Selection and Characterization of RNAs Replicated by Qβ Replicase†

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ABSTRACT: RNAs replicated by Qβ replicase were isolated from two random sequence RNA populations (one 56 nucleotides in length, the second 83) using a replication/dilution protocol. The selected molecules were cloned and sequenced, generating a data set of 54 replicatable RNAs. Four molecules that dominated their respective selection pools were characterized. All four of the RNAs bound with higher affinity to Qβ replicase than did the random populations from which they were selected. Deletion analyses on two of the molecules indicated that internal regions of the RNAs were responsible for the specific binding of Qβ replicase. Truncated molecules representing the minimized RNA binding sites could inhibit replication of the full-length molecules, apparently by obstructing their binding to the replicase. The binding regions of the two RNAs were dominated by extended runs of pyrimidines. Similar C/U-rich regions existed in 85% of the sequences in the data set as well as in all of the previously published replicatable sequences. Mutation of the polypyrimidine domain of one of the replicatable sequences reduced the affinity of the molecule for Qβ replicase by 10-fold and completely abolished its ability to be replicated.

Qβ replicase is the RNA-dependent RNA polymerase responsible for producing replicas of the single-stranded RNA genome of the coliphage Qβ (Haruna & Spiegelman, 1965a). The replicase displays high specificity during infection, replicating the phage RNA while ignoring the extraordinary excess of bacterial RNA in the host cell. Such specificity is observed *in vitro* as well; very few of the RNAs tested have exhibited any template activity in the presence of the phage polymerase (Haruna & Spiegelman, 1965b; Weissmann *et al.*, 1968). Despite the level of specificity exhibited by Qβ replicase and the fact that the enzyme has been studied for nearly 30 years, the precise RNA features required for replication are still unknown.

In addition to the Qβ genome, there have been nine published sequences of RNAs that are efficiently replicated by Qβ replicase (Schaffner *et al.*, 1977; Biebricher, 1987; Priano *et al.*, 1987; Munishkin *et al.*, 1988, 1991; Moody *et al.*, 1994). Attempts to define the minimal requirements for replication have been made by doing comparative analyses on different sets of these published sequences (Kuppers & Sumper, 1975; Voronin, 1992). The only obvious conserved elements among the replicatable molecules are (1) a GGG at the 5′ terminus and CCC at the 3′ terminus, (2) the potential to involve many nucleotides in intramolecular base-pairing, and (3) the ability to form a stable hairpin loop near the 5′ ends of both the plus and minus strands (Zamora *et al.*, 1995). The C's at the 3′ ends most likely reflect a mechanistic requirement for efficient complementary strand initiation (Blumenthal, 1980). The intramolecular secondary structures are probably used to reduce duplex formation between the complementary products of replication, as double-stranded RNA has been shown to be rejected as a template by the phage polymerase (Weissmann *et al.*, 1968).

The three shared characteristics detected among the replicatable molecules are clearly not sufficient to explain the specificity exhibited by Qβ replicase. All of the replicated RNAs must satisfy some minimal criteria required by the enzyme and thus share more common traits than are currently understood. Because the available data set is small and the replicated molecules are of widely varying length, subtle sequence or structure similarities that are absolutely required by the polymerase would be very difficult to identify.

To provide a more consistent set of sequences for comparative analysis, a serial replication/dilution procedure was used to select efficiently replicated molecules from a population of RNAs of identical length but random sequence. The selected RNAs were cloned and sequenced, providing a data set adequately similar in size and replication efficiency to analyze in an attempt to better understand the minimal RNA elements required for replication by Qβ replicase. Characterization of several of the selected RNAs provides insights into the molecular mechanism that results in the replication of specific RNA molecules by the phage polymerase.

MATERIALS AND METHODS

Qβ Replicase and Synthetic Oligonucleotides. The Qβ replicase used in these experiments was a generous gift from Gene-Trak, Inc. The protein preparation has been characterized in a previous report (Moody *et al.*, 1995). Synthetic deoxyoligonucleotides were purchased from Operon Technologies, Inc.

Selection of Replicatable Molecules. Although a more detailed description is provided elsewhere (Chen *et al.*, 1996), a brief consideration of the protocols used for selecting and cloning the molecules that were replicated by Qβ replicase is provided. Two RNA libraries, transcribed from synthetic DNA templates by T7 RNA polymerase (Milligan *et al.*, 1987), were used to initiate the selection process. The sequences of the two RNA populations were

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5′ GGG$(N)_{50}$CCC 3′

5′ GGG$(N)_{77}$CCC 3′

where N represents a position in the sequence with an equal likelihood of being any one of the four nucleosides.

