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SHORT COMMUNICATION

Tandem Placement of a Coronavirus Promoter Results in Enhanced mRNA Synthesis from the Downstream-Most Initiation Site

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Insertion of the 17-nucleotide promoter region for the bovine coronavirus N gene as part of a 27-nucleotide cassette into the open reading frame of a cloned synthetic defective-interfering (DI) RNA resulted in synthesis of subDI RNA transcripts from the replicating DI RNA genome. Duplicating and triplicating the promoter sequence in tandem caused a progressive increase in the efficiency of subgenomic mRNA synthesis despite a concurrent decrease in the rate of DI RNA accumulation that was not specific to the promoter sequences being added. Although initiation of transcription (leader fusion) occurred at each of the three promoter sites in the tandem construct, almost all of the transcripts were found as a product of the most downstream (3′-most on the genome) promoter. These results show that enhancement of subgenomic mRNA synthesis is a property that can reside within sequence situated near the promoter. A possible role for the plus strand in the downstream promoter choice is suggested. © 1996 Academic Press, Inc.

During bovine coronavirus replication, a 3′-coterminal nested set of subgenomic mRNAs that coordinately peak in abundance at around 6 hr postinfection is made. At this time, the mRNA species are not equal in number, but rather the shorter 3′-proximal species are progressively more abundant. The molar differences range from 1 for genome (mRNA 1) to 1000 for mRNA 7 (N gene mRNA), the smallest mRNA species (*1*). Although exceptions occur, this general pattern of mRNA regulation holds true for all coronaviruses (reviewed in *2*).

Explanations for the differing mRNA levels have reflected views on how mRNAs are generated. In the leader-primed transcription model, mRNAs are generated from a genome-length minus strand (antigenome) by priming from a free leader at intergenic promoter sites (*3, 4*). The original model predicted that the degree of base complementarity between the promoter region and the priming free leader would determine promoter strength. Although this prediction was supported by the degree of base pairing and mRNA accumulation rates in murine hepatitis virus (MHV) (*5, 6*), the same correlation was not found in infectious bronchitis virus (*7*), calling this mechanism of regulation into question. In addition, Makino and coworkers (*8–10*) and Spaan and coworkers (*11*) have shown with cloned replicating defective-interfering (DI) RNAs of MHV that the rate of mRNA synthesis from an engineered intergenic promoter does not necessarily correlate with the degree of sequence complementarity. Sequences distantly upstream from the promoter in MHV, furthermore, may enhance transcription (*12*).

A second model used to explain the origin of differing mRNA levels (*13*) was based on the observations that subgenomic mRNA-length minus strands function as components of replicative intermediates (*13, 14*). This model predicted that subgenomic minus-strand RNAs are generated by an interruption of minus-strand synthesis at attenuating intergenic sites. A greater abundance of 5′-proximal minus-strand templates would be expected that would, in turn, generate progressively more abundant 3′-proximal mRNAs. Transcription initiation would result from the use of 5′-terminal AGAUUUG promoter motifs or perhaps from a spliced antileader on the subgenomic minus strands (*13*).

A third model was also based on the presence of subgenomic minus-strand RNAs (*1, 14, 15*) but predicted that mRNAs, once generated (by whatever mechanism), would undergo amplification by replication through the use of terminal promoter sequences (*1, 14*). Conceivably, for either model 1 or 2, subgenomic molecules possessing intergenic promoters could function as templates for transcription thus creating a cascading effect that would preferentially amplify 3′-proximal mRNAs (*16*). For models 1 and 2, flanking sequences at intergenic promoter sites might be expected to influence transcription rates.

