RNA (2000), *6*:698–707. Cambridge University Press. Printed in the USA.

CCA initiation boxes without unique promoter elements support in vitro transcription by three viral RNA-dependent RNA polymerases

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ABSTRACT

It has previously been observed that the only specific requirement for transcriptional initiation on viral RNA in vitro by the RNA-dependent RNA polymerase (RdRp) of turnip yellow mosaic virus is the CCA at the 3′ end of the genome. We now compare the abilities of this RdRp, turnip crinkle virus RdRp, and Q*β* replicase, an enzyme capable of supporting the complete viral replication cycle in vitro, to transcribe RNA templates containing multiple CCA boxes but lacking specific viral sequences. Each enzyme is able to initiate transcription from several CCA boxes within these RNAs, and no special reaction conditions are required for these activities. The transcriptional yields produced from templates comprised of multiple CCA or CCCA repeats relative to templates derived from native viral RNA sequences vary between 2:1 and 0.1:1 for the different RdRps. Control of initiation by such redundant sequences presents a challenge to the specificity of viral transcription and replication. We identify 3′-preferential initiation and sensitivity to structural presentation as two specificity mechanisms that can limit initiation among potential CCA initiation sites. These two specificity mechanisms are used to different degrees by the three RdRps. The finding that three viral RdRps representing two of the three supergroups within the positive-strand RNA viral RdRp phylogeny support substantial transcription in the absence of unique promoters suggests that this phenomenon may be common among positive-strand viruses. A framework is presented arguing that replication of viral RNA in the absence of unique promoter elements is feasible.

Keywords: Q*β* bacteriophage; RNA-dependent RNA polymerase; turnip crinkle virus; turnip yellow mosaic virus

INTRODUCTION

The genomes of positive-strand RNA viruses replicate in the cytoplasms of host cells through negative-strand RNA intermediates, catalyzed by the activities of virus-encoded RNA-dependent RNA polymerases (RdRp) or replicases. Successful replication and amplification of the genome of a positive-strand RNA virus requires accurate selection of the initiation sites for the transcription of negative and positive strands, as well as discrimination of viral RNAs as templates from competing host RNAs. Purified or partially purified viral RdRps typically display in vitro the expected preference for the use of cognate viral genomic RNAs as templates for full-length transcription (e.g., Blumenthal & Carmichael, 1979; Hardy et al., 1979; Singh & Dreher, 1997).

The observed specificity of template usage is most easily imagined—and usually considered—to arise from the direct recognition in a lock-and-key manner of unique *cis*-acting control elements, or promoters, present in viral RNAs in the form of specific sequence or structural features. Early in vitro studies with brome mosaic virus (BMV) provided strong support for the presence of specific, unique *cis*-acting elements within the ~200-nt-long 3′-tRNA-like structure of BMV RNAs (Dreher et al., 1984; Miller et al., 1986). It has recently been confirmed that a bulge-containing stem-loop provides this function within the tRNA-like structure to control BMV negative-strand transcription (Chapman & Kao, 1999). On the other hand, identification of the precise *cis*-acting sequences controlling the transcription of other positive-strand viral RNAs has been less successful. Although a branched stem-loop structure

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towards the middle of Qβ genomic RNA has been identified as a site that interacts with Qβ replicase to support minus-strand synthesis (the M site; Meyer et al., 1981), there do not appear to be precise sequence determinants associated with this site (Schuppli et al., 1998), and several smaller RNAs amplified by Qβ replicase lack identifiably similar features (Zamora et al., 1995). At present, it thus seems that Qβ replicase may respond to rather generic, nonunique *cis*-acting signals, although further studies may discern more specific features present in templates transcribed by Qβ replicase.

Similarly, it has recently been shown that turnip yellow mosaic virus (TYMV) RdRp is able to transcribe 3′ fragments of the genome in vitro without the requirement of unique promoter elements (Deiman et al., 1998; Singh & Dreher, 1998). Instead, complementary strand synthesis is under the control of a –CCR– "initiation box" (Singh & Dreher, 1998) whose sequence is present 415 times in the genomic RNA and at the 3′ ends of cellular tRNAs. Unstructured RNAs containing multiple –CCA– repeats, but no other viral sequences, support similar levels of complementary strand synthesis by TYMV RdRp as do *bona fide* viral 3′-RNA fragments. We have argued that specificity in initiation site selection can be achieved despite such a redundant specific requirement by the modulating effect of nonspecific secondary or tertiary structure that determines whether –CCR– triplets are sterically more or less favorable for supporting initiation (Singh & Dreher, 1998).

To determine whether this mode of template usage is unique to TYMV or more widespread among positive-strand RNA viruses, we have compared the RdRps from two positive-strand RNA viruses only distantly related to TYMV, Qβ bacteriophage and turnip crinkle virus (TCV), to TYMV RdRp. Our results indicate that TYMV RdRp is not an isolated case among the positive-strand RNA viruses in utilizing specificity mechanisms other than unique recognition elements in controlling RNA replication. However, the relative importance of these mechanisms and their detailed properties vary between different viruses.

RESULTS

Rationale of this study

Stimulated by our discovery that TYMV RdRp is able to initiate complementary strand synthesis from –CCR– triplets in the absence of conventional unique promoter elements, a survey of the literature suggested two accessible positive-strand viral RdRp systems that might share some of these properties with TYMV: Qβ bacteriophage and TCV. Qβ replicase is well known to prefer templates with C-rich termini (Blumenthal & Carmichael, 1979) present in non-base-paired form (Zamora et al., 1995). Although promoter-like elements have been described in a number of RNA templates transcribed or amplified by Qβ replicase (Meyer et al., 1981; Nishihara et al., 1983; Brown & Gold, 1996; Schuppli et al., 1998), these cannot be classified as a defined sequence or structure, unlike, for example, the unique promoter recognized by T7 RNA polymerase (Li et al., 1996).

