer steps in manipulation as possible sources of intersample variability, and would be a reliable measure of transcription in situations such as this, in which RNA replication is unaffected by the mutation in question.

The levels of CAT and LUC activities were approximately the same from one minigenome to the next, consistent with the results of the Northern blot analyses (Table 1). Taking these values of enzyme activity as arbitrary units, the LUC activity of each sample was divided by the associated CAT activity to obtain a ratio of expression of the downstream to expression of the upstream gene (Table 1). The assumption was made that expression of the upstream CAT gene should be invariant and would serve as an internal standard. Relating each LUC activity to its internal CAT standard should normalize the values and help detect small effects on the expression of the downstream gene. A lower value for this LUC/CAT ratio would reflect greater attenuation, specifically the decreased expression of the downstream gene relative to the upstream one. As shown in Table 1, these values were very similar from experiment to experiment and among the different minigenomes. To facilitate comparison, these values were then normalized within each experiment relative to that for the N/P minigenome as 1.00 (Table 1). Within each experiment, the largest value was only 1.27- to 1.41-fold greater than the lowest one, illustrating the similarity among the different mutants. The P/M mutant exhibited the greatest LUC/CAT ratio in three of the four experiments, suggesting that the level of attenuation across this junction might be somewhat lower. The M/SH, SH/G(−1), and F/M2 mutants tended to have lower ratios. Since these include two of the longest intergenic regions, it is tempting to suggest that there might be a slight increase in the level of attenuation associated with increased length. However, in general, these differences were inconsistent and small, and the overall pattern among the different intergenic regions was one of close similarity. Specifically, when the results of the four experiments were averaged, the highest normalized mean LUC/CAT ratio (Table 1, right-hand column) was only 1.27-fold greater than the lowest one and all eight values were within 1 standard deviation of this mean.

Another minigenome was constructed in which the intergenic region was a nonnatural 10-nucleotide sequence which differed from the naturally occurring ones in being rich in G and C residues and in being a palindrome, with the potential of forming a stem (Fig. 1). In addition, a minigenome in which the intergenic nucleotides were deleted altogether was constructed. These were compared with RSV-CAT-G/F with regard to the expression of CAT and LUC enzymes following transfection and following passage of medium supernatants to fresh cells (Table 2). Enzyme expression was greater in the passage than in the transfection, which has been noted before and is due to increased replication of the minigenome during serial passage (21, 25). Expression of the upstream CAT gene

TABLE 2. Expression of CAT and LUC enzymes during transfection and passage of the RSV-CAT-LUC minigenome containing a nonnatural intergenic sequence (stem IG)[a] or in which the intergenic sequence had been deleted (no IG)[b]

Construct	CAT activity[c]			LUC activity[c]			LUC/CAT ratio[d]				Normalized ratio[e]	
	1	2	Pass.[f]	1	2	Pass.[f]	1	2	Mean (1+2)	Pass.	Mean (1+2)	Pass.
(G/F) IG	7.47	9.96	25.64	164.7	194.1	498.3	22.05	19.49	20.77	19.43	1.00	1.00
No IG	7.54	5.62	18.14	102.5	70.24	225.0	13.59	12.50	13.05	12.4	0.63	0.64
Stem IG	8.04	7.08	21.94	128.3	109.6	308.0	15.96	15.49	15.73	14.0	0.76	0.72
No IG (expt 2)	9.79	9.31	32.73	167.1	146.8	467.8	17.07	15.77	16.42	14.3	0.79	0.74
Stem IG (expt 2)	14.24	15.75	16.85	236.8	246.9	198.4	16.63	15.68	16.15	11.8	0.78	0.61
RSV control[g]	<0.1	<0.1	<0.1	<0.01	<0.01	<0.01	ND[i]	ND	ND	ND	ND	ND
RNA control[h]	<0.1	<0.1	<0.1	<0.01	<0.01	<0.01	ND	ND	ND	ND	ND	ND

