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Characterization and Engineering of Sequences Controlling In Vivo Synthesis of Brome Mosaic Virus Subgenomic RNA

ROY FRENCH† AND PAUL AHLQUIST*

Institute for Molecular Virology and Department of Plant Pathology, University of Wisconsin—Madison, Madison, Wisconsin 53706

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Expression of brome mosaic virus (BMV) coat protein and internal genes of many other positive-strand RNA viruses requires initiation of subgenomic mRNA synthesis from specific internal sites on minus-strand genomic RNA templates. Biologically active viral cDNA clones were used to investigate the sequences controlling production of BMV subgenomic RNA in vivo. Suitable duplications directed production of specifically initiated, capped subgenomic RNAs from new sites in the BMV genome. Previously implicated promoter sequences extending 20 bases upstream (−20) and 16 bases downstream (+16) of the subgenomic RNA initiation site directed only low-level synthesis. Subgenomic RNA production at normal levels required sequences extending to at least −74 but not beyond −95. Loss of an $(rA)_{18}$ tract immediately upstream of the −20 to +16 "core promoter" particularly inhibited subgenomic RNA synthesis. The −38 to −95 region required for normal initiation levels contains repeats of sequence elements in the core promoter, and duplications creating additional upstream copies of these repeats stimulated subgenomic RNA synthesis above wild-type levels. At least four different subgenomic RNAs can be produced from a single BMV RNA3 derivative. For all derivatives producing more than one subgenomic RNA, a gradient of accumulation progressively favoring smaller subgenomic RNAs was seen.

Many positive-strand RNA viruses, with both single and multicomponent genomes, encode two or more genes on a single genomic RNA. In most of these cases, the internal genes are not translated directly from the genomic RNA. Rather, each internal gene is translated from its own subgenomic mRNA, a partial, 3′-coterminal copy of the genomic RNA whose 5′ end is situated to provide ribosomal access to the relevant gene. This strategy allows the potential to independently regulate, in time and amount, the expression of two or more genes from a single genomic RNA.

In recent years, many positive-strand RNA viruses which produce subgenomic mRNAs have been shown to share substantial sequence similarities in nonstructural proteins involved in viral RNA synthesis (8, 15, 19). The viruses related by these similarities include the animal alphaviruses and also a wide range of plant viruses. One of the plant viruses so related is brome mosaic virus (BMV), an isometric virus of cereals (24). The BMV genome is divided among RNAs 1, 2, and 3 of 3.2, 2.9, and 2.1 kilobases, which have been completely sequenced (3, 7). Monocistronic RNAs 1 and 2 encode genes which are both required and, together, sufficient to induce viral RNA synthesis (18, 22). RNA3 encodes two genes, the 5′-proximal 3a nonstructural protein which is translated directly from RNA3 and also the 3′-proximal coat gene, which is translated from a subgenomic mRNA designated RNA4 (7, 34). Complete BMV cDNA clones have been constructed from which infectious transcripts can be produced (5, 21). Among other studies, these transcripts have been used to characterize the *cis*-acting sequences required for amplification of RNA3 in BMV-infected cells (17).

Previous studies have shown that an RNA-dependent RNA polymerase from BMV-infected cells will synthesize RNA4 in vitro from negative-strand RNA3 templates, demonstrating that RNA4 production is by partial transcription rather than by cleavage of RNA3 positive strands (29). In these studies, negative-strand RNA3 templates extending no more than 20 bases upstream of (i.e., 3′ to) the RNA4 initiation site were able to direct RNA4 production in vitro. Other studies show that deletions and foreign gene insertions within 17 bases downstream of the RNA4 start site do not interfere with subgenomic mRNA production in vivo, allowing the expression of suitably inserted foreign genes by the subgenomic mRNA pathway (18). Taken together, these results suggest that the 37-base region so defined constitutes at least a functional core of the promoter which directs synthesis of subgenomic RNA4 from negative-strand RNA3 templates (18).

In this study, we have investigated the nature and behavior of sequences influencing RNA4 production in vivo, examining the role of additional upstream sequences, positional influences on promoter function, and other effects. We have also analyzed the organization of sequence elements within the promoter and their relation to subgenomic mRNA initiation sites in other viruses, including the animal alphaviruses.

MATERIALS AND METHODS

Wild-type BMV cDNA clones. Plasmids pB1TP3, pB2TP5, and pB3TP8 contain cDNA copies of BMV RNAs 1, 2, and 3, respectively, from which properly initiated, capped, and infectious transcripts of the complete BMV genome can be produced with T7 RNA polymerase (21). These transcripts were used to provide wild-type BMV components as needed for the various combinations inoculated. Other plasmids used in construction of RNA3 derivatives were pB3TP7 (21) and pB3PM1 (6). These plasmids contain the same BMV RNA3 cDNA region as pB3TP8, but in pB3TP7 and pB3PM1 this cDNA is linked to a T7 promoter with a single intervening G residue and linked directly to an *Escherichia coli*

* Corresponding author.
† Present address: Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583.