In the case of TCV RdRp, promoter-like elements have been described, particularly a stem-loop at the 3′ end of TCV-associated satellite RNA C (+) strand (Song & Simon, 1995; Stupina & Simon, 1997; Carpenter & Simon, 1998). However, TCV RdRp is able to initiate complementary strand synthesis from a number of C-rich sites within truncated forms of satellite C negative-strand RNA (Guan et al., 1997). The ability of C-rich tracts to act as independent internal initiation sites suggested some similarity to properties of TYMV RdRp. Note that all three enzymes initiate RNA synthesis from natural templates opposite a 3′ site comprising two or more C residues.

Our comparison of TYMV, TCV, and Qβ RdRps addressed two questions: first, how efficiently are these RdRps able to utilize unstructured templates composed of CCA or related repeats? Second, if such RNAs are active templates, can a mechanism that produces a preferred initiation site (specificity) be discerned? To answer the first question, we tested the template activities of RNAs with a 5′-GGA sequence followed by 12 CCA, 9 CCCA, or 7 CCCCA repeats (Fig. 1). To test the influence of secondary structure on initiation site selection, derivatives of CCA12 RNA bearing oligo(U): oligo(A) stems were used as templates (RNAs S6L6, S6L9, S9L6, and S9L9; Fig. 1). All these RNAs are 39 nt long, except CCCCA7 (38 nt). The use in RdRp incubations of [α-^{32}P]CTP, which is incorporated only at the 3′ end for all RNAs except products templated by S9L6 and S9L9 RNAs, ensures that all products are labeled to identical specific activity and that premature termination products are not seen. The reaction conditions used in these studies were those typical of each RdRp. Special conditions known to relax template specificity, such as addition of manganese salt (Blumenthal & Carmichael, 1979), were avoided.

Each RdRp can utilize multiple initiation sites on unstructured C-rich RNAs

For each RdRp, the unstructured RNAs CCA12, CCCA9, and CCCCA7 were compared as templates with appropriate positive-control RNAs. All three enzymes were able to transcribe CCA12 RNA, using multiple initiation sites, with initiation occurring opposite the 3′-most C of each CCA triplet (verified for Qβ replicase reactions by sequencing products with ribonuclease; S.Yoshinari, not shown). However, the efficiencies relative to positive-control RNAs of similar length, and the spectrum of initiation sites, differed between the enzymes.

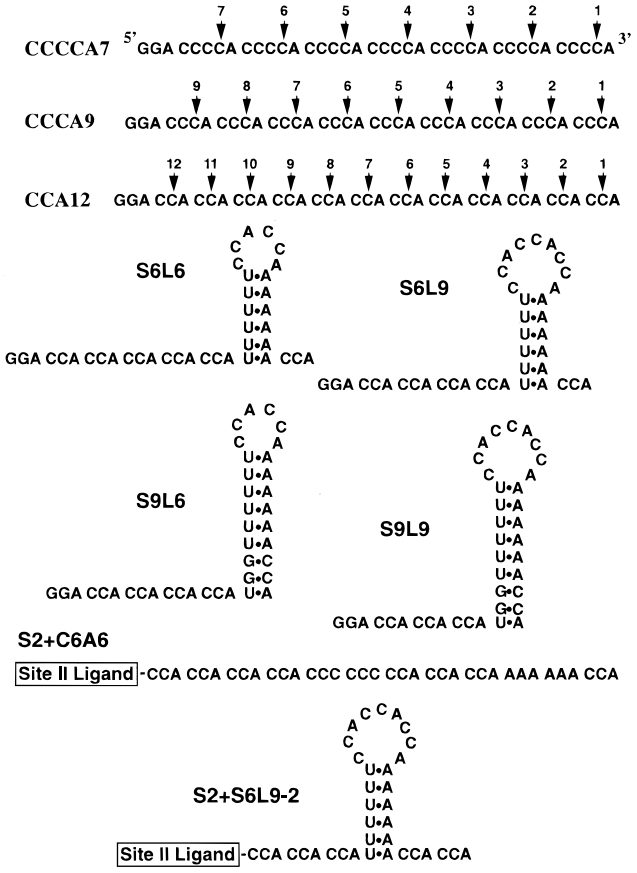

FIGURE 1. The sequences and expected foldings of template RNAs used in this study. The repeat elements are numbered in the linear RNAs with arrows placed at the major initiation site within each potential initiation box.

TYMV RdRp transcribed CCA12 RNA almost as efficiently as TY-41 RNA (41 nt long), which comprises the 3′ half of the tRNA-like structure that is present at the 3′ end of TYMV genomic RNA (Fig. 2A). TY-41 RNA is transcribed with similar efficiency as the entire 83-nt-long TYMV tRNA-like structure (Singh & Dreher, 1997). Little initiation occurred from the –CCA– triplets towards the 3′ end of CCA12 RNA, with most initiation events occurring towards the middle of the template (Figs. 2A, 3A). These results are similar to those previously reported for L40, an RNA that differs only in the 5′-terminal sequence (Singh & Dreher, 1998).

Qβ replicase utilized CCA12 over two times more efficiently than DN3 RNA (Fig. 2C), a 34-nt-long replicon that can be amplified in vitro (Zamora et al., 1995). Qβ replicase showed a strong preference for initiation from the 3′-penultimate –CCA– triplet, with less initiation occurring from internal sites (Figs. 2C, 3C). The doublet product bands arise principally as a result of untemplated 3′-terminal A addition to most, but not all, products (Bausch et al., 1983; Yoshinari & Dreher, 2000).