[a] See Fig. 1.
[b] Transfections were performed in duplicate (numbered 1 and 2), and the medium supernatants were combined and passaged to fresh cells (Pass.).
[c] CAT activity is expressed as percent conversion of [^{14}C]chloramphenicol measured by thin-layer chromatography; LUC activity is expressed as luminometer light units.
[d] The LUC and CAT activities were taken as arbitrary units and expressed as a LUC/CAT ratio for the purposes of comparison.
[e] The transfection means and the passages were normalized separately relative to G/F as 1.00.
[f] Increased expression during passage is typical and reflects a slight replicative advantage of the minigenome.
[g] Negative control consisting of RSV-infected cells which did not receive minigenome.
[h] Negative control consisting of uninfected cells transfected with the G/F minigenome.
[i] ND, not determined.

was not affected in either of these two modified minigenomes, whereas expression of the downstream LUC gene was slightly reduced.

Measurement of transcriptional polarity across the RSV-CAT-LUC gene pair separated by different intergenic regions. In the experiments described above, the level of CAT mRNA with respect to LUC mRNA was measured by two different enzyme assays or with two different riboprobes and was expressed as a ratio of arbitrary units. While this could be used to compare both the overall level of expression and the degree of polarity in one minigenome with respect to another, it was not a reliable measurement of the relative molar amounts of the two mRNAs. Therefore, four of the RSV-CAT-LUC minigenomes described above were modified by removing a 511-bp fragment of LUC and replacing it with a second copy of the CAT ORF, specifically a 631-bp fragment containing 94% of the CAT sequence (Fig. 3). In this way, both subgenomic RNAs could be detected with the CAT riboprobe and the relative levels of expression of the downstream and upstream genes could be directly compared. The four intergenic regions compared included two of the longest ones [F/M2, 46 nucleotides; SH/G(−1), 43 nucleotides], the shortest one (N/P, 1 nucleotide), and one of an intermediate length (NS1/NS2, 19 nucleotides). The SH/G(−1) intergenic region was of additional interest because its authentic counterpart was the only one which was not associated with a readthrough mRNA in previous studies (5, 11).

The positive-sense mRNAs expressed by the four different minigenomes were compared by Northern blot hybridization of total intracellular RNA (Fig. 4); enzyme activities were not measured, because the chimeric LUC(CAT) mRNA would encode an incomplete, inactive LUC protein. Consistent with the results with the previous panel of minigenomes (Table 1), the four intergenic regions were nearly indistinguishable with regard to their effect on the level of expression of the two genes (Table 3). The LUC(CAT) mRNA, representing the down-

