Virology **300,** 304–315 (2002)
doi:10.1006/viro.2002.1541

Transcriptional Termination Modulated by Nucleotides Outside the Characterized Gene End Sequence of Respiratory Syncytial Virus

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Received February 27, 2002; returned to author for revision March 25, 2002; accepted May 1, 2002

The genes of respiratory syncytial (RS) virus are transcribed sequentially by the viral RNA polymerase from a single 3′-proximal promoter. Polyadenylation and termination are directed by a sequence at the end of each gene, after which the polymerase crosses an intergenic region and reinitiates at the start sequence of the next gene. The 10 viral genes have different gene end sequences and different termination efficiencies, which allow for regulation of gene expression, since termination of each gene is required for initiation of the downstream gene. RNA sequences within the previously characterized 13 nucleotide gene end, including a conserved sequence 3′-UCAAU-5′ and a tract of U residues, are important for termination. In this study, two additional sequence elements outside of the 13 nucleotide gene end were found to modulate termination efficiency: the A residue upstream of the 3′-UCAAU-5′ sequence, and the first nucleotide of the intergenic region when it follows a U_4 tract.

INTRODUCTION

Respiratory syncytial (RS) virus, the leading cause of bronchiolitis and pneumonia in infants, is a paramyxovirus within the order *Mononegavirales* (Collins *et al.,* 2001). The RS viral genome, a single strand of negative-sense RNA, contains 10 genes flanked by noncoding leader and trailer regions in the order 3′-leader-NS1-NS2-N-P-M-SH-G-F-M2-L-trailer-5′. To express these 10 genes, the viral RNA polymerase gains access to the genome at a single 3′-proximal promoter and transcribes 10 monocistronic mRNAs sequentially from 3′ to 5′ (Dickens *et al.,* 1984). According to the "stop-start" model of transcription, which is most consistent with available evidence, the polymerase must terminate transcription of each gene to reinitiate transcription of the downstream gene (Barr *et al.,* 1997b; Emerson, 1987; Harmon *et al.,* 2001; Kuo *et al.,* 1996). The level of mRNA transcription is attenuated (decreased) from 3′-proximal to 5′-proximal genes (Barik, 1992; Dickens, 1985; Dickens *et al.,* 1984).

Transcription is directed by *cis*-acting sequences in the genomic RNA of RS virus, which include the 3′-proximal transcriptional promoter and sequences at the beginning and end of each gene. Elements of the 3′-proximal promoter include the first 25 nt of the leader region and the gene start (GS) sequence of the first gene (Fearns *et al.,* 2000, 2002). Our work focuses on the events of stop-start transcription that occur as the polymerase traverses the gene junctions, each of which consists of three parts: a gene end (GE) sequence, which comprises the end of the upstream gene; an intergenic region, which lies between the transcribed regions of two genes; and a GS sequence, which comprises the beginning of the downstream gene (Collins *et al.,* 1986).

The GE sequences of RS virus, strain A2, are shown in Table 1. Sequences throughout this study are shown from 3′ to 5′ in the negative (template) sense, and the positions of the nucleotides in the GE sequence are numbered. The 10 GE sequences contain two conserved regions, the sequence 3′-UCAAU-5′ (positions 1 to 5) and a tract of 4 to 7 U residues, as well as a central region (positions 6 to 8) that is poorly conserved. The GE directs polyadenylation of mRNAs by reiterative transcription (slippage) on the U-tract template, and mRNA release (termination), based on studies of RS virus and the rhabdovirus vesicular stomatitis virus (VSV) (Barr and Wertz, 2001; Barr *et al.,* 1997a; Harmon *et al.,* 2001; Kuo *et al.,* 1996). Downstream of the U tract is an intergenic region, the length of which varies from 1 to 52 nt in RS virus, with no sequence conservation (Table 1) (Collins *et al.,* 1986; Johnson and Collins, 1988; Zamora and Samal, 1992). The intergenic sequence has not been detected in monocistronic mRNAs of the *Mononegavirales,* and therefore, the polymerase is thought to cross the intergenic region without transcribing it during stop-start transcription (Collins *et al.,* 1986; Rassa *et al.,* 2000). The GS sequence at the beginning of each gene is conserved and is thought to direct initiation and capping of mRNAs (Kuo *et al.,* 1996, 1997; Stillman and Whitt, 1999).

As mentioned before, the GE and intergenic se-

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0042-6822/02 $35.00

TABLE 1

Comparison of the GE Sequences of RS Virus

3′ Gene	GE sequence[a]	Intergenic region[b]		Termination efficiency[c]	5′ gene
	-1 1 6 13				
NS1	3′-UAA UCAAUUAUAUUUUU-5′	G	19	+	NS2
NS2	AUA UCAUUAAAUUUUU	A	26	+	N
N	AAC UCAAUUAUUUUUUU	A	1	++	P
P	AUA UCAAUGUUUUUUUU	C	9	++	M
M	UAU UCAAUUAUUUUUU	A	9	++	SH
SH	UCA UCAAUUAAUUUUU	A	44	+++	G
G	UCA UCAAUGAAUUUUU	G	52	++	F
F	AUA UCAAUAUAUUUU	G	46	+	M2
M2	CUA UCAAUAAAUUUU	C	n/a	+	L
L	AUA UCAAUAAUUUUU	A	n/a	+	n/a

[a] The GE sequences of strain A2 (Collins *et al.,* 1986) and the 3 nt upstream are shown from 3′ to 5′ in the negative sense (i.e., template strand). The nucleotide positions are numbered with respect to the conserved 3′-UCAAU-5′ sequence (positions 1 to 5), and the U tract is underlined.

[b] The first nucleotide downstream of the U tract and total length of each intergenic region is shown; n/a, not applicable. The GS sequence (3′-CCCCGUUUA-5′ for 9 of 10 genes) is not shown.

[c] Adapted from Hardy *et al.,* 1999. The termination efficiency at each GE was determined using dicistronic replicons in the presence of M2-1 protein expression (Hardy *et al.,* 1999), and was classified as high (+++), moderate (++), or poor (+) efficiency.

quences of RS virus vary. The termination efficiencies at the 10 GEs of RS virus were compared using dicistronic RNA replicons, each containing one of the authentic gene junctions surrounded by sequences from the upstream and downstream genes, and these studies showed that the varied GE sequences directed termination with different efficiencies (Hardy *et al.,* 1999, and Table 1). Termination was found to be most efficient at the SH/G gene junction, moderately efficient at the N/P, P/M, M/SH, and G/F gene junctions, and inefficient at NS1/NS2, NS2/N, F/M2, and M2/L gene junctions (Hardy *et al.,* 1999 and Table 1). Correspondingly, it appears that termination at the SH/G gene junction is most efficient in RS virus-infected cells. Although it has not been possible to measure termination directly in RS virus infected cells, it has been observed that polycistronic readthrough RNAs, which are the result of a failure to terminate, are present for every gene junction except for the SH/G gene junction (Collins and Wertz, 1983; Dickens, 1985). Taken together, these data indicate that termination at the SH/G gene junction is more efficient than at any other gene junction of RS virus. The variation of termination efficiency in RS virus may provide an additional mechanism for regulating transcription of the genes, because termination of a gene is required for initiation of the downstream gene (Hardy *et al.,* 1999; Harmon *et al.,* 2001). Therefore, identification of the specific nucleotides that affect termination efficiency is important.

