Evolution of Host Cell RNA into Efficient Template RNA by Qβ Replicase: The Origin of RNA in Untemplated Reactions

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ABSTRACT: Qβ replicase can replicate a single molecule of certain species of RNA to 10^{14} copies in minutes. This replication ability has been used for *in vitro* studies of molecular evolution and is currently being utilized as a method of amplifying RNAs that contain probe sequences. It has been observed that Qβ replicase can produce replicatable RNA even in the absence of exogenously added template RNA. The origin of this RNA has been ascribed either to contamination with replicatable RNA or to an ability of Qβ replicase to synthesize RNA *de novo* from the nucleotides present in the reaction. Technologies that employ Qβ replicase require a thorough understanding of the conditions that lead to this so-called spontaneous RNA production. We have created an expression system and purification method with which we produce gram quantities of highly purified Qβ replicase, and we have identified reaction conditions that prevent the amplification of RNA in assays that do not contain added RNA. However, when these reaction conditions are relaxed, spontaneous RNA replication is seen in up to 100% of the assays. To understand the origin of this RNA, we have cloned several spontaneously produced RNAs. Sequence analysis of one of these RNAs shows that it arose by the evolution of *Escherichia coli* tRNA into a replicatable template and not by *de novo* synthesis from nucleoside triphosphates in the reaction.

Qβ replicase is the RNA-dependent RNA polymerase that replicates Qβ bacteriophage RNA (Haruna & Spiegelman, 1965a). This enzyme is a heterotetramer composed of a phage-encoded subunit and three host-encoded proteins: S1 ribosomal protein, EF-TU, and EF-TS. The replication of Qβ phage RNA also requires host factor, an additional *Escherichia coli* protein that is not needed for the replication of other RNAs by this enzyme [reviewed in Blumenthal and Carmichael (1979)]. Because both the plus-strand and the minus-strand of Qβ bacteriophage RNA serve as templates for RNA synthesis, the enzyme is able to exponentially amplify the phage RNA (Haruna & Spiegelman, 1965c).

Although the activity of Qβ replicase is template specific (Haruna & Spiegelman, 1965a−c; Miyake et al., 1971; Blumenthal, 1980), the enzyme is capable of replicating a limited number of small RNA molecules of between 25 and 300 nucleotides in length (Banerjee et al., 1965; Kacian et al., 1972; Mills et al., 1975; Schaffner et al., 1977; Biebricher & Luce, 1993). Many of these replicating RNAs were originally isolated from *in vitro* Qβ replicase reactions performed without exogenously added RNA, and their origin continues to be an area of active interest and debate.

Some workers have argued that their Qβ replicase preparations were free from contaminating template RNA and that the origin of the replicatable RNA was *de novo* synthesis by the random polymerization of nucleotides followed by selection of the faster replicating RNA species (Sumper & Luce, 1975; Biebricher, et al., 1986; Biebricher & Luce, 1993). Hill and Blumenthal (1983) claimed that spontaneous RNA replication was template instructed and not the result of *de novo* synthesis. They removed the S1 subunit and contaminating RNA from Qβ replicase by phosphocellulose chromatography in the presence of urea and found that this Qβ replicase preparation was not capable of spontaneous RNA replication, unless the RNA contaminant was returned to the enzyme. This hypothesis is supported by the observation that some species of replicatable RNA appear to be related to *E. coli* RNA (Munishkin et al., 1981, 1991). Biebricher and co-workers (1986) prepared Qβ replicase using the methods of Hill and Blumenthal and found that, in their hands, spontaneous RNA replication did occur. They reported that this RNA replication was not due to contaminating template RNA but was due to *de novo* synthesis, facilitated by their use of greater concentrations of enzyme and nucleotides than those employed by Hill and Blumenthal.

Spontaneous generation of replicatable RNAs by Qβ replicase would interfere with the sensitivity and utility of assays that employ this enzyme. We therefore sought means of eliminating or, at least, delaying the appearance of such replication products so that they could be distinguished from the products of exogenously added detector probe/template molecules. A number of observations deriving from these efforts bear on the question of the origin of spontaneous replication products. Here we report on a new purification

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method for the production of gram quantities of highly purified Qβ replicase. The enzyme is produced from a tightly regulated expression plasmid encoding only the 65 kDa Qβ replicase subunit; thus, no phage or phage RNA is introduced into the system, except for the RNA encoding the replicase message. Expression is induced late in the growth cycle from seed stocks of transformed *E. coli* that are themselves not passaged, thus minimizing the time available for the replicase holoenzyme to evolve replicatable RNA *in vivo*. Finally, the enzyme is purified under rigorously aseptic conditions, eliminating contamination with exogenous replicatable RNAs from the laboratory environment.

We have developed a number of modifications to standard reaction conditions that suppress or significantly delay the appearance of spontaneous replication products by hours or days, while permitting the replication and detection of single molecules of exogenously added template molecules in 15 min or less. These assay conditions include elevating the Mg^{2+} concentration and lowering the Qβ replicase concentration.

Nevertheless, under relaxed reaction conditions, or during very long incubations, our enzyme preparation is still capable of spontaneously generating a variety of replicatable RNAs. In an effort to understand the origin of these RNAs, we have developed a method of cloning the products of such reactions without *a priori* knowledge of their sequence. Analysis of a number of such spontaneous replication products shows that they are related to *E. coli* nucleic acids.

