Positive-strand RNA viruses that encode multiple cis-
trons often mediate expression of 3'-encoded open read-
ing frames via RNA-templated transcription of sub-
genomic (sg) mRNAs. Tomato bushy stunt virus (TBSV) is a
positive-strand RNA virus that transcribes two such sg
mRNAs during infections. We have previously identified
a distal element (DE), located −1100 nucleotides up-
stream from the initiation site of sg mRNA2 transcription,
part of which must base pair with a portion of a core
element (CE), located just 5′ to the initiation site, for
efficient transcription to occur (Zhang, G., Slowin-
ski, V., and White, K. A. (1999) RNA 5, 550–561). Here we
have analyzed further this long distance RNA-RNA in-
teraction and have investigated the regulatory roles of
other subelements within the DE and CE. Our results
indicate that (i) the functional base-pairing interaction
between these elements occurs in the positive strand
and that the interaction likely acts to properly position
other subelements, (ii) two previously undefined subele-
ments within the DE and CE are important and essen-
tial, respectively, for efficient sg mRNA2 accumulation,
and (iii) the production of (−)-strand sg mRNA2 can be
uncoupled from the synthesis of its (+)-strand comple-
ment. These data provide important insight into the
mechanism of sg mRNA2 transcription.

Many (+)-strand (i.e. messenger-sensed) RNA viruses pos-
sest polyestronic coding organizations (1). However, this cod-
ing strategy poses a problem for translation of sequentially
encoded open reading frames (ORFs)\(^1\) within these genomes.
The difficulty arises due to the 5′ to 3′ and linear nature of
conventional ribosome scanning, which generally allows for
only the first ORF encoded in a message to be translated
efficiently (2). A coping strategy used commonly by a wide
variety of (+)-strand RNA viruses is to synthesize smaller viral
mRNAs via RNA-templated transcription (3). These so-called
subgenomic (sg) mRNAs represent 5′-terminally encoded 3′-etermal
copies of the genome that permit efficient translation of their
most 5′-proximal ORFs (3).

There is compelling evidence for two distinct mechanisms for
the transcription of sg mRNAs. The first mechanism, which has
been studied most extensively in Brome mosaic virus, involves
the initiation of transcription at a localized internal promoter
within the full-length (−)-strand of the genome (4, 5). The second
defined mechanism, which occurs in the arteriviruses, involves
the discontinuous synthesis of (−)-strands that are then used as
templates in the production of sg mRNAs, which include a portion
of the 5′-untranslated region of the genome (6, 7). A third
mechanism that has been proposed is that of premature termi-
nation of (−)-strand synthesis during the copying of the genome
and subsequent use of the 3′-truncated product as a template for
the transcription of sg mRNAs (8–10). Although there is no
compelling experimental evidence validating this latter model, it
is supported indirectly by the observation that (−)-strand sg
mRNAs do accumulate in various (+)-strand RNA viral infec-
tions (8, 11–13). However, the origin and function of these (−)-
strand sg mRNAs remain to be determined.

Tomato bushy stunt virus (TBSV) is the prototype member of
the family Tombusviridae (14). Its (+)-strand RNA genome is
4.8 kilobases in length and encodes five functional ORFs (see
Fig. 1A). The 5′-terminally encoded p33 and its readthrough
product p92 are the only viral proteins required for viral RNA
synthesis, and both are translated directly from the viral gen-
ome (15). The three ORFs encoded more 3′ in the genome are
involved in viral assembly, movement, and suppression of host
defense mechanisms and are translated from two sg mRNAs
that are synthesized during infections (Fig. 1A) (16, 17). RNA
sequence elements within the genome that are required for
transcription of the smaller sg mRNA2 have been identified
previously (18, 19). Efficient transcription of this message re-
quires sequences both at the site of initiation, termed the core
element (CE), as well as sequences some ~1100 nucleotides
(nt) upstream from the start site, termed the distal element
(DE) (Fig. 1A). The DE and CE can each be divided further into
subelements A, B, and C based on their relative positions
and/or structural properties. The sequence of the CE-C subele-
ment is highly conserved within tombusvirus genomes and is
located just 5′ to the sg mRNA2 initiation site (Fig. 1C) (18).
The DE-C element is not well conserved within the genus
Tombusvirus and displays no significant complementarity to
CE-C. In contrast, the DE-A and CE-A subelements and the
DE-B and CE-B subelements are complementary to each other,
and previous studies have provided compelling evidence that
base pairing of the former pair is required for efficient sg
mRNA2 synthesis (Fig. 1, B and C) (18). A functional role for
the DE-A/CE-A base-pairing interaction was supported by com-
parative sequence analysis of tombusvirus genomes as well as
deletion and compensatory-type mutational analyses (18).
Interestingly, long distance base-pairing interactions have also
been found to be important for sg mRNA transcription in the
unrelated virus Potato virus X (20, 21). This suggests that long
range RNA interactions involved in regulating sg mRNAs may
be a common feature in diverse groups of (+)-strand RNA
viruses. However, the functions of these far-spanning interac-
functions may be quite different within the unique contexts of these distinct viral genes.