In previous work, we selected a representative gene junction (M/SH) and characterized the effects on termination of mutations in the M GE sequence in the context of a dicistronic replicon, M/SH-B (Harmon *et al.,* 2001). Positions 1 to 8 of the M GE were changed individually to all possible nucleotides, and the length and position of the U tract were altered. Any single nucleotide change at positions 2 to 6 of the M GE reduced the efficiency of termination by half or more; any change at position 3 to 5 reduced termination to a very low level [<4%, compared to about 60% for the wild-type (wt) M GE]. By comparison, mutations at positions 1 and 7 had lesser effects on termination. Additionally, the U residues at positions 9 to 13 of the M GE were required for efficient termination. Termination at the M GE was efficient either when the WT U_6 tract of the M GE (positions 8 to 13) was present or when a U_5 tract was present with U's at positions 9 to 13. Termination also occurred with low efficiency (16%) with a U_4 tract at positions 9 to 12, but termination was reduced to a very low level when a U residue was not present at position 9 or position 12 (Harmon *et al.,* 2001).

These previous data showed that most nucleotides within the 13 nt of the characterized M GE were important for efficient termination, including the sequence 3′-CAAUU-5′ at positions 2 to 6 and the presence of U residues at positions 9 to 13 (Harmon *et al.,* 2001). Consequently, it is likely that these nucleotide positions are important for termination at the other GE sequences. Consistent with this hypothesis, the nucleotides at positions 1 to 5 are well-conserved, and the five GEs of RS virus that terminate inefficiently lack U residues at position 9 or 13 (Table 1) (Hardy *et al.,* 1999). However, additional data suggested that sequences other than positions 1 to 13 of the GE were important for efficient termination, particularly at the SH/G gene junction. When the 13 nt M GE sequence in the replicon M/SH-B was replaced with the 13 nt SH GE, the efficiency of termination was not increased as expected, but was slightly decreased from 60 to 40% (Harmon *et al.,* 2001). By contrast, a higher efficiency of termination (90 to 95%) was observed at the SH GE in the replicon SH/G, in which the SH GE was placed in its authentic sequence context, including the last 81 nt of the SH gene, the SH/G intergenic region, and 554 nt of the G gene (Hardy *et al.,* 1999). The role in termination of specific nucleotides outside the 13-nt characterized GE sequence remains poorly understood.

The variable intergenic regions of RS virus were previously reported not to modulate termination or initiation of transcription (Bukreyev *et al.,* 2000; Kuo *et al.,* 1996). Kuo *et al.* analyzed the nine intergenic sequences in the context of a single selected GE sequence, the N GE, which contains a U_6 tract (Table 1). Subsequently, studies of the paramyxovirus simian virus 5 (SV5) showed that the importance of the intergenic sequence for termination was dependent on the length of the U tract (Rassa

and Parks, 1999; Rassa *et al.,* 2000). The intergenic sequence of SV5 had little effect on termination when a U_6 tract was present in the GE; however, when a U_4 tract was present, termination was less efficient if the first intergenic nucleotide was A rather than G or C.

In the present study, we investigated whether sequences outside the characterized GE sequence (positions 1 to 13) were important for termination in RS virus. These studies identified two previously unrecognized nucleotides that modulated the efficiency of termination in RS virus: (i) the presence of an A residue at position −1, upstream of the characterized GE sequence, increased the efficiency of termination; and (ii) the first nucleotide of the intergenic region was important for termination when the GE contained a U_4 tract. These data allowed the prediction of a consensus GE sequence for RS virus and provided further insight into the diverse RNA sequences that control termination in RS virus.

RESULTS

Sequences upstream of the SH/G gene junction involved in termination

Transcriptional termination is more efficient at the SH/G gene junction of RS virus than at any other gene junction (Collins and Wertz, 1983; Hardy *et al.,* 1999). To investigate whether sequences outside the characterized SH GE (positions 1 to 13) contributed to efficient termination at the SH/G gene junction, we analyzed transcription of a dicistronic RNA replicon, SH/GFP (Fig. 1A). This replicon contained the SH/G gene junction flanked by two genes, the SH gene (gene 1) and the green flourescent protein (GFP) gene (gene 2). Transcription from SH/GFP was analyzed by metabolic labeling in HEp-2 cells in the presence of N, P, L, and M2-1 protein expression (Fig. 1B, lane 1). Termination was quantitated by calculating the termination efficiency at the end of gene 1, as described under Materials and Methods. For the replicon SH/GFP, the termination efficiency was approximately 85% (Fig. 1C).

To determine whether sequences upstream of the 13-nt SH GE sequence were involved in efficient termination, we replaced individual segments of the SH gene with heterologous nonviral sequences, as shown in Fig. 1A. In the replicon SH-12, replacement of the 12-nt segment just upstream of the SH GE (positions −1 to −12) decreased termination, as shown by the increased readthrough RNA synthesis and decreased mRNA1 and mRNA2 synthesis (Fig. 1B, lane 2). The termination efficiency was decreased from 85% in SH/GFP to 45% in SH-12 (Fig. 1C). Termination was not affected by replacement of segments upstream of position −12 in the replicons SH-24, SH-36, SH-48, and SH-56 (Fig. 1C). These results showed that the efficiency of termination was affected by the 12 nt just upstream of the SH GE, but not by sequences upstream of position −12.

We investigated whether the SH/G gene junction was the only gene junction that could direct highly efficient termination in the context of the upstream SH gene sequence. The SH/G gene junction in SH/GFP was replaced with the N/P gene junction (Fig. 1A, replicon SH-NP), which was previously shown to direct termination with moderate efficiency, about 70%, in its natural sequence context (Hardy *et al.,* 1999). In the context of the replicon SH-NP, termination at the N/P gene junction was 90% efficient, slightly more efficient than in SH/GFP (Fig. 1C), which showed that highly efficient termination could be directed by a gene junction other than SH/G and implied some element outside the conserved gene junction increased termination. An A-to-G change at position 8 of the SH GE, which reduced termination at the M GE to very low levels, with termination efficiency <4% (Harmon *et al.,* 2001), reduced termination in the replicon SH+8G to a lesser extent (termination efficiency = 50%, Fig. 1C). Taken together, these results indicated that a sequence within the 12 nt upstream of the SH GE was able to increase the efficiency of termination at a gene junction.

Termination at the M/SH gene junction increased by short upstream sequences

Because mutation of the 12 nt upstream of the SH GE decreased the termination efficiency at the SH/G gene junction (Fig. 1), we used a complementary approach to test the effect of this 12-nt sequence on termination at another gene junction (M/SH) in a dicistronic replicon. As shown in Fig. 2A, the 12-nt segment of the M gene just upstream of the M GE in the replicon M/SH-term was replaced with the 12-nt segment from upstream of the SH GE to generate the replicon M/SH-12. The termination efficiency was increased from 64% in M/SH-term to 84% in M/SH-12, showing that the 12 nt from upstream of the SH/G gene junction increased termination at the M/SH gene junction (Fig. 2C).

To further localize the nucleotides responsible for this increase in termination efficiency, the replicon M/SH-5 was constructed, in which positions −1 to −5 upstream of the M GE matched the SH gene sequence, but positions −6 to −12 did not (Fig. 2A). Again, the termination efficiency of M/SH-5 was increased compared to M/SH-term (Fig. 2C), suggesting that the five nucleotides upstream of the GE modulated the termination efficiency. In these experiments, quantitation of termination efficiency was facilitated by the presence of two tandem GE sequences at the end of gene 2 in the replicon M/SH-term, which allowed readthrough RNAs to be distinguished clearly from replication products (see Materials and Methods).