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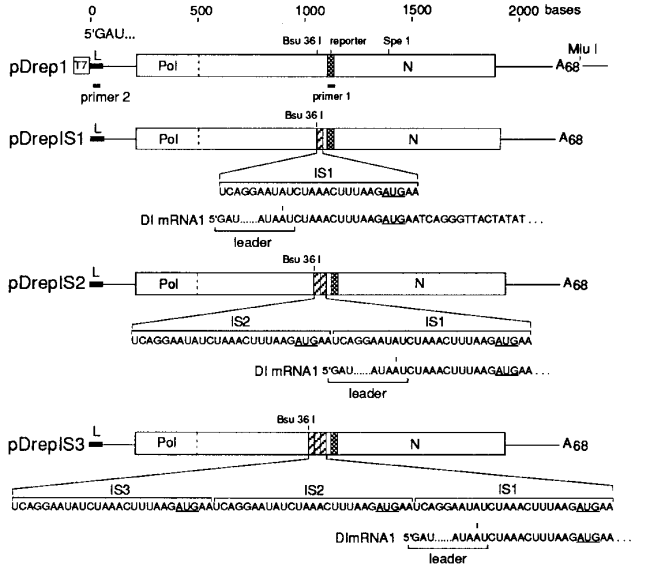

FIG. 1. Structures of the mutated DI RNA genome and of the most abundant subgenomic transcript (mRNA1) arising from each construct. pDrep1 is the cloned reporter-containing BCV DI RNA, and the diagram shows the 5′-terminal bases in the T7 polymerase-generated transcript and positions of the 30-nt reporter sequence, the *Bsu*36.1 restriction endonuclease site, and primers 1 and 2 used for RT–PCR amplification of leader-RNA fusion regions within the DI RNA and subDI RNA transcripts. Structures of pDrepIS1, pDrepIS2, and pDrepIS3 contain 1, 2, and 3 intergenic sequences, respectively. The potential codons for the initiation of translation on the subDI RNA transcripts are identified with an underline.

In this study we have directly examined the effect of a multimerized internal promoter (termed intergenic sequence, IS) on mRNA abundance. For this, a cDNA clone of a naturally occurring bovine coronavirus (BCV) DI RNA engineered to contain a reporter, an in-frame 30-nt sequence from the N gene of porcine transmissible gastroenteritis virus (TGEV), and called pDrep1 (*17*) was used for intergenic sequence insertion (Fig. 1). We have shown that T7 RNA polymerase-generated transcripts of pDrep1 linearized at the *Mlu*1 site undergo replication and subsequent packaging after transfection into helper virus-infected cells (*17*). To make pDrepIS1, two 27mer oligonucleotides, A1 (5′TCAGGAATATCTAAACTTTAAGATGAA-3′) and A2 (5′TGATTCATCTTAAAGTTTAGATATTCC3′), were annealed (creating a *Bsu*36.1-compatible site at its 5′ end, virus sense) and inserted at the unique *Bsu*36.1 site 52 nt upstream of the reporter sequence in pDrep1 (Fig. 1). Upon insertion the *Bsu*36.1 site was regenerated at the 5′ end. To construct pDrepIS2 and pDrepIS3, oligonucleotides A1 and A2 were phosphorylated, annealed, and ligated with *Bsu*36.1-digested, dephosphorylated pDrepIS1. pDrep-gpD81 was generated by cloning an 87-nt blunt-ended fragment (5′ccAAATATGCCTTG-GTGGATGCCTCTCTCAAGATGGCCGACCCCAATCGC-TTTCGCGGCAAAGACCTTCCGgtcctggacgggaatt 3′; lowercase letters refer to primer sequences used in the initial cloning) encoding the 1- to 23-aa neutralizing epitope of herpes simplex virus (HSV) gD (*18*) into the mung bean nuclease blunt-ended *Bsu*36.1 site of pDrep1 (*17*). A 3-nt overdigestion at the *Bsu*36.1 site caused an in-frame insertion of 81 rather than 84 nt.

