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Sequence elements controlling expression of *Barley stripe mosaic virus* subgenomic RNAs in vivo

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Abstract

*Barley stripe mosaic virus* (BSMV) contains three positive-sense, single-stranded genomic RNAs, designated α, β, and γ, that encode seven major proteins and one minor translational readthrough protein. Three proteins (αα, βα, and γα) are translated directly from the genomic RNAs and the remaining proteins encoded on RNAβ and RNAγ are expressed via three subgenomic messenger RNAs (sgRNAs). sgRNA1 directs synthesis of the triple gene block 1 (TGB1) protein. The TGB2 protein, the TGB2' minor translational readthrough protein, and the TGB3 protein are expressed from sgRNAβ2, which is present in considerably lower abundance than sgRNA1. A third sgRNA, sgRNAγ, is required for expression of the γb protein. We have used deletion analyses and site-specific mutations to define the boundaries of promoter regions that are critical for expression of the BSMV sgRNAs in infected protoplasts. The results reveal that the sgRNA1 promoter encompasses positions −29 to −2 relative to its transcription start site and is adjacent to a cis-acting element required for RNA replication that maps from −107 to −74 relative to the sgRNA1 start site. The core sgRNAβ2 promoter includes residues −32 to −17 relative to the sgRNAβ2 transcriptional start site, although maximal activity requires an upstream hexanucleotide sequence residing from positions −64 to −59. The sgRNAγ promoter maps from −21 to +2 relative to its transcription start site and therefore partially overlaps the γa gene. The sgRNAβ1, β2, and γ promoters also differ substantially in sequence, but have similarities to the putative homologous promoters of other Hordeiviruses. These differences are postulated to affect competition for the viral polymerase, coordination of the temporal expression and abundance of the TGB proteins, and constitutive expression of the γb protein.

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Introduction

The synthesis of subgenomic messenger RNAs (sgRNAs) is a common strategy employed by positive-sense RNA viruses to mediate expression and regulation of 3' proximal open reading frames (ORFs) on multicistronic genomic RNAs. Three general mechanisms have been proposed for sgRNA synthesis (White, 2002). The most commonly accepted mechanism, internal initiation from negative-sense RNA templates (Miller et al., 1985), appears to be operating during sgRNA synthesis by the Bromoviruses (Seigel et al., 1997, 1998), Alfamoviruses (Van Der Kuyl et al., 1990), Turnip crinkle virus (TCV) (Wang and Simon, 1997), and several other viruses (Miller and Koev, 2000). In contrast, a discontinuous mechanism of transcription that produces sgRNAs with 5' and 3' sequences identical to the genomic RNAs has been reported for the large Coronaviruses (Sawicki and Sawicki, 1998) and Arteriviruses (Pasternak et al., 2001). These sgRNAs arise from minus-strand subgenomic templates produced via polymerase jumping during transcription from genomic RNA templates. A third mechanism, premature termination, has been reported for Flock house virus (FHV) (Zhong and Ruekert, 1993; Lindenbach et al., 2000), Red clover necrotic mosaic virus (RCNMV) (Sit et al., 1998), and Tomato bushy stunt virus (TBSV) (reviewed by White, 2002). In these cases, truncated negative-sense RNAs that had been generated by premature termination at regions of secondary structure of genomic RNA are postulated to serve as templates for the positive-sense sgRNAs. In the case of FHV and TBSV, cis-acting sequences within the genomic RNA are postu-
lated to mediate long distance interactions that contribute to premature polymerase termination during transcription of the negative-sense templates (White, 2002), whereas in the case of RCNMV, termination during transcription from the RNA 1 template requires base pairing of trans-activator sequences residing on RNA 2 with trans-activator binding sequences on RNA 1 (Sit et al., 1998). Other less well-defined examples of premature termination appear to occur during synthesis of two classes of 5' coterminal sgRNAs appearing in Citrus tristiza virus (CTV) infected plants (Che et al., 2001). Although the possible functions of the CTV sgRNAs have not been resolved, the synthesis of the two species appears to be regulated because they exhibit some differences in the timing of their appearance and they are the earliest and the most abundant sgRNAs synthesized.

The core sgRNA promoters have been mapped and characterized in a variety of plant RNA viruses that synthesize either single or multiple sgRNAs (Miller and Koev, 2000). These promoters range in size from less than 30 nucleotides (nt) (Johnston and Rochon, 1995; Wang et al., 1999) to nearly 150 nt (Van Der Vossen et al., 1995; Koev and Miller, 2000), and they normally reside upstream of, or encompass only a few nucleotides downstream of, the transcription initiation site. However, a few promoters include substantial regions downstream of the transcription initiation site (Balmori et al., 1993; Koev and Miller, 2000). For example, the Beet necrotic yellow vein virus (BNYVV) RNA3 sgRNA promoter occupies more than 100 nt downstream of the transcription start site (Balmori et al., 1993) and the Barley yellow dwarf virus (BYDV) sgRNA2 promoter resides entirely within the sgRNA. In addition to this complexity and variation among RNA viruses, long distance interactions have also been noted in several viruses (Miller and Keov, 2000). In the case of Tobacco mosaic virus (TMV), the 3' untranslated region (UTR) contains three pseudoknot structures whose ectopic placement appears to redistribute polymerase activity to the closest upstream sgRNA promoter (Shivprasad et al., 1999). In contrast, optimal activity of the TBSV sgRNA2 promoter requires long distance cis interactions with complementary upstream sequences separated by more than 1000 nt (Zhang et al., 1999; Choi and White, 2002).

Promoter elements within viruses expressing more than one sgRNA can also vary considerably in their core sequences, and in their sizes and positioning relative to the transcription start sites of the sgRNAs. In one well-studied case, the BYDV sgRNA1 promoter has been mapped from −75 to +21 relative to its transcription initiation site, while the sgRNA2 and sgRNA3 promoters map between positions +1 and +143, and −6 and +38, respectively (Koev and Miller, 2000). Thus, the BYDV sgRNA1 and sgRNA3 promoters occupy sequences that overlap the transcription initiation site, whereas the sgRNA2 promoter resides entirely within the sgRNA transcript. Aside from a conserved hexanucleotide shared between the sgRNA1 and sgRNA2 promoters, little or no similarity exists between the three promoters. Several lines of evidence suggest that both primary sequence and secondary structure, including two stem loop structures, function during the regulation of BYDV sgRNA1 synthesis.

In contrast to the well-defined viruses described above, only rudimentary information is available to define sequences affecting expression of the sgRNAs of a number of other viruses, including Barley stripe mosaic virus (BSMV). BSMV is a Hordeivirus whose genome is divided into three positive-sense, single-stranded RNAs, designated α, β, and γ (Fig. 1). The α and γ RNAs are required for replication, while RNAβ is essential for cell-to-cell movement. The replicase proteins αα and γα are translated directly from their respective genomic RNAs (Petty et al., 1990). In addition, RNAγ also encodes a small cysteine-rich protein, designated yβ, that is translated from sgRNAγ and is dispensable for BSMV replication. The coat protein, βα, is translated directly from RNAβ and, following an intergenic region, the overlapping viral movement genes are arranged in a “triple gene block” (TGB). The three major TGB proteins (TGB1, 2, and 3) and one minor protein, TGB2′, are translated from two sgRNAs, designated sgRNAβ1 and sgRNAβ2. The TGB1 protein is expressed from sgRNAβ1, while the other overlapping proteins TGB2′, TGB2, and TGB3 are translated from sgRNAβ2 (Zhou and Jackson, 1996b). The TGB2′ protein is a translational readthrough product of the TGB2 ORF, and the TGB3 protein is translated by leaky scanning of the TGB2 start codon (Zhou and Jackson, 1996b). The transcription initiation sites of the three BSMV sgRNAs have been mapped and their relative abundance has been determined (Gustafson et al., 1987; Zhou and Jackson, 1996b). Northern blot analyses of nucleic acids extracted from BSMV-infected protoplasts have demonstrated that sgRNAγ and sgRNAβ1 accumulate to high levels, whereas sgRNAβ2 is present in much lower abundance (Zhou and Jackson, 1996a, 1996b). Limited evidence from protoplasts also indicates that sgRNAβ1 and β2 expression is regulated temporally and that sgRNAγ is expressed constitutively (Zhou and Jackson, 1996b).