In separate experiments, the RNA populations were incubated for 1 h at 37 °C in 50 μL reactions containing 150 nM Qβ replicase, 500 μM NTP's (500 μM ATP, 500 μM CTP, 500 μM GTP, and 500 μM UTP), and replicase buffer [84 mM Tris-HCl (pH 7.4), 12 mM $MgCl_2$]. The replication products were separated by electrophoresis through an 8% acrylamide/7 M urea gel. The correct-sized RNA products of the replication reaction (56 or 83 nt for the two different selections) were acrylamide gel band purified as described (Chen *et al.*, 1996). One percent of the purified RNA was used to initiate a subsequent round of replication. This cycle of replication, purification, and dilution was continued until the overall level of dilution was sufficient to rid the population of all of the molecules that had been in the initial, random population. The result was a final population of RNAs consisting entirely of sequences that had arisen via replication by Qβ replicase. Those sequences that were most efficiently replicated under the conditions of the experiment were the most abundant.

Cloning of the Replicatable Molecules. Cloning of the sequences that had been selected necessitated the addition of homopolymer tails to provide annealing sites for PCR (Chen *et al.*, 1996). Poly(A) tails were added to the 3′ ends of the selected RNAs using poly(A) polymerase. The modified RNAs were reverse transcribed by SuperScript II (purchased from Gibco BRL) using a primer with the sequence 5′ d[GGGATCC$(T)_{20}$GG] 3′. The resulting cDNA was gel band purified as the RNA was above. A poly(dA) tail was then added to the 3′ ends of the cDNAs by terminal deoxynucleotidyl transferase. The modified cDNA population was PCR amplified using the same primer as used in the cDNA synthesis step above, and the resulting DNA was purified. The DNA copies of the selected RNAs were ligated into the *Bam*HI site of pUC18, the plasmids were used to transform electroporation competent DH5α, and the resultant clones were sequenced by standard methods (Maniatis *et al.*, 1982; Schneider *et al.*, 1992).

Production of RNAs for Analysis. RNAs used for characterization were transcribed from synthetic DNA templates using T7 RNA polymerase as described (Milligan *et al.*, 1987). The *Nco*I truncated RNAs [77#1(+)z and 77#1(−)z] were transcribed from DNA templates that were produced by restricting the gel purified PCR products of 77#1(+)y and 77#1(−)x overnight at 37 °C with 20 units of *Nco*I.

Structure Probing. The single-strand-specific base-modifying agents 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT), dimethylsulfate (DMS), and kethoxal were used to assess the structures of 50#1(+), 50#2(+), 77#1(+), and 77#1(−) (Stern et al., 1988). Separate tubes containing 10 pmol of one of the RNAs were incubated at 45 °C for 5 min in 50 μL of Qβ replicase buffer. The samples were cooled to 25 °C, and CMCT to a final concentration of 15 μg/μL, DMS to a final concentration of 4 ng/μL, or kethoxal to a final concentration of 0.8 μg/μL was added to the RNA solutions. The modifying reactions were incubated at 37 °C for 7 min and then cooled to 0 °C. Seventy-five microliters of DMS stop buffer (1 M Tris acetate, pH 7.5, 1.5 M sodium acetate, 0.1 M EDTA, and 1 M 2-mercaptoethanol) was added to the DMS samples. The kethoxal reactions were stopped with the addition of potassium borate (pH 7.5) to a final concentration of 20 mM. Two hundred microliters of ethanol was added to each of the samples. Twenty minutes of incubation on ice was used to precipitate the RNA. The precipitate was pelleted by centrifugation. The lyophilized samples were dissolved in 50 μL of water, and 0.4 pmol of each RNA was reverse transcribed using a ^{32}P-labeled primer that was complementary to the 10 3′-most bases of each RNA. The reaction products were separated by electrophoresis through a 10% acrylamide/7 M urea gel and visualized by autoradiography.

Kinetics. The replication rate data for 50#1(+), 50#2(+), 77#1(+), and 77#1(−) were generated by incubating approximately 100 of each of the RNA molecules with 100 nM Qβ replicase, 500 μM NTPs, and 0.5 μM [α-^{32}P]GTP in 60 μL of replicase buffer at 37 °C. Five microliter aliquots at various time intervals were removed, mixed with 5 μL of formamide, and heated to 90 °C for 5 min. The samples were cooled to −20 °C. After the last aliquot had been removed and heated to 90 °C, the replication products were separated by electrophoresis on an 8% acrylamide/7 M urea gel. The RNA was visualized by autoradiography, RNAs of the appropriate length were removed, and the total RNA was quantitated by scintillation counting.

Binding. Binding curves of Qβ replicase for the various RNAs in the absence of nucleotides were generated by filter binding assays using nitrocellulose filters (Carey *et al.*, 1983). Twenty microliter binding reactions containing replicase buffer, 100 pM γ-^{32}P-labeled RNA, and varying replicase concentrations were incubated at 37 °C for 5 min. The binding reactions were vacuumed through nitrocellulose filters (Millipore 25-mm HAWP) that had been prewet with replicase buffer. Five milliliters of wash buffer (replicase buffer + 200 mM NaCl to reduce background RNA binding to the filters) was used to wash the nitrocellulose filters of any non-protein-bound RNAs that remained. The filters were dried under an infrared lamp for 10 min. The bound RNA was quantitated by scintillation counting.