TCV RdRp transcribed CCA12 RNA with considerably less efficiency than SatC-43 RNA (Fig. 2B), which comprises the 3′ 43 nt of satellite RNA C positive strand, including the stem/loop that has previously been associated with promoter activity (Song & Simon, 1995). SatC-43 purified by denaturing electrophoresis supported 5.6-fold higher initiation than CCA12 RNA, while SatC-43 purified without denaturation supported 17-fold higher initiation than CCA12 RNA (Fig. 2B). Presumably, SatC-43 RNA exists in two alternative conformations, as observed for the Qβ replicase template SV-11 RNA (Zamora et al., 1995). A marked preference for initiation from the 3′-most CCA triplet was observed, but initiation also occurred at multiple internal sites, with a second peak of initiation preference towards the middle of the RNA (Fig. 3B), as observed with TYMV RdRp.

Control experiments verified that the profile of products seen in Figure 2 was not the result of fragmentation of either the template or the product. Template RNAs were carefully purified and shown by postlabeling at the 5′ end with [γ-^{32}P]ATP to remain intact during storage. After incubation with each of the three RdRps under mock transcription conditions, more than 95% of selected template RNAs remained full-length (not shown). T7 transcripts made to mimic the products templated by CCA12 RNA were demonstrated to remain intact during sample preparation for electrophoresis (see Materials and methods). To provide support for the theory that RNAs such as CCA12 support internal initiation rather than premature termination, we have observed a similar profile of products when labeling with other NTPs, such as [α-^{32}P]UTP (Singh & Dreher, 1998), [γ-^{32}P]GTP, or [α-^{32}P]ATP, the last being added in a nontemplated manner by Qβ replicase to the 3′ end of transcripts (Bausch et al., 1983; Yoshinari & Dreher, 2000).

As –CCA– repeats may not be optimal for initiation by each RdRp, CCCA9 and CCCCA7 RNAs were also tested as templates. These RNAs were recognized as templates for multiple initiations by each enzyme (Fig. 2). TCV RdRp showed maximal activity with CCCA9, which supported threefold and ninefold fewer initiation events than SatC-43 RNA prepared from denaturing and nondenaturing gels, respectively. The distinctive profiles of relative initiation frequencies observed with CCA12 RNA were largely preserved with the other linear RNAs, except that initiation from the 3′-most repeat by Qβ replicase became more significant as the repeat length increased. The ratios of initiation from the 3′-penultimate repeat to that from the 3′-most repeat (box 2:box 1; see Fig. 1) were 8.1, 2.8, and 1.5 for CCA12, CCCA9, and CCCCA7 RNAs, respectively.

Effect of secondary structure on initiation site selection from –CCA– triplets

Because the observed ability of each of the three viral RdRps to initiate from multiple C-rich sites in an RNA