To begin to determine how expression of the three BSMV sgRNAs may be mediated, we have delineated the boundaries of the promoters based on the expression of sgRNAs in infected protoplasts. In addition, we have mapped a cis-acting element adjacent to the sgRNAβ1 promoter that is required for RNAβ replication (Zhou and Jackson, 1996b). The three BSMV sgRNA promoters have also been repositioned into β and γ RNAs that either contain or lack the native promoters. The results indicate that the analogous BSMV sgRNA promoters have some sequence similarity to predicted promoter regions of other Hordeiviruses. Nevertheless, the three sgRNA promoters do not share extensive sequence similarity, nor are recognizable common structural elements evident. Although the BSMV core promoter sequences failed to function when inserted ectopically into RNAβ and RNAγ, core promoters introduced with additional flanking sequences were active,
of the TGB1, TGB2, TGB2/H11032 interfered with the ORF. The intergenic region between the TGB1 protein is expressed from sgRNAβ1 and the TGB2, TGB2', and TGB3 proteins are translated from sgRNAβ2. The genomic RNA-y serves as a messenger for translation of the γ protein, which is expressed from sgRNAγ. All genomic and subgenomic RNAs are capped (black circle) at the 5’ terminus, contain an internal poly (A) tail (An), and possess a conserved tRNA-like structure (black rectangle) at the 3’ terminus.

Results

Mapping sequences required for expression of sgRNAγ

In a previous study, we had determined that the sgRNAγ transcription start site begins at nt 2054 on RNAγ (Gustafson et al., 1987). This site resides within the 42-nt intergenic region between the γ and yb ORFs (Fig. 1). To analyze the sgRNAγ promoter, BY-2 protoplasts were transfected with in vitro transcripts, and the RNAs synthesized during infection were evaluated by Northern blot analyses. Because the γ stop codon is located 18 nt upstream of the sgRNAγ transcriptional start site, we predicted that the sgRNAγ promoter would overlap the γ ORF. The γ protein encodes the viral polymerase and hence is essential for viral replication. Therefore, engineering deletions to map the sgRNAγ promoter could have interfered with the γ ORF and might have affected replication. To circumvent this problem, protoplasts were transfected with two RNAγ derivatives. One RNA, γKpnI/HpaI, provided a source of the γ replicate protein and also contained a 350-nt deletion (from positions 2112 to 2461 on RNAγ) to remove the majority of the yb ORF, and a second derivative designed to assess promoter activity contained deletions engineered within the putative sgRNAγ promoter (Fig. 2A). The deletion present in γKpnI/HpaI RNA allowed us to distinguish between the two RNAγ derivatives based on their sizes. Protoplasts were transfected with RNAα, RNAγ, and RNAγKpnI/HpaI, and nucleic acid was extracted from protoplasts at 20 h posttransfection. Each of the three RNAs replicated to wild-type (wt) levels in protoplasts and synthesized the predicted sgRNAs (data not shown). Therefore, in subsequent mapping experiments, but this activity was dependent on the context into which the sequences were inserted.

Fig. 1. Illustration of the BSMV genomic and subgenomic RNAs. RNAα serves as the mRNA for the α protein, which contains the capping and helicase subunits of the RNA-dependent RNA polymerase (RdRp). RNA β encodes the β protein (coat protein) and the “triple gene block” composed of the TGB1, TGB2, TGB2’, and TGB3 proteins. The β protein is translated from the genomic RNA; the TGB1 protein is expressed from sgRNAβ1 and the TGB2, TGB2’, and TGB3 proteins are translated from sgRNAβ2. The genomic RNAγ serves as a messenger for translation of the γ protein, which is expressed from sgRNAγ. All genomic and subgenomic RNAs are capped (black circle) at the 5’ terminus, contain an internal poly (A) tail (An), and possess a conserved tRNA-like structure (black rectangle) at the 3’ terminus.

Fig. 2. Identification of the boundaries of the sgRNAγ promoter. (A) The schematic illustration shows the two γ RNAs used in conjunction with RNAα to transfected protoplasts. The γ KpnI/HpaI RNA provided a source of the γ protein, while the second RNAγ was used to construct deletions within the region surrounding the sgRNAγ transcription start site (note arrow). Tobacco BY-2 protoplasts were cotransfected with the α and γ KpnI/HpaI RNAs plus RNAγ derivatives with deletions originating at either −152 or +57 relative to the sgRNAγ transcription start site. The deletions are shown above the blot. Total nucleic acid was extracted 20 h posttransfection, separated on 1% agarose gels, and transferred to nylon membranes. Northern blot analyses were conducted with a γ-specific riboprobe designed to detect the presence of the genomic RNAγ and sgRNAγ. The probe was derived from the KpnI/Hinfi III (2111 to 2444 nt) fragment of RNAγ and hence does not hybridize to the genomic or sgRNAs generated from the γ KpnI/HpaI RNA. (B) Analysis of RNAγ derivatives containing small deletions in the region spanning −30 to +10 relative to the sgRNAγ transcription start site. The sequence shows the intergenic region in the negative sense, with the arrow representing the sgRNAγ transcription start site.
protoplasts were transfected with RNAα, RNAγ*Kpnl/HpaI, and an RNAγ derivative that contained a deletion within the putative sgRNAγ promoter.

To identify starting points for analysis of the sgRNAγ promoter region, two RNAγ derivatives were constructed. One contained a large-scale deletion in the γa ORF from nt 593 to 1899 and the other had a smaller deletion from positions 2111 to 2262 in the γb ORF. When these RNAs were transfected individually into protoplasts along with RNAα and RNAγ*Kpnl/HpaI, both of the deletion derivatives were able to replicate and synthesize sgRNAγ at levels comparable to wt RNAγ (data not shown). These results indicate that the deleted regions are not required for sgRNAγ synthesis and that they do not contain regulatory elements that affect replication. Therefore, a more refined analysis of the sgRNAγ promoter was initiated by evaluation of deletions beginning at position −152 (position 1902 on RNAγ) and extending to positions −119, −78, −38, and −18 upstream of the transcription start site. Two additional deletions beginning at position +57 and extending to +27 and +11 were also generated. To specifically visualize the replication of these RNAγ derivatives and the presence of sgRNAγ, a 32P-labeled riboprobe was used that is complementary to the KpnI/HpaI region that had been eliminated in the γ*KpnI/HpaI RNA used to mediate replication of the test RNAs. This probe thus recognizes only the RNAγ and sgRNAγ derivatives designed to assess promoter activity. As shown in Fig. 2A, Northern blot analysis revealed that each of the RNAγ test derivatives were able to replicate in trans and that the γRNAs containing the deletions −152/−119, −152/−78, −152/−38, +57/+27, and +57/+11 were able to synthesize sgRNAγ. However, when the region between −152 and −18 was deleted, sgRNA synthesis was not evident. Therefore, these results indicated that the sgRNAγ promoter resides between positions −38 and +11 relative to the sgRNAγ transcriptional start site.