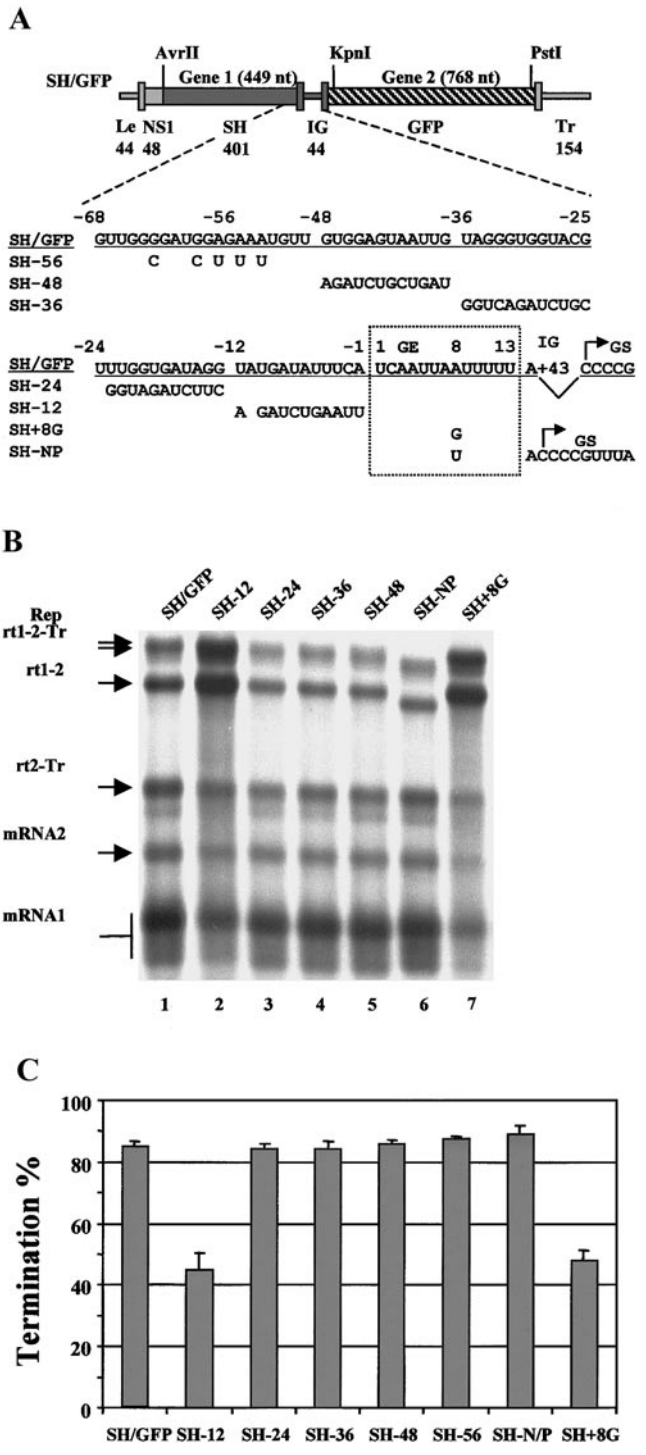

FIG. 1. Sequences upstream of the SH/G gene junction involved in termination. (A) The dicistronic RNA replicon SH/GFP, shown schematically, contains the SH/G gene junction of RS virus. The WT SH/G gene junction sequence (underlined) is aligned with nucleotides that were changed in mutant replicons. Negative numbers above the sequence refer to the number of nt upstream of the previously characterized SH GE, which is enclosed in a dashed box. Only the first nucleotide of the 44 nt SH/G intergenic (IG) region and the first 5 nt of the G GS are shown. Le, leader; Tr, trailer. (B) Viral RNA synthesis directed by SH/GFP replicons. HEp-2 cells were infected with MVA-T7 and transfected with plasmids to express the indicated dicistronic replicon and the N, P, L, and M2-1 proteins of RS virus. RNA synthesis directed by RS viral polymerase was metabolically labeled with [^{3}H]uridine in the presence of actinomycin-D and analyzed by denaturing agarose gel electrophoresis. RNAs synthesized from the SH/GFP template included the replication products (Rep), monocistronic mRNA1 and mRNA2, and three polycistronic readthrough (rt) RNAs, which were produced when polymerase failed to terminate at the GE of gene 1, gene 2, or both. The mRNA1 doublet resolved to a single, faster migrating band when the poly(A) tails were removed by RNase H cleavage (data not shown). (C) The average termination efficiency was calculated as the ratio of mRNA1 to total gene 1 transcription (mRNA1 plus readthrough RNAs rt1-2-Tr and rt1-2). Error bars represent the standard deviation from the mean of three independent experiments.

Extension of the GE consensus sequence

An A residue was noted to be present at position −1 in 8 of the 10 GEs of RS virus, all except the N and M GEs, where position −1 is C and U, respectively (Table 1). This observation, combined with the data in Fig. 2, suggested that an A residue at position −1 was part of the GE sequence. To test this hypothesis, we changed the U at position −1 upstream of the M GE to G, A, or C in the replicon M/SH-Eco (Fig. 3A), which was identical to M/SH-term (Fig. 2), except for the presence of an EcoRV site near the end of gene 2. Termination was highest when position −1 of the GE was A (Fig. 3B). Thus, the A residue at position −1 increased the efficiency of termination, and it is present at 8 of the 10 gene junctions, indicating that it is part of the GE consensus sequence of RS virus.

The 7-nt consensus sequence for positions −1 to 6 of the GE is 3′-AUCAAUU-5′, based on the results in Fig. 3 and previous data (Harmon *et al.,* 2001). In 9 of the 10 GEs of RS virus, 6 or 7 of these positions match the consensus sequence (Table 1). Differences from the consensus tend to occur at either position −1 or position 6, but not at both. To further investigate the role of positions −1 to 6 in signaling termination, we determined the effects of combined point mutations in the M GE (Fig. 4). Position −1 of the M GE in the replicon M/SH-Eco (Fig. 3A) was made U or A, while position 6 was made U, G, or A, as found in the viral GEs (Table 1). Analysis of RNA synthesis from these replicons is shown in Fig. 4A, and the termination efficiency is shown in Fig. 4B.

Of the mutant GE sequences analyzed, the GE containing the consensus sequence 3′-AUCAAUU-5′ terminated the best (Fig. 4A, lane 2, and Fig. 4B). The termination efficiency was decreased by 20% or more when either position −1 or position 6 was different from the consensus sequence (Fig. 4A, lanes 1, 6, and 8). When both positions −1 and 6 differed from the consensus, the termination efficiency was further decreased (Fig. 4A, lanes 5 and 7). Thus, a nonconsensus nucleotide at either end of the 3′-AUCAAUU-5′ sequence had a milder effect on termination when a consensus nucleotide was present at the other end. These data showed that mutations at positions −1 and