Inhibition. 10 nM Qβ replicase was incubated for 5 min at 25 °C in 10 μL reactions containing replicase buffer and varying concentrations (600−6 nM) of 77#1(+)z, 77#1(−)z, 77#1(+)x, or 77#1(−)y RNAs. To each of the above reactions were added 10 μL of replicase buffer containing 10 pM full-length RNA [77#1(+) or 77#1(−)], 500 μM NTPs, and 0.5 μM [α-^{32}P]GTP. The replication reactions were incubated at 37 °C for 15 min. Ten microliters of formamide was added, and each reaction was incubated at 90 °C for 5 min. The replication products were separated by electrophoresis through an 8% acrylamide/7 M urea gel. The radioactive products were visualized by autoradiography.

Competition. γ-^{32}P-Labeled 77#1(+) or 77#1(−) (40 nM) was incubated at 25 °C for 3 min with varying concentrations of nonlabeled RNAs in 10 μL of replicase buffer. To each reaction was added 10 μL of replicase buffer containing 20 nM Qβ replicase. The binding reactions were incubated at 37 °C for 10 min. The replicase/RNA complexes were separated from the nonbound RNAs by nitrocellulose filter binding and washing as above. The filters were dried under an infrared lamp, and the bound radiolabeled RNA was quantitated by scintillation counting.