For transfection and transcription analysis, cells infected with BCV were transfected as previously described (*17*), except that the helper virus was a stock that contained no wild-type DI RNA (as determined by Northern analysis), and 1 μg RNA per 35-mm plate was used for transfection. At 24 hr posttransfection supernatant was collected and used to infect fresh HRT cells from which cytoplasmic RNA was isolated at 9 hr postinfection. RNA was analyzed by quantitative Northern blot hybridization using the AMBIS photoanalytic imaging system (AMBIS, Inc., San Diego, CA) following hybridization with end-labeled oligonucleotide 8 which hybridizes to the plus strand of the TGEV reporter sequence (*17*).

For primer extension analysis, approximately 1 pmol of oligonucleotide 8, 5′-end-labeled with ^{32}P to a specific activity of 1.3×10^6 cpm/pmol (Cerenkov counts), was used with 2.5 μg of cytoplasmic RNA for the primer extension reaction (*19*). Products were analyzed on a DNA sequencing gel of 6% polyacrylamide and quantitated by scanning the autoradiogram with a BioRad imaging densitometer.

For sequencing and cloning of RT–PCR products, cDNA synthesis from cytoplasmic RNA and sequencing of asymmetrically amplified PCR products were carried out as previously described (*20*) except that oligonucleotide 8b, 5′CATGGCACCATCCTTGGCA3′, a 3′-truncated version of oligonucleotide 8, was used for the reverse transcriptase reaction. For sequencing the 5′ end of DI RNA subgenomic mRNAs, oligonucleotide L(−), 5′GCG-GGATGCACGCACGCAAATCGCTC3′, which binds to bases 7 to 33 of BCV leader plus strand, and oligonucleotide 8 were used. For sequencing genomic DI RNA, oligonucleotide N(−), 5′AGAGCGTCCTTTGGAAATCGTTCT-GG3′, which binds to bases 34 to 59 in the N ORF, and oligonucleotide 8 were used. Cloning of PCR-amplified products was carried out as recommended by the manufacturer for TA cloning (Invitrogen).

From studies of genomic sequences for the mammalian coronaviruses BCV (Table 1), MHV, and TGEV, the heptameric intergenic sequence UCUAAAC has emerged as a consensus element postulated to be the core promoter for directing viral subgenomic transcription. This was directly tested by Makino and Joo (*9*) in a study that demonstrated the UCUAAAC heptad to be sufficient for subgenomic transcription from a site within the ORF of a replicating DI RNA of MHV. Promoter activity, however, became optimal when an intergenic sequence of 18 nt from the N gene, a region showing full complementarity to the 3′ end of MHV genomic leader, the postulated primer of transcription in the leader priming model (*3, 4, 23*), was used.

BCV is a close relative of MHV (*24, 25*), yet differs

TABLE 1

Intergenic Sequences on the Bovine Coronavirus Genome

Gene immediately downstream of the intergenic sequence	Intergenic sequence[a]
Pol	5′auaaUCUAAACuuuaua3′[b]
	. .....
N	5′aauaUCUAAACuuuaag3′
M	5′uuaaUCCAAACauuaug3′
9.6 kDa	5′caaaUCCAAACauuaug3′
12.7 kDa	5′uuuaggUAgACcuuaua3′
S	5′auaaUCUAAACauguug3′
HE	5′gaagaCUAAACucagug3′
Consensus	5′UCUAAAC3′
27mer cassette	5′ucaggaauaUCUAAACuuuaagaugaa3′[c]

[a] Documented in Refs. *20–22.*

[b] The 5′-terminal 65-nucleotide BCV genomic leader terminates at base 6 in this sequence. Bases identical between the extended genomic leader and the intergenic sequence immediately preceding the N gene are identified with dots.