These observations lead us to conclude that the spontaneous replication products derive from low levels of *E. coli* nucleic acids that contaminate the Qβ replicase preparation. These poorly replicating RNAs then evolve into replicatable species under relaxed conditions or long incubations of untemplated reactions. There appears to be no necessity to invoke the more exotic hypothesis that any amplifiable RNAs arise from the random untemplated polymerization of nucleotides in the reaction.

MATERIALS AND METHODS

Materials. The plasmid pDMQβ6BP, which includes the Qβ bacteriophage region encoding the 65 kDa subunit of Qβ replicase, was the generous gift of Dr. Don Mills (Dept. of Microbiology and Immunology, SUNY, Brooklyn, NY). The pPL-λ expression vector, containing the strong, tightly regulated leftward promoter of bacteriophage λ, was purchased from Pharmacia (Piscataway, NJ). Recombinant molecules using the pPL-λ vector were grown in the *E. coli* strain (λ lysogen) N99cI$^+$, which was supplied by Pharmacia. Oligonucleotides were synthesized on an Advanced Biosystems Inc. synthesizer and purified by polyacrylamide gel electrophoresis. Polymerase chain reactions (PCR) were performed on a Perkin-Elmer-Cetus DNA thermal cycler. Poly(cytidylic acid) (Poly C), SDS−PAGE phastgels, and fast flow Q-Sepharose and S-Sepharose chromatography resins were purchased from Pharmacia.

Cloning of the 65 kDa Subunit of Qβ Replicase in pPL-λ. The polymerase chain reaction (Mullis et al., 1986) was used to amplify DNA encoding the 65 000 MW subunit of Qβ replicase so that it could be cloned into the pPL-λ expression plasmid. Synthetic oligonucleotides with the sequences 5′-CTAGATAAGTAACACAGGAAACAGAATGTCTAAGACAGCATCTTCG and 5′-CGAACCCTCCCAGGTTACGCCTCG were used as 5′ and 3′ primers, respectively, for PCR amplification of the replicase subunit. One microgram of the purified PCR product was treated with 5 units of Klenow fragment of *E. coli* DNA polymerase I in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 μg/mL bovine serum albumin (BSA), and 500 μM dNTPs at 37 °C for 30 min in order to create blunt-ended DNA molecules. The reaction products were recovered and suspended at a concentration of about 30 ng/μL. The expression vector pPL-λ was prepared by restriction with *Hpa*I, which cuts the plasmid DNA once (in the N gene of λ phage) and results in blunt ends. The 5′ terminal phosphates were removed with calf intestinal alkaline phosphatase (CIAP). The prepared pPL-λ DNA vector was ligated with the prepared PCR product and then used to transform freshly prepared N99cI$^+$ competent cells. The transformations then were plated onto Luria broth agar plus 100 μg/mL ampicillin (LB/amp medium). Transformed colonies containing the Qβ replicase gene were identified, and clones containing the gene for the 65 kDa subunit of Qβ replicase were checked for correct orientation by restriction analysis with *Bam*HI and *Eco*RV plus *Pvu*II. Clones containing the gene in the correct orientation then were restricted with *Xba*I and *Bst*BI enzymes. These two restriction sites are created only if the two junctions between insert and vector are formed correctly (Figure 1). Several clones were obtained with the insert in the correct orientation, and one clone, designated pPLQβTac2, was chosen for subsequent analysis to determine the level of active Qβ replicase expression.

Expression and Purification of Qβ Replicase. The N99cI$^+$ *E. coli* host strain containing the pLQβTaq2 expression plasmid was maintained as a 15% glycerol stock in LB/amp medium and stored at −70 °C. Cell paste for Qβ replicase purifications was prepared by inoculating TSBYE medium containing 100 μg/mL ampicillin with fresh colonies from LB/amp plates. Cultures were incubated at 30 °C until they had grown to an A_{600} of 1, and then expression of Qβ replicase was induced with 120 μg/mL naladixic acid. Cells were harvested at 3 h postinduction, and the cell paste was stored frozen (−20 °C) until needed. Qβ replicase was purified from 3 kg of cell paste prepared from a total fermentation volume of 1000 L.

The cell paste was suspended in 6 L of 50 mM Tris-HCl (pH 7.8), 55 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 500 mM NaCl. The cell suspension was placed on ice, and the cells were ruptured by twice passing the suspension through a Manton Gaulin homogenizer at 15 000 psi. Poly(ethylene imine) (PEI) was added slowly to the chilled cell lysate to a final concentration of 0.3%, and the resulting precipitate was removed by centrifugation at 13000*g*. The clarified cell lysate was diluted with 4 vol of a solution containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 1 mM ethylenediaminetetraacetic acid (EDTA) and applied at flow rate 4 L/h to a 4.5 L Q-Sepharose column (previously equilibrated with the cell lysate dilution buffer plus 100 mM NaCl). The column was then washed with 10 column vol of 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 150 mM NaCl until the absorbance (280 nm) of the column effluent was less than 0.3. The enzyme was eluted with the preceding wash solution containing 250

