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RNA Sequence and Secondary Structural Determinants in a Minimal Viral Promoter that Directs Replicase Recognition and Initiation of Genomic Plus-strand RNA Synthesis

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Viral RNA replication provides a useful system to study the structure and function of RNAs and the mechanism of RNA synthesis from RNA templates. Previously we demonstrated that a 27 nt RNA from brome mosaic virus (BMV) can direct correct initiation of genomic plus-strand RNA synthesis by the BMV replicase. In this study, using biochemical, nuclear magnetic resonance, and thermodynamic analyses, we determined that the secondary structure of this 27 nt RNA can be significantly altered and retain the ability to direct RNA synthesis. In contrast, we find that position-specific changes in the RNA sequence will affect replicase recognition, modulate the polymerization process, and contribute to the differential accumulation of viral RNAs. These functional results are in agreement with the phylogenetic analysis of BMV and related viral sequences and suggest that a similar mechanism of RNA synthesis takes place for members of the alphavirus superfamily.

Keywords: RNA replication; viral replicase; RNA promoter; RdRp; brome mosaic virus

Introduction

RNA-dependent RNA polymerases (RdRps) are an interesting class of enzymes for several reasons: (1) RdRps are key enzymes in the replication of viral pathogens, including the causative agents of measles, the common cold, and viral hepatitis (Van Dyke & Flanagan, 1980; Neufeld et al., 1991; Poch et al., 1990; Behrens et al., 1996; Yuan et al., 1997); (2) cellular RdRps affect gene expression in enucleated cells and in gene silencing (Volloch et al., 1996; Bingham, 1997; Schiebel et al., 1998; Cogoni & Macino, 1999); and (3) RdRps are the only class of template-dependent polymerases that can initiate synthesis de novo from the end of the template (for reviews, see Buck, 1996; O'Reilly & Kao, 1998). We study the mechanism of RNA synthesis and promoter recognition using virally encoded replicases.

Abbreviations used: RdRp, RNA-dependent RNA polymerase; BMV, brome mosaic virus; NOE, nuclear Overhauser effect spectroscopy.

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The virally encoded RdRp, along with viral and cellular proteins make up the replicase of positive-sense RNA viruses (Hardy et al., 1979; Quadt & Jaspers, 1990; Buck, 1996). In one cycle of RNA replication, the replicase uses the genomic plus-strand RNA as the template to generate the complementary minus-strand RNA, which in turn serves as the template for generating multiple copies of genomic, and in some viral species, subgenomic plus-strand RNAs. Viral RNA replication requires the specific recognition of RNA features by the replicase, a process that is only beginning to be elucidated.

We study RNA synthesis using the model system brome mosaic virus (BMV), a plant-infecting member of the alphavirus-like superfamily of positive-strand RNA viruses (Goldbach et al., 1991). BMV has three genomic RNAs, designated as RNA1 (3.2 kb), RNA2 (2.8 kb), and RNA3 (2.1 kb). RNA1 and RNA2 are monocistronic and encode the 1a and 2a proteins, respectively, which are essential for the replication of the BMV genome (Ahlquist, 1992). RNA3 is dicistronic and encodes the 3a movement protein and the coat protein that is expressed via a subgenomic RNA. An in vitro
system using highly enriched BMV replicase preparations from infected barley has been developed to study initiation of RNA synthesis from minimal templates. Using this system, accurate initiation of minus-strand, genomic plus-strand and subgenomic RNAs in vitro has been demonstrated (Dreher & Hall, 1988; Miller et al., 1985; Sun et al., 1996; Adkins et al., 1997; Siegel et al., 1997, 1998; Chapman et al., 1998; Sivakumaran & Kao, 1999).

The initiation of genomic plus-strand RNA synthesis from the 3’ end of the minus-strand template requires one nucleotide 3’ of the initiation nucleotide (Sivakumaran & Kao, 1999). This minus-strand template is recognized by the BMV replicase in a species-specific manner; the BMV replicase does not efficiently recognize the (−)-strand RNA of the closely related cucumber mosaic virus (CMV) or tobacco mosaic virus (Sivakumaran & Kao, 1999; Chapman & Kao, 1999). In this paper, the secondary structure and sequence determinants governing the specific recognition of the RNA directing genomic plus-strand RNA synthesis was investigated. Using biochemical analysis and nuclear magnetic resonance spectroscopy (NMR), we found that efficient RNA-directed genomic plus-strand RNA synthesis can take place with more than one RNA secondary structure. Furthermore, the template sequence appears to determine the efficiency of RNA synthesis.

Results

Proposed secondary structure required to direct genomic plus-strand synthesis

We have demonstrated that accurate initiation of genomic plus-strand synthesis from input minus-strand templates of 27 nt was comparable to those from longer templates (Sivakumaran & Kao, 1999). The removal of two additional 5’ template nucleotides from the 27 nt RNA reduced synthesis to 30%. Therefore, the 27 nt RNA B2(−)26G represents a minimal functional promoter and template (to be named an endscript since the promoter is also the template). We seek to use endscript B2(−)26G to determine the features that enable efficient plus-strand RNA synthesis.

Multidimensional NMR is a powerful method for detailed structural analysis of molecules in solution. By virtue of its small size (27 nt), B2(−)26G is amenable to studies with proton NMR. NMR analysis requires the RNA to be in one predominant conformation. Since the regions forming intramolecular base-pairing can have high potential to form a dimeric conformation through intermolecular base-pairing, it is important to ensure that the RNA forms a stable monomeric conformation. To examine this, UV melting experiments were done with B2(−)26G over a 30-fold concentration range. Melting temperature ($t_m$) for the major transition was estimated at 55.6(±0.5)°C, and did not change appreciably over a concentration ranging from 2 to 60 μM, indicating that B2(−)26G has a monomeric conformation. In addition, native gel analysis was performed to determine the number of electrophoretically distinct conformations of B2(−)26G that may exist. As a control, a 17 nt RNA that exists both as a monomer and a dimer was used (Figure 1(a), lane 1). B2(−)26G was found to migrate predominantly as a single band (Figure 1(a), lane 2), indicating that it does not form a dimer. Also, when tested in salt concentrations ranging from 50 to 200 mM, the mobility did not change, indicating that its conformation is stable in the range of salt conditions used for NMR studies.