To analyze the sgRNAγ promoter more precisely, smaller deletions were generated in the region between positions −30 and +10 (Fig. 2B). The γ RNAs containing the deletions −30/−28, −30/−22, −30/−21, +10/+4, +10/+3, +10/+2, and +10/+1 were transfected into protoplasts along with the α and γ KpnI/HpaI RNAs. Northern blot analysis demonstrated that the RNAs containing deletions −30/−28 and −30/−22 replicated and were capable of synthesizing sgRNAγ, but that removal of the residue at position −21 eliminated sgRNAγ synthesis (Fig. 2B). Our interpretation of these results is that the C at position −22 is dispensable for sgRNAγ synthesis, but that the A at position −21 is required for promoter activity. The RNAs used for deletion analysis starting at position +10 were all able to replicate, but only the +10 to +4 or +3 deletions were able to synthesize sgRNAγ (Fig. 2B). When the next residue at +2 was deleted, sgRNAγ was not evident, and hence, the A at position +2 is required for sgRNAγ synthesis, whereas the A at +3 is dispensable. Therefore, these results indicate that the sgRNAγ promoter boundaries map to positions −21 and +2 relative to the sgRNAγ transcription start site. The sgRNAγ promoter thus overlaps the sgRNAγ transcription initiation site and the last two codons of the γa polymerase protein.

Defining the sgRNAβ1 promoter and the RNAβ cis-acting element boundaries

RNAβ is dispensable for replication and therefore, substantial deletions could be engineered into the RNA to facilitate analysis of the sgRNAβ1 promoter. The sgRNAβ1 transcription start site had previously been mapped to nt 789 in the 118-nt intergenic region between the βa and TGB1 ORFs (Zhou and Jackson, 1996b). In addition, we had identified a cis-acting element required for RNAβ replication within this intergenic region (Zhou and Jackson, 1996a). Therefore, careful consideration of the cis-acting element had to be taken into account because of the possibility that the sgRNAβ1 promoter boundaries might reside within this region. Hence, we devised a strategy to identify the cis element and to map the sgRNAβ1 promoter by transfecting BY-2 protoplasts with the α and γ RNAs and an RNAβ mutant to evaluate promoter activity. Northern blot analysis with a β-specific probe was then used to assess replication and sgRNAβ1 synthesis.

Identification of the sgRNAβ1 promoter initially was complicated because the 18S rRNA masked sgRNAβ1 in Northern blots (data not shown). To circumvent this problem, we constructed mutations in a cDNA clone that contained a 1427-nt deletion (βS/B) which removed the majority of the TGB ORFs. This deletion did not affect RNAβ replication, in agreement with our previous results (Zhou and Jackson, 1996a) and also clearly resolved sgRNAβ1 and the rRNAs to permit detection of sgRNAβ1 (data not shown). It is important to note that the βS/B RNA deletion also eliminated the sgRNAβ2 promoter to mitigate possible competition effects that might have affected synthesis of sgRNAβ1. To define the starting points for mapping the sgRNAβ1 promoter, we used two RNAβ derivatives. One of these, βΔ1.6, contained a deletion from position 295 to 633, and the other, βΔ2.0, eliminated nts 802 to 1375 on the genomic RNA sequence. Northern blot analysis of RNA isolated from protoplasts transfected with the α and γ RNAs and the βΔ1.6 or βΔ2.0 RNAs indicated that the deletions had no discernable effect on sgRNAβ1 synthesis (data not shown). Therefore, the BstBI site at −156 (RNAβ nt 633) and the NcoI site at +14 (nt 802) relative to the sgRNAβ1 transcription start site were used as starting points for analysis.

Initially, deletions were generated from −156 to −107, −74 and −34 relative to the sgRNAβ1 transcription initiation site. As shown in Fig. 3A, the −156/−107 deletion mutant was able to replicate and to synthesize sgRNAβ1, but the −156/−74 and −156/−34 deletions reduced replication to barely detectable levels. To confirm the replication of RNAs α and γ, the blots were stripped and reprobed with
further de
required for replication (Zhou and Jackson, 1996a). To
represents the sgRNA
site. (C) Fine-scale mapping of the sgRNA
mapping of the sgRNA
2, except that the blots were probed with a
cis-acting element. Protoplasts were transfected with wt
derivatives containing the 1427 nt S/B (\(\beta\) Sal/\(Bgl\II\))
deletion. Total nucleic acid was extracted and blotted as described in Fig.
2, except that the blots were probed with a \(\beta\)-specific riboprobe (\(\beta\) Nco/ SalI). The numbers above the lanes correspond to deletions at positions
relative to the sgRNA\(\beta\)1 transcription start site. (A) Large-scale deletion
mapping of the sgRNA\(\beta\)1 promoter and the RNA\(\beta\) cis-acting element
required for replication. (B) Deletion mapping of the sgRNA\(\beta\)1 promoter
using 10-nt deletions at positions corresponding to the transcription start
site. (C) Fine-scale mapping of the sgRNA\(\beta\)1 promoter. The sequence
represents the negative-sense orientation of the intergenic region between
the \(\beta\)a and TGB1 ORFs which contains the sgRNA\(\beta\)1 promoter. The arrow
represents the sgRNA\(\beta\)1 transcription start site.

\[\begin{align*}
A \\
RNA\(\beta\)- \\
sgRNA\(\beta\)1 \\
B \\
RNA\(\beta\)- \\
sgRNA\(\beta\)1 \\
C \\
\text{AUCAAAACGAAAUAUGCGCAGAUAGUCUCAAUACUGAAUGC} \\
\text{RNA\(\beta\)-} \\
\text{sgRNA\(\beta\)1} \\
\end{align*}\]

\(\alpha\)- and \(\gamma\)-specific probes (data not shown). These results
indicated that the \(-156/-74\) and \(-156/-34\) deletions de-
stroyed a cis-element within the intergenic region that is
required for replication (Zhou and Jackson, 1996a). To
further define the RNA\(\beta\) cis-acting element, two additional
derivatives were constructed that contained deletions span-
nning \(-107/-74\) and \(-74/-34\). Northern blot analysis using a
\(\beta\)-specific probe revealed that replication of \(-107/-74\), similar to that of the \(-156/-74\) and \(-156/-34\) mutants,
was undetectable. However, the \(-74/-34\) deletion mutant
replicated to low levels and produced small amounts of
gRNA\(\beta\)1 (Fig. 3A). These results suggest that the cis-
acting element required for RNA\(\beta\) replication resides within
the 118-nt intergenic region between positions \(-107\) and
\(-74\). However, sequences within the \(-74\) to \(-34\) region
also contribute to efficient replication of RNA\(\beta\).

The sgRNA\(\beta\)1 promoter activity was examined in more
detail by engineering 10-nt deletions in \(\beta S/B\) from positions
\(-60\) to \(+1\) relative to the sgRNA\(\beta\)1 transcription start site.
Northern blot analyses revealed that the \(-60/-51\), \(-50/
-41\), and \(-40/-31\) deletions did not affect genomic RNA
replication and abundant amounts of sgRNA\(\beta\)1 were syn-
thesized. However, when the \(-30/-21\), \(-20/-11\), or
\(-10/+1\) regions were deleted, genomic RNA replicated to
wt levels but sgRNA\(\beta\)1 failed to accumulate (Fig. 3B). To
further resolve the nature of the active sgRNA promoter
element, we engineered smaller deletions from positions
\(-30\) to \(+14\). As shown in Fig. 3C, when the A residue at
\(-30\) was deleted, sgRNA\(\beta\)1 was evident, but deletion of the
adjacent U at \(-29\) effectively eliminated promoter activity
without obvious effects on replication of the genomic
RNAs. In contrast, deletions that spanned \(+14/+2\), \(+14/
+1\), and \(+14/-1\) retained promoter activity (Fig. 3C).
However, when the next residue, a U at \(-2\), was deleted,
promoter activity was disrupted. These results suggest that
the core promoter sequence required for sgRNA\(\beta\)1 syn-
thesis resides immediately upstream of the sgRNA\(\beta\)1 tran-
scription start site between positions \(-29\) and \(-2\). How-
ever, an alternative possibility that needs further exploration
is that the \(+14/+1\) deletion could have generated an alter-
native transcription initiation site at the upstream C residue.
Similar alternative sites have previously been identified in
in vitro studies with the Brome mosaic virus sgRNA pro-
moter (Stawicki and Kao, 1999).