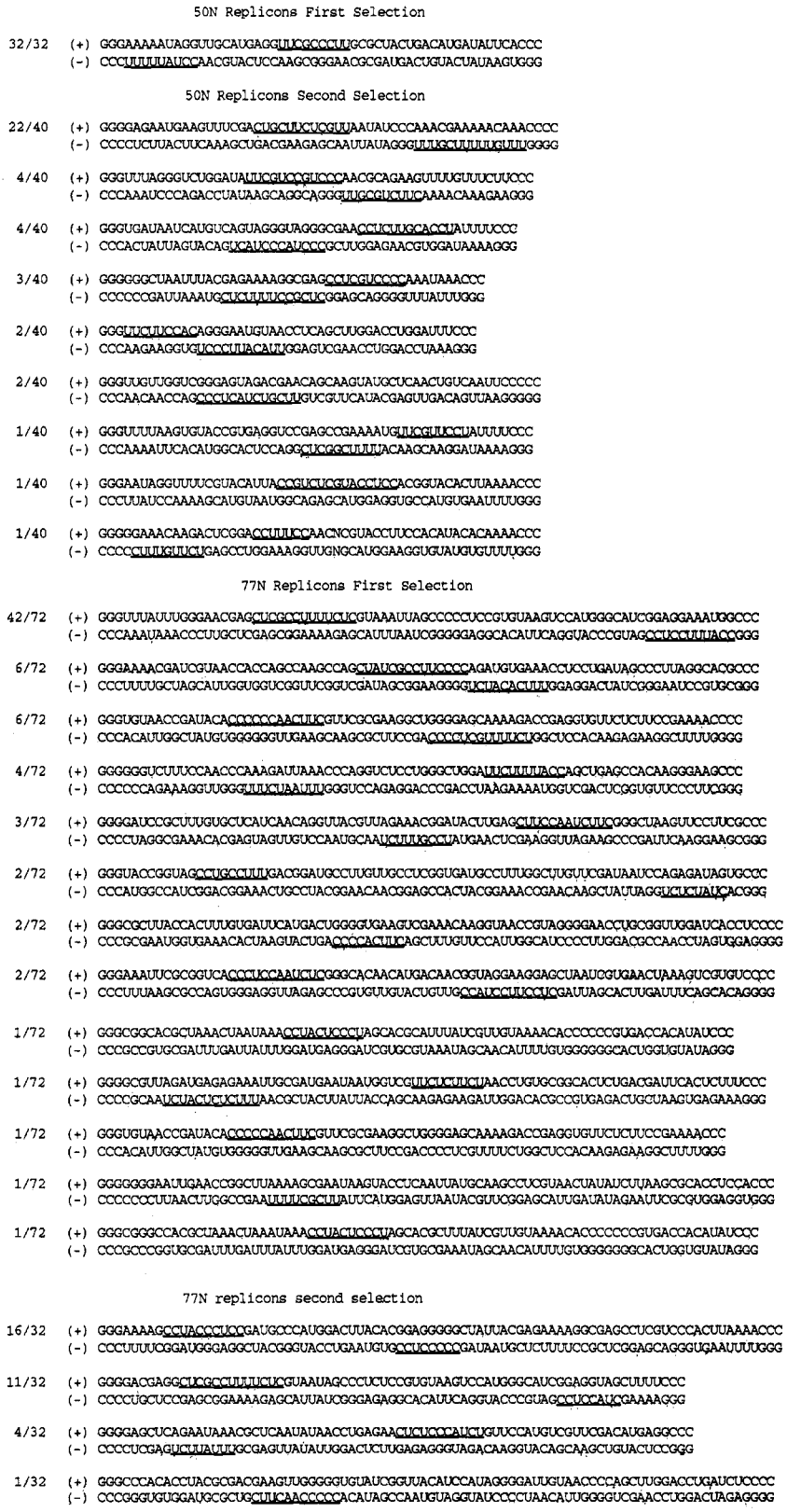

FIGURE 1: Four individual selections were performed, two using the 50N population and two using the 77N population. With the exception of the first 50N selection, each of the experiments generated multiple sequences. The complementary strands for each molecule that was sequenced is displayed because both strands must be active templates for the sequences to withstand the selection. The (+) and (−) to the left of each sequence is merely a notation used to identify the two strands. The (+) strands are listed in the 5′ to 3′ direction while the (−) strands are shown in the 3′ to 5′ direction. The column to the left of each replicatable pair indicates the fraction of sequenced clones that possessed one of the two complementary strands. The C/U-rich regions observed in the comparative analysis are underlined.

RESULTS

Selection of RNAs Replicated by Qβ Replicase. The set of published sequences of RNAs replicated by Qβ replicase has proven to be insufficient for use in comparative analysis to determine the minimal RNA elements necessary for replication. We chose to improve the data set by selecting

replicatable molecules from populations consisting of RNAs with identical lengths but degenerate sequences. Incubation of 10^{13} RNA molecules representing approximately 10^{12} unique sequences with Qβ replicase and NTPs provided amplification of those sequences that were substrates for the replicase. A fraction of the resultant RNA population was then used in a second replication reaction. Repeated cycles of dilution and replication ultimately resulted in an RNA population consisting entirely of RNAs that were efficiently replicated by Qβ replicase. Two degenerate sequence RNA populations, varying in the size of their randomized region (see Materials and Methods), were used to initiate multiple selection experiments. The RNAs from the final populations were cloned and sequenced.

Analysis of the predicted secondary structures (Zuker, 1989) of the molecules from four different selections revealed several shared characteristics (Figure 1). With few exceptions, each of the sequences had the potential to involve the majority of its bases in intramolecular base-pairing. Of particular interest was the prevalence of UUCG tetraloops, a structure that has been found to stabilize hairpins (Tuerk et al., 1988). The second structural motif that was commonly shared was a relatively stable hairpin loop structure near the 5′ terminus of each molecule. Once again, tetraloops appeared to play a major role in stabilizing this structure in a great number of the RNAs, especially those in the 50N selections.

Comparison of the optimal and suboptimal structures predicted for each of the RNAs revealed no commonly shared structural elements except for the UUCG tetraloops. A search for primary sequence homologies between the RNAs likewise yielded nothing that was statistically significant. However, there was a primary sequence motif that was highly conserved. Forty-six out of the 54 RNA molecules, including all but one of the sequences that appeared in more than one clone, possessed an 8−15 nucleotide segment where at least 80% of the positions were pyrimidines (Figure 1). At least one of the complementary strands of all of the selected molecules had this motif. The latter observation is important, because it is possible that some of the molecules could have been selected on the basis of the superior replication of a single strand coupled to a lesser rate for the complementary strand. The polypyrimidine tract was not the same distance from the 3′ end for each molecule, but it did tend to be in a centralized location. The secondary structures predicted around each of these regions were highly variable, though, in every case, at least some part of the polypyrimidine was unpaired.

Structure Probing. Four of the sequences, each of which had dominated their respective selections, were chosen for characterization: the plus strands of the most frequently cloned molecules from the two 50N selections and the two complementary strands of the most prevalent RNA sequenced from the first 77N selection. The single-strand-specific base-modifying reagents DMS, CMCT, and kethoxal were used to examine the secondary structures of the four RNAs. Figure 2 provides a summary of the relative sensitivities of the bases to each of the reagents overlaid on the secondary structures for each of the molecules that best supported the data. The only major deviation between the computer prediction and the base-sensitivity experiments occurred with 77#1(+), though its structure was predicted as a suboptimal folding of the RNA. As predicted by the structural analysis