The most stable secondary structure predicted for B2(−)26G by the Zuker fold program (Jaeger et al., 1989) is shown in Figure 1(b). We sought to confirm or modify this predicted structure using the results obtained from NMR analysis. Although solving a complete tertiary structure at atomic resolution using NMR is a time-consuming process, one-dimensional (1D) imino NMR and two dimensional NMR spectra of an RNA molecule can be used to determine RNA secondary structure (Varani & Tinoco, 1991). When the imino protons are involved in base-pairing or forming an unusual conformation, they are usually protected from fast proton exchange with the water solvent, resulting in sharp imino proton peaks. If those protected imino protons are located within a 5 Å space, the crosspeaks between imino peaks can be observed by two-dimensional nuclear Overhauser effect spectroscopy (2D NOESY), where protected imino protons in guanine and uracil bases will result in a NOE in a NOESY spectrum (Varani & Tinoco, 1991) and allow the assignment of nucleotides in base-pairs. As seen in Figure 1(b), the proposed secondary structure contains eight base-pairs with a U-U mismatch and a CUU tri-loop. If this structure is the major conformation in solution, we should be able to see major imino peaks from the two A-U, four G-C, and one G-U base-pair. The terminal A-U base-pair might not be stable due to increased flexibility caused by the adjacent 5’ and 3’ overhangs. More than eight imino peaks are observed in the 1D imino spectrum (Figure 1(c)), which indicates that the 27 nt RNA may have unusually protected imino peaks or some minor conformation that can be clarified by analyzing 2D water NOESY crosspeak pattern.

The crosspeak pattern shown in the 2D H$_2$O NOESY (Figure 1(d)) would allow us to discern between G-C and A-U base-pairs using the characteristic crosspeaks between the uracil imino and adenine H2 in the A-U base-pair, and the crosspeaks between guanine imino and cytosine amino protons in the G-C base-pair, respectively. The expected G-U wobble base-pair was discerned by its characteristic strong crosspeaks between G and U imino protons. The connectivity between weaker A-U base-pairs was also observed (gray circles with dotted lines in Figure 1(d)), which might be either the minor conformation of A-U base-pairs or unstable A-U base-pairs from the terminal stem or...
A viral RNA promoter

Effect of mutations on synthesis from B2(−)26G endscript

Mutational analysis of B2(−)26G was performed to determine the features required for recognition by the BMV replicase. Mutant endscripts were all quantified after transcription and purification as described by Adkins et al. (1998). In essence, one picomole of each endscript was subjected to RNA synthesis assay and the amount of product synthesized was normalized to that produced by wild-type B2(−)26G. B2(−)26G yielded a predominant product of 26 nt, and some minor amounts of RNA one nucleotide longer and shorter due to non-templated nucleotide addition and premature termination, respectively (Figure 2(a), lanes 2-3). All three products initiated from the +1 cytidylate, since its change to a guanylate abolished all synthesis (Figure 2(a), lanes 4-5). Products directed by several of the mutant endscripts are shown in Figure 2(a) (lanes 6-15). Approximately 50 mutant endscripts were characterized according to the following scheme. To get an impression of the sequences important for RNA synthesis, a series of three nucleotide transitions starting at the 3′ end up to position +21 were carried out. In general, mutations within the first seven positions relative to the 3′ end of the template have a more severe effect on RNA synthesis than mutations from position +8 to +15 (Figure 2(b)). Some mutations near the 5′ terminus also decreased RNA synthesis. We have previously analyzed the effect of changes in positions between +17 and +24 and found that sequence within this region is required for efficient RNA synthesis in a yet undetermined mechanism (Sivakumar & Kao, 1999). We decided to focus on position +2 to +8 because this sequence is likely to be directly involved in the initiation process (Sun et al., 1996; Sun & Kao, 1997a,b). We consequently tested every possible single nucleotide substitution from position +1 to +9, along with at least one change at position +10 to +21. In contrast to the BMV subgenomic promoter, where changes at key positions drastically reduced RNA synthesis (Siegel et al., 1997), we found that none of the substitutions prevented RNA synthesis except for a C/G change at the initiation site. However, position +4 to +7 were comparatively sensitive to substitutions. Interestingly, while single nucleotide substitutions of either the +6 or +7 guanylates with cytidylates resulted in low levels of synthesis, replacement of both guanylates with cytidylates in

Figure 1. Analysis of the secondary structure of endscript B2(−)26G. (a) Visualization of RNA conformations by electrophoresis through a non-denaturing 12% polyacrylamide gel. Between 30 and 60% of the imino protons from B2(−)26G as predicted by the mfold program. (c) One-dimensional NMR spectrum of RNA purified by denaturing gel electrophoresis were heated to 90°C for two minutes, then allowed to cool over a 30 minute period to room temperature. The samples were then electrophoresed on a 17 cm x 15 cm x 0.8 cm gel at 200 V until the bromophenol blue migrated to the middle of the gel. The RNAs are SL13, an RNA that exists in equilibrium between an intramolecularly folded hairpin and an intermolecularly folded dimer (lane 1), B2(−)26G (lane 2), +3 U/A (lane 3), +6,7 G/C, +9G/A. (b) The secondary structure of B2(−)26G as predicted by the mfold program. (c) One-dimensional NMR spectrum of the imino protons from B2(−)26G taken at 20°C by a Bruker 600 MHz spectrometer. B2(−)26G, at 0.7 mM, was purified and prepared in 10% deuterium/90% water as described in Experimental Procedures. (d) The two-dimensional NOESY spectrum of a 2.0 mM B2(−)26G sample taken at 20°C from a Bruker 500 MHz spectrometer. The identity of the nucleotide responsible for the signals in the diagonal plane is indicated on the left-hand side of the Figure. Where noted with a gray spot, the origin of the signal was not determined with certainty and could have arisen from nucleotides in the loop of unbase-paired regions. The vertical and horizontal lines were added to the output containing the NMR signals to identify the connectivity of two nucleotides. The two crosspeaks associated with U4 and indicated by squares were observed in spectra with different mixing time than the one shown.
The mutations in B2(−26G) include some that are expected to affect the two stems (A and B), the 3 nt loop, and the mismatched region within the predominant structure of B2(−26G) (Figure 1(b)). Mutations that should affect at least a portion of the B2(−26G) structure while retaining the ability to direct synthesis are summarized in Figure 3(a).