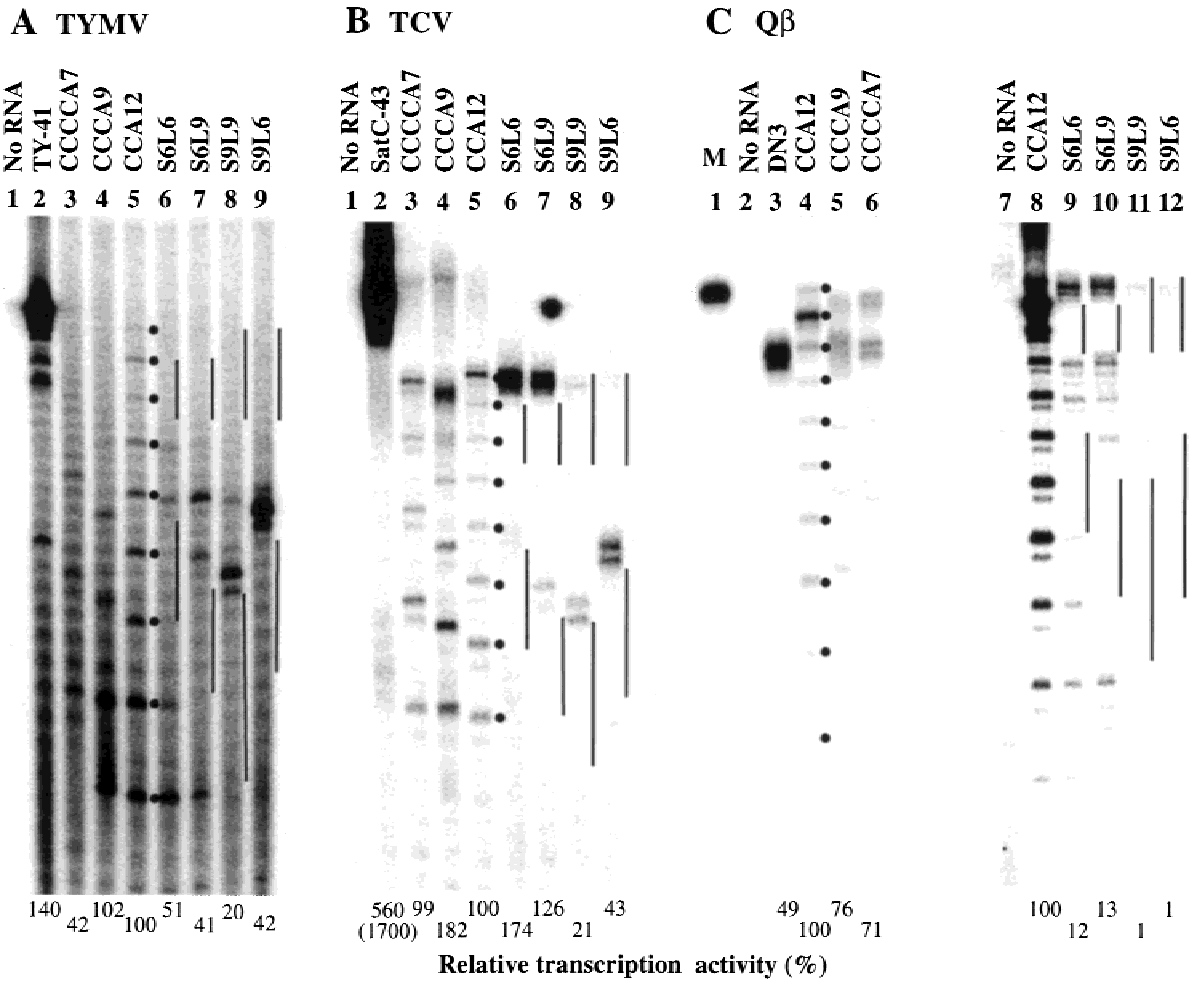

FIGURE 2. Transcription of C-rich linear and structured RNAs by TYMV, TCV, and Qβ RdRps. Products were labeled with [α-^{32}P]CTP, RNase-treated as described in Materials and methods, and separated by denaturing PAGE (12.5%). The template concentrations were 0.6 μM, 3.6 μM, and 0.1 μM with TYMV, TCV, and Qβ RdRps, respectively. The dots next to lanes indicate products initiated from separate –CCA– triplets. The bars indicate the regions expected to be base paired. The transcription activities of templates relative to CCA12 RNA for each enzyme are shown below each lane (averages of three experiments). These activities are corrected for the number of CMPs incorporated into each product, and represent cumulative initiation from the C_nA repeats present in each RNA. Note that [α-^{32}P]CTP is incorporated only at the 3′ end of the product strand for all C-rich RNAs, except products templated by S9L6 and S9L9 RNAs; products templated by TY-41, SatC-43, and DN3 RNAs are expected to contain 11, 13, and 7 CMP residues, respectively. The (1700) below lane B2 refers to the relative activity of RNA purified without denaturating conditions.

represents a threat to the efficacious and specific replication of a genomic RNA, each enzyme should possess properties that can be used as specificity filters to ensure that only appropriate initiation occurs on viral templates. The preferences for initiation at or adjacent to the 3′ end observed for TCV RdRp and even more strongly for Qβ replicase (Fig. 2) represent one mechanism for suppressing alternative initiations. The inability to initiate from a site involved in base pairing, observed for TYMV RdRp (Singh & Dreher, 1998) and deduced for Qβ replicase (Zamora et al., 1995; Schuppli et al., 1997), is another mechanism that limits initiation from inappropriate sites in template RNAs. We have recently observed with TYMV RdRp that secondary structure can also impose preferences for initiation among non-base-paired –CCA– potential initiation sites (Singh & Dreher, 1998). To determine whether such a mechanism is operative with Qβ replicase and TCV RdRp, we have tested the structured derivatives of CCA12 RNA shown in Figure 1 as templates.

S6L6 and S6L9 RNAs have a U_6:A_6 stem closed by a 6- or 9-nt-long loop, respectively. A single CCA triplet is present non-base-paired at the 3′ end, and five or four non-base-paired CCA triplets are present upstream of the stem-loop (Fig. 1). As observed previously (Singh & Dreher, 1998), such a stem-loop alters the selection of initiation sites by TYMV RdRp, resulting in preferred initiation from the middle and 5′ end of the loop (Fig. 2A,

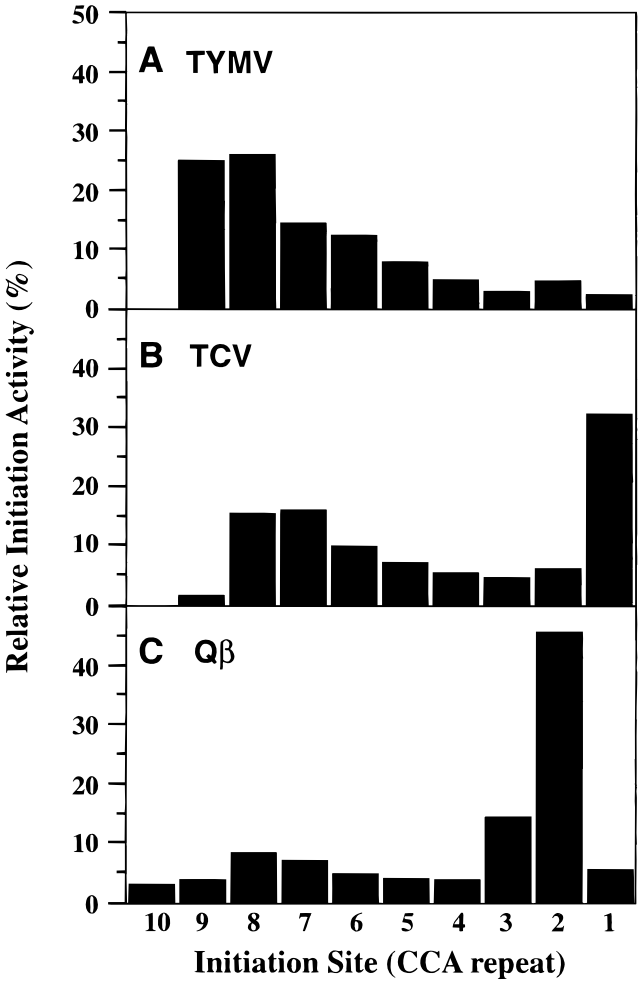

FIGURE 3. Distribution of initiation sites within CCA12 RNA utilized by TYMV, TCV, and Qβ RdRps. The relative initiation activity (percentage of total initiation) from each CCA repeat is shown (average of three experiments). The CCA repeats are numbered from the 3′ end.

lanes 6 and 7). The lack of a 3′-end preference by TYMV RdRp is demonstrated by the low initiation from the 3′ end. Variants of S6L6 and S6L9 RNAs in which the 3′-most CCA is expected to be base paired in an extension of the U_6:A_6 stem were transcribed almost exclusively from the 5′ end of the loop (Fig. 2A, lanes 8 and 9). No initiation was observed from the base-paired 3′-CCA.