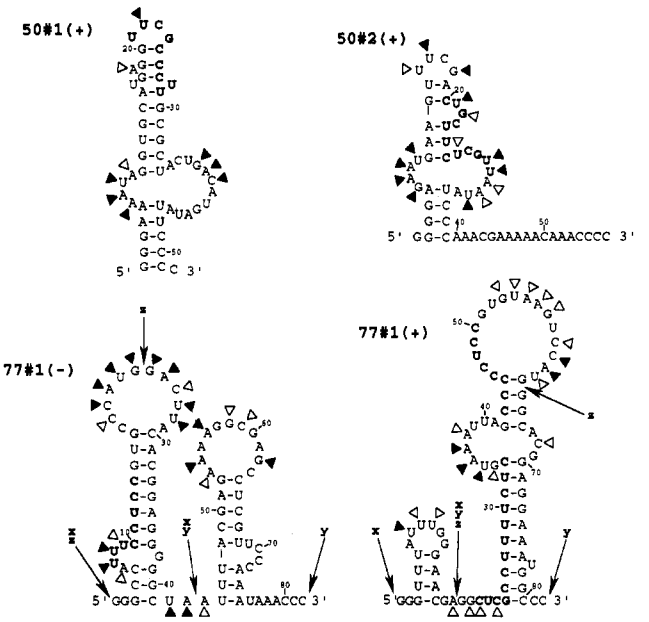

FIGURE 2: Secondary structures of four of the replicatable sequences. The structures are the most amenable to the chemical modification data. Closed triangles indicate bases that were very sensitive to CMCT, DMS, or kethoxal; open triangles indicate bases that were merely sensitive to the base-modifying reagents. The large arrows on 77#1(+) and 77#1(−) indicate positions that ended and initiated the truncated RNAs used in the deletion analysis shown in Figure 4.

performed on the RNAs above, the lone unifying structural feature among the RNAs was a hairpin loop structure near the 5′ ends of the molecules. Also predicted by the folding program was the fact that the majority of the bases in each of the molecules were involved in intramolecular Watson−Crick interactions. As indicated in the introduction, both of these features have been observed by previous researchers.

Replication of the Selected RNAs. The same four molecules were tested for their ability to be replicated by Qβ replicase. For each sequence, approximately 100 RNA molecules were used to initiate a 60 μL replication reaction. [α-^{32}P]GTP was added to the reactions, providing a way of quantitating the product RNA. Aliquots were taken at various time points, and the replication products were separated on a denaturing 8% polyacrylamide gel. Bands representing RNAs of the appropriate size were removed and analyzed by scintillation counting. The observation that each of the sequences yielded measurable product from 100 input molecules (a feat that requires a 10^9-fold amplification) in under 10 min was ample proof that the RNAs were being replicated (Figure 3). In addition, each of the RNAs exhibited the phases common among replicated sequences: an extended period where no product was observed as the limited RNA population was exponentially amplified at an RNA quantity that was too small to detect, a rapid appearance of product as the RNA quantities exceeded the detection limit, and a plateau of product synthesis as the RNA concentration became great enough to slow replication through duplex formation between the complementary RNA products of replication (Biebricher, 1987). The lengths of time required to observe the products of replication for the four selected RNAs were very similar to published results of previously characterized replicatable molecules (Table 1).

Binding to Qβ Replicase. The prevailing view in the literature is that high affinity binding to Qβ replicase is an

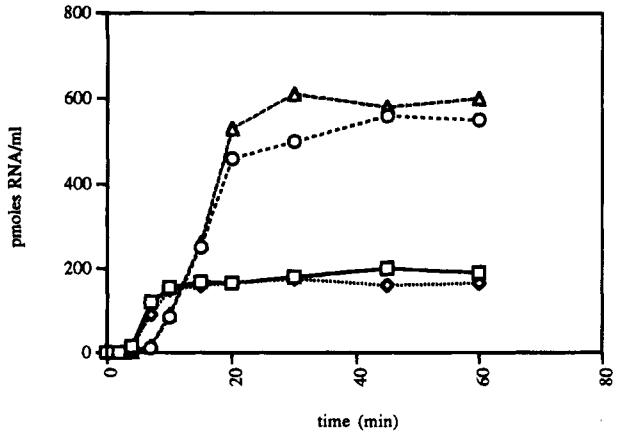

FIGURE 3: Replication profiles of the four RNAs chosen for characterization. One hundred molecules were used to initiate 60 μL replication reactions. The symbols plot RNA product synthesis over time. Squares are for 50#1, diamonds for 50#2, circles for 77#1(+), and triangles for 77#1(−).

Table 1: Comparison of Kinetic Data[a]

	response time (min)
MDV-1(−)	6
MAR 57	22
MAR 72	6.5
50#1(+)	6
50#2(+)	6
80#1(+)	11
80#1(−)	11

[a] The response time is the time required to detect product (approximately 10 pmol of RNA) when the replication reaction is initiated by approximately 100 RNA molecules.

important factor in determining which RNAs will be replicated (Werner, 1991). Because the RNAs identified in this study were isolated from random populations, it was possible to determine if there was a correlation between replication efficiency and binding affinity. Filter binding assays (Carey *et al.*, 1983) were done to determine the equilibrium dissociation constants (K_d's) in the absence of NTPs between Qβ replicase and 50#1(+), 50#2(+), 77#1(+), and 77#1(−) as well as the 50N and 77N degenerate sequence RNA populations. The four molecules that were isolated based on their replication efficiency had affinities

Table 2: Dissociation Constants for Replicatable RNAs and the Random Populations from Which They Were Selected

	K_d (nM)
50N	~200
50#1(+)	30
50#2(+)	30
77N	~200
77#1(+)	20
77#1(−)	20

for Qβ replicase that were approximately 10-fold greater than the populations from which they were selected as witnessed by their lower K_d's (Table 2). These results suggest that the RNAs were selected for replication based, at least partially, on their ability to bind the phage enzyme.