A change to an adenylate (+3U/A) resulted in RNA synthesis of 80% despite a predicted destabilizing effect on stem A (Figure 2(a), lanes 6-7, 2(b), and 3(a)). A change of the +9 guanylate to an adenylate (+9G/A), expected to weaken stem B, resulted in 73% synthesis while changes at positions +10 and +14 expected to weaken stem B resulted in increased synthesis (Figure 3(a)). The results indicate that the RNA secondary structure of B2(−26G) can be changed without debilitating plus-strand RNA synthesis by the BMV replicase.

Several mutations that are expected to preserve the secondary structure in B2(−26G) were found to have a detrimental effect on RNA synthesis. A change of +2 adenylate to a guanylate, which should retain a wobbled G-U base-pair instead of a Watson-Crick A-U base-pair, resulted in 8% synthesis (Figures 2(b) and 3(b)). A transition of the +3 uridylate to a cytidylate resulted in 18% synthesis, while a replacement of three base-pairs in stem A with three G-C base-pairs abolished synthesis. Changes in loop 1 also greatly reduced RNA synthesis (Figure 3(b)). Lastly, since nucleotide substitutions that disrupted stem A had detrimental effects on RNA synthesis, we made a compensatory change to the +6 G/C endscript by replacing the +18 cytidylate with a guanylate. This double mutant, SS1, should maintain a predicted secondary structure similar to B2(−26G). However, RNA synthesis from SS1 was at 21%. A similar change of +7 G/C along with the complementary change at +17 C/G in endscript SS2 resulted in only 26% of the activity of B2(−26) (Figure 4(a)). Consistent with the findings that mutations may alter B2(−26G) structure without significant loss of activity (Figure 3(a)), these results indicate that the preformed, predominant secondary structure of B2(−26G) is not essential for directing plus-strand RNA synthesis.

The structures predicted by the mfold program for select mutant endscripts were examined to better understand the RNA determinants in B2(−26G) required for initiating RNA synthesis. The predicted secondary structures of the endscripts directing the initiation of BMV RNA1 and RNA3...
are shown along with several derivatives of B2(−26G) (Figure 4(a)). All endscripts capable of directing RNA synthesis tend to have either an exposed 3' initiation nucleotide, or have it present in a relatively weak portion of a stem that is rich in A·U and G·U base-pairs. Also, all except B3(−26G) possess a loop region rich in pyrimidines. If such a loop motif is necessary for RNA synthesis, it is not sufficient since SS1, SS2, and several other mutant endscripts that retain such a motif are debilitated for RNA synthesis. Finally, we note that while the RNAs are predicted to vary in the lengths of the stems from six (assuming a uridylate-uridylate base-pair in several of the endscripts) to nine base-pairs, they are all capable of directing RNA synthesis (Figure 4(a)).

To examine whether the predicted stability of the secondary structure is correlated to the ability to direct RNA synthesis, a plot of the calculated ΔG from the mfold program for all mutants endscripts were plotted against their ability to direct RNA synthesis (Figure 4(b)). Although the calculated ΔG based on the mfold program is only partially accurate due to the lack of thermodynamic information of the loop region, we should be able to determine whether there is an overall correlation between stability of endscript secondary structures and the ability to direct RNA synthesis. Two extremely unstable endscripts did result in inefficient RNA synthesis (Figure 4(b)). Nonetheless, no consistent correlation between RNA stability and the ability to direct RNA synthesis was observed. For example, endscript +10 C/U (ΔG = −3.4 kcal/mol) is predicted to have lower stability than B2(−26G) (ΔG = −3.9 kcal/mol), but was nonetheless more efficient in directing RNA synthesis. In addition, +8 U/G (ΔG = −8.8 kcal/mol), was observed to direct RNA synthesis nearly twice as efficiently as B2(−26G). Hence the stability of the RNA secondary structure was not a consistent indicator of the ability to direct synthesis.

Biochemical and biophysical studies on the conformations of endscript RNAs

Our mutational results are inconsistent with the possibility that the interaction between the BMV replicase and B2(−26G) is primarily determined by the predominant solution structure of the RNA. Therefore, we wanted to perform in-depth analyses of select endscripts using NMR and RNA melting experiments. Three mutant endscripts were selected from those described in Figure 2(b): (1) +3U/A contains a change of the +3 uridylate to adenyate; (2) +6,7 G/C has both the +6 and +7 guanylates changed to cytidylates; and (3) RNA +9 G/A has the +9 guanylate changed to an adenyate. Although each mutation should affect the stability of the different regions within B2(−26G), the ability to direct RNA synthesis was at least 70% of that of B2(−26G) (Figure 3(a)).

Native gel electrophoresis was performed to examine the structures of the three mutant endscripts. In a 12% native gel, each of the three mutant endscripts exists predominantly in a single electrophoretic conformation (Figure 1(a)). However, the mobility of the three mutant RNAs differ from each other and from that of B2(−26G). The mobility of +3 U/A, +9 G/A, and +6,7 G/C in comparison to B2(−26G) were, respectively: slightly reduced, drastically reduced, and increased (Figure 1(a), lanes 2-5). In a denaturing gel, all the three RNAs co-migrated with B2(−26G). These results suggest that the differences in mobility of the RNAs are likely due to changes in the RNA conformation.

Next, we examined the melting profiles of the RNAs. The shape of the melting curve for B2(−26G), +3 U/A and +9 G/A suggests that they melted with multiple step transitions, while +6,7 G/C undergoes a single step transition between two states (Figure 5(a)). With a crude estimate of the relative increase in absorbance and the magnitude of the slope for each transition, it seems that B2(−26G), +3U/A and +9G/A underwent a relatively sharp major transition and then a broad minor transition at higher temperatures (Figure 5(a)). The multiple transitions might be due to differential melting of secondary structure elements or the unstacking of the bases in the single-stranded portions of the RNAs. The absence of multiple transitions in +6,7G/C clearly shows that it has distinct thermodynamic properties from the other endscripts, implying a different conformation. In addition, even among B2(−26G), +3 U/A and +9 G/A, the overall shapes of the melting curves look different, which indicates that these RNAs undergo different unfolding processes. These results are consistent with the native gel analysis and suggest that the three mutant endscripts are altered in their secondary structures in comparison to B2(−26G) and to each other.
To characterize the changes in the structures of the mutant endscripts more precisely, 1D imino spectra were taken at different temperatures (Figure 5(b)). The quality of the ca 12 peaks characteristic of B2(26G) were significantly affected by temperature. The peaks were relatively sharp at 20°C (data not shown), but became increasingly broad at 30°C and 40°C, indicating that exchange with the environment of some of the imino protons increased at higher temperatures (Figure 5(b)).