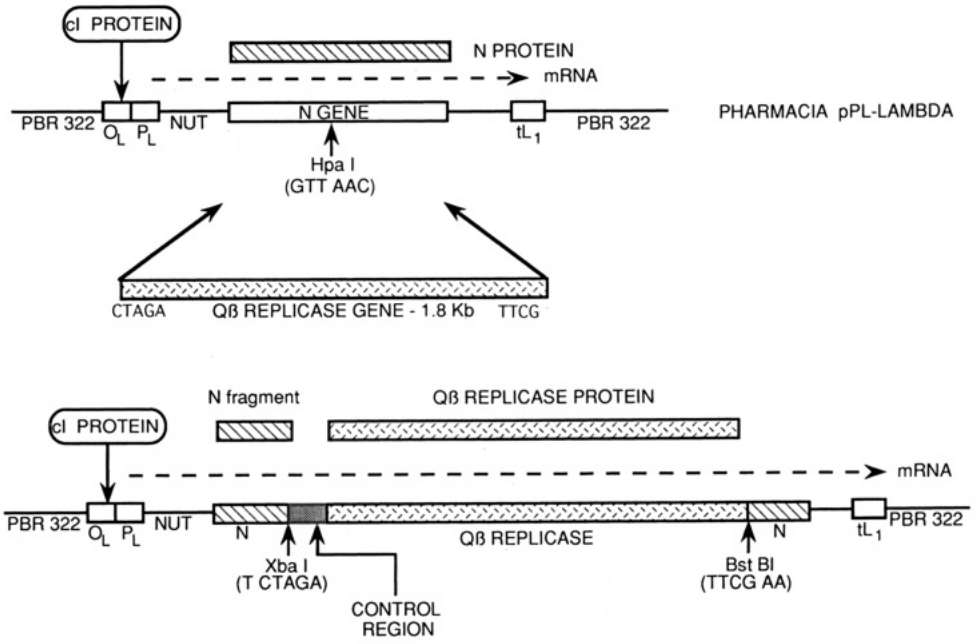

FIGURE 1: Design of the Qβ replicase expression plasmid. A PCR DNA molecule was treated to produce blunt ends and then inserted into the N gene of the pPL-λ plasmid. Transcription is directed from the P_L promoter to produce an mRNA that directs the translation of a small fragment of the λ phage N protein and the Qβ replicase protein.

mM NaCl. Column fractions were assayed for activity by a polyC assay (as described in the next section), and those fractions containing 50% or more of the activity of the peak fraction were pooled for further purification.

The pooled Q-Sepharose fractions were diluted with 1.5 vol of a solution containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, and 1 mM EDTA and applied to a 450 mL bed volume S-Sepharose column previously equilibrated with this solution containing 100 mM NaCl. The column was washed with the equilibration buffer until the absorbance at 280 nm of the column effluent had a value of less than 0.3. The enzyme was eluted using the equilibration buffer containing 250 mM NaCl. Fractions containing 30% or more of the activity found in the peak fraction were pooled. Glycerol was added to 50% (v/v), and the enzyme was stored at −20 °C.

Assays. The activity of Qβ replicase in various crude and purified fractions was determined by measuring the incorporation of [α-^{32}P]GTP into the polyG produced from the poly(cytidylic acid) template (PolyC assay) (Feix & Sano, 1975). PolyC assays on 2.5 ul aliquots of Qβ replicase fractions were conducted in a final volume of 25 μL, which contained 90 mM Tris-HCl (pH 7.8), 14 mM $MgCl_2$, 2 mM GTP, 240 μg of polycytidylic acid, 2.4 ng of rifampicin, and 2 μCi of [α-^{32}P]GTP [rifampicin, which has no effect on Qβ replicase, was included in polyC assays to eliminate interference from host-cell RNA polymerase activity in crude fractions (Kamen, 1972)]. The reaction was started by the addition of the Qβ replicase fraction to the assay reagent mixture, which had been prewarmed to 37 °C. Ten microliter aliquots were removed after a 10 min incubation and spotted onto DE81 filter paper. The filters were washed 4 times with 0.5 M phosphate buffer (pH 7). Five milliliters of water was added to each filter, and the Cherenkov radiation of the incorporated [α-^{32}P]GTP was counted in a scintillation counter.

Protein concentrations were determined by the Bradford method using Bio-Rad protein assay dye reagent and by measuring the absorbance at 225 nm (Stoscheck, 1990). Bovine serum albumin was used as a standard for both methods. Gel analysis of proteins was performed as described by Laemmli using the Pharmacia Phastgel system. RNA produced in Qβ replicase amplifications was analyzed by electrophoresis in 5% polyacrylamide TBE gels containing 7 M urea (Sambrook et al., 1989).

Qβ Replicase RNA Amplification. The ability of Qβ replicase to amplify template RNA was measured in the following standard 200 μL assay containing each nucleotide (ATP, GTP, CTP, and UTP) at a concentration of 0.6 mM, 5 μg of Qβ replicase, and propidium iodide (at 1 μg/mL) in a buffer composed of 116 mM Tris-HCl (pH 7.5), 21.5 mM $MgCl_2$, 18.8 mM NaCl, 0.25% NP-40, and 12.5% glycerol. Initiation of 96 multiple assays was synchronized by keeping all reagents on ice while the reaction components were assembled. Replication was initiated by the rapid warming (less than 1 min) of the reaction mixture to 37 °C. The increase in fluorescence associated with the production of RNA in the presence of propidium iodide was monitored every 40 s in a custom-built temperature-controlled fluorimeter capable of the simultaneous monitoring and analysis of 96 fluorescence assays (L. Burg, unpublished data).