Although the structured RNAs supported quite high amounts of transcription by TYMV RdRp (20–51% relative to CCA12 RNA), they were poor templates for Qβ replicase (1–13% relative to CCA12 RNA; Fig. 2). The presence of secondary structure did not alter initiation-site preference among the remaining –CCA– triplets, except that inclusion of the 3′-CCA in the stem clearly inhibited initiation from this site. A little 3′ initiation was observed from S9L6 and S9L9 RNAs, perhaps because of the transient melting of the stem (Fig. 2C, lanes 11 and 12).

S6L6 and S6L9 RNAs were better templates for TCV RdRp than CCA12 RNA, with initiation occurring almost exclusively from the 3′ end (Fig. 2B, lanes 6 and 7). Integration of the 3′-CCA triplet into the base-paired stem inhibited initiation from that site, although as with Qβ replicase, a small amount of initiation was observed from the 3′-terminal CCA triplet of S9L6 and S9L9 RNAs (Fig. 2B, lanes 8 and 9). The inaccessibility of the 3′ ends of these RNAs for initiation resulted in preferential initiation from the 5′-most CCA triplets of the loops of these RNAs, reminiscent of the behavior of TYMV RdRp. Secondary structure has thus introduced marked selectivity among non-base-paired potential initiation sites for both the TYMV and TCV RdRps.

Effect of other transcriptional enhancers on the activities of CCA initiation boxes

Because transcription-enhancing elements have been described for both Qβ replicase and TCV RdRp, it was of interest to further compare the relative importance of such elements to unstructured C-rich regions in directing initiation. Chimeric RNAs with a $(CCA)_{12}$ tract appended to the 3′ end of elements known to enhance transcription were studied as templates.

The chimeric template used with TCV RdRp, C43+A+12CCA RNA, contained at its 5′ end SatC-43 RNA, with an additional A residue to act as spacer to the $(CCA)_{12}$ 3′-terminal domain. Initiation occurred from both within the $(CCA)_{12}$ tract (predominantly from the 3′ end) and from the normal initiation site directed by SatC-43 RNA (Fig. 4A, lane 5), with relative activities similar to those observed from separate transcription of SatC-43+A RNA (Fig. 4A, lane 4) and CCA12 RNA (Fig. 4A, lanes 1 and 2). This indicates that the two halves of the chimeric RNA act independently to direct initiation. The presence of the SatC-43 domain was clearly able to counteract the 3′ preference observed for TCV RdRp in Figure 2B, directing strong internal initiation adjacent to the SatC stem-loop, some 38 nt from the 3′ terminus. This domain did not, however, overcome the 3′ preference for initiation that occurred within the $(CCA)_{12}$ tract. It is not known why the addition of the 3′-A to SatC-43 RNA decreased transcription 4.5-fold (compare Fig. 4A, lanes 3 and 4).

For experiments with Qβ replicase, the site II ligand (GGGCUCCCUAUUUCUUGCUCGCU) was placed upstream of the $(CCA)_{12}$ tract, yielding the chimeric S2+12CCA RNA. The 23-nt-long pyrimidine-rich site II ligand RNA is a tight-binding ligand of the EF-Tu subunit of Qβ replicase that can activate the transcription of a short CCC-3′-ended RNA (Brown & Gold, 1996). The pattern of initiation sites within the $(CCA)_{12}$ tract was not influenced by the presence of the Site II ligand sequence (Fig. 4B, compare lanes 1 and 2). In this context, site II ligand did not act as a transcriptional enhancer, as over-