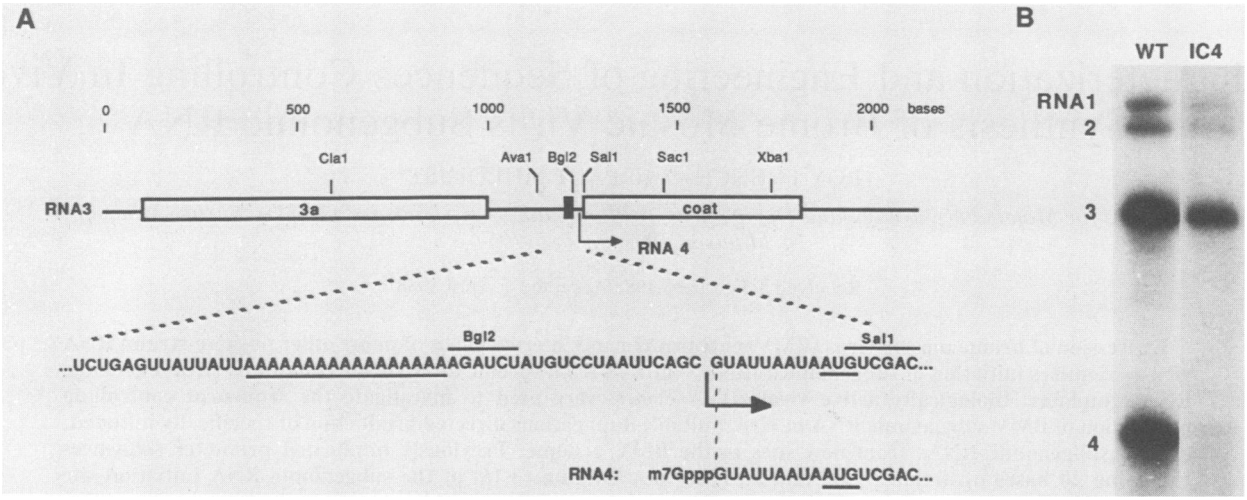

FIG. 1. Effect of deleting the oligo(A) region of BMV RNA3. (A) Schematic map of BMV RNA3 and sequence surrounding the start of RNA4. Noncoding regions are shown as a single line, the 3a and coat genes are shown as open boxes, the oligo(A) is shown as a small filled box, and a bent arrow marks the start of sequences encoding subgenomic RNA4. Within the sequence shown, the *Bgl*II and *Sal*I sites bounding the core promoter discussed in the Introduction are overlined and the coat gene initiation codon and the oligo(A) tract are underlined. All but one of the A residues in this tract were deleted to generate the derivative IC4. In keeping with usual practice, the sequence of the packaged, coding RNA strand is shown, but it should be noted that the complementary sequence serves as template for subgenomic RNA synthesis. (B) RNA blot hybridization analysis of progeny RNA from protoplasts inoculated with in vitro transcripts from BMV RNA1 and RNA2 cDNA clones (pB1TP3 and pB2TP5) and transcripts from either wild-type RNA3 cDNA clone pB3TP8 (WT) or deletion derivative pB3IC4 (IC4). After incubation of inoculated protoplasts at 24°C for 20 h, total nucleic acids were extracted, electrophoresed in a 1% agarose gel, and transferred to a Zetaprobe (Bio-Rad Laboratories) membrane. The membrane was then probed with ^{32}P-labeled RNA complementary to the conserved 3′-terminal 200 bases of BMV RNAs 1 to 4 and autoradiographed.

promoter, respectively. All of the above plasmids except pB3PM1 are derivatives of pUC119 (provided by J. Vieira), and so they contain the single-stranded DNA (ssDNA) replication origin of M13, allowing recovery of ssDNA forms after superinfection with suitable helper bacteriophage such as M13K07 (also provided by J. Vieira).

Plasmid constructions. Standard methods for plasmid construction were used with minor modifications (26). To join noncomplementary ends from different restriction fragments, 3′ protruding ends were trimmed to bluntness with T4 DNA polymerase and 3′ recessed ends were extended to bluntness with either the large fragment of DNA polymerase I or T4 DNA polymerase. The following plasmids were constructed.

(i) pB3IC4. *E. coli* RZ1032 (Dut$^-$ Ung$^-$) (23) was transformed with pB3TP8, and dUTP-containing ssDNA was isolated by superinfection with M13K07. This ssDNA was used as a template for oligonucleotide-directed mutagenesis (23) to delete 17 of the 18 residues of the pB3TP8 intercistronic oligo(A) tract, and the product clones were screened by dideoxynucleotide sequencing (11). The mutagenic oligonucleotide used was d(GACATAGATCTAATAATAAC). A single residue from the oligo(A) was retained in pB3IC4 to preserve the *Bgl*II site bounding the "core promoter."

(ii) Clones SG5, -5R, -7, -9, -10, -11, -13, -14, -15, -21, and -22. For the following clones, copies of the *Ava*I-*Sac*I fragment of pB3TP7 were inserted as duplications in the indicated sites of the RNA3 cDNA region of pB3TP7 (see Fig. 6A): pB3SG5 (SG5), *Xba*I; pB3SG7 (SG7), *Cla*I; pB3SG9 (SG9), *Cla*I and *Xba*I; pB3SG10 (SG10), *Xba*I and *Stu*I; pB3SG13 (SG13), *Cla*I, *Xba*I, and *Stu*I; pB3SG14 (SG14), *Cla*I, *Nsi*I, *Xba*I, and *Stu*I. Restriction analysis was used to select those insertions with the same orientation as the surrounding cDNA. Multiple rounds of cloning were required to construct the larger derivatives. For other clones, the following pB3TP7 fragments (see Fig. 2 and 3) were duplicated at the *Xba*I site of pB3TP7 cDNA: pB3SG5R (SG5R), the *Ava*I-*Sac*I fragment in reverse orientation; pB3SG11 (SG11), the *Ava*I-*Sal*I fragment; pB3SG15 (SG15), the *Bgl*II-*Sac*I fragment; pB3SG21 (SG21), the *Bst*XI-*Sac*I fragment; pB3SG22 (SG22), the *Dde*I-*Sac*I fragment.

(iii) pB3SG6 and pB3SG6R. pB3BB1 is a pB3TP7 derivative in which a deletion from base 1168 to the downstream *Sac*I site has been introduced (17). The *Ava*I-*Sac*I fragment of pB3TP8 was introduced into the *Xba*I site of pB3BB1 in the same orientation as the surrounding cDNA to create pB3SG6 (SG6) and in the reverse orientation to create pB3SG6R (SG6R).

(iv) pB3IC2, pB3IC3, pB3IC5, and pB3IC6. In separate constructions, a *Bam*HI linker (CGCGGATCCGCG; New England BioLabs) was inserted in the *Ava*I and *Bgl*I sites of pB3PM1 after filling out the 3′ recessed ends with Klenow DNA polymerase. The small *Pst*I-*Bam*HI fragment of each of these plasmids was ligated with the large *Pst*I-*Bam*HI fragment of pB3BC1 (17) to yield intermediate plasmids with the cDNA duplications of IC2 and IC3, respectively. The small *Pst*I-*Cla*I fragment of each plasmid was then exchanged for the small *Pst*I-*Cla*I fragment of pB3TP8 (providing a phage T7 promoter in place of an *E. coli* promoter for in vitro transcription) to yield IC2 and IC3. In two additional constructions, the large *Eco*RI-*Hin*cII fragment of pB3HS1 (17) was ligated with the small *Ava*I-*Eco*RI fragment of pB3TP8 or pB3IC4 (Fig. 1). The large *Bgl*II-*Eco*RI fragments of the resulting plasmids were ligated individually with the small *Bam*HI-*Eco*RI fragment of pB3BC1 to yield IC5 and IC6, respectively.

In vitro transcription, protoplast inoculations, and RNA analysis. Barley protoplasts were isolated as described by Loesch-Fries and Hall (25). In vitro transcription in the

presence of m^7GpppG or GpppG, protoplast inoculation and incubation, viral RNA extraction and analysis, and densitometry of the resulting autoradiographs were carried out as described previously (17). Each plasmid construct was tested in at least three, and usually many more, independent experiments. Probes for positive-strand RNA were a ^{32}P-labeled SP6 transcript from a subclone, pB3HE1, containing the 200-base *Hin*dIII-*Eco*RI fragment of 3′-terminal RNA3 cDNA sequences from pB3TP8 or (see Fig. 6) a similar in vitro transcript complementary to the *Bgl*II-*Sac*I region of RNA3. Denaturing electrophoresis for negative-strand RNA analysis was carried out on formaldehyde gels (26). Probes for negative-strand RNAs were in vitro transcripts from subclones covering the *Ava*I-*Sac*I region of BMV RNA3.