Cloning Products of Qβ Replicase Amplification. Qβ replicase amplification reactions result in the production of two complementary RNA strands. Only the 5′-GG and 3′-CC sequences are conserved on the known replication templates. A method for cloning such molecules was devised by Chetverin and co-workers (Munishkin et al., 1991). We used a modification of this method combined with vectors designed to give cloned molecules in an immediately useful form.

Briefly, the RNA products of Qβ replicase were purified by phenol extraction and ethanol precipitation. PolyA polymerase was then used to add A residues to the 3′ ends of the purified RNA. An oligonucleotide, AAGCTTGAAAT TAATACGACTCACTATAGGGTGCAGTTTTTTTTTTT-TTTTGG, was used to prime mu MLV reverse transcriptase

A.

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AAGCTTGAAA TTAATACGAC TCACTATAGG GTGCAGTTTT TTTTTTTTT GG
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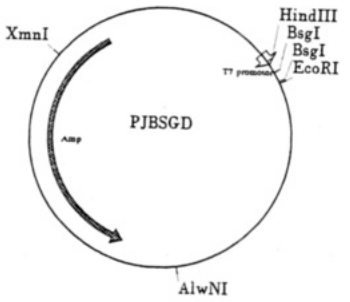
B.



C.

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1   AAGCTTGAAT TAATACGACT CACTATAGGG ATATAACTAG TATCTGCACG GATCCGTCGA
    TTCGAACTTA ATTATGCTGA GTGATATCCC TATATTGATC ATAGACGTGC CTAGGCAGCT
61  CCCGGGACTA CTTGAGTCTG CACGAATTC
    GGGCCCTGAT GAACTCAGAC GTGCTTAAG
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FIGURE 2: Cloning and expression of replicatable RNA. (A) This shows the nucleotide sequence of the primer used for cDNA synthesis. The template was RNA produced by Qβ replicase and subsequently tailed with *E. coli* polyA polymerase (Pharmacia). The two 3′-G residues position the primer at the CCA 3′ end of the RNA. The *Bsg*I recognition sequence is underlined, and the site at which it cuts is marked by a vertical arrow. After cDNA synthesis, RNase H digestion and annealing followed by Klenow treatment to render flush ends, the products were ligated to *Hinc*II-cut pGem 4Z (Promega). The ligated products were introduced into transformation competent *E. coli* DH5-α. White ampicillin resistant colonies were picked from X-Gal-containing LB plates and tested for plasmids containing *Bsg*I excisable fragments. Such plasmid inserts were sequenced, and their *Bsg*I excisable fragments were subcloned into pJBSGD for transcription to produce templates for Qβ replicase. The first nucleotide of the resulting transcripts corresponds to the 5′ terminal nucleotide of the original RNA product of Qβ replicase, and the last nucleotide corresponds to its 3′ terminal C. (B) This shows a map of pJBSGD, the vector used for making plasmid clones for transcription of Qβ replicase replication products. (C) Shown here is the nucleotide sequence inserted at the *Hin*dIII and *Eco*RI sites of pUC8 to generate pJBSGD. The T7 promoter ends at nucleotide 27, and the following G residue encodes the first nucleotide of transcripts inserted by the method described. The two recognition sequences for *Bsg*I are underlined and their corresponding cut-sites are marked with arrows. *Bsg*I cutting of this DNA leaves a vector fragment that terminates with a 3′-GG extension on both strands. The *Sma*I site was used to linearize plasmids prior to transcription.

to generate cDNA to both strands of polyA-tailed RNA. This was followed by heat denaturation and ribonuclease treatment to destroy RNA templates. The resulting cDNA strands were annealed to produce double-stranded DNA with 5′ extensions containing the oligo(dT) priming sequence and the recognition site for the class 2S restriction endonuclease, *Bsg*I (underlined in the preceding sequence). After Klenow treatment with all four dNTPs to complete the double-stranded molecule, the products were cloned in the *Hinc*II site of pGem 4Z (Promega). Transformation of *E. coli* DH5-α and plating on LB agar containing X-Gal was followed by screening individual white colonies for plasmids from which *Bsg*I excised a fragment. The nucleotide sequences of such inserts were determined, and the *Bsg*I fragments were subcloned in both orientations in a plasmid vector, pJBSGD (Figure 2). The plasmid pJBSGD is derived from pUC8 by inserting an oligonucleotide between the *Hin*dIII and *Eco*RI sites. The oligonucleotide, AAGCTTGAAATTAATACGACTCACTATAGGGATATAACTAGTATCTGCACGGATCCGTCGACCCGGGACTACTTGAGTCTGCACGAATTC, contains two *Bsg*I sites oriented so that the product of cutting with this enzyme is a vector fragment that has a two-nucleotide 3′-GG extension at each end. These ends are not compatible for ligation to each other, but are compatible with the ends produced by *Bsg*I cutting of the double-stranded DNA resulting from the cloning procedure described earlier. When such products are cloned in pJBSGD, the resulting plasmid can be cut with *Sma*I and the linearized plasmid transcribed with T7 RNA polymerase, making use of the promoter that is also encoded by the oligonucleotide. Transcripts are identical to the original Qβ replicase products except for the terminal A residue, which is not required for replication. For convenience, versions of these vectors with stuffer fragments inserted between the *Bam*HI and *Xho*I sites were used. All *Bsg*I digestion products were isolated by gel electrophoresis prior to use in ligation reactions.