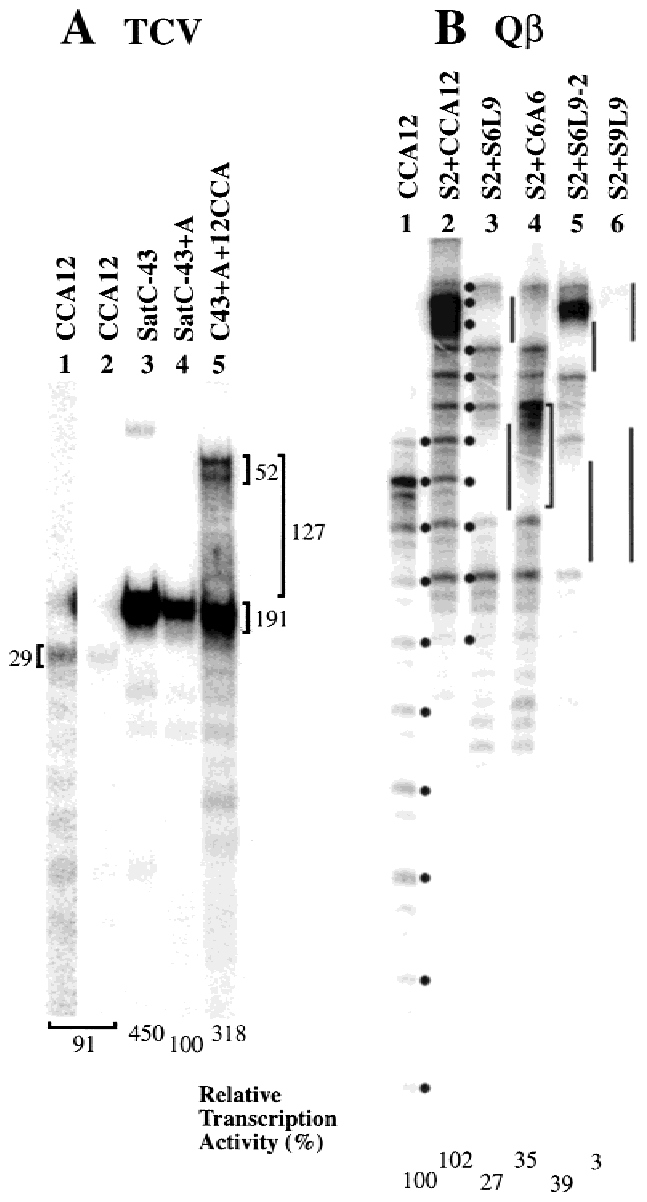

FIGURE 4. Transcription of chimeric RNAs comprising a transcriptional enhancer and a 3′ C-rich domain. The indicated templates were incubated with TCV RdRp (**A**) or Qβ replicase (**B**), and the products were analyzed after RNase treatment as for Figure 2, except that **A** represents separation by 10% native PAGE. The TCV reactions contained 0.6 μM RNA. In **B**, the bars indicate regions expected to be base paired, and the C_8 tract of S2+C6A6 RNA is indicated with a bracket. The relative molar transcription activities of each template for each RdRp are given below each lane, and to the side of the bands for initiation from particular regions of the template RNA in lanes A1 and A5. Note that the S2 domain of the RNAs in lanes B2–B6 templates an additional three CMP residues compared to CCA12 RNA.

all transcription from the chimeric RNA was the same as that from CCA12 RNA.

Derivatives of S2+12CCA RNA with U_6:A_6 stems within the CCA repeat portion of the chimeric RNA (S2+S6L9 and S2+S6L9-2 RNAs), likewise, showed a similar spectrum of initiation sites to that observed from the related RNAs in Figure 2, except that less initiation was observed from the 3′-most CCA repeat (compare Fig. 4B, lane 3 to Fig. 2C, lane 10). The presence of U_6:A_6 stems did not alter the initiation hierarchy from the remaining –CCA– triplets (Fig. 4B, compare lane 2 with lanes 3 and 5). Inclusion of the 3′-most –CCA– triplet in a base-paired stem resulted in very little transcription (Fig. 4B, lane 6) and no preferential initiation from the internal loop as observed with TYMV RdRp.

S2+S6L9 RNA, which has a non-base-paired –CCA– triplet downstream of a 6-bp stem, supported very little initiation from the 3′ end (Fig. 4B, lane 3). This suggests that an isolated –CCA– provides low transcriptional strength; adjacent C-rich sequences seem to provide transcriptional enhancement. This is directly illustrated by the increased initiation from S2+C6A6 RNA, which differs from S2+S6L9 RNA by possessing C_6 in place of U_6 (Fig. 1). The presence of eight consecutive C residues promotes initiation from the position of CCA repeat number 6 by 3.6-fold (Fig. 4B, compare lane 4 to lane 3).

DISCUSSION

Short C-rich motifs as initiation boxes directing positive-strand-RdRps

Our results show that the ability of short C-rich boxes to serve as independent sites for transcriptional initiation is not limited to the TYMV system, but is also observable in vitro with Qβ replicase and TCV RdRp. None of these enzymes requires unusual reaction conditions, such as the addition of Mn^{2+}, to support this activity. Association of this property with Qβ replicase, an enzyme capable of high-fidelity genome amplification in vitro, argues that the phenomenon is not an in vitro artefact. Within the phylogeny of positive-strand RNA viral RdRps, the Qβ and TCV enzymes are among the most divergent members of supergroup 2, and TYMV RdRp falls into supergroup 3 (Koonin & Dolja, 1993). It may thus be concluded that the ability to respond to initiation boxes related to –CCA– is not of limited occurrence among the positive-strand RNA viruses.

In demonstrating that RNAs comprised of simple repeated sequence elements such as –CCA– are able to support significant levels of transcriptional initiation at multiple sites by three unrelated viral RdRps, we show that short initiation boxes can exert a strong influence on viral RNA synthesis. Important transcriptional control can thus be provided by small initiation boxes, and transcription in some viral systems is not only dependent on unique *cis*-acting elements that are usually thought to control positive-strand RNA viral transcription (Lai, 1998).

The importance of initiation boxes as transcriptional control elements may vary widely among different viruses. From our comparison of three polymerases,

–CCA– initiation boxes are able to strongly direct transcription by the TYMV and Qβ RdRps, while short C-rich initiation boxes are considerably weaker in directing transcription by TCV RdRp than is the stem-loop at the 3′ end of satellite C RNA (SatC-43 RNA) (Figs. 2 and 4). Among some positive-strand RNA viruses, the role of initiation boxes may be weaker still, with a role restricted to specifying the site for RNA initiation rather than conferring transcriptional strength.

Although we have shown that a number of viral RdRps respond strongly to C-rich initiation boxes, other positive-strand viral genomes terminate in sequences other than $CC(A)_{3'}$. It seems possible that for at least some of these viruses, non-C-rich initiation boxes are utilized as primary transcriptional elements. Experimental support for this contention is suggested by the finding that as few as three specific nucleotides ($–UAC_{3'}$) are required to support the replication in vivo of negative-sense RNA2 of flock house nodavirus (Ball, 1994; L.A. Ball, pers. comm.). Interestingly, nodavirus RdRps are classified within supergroup 1 (Koonin & Dolja, 1993), distinct from the TYMV, TCV, and Qβ RdRps. A reliance on minimal *cis*-acting template requirements in negative-sense genomes may be a means to support the high rates of positive-strand synthesis characteristic of positive-strand RNA viruses.