[c] Underlined region in the 17-nucleotide intergenic sequence found immediately preceding the N gene. The engineered start codon for subgenomic mRNA is boxed.

from MHV in two respects with regard to the relationship between genomic leader and intergenic sequence. (i) The region of contiguous sequence identity between the extended genomic leader and the intergenic sequence preceding the N gene is 12 not 18 nt (Table 1). (ii) Repetitive UCUAA sequences, thought to yield alternative fusion sites in MHV (*6, 26–28*), do not exist in the BCV leader or its flanking sequence and hence may be a sequence phenomenon related to the invariant mRNA fusion junction patterns found in BCV (*20*). To determine, therefore, whether the analogous region in BCV could direct subgenomic transcription, the analogous 17-nt intergenic sequence from the BCV genome (a base was deleted from the 3′ end to conform to the homologous region as defined in Ref. *7*) (Table 1) was inserted as part of a 27-nt cassette into the unique *Bsu*36.1 site of pDrep1 to form pDrepIS1 (Fig. 1), and transcripts were tested for replication and subgenomic transcription. The cassette was designed to maintain the DI RNA ORF, a *cis*-acting requirement for BCV DI RNA replication (*17*), and to insert an AUG start codon just downstream of the leader on the subgenomic transcript. Both replication of the DI RNA and generation of subgenomic mRNA from pDrepIS1 were observed (results were similar to those in Fig. 2A, lane 2).

To determine what effects multimerization of the promoter sequence would have on subgenomic mRNA synthesis, the cassette was placed in two (pDrepIS2) and three (pDrepIS3) adjacent copy repeats within the pDrep1 ORF sequence, and the rates of subgenomic mRNA and DI genome accumulation for these were compared to those for pDrep1 and pDrepIS1 (Fig. 2A, lanes 1 through 4). Quantitative Northern analysis using the reporter-detecting probe demonstrated that synthetic transcripts of pDrepIS1, pDrepIS2, and pDrepIS3 replicated and generated subgenomic mRNA and that progeny genomes were packaged. Whereas the accumulated amounts of subgenomic mRNA remained nearly the same for pDrepIS1, pDrepIS2, and pDrepIS3, the amounts of DI RNA genome progressively decreased (Fig. 2A, lanes 2–4), resulting in subgenomic to genomic RNA molar ratios of 0.25, 0.65, and 1.20, respectively. This demonstrated an apparent progressive increase in the efficiency of transcription (Fig. 3). To determine whether the inhibition in DI RNA accumulation was a function of the subgenomic transcription process or was possibly the result of another function of an inserted sequence, an 81-nt sequence with no known promoter-like motif was added at the *Bsu*36.1 site to form pDrep-gpD81, and transcripts were tested for replication. An inhibition of DI RNA replication equivalent to that for pDrepIS3 but without subgenomic transcription was found for pDrep–gpD81 RNA (Fig. 2B), suggesting that the inhibition of replication in pDrepIS constructs was not necessarily a result of the transcription but was due to an added sequence at this site in the DI RNA genome. By probing a blot identical to that in Fig. 2A, lanes 1–4, with an oligonucleotide recognizing the N gene sequence, and hence all viral mRNA species, it was determined that virus from passage 1 replicated nearly equally well in all infected plates (Fig. 2A, lanes 5–8).

The pattern of Northern blot analysis (Fig. 2A) suggested that the subgenomic transcripts of pDrepIS1, pDrepIS2, and pDrepIS3 were the same size since incremental differences of 27 nt would have been revealed in an agarose gel of this design. These differences, for example, could be discerned among the DI genomic RNAs. In the first approach used to characterize the leader-mRNA fusion sites for subgenomic mRNAs, RT–PCR analysis (Fig. 2C) was carried out on RNA samples prepared from the first virus passage, the same RNA used for Northern blot hybridization analysis (Fig. 2A). As suggested by Northern hybridization data, there was revealed a preferential usage of the 3′-most promoter for leader fusion. The identity of the abundant DI mRNA band 1 in Fig. 2C, lanes 2, 3, and 4, was further ascertained by direct PCR sequencing, and that of DI mRNAs 1 and 2 by the sequencing cDNA-cloned cDNA products (data not shown). The DI mRNA 3 band could not be cloned as cDNA. These results showed that the downstream-most promoter site was predominantly used by pDrepIS2 and pDrepIS3 and that the junction sequence in each subgenomic transcript demonstrated no heterogeneity and was therefore the same as for N mRNA (*20*; Fig. 1, and data not shown). The DI mRNA 3 fusion site could not be confirmed by sequencing but the position