In a previous study we confirmed the functional requirement for the DE-A/CE-A base-pairing interaction (18); however, these analyses did not determine whether this interaction occurred in the (+)- or (−)-strand of the genome nor did they investigate the specific role of the interaction in sg mRNA2 transcription. To address these questions and to determine the possible roles of other yet uncharacterized subelements, we have carried out additional analyses of the DE and CE. Our results indicate that the DE-A/CE-A base-pairing interaction is functional in the (−)-strand and that the DE-C and CE-C subelements represent newly defined components that are important and essential, respectively, for efficient sg mRNA transcription. Additionally, by localizing both the DE and CE at different positions within the genome, we have deduced that the likely function of the DE-A/CE-A interaction is to position other subelements optimally. Furthermore, mutational analysis of the initiating nucleotide for sg mRNA2 transcription has provided clues to the origin of (−)-strand sg RNAs and, together with other results, provided important new insight into the mechanism of sg mRNA2 transcription.

**Experimental Procedures**

**Plasmid Construction**—The plasmid T-100, containing a full-length cDNA copy of the wild type (WT) TBSV genome, has been described previously (14). Construction of mutant derivatives of T-100 containing modifications to the DE and/or CE (+ i.e. Psg20/26 and Psg1) have also been described (18). All mutant viral constructs used in this study were generated by standard recombinant DNA cloning techniques and polymerase chain reaction (PCR)-based oligonucleotide-mediated mutagenesis (22) and are derivatives of the previously described constructs pT-100, Psg20/26, or ΔPsg1. Additionally, new mutant constructs were sequenced across PCR-amplified regions to confirm that only the desired modifications were present.

Mutants Psg20/26m1 through Psg20/26m6, as well as Psg20/26m1/4, Psg20/26m2/5, and Psg20/26m3/6, were derivatives of Psg20/26 (18). For Psg20/26m1 through Psg20/26m3, primer pairs PM1 (5′-CAACCTGCAGCTCTAATTTAGTGTGTCCTGCGGCTCTC-AGAAGGGGCCUCUU) or PG50 (5′-GCCCCCAAGCGTGCAAGGGAAGAGAAATACACA1CACT-TAG-AGAAGGGGCCUCUU) and Psg20/26m4 through Psg20/26m6, primer pairs PB30 and PG51 (5′-CCGGTCGACCGAGGCTGCTTGCTCTGGCGAAAGATCGGTT-AGAAGGGGCCUCUU), or PG52 (5′-CCGGTCTCTGAGCTAAATTCTGTGTTTCTTGGTAGTTGAG) that maintained the A5′-3′ strand viral RNAs were precipitated in ethanol with 0.1M sodium acetate rather than 2 M ammonium acetate. For (−)-Strand RNAs were detected by Northern blotting following electrophoretic separation of glyoxal-treated samples (22). Minus-sensed viral RNAs were detected with a radiolabeled oligonucleotide probe (P9) complementary to the 3′-end-labeled oligonucleotide probe (P9) complementary to the 3′-terminal 23 nt of the TBSV genome (23). (−)-Strand RNAs were detected by Northern blotting following electrophoretic separation of glyoxal-treated samples (22). Minus-sensed viral RNAs were detected with a radiolabeled oligonucleotide probe (P9) complementary to the 3′-terminal 23 nt of the TBSV genome (23).
we sought to answer this question regarding polarity by introducing base substitutions into DE-A or CE-A that would progressively and preferentially destabilize the DE-A/CE-A base-pairing interaction in either the (+) or (−)-strand (Fig. 2A). These mutations were introduced into the previously described mutant genome Psg20/26 that is amenable to modification due to the introduction of convenient restriction enzyme sites near the DE-A and CE-A subelements (18). Psg20/26 lacks both the DE-B and CE-B subelements (Fig. 2A) but maintains sg mRNA2 accumulation at ~80% that of the WT genome (18). Preferential (−)-strand destabilization was accomplished by introducing one, two, or three GU base pairs into DE-A/CE-A (via nucleotide substitutions) within the (+)-strand of Psg20/26, thereby creating mutant derivatives Psg20/26m1, Psg20/26m2, and Psg20/26m3, respectively (Fig. 2A). These non-Watson-Crick GU base pairs were predicted to be less destabilizing to the (+)-strand interaction than to the corresponding (−)-strand interaction that would contain complementary CA mismatches (Fig. 2A). Conversely, one, two, or three CA mismatches were introduced into DE-A/CE-A in the (−)-strand of Psg20/26 to preferentially destabilize the (−)-strand interaction, thereby creating Psg20/26m4, Psg20/26m5, and Psg20/26m6, respectively (Fig. 2A).