RESULTS

Cloning of the 65 kDa Subunit of Qβ Replicase in pPL-λ. When Qβ replicase is produced in *E. coli* infected by Qβ phage, the cells also contain Qβ RNA and/or 6S RNA, which can serve as a template for RNA amplification. This potential contamination can be avoided by cloning the gene encoding Qβ replicase into a plasmid lacking other Qβ phage nucleotide sequences. Although the gene encoding the Qβ phage subunit of Qβ replicase previously has been cloned into a plasmid expression system (pDMQβ6BP), we sought to improve the yield of active Qβ replicase by placing the gene under the control of stronger transcription and translation signals. High-level expression of this 65 kDa polypeptide in *E. coli* could possibly cause cell death by recruiting host proteins essential for protein synthesis (ribosomal protein

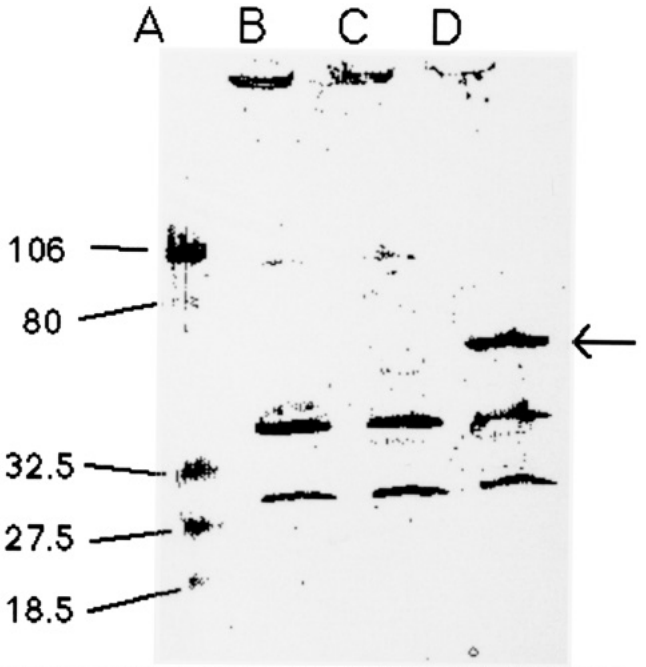

FIGURE 3: Western blot analysis of expression of the Qβ bacteriophage-encoded Qβ replicase subunit in *E. coli* N99cI$^+$ cells. Ten milliliter cultures of N99cI$^+$ cells and N99cI$^+$ cells containing the pLQβTaq2 expression plasmid were grown at 30°C in LB medium (plus ampicillin for cells containing pLQβTaq2) until early log phase (A_{600} = 0.16) and then induced with 100 μg/mL naladixic acid. Cells were pelleted and frozen (−20 °C) immediately prior to induction or after 3 h of incubation. The frozen cell pellets were suspended in Laemmli loading buffer to a density equivalent to A_{600} = 7. The samples were boiled for 1 min, and 1 μL was applied to a 10−15% SDS Phastgel (Pharmacia). After electrophoresis, the proteins in the gel were electroblotted onto nitrocellulose and stained with a 1:10 000 dilution of a rabbit anti-Qβ replicase subunit polyclonal serum. Lane A, prestained molecular weight markers; lane B, uninduced N99cI$^+$ host cells; lane C, uninduced N99cI$^+$ pLQβTaq2 cells; lane D, induced N99cI$^+$ pLQβTaq2 cells. The arrow indicates the position of the Qβ bacteriophage-encoded subunit of Qβ replicase. The image shown is a DeskScan black and white drawing scan of the stained Western blot.

S1, elongation factor TU, and elongation factor TS) and assembling them into Qβ replicase holoenzyme. Therefore, we chose to express the 65 kDa subunit from the tightly regulated pL promoter of bacteriophage λ. This strong promoter is tightly controlled by the cI protein of λ (Shatzman & Rosenberg, 1987). The resulting expression plasmid is designated pPLQβTaq2 (Figure 1).

Expression and Purification of Qβ Replicase. The expression of Qβ replicase by pPLQβ Taq2 was examined by inducing N99cI$^+$ cells containing this plasmid with 50 μg/mL naladixic acid. Western blot analysis shows that Qβ replicase production was tightly regulated by this expression system. Qβ replicase was detected in the induced cells, but was not detected in uninduced cells (Figure 3). When known amounts of purified Qβ replicase were included in the Western blots, it was possible to determine that the concentration of Qβ replicase in induced cells was approximately 10 mg/L of cell culture (data not shown). Cells containing the Qβ replicase were lysed, and the enzyme was purified by a simple three-step method of PEI precipitation, followed by Q-Sepharose and S-Sepharose ion-exchange chromatography. The yield of Qβ replicase from this method was 74% (Table 1) and it was 85% pure (as judged by densitometry of Coomassie-stained SDS−PAGE gels).

Table 1: Purification of Qβ Replicase from *E. coli* N99cI$^+$ pPLQβTaq2 Cells

fraction	protein (mg/mL)	specific activity[a]	purification (-fold)	recovery (%)
PEI supernatant	24.7	6	1	100
Q-Sepharose	5.9	33	6	99
S-Sepharose	0.7	434	74	38

[a] Specific activity was determined as the nanomoles of GTP incorporated into poly(cytidylic acid) per minute per milligram of protein.