For ease of description, several of the imino peaks have been named in the spectra of B2(26G) (Figure 5(b)). Of interest is the U8-U16 interaction at approximately 11 and 9.7 ppm. This interaction appeared to be more sensitive to temperature changes compared to interactions associated with G-C base-pairs adjacent to the U8-U16 interaction. However, missing were the base-pairs at the bottom of stem A, as exemplified by the absence of the U5 peak at 14.4 ppm. At 40°C, all the peaks were significantly broadened in comparison to those from B2(26G), indicating that the mutation in +3U/A RNA destabilizes the structure of the RNA. Endscript +6,7 G/C gave a number of sharp peaks that do not correspond to the ones observed in B2(26G) (Figure 5(b)), indicating that +6,7 G/C exists in a different structural conformation than B2(26G). The structure predicted for +6,7 G/C by the mfold program was different from that for B2(26G) (Figure 4(b)). In addition, since the peaks from +6,7 G/C remain well defined at 40°C, its conformation is more...
stable than that of B2(−)26G. This observation is consistent with its UV melt properties (Figure 5(a)). Endscript +9 G/A RNA, like +3 U/A RNA, retained a subset of the imino peaks observed in B2(−)26G, mostly corresponding to those in stem A (Figures 1(d) and 5(b)). However, these peaks are different from those observed in +3 U/A RNA. Most notably, the U8-U16 signal was drastically broadened, indicating the absence of the U8-U16 interaction adjacent to the mutation at position +9. Compared with the spectrum taken at 20°C (data not shown), most peaks were shifted and broadened significantly at 30°C (Figure 5(b)). At 35°C, all the peaks were severely broadened, indicating a rapid exchange of the imino protons with the environment due to destabilization of its conformation. Taken together, the biophysical characterization strongly suggest that the predominant structure in B2(−)26G RNA was significantly altered by the introduced mutations. Therefore, the ability of endscripts to direct RNA synthesis by the BMV replicase under conditions that affect RNA conformation is of interest.

We examined the effect of temperature on RNA synthesis using B2(−)26G and the three mutant endscripts. Since temperature should affect both the RNA conformation and the activity of the BMV replicase, all syntheses are normalized to that of B2(−)26G at each assay temperature. While increasing the temperature from 30°C to 43°C did result in reduced synthesis (Figure 6), none of the mutant endscripts were differentially affected in comparison to B2(−)26G. Altogether, these biochemical and biophysical results indicate that multiple RNA secondary structures are acceptable for efficiency of RNA synthesis by the BMV replicase. These results also suggest that the template sequence may contribute more significantly to the level of RNA synthesis than its specific RNA structure.

The effect of nucleotide changes on template-RdRp interaction

Mutations in B2(−)26G could affect RNA synthesis either through modulation of the polymerization process, or by preventing template-replicase interaction. To examine the latter possibility, competition assays were performed with several endscripts. This assay determines the ability of competitor RNAs to reduce synthesis directed by an efficiently recognized subgenomic RNA, −20/15 (Siegel et al., 1998; Sivakumaran & Kao, 1999). Competitor RNAs were added at 10 and 20 molar excess of −20/15 and the amount of synthesis directed by −20/15 was quantified. As a result, the amount of RNA synthesis directed by −20/15 was quantified. As a
negative control, we tested RNA B2(−)Δ3′, which has the three 3′ nucleotides deleted. In comparison to a reaction lacking a competitor, B2(−)Δ3′ reduced synthesis from −20/15 to 86 % at ten molar excess and to 76 % at 20 molar excess (Table 1). This mild reduction in −20/15 synthesis is consistent with our previous observations that the initiation site is required for recognition by the BMV replicase (Sivakumaran & Kao, 1999). In contrast, three functional RNAs, B1(−)26G, B2(−)26G, and B3(−)26G encoding the 3′ ends of BMV RNA1, RNA2, and RNA3, reduced synthesis from −20/15 more severely, to 52, 63, and 63 % at ten molar excess, respectively (Table 1). CMV2(−)26G, the equivalent 3′ end of minus-strand RNA2 of CMV, reduced synthesis to 49 %, a more effective level of inhibition than seen with BMV RNAs (Table 1). While CMV2(−)26G has the same 3′ three nucleotides as BMV RNAs and can interact with the BMV replicase, it was unable to direct efficient synthesis by the heterologous BMV replicase (Sivakumaran & Kao, 1999). Endscripts +3 U/A, +9G/A, +6,7 G/C, and B3(−) + 4U/C were at least as capable competitors as B2(−)26G (Table 1). These results suggest that the different levels of plus-strand RNA synthesis observed from the different endscripts is not due to a lack of recognition by the BMV replicase. Although it is possible that some RNA synthesis may be negatively affected by a replicase-endscript interaction that is too tight. Nonetheless, these competition results suggest that polymerization of nucleotides following the initial binding of the replicase to the initiation sequence contributes to the efficiency of RNA synthesis.

### Template guanylates can reduce plus-strand RNA synthesis

We had previously reported that the BMV replicase could utilize DNA templates (Sivakumaran & Kao, 1999; Siegel et al., 1999). Furthermore, guanylates present within the first four positions of the initiation cytidylate were found to be detrimental to RNA synthesis from DNA endscripts (Sivakumaran & Kao, 1999). In our mutational analysis we observed that guanylates introduced near the initiation cytidylate of RNA B2(−)26G had a similar inhibitory effect (Figure 2(b)). Replacement of the native nucleotide with a guanylate from the +2 to +5 positions allowed synthesis between 8 to 44 % of B2(−)26G. The detrimental effect of guanylates decreased as distance from the initiation cytidylate increased (Figure 7). From position +6 to +14, guanylates did not significantly affect RNA synthesis and sometime increased synthesis, as evidenced by guanylates at position +8 and +10 (Figure 7). However, guanylates at position +15 to +18 reduced RNA synthesis (Figure 7). The changes within position +15 to +18 likely affected a sequence previously observed to be important for RNA synthesis (Sivakumaran & Kao, 1999). Altogether, these results suggest that the identity of nucleotides at specific positions may affect the efficiency of RNA synthesis by the BMV replicase in vitro.