Deletion Analysis. A deletion analysis was used to localize the site of interaction between the polymerase and two of the replicatable RNAs [77#1(+) and 77#1(−)]. Initial truncated forms of the two RNAs were designed to represent structural domains predicted by the structure probing experiments. Binding curves of the first truncated forms of each RNA narrowed the binding sites to the 3′ portion of 77#1(+) and the 5′ half of 77#1(−) (Figure 2 provides the truncated RNAs, and Figure 4 gives the binding curves). Further truncation revealed that an internal region of 77#1(+) [77#1(+)z] and 20 nucleotides at the 5′ end of 77#1(−) [77#1(−)z] could bind Qβ replicase almost as well as the full-length molecules (Figure 4). It was interesting that the regions seemed to be independent of the predicted secondary structures for the molecules and that the primary sequences of the two binding domains possessed the polypyrimidine region observed in the comparative analysis.

Inhibition by the Truncated RNAs. If the truncated RNAs bound the replicase at a site on the enzyme required for template recognition, then the truncated molecules would inhibit replication of the full-length replicatable molecules by obstructing their binding to the replicase. Varying quantities of the truncated RNAs [77#1(+)z and 77#1(−)z] were pre-incubated with the replicase. The truncate bound polymerases were then tested for their ability to replicate full-length 77#1(+) and 77#1(−). Figure 5 provides the inhibition efficiencies of the two binding site mimics as well as two of the truncates identified in the deletion analysis as

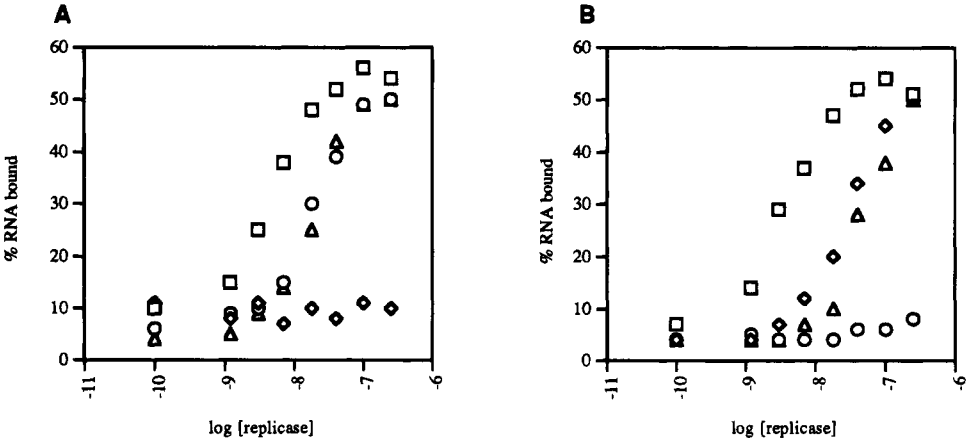

FIGURE 4: Binding curves of RNAs used in the deletion analysis. Various truncated forms of 77#1(+) and 77#1(−) (see Figure 2) were tested for their ability to bind Qβ replicase. The equilibrium binding of 77#1(+) RNA and its derivatives by Qβ replicase are shown in panel **A**; 77#1(−) and its derivatives are shown in panel **B**. Squares represent the binding of the full-length molecules; diamonds, the 5′ truncates, 77#1(+)x and 77#1(−)x (the regions from x to x in Figure 2); circles, the 3′ truncates, 77#1(+)y and 77#1(−)y (y to y in Figure 2); and triangles, the *Nco*I truncates, 77#1(+)z and 77#1(−)z (z to z in Figure 2).

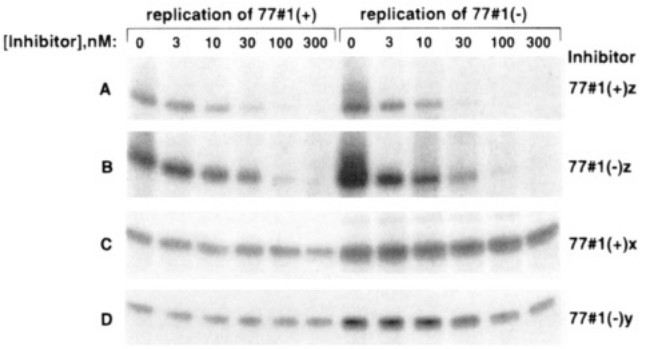

FIGURE 5: Inhibition of replication by 77#1(+)z, 77#1(−)z, 77#1(+)x, and 77#1(−)y. The replication products from a reaction initiated by 77#1(+) are shown in the six lanes on the left half of each gel. The replication products from 77#1(−) are shown on the right half. The identity of each inhibitor is shown in the right-hand column. The inhibitor concentration for each lane is shown above.

being unable to bind the replicase [77#1(+)x and 77#1(−)y]. The binding-site-specific RNAs inhibited the replicase with K_i's approximately equal to their K_d's. This implied that binding of the truncated RNAs blocked a binding site on the replicase that was required for replication. In contrast, the nonbinding truncates, 77#1(+)x and 77#1(−)y, provided no inhibition of the replicase, illustrating that the inhibition was specific and not merely due to nonspecific RNA−protein or RNA−RNA interactions.