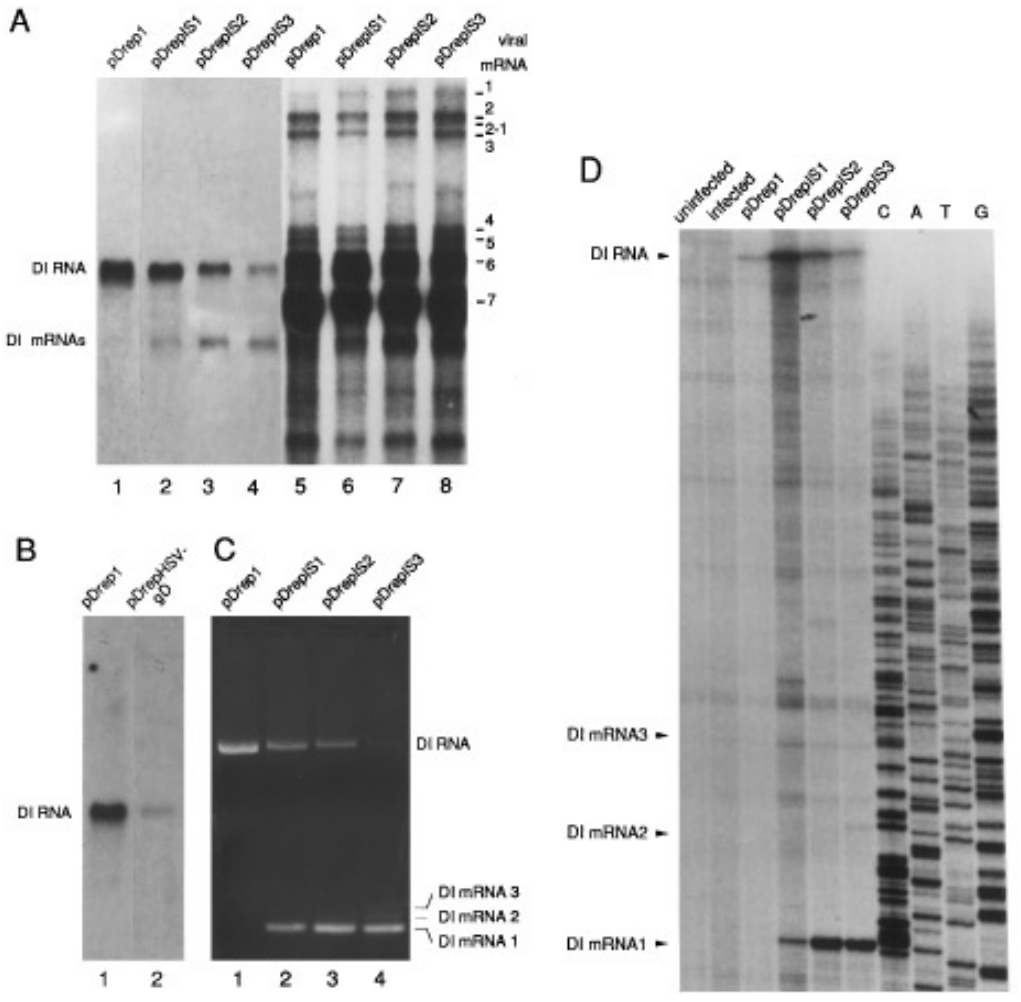

FIG. 2. Effect of tandemizing the internal promoter on accumulation of DI genomic RNA and subgenomic mRNA. (A) Northern blot analysis showing relative amounts of DI genomic RNA and subgenomic mRNAs. Equal amounts of cytoplasmic RNA from cells infected with passage 1 virus harvested from the respective transfection experiment were analyzed with ^{32}P-labeled oligonucleotide probe. Lanes 1 through 4 were probed with reporter plus-strand-detecting probe (oligonucleotide 8) to identify DI RNA genome and subgenomic transcripts, and lanes 5 through 8 were probed with N plus-strand-detecting probe (oligonucleotide N+) to identify helper virus RNA as well as DI RNA species (*17*). (B) Northern blot analysis showing inhibition of DI RNA replication as a function of the 81-nt HSV gD (nonpromoter) insert. Probing was done as described in A, lanes 1 through 4. (C) RT–PCR analysis showing the relative amounts of genomic DI RNA and subgenomic mRNAs. Cytoplasmic RNA described in A was used in an RT–PCR with primers 1 and 2, and the products were analyzed on an agarose gel of 3% and stained with ethidium bromide. (D) Primer extension analysis. Lanes 1 through 6, radiolabeled primer (oligonucleotide 8) which binds to the reporter sequence 52 nt downstream from the IS1 promoter sequence was extended on cytoplasmic RNA obtained from cells as indicated. The extended products were then electrophoresed on a denaturing sequencing gel of 6% polyacrylamide. Lanes 7 through 10, products of dideoxynucleotidyl DNA sequencing using oligonucleotide 8 as the radiolabeled primer and pDrepIS3 DNA as the template. The predicted mRNA positions were determined from the DNA sequence.

of the PCR band (Fig. 2C) indicated its fusion site would be at or near the upstream-most position.