### Phylogenetic analysis of viral template sequence

To determine whether guanylates near the initiation site have a possible effect in vivo, we examined the distribution of guanylates in the sequences of BMV and other representatives of the alphavirus family. Sequences immediately following the initiation of genomic and subgenomic plus-strand RNAs generally lacked a guanylate in the first four positions (Table 2). At the +5 position, some animal-infecting, but no plant-infecting RNA viruses, had a guanylate in their RNAs. Guanylates were then found in increasing frequency starting at the +6 position. The absence of template guanylates near the initiation nucleotide may be due to a requirement for replicase-promoter recognition, or because the

| RNA competition | 1:10 | 1:20 |
|-----------------|------|------|
| None (100%)     |      |      |
| B2(−)Δ3′        | 86 ± 11 | 76 ± 7.7 |
| B2(−)26G WT     | 63 ± 10 | 57 ± 7.5 |
| B2(−)4U/C       | 52 ± 8  | 47 ± 4  |
| B2(−)3U/A       | 62 ± 4.1 | 56 ± 2.6 |
| B2(−)9G/A       | 39 ± 2.1 | 30 ± 3.5 |
| B2(−)6,7GG/CC   | 60 ± 3.5 | 58 ± 9.8 |
| B3(−)26G WT     | 63 ± 2.3 | 49 ± 8  |
| B3(−)4U/C       | 76 ± 11 | 46 ± 11 |
| CMV2(−)26G WT   | 49 ± 1.2 | 37 ± 5  |

**Figure 7.** Correlation between guanylate substitutions and their effect on RNA synthesis. Guanylates were introduced at the different positions of the template as described in Experimental Procedures. All results are normalized to the amount of RNA synthesis directed by B2(−)26G (100 %). The effect of three independently tested guanylate substitutions on viral RNA synthesis is shown with error bars denoting one standard deviation of the mean. Guanylates present in the wild-type sequence are shown without error bars.
interaction of the nascent RNA with the template is affected by tighter base-pairing. In the latter case cytidylates near the initiation site should also be detrimental to synthesis. While we did observe that substitutions with cytidylates near the initiation cytidylate did have detrimental effects, the effects were not to the same degree as the guanylate substitutions. However, in examining the 3' ends of genomic plus-strand templates of approximately 60 viral templates, we observed a similar striking bias against guanylates and cytidylates from position ‡2 to ‡5, followed by a more normal distribution of these two nucleotides (Figure 8). This trend appears to be unique for templates directing the synthesis of plus-strand RNAs, since minus-strand synthesis does not follow the same trend (Figure 8). Together, the phylogenetic and mutational results are consistent with the testable hypothesis that the strength of the interaction between the template and the nascent RNA near the initiation site contributes to the efficiency of plus-strand RNA synthesis.

Sequence affects RNA synthesis

The observation suggesting that the primary sequence of BMV RNAs can affect RNA synthesis prompted us to examine the BMV RNA sequences more closely. The templates for synthesis of BMV RNA2 and RNA3 are identical in the first five positions and RNA1 and RNA2 are identical at the first 26 positions except at +4, where RNA1 has a cytidylate and RNA2 a uridylate (Figure 9a). In vitro, B1(+)26G directs only 42% of the amount of product as B2(+)26G (Figure 9b, lanes 1-2 and 3-4). In vivo, RNA1 is known to accumulate at 68% of RNA2 (Marsh et al., 1991). To determine whether the +4 position is important in regulating the efficiency of RNA synthesis, we made B3(+)26G with a +4U/C change and quantified the amount of synthesis relative to B2(+)26G. Synthesis directed by B3(+)26G was 114% of that from B2(+)26G (Figure 9b, lanes 1-2 and 5-6). B3(+)26G with a +4U/C changed decreased synthesis to 52% of that from B3(+)26G (Figure 9b, lanes 5-6 and 7-8). These results provide another example that the identity of a template nucleotide near the initiation site can affect the efficiency of RNA synthesis.

Discussion

An understanding of the rules guiding RNA synthesis is lacking in all RNA viruses. Determining these rules will provide powerful guides for developing strategies to inhibit viral infections and to compare the mechanism of RNA synthesis by other polymerases. We have previously demonstrated that initiation of BMV genomic plus-strand synthesis can be accurately directed by minimal
minus-strand templates in vitro (Sivakumaran & Kao, 1999). A more detailed characterization of a functional endscript of 27 nt, B2(−)26G, was undertaken in the present study. In this work we demonstrated that: (1) the preformed RNA secondary structure of B2(−)26G is not specifically required for template recognition by the BMV replicase; (2) the RNA promoter for RNA synthesis requires nucleotides −1, +1 and +2 as a determinant for replicase binding; (3) template sequence beyond the +3 position likely affects RNA synthesis by modulating the polymerization process; and (4) phylogenetic analysis of the viral sequences indicate that the in vitro requirements described likely reflect in vivo requirements. The results are placed below in context of the mechanism of viral RNA replication.

Genomic and subgenomic plus-strand syntheses

The BMV subgenomic promoter is recognized by the BMV replicase in a sequence-specific manner (Siegel et al., 1997). Results herein demonstrate that the endscript directing genomic plus-strand synthesis also interacts with the viral replicase via a mechanism that does not depend on a specific preformed RNA structure. For the subgenomic promoter, the recognition nucleotides include the initiation cytidylate (+1) and at least four positions 3′ of the initiation site (positions −17, −14, −13, and −11) (Siegel et al., 1998) in a region determined to be important for BMV subgenomic RNA synthesis in infected cells (French & Ahlquist, 1988). For the genomic endscript, interaction with the replicase requires the initiation cytidylate and the neighboring −1 and +2 nucleotides. The sequence 3′ to the subgenomic core promoter has been demonstrated to modulate the level of RNA synthesis (Marsh et al., 1988; Adkins et al., 1997). In the genomic endscript, 5′ sequence from +17 to +24 affect the level of RNA synthesis. Finally, both templates are rich in adenylates and uridylates. With the genomic endscript the A+U-rich nature of the template could affect synthesis, but not binding to the replicase (Figure 2(b) and Table 1).