Primer extension. Approximately 10^5 protoplasts were inoculated with transcripts of wild-type RNA1 and RNA2 cDNA clones (pB1TP3 and pB2TP5) and transcripts of the appropriate RNA3 cDNA clone (see Fig. 3). After incubation at 24°C for 20 h under continuous light, total nucleic acids were isolated and dissolved in 15 μl of deionized distilled water. A 1.5-μl portion of this nucleic acid was mixed with 1 ng of 5′-^{32}P-labeled d(GCGAGTCATCTTACC) (complementary to bases 28 to 42 of BMV RNA4) in a total volume of 4 μl containing 1.5 U of reverse transcriptase (Life Sciences, Inc.), deoxynucleotides at 25 μM each, 25 mM Tris chloride (pH 8.3), 5 mM $MgCl_2$, 25 mM KCl, and 5 mM dithiothreitol. After incubation for 30 min at 37°C, the products were denatured at 95°C for 3 min in an equal volume of 90% formamide–20 mM EDTA–0.1% xylene cyanol–0.1% bromophenol blue and 3-μl portions were electrophoresed on a 12% polyacrylamide–8.3 M urea gel. Marker lanes were prepared similarly except that each reaction also contained 50 μM of a single dideoxynucleotide, and the template was ssDNA prepared from phage particles isolated after superinfection of pB3SG6-transformed *E. coli* with helper phage M13K07 (provided by J. Vieira). Sequence comparisons were made with the assistance of software from the University of Wisconsin Genetics Computer Group.

RESULTS

Contribution of intercistronic oligo(A) to efficient subgenomic mRNA synthesis in vivo. Previously, we have shown that extensive deletions and substitutions of sequences 3′ to the *Sal*I site of BMV RNA3 (Fig. 1A) do not markedly affect subgenomic mRNA synthesis in vivo (17, 18). Thus, initiation of BMV subgenomic RNA synthesis does not require more than 16 bases downstream of the RNA4 transcription start site (i.e., 3′ to the start of RNA4 sequences in virion-sense RNA3). In this study, infectious in vitro transcripts of cloned BMV cDNA (5, 21) were used to investigate the involvement of sequences upstream of the RNA4 start in subgenomic RNA synthesis. One notable feature of the BMV RNA3 sequence upstream of the RNA4 start site is in an oligo(A) tract (Fig. 1A), which is conserved at equivalent genomic positions in other members of the bromovirus group (2; R. Allison, M. Janda, and P. Ahlquist, unpublished results). This tract has a heterogeneous length of 16 to 22 bases in natural RNA3 populations from virions (7), and is 18 bases long in pB3TP8, the biologically active, wild-type RNA3 cDNA clone used in this study (21; M. Janda and P. Ahlquist, unpublished results).

Unlike nearby upstream sequences, this tract does not contribute to replication or stability of BMV RNA3 in vivo (17). To test the specific contribution of the oligo(A) to subgenomic mRNA synthesis, oligonucleotide-directed mutagenesis was used to remove all but one A residue of this tract from pB3TP8, generating the derivative IC4 (Fig. 1A). When transfected into protoplasts with RNA1 and RNA2 cDNA transcripts, transcripts of the IC4 RNA3 derivative were replicated (Fig. 1B), but accumulation of RNA4 relative to the BMV genomic RNAs was reduced more than 10-fold, as measured by densitometry of the Fig. 1B autoradiograph and others. Since the RNA4 produced in infection with derivative IC4 is identical to wild-type RNA4, this reduced RNA4 accumulation must reflect reduced synthesis rather than altered stability. Thus, deletion of the oligo(A) dramatically reduces RNA4 synthesis in vivo.

Insertion of a functional subgenomic promoter at a new site in BMV RNA3. The role of sequences 5′ to the oligo(A) in subgenomic RNA synthesis would be difficult to study by applying deletion or other mutagenic approaches to wild-type RNA3, since deletion of some sequences 5′ to the oligo(A) substantially inhibits RNA3 replication (17). To separate the effects of sequence changes on subgenomic RNA production from effects on genomic RNA3 amplification, we investigated whether an independent subgenomic RNA initiation site could be established in RNA3 by duplicating appropriate sequences at a new location. Accordingly, an *Ava*I-*Sac*I fragment of cDNA spanning the RNA4 start site was duplicated by insertion in the *Xba*I site of RNA3, creating derivative SG5 (Fig. 2A). When transfected into protoplasts along with wild-type RNA1 and RNA2 transcripts, SG5 transcripts were replicated and directed the production of two subgenomic RNAs as intended (Fig. 2B). In a further derivative, SG6, the activity of the subgenomic promoter in its wild-type context was abolished by deleting the sequence immediately surrounding the RNA4 transcription start site, leaving only the new promoter active (Fig. 2). As expected, two subgenomic RNAs were also produced if only the *Ava*I-*Sal*I fragment was duplicated at the *Xba*I site (derivative SG11, Fig. 2B).

In comparing relative accumulation of the various subgenomic RNA species, it was useful to establish an internal standard to account for any variations in transcript inoculum production and protoplast inoculation efficiency (17). RNA3 was chosen as the most appropriate internal standard, since both genomic RNA3 and subgenomic RNAs are copied from the same negative-strand RNA3 template (see reference 29 and below). Relative to RNA3 production, SG5-, SG6-, and SG11-directed accumulation of the new subgenomic RNA4′ actually exceeds that of wild-type RNA4 (Fig. 2B), suggesting that the *Ava*I-*Sal*I fragment transferred in these constructions contains all the information which specifically contributes to subgenomic RNA production. For both SG5 and SG11, the amount of subgenomic RNA4 produced from the wild-type 5′-proximal initiation site is reduced relative to that produced from the newly introduced promoter at the *Xba*I site (Fig. 2B). This effect and the relative activity of individual promoters in other multiple subgenomic promoter constructs are discussed further below.

For both SG5 and SG6, parallel constructions were made in which the *Ava*I-*Sac*I fragment was inserted in the opposite orientation (SG5R and SG6R, Fig. 2). In both cases, this inversion resulted in severe inhibition of RNA3 accumulation. If this inhibition is due to the potential of the inverted region to base pair with its noninverted counterpart, then secondary structure throughout the viral RNA may be under considerable constraints. However, the mechanism of inhibition is not clear, since elongation by an in vitro BMV RNA polymerase extract is largely unaffected by large double-