**Delineating the sequences required for sgRNA\(\beta\)2 expression**

The transcription start site of sgRNA\(\beta\)2 has previously
been identified within the 3’ end of the TGB1 ORF at
position 2279 in RNA\(\beta\) and is 1490 nt downstream of the
sgRNA\(\beta\)1 transcription start site (Zhou and Jackson, 1996b). Since sgRNA\(\beta\)2 is expressed at very low levels in
infected protoplasts (Zhou and Jackson, 1996a, 1996b), we
attempted to increase its abundance to improve the ease and
reproduceability of promoter analysis. For this purpose, the
clon\(e\) \(\beta1-34/+14\) was created by removing nucleotides
755 through 802 (\(-34/+14\) relative to the sgRNA\(\beta\)1 tran-
scription start site) on RNA\(\beta\) (Fig. 4A). As expected, this
48-nt deletion abrogated sgRNA\(\beta\)1 synthesis and also re-
sulted in easily detectable levels of sgRNA\(\beta\)2 without af-
and 2287 to 2434. RNAs containing these deletions were

fected RNAβ replication (data not shown). This result
implies that competition between the two promoters has a
major role in regulating the differential rates of synthesis
of the two sgRNAs.

To initially define sequences flanking the sgRNAβ2 pro-
moter, two large deletions were generated in the β1-34/+14 clone that eliminated RNAβ positions 1705 to 2109
and 2287 to 2434. RNAs containing these deletions were
individually transfected into protoplasts along with the β
and γ RNAs, and nucleic acids were extracted at 20 h
posttransfection. Northern blot analyses using a β-specific
probe revealed the presence of sgRNAβ2 in both deriva-
tives, indicating that neither of the deletions affected
gRNAβ2 promoter activity (data not shown). Therefore,
we began to focus on the region between −179 (nt 2109)
and +9 (nt 2288) relative to the sgRNAβ2 transcription
initiation site.

Analysis of four deletions extending from position −179
to positions −110, −76, −52, and −28 relative to the
transcription start site revealed that the mutant RNAs were
able to replicate in protoplasts, but only mutants containing
the deletions −179/−110 or −179/−76 were able to direct
synthesis of sgRNAβ2 (Fig. 4A). More detailed analyses
performed on the region between −70 and −52 demon-
strated that the −70/−65 and −58/−53 mutants transcribed
gRNAβ2, but that promoter activity was destroyed by the
−64/−59 deletion (Fig. 4A). These results thus indicate that
an upstream element residing between −64 and −59 rela-
tive to the sgRNAβ2 transcription start site is required for
gRNAβ2 promoter activity. However, in the presence of
this element, deletions between positions −40/−32 and
+10/−17 nearly eliminated sgRNAβ2 synthesis (Fig. 4B).
In contrast, Northern blot analyses revealed that deletions
between −52/−47, −46/−41, −40/−35, −40/−34, and
−40/−33 had only minor effects on RNAβ replication
and sgRNAβ2 synthesis in transfected protoplasts (Fig. 4B
data not shown). Similarly, the deletions +11/−9, +11/−15,
and +10/−16 were able to replicate and synthesize
gRNAβ2. These results demonstrate that the sgRNAβ2
promoter maps from the G at −32 to the C at −17 and
hence does not encompass the transcriptional start site.

**Ectopic expression of the sgRNA promoters**

The sgRNAβ1, sgRNAβ2, and sgRNAγ promoter frag-
ments were initially inserted into RNAβ to determine
whether they could function ectopically and to evaluate
their competition with a native sgRNA promoter present
on the same RNA. For this purpose, fragments encompassing
each of the three sgRNA promoters were PCR-amplified
and inserted into RNAβ β S/B or β1−34/+14 at position
1134 (344 nt downstream of the sgRNAβ1 promoter). The
β S/B clone provided a background that contained the
native sgRNAβ1 promoter (Fig. 5A), while the β1−34/+14
clone lacked the sgRNAβ1 promoter (Fig. 5B). Two frag-
ments that encompassed the sgRNAβ1 promoter were in-
serted ectopically into the two RNAβ derivatives. The
larger fragment β1a consisted of 266 nt amplified from
positions 599 to 864 on the genomic RNA and a smaller
150-nt fragment β1b was derived from positions 715 to 864.
A 295-nt fragment (β2) overlapping the sgRNAβ2 pro-
moter was derived from positions 2080 to 2374, and a
276-nt fragment (γ) was amplified from positions 1864 to
2139 to encompass the sgRNAγ promoter.

Northern blot analysis of RNA isolated from protoplasts
transfected with RNAs α and γ, and RNAβ derivatives

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**Fig. 4.** Defining the boundaries of the sgRNAβ2 promoter. Protoplasts
were transfected with the α and γ genomic RNAs and RNAβ derivatives
containing a 48-nt deletion (β1−34/+14) to eliminate sgRNAβ1 promoter
activity. The deletions are numbered according to the sgRNAβ2 transcrip-
tion start site, which is illustrated by the arrow. Total nucleic acids were
extracted at 20 h posttransfection and processed as described in Fig. 2,
extcept that hybridizations were performed with a β-specific riboprobe (β
SspUBg/III) derived from sequence upstream of the sgRNA2 promoter
region. (A) Large-scale deletion mapping of the sgRNAβ2 promoter is
shown on the blot to the left. To define the region between −70 and −53
further, 6-nt deletions were constructed and are shown on the blot to the
right. (B) Small-scale mapping of the sgRNAβ2 promoter. The sequence
represents the negative-sense region of the TGB1 ORF that contains the
sgRNAβ2 promoter.
Protoplast transfections and RNA blots were carried out as described in the native promoters and the ectopic promoter insertion sites, respectively. Genomic RNAs are as described in the legend to Fig. 1. N and E represent Fig. 5. Ectopic expression of the sgRNA

72

promoters in RNA

/H9252/ H9253 derivatives. The designations on the

/H9252/ H9253 , sgRNA

1, and sgRNA

2

originated from the sgRNA

of A and B refer to a consistently observed band of unknown origin that therefore should detect all genomic and sgRNAs. Asterisks along the side sgRNA (Fig. 5A). However, transcription from the ectopic

RNA

/H9253/ H9252/ H9253 tubes of BY2 protoplasts were transfected with RNAs

/H9253/ H9252/ H9253 sgRNA after protracted exposure of the blots (Fig. 5A, replicate and synthesize sgRNA

2339, which is located 285 nt downstream of the transcription start site of the native sgRNA

2 promoter. However, RNA

/H9253/ H9252/ H9253 replication and synthesis of native sgRNA

1 or sgRNA

2 promoter when positioned downstream of the wt sgRNA

1 promoter. (C) Activity of the sgRNA

34/-c-2785). (B) Expression of the sgRNA

1, sgRNA

2, and sgRNA

γ promoters in the RNA

β S/B derivative (β1−34/+14), which contains a 48-nt deletion inactivating the native sgRNA

1 promoter. (C) Activity of the sgRNA

1, sgRNA

2, and sgRNA

γ promoters inserted into the γ ORF at nt 2339 of RNA

γ. Protoplasts were coinoculated with RNA

α and RNA

γ containing the promoter fragments and extracted at 20 hpi. RNA blots were probed with a riboprobe that anneals to the conserved 3’ end of BSMV RNAs and therefore should detect all genomic and sgRNAs. Asterisks along the side of A and B refer to a consistently observed band of unknown origin that originated from the sgRNA

γ promoter insertions.