The sequences directing plus-strand syntheses are part of the intermediate synthesized during viral replication. Given sufficient time, these intermediates will either associate intermolecularly with the complementary template to form stable double-stranded RNAs, or fold intramolecularly into a highly structured state (unless there are proteins that affect RNA structure). Since there are no sequences in common for genomic plus and subgenomic RNA synthesis except for the initiation cytidylate, it is likely that both promoters are recognized before stable RNA structures can be formed in the nascent RNA. Based on these and other observations (Bujarski et al., 1994; Adkins

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Figure 8. A selection of 60 plus-strand viral RNA templates reveals a propensity against guanylate and cytidylate proximal to the genomic plus-strand initiation site. Genomic minus-strand initiation sequences of the same templates do not exhibit such a preference. Sequence data were obtained from NCBI Entrez. Only plus-sense RNA viruses with complete genomic sequences and with plant tropism were included. For database accession numbers, see Experimental Procedures.

Figure 9. Effect of nucleotide changes on the differential accumulation of BMV RNAs. (a) Comparison of the 3′ minus-strand RNA sequences of BMV. The non-templated guanylate at the 3′ end is shown in lower case g. The arrow denotes the initiation cytidylate. The +4 U/C change in B3(−)26G endscript is shown in bold. (b) RNA synthesis directed by B1(−)26G, B2(−)26G, B3(−)26G and B3(−) + 4 U/C. RdRp reaction products were separated by 12% denaturing PAGE and visualized by autoradiography. The amount of RNA synthesis from various templates relative to B2(−)26G are summarized at the bottom of the autoradiogram. Each number presented is an average from three independent trials.
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et al., 1997; Siegel et al., 1997, 1998; Sivakumaran & Kao, 1999), we propose that the initiation of BMV genomic and subgenomic plus-strand RNA synthesis takes place while the replicase is still associated with the nascent RNA (Figure 10). In this model, when minus-strand RNA synthesis reaches the BMV RNA3 intercistronic sequence encoding the subgenomic promoter, the replicase is faced with a roadblock that signals the replicase to either continue copying the template RNA or reverse course and de novo initiate subgenomic RNA synthesis from the nascent RNA. Overcoming this roadblock may depend on the ability of the replicase to successfully pass the intercistronic sequence, which in several bromoviruses contain an unusual homopolymeric sequence (Marsh et al., 1988; Allison et al., 1988, 1989; Romero et al., 1992) or contain stable stem-loop structures in other viruses (Zavriev et al., 1996; Koev et al., 1999), or stable RNA-RNA interactions (Kim & Hemenway, 1999; Sit et al., 1998). The replicases that cannot pass the intercistronic sequence would release the template RNA and initiate subgenomic RNA synthesis from the recently synthesized nascent RNA. The precise initiation position would be determined by the specific key nucleotides present in the subgenomic core promoter (Siegel et al., 1997). Bujarski et al. (1994) have noted that the BMV intercistronic sequence is a region for frequent template switching, and Nagy et al. (1999) have demonstrated that the AU content of a template may promote recombination, a process that depends on template switching. Further support for this hypothesis include the observations by French & Ahlquist (1988), who by placing a series of BMV subgenomic promoters at different positions found that promoters closer to the start site of minus-strand RNA synthesis directed more subgenomic RNA products.

The replicase molecules that could successfully pass through the intercistronic sequence would proceed forward generating full-length minus-strand RNAs. Upon reaching the end of the viral template, the replicase adds a non-templated nucleotide to the nascent minus-strand RNA by a terminal transferase activity (Siegel et al., 1997). The recognition of the initiation site (nucleotides −1, +1 and +2) signals the replicase to initiate genomic plus-strand synthesis from the nascent minus-strand RNA template. The differential accumulation of subgenomic and genomic RNAs in vivo (Marsh et al., 1991) could be due to a larger number of replicase molecules stalling at the intercistronic promoter region and reversing course to initiate subgenomic RNA synthesis.

The hypothesis that plus-strand RNAs are synthesized from the nascent minus-strand RNA without releasing the polymerase has been proposed for several viral systems. In Coronaviruses, minus-strand RNAs approximately the length of subgenomic RNAs were produced during infection (Sethna et al., 1989) and these replication intermediates were found to be transcriptionally active (Stawicki & Stawicki, 1990), suggesting that incompletely synthesized minus-strand RNA may direct plus-strand RNA synthesis. In the polycistronic red clover necrotic virus, the interaction in trans between two RNA sequences may prevent the progression of the viral replicase and induce subgenomic RNA synthesis (Sit et al., 1998). For the polycistronic potato virus X RNA, a series of repeated sequences that resemble transcription pause/termination sites of DNA templates may act to slow the progression of the replicase during minus-strand synthesis and thereby induce the initiation of plus-strand RNA synthesis (Kim & Hemenway, 1999).

**Minus versus plus-strand RNA synthesis**

The sequence-specific requirements for plus-strand genomic and subgenomic syntheses are
apparently in contrast to the requirements for genomic minus-strand synthesis. Structures at the 3′ end of the viral genomic RNA that are required to direct genomic minus-strand synthesis have been demonstrated in several systems, including BMV (Dreher & Hall, 1988; Pogue & Hall, 1992; Filipenkov et al., 1996; Song & Simon, 1995; Draper, 1995; Hong et al., 1998; Chapman & Kao, 1999). There may be major differences in the mode of recognition of promoters for minus versus plus-strand RNA synthesis. For plus-strand RNA viruses the genomic RNA is present in the infected cell prior to its translation and assembly of the replicase. Since all molecules prefer to exist in a lower energy state, the viral genomic RNA would possess highly ordered secondary and tertiary structures, which may be formed with the assistance of sequence-non-specific RNA-folding chaperons or sequence-specific RNA-binding cofactors (Weeks, 1997), such as the BMV 1a protein (Sullivan & Ahlquist, 1999). Hence, the replicase needs to recognize the highly structured genomic RNA to initiate minus-strand synthesis. The differences in the incidence of guanylates and cytidylates (Figure 8) provide another indication of the differences between minus and plus-strand RNA synthesis. The initiation of minus-strand RNA synthesis may also be affected by upstream sequence (Carpenter et al., 1995; Sullivan & Ahlquist, 1999; Chapman et al., 1998).