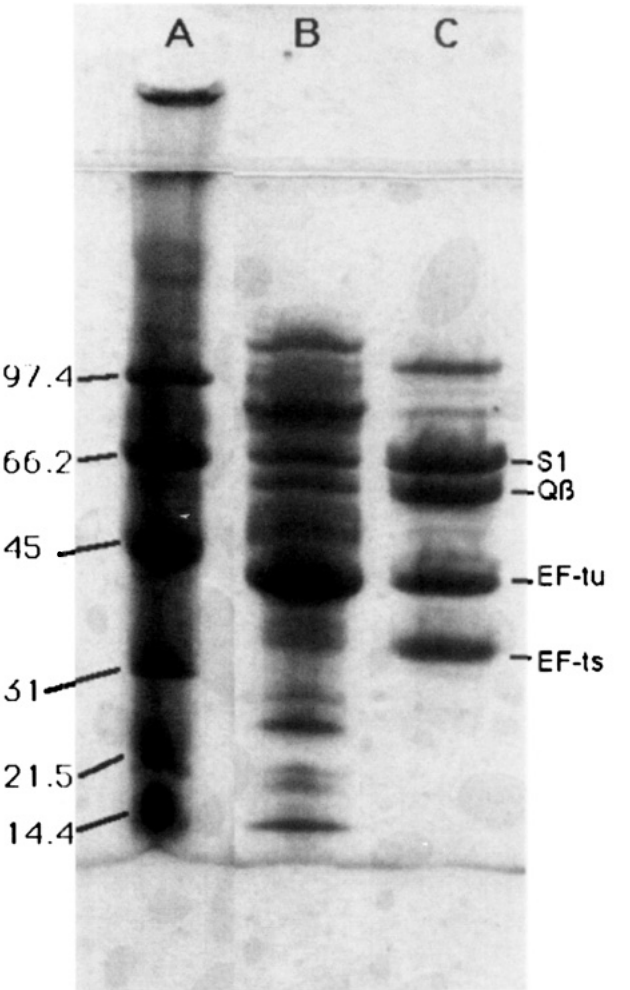

FIGURE 4: Purification of Qβ replicase. Coomassie-stained 10−15% SDS Phastgel (Pharmacia) containing fractions from the Qβ replicase purification. Samples were prepared by mixing 20 μL of the fraction with 5 μL of 5× Laemmli loading buffer and boiling for 1 min. Lane A, molecular weight markers; lane B, Q-Sepharose-pooled fractions; lane C, S-Sepharose-pooled fractions. The image shown is a DeskScan black and white photo scan of the stained gel.

To determine whether the presence of the low levels of contaminating proteins found in standard preparations (Figure 4) would alter the behavior of Qβ replicase in the assay, enzyme preparations that were free from detectable levels of these protein contaminants were prepared by changing the NaCl concentrations employed in Q-Sepharose and S-Sepharose chromatography. Examination of these special Qβ replicase preparations did not reveal any parameters that could differentiate them from the standard preparation (M. D. Moody, unpublished data).

Metal Content of the Qβ Replicase Preparation. Because metal ions (Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}) can reduce the

fidelity of DNA polymerases (Loeb & Kunkel, 1982) and Mn^{2+} can influence the specificity of Qβ replicase (Haruna & Spiegelman, 1965; Palmenberg & Kaesberg, 1974), the concentrations of these metals, as well as others (Mg^{2+}, Cu^{2+}, Cd^{2+}, Fe^{2+}, and Pb^{2+}), were determined in the pooled S-Sepharose fractions. Analysis by inductively coupled plasma spectroscopy analysis (Chemical Analysis Laboratory, University of Georgia, Athens, GA) showed that Mg^{2+} was present at approximately 12 mM, while the concentration of the other metals was below the micromolar limits of detection.

Qβ Replicase Replication of an MDV-1-Based Detector Probe RNA. A detector probe molecule was constructed by inserting, into MDV-1, a 35-base sequence complementary to a *Chlamydia trachomatis* rRNA. This probe RNA, named C29 RNA, served as a model template for the Qβ RNA synthesis experiments to be described here (L. Burg, unpublished data). The replication of C29 was measured using a fluorimeter that monitors the real time fluorescence of 96 assays. Fluorescent measurements showed a relatively flat baseline followed by increasing fluorescence produced by the interaction of propidium iodide (PI) and newly made RNA. The time in minutes at which the fluorescence from the interaction of PI with the newly made RNA intersected with the baseline was referred to as the response time. Figure 5A shows the results of C29 amplifications using eight different levels of template RNA. There was a linear relationship between the log of the starting number of RNA molecules and the response time (Figure 5B). The rate of change in fluorescence calculated 2 min after the response time was reported as the slope. Figure 5A shows that the slope is generally independent of the input C29 RNA concentration.

The effect of Qβ replicase concentration on the response time and slope was examined by varying the Qβ replicase concentration from 14 to 100 nM in amplifications initiated with 10^7 copies of C29 RNA. These experiments revealed a linear relationship between Qβ replicase concentration and slope (Figure 6). Response time, in contrast, was relatively insensitive to Qβ replicase concentration. There was no change in the response time when the Qβ replicase concentration was reduced from 100 to 30 nM, and further reduction to 14 nM increased response time by only 1 min (Figure 6). The response time and the slope respond differently to changes in Qβ replicase concentration because the RNA amplification proceeds through exponential and linear phases (Haruna & Spiegelman, 1965c). RNA amplification is exponential at first because the number of Qβ replicase enzyme molecules exceeds the number of template RNA molecules, and thus, every daughter strand serves as a template. When the concentration of RNA molecules becomes equal to or greater than the concentration of active Qβ replicase enzyme molecules, then the reaction is limited by the enzyme concentration and the rate of RNA production becomes linear until it begins to plateau (Biebricher et al., 1983). Because the detection threshold of the instrument is approximately 3.5×10^{11} molecules of C29 and the standard assay contains approximately 1×10^{13} Qβ replicase molecules, the enzyme concentration was not yet limiting when the response time was measured. Since the doubling time of C29 RNA was approximately 20 s, the C29 concentration becomes greater than the Qβ replicase concentration approximately 1.5 min after the response time (under standard conditions) (L. Burg, unpublished data). Because the slope measurement is calculated from data points in the early linear phase of RNA replication, the slope is sensitive to the enzyme concentration.