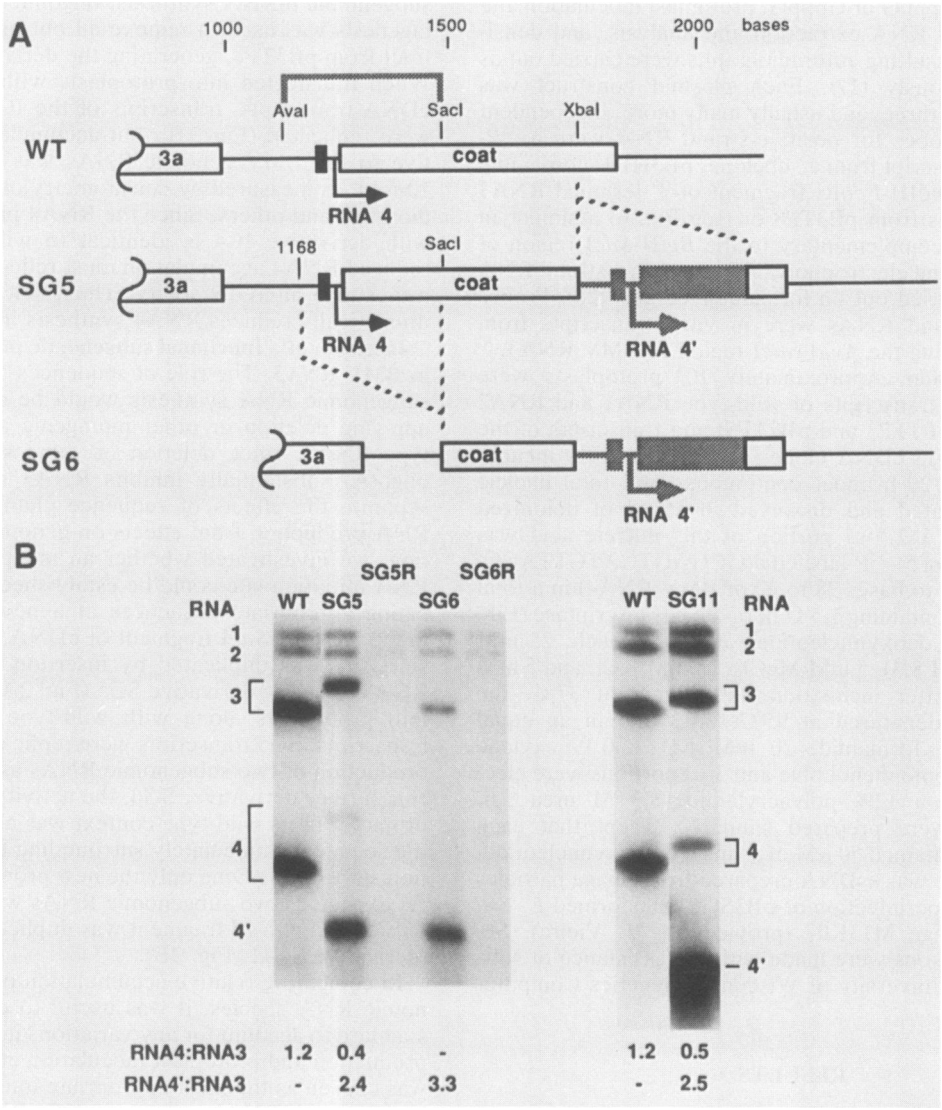

FIG. 2. Creation of a new subgenomic RNA initiation site in RNA3. (A) Schematic diagram of the regions surrounding the coat gene cDNA in plasmids pB3TP8 (WT), SG5, and SG6. The *Ava*I-*Sac*I fragment duplicated in SG5 is bracketed over the WT sequence, and shaded in its new locations in SG5 and SG6. Initiation sites for subgenomic RNAs 4 and 4′ are shown by bent arrows. Plasmids SG5R and SG6R (not diagrammed) correspond to SG5 and SG6, respectively, with the shaded fragment inverted. SG11 (not diagrammed) is similar to SG5 except that the smaller *Ava*I-*Sal*I fragment (Fig. 1A) is duplicated. (B) Analysis, as described for Fig. 1B, of viral RNA from protoplasts inoculated with transcripts of wild-type RNA 1 and 2 cDNA clones and of the indicated RNA3 clones. Molar ratios of RNA4:RNA3 and RNA4′:RNA3, given where applicable, are averages of densitometry results from autoradiographs of three or more independent experiments.

stranded regions in the interior of the RNA template (1). For SG6R, in which the inverted repeat is only 26 bases long, a low level of RNA3 accumulation could be seen on the original autoradiograph of Fig. 2 and on overexposures of this and similar films. For SG5R, which contains a 330-base inverted repeat, neither accumulation of RNA3 nor production of RNA4 was ever detected. Attempts to detect any internally initiated pseudosubgenomic RNA species of negative polarity were unsuccessful with both constructions.

The sizes of the subgenomic RNA species observed in protoplast infections with derivatives SG5 and SG6 were consistent with initiation from the expected sites in the duplicated promoter sequences. To confirm that the initiation site of subgenomic RNA synthesis was unchanged after the subgenomic promoter was moved to a new context, primer extension was used to map the 5′ ends of subgenomic RNAs from protoplast infections with wild-type, SG5, and SG6 RNA3 (Fig. 3). In all three cases, an identical pattern of two major strong-stop cDNA species was observed, confirming that the same start site within the subgenomic promoter is used when the promoter is located in its wild-type context, a 3′-proximal position (derivative SG6), or both simultaneously (derivative SG5). The appearance of a cDNA doublet in such primer extension experiments is associated with the presence of a 5′ cap on the RNA template (6, 20). The appearance of this doublet in the SG6 primer extension confirms that subgenomic RNA produced from the new initiation site in RNA3 is capped, as is wild-type RNA4 (16).

Effects of 5′ deletions on subgenomic promoter function. A

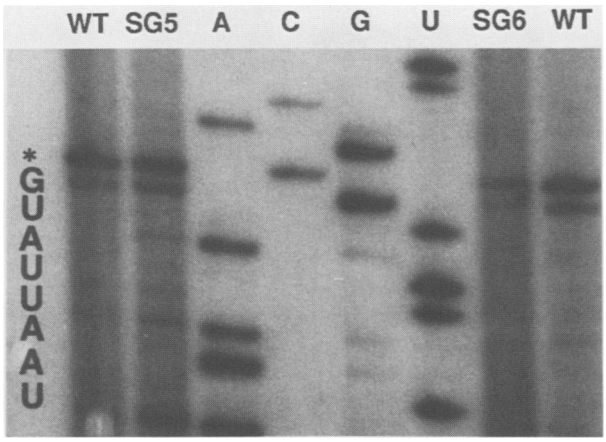

FIG. 3. Subgenomic RNA initiation site(s) in RNA3 derivatives SG5 and SG6. A 5′-^{32}P-labeled deoxyoligonucleotide complementary to bases 28 to 42 of BMV RNA4 was annealed with total RNA extracted from appropriately transfected protoplasts and extended with reverse transcriptase in the presence of deoxynucleotides. The resulting cDNA products were fractionated on a 12% polyacrylamide–8.3 M urea gel and autoradiographed. Protoplasts were inoculated with transcripts of BMV RNA1 and RNA2 cDNA clones and transcripts of either pB3TP8 (WT) or the SG5 or SG6 cDNA clones (Fig. 2), as indicated. The center lanes A, C, G, and U each contained the complementary dideoxynucleotide and were prepared by extension of the same 5′-^{32}P primer on ssDNA containing the SG6 cDNA sequences (see Methods). The letters at left follow the sequence of RNA4 from the sequencing lanes, reading 3′ to 5′ from the bottom of the gel. As shown, the lower strong-stop cDNA band of the protoplast lanes corresponds to the first residue (G) of RNA4, whereas the upper strong-stop band (*) is associated with the presence of the m^{7}G capping group (see text).

new subgenomic promoter having been established distal to the intercistronic region in constructs SG5 and SG6, it was possible to use deletion analysis to investigate the 5′ limits of sequences contributing to subgenomic RNA synthesis without significant effects on genomic RNA3 accumulation. A series of deletions was generated which removed various lengths of sequence from the 5′ end of the new promoter insertion in SG5 RNA3 (Fig. 4A). In protoplast infections, these deletions induce a clear and progressive decline in accumulation of the new, smaller subgenomic RNA species (Fig. 4B). The decreased accumulation must reflect reduced synthesis since the smaller subgenomic RNA itself, and thus its stability, is unchanged in the various constructions. The successive activity reductions observed show that each of the three segments removed in Fig. 4 contributes to subgenomic RNA production. Notably, the *Bgl*II-*Sal*I core promoter segment discussed in the Introduction induces only a low level of subgenomic RNA synthesis in vivo (SG15, Fig. 4), and requires the presence of from 54 to 75 additional upstream bases for full activity (SG5 and SG21, Fig. 4).

