Supporting Information

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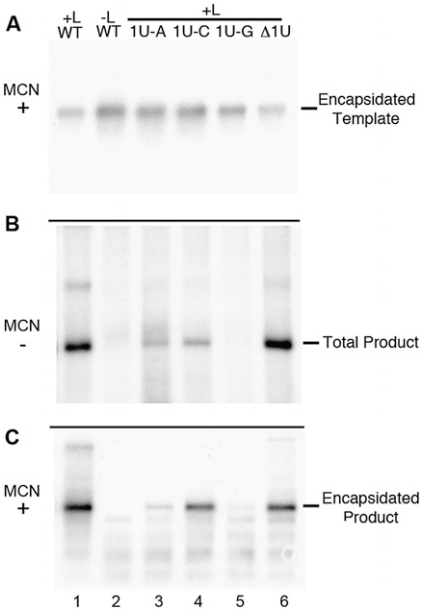

Fig. S1. Northern blot analysis to determine impact of mutating the 3′ terminal nucleotide of the template on RSV RNA replication. (*A*) Northern blot analysis of nuclease-resistant negative sense minireplicon RNA expressed by T7 polymerase in MCN-treated RNA samples. (*B*) Replication products expressed from the mutant minireplicons in total RNA samples (note that because of experimental variation, the Δ1U product levels are somewhat overrepresented in this blot; Fig. S2). (*C*) Nuclease-resistant minireplicon replication products in MCN-treated RNA samples.

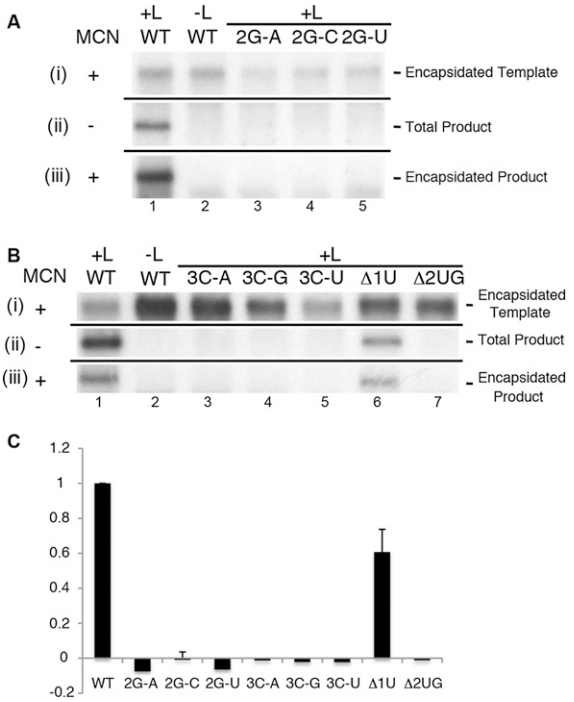

Fig. S2. Impact of mutating template positions 2 and 3 on RSV RNA replication. (*A* and *B*) Northern blot analysis of nuclease-resistant negative sense minireplicon RNA expressed by T7 polymerase in MCN-treated RNA samples (*i*). Replication products expressed from the mutant minireplicons in total (*ii*) and MCN-treated RNA samples (*iii*). (*C*) Quantitation of replication products generated from the mutant minireplicons in total RNA samples. Each RNA value was calculated relative to the WT value (set at 1.0). Each error bar represents the standard error of the mean from at least three independent experiments.

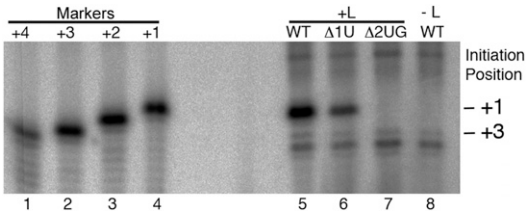

Fig. S3. Impact of deleting template positions 1 and 2 on RNA replication initiation. Primer extension analysis of RNA generated from the Δ2UG minireplicon, using a primer that hybridized at positions 24–48 relative to the +1 initiation product (Fig. 1, primer 1). The Δ2UG RNA was compared with RNA generated from WT and Δ1U minireplicons (compare lanes 5, 6, and 7). Lane 8 is a negative control of RNA from cells transfected with plasmid encoding the WT minireplicon but no L polymerase plasmid. Molecular weight markers present in lanes 1–4 are end-labeled primers, representing products initiated from position +4 to +1 of the template, respectively.