Equilibrium Binding Competition. The idea that the truncated RNAs were inhibiting the replication of the full-length molecules by blocking a binding site on Qβ replicase was supported by binding competition experiments. These experiments relied on nitrocellulose filter binding to separate protein-bound RNA from free RNA. Qβ replicase binding competitions between radiolabeled 77#1(+) or 77#1(−) and varying quantities of cold RNA (both truncated and full-length molecules) in the absence of NTPs were performed. The two truncated binding RNAs, 77#1(+)z and 77#1(−)z, competed for binding with each of the full-length molecules as well as the full-length 77#1(+) did (Figure 6). As expected, the nonbinding truncates did not affect the binding of the full-length molecules to the polymerase. These data support the hypothesis that the truncated RNAs inhibited replication by blocking the template binding site of the replicase.

Mutational Analysis of 77#1(−). The primary conserved element between the replicated molecules was the presence of a C/U-rich domain. To test if this RNA element was essential for replication, a mutant of 77#1(−) was designed that disrupted the pyrimidine tract without altering the predicted secondary structure of the molecule (Figure 7A). The affinity of the mutant RNA for Qβ replicase was 10-fold less than that of 77#1(−) (Figure 7B). Separate replication reactions were initiated by 100 molecules of 77#1(+), 77#1(−), and the mutated 77#1(−) [77#1(−)mut]. Both 77#1(+) and 77#1(−) provided product in 15 min, but 77#1(−)mut yielded no product even after 1 h (Figure 7C). These results provide a link between the polypyrimidine tracts of the replicatable molecules, binding by Qβ replicase, and replication efficiency.

DISCUSSION

The specific RNA requirements for replication by Qβ replicase have long been a mystery. We performed a series of selections designed to generate a set of efficiently replicated sequences. Careful examination of the replicated RNAs indicated that the structural elements previously identified, namely, a high degree of secondary structure and a hairpin loop near the 5′ termini of the RNAs, were generally upheld. Many of the predicted hairpins possessed UUCG tetraloops, a motif that has been shown to significantly enhance the stability of hairpins. Because heteroduplex RNA is inactive as a template for Qβ replicase, tetraloops probably represent an efficient means for stabilizing the intramolecular structures necessary to inhibit duplex formation during product strand synthesis (Axelrod *et al.*, 1991).

The more interesting conserved element was observed at the primary sequence level. A polypyrimidine-rich region of at least eight nucleotides was present in 85% of the sequenced clones and in at least one of the two complementary strands for every one of the replicatable pairs. Truncated versions that included the polypyrimidine motifs of two of the replicatable RNAs could bind to Qβ replicase almost as well as the full-length molecules. The truncated RNAs could

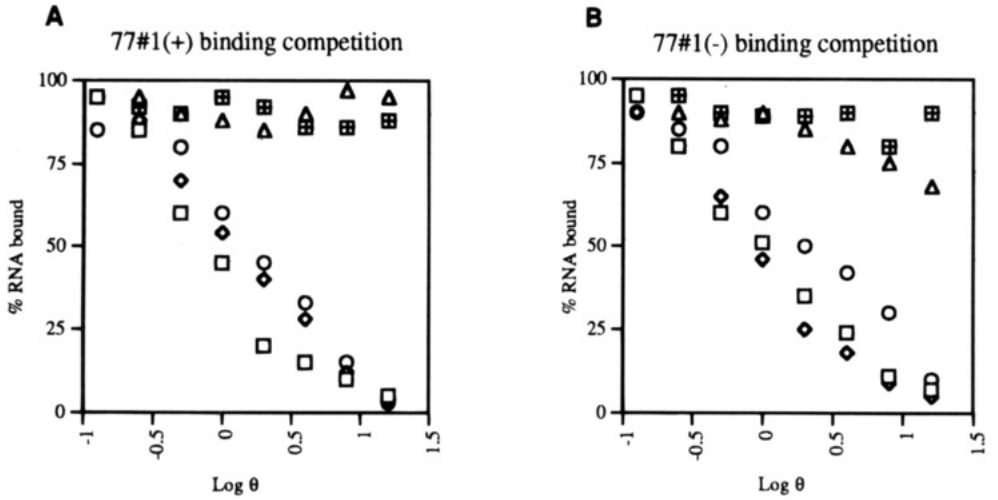

FIGURE 6: Equilibrium binding competition experiments. Panel **A** plots the binding of radio-labeled 77#1(+) in the presence of other unlabeled RNAs. Panel **B** is a graph showing the binding of radiolabeled 77#1(−) in the presence of the same RNAs. Theta is the ratio of cold competitor RNA to labeled RNA. Open squares show competition with 77#1(+); diamonds, 77#1(+)z; circles, 77#1(−)z; triangles, 77#1(+)x; and crossed squares, 77#1(−)y.