Stimulation of RNA4 production by upstream insertions. During these and previous experiments, we noted that upstream duplication of certain sequences in the RNA3 intercistronic region unexpectedly increased accumulation of RNA4 relative to RNA3. For example, in derivative IC3, nearly the entire intercistronic region is duplicated at a site over 220 bases 5′ to the RNA4 start, giving a clearly visible increase in RNA4 over a parallel infection with wild-type RNA3 (Fig. 5). As in some previous comparisons, sequences 3′ to the RNA4 start site were unchanged, so that the increased RNA4 accumulation must result from increased initiation of RNA4 synthesis and not from effects on elongation or stability of RNA4. To locate the specific sequences responsible for this effect, subfragments of the IC3 duplication were inserted in derivatives IC2, IC5, and IC6 (Fig. 5). RNA4 production by these derivatives shows that while sequences 5′ to the *Ava*I site have little effect (mutant IC2), significant stimulation of RNA4 production is conferred by duplicating the *Ava*I-*Bgl*II fragment (mutant IC5). Removal of the oligo(A) tract from the IC5 duplication reduces but does not eliminate RNA4 stimulation (mutant IC6). Thus, as well as contributing substantially to the full activity of subgenomic RNA synthesis in its natural position proximal to the RNA4 start site (Fig. 1, 2, and 4), the *Ava*I-*Bgl*II sequence is able to stimulate subgenomic RNA production from an upstream site considerably distant in primary sequence from the RNA4 start site.

Production of subgenomic mRNAs from multiple sites in individual RNA3 derivatives. To determine whether the ability of the *Xba*I site to support subgenomic promoter function was unusual, the *Ava*I-*Sac*I fragment of RNA3 was duplicated at a *Cla*I site 5′ to the normal RNA4 start site, creating RNA3 derivative SG7 (Fig. 6). As with constructs SG5 and SG11 (Fig. 2), both the wild-type and newly inserted subgenomic promoter are functional in SG7. Moreover, in all of these cases the smaller subgenomic RNA accumulates to a significantly higher level in infected cells.

A further series of constructions was then made to test whether more than two functional subgenomic promoters could be introduced into a single RNA3 derivative (Fig. 6A). In addition to the subgenomic RNA4 promoter in its wild-type context, these constructions carried either two (SG9, SG10) or three (SG13, SG14) additional copies of the *Ava*I-*Sac*I region at various combinations of the 5′-proximal *Cla*I site and the 3′-proximal *Xba*I, *Stu*I, and *Nsi*I sites. The *Ava*I-*Sac*I fragment was used as the promoter insertion in these constructions in preference to the smaller, functional *Ava*I-*Sal*I fragment (Fig. 2), because the additional sequence downstream of the initiation site made all resulting subgenomic RNA species readily differentiable in size by gel electrophoresis.

For each of the new RNA3 derivatives, the predicted subgenomic RNAs were produced in protoplasts coinfected with wild-type RNAs 1 and 2, showing that all copies of the subgenomic promoter were active (Fig. 6B). Moreover, as shown by the histogram of Fig. 6A, a distinct and consistent gradient favoring greater accumulation of subgenomic RNAs from 3′-proximal initiation sites is observed for each of the new derivatives, as previously seen with SG5 and SG11 (Fig. 2). In principle, if each subgenomic RNA gives rise to a corresponding negative-strand RNA, longer subgenomic negative strands might serve as additional templates for production of smaller subgenomic positive strands, contributing to their preferential accumulation. To examine this possibility, RNA samples from protoplasts infected with all constructs shown in Fig. 6 were electrophoresed on a denaturing gel, transferred to a nylon membrane, and probed for negative strands. For each infection, levels of subgenomic-sized negative strands were found to be small compared with the levels of genomic RNA3 negative strand (results not shown). Consequently, this effect could make only a minor contribution to the differential subgenomic RNA production. Other possible mechanisms underlying the observed gradient are considered in the Discussion.

Imperfect direct repeats in the BMV subgenomic promoter and homologies with corresponding regions in other viruses. Computer-assisted inspection reveals that the 120-base

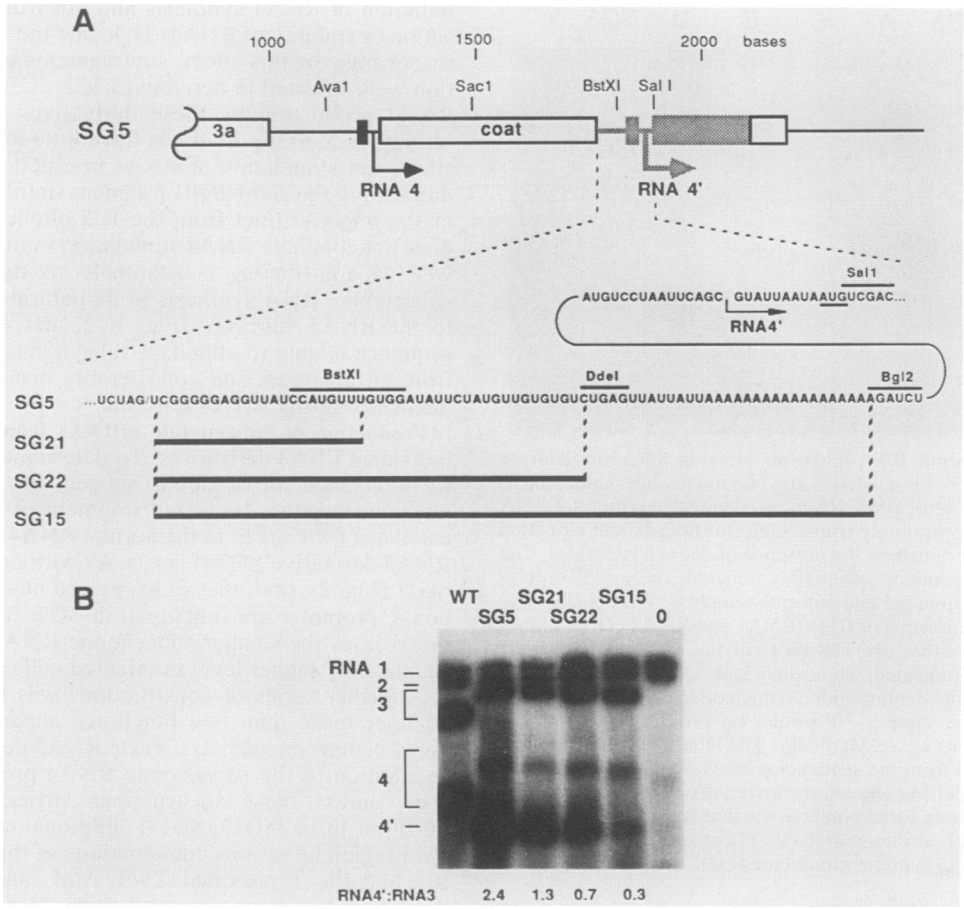

FIG. 4. Effects of 5′ deletions on subgenomic promoter activity. (A) Sequence surrounding the 3′-proximal subgenomic RNA initiation site in derivative SG5 (Fig. 2A) and map of deletions in derivatives SG21, SG22, and SG15. (B) Analysis, as described for Fig. 1B, of viral RNA from protoplasts inoculated with transcripts of RNA 1 and 2 cDNA clones and transcripts of the indicated RNA3 clones. Values given for the molar ratios of RNA4′:RNA3 are the averages of two or more independent experiments.