Fig. 5. Ectopic expression of the sgRNA

γ, sgRNA

β1, and sgRNA

β2 promoters in RNA

β and RNA

γ derivatives. The designations on the genomic RNAs are as described in the legend to Fig. 1. N and E represent the native promoters and the ectopic promoter insertion sites, respectively. Protoplast transfections and RNA blots were carried out as described in

containing the native sgRNA

β1 promoter with the smaller sgβ1 (150 nt), sgβ2, or the sγ fragment insertions at position 1134 revealed that the β RNAs were able to replicate and synthesize sgRNA

β1 from the native promoter (Fig. 5A). However, transcription from the ectopic sgRNA

β1 and sgRNA

β2 promoters could be detected only after protracted exposure of the blots (Fig. 5A, β1 and β2 lanes, and data not shown). The RNA

β derivative containing the 48-nt deletion in the intergenic region was able to replicate and to express small amounts of the ectopic sgRNAs from the smaller 150-nt sgRNA

β1 fragment and sgRNA

β2 (Fig. 5B, β1 and β2 lanes), but both the RNA

β derivatives failed to replicate when the larger 266-nt sgRNA

β1 fragment was inserted (data not shown). In marked contrast, the 276-nt ectopic RNA

γ fragment was active following insertion into both of the RNA

β derivatives and ectopic sgRNAs of the appropriate sizes were easily detectable in both backgrounds (Figs. 5A and B, γ lanes). These results demonstrate that the three BSMV sgRNA promoters can function ectopically in RNA

β, but that the extent of their activity varies depending on their flanking sequences and the genomic RNA context into which they are inserted. The results also provide evidence that the ectopic sgRNA

γ promoter competes for the BSMV replicase complex more efficiently than an ectopic sgRNA

β1 or sgRNA

β2 promoter when positioned downstream of the wt sgRNA

β1 promoter.

Each of the four promoter fragments (β1a, β1b, β2, and γ) were also inserted into the γ cDNA clone at position 2339, which is located 285 nt downstream of the transcription start site of the native sgRNA

γ (Fig. 5C). As seen with RNA

β, the presence of the sgRNA

β2 fragment (β2) and the sgRNA

γ promoter derivative (γ) permitted abundant RNA

γ replication and synthesis of native sgRNA

γ (Fig. 5C, β2 and γ lanes). Both the sgRNA

β2 and the sgRNA

γ promoters were also able to direct synthesis of easily detectable amounts of ectopic sgRNA. Again, transcription from the β2 promoter was less than half of that from the native sgRNA

γ promoter, and the ectopic sgRNA

γ promoter also appeared to be substantially more active than the corresponding sgRNA

β2 promoter. However, RNA

γ replication
was nearly eliminated when either the smaller 150-nt sgRNAβ1 promoter (β1, Fig. 5C) or the larger 266-nt sgRNAβ1 promoter fragments were inserted (data not shown). These results suggest that the ectopic sgRNAβ2 promoter competes less efficiently for the BSMV replicase complex than the wt sgRNAγ promoter. The deleterious effects of the sgRNAβ1 fragment insertions in RNAγ also suggest that sequence, context effects, and perhaps competition for replicase can have drastic effects on replication.

To determine if the three minimal sgRNA promoters were sufficient for ectopic transcription, we inserted each of them into position 1134 of the RNAβ β S/B derivative and the 48-nt deletion derivative (β1–34/+14). Similar insertions were made into RNAγ at position 2339. Ectopic sgRNA expression was not detected following protoplast transfection with any of the derivatives, although the genomic RNAs were able to replicate (data not shown). This result suggests that although the minimal promoter sequences identified by deletion analysis are required for activity in their native context, each of the promoters requires additional flanking sequences to provide optimal promoter activity from the ectopic positions tested in these experiments.

**Sequence comparisons of the three BSMV sgRNA promoter regions**

The comparisons shown in Fig. 6A indicate that the three BSMV sgRNA promoters share little obvious sequence relatedness. We also conducted a more refined gapped alignment and were still unable to detect major blocks of similarity (data not shown). Additional comparisons of the 238 nt at the 3’ common termini of the genomic RNAs and at the diverse 3’ ends of the three minus-strand RNAs also indicated that the four replication promoters within the genomic RNAs do not contain substantial regions of similarity to the three BSMV sgRNA promoters (data not shown). However, comparisons of the three sgRNA promoters of BSMV with the putative promoters of *Poa semilatent virus* (PSLV) and *Lychnis ringspot virus* (LSRV) revealed that the analogous *Hordeivirus* sgRNA promoters share a number of blocks of identical sequence (Fig. 6B). Furthermore, the BSMV and PSLV sequences share a higher degree of similarity with each other than either does with LRSV. In the case of the BSMV sgRNAγ promoter, nearly 80% of the PSLV residues are identical (19/24) to the BSMV sequence, whereas a gapped alignment between BSMV and LRSV indicated a considerably lower degree of similarity (14/24 residues) within the aligned region. These results buttress the existing strong evidence for a common origin of the *Hordeiviruses* and support biological evidence, suggesting that BSMV and PSLV are more closely related to each other than to LRSV (Hunter et al., 1989).

To obtain additional information about the relationship of the *Hordeivirus* sgRNA promoters, sequence comparisons were conducted with other TGB-containing viruses. Initial comparisons using the NCBI BLAST program failed to reveal significant matches with other viral promoter sequences. To obtain more definitive comparisons, we analyzed the 300 nucleotides surrounding the start codons of the TGB proteins of *Beet necrotic yellow vein virus*, *Peanut clump virus* (PCV), *Potato mop top virus* (PMTV), *Potato virus M* (PVM), and *Potato virus X* (PVX). No obvious similarity was detected in comparisons of the sgRNAβ2 promoter regions, and only short blocks of common sequence were noted in the regions surrounding the putative sgRNAβ1 promoters. These common blocks primarily consisted of strings of A residues located at various positions upstream of the translational start site and hence are unlikely to represent conserved promoter elements. Thus, the results indicate that the sequence conservation noted among the TGB proteins of the *Hordeiviruses* does not extend to the analogous TGB promoter regions of more distantly related viruses.

We also conducted comparisons of the BSMV sgRNAγ promoter with putative promoter regions of other viruses. Interestingly, the PCV p15, which encodes a cysteine-rich protein with some similarity to BSMV γb, and the BNYVV TGB1 sequences have a high degree of similarity to the BSMV sgRNAγ promoter. In these cases, 16 (PCV) or 14 (BNYVV) residues of 24 are identical to the BSMV sgRNAγ sequence. These regions of similarity include two blocks (ACACACUU and ACUA) within the 20 residues upstream of the transcriptional start site of the BSMV sgRNAγ, and they surprisingly share more than 60% identity (15/24 residues) with a region of the TMV 30 K movement protein promoter (Grdzelishvili et al., 2000). Despite these intriguing similarities, the significance, if any, of these common sequences is conjectural and additional analyses are needed to determine their importance in sgRNA synthesis.