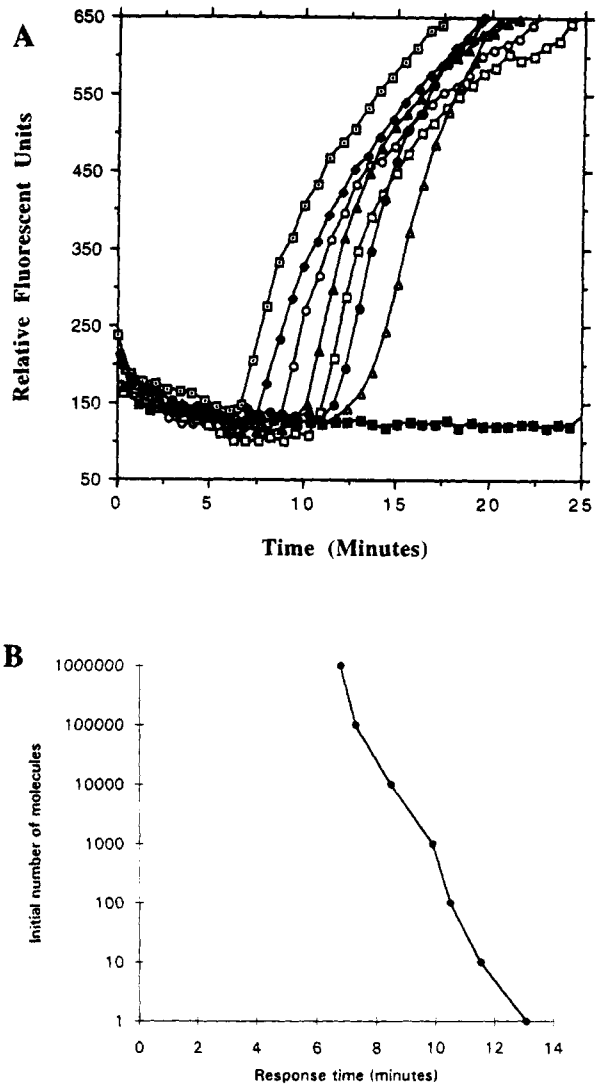

FIGURE 5: (A) Real time measurement of Qβ replicase-catalyzed amplification of replicatable RNA. A replicatable RNA molecule, C29, was amplified by Qβ replicase under the standard assay conditions described in Materials and Methods. The input level of RNA was varied in 10-fold dilutions from 10^6 molecules to 1 molecule. The replication of C29 was detected by monitoring the fluorescence of RNA-associated propidium iodide. The presence of RNA is first detected when the fluorescence increases above background levels. The time when detection occurs is designated as the response time. The fluorescence of all eight reactions was monitored simultaneously. Legend: ⊡, 1×10^6 input C29 molecules; ◆, 1×10^5 input C29 molecules; ○, 1×10^4 input C29 molecules; ▲, 1×10^3 input C29 molecules; □, 1×10^2 input C29 molecules; ●, 10 input C29 molecules; △, 1 input C29 molecule; ■, 0 input C29 molecules. (B) Plot of the relationship between the input number of C29 RNA molecules and the response times. The data are from the reactions described in part A.

Amplification of RNA in Untemplated Qβ Replicase Reactions. Qβ replicase reactions, to which no template RNA is added, sometimes give rise to spontaneously produced RNA. We refer to such RNA as false positive RNA. To determine whether our Qβ replicase preparation would perform free from spontaneous/false positive RNA replication, we incubated our Qβ replicase preparation with and without C29 RNA. We found that when no template

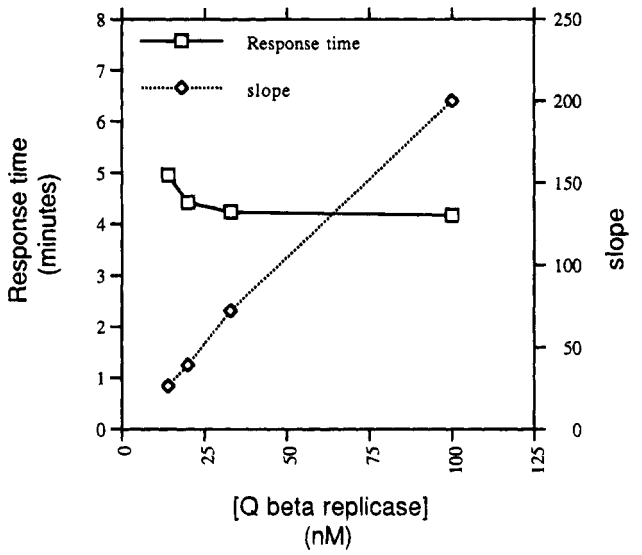

FIGURE 6: Relationship between the concentration of Qβ replicase and the amplification of C29 RNA. The amplification of C29 RNA (10^7 input molecules) was performed under standard conditions, except that the $MgCl_2$ concentration was 11.25 mM and the concentration of Qβ replicase was varied from 14 to 100 nM. Response time and slope were determined as described in Materials and Methods.