3′ preference and conformational filtering are two mechanisms that can produce specific initiation by RdRps that respond to redundant –CCA– initiation boxes

If viral RdRps are found to respond strongly to short initiation boxes that are common within viral RNAs (–CCA– is present 271 times within TYMV RNA), there must be some additional specificity mechanisms to permit appropriate specific end-to-end replication of viral RNAs. We have reported previously (Singh & Dreher, 1998) that nonspecific secondary structure can modulate the availability of –CCA– potential initiation sites to TYMV RdRp, resulting in preferential initiation from certain sites. This principle is again demonstrated for TYMV RdRp in Figure 2A, and has been extended to TCV RdRp, which produced very similar products from S9L9 and S9L6 RNAs, as did TYMV RdRp (Fig. 2). Transcription from essentially a single site on these RNAs, which have three or four –CCA– potential initiation boxes, arises from the lack of initiation from a base-paired –CCA– and from differential initiation among non-base-paired –CCA– boxes that lie within different conformational environments.

Qβ replicase differs from the TYMV and TCV RdRps in having a reduced or different ability to discriminate the more subtle conformational differences of non-base-paired –CCA– boxes, but shares the inability of the other RdRps to initiate from a base-paired –CCA– (Figs. 2C and 4B). This requirement for an available 3′ initiation site has been discerned previously (Zamora et al., 1995), and is the probable reason that the transcription of Qβ-plus-strand RNA requires the Hfq (HF-1) host-factor protein. The Hfq protein binds to the 3′ region of Qβ RNA to make the 3′ end available for initiation by the replicase (Miranda et al., 1997), and Qβ RNA variants that no longer require the Hfq protein for replication are thought to have an altered 3′ conformation in which the $–CCA_{3'}$ is no longer base-paired (Schuppli et al., 1997). Further experiments will be needed to discern whether Qβ replicase responds differently to non-base-paired initiation boxes that are present in various conformational environments.

The present studies have identified a built-in tendency for initiation from the 3′ end as a second mechanism that can produce a specific outcome from an RdRp responding to a redundant initiation box. Although not evident with TYMV RdRp, different forms of 3′-end preference are exhibited by the TCV and Qβ RdRps. 3′-end preference is stronger with Qβ replicase than with TCV RdRp (Figs. 2 and 3). With TCV RdRp, this preference results in initiation from the 3′-most CCA, whereas with Qβ replicase, initiation is curiously preferred from the 3′-penultimate –CCA– (Fig. 2C).

A model for the replication of positive-strand RNA viral genomes

We believe that our results permit a new view of the *cis*-acting factors that contribute to the replication of positive-strand RNA viral genomes. Compatible views have been suggested, though to our knowledge not explicitly described, in previous studies of Qβ RNA replication. We suggest that for some viruses, such as TYMV and perhaps Qβ, the transcriptional steps that contribute to replication are controlled to a significant extent by small, redundant initiation boxes. Specific initiation site selection can result from built-in properties of the RdRp: conformational filtering and/or preferential initiation from the 3′ end. These mechanisms result, respectively, in preferential transcription from initiation boxes that are non-base-paired and perhaps even in a particular conformational environment, and in transcription from an initiation box at or adjacent to the 3′ end of the template. This type of control over initiation may be restricted to positive-strand synthesis with some viruses, such as flock house nodavirus.

The novel aspect of this view of viral replication is the decreased emphasis on unique promoter elements that direct RdRp action by a specific recognition mechanism. Almost certainly, this type of specific transcriptional control over genome replication does exist among positive-strand RNA viruses, because it has been demonstrated for the transcriptional synthesis of the subgenomic coat protein mRNA by BMV RdRp (Siegel et al., 1998), and appears to control negative-strand synthe-

sis by this enzyme (Chapman & Kao, 1999). It seems likely that a continuum will be found to exist among the positive-strand RNA viruses between the extremes of transcriptional control by small initiation boxes as the sole specific elements, and control by unique sequence or structural elements. Indeed, we have seen part of such a gradation among the three RdRps examined in this study. TYMV and Qβ RdRps respond strongly to –CCA– boxes in linear C-rich RNAs, and a demonstrated transcriptional enhancer (Brown & Gold, 1996) does not strongly influence the pattern or efficiency of initiation by Qβ replicase (Fig. 4B). The introduction of a U_6:A_6 stem into CCA12 RNA decreased transcription by TYMV rather moderately (Fig. 2A), but resulted in a large decrease in transcription by Qβ replicase (Fig. 2C). This suggests that an individual –CCA– initiation box provides more transcriptional strength towards TYMV RdRp than towards Qβ replicase, which may require a variety of transcriptional enhancers to provide full activity to a –CCA– initiation box. Such enhancers could be adjacent C-rich sequences (as in CCA12 RNA or poly(C) RNA), the M site present in Qβ virion RNA (Schuppli et al., 1998), or RNA elements selected in vitro for binding to Qβ replicase, the site I and site II ligand RNAs (Brown & Gold, 1996). Note that none of these elements is a unique feature with the specificity of a T7 promoter. No such elements that promote transcription have yet been described in TYMV RNA, but they have been described for TCV (Song & Simon, 1995; Nagy et al., 1999), and, indeed, initiation boxes alone are not sufficient to produce the level of transcription by TCV RdRp that is supported by such elements (Fig. 2B).