Psg20/26m4 through Psg20/26m6, which were predicted to be preferentially destabilized in the (−)-strand interaction, showed a more pronounced progressive maximal decrease in sg mRNA2 accumulation to ~20% that of the Psg20/26 control. These data indicate that the (+)-strand DE-A/CE-A interaction is more sensitive to disruption than its (−)-strand counterpart and thus favors a functional role for this interaction in the (−)-strand.

Alternatively, the more prominent inhibitory effects observed for Psg20/26m4 through Psg20/26m6 could be related to the changes in nucleotide identities in DE-C (as opposed to their base-pairing potential). To address this possibility we systematically restored base-pairing competence for Psg20/26m4, Psg20/26m5, and Psg20/26m6 by introducing additional substitutions into DE-A that would regenerate base pairing while maintaining the original substitutions in CE-A, thus creating Psg20/26m1/4, Psg20/26m2/5, and Psg20/26m3/6, respectively (Fig. 2A). Analysis of these mutants showed that sg mRNA2 levels were near Psg20/26 control levels in all cases (Fig. 2C). Since the original U to C substitutions were retained in these mutants, the dominant functional property is likely (−)-strand base pairing rather than nucleotide identity.

Analysis of Viral Genomes Containing Localized DE/CEs—In the TBSV genome the DE and CE are separated by ~1100 nt. The reason for the long distance positioning of these two elements is not clear but may be related to the mechanism(s) by which they operate. In an attempt to gain insight into the function of these elements, we created a series of mutants in which the two elements were positioned directly adjacent to one another in the primary sequence. In these mutant genomes, the ~1100-nt-long sequence normally separating the DE and CE was reduced to a 6-nt-long restriction enzyme site (XbaI, Fig. 3A). For construction of the first set of mutants (C-series, Fig. 3B), the previously generated mutant genome ΔPsg1 was used as the base construct (18). In ΔPsg1, sg mRNA1 synthesis is completely inactivated by five nucleotide substitutions in and near its site of initiation (18). Additionally,
and more relevant to this work, the DE is deleted from its
natural upstream position (Fig. 3A), resulting in only very
low levels of sg mRNA2 accumulation (~10% that of T-100, Fig.
3C). To determine whether sg mRNA2 synthesis could be re-
stored by reintroducing the DE proximal to CE in ΔPsg1, the
entire DE sequence was inserted just 5' to the CE (Fig. 3B).
The ΔPsg1 derivative generated, Psg55C (Fig. 3, A and B), was
then inoculated into protoplasts to assess the effect on sg
mRNA2 accumulation. Northern blot analysis of accumulating
viral RNAs revealed a considerable increase in sg mRNA2
levels to ~50% that of the T-100 control (Fig. 3C). Additional
mutants were also constructed in which various subelements
were deleted. In Psg51C and Psg50C, either the DE-B/CE-B
subelements or both the DE-B/CE-B and DE-A/CE-A subelements,
respectively, were deleted from their localized positions (Fig. 3A). These
modifications resulted in relative sg mRNA2 levels of ~25 and ~40%,
respectively (Fig. 3C). These results suggest that neither the DE-B/CE-B nor the DE-A/CE-A subelement interactions are required for the DE-mediated in-
crease in sg mRNA2 accumulation observed and instead impli-
cate DE-C as a possible key subelement for improved activity.