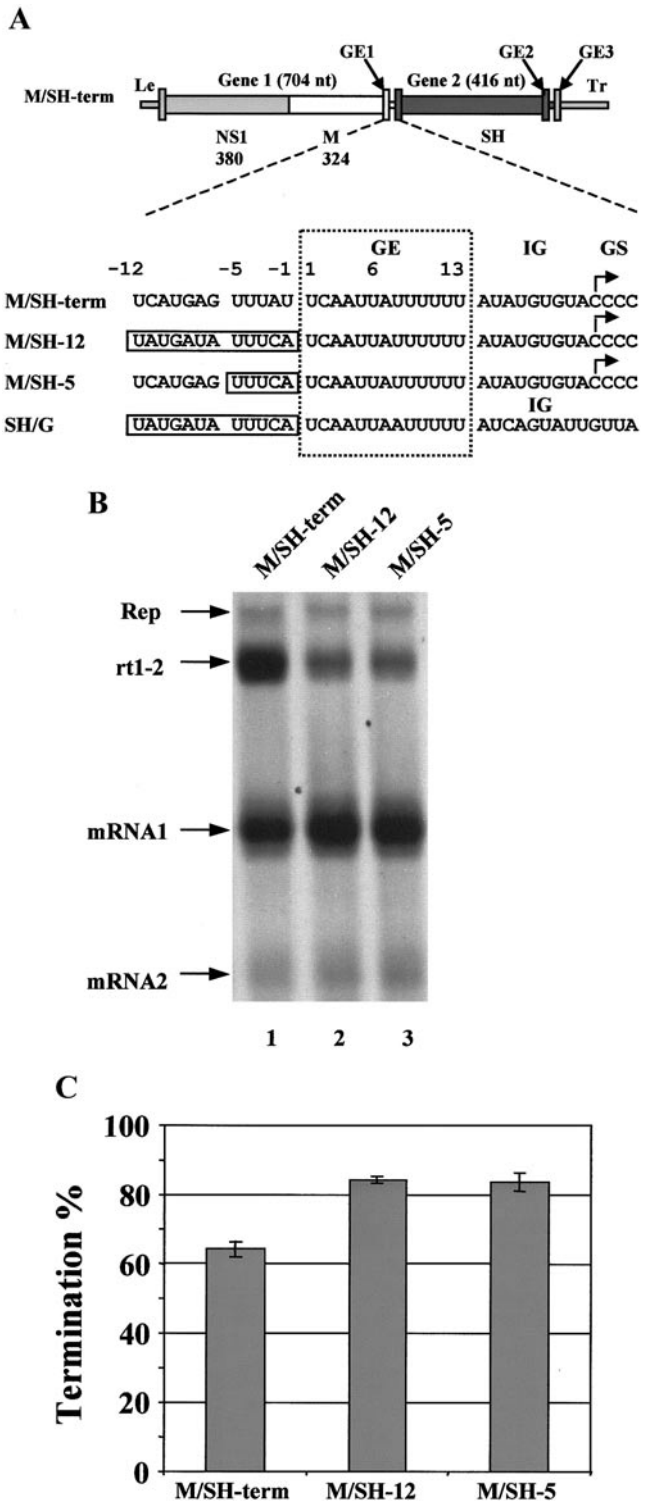

FIG. 2. Termination at the M/SH gene junction increased by short upstream sequences. (A) Termination at the M/SH gene junction was analyzed using the dicistronic replicon M/SH-term, which is shown schematically. Gene 2 of M/SH-term contained the entire SH gene sequence and had two GE sequences at the end of gene 2 (GE2 and GE3). The WT RNA sequences near the end of gene 1 (GE1) from M/SH-term, two mutant replicons, and the SH/G gene junction are shown. Within the 12 nt upstream of the GE, sequences from the SH gene are enclosed in boxes. IG, intergenic. (B) Viral RNA synthesis directed by replicons was analyzed as described in Fig. 1B. The readthrough transcripts rt1-2-Tr and rt2-Tr (Figs. 1 and 5) were not observed due to more efficient termination at the end of gene 2. (C) The average termination efficiency at the M/SH gene junction in RNA replicons was calculated as in Fig. 1C.

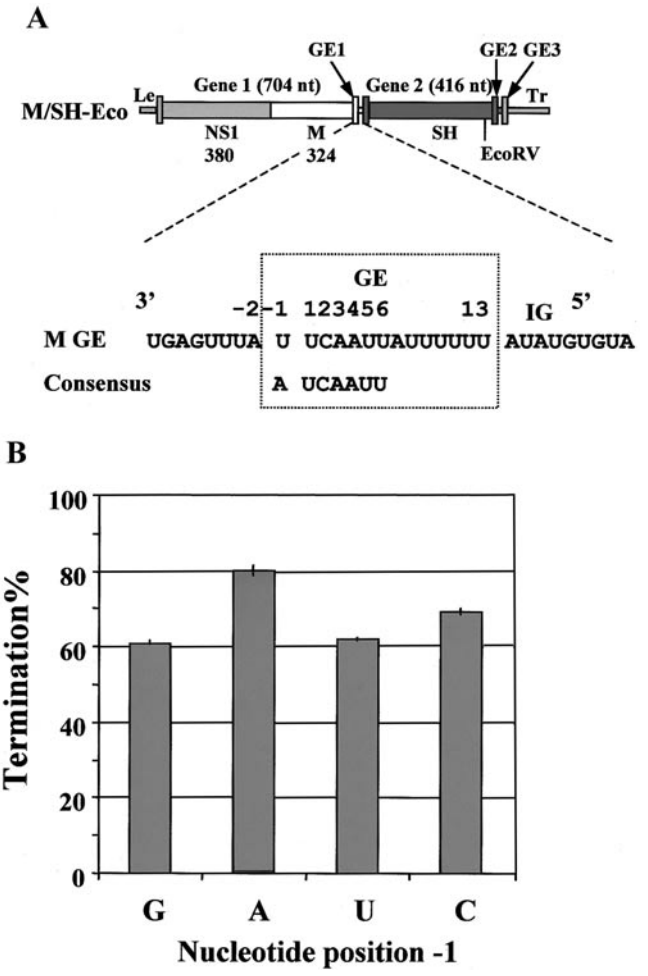

FIG. 3. Effects of nucleotide substitutions at position −1 of the M GE. (A) The dicistronic replicon M/SH-Eco was identical to M/SH-term (Fig. 2A), except for the introduction of an *Eco*RV site 12 nt upstream of GE2. The WT M GE sequence and the proposed consensus sequence for positions −1 to 6 of the GE are shown. IG, intergenic. (B) The effect on termination efficiency of changing position −1 upstream of the M GE in the replicon M/SH-Eco was analyzed as in Fig. 1C.

6 had mild but cumulative effects on termination. By contrast, termination was reduced to very low levels by an A-to-U change at position 3 in the middle of this sequence, even when all other positions matched the consensus (Fig. 4A, lane 4).

We also investigated whether combined point mutations at positions 1, 2, and 4 of the M GE sequence had cumulative effects on termination. Nucleotides at these positions were changed individually or in combination in the replicon M/SH-B, as shown in Fig. 4C. The U-to-G change at position 1 and the C-to-A change at position 2

