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Mechanistic analysis of RNA synthesis by RNA-dependent RNA polymerase from two promoters reveals similarities to DNA-dependent RNA polymerase

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ABSTRACT

The brome mosaic virus (BMV) RNA-dependent RNA polymerase (RdRp) directs template-specific synthesis of (−)-strand genomic and (+)-strand subgenomic RNAs in vitro. Although the requirements for (−)-strand RNA synthesis have been characterized previously, the mechanism of subgenomic RNA synthesis has not. Mutational analysis of the subgenomic promoter revealed that the +1 cytidylate and the +2 adenylate are important for RNA synthesis. Unlike (−)-strand RNA synthesis, which required only a high GTP concentration, subgenomic RNA synthesis required high concentrations of both GTP and UTP. Phylogenetic analysis of the sequences surrounding the initiation sites for subgenomic and genomic (+)-strand RNA synthesis in representative members of the alphavirus-like superfamily revealed that the +1 and +2 positions are highly conserved as a pyrimidine–adenylate. GDP and dinucleotide primers were able to more efficiently stimulate (−)-strand synthesis than subgenomic synthesis under conditions of limiting GTP. Oligonucleotide products of 6-, 7-, and 9-nt were synthesized and released by RdRp in 3–20-fold molar excess to full-length subgenomic RNA. Termination of RNA synthesis by RdRp was not induced by template sequence alone. Our characterization of the stepwise mechanism of subgenomic and (−)-strand RNA synthesis by RdRp permits comparisons to the mechanism of DNA-dependent RNA synthesis.

Keywords: alphavirus; DdRp; RdRp; viral polymerase

INTRODUCTION

The recent discovery of conservation in the structures of several different polymerases suggests conservation in the mechanism of nucleic acid synthesis (Joyce & Steitz, 1995; Hansen et al., 1997). The best characterized of these polymerases are the DNA-dependent RNA polymerases (DdRp) responsible for transcription. DNA-dependent RNA synthesis has been divided into a number of biochemically distinct steps: binding of the DdRp to the promoter, formation of a transcriptionally active open complex, synthesis of the first phosphodiester bond, abortive RNA synthesis, promoter clearance, processive elongation, and termination (McClure, 1985). Viral RNA replication is mediated by RNA-dependent RNA polymerases (RdRp). For a positive-sense RNA virus, the genomic (+)-strand RNA serves as a template for synthesis of (−)-strand RNA which, in turn, serves as a template for synthesis of additional copies of genomic (+)-strand RNA and, in many viruses, (+)-strand subgenomic RNAs.

Our laboratory studies the mechanism of RNA-dependent RNA synthesis using a model positive-sense RNA virus, brome mosaic virus (BMV). BMV is the type member of the bromovirus group of plant viruses in the alphavirus-like superfamily of positive-sense RNA viruses (Goldbach et al., 1991). BMV has three genomic RNAs designated RNA1 (3.2 kb), RNA2 (2.8 kb), and RNA3 (2.1 kb). Monocistronic RNA1 and RNA2 encode proteins 1a (containing putative methyltransferase and helicase domains) and 2a (containing polymerase-like domains), respectively (Ahlquist, 1992). In conjunction with cellular proteins, 1a and 2a compose the template-specific BMV RdRp. The dicistronic RNA3 encodes the 3a movement protein and the coat protein, whose translation is directed by the subgenomic RNA4 (0.88 kb). Synthesis of subgenomic RNA4 is by internal initiation from a (−)-strand copy of RNA3 (Miller et al., 1985).

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BMV RNA synthesis is amenable to biochemical studies because the viral RdRp can use exogenously added templates containing BMV promoter sequences. Accurate initiation of (−)-strand RNA synthesis from input (+)-strand templates has been demonstrated (Hardy et al., 1979; Miller & Hall, 1983; Quadt & Jaspars, 1990; Kao & Sun, 1996). Several steps in (−)-strand RNA synthesis have been defined, including initiation (Dreher et al., 1984; Miller et al., 1986; Dreher & Hall, 1988; Kao & Sun, 1996), primer-induced RNA synthesis (Kao & Sun, 1996), synthesis of abortive initiation products of up to eight nucleotides accumulating at a 10-fold molar excess to full-length RNA (Sun et al., 1996), and the transition of the RdRp from initiation to elongation (Sun & Kao, 1997a, 1997b). In contrast, the mechanism of subgenomic RNA synthesis has not been studied carefully. We have used short regions of (−)-strand RNA3 to refine previous characterizations of the subgenomic promoter (French & Ahlquist, 1988; Marsh et al., 1988) and determine how the RdRp recognizes the promoter (Adkins et al., 1997; Siegel et al., 1997). We present a detailed analysis of the mechanism of subgenomic (+)-strand RNA synthesis, including initiation, termination, and a comparison to (−)-strand synthesis. Finally, RNA-dependent RNA synthesis is contrasted with DNA-dependent RNA synthesis.

RESULTS

Sequence requirements for initiation of subgenomic RNA synthesis

BMV subgenomic RNA synthesis in vivo initiates at the (−)-strand complement of nt 1242 of RNA3 using a cytidylate as the first templated nucleotide (Dasgupta & Kaesberg, 1982). Short regions of (−)-strand RNA3 can direct accurately initiated subgenomic synthesis (Adkins et al., 1997). We refer to these constructs as proscripts to denote the fact that the promoter and template regions are distinct for subgenomic RNA synthesis. To determine the sequence context required for subgenomic initiation, a series of proscripts was synthesized that contains mutations surrounding the subgenomic initiation site and directs BMV RdRp synthesis of 13-nt products. The predominant RdRp product from all functional proscripts was 14-nt due to the nontemplated addition of one nucleotide, a phenomenon observed previously with the BMV RdRp (Siegel et al., 1997). Relative percent activity of the mutant proscripts was determined by comparison of the amount of product synthesized from them to that synthesized from wild-type proscripts. All values represent the mean of four independent experiments.

Transversion of the initiation cytidylate to a guanylate was shown previously to abolish the ability of the proscript to direct RNA synthesis (Adkins et al., 1997; Siegel et al., 1997). However, the BMV RdRp can inefficiently initiate synthesis using a uridylate as the first templated nucleotide (Siegel et al., 1997). Therefore, we addressed the roles of the neighboring nucleotides. Proscripts containing all possible nucleotide replacements at the +2 position were assayed to determine the requirements for this position (Fig. 1A). Mutation of the +2 adenylate to a guanylate abolished the ability of the proscript to direct RNA synthesis, whereas a change to a cytidylate or uridylate directed 37% or 66% of the wild-type level of RNA synthesis, respectively (Fig. 1A,B, lanes 6–8). Thus, an adenylate at the +2 position is preferred for subgenomic RNA synthesis, although no predictions can be made for the base functional groups required at +2.

Nucleotide requirements at the +3 and +4 positions for BMV subgenomic RNA synthesis were evaluated next. Mutation of the +3 uridylate to a cytidylate or guanylate reduced the ability of the template to direct RNA synthesis to 64% or 58%, respectively, of the wild-type level (Fig. 1A,C, lanes 3, 4). Mutation of the +4 adenylate to a cytidylate had no adverse effect on its ability to direct RNA synthesis (Fig. 1A,C, lane 5). Mutation of the +4 adenylate to a guanylate reduced the ability of the template to direct RNA synthesis to 57% of the wild-type level (Fig. 1A,C, lane 6). Thus, the effect of template sequence on RNA synthesis appears to decrease as the distance from the initiation nucleotide increases (Fig. 1A).

We next analyzed the effect of nucleotide changes at the −1 position. Mutation of the −1 guanylate to an adenylate reduced activity to 82% of the wild-type proscript, whereas a change to a uridylate had no effect (Fig. 1A,B, lanes 4, 5), suggesting that the identity of the −1 nucleotide does not affect the efficiency of subgenomic RNA synthesis. Quite interestingly, synthesis of RdRp products from alternate initiation sites in proscripts containing −1 mutations was observed, albeit at less than 5% of the amount from the authentic initiation site (Fig. 1B, lanes 4, 5). The novel 15-nt RdRp product from proscript −1G/U was apparently initiated at the −1 position using a templated uridylate. The novel 16-nt RdRp product from proscript −1G/A was apparently initiated at the −2 cytidylate. We note that in proscript −1G/A, the novel 16-nt and predominant 14-nt RdRp products each arise from a cytidylate–adenylate pair in the template. These results demonstrate that there is some flexibility in recognition of the initiation site by RdRp.