In the second approach to characterize leader-mRNA fusion sites, a primer extension experiment was done on the RNA using radiolabeled oligonucleotide 8 which binds to the DI RNA reporter sequence. Since it can be expected that the primer would bind alike to DI mRNAs and DI genomic RNA, extended products should reflect the relative amounts of template species. Except for the pDrep1 RNA, RNA samples in this experiment were the same as those used in the Northern blot in Fig. 2A. The extended products (Fig. 2D) showed the following: (i) There was a progressive decrease in the amount of DI genomic RNA accumulated with each additional promoter sequence; (ii) there was an apparent progressive increase in the efficiency of transcription as judged by the DI mRNA to genome ratios of 0.56, 1.63, and 3.24, for pDrepIS1, pDrepIS2, and pDrepIS3, respectively; and (iii) the vast majority of transcripts came from the downstream-most (DI mRNA 1) position in pDrep1S2 and pDrepIS3. For pDrepIS2 no DI mRNA 2 was found, and for pDrepIS3 little DI mRNA 2 and no DI mRNA 3 transcripts were found. Although these results support the trend identified by Northern analysis (Fig. 2A), they cannot be given a strict quantitative interpretation since shorter products are favored in enzymatic extension re-

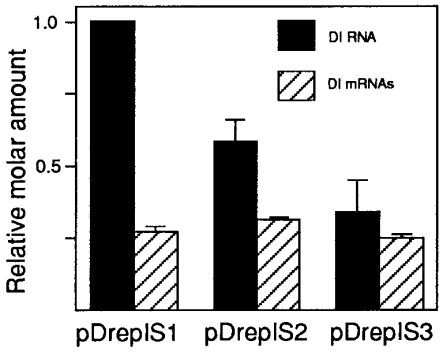

FIG. 3. Relative molar amounts of DI genomic RNA and subgenomic mRNAs as determined by Northern blot analysis. Plotted values represent the means from two separate preparations of synthetic transcripts analyzed as described in Fig. 2A, lanes 2–4. Within each experiment the number of counts in individual RNA bands was compared to that in the pDrepIS1 genome.

actions. To ensure that the replicating molecules had not acquired mutations in the unused promoter regions that might have harmed promoter function, asymmetric RT–PCR sequencing was carried out on cytoplasmic DI genomic RNA bands in Fig. 2A with oligonucleotides 8 and N(−). No mutations were found (not shown).