Template sequence for initiation and elongation

An interesting conclusion from our analysis of the BMV genomic minus-strand RNA endscript is that template sequence may regulate the amount of RNA synthesis in a manner independent of replicase-promoter interaction. This is different from the general notion of promoter-strength, which implies a modulation of the frequency of initiation events, and necessitates an emphasis on the frequency of successful transitions into processive elongation. Transcription studies using DNA templates indicate that the template sequence could affect the proportion of RNAs that are successfully transcribed to completion (Keene & Luse, 1999; Nuelder et al., 1997; Von Hippel & Yager, 1991). A similar strategy may regulate RNA synthesis from RNA templates. An assumption of this hypothesis is that the viral sequence contains nucleotides that are selected at specified positions to enable proper transition from initiation to elongation phase of RNA synthesis and the optimal level of RNA accumulation. The difference in BMV RNA1 and RNA2 at the +4 position provides an example where a single-nucleotide change in the viral RNA can account for a difference in RNA accumulation (Figure 9).

The BMV replicase is known to increase its affinity for interaction with the template RNA as synthesis progresses (Sun & Kao, 1997a,b). The increased affinity may affect the stability of the template-nascent RNA duplex interaction, i.e. too stable an interaction during initiation may prevent the replicase from making the transition from the initiation site, resulting in decreased RNA synthesis. Consistent with this testable hypothesis, we observed that guanylates and cytidylates negatively affected RNA synthesis when they were within four positions of the initiation nucleotide. Inconsistent with this hypothesis, however, is the fact that substitutions with guanylate and cytidylates do not have identical effects (Figure 2(b)). Stability of the template-nascent strand duplex is also affected by the contributing effects of the neighboring nucleotides (Turner et al., 1988), and a cytidylate substitution at a given position may not have the same thermodynamic effect as a guanylate. Alternatively, thermodynamic parameters may not be the sole factor affecting viral RNA synthesis. Another possible effect could be that the template sequence might also have evolved to influence translation.

The transition from initiation to elongation phase during BMV RNA synthesis takes place at positions +8 to +10 (Sun & Kao, 1997a,b; Adkins et al., 1998). The observation that guanylates and cytidylates are naturally enriched between position +6 to +10 suggests that these positions are acting in a manner different than the positions required for initiation. Perhaps a tighter template-nascent RNA interaction is required for efficient transition from initiation to elongation (Figure 2(b)). Consistent with this, the introduction of a guanylate at position +8 or +10 in B2(−)26G increased RNA synthesis above wild-type levels (Figure 2(b)).

Concluding comments

Our results indicating that viral replicase-promoter interaction being independent of the initial RNA secondary structure may be interpretable by two general models. First, the recognition of the promoter may be directed by the sequence of the RNA. Sequence-specific recognition has been previously demonstrated for the bacteriophage T4 translational repressor RegA protein (Webster & Spicer, 1990; Brown et al., 1997). Second, it is possible that the recognition of the genomic minus-strand RNA by the viral replicase could take by inducing tertiary interaction between protein and RNA which is tolerant of some differences in the RNA secondary structure. An induced-fit mechanism has been suggested for a number of RNA-binding proteins, including the splicing factor, U1A, the ribosomal S15 protein, and the binding of the hepatitis B encapsidation signal by the P protein (Gubser & Varani, 1996; Batey & Williamson, 1999; Beck & Nassal, 1997). There is also evidence for an induced fit mechanism for the recognition of the BMV subgenomic promoter by the BMV replicase. However, in our studies, the contacts in the RNA will induce a conformational change in the replicase, resulting in different nucleotide moieties being recognized (Adkins & Kao, 1998; Stawicki & Kao, 1999). A subsequent change in the RNA sec-
ondary or tertiary structure after replicase binding to the RNA has not been ruled out. This induced fit mechanism is compatible with the ability of the replicase to recognize the subgenomic and genomic promoters without the release of the replicase during minus-strand RNA synthesis.

**Experimental Procedures**

**NMR spectroscopy**

Wild-type and mutant endscripts were prepared for NMR analysis by *in vitro* transcription using phage T7 RNA polymerase and synthetic DNA templates (Milligan et al., 1987; Wyatt et al., 1991). The transcribed RNAs were purified by denaturing polyacrylamide gel electrophoresis and eluted from the gel slices using a electroelution trap (Scheicher & Schuell). The eluted RNA was concentrated by ethanol precipitation and dialyzed against the NMR buffer (10 mM sodium phosphate, 0.1 mM EDTA, 100 mM NaCl, pH 6.5) for 48 hours. Finally, the samples were dried down and dissolved in the same volume of 90% H2O/10% D2O. The RNA samples are at 0.4 to 1 mM for 1D spectra and 0.1 to 0.5 mM for 2D spectra. In analysis of the mutant endscripts, all of the samples were indirectly referenced to DSS using the water resonance with the excitation maximum set around the middle of the usual chemical shift range of the imino protons (12.5 ppm) (Plateau & Gueron, 1982). A spectral width of 12,000 Hz was used and 4096 complex data points were collected with a relaxation delay of 2 s. Depending on the sample concentration, 32 to 1024 scans were taken. In analysis of the mutant endscripts, all of the gains were set at the same level. The 2D H2O NOESY spectra were collected independently using a Bruker AMX-600 spectrometer equipped with a Bruker triple resonance triple-axis gradient probe. Solvent suppression was achieved using the jump-return method with the relaxation maximum set around the middle of the usual chemical shift range of the imino protons (12.5 ppm) (Plateau & Gueron, 1982). A spectral width of 12,000 Hz was used and 4096 complex data points were collected with a relaxation delay of 2 seconds. Depending on the sample concentration, 32 to 1024 scans were taken. In analysis of the mutant endscripts, all of the gains were set at the same level. The 2D H2O NOESY spectra were collected independently using a Bruker AMX-600 spectrometer equipped with a Bruker triple resonance triple-axis gradient probe and a Bruker DRX-500 spectrometer equipped with a Nalorac triple resonance z-axis gradient probe. The results from the two spectrometers were consistent. H2O NOESY spectra were collected at mixing times 200 ms and 300 ms at 20 °C. A total of 400 to 440 t1 blocks were taken, for each block, 32 scans were taken with a relaxation delay of two seconds. Solvent suppression was accomplished by replacing the last pulse in NOESY pulse sequence with a jump-return pulse sequence and applying z-gradient pulse during the mixing time. Proton chemical shifts were indirectly referenced to DSS using the water resonance. All NMR spectra were processed using FELIX 95.0 (Bioysm Technologies, Inc.).