To further test this latter concept, the DE-C subelement was
deleted from the Psg55C and Psg51C contexts to generate
Psg36 and Psg56, respectively (Fig. 3A). Both of these DE-C-
lacking genomes exhibited reduced levels of sg mRNA2 accumu-
lation similar to that of ΔPsg1 (Fig. 3C), supporting an
important role for DE-C within this CE-localized context.

Next the same group of localized DE/CE segments (Fig. 3A)
was repositioned near the normal DE locale. The structure of the
localized DE/CEs in this second set of mutants was identi-
cal to those in the first; however, the genomic context differed.
Although these mutants were also derivatives of ΔPsg1, their
genomes were ~1100 nt shorter than ΔPsg1 due to the absence
of the normal intervening sequence between the DE and CE in
these constructs (Fig. 3D). Consequently the genomes of these
mutants exhibit increased electrophoretic mobility (Fig. 3E).
This particular genomic structure was chosen over an alterna-
tive in which the CE would be deleted from its normal location
and inserted just 3' to the DE (while maintaining the original
~1100 nt separation between the DE and CE) because the resulting sg mRNA trans-
scribed from such a mutant would be ~1100 nt longer than the
normal sg mRNA2. This highly modified sg mRNA would be
predicted to have significantly altered properties that would
invalidate any comparisons with WT sg mRNA2. The analysis
of Psg55D, which contained the entire CE element positioned
near the DE locale, revealed an average sg mRNA2 level ~70%
that of T-100 (Fig. 3E). Deletion of the DE-B/CE-B or DE-B/
CE-B and DE-A/CE-A subelements in Psg51D and Psg50D,
respectively, resulted in rather modest decreases in sg mRNA2 accumulation levels to ~60% and ~40%, respectively (Fig. 3E). This lack of strong dependence on these subelements is similar to that witnessed for the corresponding mutants localized at the CE (Fig. 3C) and again suggests that the DE-B/CE-B and DE-A/CE-A subelements are dispensable for the basal activity observed (i.e. >3-fold over that of ΔPsg1) for these localized structures. Interestingly, the deletion of the DE-C subelement in Psg36D and Psg56D did not lead to any major decreases in sg mRNA2 accumulation (Fig. 3E). These latter results are in contrast to those observed for Psg36C and Psg56C where deletion of DE-C resulted in levels of sg mRNA2 accumulation similar to that of Psg1 (Fig. 3C). This finding indicates that the requirement for DE-C is context-dependent.

Analysis of the CE-C and DE-C Subelements and Initiating Nucleotide—To explore the role of the newly defined and previously uncharacterized CE-C subelement, two mutant genomes were constructed within a Psg20/26 context. In the first mutant, Psg20/26ΔCEC, the entire CE-C subelement (5’-AGGGGCCCUCUU) was deleted; however, the initiating guanylate located just 3’ to CE-C (see Fig. 1C) was maintained (Fig. 4A). For the second mutant, Psg20/26mCEC, the sequence of CE-C was mutated to 5’-GCUCUCGUGAG to maintain the original nucleotide composition but not the primary sequence (Fig. 4A). Again the original initiating guanylate just 3’ to CE-C was maintained. When tested for their ability to direct sg mRNA2 transcription, both showed very low levels of sg mRNA2 accumulation (Fig. 4B). This result defines CE-C as a new subelement that is essential for efficient sg mRNA2 accumulation.