One possible determinant for the strong initiation of transcription from the IS1 promoter (fusion) site might have been a linkage between the transcription initiation and the closely associated translation start site. This possibility was tested by converting the AUG at base 23 in the IS1 promoter to ACG in the pDrepIS1 construct (Fig. 1) by site-directed mutagenesis. Subgenomic transcription rates were identical with the AUG and ACG codons at this site (data not shown). Thus, the mutation did not prevent subgenomic transcription, making the translation start codon an unlikely cause for the preference of downstream transcription initiation in pDrepIS2 and pDrepIS3.

Our studies with a BCV DI RNA show results consistent with those of Makino and coworkers (*8–10, 29*) and Spaan and coworkers (*11, 30*) with MHV DI RNA in which it was shown first that synthesis of a subgenomic mRNA can be induced by insertion of an intergenic promoter sequence into the DI RNA and second that enhancement of downstream subgenomic mRNA synthesis resulted when two or more promoters were placed in close proximity. This pattern of mRNA synthesis from engineered DI RNAs of two separate coronaviruses, therefore, reflects the general pattern observed from the coronavirus genome and would seem to validate the use of DI RNA molecules for deciphering the mechanisms of mRNA abundance regulation. The results of our experiments did not allow us to discern among the postulated templates for transcription, which include the DI antigenome, nonreplicating subgenomic minus strands, and replicating subgenomic minus strands (corresponding to the three models of transcription described above). They do, however, demonstrate that a local sequence surrounding a promoter region in the DI RNA genome can direct an enhanced synthesis of subgenomic mRNA when synthesis of that RNA is quantitated as a ratio of DI template to subDI mRNA.

How might the downstream initiation site be determined in a tandem construct? An explanation we favor is a scheme that is most consistent with the model in which minus-strand RNA synthesis is attenuated at intergenic sites to yield subgenomic minus-strand templates for transcription (*13*; model 2 above). The nascent mRNA might then continue to amplify (*1, 14*; model 3 above). We speculate that the downstream selection involves the positive-strand RNA in a template that is at least partially double-stranded. This view stems first from the observation that nearly all coronavirus minus-strand RNAs, genomic and subgenomic, are found as components of membrane-bound replication complexes in which the plus and minus strands exist in nearly equimolar amounts (*31*) as apparent components of transcriptionally active double-stranded molecules (*13, 32*). In addition, at least one viral protein, N, and a 55-kDa cellular protein have been shown to bind to plus-strand leader in the region of the UCUAAAC consensus sequence (*33–35*), suggesting that they may take part in transcription initiation. The potential of N to multimerize (*24, 26*) might also contribute to protein–protein interactions of the kind postulated for assembly of coronavirus transcription complexes (*11, 12*). Many features of this view find precedent in poliovirus for which both viral and cellular proteins bind to the 5′ terminus of the plus-strand genome and contribute mechanistically to initiation of plus-strand synthesis (*37, 38*).

Thus, transcription factors could bind to plus strands at intergenic sites and interact with polymerase and other cofactors to initiate transcription on nascent subgenomic minus strands as they are generated. In this scheme, the most downstream site in the promoter series would be chosen because it would have associated with it the first assembled complex encountered by the approaching polymerase during minus-strand synthesis. On the basis of recent studies with high-frequency leader recombination in BCV (*39*), we further envision that the polymerase might switch templates near the region of the intergenic promoter to copy a leader (possibly one on genomic or subgenomic mRNAs) and thereby generate an antileader. The nascent antileader-containing minus strands would then serve as templates for synthesis of leader-containing subgenomic plus strands and could conceivably be repeatedly used as template were mRNA to replicate. A recent demonstration of *in vivo* transcription from the termini of subgenomic minus-strands would seem consistent with this model (*40*). Our experimental system, furthermore, may reflect a mechanism used by BCV in a natural case of strong downstream selection between two adjacent promoters (*20*). During synthesis of the

mRNA for the BCV 12.7-kDa putative nonstructural protein, the nonconforming GGUAGAC promoter sequence is chosen over a conforming UCCAAAC promoter sequence mapping just 15 nucleotides upstream. Possibly a locally high concentration of transcription factors is responsible for this choice.

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