Relaxed requirement for the +2 nt for (−)-strand synthesis

We next examined whether the +2 nt in the promoter for (−)-strand synthesis had a similar preference. For this analysis, two templates (B1–242 +2 C/A and B1–242 +2C/U) directing synthesis of a 242-nt (−)-strand RNA1 product containing a change of the +2 cytidylate to an adenylate or uridylate were used. Synthesis from

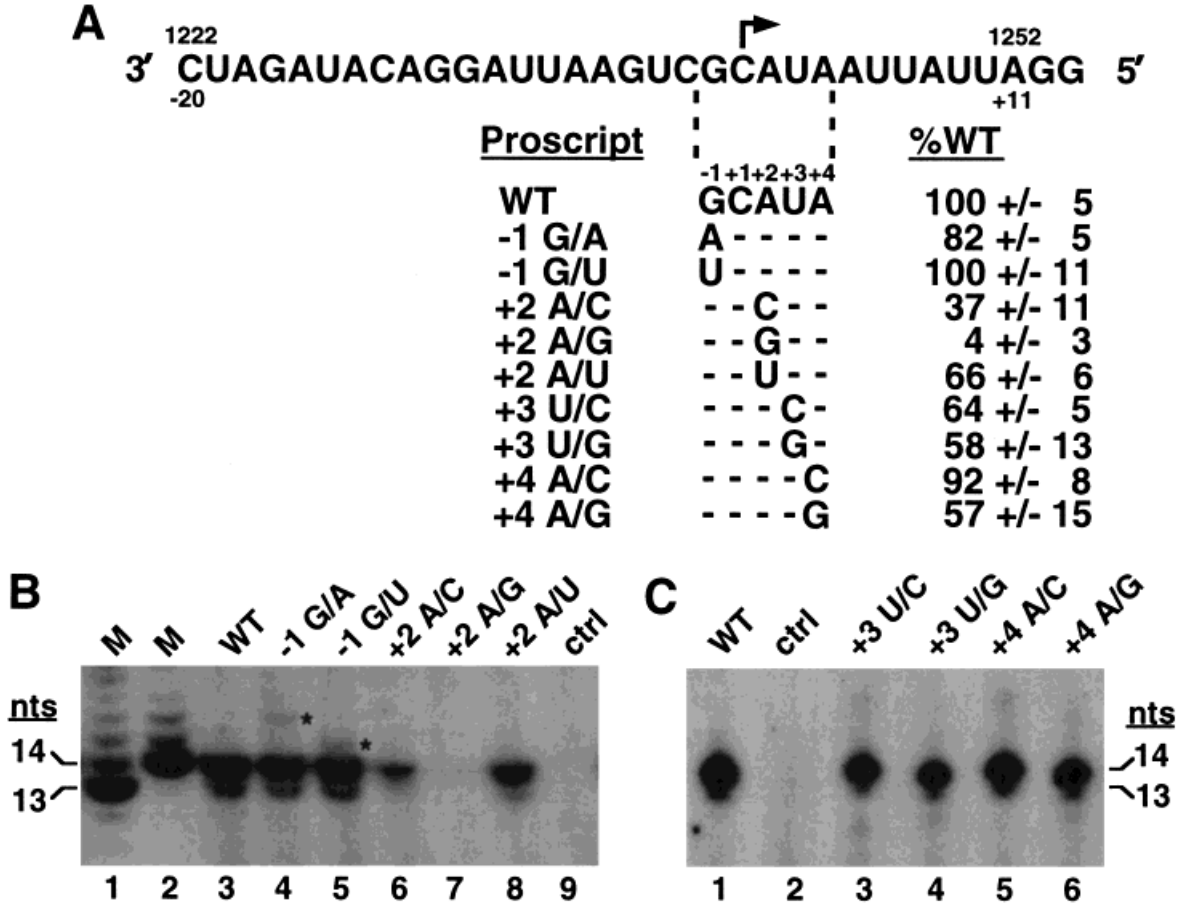

FIGURE 1. Mutational analysis of sequence for the initiation of subgenomic RNA synthesis. **A:** Complete sequence of the wild-type (WT) 33-nt proscript encompassing the (−)-strand complement of nt 1222–1252 of RNA3 representing nt −20 to +11 relative to the initiation site (indicated with arrow) for subgenomic RNA synthesis. Two additional guanylates incorporated by T7 DdRp are shown at the 5′ end of the proscript. The initiation sequence (−1 to +4) is expanded below the WT proscript. Proscripts are named for the mutation shown in the sequence. WT nucleotides are represented with a dash. Relative amounts of RdRp products normalized to WT following quantitation with a phosphorimager are presented to the right of the sequence. Values represent the mean and standard deviation from four independent experiments. **B:** Autoradiograph of RdRp products synthesized from proscripts containing mutations at −1 or +2 nt of template for subgenomic synthesis and analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. Lanes 1 and 2 contain 13- and 14-nt RNAs synthesized by T7 DdRp. Bands of higher molecular weight represent products of reiterative transcription by T7 DdRp. Proscripts used in reactions (lanes 3–8) and a control (ctrl) reaction to which no template was added (lane 9) are noted above the autoradiograph. Relative sizes of the T7 markers (in nt) are indicated at the left of the autoradiograph. RdRp products initiated from alternative sites are indicated with an asterisk (*) to the right of their position. **C:** Autoradiograph of RdRp products synthesized from proscripts containing mutations at +3 and +4 nt of template for subgenomic RNA synthesis. Proscripts used in reactions (lanes 1, 3–6) and a control (ctrl) reaction to which no template was added (lane 2) are noted above the autoradiograph.

B1–242 +2 C/A or B1–242 +2 C/U was compared with synthesis from a second template (B3–198), directing synthesis of a 198-nt (−)-strand RNA3 product, present at the same molar concentration in the same reaction. A change of the +2 cytidylate to a uridylate reduced synthesis to 31% (similar to what we have observed previously; Sun et al., 1996), whereas a change to an adenylate increased synthesis to 157% of wild-type B1–242 and B3–198 (data not shown). This result suggests that the identity of the +2 nt for (−)-strand synthesis is not as critical as it is for subgenomic synthesis.

High UTP concentration is required for subgenomic RNA synthesis

A requirement for high GTP concentration during (−)-strand RNA synthesis was observed previously (Kao & Sun, 1996). The preference for a +2 adenylate (noted above) and the inefficient synthesis of subgenomic RNA in vitro when [α-^{32}P]UTP is used as the radiolabel instead of the usual [α-^{32}P]CTP (data not shown) suggest that the second nucleotide incorporated during subgenomic RNA synthesis (UTP) may also have special requirements. To examine this possibility, the UTP requirements for subgenomic and (−)-strand RNA syn-

thesis were compared using up/45 (directing synthesis of a 207-nt subgenomic RNA) and B3–198. Both RNAs were used by the BMV RdRp when present individually in reactions containing 50 μM UTP (Fig. 2, lanes 2, 3). Furthermore, an equimolar mixture of the two RNAs in reactions containing 50 μM UTP yielded very similar amounts of products (Fig. 2, lane 4) to the reactions containing either RNA alone (Fig. 2, lanes 2, 3), demonstrating approximately equal promoter use by the BMV RdRp. Reduction of the UTP concentration to 1.6 μM decreased synthesis from both templates, but by different amounts. In three independent experiments, subgenomic RNA synthesis was reproducibly more diminished than (−)-strand synthesis by 4–10-fold. In the experiment shown (Fig. 2, lanes 5, 6), (−)-strand synthesis was reduced to 13% of the levels observed at 50 μM UTP, whereas subgenomic synthesis was reduced to undetectable levels. These results provide additional evidence for the preference of a +2 adenylate in the template for subgenomic RNA synthesis and suggest that the synthesis of the first phosphodiester bond in subgenomic RNA requires high concentrations of both GTP and UTP.

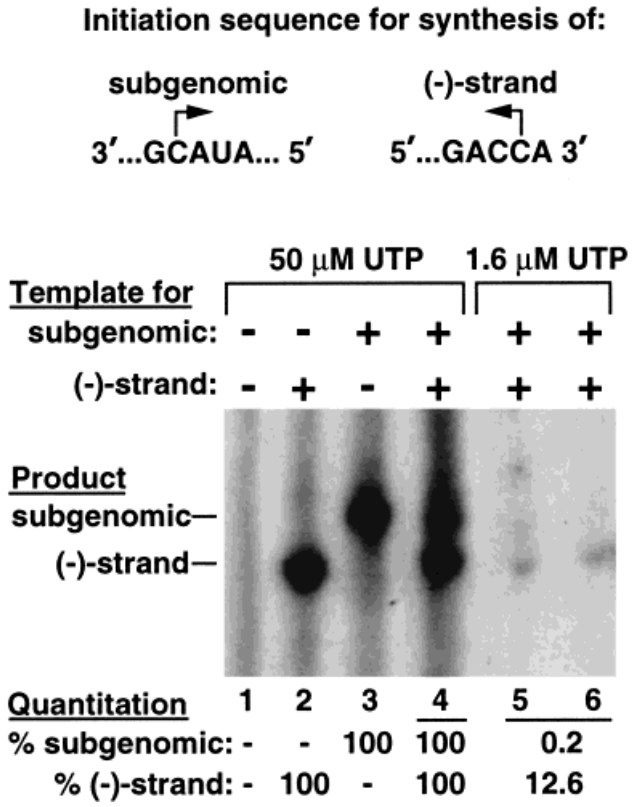

FIGURE 2. UTP concentration requirement for synthesis of subgenomic and (−)-strand RNA in vitro. Sequences surrounding the initiation sites (indicated with arrows) in templates for subgenomic (up/45; directing synthesis of a 207-nt subgenomic RNA) and (−)-strand [B3–198; directing synthesis of a 198-nt (−)-strand RNA3] synthesis are shown. RdRp products from these templates were treated with S1 nuclease and then analyzed by electrophoresis on a 5% denaturing polyacrylamide gel followed by autoradiography. Templates and UTP concentrations used in RdRp reactions are indicated above the autoradiograph. Positions of the subgenomic and (−)-strand products are indicated to the left of the autoradiograph. Relative amounts of RdRp products normalized to those synthesized at 50 μM UTP are shown below the autoradiograph.