The requirement for the DE-C subelement in the more natural Psg20/26 context was also examined by analyzing mutant Psg52 in which the DE-C was deleted (Fig. 4A). Deletion of this subelement led to a significant decrease in the relative accumulation of sg mRNA2 (to ~50% that of Psg20/26, Fig. 4C). This notable reduction implicates DE-C as being important for optimal sg mRNA2 accumulation levels when it is positioned distally in a WT-like genomic context.

To investigate the role of the initiating nucleotide located just 3’ to CE-C, the guanylate present in the control genome Psg35G was mutated to an A, U, or C, generating the Psg35G-derivatives Psg34A, Psg34U, and Psg34C, respectively (Fig. 5A). Each of the three substitutions led to dramatic decreases (>10-fold) in sg mRNA2 accumulation levels (Fig. 5B). In contrast, when (+)-strand viral RNAs were analyzed for the same infections there was no corresponding decrease in the relative (+)-strand sg mRNA2 accumulation levels, and, in two cases, relative levels instead increased (Fig. 5C). These observations indicate that the initiating nucleotide is specifically required for efficient accumulation of sg mRNA2 (+)-strands and suggests that it can also influence, albeit to a lesser degree, the relative abundance of sg mRNA2 (-)-strands.
DISCUSSION

Role of the DE-A/CE-A Interaction—In this study we have carried out a more detailed analysis of the RNA subelements implicated in the regulation of sg mRNA2 transcription in TBSV. Previous results from our laboratory have confirmed the importance of the DE-A/CE-A base-pairing interaction for efficient sg mRNA2 transcription (18). Our current results suggest that this interaction is functional primarily in the (+)-strand (Fig. 2B). Strengthening of this interaction by the substitution of AU with GC base pairs did not dramatically alter sg mRNA2...
accumulation, although slightly elevated levels were observed when three GC base pairs were introduced (Fig. 2C). This suggests that this interaction may already be at its minimally required stability for near-optimal activity. The (+)-strand activity defined for the DE-ACE-A interaction also likely extends to the DE-B/CE-B interaction that was proposed to play an auxiliary role in promoting and/or stabilizing the DE-ACE-A interaction (18). Additional support that these two interactions occur in the (+)-strand comes from previous comparative sequence analyses of corresponding elements in other species of the genus Tombusvirus (18). Sequence comparisons of the predicted secondary structures revealed a preponderance of GU, GA, and GG non-Watson-Crick base pairs within the analyzed helices. Such noncanonical base pairs are common in rRNA secondary structure and are predicted to be significantly more stable than their corresponding (−)-strand counterparts (26). Taken together, these data support the functional interaction of DE-A/CE-A, and by extension DE-B/CE-B, in the (+)-strand of the viral genome.

What is the function of the DE-A/CE-A interaction in the (+)-strand? A similar 8-base pair-long secondary structure is predicted to form in Red clover necrotic mosaic virus (RCNMV), however it occurs just 2 nt upstream from the initiation site of sg mRNA synthesis (10). Another difference in the RCNMV interaction is that it occurs in trans as it involves base pairing between the two RNA segments comprising the bipartite genome. For the RCNMV interaction, it was proposed that formation of this helix acts to stall the viral polymerase during (−)-strand synthesis and that this, in turn, causes it to terminate prematurely and generate (−)-strand sg mRNAs (10). These (−)-strand sg mRNAs would then be used as templates for (+)-strand sg mRNA transcription. Our previous studies showed that the DE-A/CE-A interaction is essential for enhanced sg mRNA2 synthesis when the DE and CE are separated by −1100 nt within the genome (18). However, our current results indicate that when the DE and CE are placed directly adjacent to each other, there is no strict requirement for the DE-A/CE-A interaction. Localized elements containing only the DE-C/CE-C subelements were still able to direct notable sg mRNA2 transcription (Fig. 3, C and E). This observation suggests that the primary role of the DE-A/CE-A interaction within the context of the WT genome may be in the appropriate relative positioning of other subelements, such as DE-C and/or CE-C, thereby facilitating their activities (see below). Nevertheless, additional roles for this interaction cannot be precluded currently.