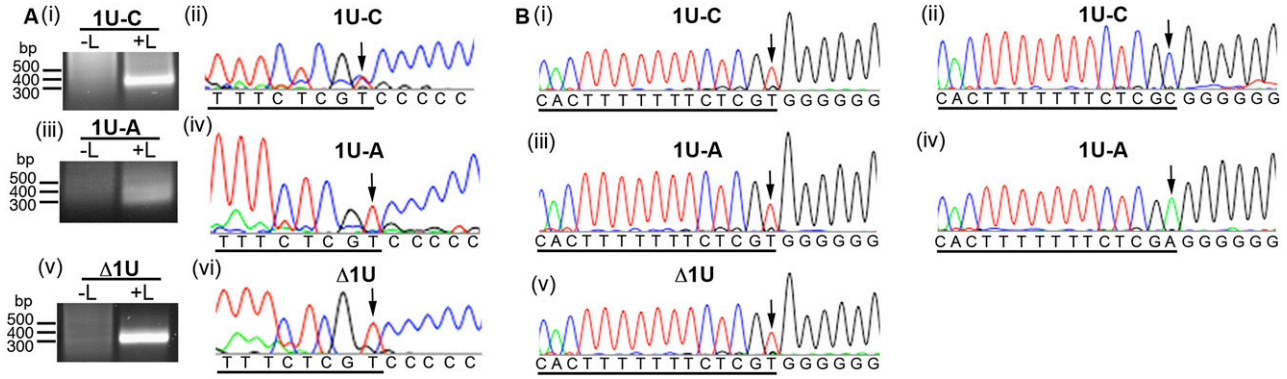

Fig. S4. Further analysis of replication products by 5′ RACE. (*A*) Examples of 5′ RACE analyses in which the cDNA was tailed with dCTP. (*i, iii,* and *v*) Agarose gel electrophoresis of the 5′ RACE product generated from RNA from cells transfected with plasmid encoding the 1U-C, 1U-A, and Δ1U minireplicons, as indicated, and either lacking or containing the L polymerase plasmid; *ii, iv,* and *vi* show the corresponding sequence traces derived from the bulk 5′ RACE product. (*B*) Examples of sequence traces derived from individual 5′ RACE DNA clones (tailed with dGTP). 5′ RACE analyses were performed on RNA from cells transfected with plasmid encoding 1U-C (*i* and *ii*), 1U-A (*iii* and *iv*), and Δ1U (*v*). The majority of clones showed initiation at position 1 with a nontemplated WT nucleotide, as exemplified by *i, iii,* and *v;* however, a small proportion of clones derived from the 1U-C and 1U-A transfections demonstrated templated initiation, as exemplified by *ii* and *iv.* All sequence traces are presented as template sense DNA. TrC sequence is underlined, with position 1 relative to the WT template indicated by an arrow.

Table S1. Sequences of clonal isolates of RSV replication products generated from WT and mutant templates

Template	Transfection	Replication product sequence (nucleotides 1–6)	Frequency*
WT	1	5′ ACGAGA	11/16
		5′ UCGAGA[†]	3/16
		5′ ACGGGA	1/16
		5′ UACGAGA	1/16
WT	2	5′ ACGAGA	16/18
		5′ ACGAG–[‡]	2/18
Δ1U	1	5′ ACGAGA	34/34
Δ1U	2	5′ ACGAGA	24/24
1U-A	1	5′ ACGAGA	10/12
		5′ UCGAGU	1/12
		5′ ACGGGA	1/12
1U-A	2	5′ ACGAGA	19/24
		5′ UCGAGA	1/24
		5′ AGGAGA	1/24
		5′ ——GAGA	1/24
		5′ —— AGA	1/24
		5′ ————A	1/24
1U-C	1	5′ ACGAGA	18/23
		5′ GCGAGA	1/23
		5′ AUGAGA	1/23
		5′ ACGAGG	1/23
		5′ UGACGAGA	1/23
		5′ GACGAGA	1/23
1U-C	2	5′ ACGAGA	15/23
		5′ GCGAGA	2/23
		5′ ACGGGA	1/23
		5′ ACGAG–	1/23
		5′ GCGAG–	1/23
		5′ –CGAGA	2/23
		5′ UCACGAGA	1/23

*Frequency is given as number of clones with given sequence/ total number of sequenced clones for each transfection.

[†]Substitution mutations relative to WT sequence are underlined.

[‡]Deletions are indicated by hyphen. Note that mutations might have been created during the process of T7 transcript generation, or during 5′ RACE, in addition to being introduced by RSV polymerase.