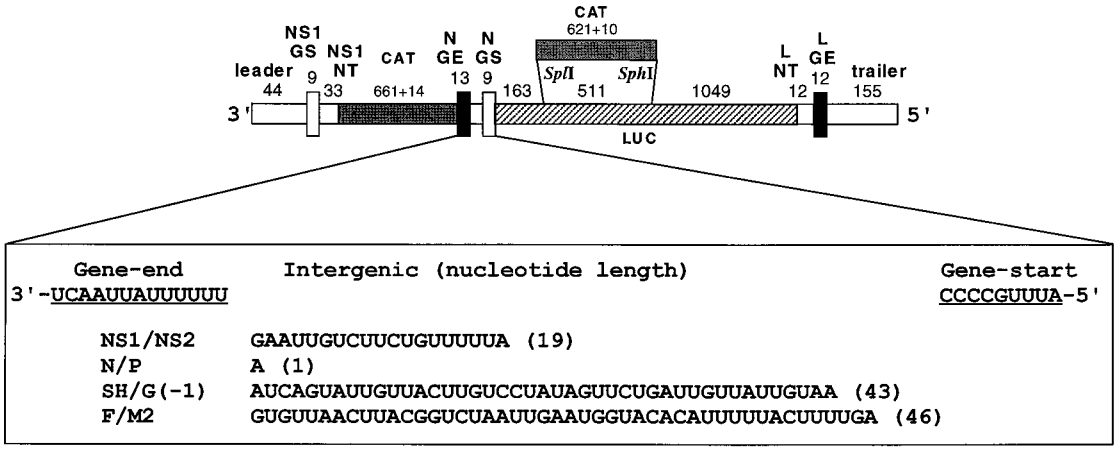

FIG. 3. Modification of the RSV-CAT-LUC minigenome by removal of the 511-nucleotide *Spl*I-*Sph*I fragment of the LUC ORF and replacement with a 631-nucleotide fragment of the CAT ORF to give a LUC(CAT) chimeric ORF. This was done so that the CAT-specific riboprobe would detect both subgenomic mRNAs with approximate equivalence. The nucleotide lengths of the CAT ORF and the transferred CAT fragment are each given as a sum in which the first value indicates the number of CAT-specific nucleotides and the second indicates the non-CAT nucleotides contributed by flanking restriction sites and spacer sequence. Four RSV-CAT-LUC minigenomes were modified in this way, and their intergenic sequences are shown underneath. As described in the legend to Fig. 1, SH/G(−1) is a version of the SH/G intergenic sequence with a single-nucleotide deletion.

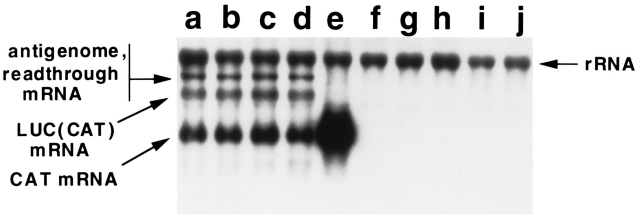

FIG. 4. Measurement of transcriptional polarity across the CAT-LUC gene pair in minigenomes representing four different intergenic regions. The RSV-CAT-LUC minigenome was modified so that the LUC ORF contained the insertion of a second copy of the CAT ORF, allowing its encoded chimeric mRNA to be compared directly with the CAT mRNA by hybridization. Cells were transfected with the minigenome, and 30 h later, medium supernatants were passaged to fresh cells. Total intracellular RNA was harvested 30 h later and analyzed by Northern blot hybridization with a negative-sense CAT riboprobe. The original transfections were prepared as follows: RSV-infected (lanes a to d) or uninfected (lanes g to j) cells were transfected with RSV-CAT-LUC(CAT) minigenome containing the following intergenic region: NS1/NS2 (lanes a and g), N/P (lanes b and h), SH/G (lanes c and i), or F/M2 (lanes d and j). An additional well of RSV-infected (lane e) or uninfected (lane f) cells was transfected with the monocistronic RSV-CAT-H-15 minigenome (21), which encodes the CAT subgenomic mRNA.

stream gene, was synthesized at 0.30 to 0.36 the level of the CAT mRNA. The amount of LUC(CAT) mRNA relative to CAT mRNA was slightly smaller for minigenomes containing the two longer intergenic regions (0.31 and 0.30) than for those containing the shorter intergenic regions (0.35 and 0.36). This might indicate a modest increase in polarity associated with increased intergenic length, but the effect was very small. It should be noted that measurements of transcriptional polarity here might be slightly exaggerated because (i) the copy of the CAT ORF which was inserted into the LUC ORF contained only 94% of the CAT sequence present in the upstream gene, which would make the LUC(CAT) mRNA seem slightly less abundant when hybridized with probe which had been fragmented by radiolysis; and (ii) the LUC(CAT) mRNA was 2.7 times longer than the CAT mRNA, and its abundance could be disproportionately affected by intragenic polymerase termination or by degradation. With these caveats, this experimental result assigns a value to the level of RSV transcriptional polarity across two genes.

Aliquots of the RNAs described above were subjected to oligo(dT) chromatography, and the bound RNAs were eluted and analyzed by Northern blot hybridization (results not shown). The chromatography step removed the antigenome and made it possible to determine the amount of CAT-LUC-(CAT) readthrough mRNA. The level of readthrough mRNA was compared with that of LUC(CAT) mRNA, the latter chosen because the polymerase must traverse the same extent of the minigenome for the synthesis of each of these two mRNAs. The level of readthrough mRNA was 0.10 to 0.13 that of LUC(CAT) mRNA (Table 3). This value did not vary greatly among the different intergenic regions, although the two longer ones were associated with slightly lower levels of readthrough mRNA. This assigned a value to the level of transcriptional readthrough across a single RSV intergenic region, showed that this was largely independent of the nature of the intergenic region for the ones tested, and showed that the low abundance of the SH/G readthrough mRNA in RSV-infected cells is not an attribute of that intergenic region.