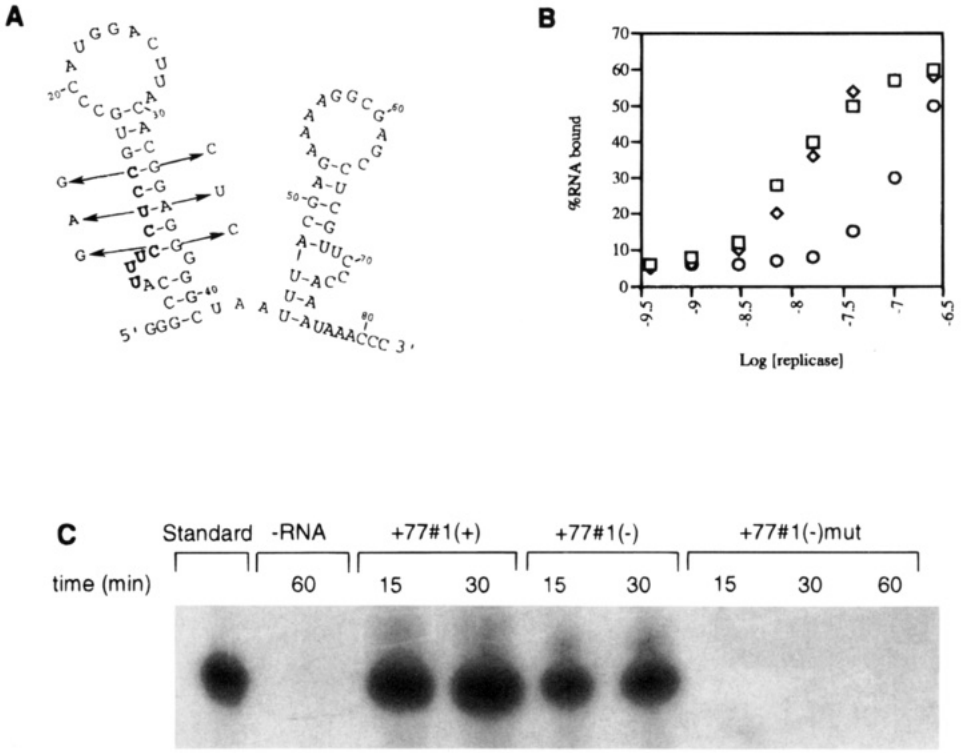

FIGURE 7: Mutational analysis of 77#1(−). Panel **A** shows the predicted structure of 77#1(−). Lines indicate nucleotides that were mutated to yield 77#1(−)mut. The predicted secondary structural stabilities for the two molecules were virtually the same. Panel **B** displays the binding curves of 77#1(+) (squares), 77#1(−) (diamonds), and 77#1(−)mut (circles). Panel **C** is an acrylamide gel displaying the replication products of 77#1(+), 77#1(−), and 77#1(−)mut at replication time points indicated above the gel.

inhibit replication, apparently by blocking template binding by the replicase. Mutation of the C/U-rich region of one of the replicatable RNAs reduced its binding affinity to Qβ replicase and abolished its capacity to be replicated. These data, coupled to the finding that Qβ replicase can bind C/U-rich motifs with high affinities (Brown & Gold, 1995), suggest that recognition of replicatable sequences is accomplished by the binding of polypyrimidines.

For these small replicatable sequences, there appears to be two very different pressures for replication. The first is that the molecules not form duplexes with the complementary strands that result from replication. This pressure apparently led to a predominance of intramolecular base-pairing with a relatively high degree of UUCG tetraloops to further stabilize the internal structures. A hairpin loop structure near the 5′ end was highly conserved among the selected RNAs. This structure has been observed in the past and is hypothesized to be a mechanism for generating single-strandedness immediately after initiation of second strand synthesis (Zamora *et al.*, 1995). Along with the selection pressure of maintaining single strands is the requirement that a site be present for binding to Qβ replicase. This requirement was apparently met by pyrimidine-rich sequences. Such a region is also present in both the plus and minus strands of all of the efficiently replicated, small RNAs that have been published as well as the minus strand of the Qβ genome (Brown & Gold, 1995). That this binding region was often involved in intramolecular base-pairing requires a mechanism for breaking the hydrogen bonds and allowing for base-specific binding by the replicase. Ribosomal protein S1, a subunit of the replicase that has been shown to have the ability to disrupt RNA secondary structure (Bear *et al.*, 1976), could be responsible for unfolding the replicatable molecules and allowing for the interaction between the replicase and the C/U rich regions.

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