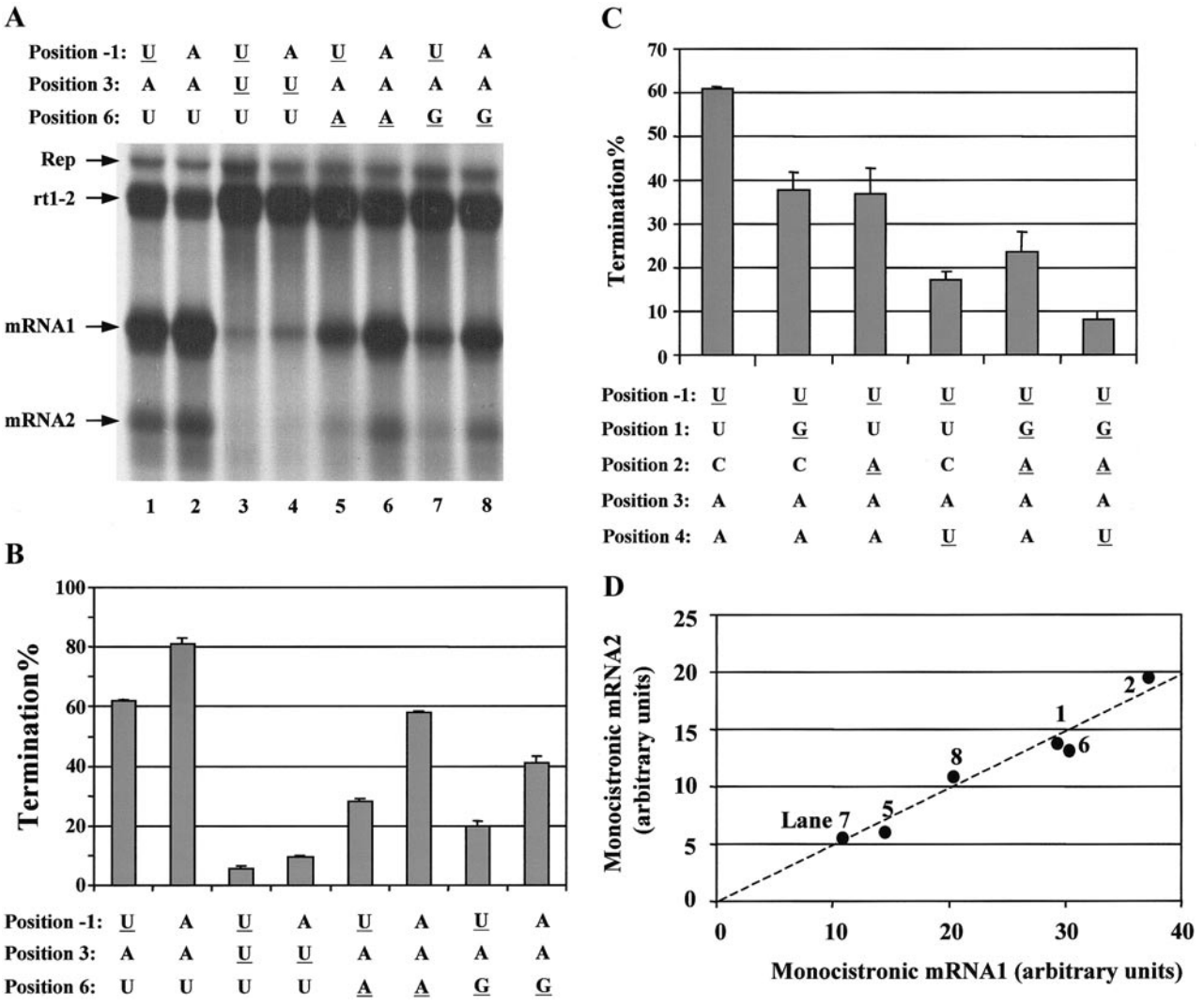

FIG. 4. Cumulative effects of nonconsensus nucleotides in the GE. (A) The indicated nucleotides were introduced at positions −1, 3, or 6 of the M GE in the replicon M/SH-Eco, which is shown in Fig. 3A. Nucleotides that differ from the proposed GE consensus sequence are underlined. Viral RNAs synthesized by the indicated replicons were analyzed as in Fig. 1B. (B) The termination efficiency was calculated for each replicon in A. (C) The effect on termination efficiency of mutations in positions 1, 2, and 4 of the M GE in the background of the replicon M/SH-B (see Fig. 5 for a schematic of this replicon). (D) The molar amounts of monocistronic mRNA1 and mRNA2 from A were quantitated by densitometry; the points are numbered according to the lanes in A.

were selected because each mutation was previously shown to reduce termination only moderately, thus allowing the cumulative effect of the two mutations to be analyzed (Harmon *et al.,* 2001). The mutations at positions 1 and 2 each reduced termination efficiency by about 25%, but the combination of these two mutations further reduced termination efficiency in a cumulative manner (Fig. 4C). The A-to-U change at position 4 reduced termination efficiency to 20%, and in combination with the other mutations, termination efficiency was further reduced to <10%. In summary, the data in Figs. 4B and 4C indicated that nucleotide changes at different positions of the GE sequence had cumulative effects on termination.

Transcription of gene 2 regulated by termination of gene 1

The efficiency of termination at the end of each gene of RS virus is thought to control the transcription of monocistronic mRNA from the downstream gene, since termination is required for downstream initiation (Harmon *et al.,* 2001; Kuo *et al.,* 1996). We further analyzed this aspect of RS viral transcription by quantitating the amounts of monocistronic mRNA1 and mRNA2 synthesized by the mutant replicons shown in Fig. 4A, because mRNA1 termination was modulated over a broad range by these mutations. The A-to-U mutation at position 3 reduced both mRNA1 and mRNA2 to very low levels, so replicons containing this mutation could not be included

in this comparison. For the other six replicons in Fig. 4A, the synthesis of monocistronic mRNA2 was proportional to the synthesis of monocistronic mRNA1 (Fig. 4D). The rate of transcriptional attenuation was approximately 50% for each of these replicons, meaning that transcription of mRNA2 was reduced by 50% compared to transcription of mRNA1. By comparison, a 64 to 70% attenuation rate was reported for another RS virus replicon (Kuo *et al.,* 1996), while a 30% attenuation rate was observed in VSV infection (Iverson and Rose, 1981). These data indicated that transcription of gene 2 by RS virus polymerase was controlled by termination of the upstream gene 1.

Importance of the first intergenic nucleotide for termination

A previous study suggested that the variable intergenic regions of RS virus did not modulate transcription when analyzed in the context of a single GE sequence, the N GE, which contained a U_6 tract (Kuo *et al.,* 1996). Studies of the paramyxovirus SV5, by contrast, showed that the first intergenic nucleotide could be important for termination, depending on the length of the U tract (Rassa and Parks, 1999; Rassa *et al.,* 2000). The U tracts at the ends of the genes of RS virus vary from four to seven residues in length (Table 1). We therefore tested whether the first nucleotide of the M/SH intergenic region was important for termination, either in the context of the WT M GE sequence, which contains a U_6 tract, or in the context of a shortened U_4 tract (Fig. 5). The first nucleotide of the M/SH intergenic region in the replicon M/SH-B was made either A (WT), G, or C, while the U_6 tract in the M GE was preserved or shortened to a U_4 tract (Fig. 5A). Because previous data suggested that U residues at positions 9 to 12 were required for termination (Harmon *et al.,* 2001), we shortened the U tract by changing the U at position 8 to A and deleting the U at position 13, which maintained U residues at positions 9 to 12.

When the GE contained the WT U_6 tract, the termination efficiency was similar whether the first intergenic nucleotide was an A or G, and somewhat lower when this nucleotide was C (Fig. 5C). However, when the GE contained the U_4 tract, the termination efficiency was 2.5-fold lower when the first intergenic nucleotide was A rather than G or C (Fig. 5C). Thus, termination was almost as efficient with a U_4 tract as with a U_6 tract, provided that the first intergenic nucleotide was G or C. These data showed that the intergenic sequence of RS virus was important for termination at a GE sequence containing a U_4 tract.