Differential primer use during subgenomic and (−)-strand genomic RNA synthesis

Differences in the roles of the +2 nt for subgenomic and (−)-strand synthesis prompted us to compare the use of the initiation nucleotide for these two types of RNA synthesis. Because GTP is used to initiate both subgenomic and (−)-strand RNA synthesis, we first analyzed the effect of GTP concentration on both types of synthesis. No difference was observed in subgenomic and (−)-strand RNA synthesis, with each first being detected at 25 μM and continuing to increase through 200 μM GTP (data not shown). We next examined the ability of mono- or dinucleotide primers to replace GTP as the initiation nucleotide. Primers have been demonstrated previously to alleviate the need for high concentrations of GTP (Kao & Sun, 1996). Primer GpU is complementary to the initiation sequence for subgenomic RNA synthesis, whereas GpG is complementary to the initiation sequence for (−)-strand RNA synthesis, as shown in Figure 3. GDP is expected to serve as a primer for both subgenomic and (−)-strand RNA synthesis.

Control reactions contained 200 μM GTP and equimolar mixtures of templates for subgenomic (up/45) and genomic (−)-strand (B3–198) synthesis and resulted in synthesis of approximately equal molar amounts of subgenomic and (−)-strand products (Fig. 3, lane 2). When GTP was reduced to 4 μM, synthesis of both subgenomic and (−)-strand products decreased to 1.5% of that observed at 200 μM GTP (Fig. 3, lanes 3, 4). The addition of GpG to reactions to final concentrations of 250–1,250 μM (Fig. 3, lanes 5–8, respectively) stimulated (−)-strand synthesis from 7- to 10-fold over the basal level, whereas subgenomic RNA synthesis remained unchanged. The addition of GpU to reactions at the same concentrations (Fig. 3, lanes, 9–12) stimulated subgenomic synthesis by about threefold over the basal level, whereas (−)-strand synthesis was unchanged. The reduced stimulation of subgenomic as compared to (−)-strand RNA synthesis is consistent with previous observations (Kao & Sun, 1996). The subgenomic product reproducibly migrated to a lower position when primed with GpU, perhaps due to the lack of 5′ phosphates on the dinucleotide primed product. The addition of GDP to reactions at 250–1,250 μM stimulated synthesis of subgenomic RNA by 1.2–3.4-fold and (−)-strand by 9.4–19-fold (Fig. 3, lanes 13–16). ADP was added at 250–1,000 μM (Fig. 3, lanes 17–19) and resulted in a 1.7-fold stimulation of (−)-strand synthesis at the 1,000 μM level (Fig. 3, lane 19), whereas no detectable

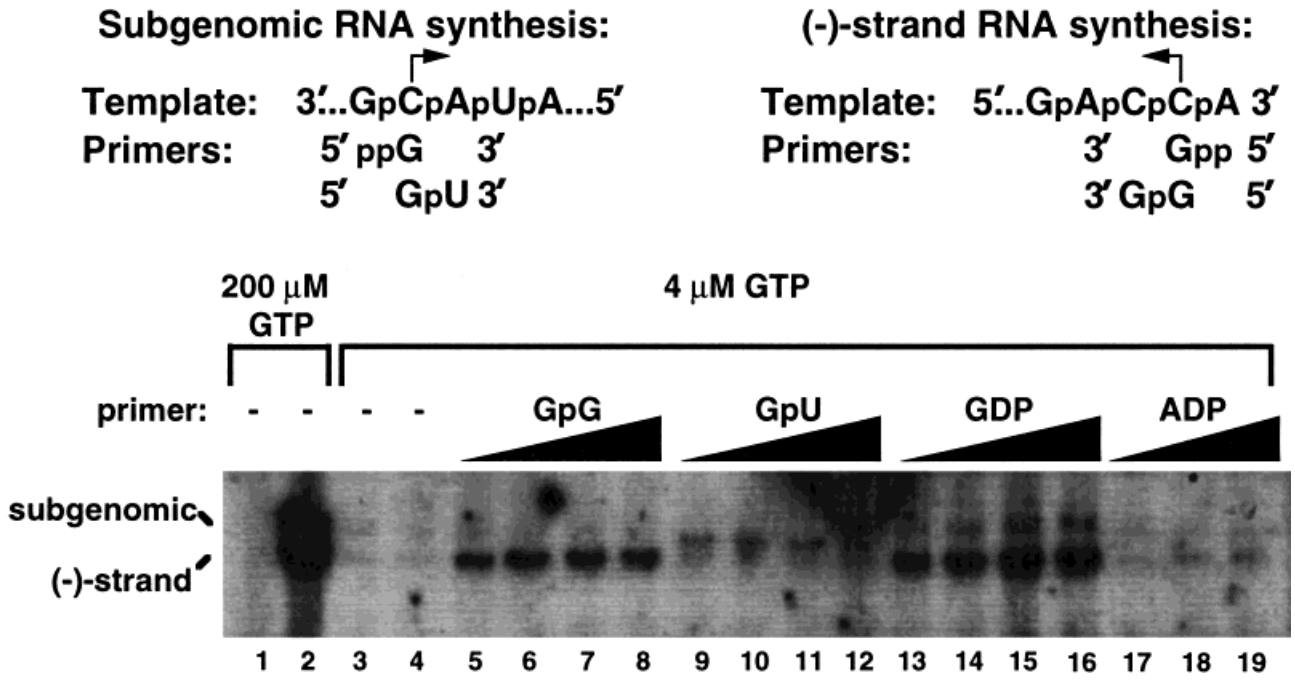

FIGURE 3. Differential primer use during subgenomic and (−)-strand RNA synthesis. Sequences surrounding the initiation sites (indicated with arrows) in templates for subgenomic (up/45; directing synthesis of a 207-nt subgenomic RNA) and (−)-strand [B3–198; directing synthesis of a 198-nt (−)-strand RNA3] synthesis are shown above relevant primers. Products from RdRp reactions were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel followed by autoradiography. An equimolar mixture of templates was included in all reactions except the control reaction (to which no template was added), whose products are shown in lane 1. Lane 2 contains the products from a standard reaction. Lanes 3–19 contain the products synthesized at 4 μM GTP with the addition of the primers indicated above the autoradiograph. Primer concentrations included in RdRp reactions were 250 μM (lanes 5, 9, 13, 17), 500 μM (lanes 6, 10, 14, 18), 1,000 μM (lanes 7, 11, 15, 19), and 1,250 μM (lanes 8, 12, 16). Positions of the subgenomic and (−)-strand products are indicated to the left of the autoradiograph.

increase in subgenomic synthesis was observed at any level. These results demonstrate that mono- and dinucleotide primers stimulate (−)-strand genomic RNA synthesis more than subgenomic RNA synthesis under conditions of limiting GTP. Furthermore, these results confirm observations in this manuscript that initiation of subgenomic and (−)-strand RNA synthesis has different requirements.

Abortive initiation during subgenomic RNA synthesis

Synthesis of abortive products during initiation of (−)-strand synthesis was observed previously (Sun et al., 1996; Sun & Kao, 1997b). Because (−)-strand synthesis initiates near the 3′ end of the genomic RNA, it is of interest to determine whether abortive initiation will occur during initiation from an internal promoter. Thus, we analyzed the products of subgenomic RNA synthesis reactions for the presence of oligonucleotides (potentially representing abortive initiation products) using high-resolution polyacrylamide gels. We used [α-^{32}P]ATP as label in these experiments due to the lack of cytidylates in the expected product prior to position +14 (Fig. 4A) and the inefficient labeling observed with UTP (noted above). Greater synthesis of full-length products was observed consistently with [α-^{32}P]CTP than with [α-^{32}P]ATP, perhaps due to ATP hydrolysis by the BMV 1a helicase-like protein, a component of RdRp (compare Fig. 4C, lanes 1 and 4, 5 and 8). Several sizes of oligonucleotides were observed during synthesis from proscript 12/26 (containing an 8-nt polyuridylate tract and directing synthesis of a 26-nt subgenomic product) (Fig. 4A,B, lanes 3–5; Fig. 4C, lane 5) and also during synthesis of full-length subgenomic RNA from (−)-strand RNA3 (Fig. 4C, lane 1). The oligonucleotides were 6-, 7-, and 9-nt in size by comparison with the T7 DdRp-generated RNAs of the sequences 5′-GUAUUA-3′, 5′-GUAUUAA-3′, and 5′-GUAUUAAUA-3′ (Fig. 4B). The RdRp-produced oligonucleotides of 6-, 7-, and 9-nt were in 12-, 7-, and 3-fold molar excess, respectively, to the full-length 26-nt product and in 20-, 8-, and 7-fold molar excess, respectively, to the full-length subgenomic RNA as determined by phosphorimager quantitation. Other sizes of oligonucleotides were present, but observed less reproducibly.