*Ava*I-*Sal*I region of BMV RNA3 implicated in directing subgenomic RNA synthesis contains several imperfect direct repeats (Fig. 7A). For example, the 30 bases overlapping and immediately 5′ to the oligo(A) have 67% identity with the *Bgl*II-*Sal*II core promoter region discussed in the Introduction, with the 3′ boundary of this duplication corresponding precisely to the coat gene start. Moreover, immediately 5′ to this duplication is a clearly related segment which again includes a single-mismatch repeat of the consensus element AUCUAUGUU. Thus, nearly all of the *Ava*I-*Sal*I promoter region consists of repeats or partial repeats of sequences related to the core promoter, punctuated in one instance by the oligo(A).

Though similar in sequence and possibly in some functions(s), the two copies of the core promoter motif flanking the oligo(A) are clearly not equivalent. Although the 3′ copy contains an active subgenomic RNA initiation site and constitutes a weak but functional promoter by itself, no subgenomic RNA initiation has been detected at the corresponding site in the 5′ copy of this motif.

As shown by the examples in Fig. 7B, the oligo(A)-core promoter region of BMV RNA3 contains similarities to subgenomic mRNA start sites in some other RNA viruses, including the animal alphaviruses as well as plant viruses such as cowpea chlorotic mottle virus (CCMV) and alfalfa mosaic virus. The sequence similarities shown overlap the BMV consensus element AUCUAUGUU, which with single mismatches is repeated three times in the BMV *Ava*I-*Sal*I fragment (Fig. 7A). No information presently exists on the functional significance of the sequence similarities observed for most of the interviral comparisons of Fig. 7B. However, we have recently observed that RNA4 synthesis proceeds correctly from CCMV RNA3 when the *trans*-acting factors for viral RNA synthesis are provided by coinfection with BMV RNAs 1 and 2 (Allison et al., unpublished results). Similarly, RNA4 is properly synthesized in coinfections with BMV RNA3 and CCMV RNAs 1 and 2. Marsh et al. (27) recently proposed somewhat different alignments for some of the viruses shown in Fig. 7B and also noted potential similarities with some other plant viruses.

DISCUSSION

These results show that BMV sequences extending 95 bases upstream and 16 bases downstream of the subgenomic RNA initiation site are sufficient in vivo to direct initiation of a new subgenomic RNA from a novel site in the viral RNA genome at wild-type or greater levels. While sequences no more than 20 bases upstream of the initiation site suffice to direct specific initiation at a low level, sequences at least as

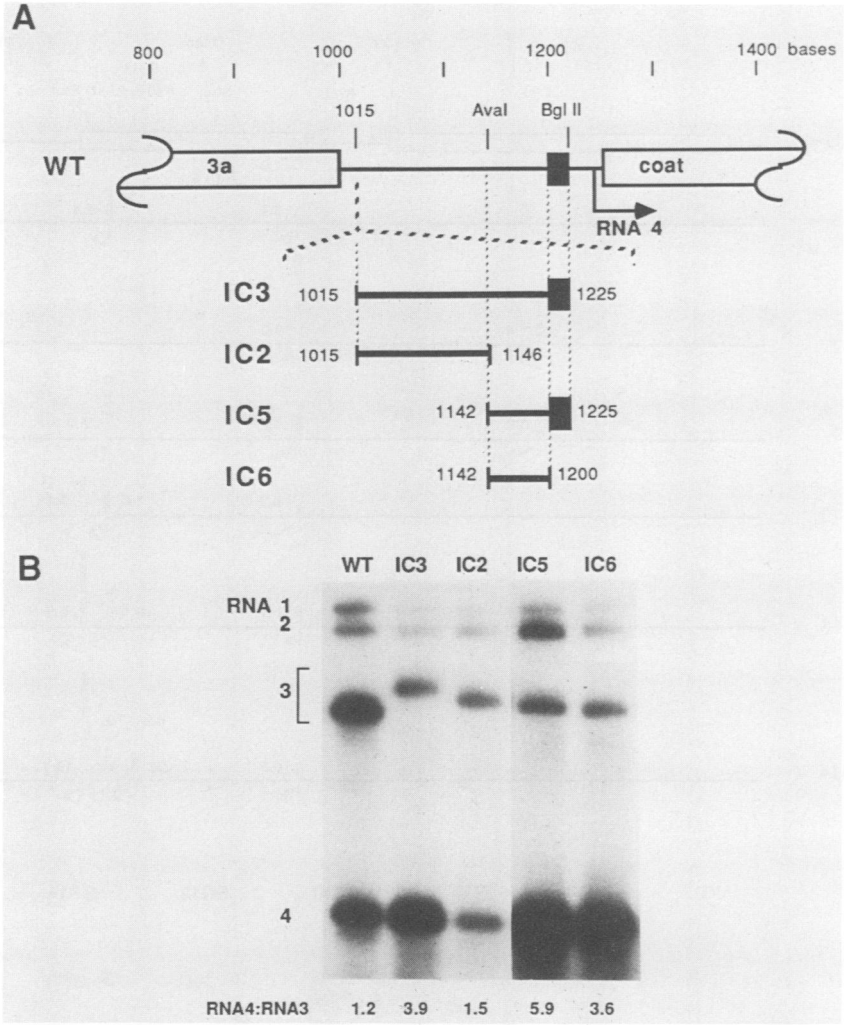

FIG. 5. Effects of upstream duplications on subgenomic promoter activity. (A) Map of the intercistronic region of wild-type RNA3 (WT) and sequences duplicated in RNA3 derivatives IC3, IC2, IC5, and IC6. In each of these derivatives, the indicated segment of intercistronic sequence is inserted as a duplication immediately 5′ to position 1015 in the RNA3 sequence. The scale at top and numbering of the duplication endpoints correspond to the published sequence of RNA3 (7). (B) Analysis, as described for Fig. 1B, of viral RNA from protoplasts inoculated with transcripts of RNA1 and RNA2 cDNA clones and transcripts of the indicated RNA3 clones. Values given for the molar ratios of RNA4:RNA3 are the averages of three independent experiments.

far as 74, but no more than 95, bases upstream are required for full subgenomic promoter activity. As previously suggested (7), the oligo(A) tract within this region is an important functional element for subgenomic RNA synthesis. Although conservation of the oligo(A) in other bromoviruses suggests that its distinctive homopolymer nature is related to its function(s), this tract may also provide needed spacing or flexibility between other elements of the promoter. For this and other reasons, linker scanning (28) or other directed substitution experiments may be more valuable than additional deletions for further study of the subgenomic promoter.