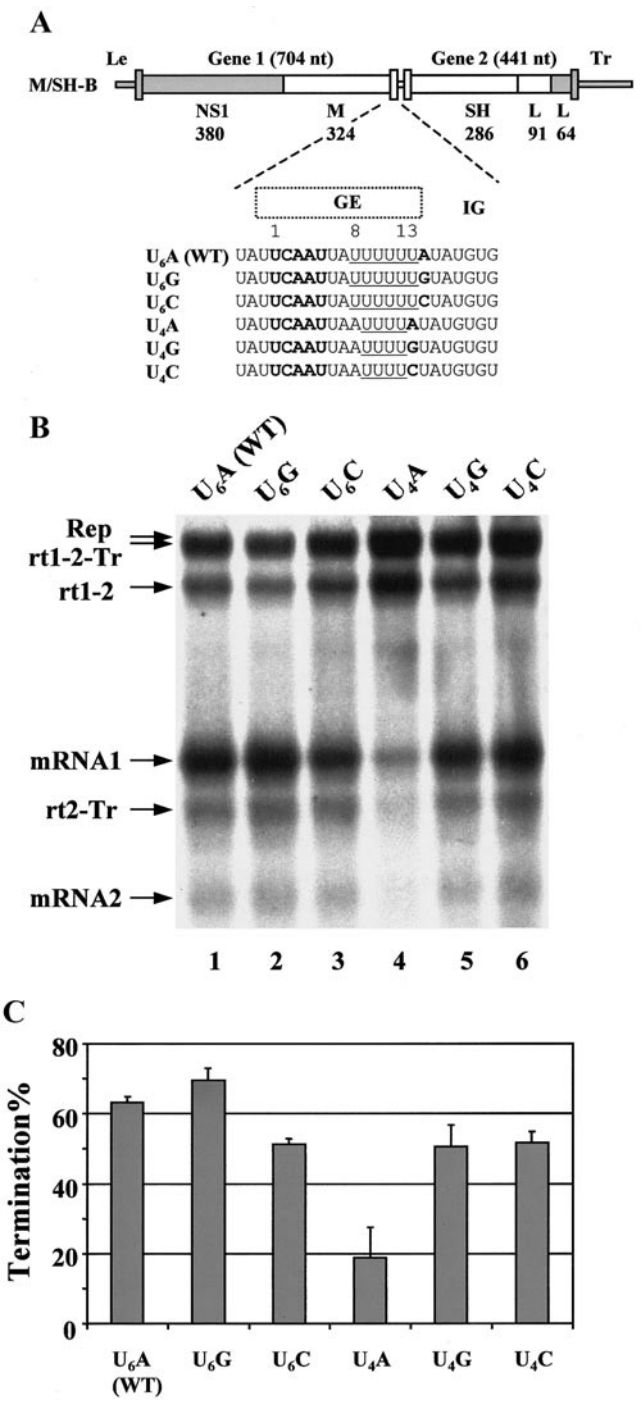

FIG. 5. Importance of the first intergenic nucleotide for termination. (A) Sequences surrounding the M GE in the dicistronic replicon M/SH-B (Harmon *et al.,* 2001) are shown, as well as mutant sequences with either a U_6 or U_4 tract and the first intergenic (IG) nucleotide as A, G, or C. (B) Viral RNA synthesis from the indicated replicons was analyzed as in Fig. 1B. (C) The termination efficiency was calculated as in Fig. 1C.

DISCUSSION

We analyzed transcriptional termination using dicistronic replicons of RS virus and found that the efficiency of termination was modulated by nucleotides outside the 13 nt of the characterized GE sequence. The GE was previously delineated as beginning with the sequence 3′-UCAAU-5′ and ending with the U tract (Table 1), based

on the conservation of the 3′-UCAAU-5′ sequence and U tract in different GE sequences of RS viruses from both subgroups A and B (Collins *et al.,* 1986, 2001; Johnson and Collins, 1988; Zamora and Samal, 1992), and reports that a 13-nt GE sequence was sufficient to signal termination of heterologous genes (Bukreyev *et al.,* 1996, 1999; Kuo *et al.,* 1996, 1997). Our previous work showed that specific sequences within the characterized 13-nt GE sequence, including the conserved 3′-UCAAU-5′ sequence and the length and position of the U tract, were important for termination (Harmon *et al.,* 2001).

Position −1 of the GE sequence

The data presented in this study indicated that the A residue at position −1 was also important for termination and should be included as part of the GE sequence. Termination at the M GE was more efficient when position −1 was A rather than G, U, or C (Fig. 3B), and mutation of positions −1 to −12 of the SH GE decreased the termination efficiency (Fig. 1). Among the viral GE sequences, the A at position −1 is present in 8 of the 10 GEs of the A2 strain (Table 1). Additionally, sequencing of the gene junctions of several RS virus strains isolated from children (Coggins *et al.,* 1998) revealed that an A was conserved at position −1 of these same eight GEs (R. Moudy, S. Harmon, W. Sullender, and G. Wertz, unpublished data). Based on these data, we propose that the consensus GE sequence of RS virus should be redefined as beginning with the sequence 3′-AUCAAUU-5′ and ending with the U tract. The first nucleotide of the intergenic region is also important for termination at GEs with U_4 tracts (see below). Our data neither support nor rule out the involvement of additional sequences in termination, although no other sequences appear to be conserved.

The consensus GE sequence of RS virus

A consensus GE sequence for efficient termination of RS virus can be predicted based on our functional analysis of the M GE (Fig. 4 and Harmon *et al.,* 2001). This consensus GE has the sequence 3′-AUCAAUU-5′ at positions −1 to 6 and U residues at positions 9 to 13. Among the GEs of RS virus, only the SH GE perfectly matches the consensus sequence at positions −1 to 6 and has U residues at positions 9 to 13 (Table 1). The close correspondence between the SH GE sequence and the predicted GE consensus sequence is consistent with the fact that the SH GE directs termination more efficiently than any other GE of RS virus (Collins and Wertz, 1983; Hardy *et al.,* 1999). The GEs of RS virus that direct termination with moderate efficiency (N, P, M, and G) contain a U tract of the predicted optimal length, and each GE differs from the 3′-AUCAAUU-5′ consensus sequence at only one position (Table 1). These four GEs vary from the consensus sequence in different ways, suggesting that suboptimal termination efficiency may have been selected at these GEs by variation from the consensus sequence. The reason that optimal termination has been selected at only the SH GE of RS virus, while moderate or poor termination has been selected at other GEs, may relate to the role of termination in regulating expression of specific genes (see below).

We previously determined the effects on termination of numerous mutations in the M GE, including all possible single nucleotide changes at positions 1 to 8 and changes in the U tract (Harmon *et al.,* 2001). In this study, we extended the analysis of the M GE to include position −1, which provided a more complete characterization of the GE sequence. It is not known whether the nucleotide changes we made in the M GE would have similar effects on the SH GE, given the nucleotide differences at positions −1 and 8 (Table 1). Available evidence, however, suggests that multiple nucleotide changes in the GE have cumulative effects. We showed that nonconsensus nucleotides at positions −1, 3, and 6 (Fig. 4B) or positions 1, 2, and 4 (Fig. 4C) had cumulative effects on termination. Thus, our mutational analysis of the M GE may be generally useful for predicting the efficiency of termination directed by any GE sequence.