Although some endogenous BMV RNA was present in the RdRp preparation and directed synthesis of high

molecular weight products in the absence of added template, no oligonucleotides were synthesized unless a (−)-strand RNA3 template was added (Fig. 4C, lanes 13, 14). The oligonucleotide products were judged to be correctly initiated based on the following lines of evidence. Labeling of both oligonucleotides and full-length products was significantly reduced or eliminated when GTP, the initiating nucleotide, was omitted from

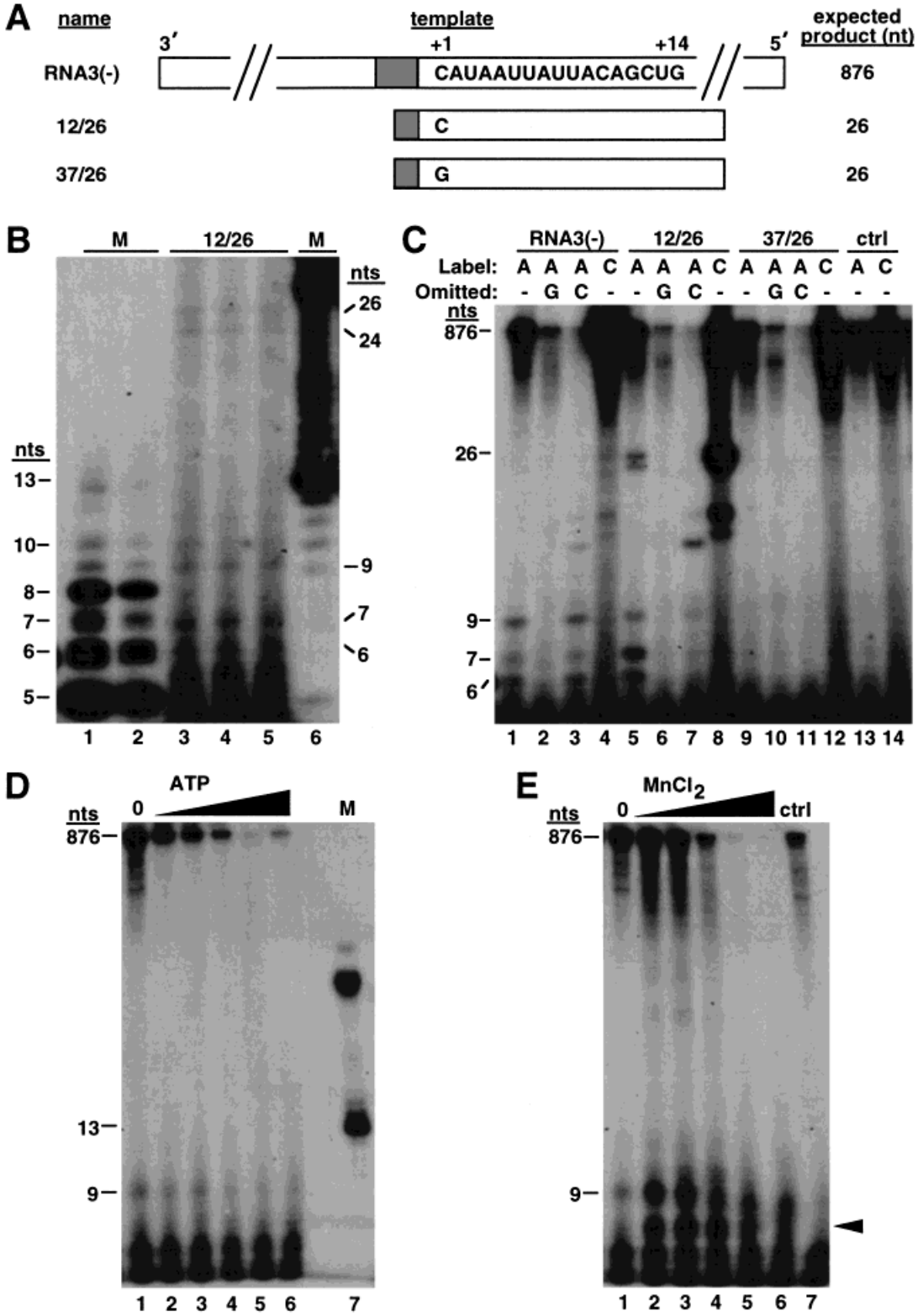

FIGURE 4. (*Legend on facing page.*)

the reactions (compare Fig. 4C, lanes 2, 6 with lanes 1, 5). Correctly initiated RNAs will not contain a cytidylate until position +14. Thus, abortive products should lack cytidylates. We found that oligonucleotide synthesis was unaffected when CTP was omitted from reactions, whereas synthesis of full-length products was abolished (although some higher molecular weight products, 14 nt and 17 nt, were seen, presumably due to contaminating CTP) (compare Fig. 4C, lanes 3, 7 with lanes 1, 5). Full-length products and some potential pause products were labeled using [α-^{32}P]CTP, whereas the oligonucleotides were not (compare Fig. 4C, lanes 4, 8 with lanes 1, 5). Finally, a proscript (37/26) containing a transversion of the +1 cytidylate to guanylate failed to direct synthesis of either oligonucleotide or full-length products (Fig. 4C, lanes 9–12).

Both the full-length 26mer and a prematurely terminated 24mer were observed from the 12/26 template when [α-^{32}P]ATP was used as label (Fig. 4B, lanes 3–5; Fig. 4C, lane 5). This is likely due to limiting ATP (242 nM) in the form of [α-^{32}P]ATP. This observation raised the concern that limiting ATP might by responsible for the production of the oligonucleotides. To examine this possibility, successively higher concentrations of unlabeled ATP were added to RdRp reactions. Synthesis of the oligonucleotides and elongated products responded in a similar manner to the addition of increasing amounts of unlabeled ATP (Fig. 4D). The 9-nt RNA and elongated products were detectable when the ATP concentration was increased to 30 μM, indicating that the synthesis of oligonucleotides is an innate property of the BMV RdRp and not due simply to limiting substrates (Fig. 4D).

Magnesium is required for the elongation phase of (−)-strand RNA synthesis, whereas manganese will suffice for initiation (Sun et al., 1996). We therefore examined the effect of manganese on the synthesis of oligonucleotide and full-length products from the subgenomic promoter (Fig. 4E). At 1–2 mM, $MnCl_2$ increased synthesis of full-length products by 8–28% and the synthesis of the 9-nt RNA by 95–127%. Addition of $MnCl_2$ to more than 2 mM reduced synthesis of both oligonucleotides and elongated products, although elongated products were more sensitive at lower $MnCl_2$ concentrations (Fig. 4E, lanes 4–7). The addition of $MnCl_2$ also resulted in the appearance of a novel oligonucleotide product (compare Fig. 4E, lanes 1 and 2–4). These results correspond to previous observations from our laboratory for initiation of (−)-strand RNA synthesis (Sun et al., 1996).

We next determined whether the oligonucleotides produced during subgenomic RNA synthesis were released by RdRp and thus represented abortive products (Fig. 5). Reactions lacking CTP should arrest RdRp on the template RNA. These arrested complexes were fractionated by passage through Sephadex CL-6B spin columns. Sixteen consecutive fractions were collected and divided into two sets, one of which was analyzed for RNA products and the second of which was assayed for RdRp activity. Elongated RNAs and RdRp activity were found in fractions 2 and 3, whereas the 6-, 7-, and 9-nt oligonucleotides were found in fractions 8–13 (Fig. 5B,C). These results demonstrate that the elongated RNAs remain in a ternary complex with the RdRp, whereas the oligonucleotides are released from the RdRp complex and hence represent the products of abortive initiation.