The upstream boundary of RNA3 sequences controlling subgenomic RNA synthesis is very close to, or possibly overlapping, the boundary of an adjacent sequence region required for efficient amplification of genomic RNA3 in infected cells (17). Thus, despite its relatively large size compared with some other tripartite plant RNA viruses, nearly the entire 250-base BMV intercistronic region has been shown to be functional and, specifically, to contribute to production of either RNA3 or RNA4. As noted before, juxtaposition of sequences controlling production of these two coencapsidated RNAs might facilitate regulation of their equimolar production from a common negative-strand template (17).

The ability of a relatively small sequence segment to direct production of new subgenomic RNAs from a variety of sites in the BMV genome further enhances prospects for engineered alteration of the virus for a wide variety of experimental purposes. The capacity of such a flexible gene expression element to facilitate rearrangement of genes within a viral RNA genome has apparently also been used in natural virus evolution, as evidenced by the diverse genetic organizations and varied use of subgenomic RNAs in viruses with RNA synthesis genes related to those of BMV (8, 15, 19).

Preferential accumulation of smaller subgenomic RNAs. In infections with RNA3 derivatives carrying multiple subgenomic promoters, smaller subgenomic RNAs always accumulated preferentially (Fig. 2 and 6). Since the observed

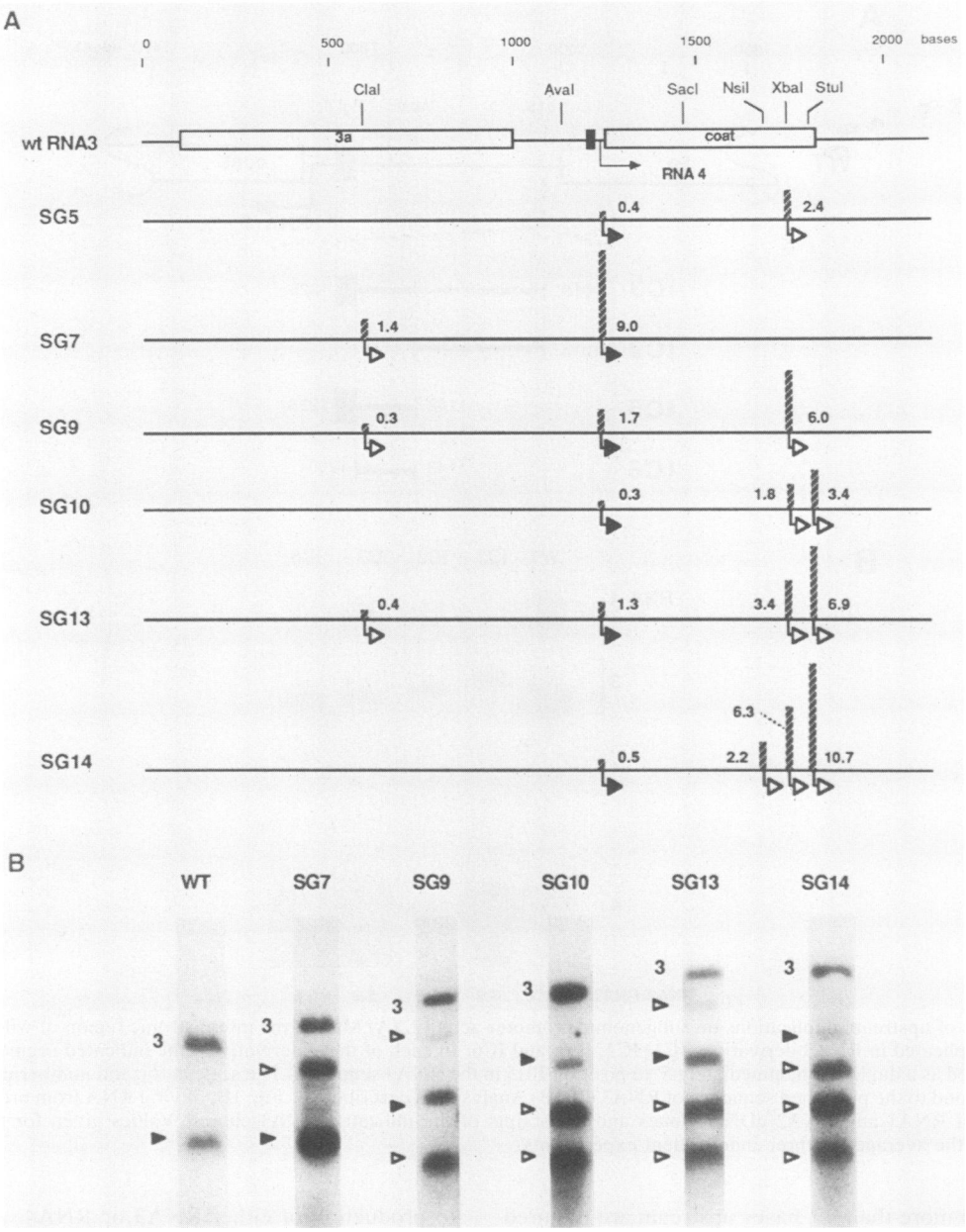

FIG. 6. Production of multiple subgenomic RNAs from individual RNA3 derivatives. (A) Schematic map of RNA3 derivatives SG5, SG7, SG9, SG10, SG13, and SG14. On each line, open-headed arrows indicate the sites of insertion of additional copies of the *Ava*I-*Sac*I fragment spanning the RNA4 initiation site. Above each arrow is given the molar ratio of that subgenomic RNA to the genomic RNA3 derivative, averaged from two or more independent experiments. Each value is also represented as a histogram bar for visual comparison. Arrows with filled heads represent the unaltered RNA4 promoter in its wild-type context. The average ratio of subgenomic RNA4 to genomic RNA3 production in wild-type infection under these conditions is about 1.2 (Fig. 2). (B) Analysis of viral RNA produced in protoplasts inoculated with transcripts of RNA1 and RNA2 cDNA clones and transcripts of the indicated RNA3 clones. Procedures were as in Fig. 1B except that the ^{32}P-RNA probe was complementary to the *Bgl*II-*Sac*I region of RNA3 to avoid generating signals from RNAs 1 and 2, which would not be electrophoretically resolved from the larger RNA3 derivatives. Since the region probed is part of the *Ava*I-*Sac*I fragment duplicated in the promoter insertions, the direct intensity of the autoradiograph underestimates the prevalence of the smaller subgenomic RNAs. Suitable corrections were made in calculating the subgenomic:genomic RNA ratios given in Fig. 6A. The upper band in each lane (band 3) corresponds to the genomic RNA3 derivative, while subgenomic RNA bands are marked with open and closed arrowheads according to the conventions of Fig. 6A. For SG5, see Fig. 2 and 4.

levels of subgenomic negative-strand RNAs are small relative to genomic RNA3 negative strand, and since even these may be overestimated because of artifacts of isolation (10, 32), mechanisms other than differential template populations must be involved in this differential accumulation. One contributing mechanism appears to be stimulation of downstream promoters by additional upstream copies of the *Ava*I-*Bgl*II sequences (Fig. 5). Recent experiments also suggest that additional downstream copies of the *Sal*I-*Sac*I sequences may reduce production of subgenomic RNA from