TABLE 3. RSV transcriptional polarity: expression of CAT mRNA, LUC(CAT) chimeric mRNA,[a] and readthrough mRNA by minigenomes representing four naturally occurring intergenic regions[b]

RNAs compared	Ratio in:			
	NS1/NS2	N/P	SH/G(−1)	F/M2
CAT mRNA/antigenome	3.1	3.89	4.33	4.54
LUC(CAT) mRNA/antigenome	1.13	1.35	1.13	1.33
LUC(CAT) mRNA/CAT mRNA	0.36	0.35	0.31	0.30
Readthrough mRNA/LUC(CAT) mRNA[c]	0.13[c]	0.11[c]	0.10[c]	0.10[c]

[a] LUC(CAT) was a chimeric version of the LUC mRNA in which a 511-bp fragment was replaced with a 631-bp fragment of the CAT ORF (Fig. 3).
[b] RNAs were measured by phosphorimagery of Northern blots hybridized with CAT riboprobe and expressed as ratios of the indicated species.
[c] This comparison involved oligo(dT)-selected mRNA.

DISCUSSION

A dicistronic RSV-CAT-LUC minigenome RNA, which was synthesized in vitro and transfected into RSV-infected cells, was used to evaluate the effects of the various RSV gene junctions on sequential transcription. The results showed that the intergenic regions have little or no influence on RNA replication or, surprisingly, on gene transcription.

Previously, there was little direct information about the role of intergenic regions in gene expression by the nonsegmented negative-strand RNA viruses. The prototypic viruses of the group, VSV and SeV, have consensus-type di- or trinucleotide intergenic regions. Because these sequences are conserved and are situated between the GE and GS signals, it is generally assumed that they, too, are some sort of polymerase signal. Activities which occur at the gene junctions and thus might be influenced by the intergenic sequences include pseudotemplated transcription to make the poly(A) tail, termination and release of the mRNA, and initiation, 5′ capping, and methylation of the new mRNA. Time course analysis of in vitro transcription by detergent-disrupted VSV virions indicated that the gene junctions are sites where pausing and polymerase disengagement occur, the latter conferring polarity (17). Naturally occurring intergenic point mutations in VSV and SeV were associated with increased levels of readthrough mRNAs (16, 23). Mutational analysis of conserved intergenic sequences has been described in preliminary work on human parainfluenza virus type 3, a close relative of SeV (13). Specifically, in a dicistronic minigenome system comparable to the one described here, the integrity of the intergenic triplet appeared to be critical for expression of the downstream gene and also appeared to affect the level of expression of the upstream gene (13). Analysis had not been reported previously for viruses, including RSV and the other examples described in Introduction, that have nonconsensus-type intergenic regions.

The eight naturally occurring intergenic regions of strain A2 were inserted into RSV-CAT-LUC and compared in parallel. Northern blot analysis showed that the minigenomes were similar with regard to both the absolute and the relative levels of synthesis of the two subgenomic mRNAs (Fig. 2). The relative levels of expression of the CAT and LUC genes were examined in greater detail by measuring enzyme activities of replicate samples (Table 1). The efficiency of expression of the downstream gene was slightly lower for two of the longer intergenic regions [SH/G(−1) and F/M2], raising the possibility that they were associated with a slight increase in transcriptional polarity. To cross those intergenic regions which are longer than just a few nucleotides, the polymerase presumably must be able to move along the template without transcribing. One possibility is that it is more prone to disengagement in this mode. However, the association between intergenic length and

polarity was not consistent or statistically significant, and in any case the effect was very small. Thus, the structural differences in the eight intergenic regions tested did not appear to modulate either the absolute or the relative level of expression of the CAT and LUC genes.