FIGURE 4. Oligonucleotides synthesized during initiation of subgenomic RNA synthesis. **A:** Diagrams of the three RNAs used in RdRp assays. Full-length (−)-strand RNA3 [RNA3(−)] and proscript 12/26 should direct RNA synthesis, whereas proscript 37/26 has a mutated initiation site. The shaded box represents the location of the polyuridylate tract. The sequence of the first 14 nt of all three templates is shown on RNA3(−) and the length of expected RdRp products is shown on the right. **B:** Predominant oligonucleotides synthesized from proscript 12/26 are 6-, 7-, and 9-nt in size. Autoradiograph of RdRp products synthesized from proscript 12/26 and analyzed by electrophoresis on a 24% denaturing polyacrylamide gel. The molecular weight markers used in this experiment were generated by T7 DdRp and are of the expected sequence of 8- and 13-nt RNAs correctly initiated by the BMV RdRp. Lanes 1 and 2 contain the 8-nt marker (and smaller T7 DdRp-generated abortive and larger T7 DdRp-generated reiterative products) with twice the amount being loaded in lane 1. Lane 6 contains the 13-nt marker and smaller T7 DdRp-generated abortive and larger T7 DdRp-generated reiterative products. Lanes 3–5 contain the products from RdRp reactions containing proscript 12/26 as template. Positions (in nt) of the size markers and T7 abortive products are indicated to the left of the autoradiograph. Positions of the full-length 26-nt product, a prematurely terminated 24-nt product, and the 6-, 7-, and 9-nt oligonucleotides synthesized by RdRp are indicated to the right of the autoradiograph. **C:** Oligonucleotides are accurately initiated from the subgenomic promoter. Template and radiolabel ([α-^{32}P]ATP or [α-^{32}P]CTP) included in and NTPs omitted from reactions are indicated above the autoradiograph of the 24% denaturing polyacrylamide gel. Lengths (in nt) of full-length and oligonucleotide products are indicated to the left of the autoradiograph. **D:** Effect of unlabeled ATP on synthesis of subgenomic and oligonucleotide RNAs from full-length (−)-strand RNA3. Autoradiograph of RdRp reaction products analyzed by electrophoresis on a 20% denaturing polyacrylamide gel with 5% stacking gel. Unlabeled ATP was included in reactions at final concentrations of 0, 1, 2, 5, 10, or 30 μM in lanes 1–6. Lane 7 contains the 13-nt marker and an unexplained higher molecular weight band. Lengths (in nt) of subgenomic, marker, and oligonucleotide RNAs are indicated to the left of the autoradiograph. **E:** Effect of $MnCl_2$ on synthesis of subgenomic and oligonucleotide RNAs from full-length (−)-strand RNA3. Autoradiograph of RdRp reaction products analyzed by electrophoresis on a 20% denaturing polyacrylamide gel with 5% stacking gel. $MnCl_2$ was included in reactions at final concentrations of 0, 1, 2, 5, 10, or 20 mM in lanes 1–6. Lane 7 contains the products from a control reaction (ctrl) to which no template was added. Lengths (in nt) of subgenomic and oligonucleotide RNAs are indicated to the left of the autoradiograph. A novel oligonucleotide induced by $MnCl_2$ is noted with an arrow to the right of the autoradiograph.

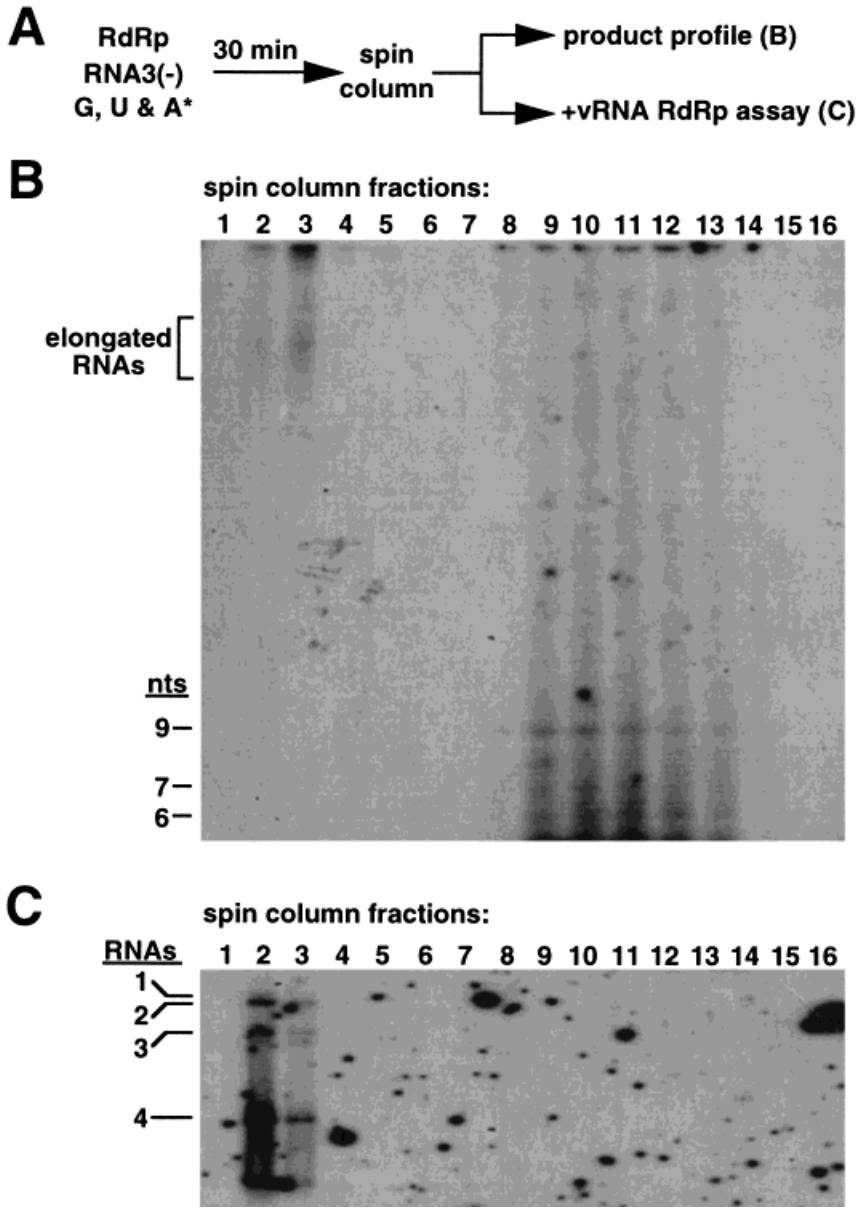

FIGURE 5. Oligonucleotides are released by RdRp during subgenomic RNA synthesis. **A:** Protocol for experiment. RdRp reactions (100 μL) lacking CTP were incubated for 30 min at 30 °C before being applied to a Sephadex CL-6B spin column equilibrated in 1× RdRp buffer lacking NTPs. Sixteen 100-μL fractions were collected, split into two aliquots, and analyzed for RdRp products (B) or RdRp activity (C). **B:** Location of elongated RNAs and oligonucleotides following spin column fractionation of the initial reaction. Fractions were phenol:chloroform extracted and ethanol precipitated (with 10 μg glycogen) prior to analysis on a 24% denaturing polyacrylamide gel followed by autoradiography. Fraction numbers are indicated at the top of the autoradiograph and the positions of elongated RNAs and oligonucleotides are shown at the left. **C:** Location of RdRp activity following spin column fractionation of the initial reaction. Fractions were tested for RdRp activity using BMV virion RNA (vRNA) as template in standard RdRp assays. RdRp products were analyzed by electrophoresis on a 1% agarose gel followed by autoradiography. Fraction numbers are indicated at the top of the autoradiograph and the positions of vRNA products are shown at the left.

Termination of BMV RNA synthesis

Termination of RNA-dependent RNA synthesis has not been analyzed previously. We tested the ability of RdRp to synthesize subgenomic or (−)-strand RNA from templates that contain extensions at their 5′ ends to determine whether synthesis is programmed to stop at the natural 5′ end of the template due to an existing sequence/structure in the template or a natural predilection of RdRp. A subgenomic RNA 250-nt longer than the authentic subgenomic RNA was synthesized by RdRp from a (−)-strand RNA3 template with a 250-nt extension at the 5′ end (Fig. 6A,B, lane 3). The 250-nt extension was derived from the Bluescript II KS(+) plas-