The inclusion of position −1 in the consensus GE sequence may also help to resolve two apparent discrepancies between previous studies. First, previous data showed that termination was reduced to a low level by the introduction of G at position 6 of the M GE (Harmon *et al.,* 2001), whereas termination was moderately efficient at the P and G GEs (Hardy *et al.,* 1999), both of which have G at position 6 (Table 1). The poor termination of the M GE with the U6G mutation likely resulted from the cumulative effects of nonconsensus nucleotides at positions −1 and 6 (Fig. 4A, lane 7). Second, when the 13-nt NS1 GE sequence was placed in the replicon M/SH-B, termination was reduced to a very low level, <4% (Harmon *et al.,* 2001), whereas the termination efficiency was 23% at the NS1 GE in a replicon that contained parts of the NS1 and NS2 genes (Hardy *et al.,* 1999). However, when the 13 nt NS1 GE was placed in the replicon M/SH-B, position −1 was effectively changed from A to U (Harmon *et al.,* 2001), which may have further decreased the termination efficiency.

The first intergenic nucleotide

The variable intergenic regions of RS virus were previously reported not to modulate termination when examined in the context of a single GE sequence with a U_6 tract (Kuo *et al.,* 1996). The data presented here, however, show that the first intergenic nucleotide was important for termination with a U_4 tract, but not with a U_6 tract (Fig. 5). Similarly, termination at the GE of SV5 was less efficient when A rather than G or C followed a U_4 tract (Rassa and Parks, 1999; Rassa *et al.,* 2000). RS virus and SV5 are not closely related phylogenetically, and the

consensus sequences upstream of the U tract are different in these viruses (Rassa and Parks, 1998). Therefore, these results raise the possibility that the intergenic region plays a similar role in termination for other paramyxoviruses as well.

The first intergenic nucleotide of RS virus may be important for termination at the four GE sequences that contain U_4 tracts, as three of these four GEs are followed by G or C (Table 1). While this article was being prepared, Sutherland *et al.* (2001) reported that the first intergenic nucleotide of the F/M2 gene junction was important for termination at the F GE, which contains a U_4 tract (Table 1). Further work is required to investigate the role of the intergenic region in transcription of RS virus. Nucleotides in the conserved intergenic region of rhabdoviruses and the nonconserved intergenic regions of paramyxoviruses have been shown to affect termination, but the reasons for these effects are unknown (Barr *et al.,* 1997b; Rassa and Parks, 1999; Rassa *et al.,* 2000; Stillman and Whitt, 1997).

The mechanism of polyadenylation in the *Mononegavirales* is thought to involve multiple cycles of realignment or slippage between the A tract in the mRNA and the U tract in the template (Barr and Wertz, 2001). Apparently, paramyxovirus RNA polymerases can carry out polyadenylation and termination efficiently on a U tract as short as four residues, depending on the first intergenic nucleotide, based on studies of RS virus (Fig. 5), SV5 (Rassa and Parks, 1999; Rassa *et al.,* 2000), and paramyxovirus GE sequence comparisons (Kolakofsky *et al.,* 1998). By contrast, the RNA polymerase of the rhabdovirus VSV requires a U_7 tract to carry out slippage, polyadenylation, and termination of mRNAs (Barr and Wertz, 2001; Barr *et al.,* 1997a; Hwang *et al.,* 1998). Although the template–mRNA duplex has not been characterized biochemically for these viruses, the available data suggest that the template–mRNA duplex involved in polyadenylation for paramyxoviruses (4 bp) is significantly shorter than the 8- to 9-bp duplex that is critical for the elongation phase of transcription of many DNA-dependent RNA polymerases (Kireeva *et al.,* 2000; Korzheva and Mustaev, 2001; Nudler *et al.,* 1997; Sidorenkov *et al.,* 1998). Slippage involving shorter duplexes (3 bp) does occur during the initiation phase of transcription by *Escherichia coli* RNA polymerase, and this is an important mechanism of gene regulation (Han and Turnbough, 1998; Liu *et al.,* 1994; Tu and Turnbough, 1997). Further studies are required to investigate how the process of slippage in RS virus is affected by the U-tract length and other sequences, including the first intergenic nucleotide.

Termination and the control of gene expression

A full appreciation of the RNA sequences that control termination in RS virus is important for understanding the broader role of termination in the viral life cycle. At some gene junctions, GE sequences that result in inefficient termination are conserved among different strains of RS virus, while at other gene junctions, GE sequences giving more efficient termination tend to be conserved (Johnson and Collins, 1988; Zamora and Samal, 1992; R. Moudy, S. Harmon, W Sullender, and G. Wertz, unpublished data). Inefficient termination at some GEs may have been selected to downregulate expression of the gene immediately downstream, since termination is required for downstream initiation, as shown previously (Hardy *et al.,* 1999; Harmon *et al.,* 2001; Hummel *et al.,* 1994; Kuo *et al.,* 1996). Extending this previous work, we showed that transcription of a monocistronic mRNA was regulated over a broad range by the termination efficiency of the upstream gene (Fig. 4C). In addition, inefficient termination at a GE reduces transcriptional attenuation at that particular gene junction, which is expected to increase expression of all genes downstream of that gene junction, other than the gene immediately downstream (Hardy *et al.,* 1999; Parks *et al.,* 2001; Wertz *et al.,* 2002). Such reduction of attenuation may be important to allow sufficient expression of genes at the 5′ end of the genome of RS virus, which has nine gene junctions and thus more potential attenuation steps than viruses with fewer gene junctions such as VSV.

Regulation of termination and gene expression has been observed in other *Mononegavirales,* including measles virus, SV5, and VSV (Cattaneo *et al.,* 1986; Hummel *et al.,* 1994; Lamb and Kolakofsky, 1996; Parks *et al.,* 2001; Quinones-Kochs *et al.,* 2001; Rassa and Parks, 1998, 1999; G. Wertz *et al.,* 2002). Unlike other *Mononegavirales,* RS virus further regulates termination by expression of the *trans*-acting M2-1 protein, which reduces nonspecific termination within genes and increases the production of readthrough mRNAs (Collins *et al.,* 1996; Fearns and Collins, 1999; Hardy *et al.,* 1999; Hardy and Wertz, 1998). The transcriptional activity of the M2-1 protein is itself modulated by phosphorylation (Cartee and Wertz, 2001; Hardy and Wertz, 2000). Taken together, these findings suggest that RS viral transcription is regulated by several diverse mechanisms besides transcriptional attenuation from 3′- to 5′-proximal genes. Further work will investigate the roles of *cis*- and *trans*-acting factors in gene regulation and replication of RS virus.

MATERIALS AND METHODS

Plasmid constructions

Plasmid pSH/GFP (Fig. 1A), which encoded a dicistronic replicon containing the SH/G gene junction, was assembled using PCR. The negative-sense replicon RNA transcribed by T7 RNA polymerase from this plasmid contained the following sequences from 3′ to 5′, in which nucleotide numbers in parentheses refer to the se-

quence of the RS virus genome, A2 strain (Accession Nos. M11486 and M75730): the RS viral leader (A2 nt 1 to 44); the first 48 nt of the NS1 gene (A2 nt 45 to 92), with a single nt change at A2 nt 90 that resulted in the creation of an *Avr*II site; nucleotides 10 to 410 of the SH gene (A2 nt 4229 to 4628); the 44 nt SH/G intergenic region (A2 nt 4629 to 4672); the 9 nt G GS sequence (A2 nt 4673 to 4682); an inserted *Kpn*I site; a 734-nt sequence containing the GFP open reading frame from pGreenLantern-1 (Life Technologies); an inserted *Pst*I site; the 13-nt SH GE sequence (A2 nt 4616 to 4628); and the RS viral trailer (A2 nt 15,068 to 15,222).