**Discussion**

During the BSMV infection cycle, the three sgRNAs vary in abundance and in their timing of expression (Zhou and Jackson, 1996a). Early in infection, the predominant species are sgRNAγ, which appears to be relatively constant throughout replication, and sgRNAβ1, whose abundance decreases as infection progresses. In contrast, the abundance of sgRNAβ2 is considerably lower than that of sgRNAβ1, but both sgRNAs appear to have similar patterns of temporal expression. These results suggest that the timing and the relative expression levels of the sgRNAs may be important in regulating aspects of the infection cycle (Zhou and Jackson, 1996a). Therefore, understanding the nature of the sgRNA promoters and the requirements for regulated expression of the mRNAs under their control will contribute substantially toward elucidating events required for systemic invasion and disease development in host plants. As a
Fig. 6. Comparison of the BSMV sgRNA promoter sequences. (A) Sequence of the three BSMV sgRNA promoters in the minus-sense orientation. The numbers above the sequence correspond to nucleotide positions relative to the respective transcription initiation sites. The underlined sequences are required for sgRNA synthesis. (B) Alignment of the sgRNA\textsubscript{H9253}, sgRNA\textsubscript{H9252}, and sgRNA\textsubscript{H9252} promoter regions of the Hordeiviruses BSMV, PSLV, and LRSV. Regions with the highest sequence similarity are shown in red. Note that the transcription start sites for the PSLV and LRSV sgRNA promoters have not been defined experimentally, although their initiation sites have been predicted previously (Savenkov et al., 1998). Alignments were performed using the MegAlign program associated with the Lasergene software package. (C) Alignment of the sgRNA\textsubscript{H9252}, sgRNA\textsubscript{H9252}, and sgRNA\textsubscript{H9253} promoter regions of BSMV with mapped (TMV and PVX) or putative (BNYVV, PCV, PMTV, and PVM) sgRNA promoter regions of other viruses. Regions identified to have high sequence similarity are shown with residues identical to the BSMV sequence highlighted in red. Alignments were performed using the MegAlign program associated with the Lasergene software package.
step toward such understanding, we have delineated the boundaries of the three BSMV sgRNA promoters.

The sgRNAγ, sgRNAβ1, and sgRNAβ2 promoters map to positions −21 to +2, −29 to −2, and −32 to −17 relative to their transcription start sites, respectively. RNAβ also requires an enhancer-like element located between −64 to −59 for production of sgRNAβ2. These three promoters are <50 nt in size and are located in close proximity to their respective transcription initiation sites. The sgRNAγ promoter lies primarily upstream of its transcription initiation site but includes the +1 nt that corresponds to the transcription start site and an additional nucleotide at the +2 position. The BSMV sgRNAβ1 and sgRNAβ2 promoters, which reside upstream of their transcription initiation sites, differ from other plant virus sgRNA promoters previously characterized because the sgRNAβ1 and sgRNAβ2 promoters appear not to overlap their transcription start sites (Miller and Koev, 2000). Independent experiments have demonstrated the importance of the +1 nt in the sgRNA promoters of a number of well-characterized viruses. For example, in vitro studies using the BMV proscript system to elucidate the minimal requirements for replicase recognition of the BMV sgRNA promoter have shown that changing the +1 nt of the promoter from a C to a G decreases sgRNA synthesis by more than 90% (Siegel et al., 1997). The importance of the +1 nt has also been shown in another Tricornavirus where changing the C to a U abolished AMV sgRNA promoter activity (Van Der Vossen et al., 1995). Similarly, when the +1 nt of the TMV MP sgRNA promoter was changed from a C to a G, sgRNA accumulation was below the limits of detection (Grdzelishvili et al., 2000). In contrast, transversion of the +1 nucleotides of the BYDV sgRNA2 and sgRNA3 promoters had no effect on sgRNA accumulation, even though both promoters span this region (Koev and Miller, 2000). Although the sgRNAγ promoter overlaps its transcription start site, we have not determined whether nucleotide substitutions at this position affect sgRNA synthesis.

Unlike many RNA viruses, the BSMV sgRNA promoters do not share substantial blocks of sequence identity within their core regions (Fig. 6A). However, the sgRNAγ and sgRNAβ2 promoters do appear to be more closely related to each other than either is to the sgRNAβ1 promoter. This probably is an anomaly due to nucleotide composition within the promoters because the sgRNAβ1 promoter sequence is particularly rich in A and U residues (73%), whereas the sgRNAβ2 and sgRNAγ promoters have a more uniform distribution of bases with an A/U content of just over 50%. In analyses comparing the sgRNAβ1 promoter with either the sgRNAβ2 or the sgRNAγ promoter, we could align no more than two identical residues in a row. However, for the sgRNAβ2 and sgRNAγ promoters, a block of five identical nucleotides (CCACU) can be aligned within the 26 residues upstream of their transcription start sites.

The three BSMV sgRNA promoters align well with the putative sgRNA promoter regions of the less well-characterized Hordeiviruses, PSLV and LRSV, whose sgRNA transcription initiation sites have not been mapped. Comparison of the three BSMV sgRNA promoters with the analogous promoters of the other Hordeiviruses reveals over 75% sequence similarity (Fig. 6B and Savenkov et al., 1998). Two blocks of identical residues were identified in the sgRNAβ1 promoter regions (AAAAAU and CUAC). Only one block of conserved sequence was observed for the sgRNAβ2 promoter (CCACU), and this same block is present in BSMV and LRSV sgRNAγ promoters. Among the three Hordeiviruses, the sgRNAγ promoter displayed the greatest degree of similarity because it contained three blocks of identical sequence of three nucleotides or longer (GAAGCU, ACCA, and ACU) as well as individual conserved residues. Additional analyses of the less conserved regions failed to indicate preferences for particular purine or pyrimidine residues. These results indicate that the abundance and timing of transcription of the three Hordeivirus sgRNAs, β1, β2, and γ, may be regulated by promoter sequences that are only distantly related. Alternatively, it is possible that short and/or long distance structural interactions that are not evident in our analyses may have profound roles in promoter timing and activity.

It appears that the sgRNAβ2 promoters of BSMV and PSLV are sufficiently similar for recognition by the BSMV replicase because in vivo studies have revealed that BSMV can support synthesis of PSLV sgRNAβ2 (Solovyev et al., 1999). In these studies, a hybrid BSMV RNAβ containing PSLV TGB1, TGB2, and most of the TGB3 sequence was able to replicate and move through the vasculature to uninoculated leaves in plants coinoculated with the α and γ RNAs (Solovyev et al., 1999). However, when a hybrid BSMV RNAβ containing the LRSV TGB1, TGB2, and TGB3 regions analogous to the PSLV sequence was coinoculated with the α and γ RNAs, the hybrid virus was unable to infect the same common hosts. These results suggest that the BSMV replicase complex can recognize the related PSLV sgRNAβ2 promoter, but it either cannot recognize the LRSV sgRNAβ2 promoter or the LRSV TGB proteins do not form sufficiently compatible interactions to mediate BSMV movement.

In comparisons of the BSMV sgRNA promoters with the analogous sequences of non-Hordeivirus TGB promoters, we were unable to detect a strong correlation between the putative promoter sequences directing sgRNAs encoding specific TGB proteins. For example, no obvious similarity was evident between the sgRNAβ1 promoter and the regions upstream of the PMTV and PCV TGB1 genes, and only limited similarity was detected with the regions upstream of the TGB1 genes of BNYVV, PVM, and PVX. Moreover, an equivalent degree of limited similarity was also observed in the regions upstream of the unrelated BNYVV p14 and the PCV TGB2 genes. Likewise, the sgRNAγ promoter shares small blocks of common sequence with the region upstream of the PCV p15 gene,
which encodes a protein closely related to the yb protein, but the promoter also has similarity to the regions upstream of the unrelated BNYVV TGB1 and TMV p30 genes. The BSMV, BNYVV, PVM, and PCV replicase proteins belong to the supergroup 3 tobamo lineage of RNA-dependent RNA polymerases (Koonin and Dolja, 1993), so in these cases it is tempting to speculate that the promoter sequences coevolved in response to the polymerase specificity rather than as a block consisting of the promoter and its associated protein. However, the RdRp proteins of BMV and PVX are also classified in the supergroup 3 tobamo lineage, yet their sgRNA promoter sequences share no apparent similarity to the BSMV promoters. Therefore, it is difficult to make a case for promoter evolution based on either promoter protein constraints or polymerase similarity.