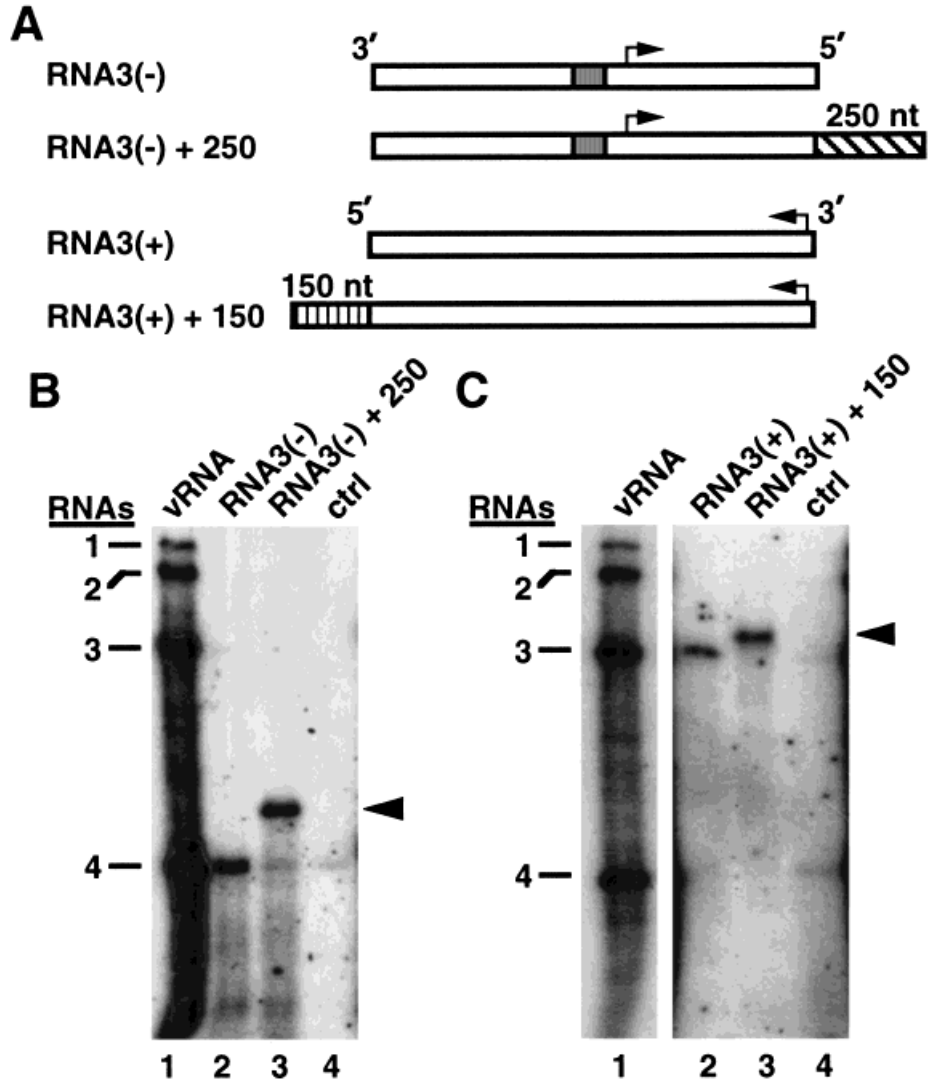

FIGURE 6. Termination of BMV RNA synthesis. **A:** Templates used for the experiments shown in B and C. RNA3(−) [wild-type (−)-strand RNA3] and RNA3(−) + 250 [(−)-strand RNA3 with a 250-nt 5′ extension of plasmid origin (diagonal stripes)] were used to examine termination of subgenomic RNA synthesis. RNA3(+) [wild-type (+)-strand RNA3] and RNA3(+) + 150 [(+)-strand RNA3 with a 150-nt 5′ extension of plasmid origin (vertical stripes)] were used to examine termination of genomic (−)-strand synthesis. Locations of the extensions (hatched boxes), initiation sites (arrows), and polyuridylate tracts (shaded boxes) are indicated. **B:** Termination of subgenomic RNA synthesis. Autoradiograph of RdRp products synthesized from the indicated templates and separated by 1% agarose gel electrophoresis. Positions of RdRp products from BMV virion RNA (vRNA) (lane 1) are shown at the left of the autoradiograph. Products of a control reaction (ctrl) containing no added template are shown in lane 4. Position of the subgenomic RNA with the 250-nt extension is indicated with an arrow at the right of the autoradiograph. **C:** Termination of genomic (−)-strand RNA3 synthesis. Autoradiograph of RdRp products synthesized from the indicated templates and separated by 1% agarose gel electrophoresis. Position of the RNA3 with the 150-nt extension is indicated with an arrow at the right of the autoradiograph. The autoradiograph of lanes 2–4 was exposed four times longer than the autoradiograph of lane 1.

mid (Stratagene) containing the BMV RNA3 cDNA. Although a faint product is visible at the location of the authentic subgenomic RNA from the reaction containing the template with the 5′ extension, it is also visible in the lane containing products from a reaction to which no template was added, indicating that it is due to endogenous template in the RdRp preparation (Fig. 6B, lanes 3, 4). We used a similar (+)-strand RNA3 template with a plasmid-derived 150-nt 5′ extension to examine termination during (−)-strand synthesis (Fig. 6A,C). A (−)-strand product 150-nt longer than the authentic RNA3 was synthesized from the template with a 150-nt 5′ extension (Fig. 6C, lane 3). These results demonstrate that the sequences at the authentic 5′ ends of the (−)- and (+)-strand templates do not cause the RdRp to terminate RNA synthesis. They further demonstrate that RdRp is not programmed to stop after synthesis of the wild-type length of RNA. In fact, an extension of 2,000 nt, generating a 4,200-nt template for (−)-strand RNA3 synthesis, led to synthesis of a

4,200-nt product (data not shown). These results do not, however, preclude the possibility that the extensions have altered a structure normally present at the 5′ end of wild-type templates that signals termination.

DISCUSSION

Subgenomic RNA is currently the only (+)-strand RNA synthesized in vitro by the BMV RdRp. Thus, we are using subgenomic RNA synthesis as a model for analysis of the mechanism of (+)-strand RNA synthesis. In this paper, we characterized subgenomic RNA synthesis with respect to sequence requirements for initiation, use of primers, synthesis of abortive products, and termination. A mechanistic comparison can now be made between synthesis from the BMV subgenomic and (−)-strand promoters (Table 1), as well as to DNA-dependent RNA synthesis.

Comparison of initiation sequence requirements for BMV subgenomic and (−)-strand RNA synthesis

In the context of truncated templates containing the subgenomic promoter, mutational analysis has demonstrated a strong preference for the +1 cytidylate (Adkins et al., 1997; Siegel et al., 1997). Similar analysis has suggested the penultimate cytidylate of genomic RNAs functions as the normal initiation site for (−)-strand synthesis (Dreher et al., 1984; Dreher & Hall, 1988). However, there may be some flexibility in the selection of the initiation site; mutation of the authentic +1 cytidylate in the template for subgenomic RNA synthesis can result in initiation from a cytidylate inserted one nucleotide 3′ or 5′ of the original initiation site (Siegel et al., 1997). We note in proscript −1G/A that the 16-nt product likely arises from initiation at the −2 cytidylate, which resembles the authentic initiation site by forming a cytidylate–adenylate pair. In proscript −1G/U, the 15-nt product likely arises from inefficient RdRp initiation from the −1 uridylate using an adenylate, as we have observed previously (Siegel et al., 1997). The observation that incorrectly initiated products are produced at low abundance indicates that the catalytic site of RdRp is positioned and somewhat sterically constrained over the authentic +1 site.

The identity of the −1 nt in the template for subgenomic synthesis does not affect the efficiency of initiation (Fig. 1A,B), whereas previous reports show replacement of the −1 adenylate with a cytidylate or uridylate reduces (−)-strand synthesis to approximately 30% of that from the wild-type sequence (Dreher et al., 1984; Dreher & Hall, 1988; Sun et al., 1996). A preference for a +2 adenylate for subgenomic synthesis was observed (Fig. 1A,B). Although the +2 nt influences (−)-strand synthesis, it can be changed from a cytidylate to a uridylate with only a moderate decrease in synthesis, whereas a cytidylate to adenylate change actually increased synthesis in vitro. We note that the cytidylate to adenylate change makes the context of the (−)-strand initiation site more similar to that of the subgenomic initiation site. Thus, although the +2 nt affects both subgenomic and (−)-strand synthesis, all mutations at +2 are detrimental to subgenomic RNA synthesis, whereas they have varying effects on (−)-strand RNA synthesis.

Conservation of initiation sequences in the alphavirus-like superfamily

Definition of the BMV subgenomic and (−)-strand initiation sequences allows a closer examination of initiation sequences of other members of the alphavirus-like superfamily. The subgenomic initiation sequences

TABLE 1. Comparison of subgenomic and genomic (−)-strand synthesis in vitro.

Characteristic	Similarities	Differences
Promoter location		Subgenomic synthesis initiates internally using a promoter distinct from template, whereas (−)-strand synthesis initiates near end of template using a promoter contained within template
Initiation sequence	Sequences surrounding initiation nucleotide influence synthesis	Subgenomic synthesis has preference for +2 adenylate
		(+)-strand initiation sequences (subgenomic and genomic) are highly conserved and very different from those for (−)-strand synthesis
NTP requirements	Require high GTP concentration	Subgenomic synthesis requires high UTP concentration
Primer use	Primers used when GTP is limiting	(−)-strand synthesis more efficiently stimulated by primers
Abortive initiation	Precedes elongation	
Termination	Sequence-independent	

of 16 representative alpha-like viruses analyzed adhered to the +1 pyrimidine and +2 adenylate rule (Table 2). Similarly, except for Barmah Forest virus (which has an additional uridylate at the 3′ end), the initiation sequences for genomic (+)-strand synthesis also followed the pyrimidine–adenylate rule (Table 2). Although the pyrimidine found at the +1 position varies between viruses, within each virus the same pyrimidine is found at the +1 position for both subgenomic and genomic (+)-strand initiation.