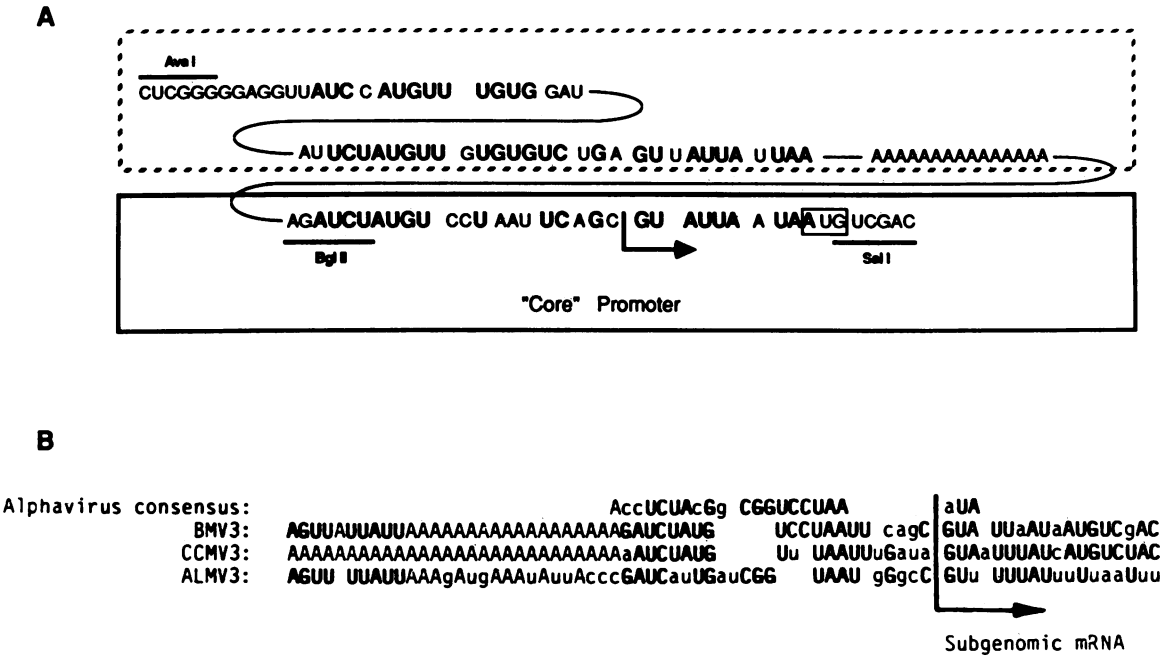

FIG. 7. (A) Alignment of the *Ava*I-*Sal*I sequence of BMV RNA3 to emphasize the presence of imperfect direct repeats. The *Bgl*II-*Sal*I core promoter sequence is shown within the indicated solid box. The initiation codon of the coat gene, overlapping the *Sal*I site, is also marked. (B) Sequence alignment showing similarity among BMV, the animal alphaviruses, CCMV, and alfalfa mosaic virus (ALMV) genomic RNAs near the start of sequences encoding subgenomic mRNAs for capsid proteins. Note that the large gap in each plant virus sequence is introduced solely to achieve alignment with the alphavirus consensus. The bent arrow shows the start of subgenomic RNA sequences, and the initiation codons of the BMV and CCMV coat genes are underlined. The alphavirus consensus was derived from the sequences of Sindbis, Middelburg, Semliki Forest, and Ross River viruses (30). The alfalfa mosaic virus RNA3 sequence is from Barker et al. (9), and the CCMV sequence is from Allison et al. (unpublished results).

upstream promoters, possibly by effects on elongation of RNA synthesis (unpublished results).

Possible role of direct repeats within the upstream sequences. In addition to other repeats shown in Fig. 7A, the consensus element AUCUAUGUU occurs three times, each with a single mismatch: once in the *Bgl*II-*Sal*I core promoter region and twice in upstream sequences required for full promoter activity. Deletions which remove each of the upstream elements in turn, along with small amounts of flanking sequences, produce successive losses of promoter activity (SG21 and SG22, Fig. 4). By analogy with extensive findings in DNA-dependent transcription (12, 31, 35), such consensus elements might constitute binding sites for a *trans*-acting factor(s) involved in initiation of subgenomic RNA synthesis, and experiments to pursue such possibilities are in progress. Interaction of *trans*-acting factors with such sites could explain the ability of artificial duplications to stimulate subgenomic RNA initiation from arbitrary sites relatively distant in primary sequence (Fig. 5). As discussed by Ptashne (31) and Schleif (33), *trans*-acting factors bound at widely separated nucleic acid sites might interact by protein-protein contacts to stimulate gene expression, looping out intervening nucleic acid sequences.

Although coat protein is the major product of BMV infection, the ability of simple direct repeats to stimulate RNA4 synthesis above wild-type levels (Fig. 5) shows that coat gene transcription is not operating at maximum potential in the wild-type virus. Presumably, this reflects the advantage of balancing synthesis of RNA4 with that of coencapsidated RNA3 and also reflects the likelihood that only a small fraction of the total RNA4 is used as mRNA during a normal infection.

The formation of direct repeats such as those in the subgenomic promoter region may be a relatively frequent event in BMV evolution. Similar direct repeats have previously been noted at other sites, such as the 3′ ends of wild-type BMV RNAs 2 and 3 (3), and de novo production of such a duplication by recombination during BMV infection has also been observed (4, 14).

Comparison of in vitro and in vivo results. The existence of an in vitro BMV polymerase extract able to carry out RNA-directed synthesis of subgenomic RNA (29) represents a valuable asset for further study of this process, particularly if it can be established that the existing in vitro system faithfully duplicates regulatory features of the in vivo reaction. Earlier studies with truncated negative-strand RNA3 templates suggested that, unlike the in vivo results reported here, subgenomic RNA synthesis in the in vitro system was not affected by sequences more than 20 bases upstream from the RNA4 start site (29). However, proximity of the core promoter sequence to the 3′ terminus of some truncated templates may have affected the results of these experiments. In a later reference to unpublished results, Marsh et al. (27) noted that the template activity of full-length negative-strand RNA3 in the in vitro BMV polymerase system was greatly reduced by an internal deletion removing the sequence complementary to the oligo(A) and adjacent upstream A/U-rich sequences. Thus, the in vitro system reproduces at least some of the sequence effects observed in vivo, and further experiments in both systems will undoubtedly contribute to unraveling the interactions by which BMV subgenomic RNAs are synthesized.

RNA1 results. In addition to subgenomic promoter manipulations within RNA3, we have recently constructed a

derivative of BMV RNA1 in which the RNA3 *AvaI-SacI* fragment has been inserted in the 3′ untranslated region. Coinoculation of this RNA1 derivative and wild-type RNA2 infects protoplasts and leads to production of a subgenomic RNA of expected size (R. French and P. Ahlquist, unpublished results). However, presumably because of the importance of the 3′-noncoding sequence in viral RNA replication (1, 13, 17), amplification of genomic RNA1 in this case is significantly inhibited. Further work on this problem is needed before the performance of the subgenomic promoter on such templates can be fully evaluated.

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