To directly compare the amount of transcription of the upstream and downstream genes, part of the LUC ORF of four of these minigenomes was replaced with a second copy of the CAT ORF. The CAT mRNA expressed from the upstream CAT gene and the LUC(CAT) mRNA expressed from the downstream chimeric gene would hybridize equally with CAT riboprobe but could be distinguished by their difference in size. Comparison of the relative molar amounts of the two mRNAs showed that transcription of the downstream LUC(CAT) gene was 0.30 to 0.36 that of the upstream CAT gene, which assigns a value to the magnitude of RSV transcriptional polarity across a single gene pair of 2.5 kb. It is unknown whether polymerase disengagement occurs at specific sites or during specific activities, such as during pseudotemplated transcription for poly(A) addition, termination, or reinitiation. The two longer intergenic regions represented in this series of minigenomes [SH/G(−1) and F/M2] were associated with slightly reduced expression of the downstream gene, as had also been observed with the more extensive minigenome panel described above. However, the difference from the values for the two shorter intergenic regions was very small. Thus, most or all of the effect of transcriptional polarity must be due to some other factor. The results with the two panels of minigenomes were consistent and showed that the various intergenic regions were very similar when compared by this transcription assay.

The complete removal of the intergenic region had no effect on the expression of the upstream gene and resulted in a very small decrease in the expression of the downstream one. This showed that the intergenic region is dispensable altogether for sequential transcription. In particular, it showed that operation of the adjacent upstream GE and downstream GS signals was independent of the sequence, length, or presence of an intergenic region. All of the intergenic regions of strain A2 and most of those of other strains sequenced to date end in A (2, 19), but apparently this feature is not significant.

Polyadenylated positive-sense RSV RNAs isolated from infected cells consist of the 10 major subgenomic mRNAs and a series of less abundant, larger species that each contain the complete sequences of two or more adjacent genes together with their intergenic region(s). They are thought to arise from transcriptional readthrough due to an inherent inefficiency of the polymerase in recognizing the GE signal (5, 7, 11). Such readthrough transcripts have been noted for essentially all of the nonsegmented negative-strand RNA viruses (5, 16, 22–24, 28, 29). In most cases, they seem to be nonessential, dead-end by-products. Transcriptional readthrough would contribute somewhat to transcriptional polarity, because it would reduce the relative expression of the subgenomic mRNA of the downstream gene of each gene pair. However, for gene overlap such as occurs for the RSV M2 and L genes, transcriptional readthrough of the overlapped GE signal is required to produce full-length mRNA, and such readthrough mRNAs are essential (10). The abundances of the various RSV readthrough mRNAs have not been quantitated but generally seem consistent with transcriptional polarity. One exception was that a readthrough for the SH/G junction has not been detected among the authentic RSV RNAs (5, 11). Here, we examined transcriptional readthrough under conditions where the two flanking genes [CAT and LUC(CAT)] remained constant and the intergenic region was varied. This assigned a value to the frequency of RSV transcriptional readthrough, 0.10 to 0.13, and showed that it was very similar for four different intergenic regions including SH/G(−1). This is consistent with the idea that the various intergenic regions do not modulate the efficiency of recognition of the adjacent, upstream GE signal and do not affect the ability of the polymerase to produce the low levels of readthrough mRNAs. The previously described low level of SH/G readthrough mRNA in infected cells presumably is due to instability or some other post-synthetic effect.

Sequences of six intergenic regions are available for comparison between the two RSV antigenic subgroups. While the level of sequence identity is low (42%), in three cases the nucleotide length is identical between subgroups (19). If the intergenic regions indeed serve no purpose, it is somewhat surprising that they have been retained and that their length has been so well conserved. However, we note that a similar situation exists for long nontranslated regions contained in several of the RSV genes. For example, the P gene has a 158-nucleotide downstream nontranslated region (not including the GE signal) that has 56% sequence identity between the subgroups but is identical in length (20). The corresponding 118-nucleotide region in the SH gene has 55% sequence identity and a 1-nucleotide difference in length between the two subgroups (9), and the corresponding 157-nucleotide region of F has 47% identity and a 3-nucleotide difference in length (18). While it is possible that these nontranslated sequences have significance, such as in mRNA stability, the lack of sequence conservation suggests that they resemble the intergenic regions in being incidental. Such nonessential regions might tend to be retained if they do not interfere with RNA replication or transcription and if their deletion does not confer a significant replicative advantage. Also, perhaps the RSV polymerase does not readily generate short deletions.

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