The initiation sequences for (−)-strand synthesis in the plant-infecting representatives of the alphavirus-like superfamily are similar to one another except for alfalfa mosaic virus. For the animal-infecting representatives, all of the initiation sequences for (−)-strand synthesis are pyrimidine-rich. Although there is a fairly high degree of conservation in the (−)-strand initiation sequences within the plant- or animal-infecting representatives, these sequences are dissimilar from those for subgenomic or (+)-strand genomic synthesis. The initiation sequences suggest that (+)- and (−)-strand synthesis are fundamentally different, whereas (+)-strand synthesis, subgenomic and genomic, shares common themes in the alphavirus-like family. The initiation pyrimidine and the +2 adenylate may participate in directing accurate/efficient (+)-strand RNA synthesis, as reported recently by Van Rossum et al. (1997) for alfalfa mosaic virus. Siegel et al. (1997) demonstrated that nucleotides within the BMV subgenomic core promoter upstream of the initiation site function in recognition of the template by RdRp and our current data indicates that the +1 and +2 nucleotides are also critical for subgenomic RNA synthesis. These two nucleotides may be contacted by RdRp and/or may be important for directing incorporation of a guanylate and uridylate. The lack of common functional groups in the

TABLE 2. Comparison of initiation sequences for genomic (+)-strand, subgenomic and genomic (−)-strand RNA synthesis in representatives of the alphavirus-like superfamily.[a,b]

		Initiation sequence for synthesis of		
Virus	RNA	Genomic (+)-strand	Subgenomic	Genomic (−)-strand
Plant-infecting				
		+1		+1
Brome mosaic[c]	1	3′ CAUC 5′		3′ ACCAGA 5′
	2	CAUU	+1	ACCAGA
	3	CAAG	3′ CAUA 5′	ACCAGA
Cowpea chlorotic mottle[c]	1	CAUU		ACCAGA
	2	CAUU		ACCAGA
	3	CAUU	CAUU	ACCAGA
Broad bean mottle[c]	1	UAUU		ACCAGA
	2	UAUU		ACCAGA
	3	UAUU	UAUU	ACCAGA
Cucumber mosaic (Q, fny)[c]	1	CAAA		ACCAGA
	2	CAAA		ACCAGA
	3	CAUU	CAAA(Q) CAAU(fny)	ACCAGA
Tobacco mosaic (vulgare)[d]		CAUA	CAAA (30K) CAAA (CP)	ACCCGG
Alfalfa mosaic[e]	1	CAAA		CGAGGG
	2	CAAA		CGAGGG
	3	CAAA	CAAA	CGAGGG
Animal-infecting				
Sindbis[f]		UAAC	UAUC	CUUUAC
Semliki forest[f]		UACC	UAAC	CCUUUA
Aura[g]		UAUC	UAUC	CUUUAU
Barmah forest[h]		UUAG	UAUC	CAUUUU
Eastern equine encephalitis[f]		UAUC	UAUC	CUUUAU
Venezuelan equine encephalitis[f]		UACC	UACC	CUUUAU
Middleburg[f]		UAAC	UAUC	CCUUAU
Ockelbo[i]		UAAC	UAUC	CUUUAC
O'Nyong-nyong[j]		UAUC	UAUC	CUUUAU
Ross river[k]		UACC	UAUC	CAUUUU

[a] Strains are indicated in parentheses where multiple strains of one virus have been sequenced.
[b] Tobacco mosaic virus produces two subgenomic RNAs: one for the movement protein (30K) and one for the coat protein (CP).
[c] Ahlquist et al., 1981; [d] Goelet et al., 1982; [e] Gunn & Symons, 1980; [f] Ou et al., 1982a, 1982b, 1983; [g] Rumenapf et al., 1995; [h] Lee et al., 1997; [i] Shirako et al., 1991; [j] Levinson et al., 1990; [k] Faragher & Dalgarno, 1986; Faragher et al., 1988.

nucleotides that can serve at the +2 position (adenylate, cytidylate, and uridylate) suggests that the +2 position may be more important for directing incorporation of the appropriate nucleotide.

Comparison of initiation requirements for subgenomic and (−)-strand synthesis

The initiation complex is formed by the intermolecular interaction of the template, RdRp, and initiation nucleotides. Although initiation generally requires a guanylate, several lines of evidence suggest that the initiation complex may be different for subgenomic and (−)-strand RNA synthesis. We observed that the stimulation of subgenomic synthesis by primers was markedly less than for (−)-strand synthesis. Several possibilities may explain these observations. Template RNA sequence and/or structure may determine priming efficiency. Perhaps a more open structure exists at the promoter for (−)-strand synthesis due to its location near the 3′ end of the template RNA or to a different conformation of the RdRp complex for subgenomic and (−)-strand synthesis. Finally, different subunits within RdRp may be required for subgenomic and (−)-strand synthesis.

Primers never restore RNA synthesis by the BMV RdRp to the levels observed with optimal concentrations of GTP. Reactions containing GTP concentrations that allow efficient (−)-strand RNA synthesis were not affected by the addition of GMP, GDP, or GpG, eliminating the possibility that these primers have a significant inhibitory effect on RNA synthesis (data not shown). This suggests that, in addition to the guanylate, one or more of the three phosphates may contribute to recognition by RdRp and is especially relevant for synthesis of the subgenomic RNA. Because the subgenomic and genomic (+)-strand RNAs are capped and capping requires the β and γ phosphates, it is tempting to speculate that the efficient use of GTP as the initiation nucleotide may be linked to capping.

Synthesis of abortive products precedes BMV RdRp elongation of subgenomic and (−)-strand RNAs

In contrast to the apparent differences in the initiation of subgenomic and (−)-strand synthesis, the transition from initiation to elongation may occur via a similar mechanism. Abortive products synthesized during initiation of subgenomic and (−)-strand genomic RNA synthesis are as long as nine nucleotides (Fig. 4) and eight nucleotides (Sun et al., 1996), respectively. Because the longest abortive product is a good indicator for the position of the transition of RdRp to productive synthesis, the transition likely occurs after the synthesis of seven to eight phosphodiester bonds for both subgenomic and (−)-strand RNA synthesis. The stoichiometry of the reactions is also similar in that a 3–20-fold molar excess of the abortive products is synthesized in comparison to full-length products. Finally, low concentrations of manganese had similar stimulatory effects on both subgenomic and (−)-strand abortive product synthesis.

Comparison of RNA-dependent and DNA-dependent RNA synthesis

RdRps and DdRps have evolved to use different templates. In addition, although both types of polymerases initiate RNA synthesis internally within the template, RdRp must also be able to initiate RNA synthesis near the 3′ ends of templates. Recent elucidation of the structures of several polymerases, including the poliovirus RdRp, revealed that the three-dimensional structures of the polymerases are more similar than their primary sequences (Joyce & Steitz, 1995; Hansen et al., 1997; Doublié et al., 1998). After characterization of both subgenomic and (−)-strand RNA synthesis by the BMV RdRp, we find that the structurally similar RdRps and DdRp share many biochemical activities and follow a highly parallel series of steps, including initiation, abortive initiation, transition to elongation, elongation, and termination (Table 3).

Initiation of RNA synthesis by both RdRp and DdRp strongly prefers a purine nucleotide, which is used at a higher concentration for initiation than for elongation (Blumenthal, 1980; McClure, 1985; Kao & Sun, 1996). BMV RdRp prefers GTP as the initiation nucleotide, but can use ATP with lower efficiency (Fig. 3) (Siegel et al., 1997). The initiation nucleotide can be considered as a primer because it is not hydrolyzed during the formation of the first phosphodiester bond. Other primers of one or a few nucleotides can substitute for the initiation nucleotide for RdRp (Fig. 3) (Blumenthal & Carmichael, 1979; Honda et al., 1986; Kao & Sun, 1996) and the T7 and *Escherichia coli* DdRps (Terao et al., 1972).

The lengths of the abortive initiation products formed by both DdRp and RdRp are strikingly similar. The maximum lengths of abortive initiation products tend to be 9–12 nt for both the T7 (Martin et al., 1988) and *E. coli* DdRps (Levin et al., 1987), whereas the maximum sizes of abortive products synthesized by RdRps are 8–9 nt (Fig. 4B) (Furuichi, 1981; Yamakawa et al., 1981; Sun et al., 1996; Sun & Kao, 1997b). Finally, the abortive products synthesized by the BMV RdRp and the T7 DdRp tend to occur after the polymerization of an adenylate or uridylate (Martin et al., 1988).