Although we have demonstrated the plausibility of the specificity mechanisms described here, they may not be sufficient to support specific viral replication in a host cytoplasm full of decoy RNAs. However, two additional factors that provide specificity can be mentioned. First, a coupling of translation and replication, which results in viral replication proteins acting predominantly *in cis* on the RNA from which they were translated, has been reported for many positive-strand viruses (e.g., Weiland & Dreher, 1993; Novak & Kirkegaard, 1994; van Rossum et al., 1996). This phenomenon is accompanied among the eukaryotic viruses by the sequestration of viral replication activity into distinct membrane-associated compartments (Francki, 1987; Restrepo-Hartwig & Ahlquist, 1996; Bolten et al., 1998) that exclude ribosomes and probably most cellular RNAs. Second, cellular RNAs are strongly associated with proteins that would likely make their 3′ ends inaccessible to viral RdRps: tRNAs are associated with factors such as eEF1A and ribosomes throughout their functional cycle (Negrutskii et al., 1994), and the poly(A) tails of mRNAs are bound by the poly(A)-binding protein (Ross, 1995). Thus, the viral RdRps that must replicate viral RNAs with 3′-tRNA-like structures or poly(A) tails may not readily be confronted with cellular RNAs. Nevertheless, additional specificity mechanisms other than reliance on unique promoter elements will likely emerge from further research on positive-strand RNA viruses, perhaps especially in the case of viruses with multipartite genomes.

MATERIALS AND METHODS

Preparation of RdRp template RNAs

RNAs used in this study were prepared by transcription with T7 RNA polymerase using DNA templates comprising a universal top strand encompassing the T7 promoter region annealed to bottom strands that contain the T7 promoter and sequence complementary to the desired RNA (Milligan et al., 1987). RNA transcripts were purified to single-base resolution by denaturing 7 M urea–10% PAGE. RNAs were quantified spectrophotometrically, using extinction coefficients calculated by the program Oligo v. 6.0 (Molecular Biology Insights, Inc.). The sequences and 3′-termini of the templates CCA12, CCCA9, and CCCCA7 were confirmed by ribonuclease sequencing (Kuchino & Nishimura, 1989) after 3′ labeling with [5′-^{32}P]pCp and T4 RNA ligase.

RdRp transcription assays

TYMV RdRp was partially purified by detergent solubilization of membranes derived from TYMV-infected Chinese cabbage leaves, followed by glycerol gradient centrifugation (Singh & Dreher, 1997). Assay reactions (25 μL) contained 21.6 μL of micrococcal nuclease-treated RdRp and 15 pmol of template RNA in the presence of 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 80 μg/mL actinomycin D, 800 U/mL rRNasin ribonuclease inhibitor (Promega), 500 μM each of ATP, GTP, and UTP, and 10 μM CTP containing 20 μCi of [α-^{32}P]CTP (Singh & Dreher, 1998). After incubation at 30 °C for 90 min, reaction products were double phenol extracted and ethanol precipitated in the presence of ammonium acetate, using 20 μg glycogen as coprecipitant.

TCV RdRp was partially purified by detergent solubilization of membranes derived from TCV-infected turnip leaves, followed by gel filtration (Peak II; Song & Simon, 1994). Reactions (25 μL) contained 10 μL of RdRp and 15 or 90 pmol of template RNA in the presence of 50 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 10 mM DTT, 100 mM potassium glutamate, 80 μg/mL actinomycin D, 4,000 U/mL rRNasin, 1 mM each of ATP, GTP, UTP, and 10 μM CTP, including 20 μCi of [α-^{32}P]CTP (Song & Simon, 1994). Reactions were incubated at 20 °C for 75 min, and treated as above.

Qβ replicase (50 nM), purified to about 80% purity after overexpression in *Escherichia coli* (Moody et al., 1994), was incubated at 37 °C for 10 min in 25-μL reactions containing 2.5 pmol (100 nM) of template RNA in the presence of 80 mM Tris-HCl (pH 7.5), 21 mM $MgCl_2$, 1 mM DTT, 200 μM each of ATP, CTP, GTP, and UTP, and including 10 μCi of [α-^{32}P]CTP (Moody et al., 1994). Moody et al. (1994) have used 21 mM $MgCl_2$ rather than the more common 10 mM to suppress run-away replication of internally deleted replicons; we have

seen no differences in the products of Figure 1 between 10 mM and 21 mM $MgCl_2$ (not shown). After incubation, nucleic acids were phenol extracted and recovered as above.

RNase treatment and analyses of radiolabeled products

Radiolabeled RdRp products obtained after ethanol precipitation were treated with a mixture of 10 μg/mL RNase A and 10 U/mL RNase T1 in 2×SSC (30 °C, 30 min) to remove the excess of single-stranded template RNAs. In turn, RNases were removed by treatment with 50 μg/mL proteinase K in 0.05% SDS and 2×SSC (30 °C, 30 min). After phenol extraction, products were ethanol precipitated in the presence of 250 pmol of DNA oligomers complementary to the template RNAs to assist in displacing RdRp products from the double-stranded form. Washed pellets were resuspended in 10 μL of 90% formamide and 10 mM EDTA, boiled for 5 min, chilled and immediately applied to 7 M urea/12.5% PAGE. For native PAGE (Fig. 4A), pellets were resuspended in 10 μL of loading buffer and applied to the gel. RdRp products were visualized and quantitated using a PhosphorImager (Molecular Dynamics). Control experiments showed no evidence of hydrolysis of $(GGU)_n$ control RNAs, or of any other RNAs, under these conditions. The spectrum of products was not altered by RNase treatment, which was performed to ensure denaturation of RNA duplexes for electrophoretic analysis.

ACKNOWLEDGMENTS

We are grateful to Dr. Michael Farrell of Vysis, Inc. for the generous gift of Qβ replicase, Hancheng Guan for preparing TCV RdRp, and Dr. Andy Ball for reviewing the manuscript. This work was supported by grants from the National Institutes of Health (GM-54610 to T.W.D.) and the National Science Foundation (MB-9630191 to A.E.S.). This is technical report No. 11554 of the Oregon Agricultural Experiment Station.

Received November 15, 1999; returned for revision January 19, 2000; revised manuscript received February 14, 2000

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