In the plasmids pSH-12, pSH-24, pSH-36, and pSH-48, 12-nt-long segments of the SH gene upstream of the SH GE in pSH/GFP were replaced with nonviral sequences using PCR. Each nonviral sequence contained an *Xba*I site and was of similar base composition to the viral sequence. In SH-56, 5 nt transversions were made in a purine-rich region of the template (A2 nt 4551 to 4563), which is found in the SH gene of bovine and human RS virus isolates. In pSH+8G, an A-to-G change was introduced at position 8 of the SH GE (A2 nt 4623) near the end of gene 1 in pSH/GFP. In pSH-NP, the SH/G gene junction in pSH/GFP (66 nt, A2 nt 4616 to 4682) was replaced with the N/P gene junction (23 nt, A2 nt 2315 to 2337).

Plasmid pM/SH-term, which encodes a dicistronic replicon containing the M/SH gene junction (Fig. 2), was similar to pM/SH-B (Harmon *et al.,* 2001), except that gene 2 consisted of the entire SH gene (including the SH GE; A2 nt 4219 to 4628), and gene 2 was followed by 23 nt of the SH/G intergenic sequence (A2 nt 4629 to 4651), an inserted G residue, the N GE sequence (A2 nt 2315 to 2327), and trailer (A2 nt 15,068 to 15,222). Thus, gene 2 was followed by two tandem GE sequences to increase the termination efficiency of gene 2. Additionally, a *Kpn*I site was inserted downstream of the GS of gene 2, between nt 9 and 10 of the SH gene (A2 nt 4227 and 4228). Plasmid pM/SH-term was assembled using PCR. In pM/SH-12, the 12 nt upstream of the M GE in pM/SH-term (A2 nt 4185 to 4196) were replaced with the 12 nt upstream of the SH GE (A2 nt 4604 to 4615). In pM/SH-5, the 2 nt upstream of the M GE in pM/SH-term, 3′-AU-5′, were replaced with the 2 nt upstream of the SH GE, 3′-CA-5′, and an additional U-to-A change was introduced at position −12.

Plasmid pM/SH-Eco was identical to pM/SH-term except that an *Eco*RV site was created in gene 2, 13 nt upstream of the SH GE, by changing A2 nt 4598 to 4603 from 3′-GAUAGG-5′ to 3′-CUAUAG-5′, using the Quikchange method and *Pfu* DNA polymerase (Stratagene). Mutations at positions −1, 3, and 6 of the M GE in pM/SH-Eco were generated by PCR. Mutations at positions 1, 2, and 4 of the M GE in pM/SH-B (Harmon *et al.,* 2001), or changes in the U-tract length and first intergenic nucleotide of the M/SH gene junction in pM/SH-B, were also generated by PCR.

Standard recombinant DNA techniques were used throughout this study, and the relevant DNA sequences of all plasmids were confirmed by dideoxy sequencing (Sambrook and Russell, 2001). All RS viral gene sequences used were derived from the A2 strain, except as noted.

Cells, transfections, and RNA analysis

HEp-2 cells, a HeLa-derived cell line (Chen, 1988) widely used for propagation of RS virus, were obtained from American Type Culture Collection and grown in minimal essential media (MEM, Life Technologies) supplemented with 5% heat-inactivated fetal calf serum (FCS, Summit Biotechnology). MVA-T7, an attenuated vaccinia virus expressing T7 RNA polymerase (Wyatt *et al.,* 1995), was obtained from B. Moss and propagated at 31°C in chicken embryonic fibroblasts (Charles River SPAFAS), which were grown in MEM with 10% FCS.

RS viral transcription from dicistronic replicons was analyzed using a vaccinia-T7 expression system, essentially as described previously (Harmon *et al.,* 2001). Briefly, HEp-2 cells were infected with MVA-T7 and transfected using Lipofectin (Life Technologies) with plasmids containing T7 promoters, which expressed a negative-sense dicistronic replicon RNA and the N, P, L, and M2-1 proteins of RS virus, all at 37°C. Because the M2-1 protein increases the production of readthrough RNAs, M2-1 protein expression was held constant in all experiments, as discussed previously (Harmon *et al.,* 2001). Transfection efficiency and GFP expression from SH/GFP replicons were monitored qualitatively using an inverted fluorescence microscope. To assay RNA synthesis from replicons, cells were exposed to [^{3}H]uridine in the presence of actinomycin-D (10 μg/ml) from 16 until 22 h after transfection. Total RNA was isolated using RNeasy columns (Qiagen), analyzed by gel electrophoresis (1.75% agarose/6 M urea/25 mM citrate), and detected by fluorography.

Quantitation of termination

The mRNA and readthrough RNA species transcribed from all dicistronic replicons were identified by annealing with specific oligonucleotides followed by digestion with RNase H, as described elsewhere (Hardy *et al.,* 1999; Harmon, 2002). To analyze termination, each RNA species was quantitated by densitometry and adjusted for uridine content as previously described (Harmon *et al.,* 2001). The termination efficiency at the end of gene 1 was calculated as follows for the replicons SH/GFP and M/SH-B: termination efficiency = 100%(mRNA1)/(mRNA1 + rt1-2 + rt1-2-Tr). The replication products that comigrated with rt1-2-Tr were ignored in this calculation because rep-

lication represented a small amount of the RNA in this band (Harmon, 2002; Harmon *et al.,* 2001).

In M/SH-term and M/SH-Eco, the two tandem GE sequences at the end of gene 2 decreased readthrough from the end of gene 2 into trailer from approximately 50% to less than 5%, as demonstrated by the absence of rt2-Tr RNA synthesis (compare Fig. 2B, lane 1 and Fig. 5B, lane 1). The decreased readthrough at the end of gene 2 allowed the readthrough transcription product (rt1-2) to be resolved from the replication products, and thus the formula for termination efficiency was simplified as follows: termination efficiency = 100%(mRNA1)/(mRNA1 + rt1-2).

In this study, we did not remove the 3′ poly(A) tails of mRNAs by annealing with oligo(dT) followed by digestion with RNase H, which was used in a previous study because it sharpened the migration of RNA bands during subsequent gel electrophoresis (Hardy *et al.,* 1999). However, this technique can result in cleavage of internal oligo(A) stretches in RNAs (Barr and Wertz, 2001). Such cleavage of readthrough RNAs could affect the quantitation of termination, a possibility we wished to avoid. The use of RNase H treatment was found to affect the quantitation of termination efficiency slightly in various replicons, but whether this was due to cleavage of readthrough RNAs and/or alteration of RNA migration is not known. For example, when RNase H treatment was used, the average termination efficiency quantitated for the previously described replicon SH/G was 95% (Hardy *et al.,* 1999), whereas when RNase H treatment was omitted, the average termination efficiency for this replicon was 90% (S. Harmon and G. Wertz, unpublished data).

To compare the amounts of monocistronic mRNA1 and mRNA2 synthesis from M/SH-Eco, the trace optical density of each mRNA was quantitated by densitometry (Harmon *et al.,* 2001), divided by the number of uridines in that mRNA, and multiplied by 1000. This calculation yielded quantities with arbitrary units that were proportional to the molar amounts of mRNA1 and mRNA2.

ACKNOWLEDGMENTS

The authors thank past and present members of the Wertz and L. A. Ball laboratories for helpful suggestions on this work. We thank Linda Wyatt and Bernard Moss for providing MVA-T7; Con Beckers for use of a fluorescence microscope; and Kristen Rogers, Sarah Ballard, and Xiaoling Tang for technical assistance. This work was supported by Public Health Service Grant AI20181 from the NIH to G.W.W.

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