Several lines of evidence suggest that abortive initiation plays significant roles in vivo and in vitro. It is likely that abortive initiation allows the dissociation of polymerase from inappropriate templates because abortive synthesis by the T7 DdRp can occur on random DNA polymers, but the polymerase does not switch to the elongation mode (Sousa et al., 1992). In addition, the abortive products may act as primers to increase

TABLE 3. Comparison of RNA synthesis by DdRp and RdRp.

Characteristic	DdRp	RdRp
Initiation	Purine[a] Primer-inducible[e] Sequence-specific[i] High K_m for initiation nucleotide[k]	Purine[b,c,d] Primer-inducible[f,g,h] Sequence-specific[j] High K_m for initiation nucleotide[g,l]
Abortive initiation	DdRp remains on template[m]	RdRp dissociates from template[n]
Template commitment	8–12 nt[o]	ca. 10 nt[h,p]
Cis-acting regulatory sequences	Enhancers[q] Repressors[q]	Activator[c] Repressors[r]
Termination	Sequence-dependent[s]	Sequence-independent[h]

[a]McClure, 1985; [b]Blumenthal, 1980; Miller et al., 1985, 1986; [c]Adkins et al., 1997; [d]Siegel et al., 1997; [e]Terao et al., 1972; Cazenave & Uhlenbeck, 1994; [f]Blumenthal & Carmichael, 1979; Honda et al., 1986; [g]Kao & Sun, 1996; [h]this work; [i]Martin & Coleman, 1987; Milligan et al., 1987; Maslak et al., 1993; Schick & Martin, 1993; [j]Siegel et al., 1997; [k]Patra et al., 1992; [l]Mitsunari & Hori, 1973; [m]Carpousis & Gralla, 1980, 1985; [n]Sun et al., 1996; Sun & Kao, 1997a; [o]Levin et al., 1987; [p]Sun & Kao, 1997b; [q]Tjian, 1996; [r]Bujarski et al., 1985; [s]Richardson, 1996.

the transition rate of polymerase into the elongation phase. Ruetsch and Dennis (1987) observed that, in the presence of primers, the transition from an initiation to a salt-resistant elongation stage occurred after synthesis of the third phosphodiester bond. Finally, Nagy et al. (1997) provided evidence for the use of abortive initiation products in repairing small deletions in the 3′ ends of turnip crinkle virus RNAs. This is a plausible mechanism for repair of the ends of genomic segments of multipartite RNA viruses and may explain the telomerase-like activity proposed to be responsible for maintenance of BMV RNA 3′ ends (Rao et al., 1989).

MATERIALS AND METHODS

RdRp activity assays

BMV RdRp was prepared from infected barley essentially as described by Sun et al. (1996). RdRp preparations used in abortive initiation studies were passed through an additional PD10 (Pharmacia) gel filtration column to remove NTPs and other low molecular weight contaminants. Standard RdRp activity assays consisted of 43-μL reactions containing 20 mM sodium glutamate, pH 8.2, 4 mM $MgCl_2$, 12 mM dithiothreitol, 0.5% (v/v) Triton X-100, 2 mM $MnCl_2$, 200 μM ATP, 500 μM GTP, 200 μM UTP, 242 nM [α-^{32}P]CTP (400 Ci/mmol, 10 mCi/mL, Amersham), equal moles (generally 1.0 pmol) template RNA, and 5–10 μL RdRp. Reactions were incubated 90 min at 30 °C unless indicated otherwise. Reaction products were extracted with phenol/chloroform (1:1, v/v) and precipitated with three volumes of ethanol and 10 μg glycogen following standard protocols (Sambrook et al., 1989). The NTP composition was modified in some experiments as indicated in the figure legends.

For abortive initiation experiments, gel filtration spin columns were used to fractionate the RdRp complex and product RNAs from 100-μL RdRp reactions lacking CTP to arrest the complex on the template RNA. Sephadex CL-6B spin columns were prepared in 1-mL syringes and used as described in Sun and Kao (1997b).

Analysis of RdRp products

Products from RdRp reactions were suspended in 1× denaturing 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] and denatured by heating at 90 °C for 3 min prior to analysis by denaturing PAGE. Products were analyzed on 20% or 24% acrylamide (19:1 acrylamide: bisacrylamide)–7 M urea gels (14 × 14 × 0.05 cm) according to published procedures (Sambrook et al., 1989). In some cases, a 5% acrylamide stacking gel (2 × 14 × 0.05 cm) was used. Products from reactions containing templates directing synthesis of 198-nt or longer products were digested with 2.5 units S1 nuclease (Promega) in the manufacturer's buffer for 10 min at 30 °C. Denaturing loading buffer was added to S1-treated products prior to analysis by denaturing PAGE on 5% acrylamide gels, whereas native loading buffer [5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol] was added to S1-treated products prior to analysis by nondenaturing electrophoresis on 1% agarose gels. All gels were exposed to film at −80 °C and the amount of label incorporated into newly synthesized RNAs was determined with a phosphorimager (Molecular Dynamics).

Synthesis of templates for RdRp

PCR was used to synthesize cDNA copies of either (−)-strand BMV RNA3 encompassing the subgenomic promoter or (+)-strand BMV RNA3 or RNA1 encompassing the (−)-strand promoter. Pairs of synthetic oligonucleotides, one of which contained a T7 promoter, were used in PCR reactions with cDNA clones of BMV RNA3 (pB3TP8) or RNA1 (pB1TP3), respectively (Janda et al., 1987). Thirty cycles of PCR were used for amplification with *Taq* polymerase, with each cycle consisting of 30 s each of denaturation at 94 °C, annealing

TABLE 4. RdRp template descriptions and oligonucleotides used for marker synthesis.[a]

Template name	Purpose	Template termini with respect to (+)-strand RNA1* or RNA3	RdRp product size (nt)
Templates for subgenomic RNA synthesis			
WT	Control for initiation sequence mutations	1222–1252	13
−1 G/A	Initiation sequence mutation	1222–1252	13
−1 G/U	Initiation sequence mutation	1222–1252	13
+2 A/C	Initiation sequence mutation	1222–1252	13
+2 A/U	Initiation sequence mutation	1222–1252	13
+2 A/G	Initiation sequence mutation	1222–1252	13
+3 U/C	Initiation sequence mutation	1222–1252	1
+3 U/G	Initiation sequence mutation	1222–1252	13
+4 A/C	Initiation sequence mutation	1222–1252	13
+4 A/G	Initiation sequence mutation	1222–1252	13
12/26	Proscript for abortive initiation experiments	1214–1265	26
37/26	Abortive initiation, +1C to G	1214–1265	26
up/45	Comparison to (−)-strand synthesis	1165–1446	207
RNA3(−)	Abortive initiation & termination experiments	1–2117	876
RNA3(−)+250	Termination experiments	1–2117+250	1,126
Templates for (−)-strand RNA synthesis			
B1–242	Comparison to subgenomic synthesis	2993–3234*	242
B1–242+2C/A	Initiation sequence mutation	2993–3234*	242
B1–242+2C/U	Initiation sequence mutation	2993–3234*	242
B3–198	Comparison to subgenomic synthesis	1920–2117	198
RNA3(+)	Termination experiments	1–2117	2,117
RNA3(+)+150	Termination experiments	1–2117+150	2,267

Name	Sequence	T7 DdRp product size (nt)
Oligonucleotides for marker synthesis[a]		
T7(−)8mer	5′-ATTAATA**CTATAGTGAGTCGTATTA**-3′	8
T7(−)13mer	5′-GGATTATTAATA**CTATAGTGAGTCGTATTA**-3′	13
T7(+)	5′-**TAATACGACTCACTATA**-3′	—

[a]T7 promoter and complementary sequences are in bold.

at 5 °C below the lowest oligonucleotide T_m, and elongation at 72 °C. PCR products were purified as described above (Sambrook et al., 1989) and used as templates for in vitro transcription. The T7 DdRp was used for all transcription reactions (Ampliscribe, Epicentre) (Table 4). Synthesis of the B3–198 template has been described previously (Sun & Kao, 1997a).

Molecular weight markers of 8 and 13 nt were synthesized by the protocol of Milligan et al. (1987) using oligonucleotides T7(+) and T7(−)8mer or T7(−)13mer (Table 4). Transcripts of full-length (−)- and (+)-strand RNA3 were synthesized from plasmids containing the cDNA of RNA3. Transcripts of (−)-strand RNA3 with a 250-nt extension at the 5′ end and (+)-strand RNA3 with a 150-nt extension at the 5′ end were synthesized from plasmids containing the cDNA of RNA3 positioned 250- or 150-nt downstream of a T7 promoter, respectively.

Prior to RdRp assays, transcripts were purified by anion exchange chromatography on Qiagen tip-20 columns using the manufacturer's protocol. Concentration of RdRp templates was determined by toluidine blue staining following denaturing PAGE and/or by using a spectrophotometer, as described previously (Adkins et al., 1997).

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