**Native gel analysis**

RNA conformation native gel analysis was performed in 12% polyacrylamide gels (40:2 acrylamide to bisacrylamide) containing 0.5× TBE (Maniatis et al., 1982). After electrophoresis for 20 minutes, 6 μl of a 6 μM sample was loaded in a dye containing 0.5× TBE, 5% (v/v) glycerol and bromophenol blue. Electrophoresis was performed at a constant 200 volts until bromophenol blue migrated for 6-8 cm. The gel was then stained with Toluidine blue to visualize the RNAs.

**RNA denaturation analysis**

The protocol used was as described by Puglisi & Tinoco (1989) using a Gilford spectrophotometer model 250 and a model 2527 thermostrommer. Briefly, the sample concentration was determined by calculations using Beer-Lambert Law with a calculated extinction coefficient for B2(−)26G of 256. After adjusting the absorbance of the samples to between 0.8 and 1.2 at 20 °C, data points were taken with a change in temperature of 0.5 deg. C/minute. The results were analyzed and plotted using a Kaleidagraph software package. t_m for each was calculated using a derivative of the raw data.

**Synthesis of RNA templates for RdRp assay**

cDNA copies of (−)-strand 3’ ends encompassing the first 26 nt of BMV RNA 1, 2 and 3 were generated by PCR amplification. Pairs of oligonucleotide primers were used, one of which contained a T7 promoter to enable transcription by T7 RNA polymerase. Following transcription, the RNAs were electrophoresed in a denaturing PAGE, and the band of correct molecular mass was identified by staining with ethidium bromide and excised with a razor. The gel was then crushed to elute the RNA in a solution of 0.3 M sodium acetate. Concentrations of RNA were determined by spectrometry and staining with Toluidine blue following denaturing PAGE.

**RNA synthesis assays**

BMV replicase was prepared from infected barley as described by Sun et al. (1996). Replicase activity assays were carried out as described by Adkins et al. (1996). Briefly, each assay consisted of a 40 μl reaction mixture containing 20 mM sodium glutamate (pH 8.2), 12 mM dithiothreitol, 4 mM MgCl2, 0.5% (v/v) Triton X-100, 2 mM MnCl2, 200 μM ATP, 500 μM GTP, 200 μM UTP, 242 nM [α-32P]CTP (400 Ci/mmol, 10 mCi/ml, Amer sham), the desired amount of template and 7 μl of RdRp. Following incubation for 90 minutes at 30 °C, the reaction products were extracted with phenol/chloroform (1:1, v/v) and precipitated with six volumes of ethanol, 10 μg of glycogen, and 0.4 M final concentration of ammonium acetate. Loading buffer (45% (v/v) deionized formamide, 1.5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, and 0.04% (w/v) xylene cyanol) was added to the products and denatured by heating at 90 °C for three minutes prior to electrophoresis on 12% acrylamide-7 M urea denaturing gels. All gels were dried and exposed to film at ~80 °C and the amount of label incorporated into newly synthesized RNAs was determined with a Phosphorimager (Molecular Dynamics).

**Phylogenetic analysis**

The template sequences used were those indicated as complete by the Entrez database. The species and accession numbers are: alfalfa mosaic virus RNA1 (L00163 J02000), alfalfa mosaic virus RNA2 (K02702 J02002), alfalfa mosaic virus RNA3 (K03542), apple chlorotic leaf spot virus (D14996), apple stem grooving virus (D14995 S47260), barley mild mosaic virus RNA1 (AJ422725), barley mild mosaic virus RNA2 (X75933), beet necrotic yellow vein mosaic
virus RNA1 (D00115), beet necrotic yellow vein mosaic virus RNA2 (X04197), beet necrotic yellow vein mosaic virus RNA3 (M36894), beet necrotic yellow vein mosaic virus RNA4 (M36896), barley stripe mosaic virus RNA alpha (J04342), barley stripe mosaic virus RNA beta (X03854), barley stripe mosaic virus RNA gamma (U13916), beets yellow mosaic virus (X37476), broad bean mottle virus RNA1 (M65138), broad bean mottle virus RNA2 (M64713), broad bean mottle virus RNA3 (M60291), brome mosaic virus RNA1 (X02380 K02706), brome mosaic virus RNA2 (X01678 K02707), brome mosaic virus RNA3 (J02042 J02043), carnation mottle virus RNA (X02996), carrot mottle mimic umbravirus RNA1 (U57305), cowpea chlorotic mottle virus RNA1 (M65139), cowpea chlorotic mottle virus RNA2 (M28817), cowpea chlorotic mottle virus RNA3 (M28818), cowpea mosaic virus RNA B (X00206), cowpea mosaic virus RNA M (X00729), cucumber mosaic virus RNA1 (D12537 D01199), cucumber mosaic virus RNA2 (D12538 D01200), cucumber mosaic virus RNA3 (D10539), cymbidium mosaic virus (U62963), grapevine virus A (X75433), olive latent virus 1 (X85989), olive latent virus 2 RNA1 (X94346), olive latent virus 2 RNA2 (X94347), olive latent virus 2 RNA3 (X76993), olive latent virus 2 RNA4 (X77115), papaya mosaic virus (D13957 D00580), peanut clump virus RNA1 (X76802), peanut clump virus RNA2 (L07269), peanut mottle virus (AF023848), pepper mottle virus (M96425), potato mop-top virus RNA3 (D16193), potato mosaic virus X (D00344), potato Y virus (A08776), raspberry bushy dwarf virus RNA1 (S51557), ryegrass mosaic virus (AF035818), soil-borne wheat mosaic virus RNA1 (L07937), soil-borne wheat mosaic virus RNA2 (L07938), soybean mosaic virus (S42280), sweet clover necrotic mosaic virus (S46028), tobacco mosaic virus (D13438), tobacco necrosis virus (D00942), tobacco rattle virus RNA1 (D00155), tobacco rattle virus RNA2 (X03686), tobacco streak virus RNA3 (X00435), tomato bushy stunt virus (M21958 M31019), turnip yellow mosaic virus (J04373) and yam mosaic virus (U42596).

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