We performed computer analyses to search for common secondary structural elements within or flanking the minimal BSMV core promoters, but were unable to identify common elements. This is surprising because in other viruses, sgRNA promoter recognition by replicase complexes often requires primary sequence as well as secondary structural elements. These requirements may facilitate diverse interactions between replicase complexes and promoters with limited sequence similarity. In BYDV, both RNA sequence and secondary structure are essential for promoter activity (Koev et al., 1999). The BYDV sgRNA1 promoter folds into two stem loops downstream of its transcription initiation site, but comparisons with the predicted structures of the sgRNA2 and sgRNA3 promoters reveal that they possess very different sized stem loops downstream of their respective transcription initiation sites (Koev et al., 1999). The TMV MP sgRNA promoter also requires a stem loop structure for promoter activity that will tolerate substantial variation in primary sequence without major effects on sgRNA synthesis (Grdzelishvili et al., 2000). In addition, chemical and enzymatic probing of the 1.45-kb sgRNA promoter of TCV has confirmed the presence of a 96-nt hairpin structure, although only a 21-nt hairpin and a 9-nt flanking single-stranded region comprise the minimal promoter (Wang et al., 1999). Thus, our comparisons suggest that BSMV promoters differ from those of a number of other viruses in that conservation of primary sequence rather than conserved structure may have a predominant role in promoter activity.

The available evidence indicates that sequences flanking the core promoter and positioning of the promoter contribute substantially to the transcription of the BSMV sgRNAs. In the case of sgRNAβ2, expression increased substantially upon removal of a 48-nt sequence required for sgRNAβ1 promoter activity. This result suggests that competition for replicase has an important role in mediating the levels of expression of sgRNAβ1 and sgRNAβ2. The ectopic expression experiments support this hypothesis because both the ectopic sgRNAγ and the sgRNAβ2 promoters directed transcription more efficiently in constructs lacking the wt promoters than in constructs with wt promoters. Positioning also appears to have variable effects on promoter activity. Accumulation of sgRNAs transcribed from promoters located close to the 3′ terminus of the genomic-sense RNA was less abundant than those of the wt sgRNA from an upstream promoter. When the sgRNAγ, sgRNAβ1, and sgRNAβ2 promoters were placed downstream of the wt sgRNAβ1 promoter in RNAβ, the ectopic sgRNAs were considerably reduced in comparisons with the wt sgRNAβ1 (Fig. 5). However, in RNAγ, the ectopic sgRNAγ promoter strength appeared to be similar to that of the wt sgRNAγ promoter. On the other hand, when the ectopic sgRNAβ2 promoter was placed downstream of the wt sgRNAγ promoter, the ectopic sgRNA accumulated to much lower levels than the wt sgRNAγ (Fig. 5C). In contrast to the larger regions encompassing the BSMV sgRNA promoters, the minimal BSMV promoter sequences were unable to function ectopically. These results suggest that positioning, flanking sequences, and, in the case of sgRNAβ2, an upstream enhancer element may contribute substantially to promoter strength. In particular, the reduction in activity resulting from placement of the BSMV sgRNA promoters near the 3′ end of the genomic RNA contrasts with many examples reported from other RNA viruses where more 3′ located ectopic promoters are preferentially transcribed (French and Ahlquist, 1988; Boccard and Baulcombe, 1993; Wang and Simon, 1997; Koev and Miller, 2000).

The majority of viruses that contain multiple sgRNA promoters on the same RNA shows a strong correlation between the abundance of their sgRNAs and their proximity to the 3′ end of the genomic RNA (Kelly et al., 1994; Wang and Simon, 1997; Grdzelishvili et al., 2000). However, BSMV sgRNAβ1 and sgRNAβ2 promoters appear not to share this correlation because sgRNAβ2, which is proximal to the 3′ terminus of RNAβ, accumulates to much lower levels than the upstream sgRNAβ1. This pattern of synthesis for TGB sgRNAs has also been demonstrated for Potato virus X (Verchot et al., 1998) and is likely the case for the multicomponent Benyviruses, Furoviruses, Pomonaviruses, and Pecluviruses and the monopartite Carlaviruses, Allexivirus, and Foveavirus (van Regenmortel et al., 2000), which contain related TGBs. During replication of BSMV, PVX, and BNYVV, two sgRNAs are generated for translation of the overlapping TGB proteins. The relatively high abundance of sgRNA1 reflects the accumulation of the TGB1 protein during the early stages of infection, whereas the 3′ proximal sgRNAβ2 is often difficult to detect (Zhou and Jackson, 1996a, 1996b; Verchot et al., 1998). It is possible that several evolutionary constraints may contribute to the low abundance of sgRNAβ2 and the analogous sgRNA in other TGB-containing viruses. One possibility is that the relative abundance of the proteins encoded by the sgRNAs may be important for the movement complex function. Alternatively, because the sgRNAβ2 promoter resides within the TGB1 ORF, optimal promoter strength may have been constrained by necessities for TGB1 protein function.
sequences residing within the same region of the genome often have evolved to permit recognition of the three different sgRNA promoters, which overlap regions encoding the γ and TGB1 ORFs, respectively. Since the BSMV sgRNA promoter regions are multifunctional, it is likely that the replicase complex has coevolved with viral cis-acting elements to permit recognition of the three different sgRNA promoters and to discriminate between the distinct elements at the 3’ ends of the positive- and negative-sense RNAs. These varied interactions may also help explain the temporal regulation of the sgRNA promoters in the same virus. For example, the diverse promoter–replicase interactions within a virus may be facilitated by varied complexes of replicase and host proteins with distinct promoter elements. Such interactions may also mediate the strength and temporal activities of the BSMV sgRNA promoters.

Materials and methods

Recombinant plasmids

The α, β (β42Spl), and γ (γ42) cDNA clones used in this study were derived from the BSMV ND18 strain (Pett et al., 1988). To define the starting points for mapping the sgRNAγ promoter, the γ cDNA clone was digested with KpnI and BsmI, the 3’ overhangs were removed with T4 DNA polymerase, and the vector was religated to generate the clone γΔKpnI/BsmI. The γAE/E cDNA clone constructed by Zhou and Jackson (1996a) was also used. To generate large-scale deletions in the sgRNAγ promoter, Spl sites were engineered by site-directed mutagenesis (Kunkel, 1985) at positions –119, –78, –38, –18, and +11 relative to the sgRNAγ transcription start site at nt 2054 using the oligonucleotides listed in Table 1. The resulting clones, γSpl–119, γSpl–78, γSpl–38, and γSpl–18 were digested with Clal (–152) and Spl, while the γSpl+11 clone was digested with KpnI (+57) and Spl. The digested clones were treated with T4 DNA polymerase to generate blunt ends and religated. These clones were designated γ−152–119, γ−152–78, γ−152–38, γ−152–18, and γ+57+11. The clone γ+57+27 was constructed by digesting the γ42BamHI clone (Pett et al., 1990) with BamHI and KpnI, blunting with T4 DNA polymerase, and religating. Smaller deletions within the sgRNAγ promoter region were engineered by site-directed mutagenesis using the γ cDNA clone to create deletions ranging in size from 2 to 10 nts in the –30 to +10 region surrounding the transcription start site. Throughout the course of this work, deletions or site-directed mutations were sequenced to ensure that the desired changes had been introduced.