Roles of the DE-C and CE-C Subelements—The DE-C, unlike the DE-A and DE-B, is not predicted to have any significant complementarity to its counterpart in the CE, CE-C (Fig. 1C). Our results indicate that the necessity of the DE-C subelement for sg mRNA2 accumulation is context-dependent. When the DE was repositioned near the normal CE location, deletion of DE-C led to a dramatic decrease in sg mRNA2 accumulation (Fig. 2B). However, when the CE was localized near the DE no such effect was observed upon deletion of CE-C (Fig. 2C). In the more natural context of Psg20/26, the DE-C was also found to be important for optimal sg mRNA2 accumulation (Fig. 4C). Based on its structural properties, a possible role for this element may simply be to not base pair with CE-C. This would in turn leave CE-C free to interact functionally with other sequences and/or proteins. This idea is consistent with the observation that there is no significant sequence identity among DE-C elements from different members of the genus Tombusvirus, yet a common feature is that they all exhibit little complementarity to their corresponding CE-C subelements (18). In contrast, the sequence comprising the CE-C is highly conserved for members of this genus and is located just 5′ to the site of initiation of sg mRNA2 (18). This subelement was found to be essential for efficient sg mRNA2 accumulation (Fig. 4B).

In RCNMV the sequence just 5′ to the initiation site of its sg mRNA interacts with a complementary viral sequence to mediate sg mRNA transcription (10). Due to the relatedness of these viruses (i.e. they are both members of the family Tombusviridae), it is possible that sg mRNA2 transcription in TBSV also requires base pairing of its similarly positioned sequence, CE-C. The lack of complementarity between CE-C and DE-C would preclude the latter from acting in this capacity. However, possible base-pairing partners for the CE-C sequence have been identified within the TBSV genomic sequence and are currently being analyzed to determine whether they participate in regulating sg mRNA2 transcription.

Role of the Initiating Nucleotide and Insights into the Mechanism of sg mRNA2 Transcription—The initiating nucleotide for sg mRNA2 synthesis has been mapped by primer extension to a guanylate just 3′ to the CE-C (27). Our results have shown that for efficient transcription of sg mRNA2 there is a strict requirement for the identity of the initiating nucleotide to be a guanylate (Fig. 5B). However, the same strict requirement was not observed for the production of (−)-strands corresponding to sg mRNA2 (Fig. 5C). These (−)-strands have been previously implicated as possible templates for the synthesis of (+)-strand sg mRNA2 (18); however, exactly how they are formed remains unclear. For example, the sg mRNA2 (−)-strands could accumulate either as dead-end products synthesized from sg mRNAs transcribed from the full-length (−)-strand of the genome or, alternatively, could represent prematurely terminated products synthesized during (−)-strand synthesis of the (+)-strand genome. In the latter case, the sg mRNA2 (−)-strands could potentially serve as intermediates by acting as templates for the transcription of (+)-strand sg mRNA2. Our finding that (−)-strand accumulation does not correlate with (+)-strand accumulation argues against the (−)-strands originating from (−)-strand sg mRNA2. This independence of (−)-on (+)-strand sg mRNA accumulation has also been observed for Flock house virus (13). For TBSV, in some cases, the relationship between the two oppositely sensed strands is actually inverse, with lower (+)-strand accumulation corresponding to higher (−)-strand accumulation, as for Psg34A and Psg34U (Fig. 5, B and C). These results support the concept that the (−)-strand sg mRNAs observed are not derived from (+)-strand sg mRNA2. Instead they could represent prematurely terminated products that arise during (−)-strand synthesis of the viral genome that, in turn, could serve as templates for sg mRNA2 synthesis. The inability of the (−)-strands generated from the mutants (Psg34A, Psg34C, and Psg34U) to direct transcription of (−)-strands could be related to the strict requirement of tombusvirus polymerases to initiate efficiently only opposite to an appropriately positioned C within promoters in viral templates (28).

Taken together, our results are consistent with the concept that sg mRNA2 transcription could occur via a premature termination mechanism. However, other alternatives, such as the observed effects being the result of post-transcriptional processes, cannot be ruled out at the present time. Additional experiments are planned to further investigate the mode by which sg mRNA2 is expressed.

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Regulatory Activity of Distal and Core RNA Elements in Tombusvirus Subgenomic mRNA2 Transcription
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