To identify the starting points for mapping the sgRNAβ promoter, we used the βA1.6 and βA2.0 clones (Pett and Jackson, 1990). Refined mapping of the sgRNAβ promoter was performed using a β cDNA clone (β5B) containing a 1427-nt deletion from SalI to BglII that removes part of the TGB1, TGB2, and TGB3 coding regions. This deletion was
engineered to increase the separation between the sgRNAβ1 and the 18S ribosomal RNA in agarose gels. KpnI sites were introduced into the βS/B cDNA clone at positions −107, −74, and −34 relative to the sgRNAβ1 transcription start site at 789 nt using site-directed mutagenesis and β1-specific oligonucleotides (Table 1). The resulting clones were digested with BsrBI (−156) and KpnI, blunted with T4 DNA polymerase, and religated to generate the β1−156/−107, β1−156/−74, and β1−156/−34 clones. Deletions were engineered in the sgRNAβ1 promoter region of the βS/B clone by site-directed mutagenesis to produce the clones β1−60/−51, β1−50/−41, β1−40/−31, β1−30/−21, β1−20/−11, and β1−10/+1 clones with a series of sequential deletions from positions −60 to +1. More detailed resolution of the sgRNAβ1 promoter region was obtained by site-directed mutagenesis of the βS/B cDNA clone to generate the clones β1−30, β1−30/−29, β1−30/−28, β1−14/+3, β1−14/+1, β1−14/−1, β1−14/−2, and β1−14/−3.

To define the cis-acting element required for RNAβ replication, two KpnI sites were created at −107 and −74, or −74 and −34, relative to the transcription start site using site-directed mutagenesis. These clones were digested with KpnI and religated to produce the clones β1−107/−74 and β1−74/−34.

The sgRNAβ2 promoter region was analyzed in a β cDNA background that contained a deletion between −34 and +14 relative to the sgRNAβ1 transcription start site and hence lacked the sgRNAβ1 promoter. Clone β1−34/+14 was constructed by digesting the β1−34KpnI clone with KpnI and NcoI, blunting with T4 DNA polymerase, and religating. Since the sgRNAβ2 transcription start site is located at nt 2279, the starting points for mapping the sgRNAβ2 promoter were defined with the clone β2PstI/EcoRI, which lacks nts 1705 to 2109, and β2 +9/SspI, lacking nts 2290 to 2434. Clone β2PstI/EcoRI was generated from the β1−34/+14 clone by digestion with PstI and EcoRI, blunting with T4 DNA polymerase, and religation. Clone β2 +9/SspI was engineered by introducing a SmaI site at position +9 relative to the sgRNAβ2 transcription start site by site-directed mutagenesis with the oligonucleotide β2 +9SmaI (Table 1). The resulting derivative was partially digested with SspI, digested with SmaI, and religated to generate the β2 +9/SspI clone. For large-scale mapping of the sgRNAβ2 promoter, the β1−34/+14 cDNA clone was used in conjunction with the β2-specific oligonucleotides listed in Table 1 to engineer unique KpnI sites upstream of the sgRNAβ2 promoter at positions −110, −76, −52, and −28. These clones were subsequently digested with EcoRI (−179) and KpnI, blunted with T4 DNA polymerase, and religated to generate the clones β2−179/−110, β2−179/−76, β2−179/−52, and β2−179/−28.

Six nucleotide deletions from −70 to −35 relative to the sgRNAβ2 transcription start site were generated by site-directed mutagenesis of clone β1−34/+14 to produce the clones β2−70/−65, β2−64/−59, β2−58/−53, β2−52/−47, β2−46/−41, and β2−40/−35. More refined mapping of the sgRNAβ2 promoter was facilitated by generating the clones β2−40/−35, β2−40/−34, β2−40/−33, β2−40/−32, β2−40/−31, β2−40/−9, β2 +10/−15, β2 +10/−16, β2 +10/−17, and β2 +10/−18 by site-directed mutagenesis of the β1−34/+14 cDNA clone.

To determine if the three sgRNA promoters can function ectopically, regions surrounding the sgRNAγ (276 nt), sgRNAβ2 (295 nt), or the sgRNAβ1 promoter (256 and 150 nt) were PCR amplified. These reactions were carried out with the oligonucleotides listed in Table 1 to introduce BamHI sites at the 5′ and 3′ termini. A BamHI site was engineered in the βS/B clone at nt 1132−1137, by site-directed mutagenesis with the β1134BamHI oligonucleotide (Table 1). The BamHI site was then introduced into RNAβ derivatives that either contained or lacked the sgRNAβ1 promoter (β1−34/+14). In addition, a BamHI site was engineered into the γ cDNA clone (containing the sgRNAγ promoter) and the γ−30/−21 cDNA clone (lacking the wt sgRNAγ promoter) at nt 2338−2343 with the γ2239BamHI oligonucleotide (Table 1). These intermediate plasmids were then digested with BamHI and ligated to each of the amplified subgenomic promoters (sgRNAγ, sgRNAβ1, and sgRNAβ2) to generate clones β1+sγ, β1+sβ1, β1+sβ2, β+sγ, β+sβ1, β+sβ2, γ+sγ, γ+sβ1, and γ+sβ2. Since the sgRNAβ1 promoter contains a MluI site, the γ+sβ1 and γ+sβ2 clones were linearized for in vitro transcription reactions at a SpeI site introduced at their 3′ termini with the BSMV3′SpeI oligonucleotide (Table 1).

**Protoplast isolation and transfection**

Protoplasts from the BY-2 tobacco cell suspension line were prepared and transfected as described previously (Watanabe et al., 1982). To map the sgRNAγ promoter, protoplasts were transfected with in vitro transcripts (Petty et al., 1988) generated from the cDNA clones α and γ KpnI/HpaI (Zhou and Jackson, 1996a), plus one of the modified γ clones described above. For analysis of the sgRNAβ1 and sgRNAβ2 promoters, protoplasts were transfected with in vitro transcripts from the α and γ cDNA clones, plus the appropriate modified β cDNA clone. Prior to use in in vitro transcription reactions, the β cDNA clones were linearized with SpeI, and the α and γ cDNA clones were linearized with MluI, except for the γ1+sβ1 and γ+sβ2 clones, which were linearized with SpeI.

**RNA extraction and analysis**

RNA was extracted from BY-2 protoplasts at 20 h post-transfection as described previously (Zhou and Jackson, 1996a). For Northern blot analysis, 5 μg of total nucleic acid was separated on 1% agarose TBE gels and blotted onto Hybond NX (Amersham Pharmacia Biotech) nylon membranes. Prehybridization, hybridization, and washes
were performed as recommended in the manufacturer’s instructions.

RNAβ- and RNAγ-specific [32P]-UTP-labeled riboprobes were generated by in vitro transcription reactions using T7 or T3 RNA polymerase (Zhou and Jackson, 1996a). Portions of the β (2434 to 2785 nt, 802 to 1375 nt) or γ (2111 to 2444 nt) cDNA clones were introduced into pBluescript KS+ (Stratagene) or pGem5ZF+ (Promega, Madison, WI) plasmids and linearized prior to being used in in vitro transcription reactions. An additional RNAβ-specific-ribobprobe (2785–2985 nt) was generated by linearizing the βc-2785 clone (D.M. Lawrence and A.O. Jackson, unpublished results) with BamHI prior to use in in vitro transcription reactions.

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