Table 2: Occurrence of Amplified RNA in Qβ Replicase Reactions Performed without Exogenously Added Template

				response time (min)		slope	
[$MgCl_2$] (mM)	[Qβ replicase] (nM)	RNA[a]	fraction positive	av	std dev	av	std dev
21.5	100	+	8/8	7.26	0.41	59	3.18
21.5	100	−	0/32				
11.2	100	+	8/8	4.24	0.17	237	10.93
11.2	100	−	24/24	28.35	2.54	69	37.25
11.2	30	+	8/8	4.06	0.17	76	1.81
11.2	30	−	1/24[b]				

[a] 10^7 copies of C29 RNA. [b] The fast response time (15.3 min) indicated that this single response was a laboratory-acquired contaminating RNA.

RNA was added, no RNA replication was observed during the 45 min assay (Table 2). Control assays demonstrated that, under these assay conditions, 10^7 copies of C29 were amplified with a response time of 7.2 min (Table 2). The response time of a single molecule of C29 input RNA is less than 15 min (Figure 5B), so that these assays can be run without false positive interference for at least 3 times longer than is necessary to detect the lowest possible concentration of detector probe.

The 0% false positive rate ($n = 32$ in this experiment) of the Qβ replicase preparation could be increased to 100% ($n = 24$) by reducing the $MgCl_2$ concentration in the assay from 21.5 to 11.2 mM (Table 2). The response times of the false positive RNA produced in the 11.2 mM $MgCl_2$ assays ranged from 11.5 to 32.7 min. In contrast to the diversity in response times of the false positive RNA, the control assays that contain 10^7 copies of C29 RNA had very similar response times (in eight assays, the average response time was 4.29 min, with standard deviation = 0.17 min). Another difference between the amplifications of C29 RNA and the false positive RNA was that the average slope measurement for C29 was 237 units (standard deviation = 10.9 units), while the slope values for the false positive amplifications ranged from 31.3 to 257.2 (average = 69.2 units, standard deviation = 37.2 units).

When the concentration of Qβ replicase was reduced from 100 to 30 nM, the response time of the false positive RNA increased to beyond 45 min, the standard length of the reaction (Table 2). The response time of the C29 RNA control, however, was not sensitive to lowering the Qβ replicase concentration (Table 2 and Figure 6). The 11.2 mM $MgCl_2$ assays (30 and 100 nM Qβ replicase) were allowed to incubate at 37 °C for an additional 5 h, and some reaction products were analyzed by gel electrophoresis (Figure 7). This analysis showed that after 5 h, the 30 nM Qβ replicase produced RNA in some of the reactions.

Cloning of Spontaneously Produced RNA. An array of 96 Qβ replicase reactions without added template RNA were set up under standard conditions (21.5 mM Mg^{+2}) in the fluorimeter and monitored for 45 min. Although no template was added, six of these reactions gave positive responses within this time period. The steep slopes of these false positives identified them as previously known laboratory contaminants, and they were not analyzed further. No signal was observed in the remaining 90 reactions during the 45 min incubation period. Without removing the rack from the fluorimeter, monitoring for 45 min periods was carried out at intervals for 21 h. By the end of that time, an additional 12 reactions were positive. In all cases, the slopes were shallow and the fluorescence plateaus were lower than with MDV or other known contaminating RNA species. RNA from 2 of these 12, which had a response time of over 200 min, was used as a template to generate cDNA that was cloned as described earlier.

DNA sequence analysis of several individual plasmid clones from one reaction showed the sequences given in Figure 8; these are referred to as the MAR3 family of sequences, which includes MAR3, MAR57, MAR58, and MAR59. The nucleotide sequences of inserts arising from the other reaction were closely related to the WS1 molecule previously identified by Schaffner and co-workers (1977) and are shown in Figure 9. These two RNA sequences are referred to as MAR72 and MAR75 and considered members of the WS1 family.

Transcripts of *Sma*I-cut plasmids encoding MAR57 RNA were used as templates for Qβ replicase reactions. These molecules can indeed function as amplifiable templates for Qβ replicase (Figure 10A). Even under these restrictive conditions (21 mM $MgCl_2$) less than 10 molecules were sufficient to give a response in about 25 min.

Origin of the MAR3 Template Family. Neither the nucleotide sequence of MAR3 nor its secondary structure, as suggested by an RNA-folding computer program, resemble previously known Qβ replicase substrates except for the 5′ terminal GG and the 3′ terminal CC, which are common to all known amplifiable templates. Searching the GeneBank for homologous nucleotide sequences revealed striking homology with *E. coli* tRNA val. An alignment of these sequences is shown in Figure 11. MAR3 contains a nucleotide sequence homologous to the 40 3′ nucleotides of this tRNA. This tRNA segment is present twice in the MAR3 molecule. The two tandem repeats of the tRNA sequence are separated by eight nucleotides and bounded by a few extra nucleotides to which the typical ends are appended. The tRNA repeat at the 5′ end of MAR3 is better

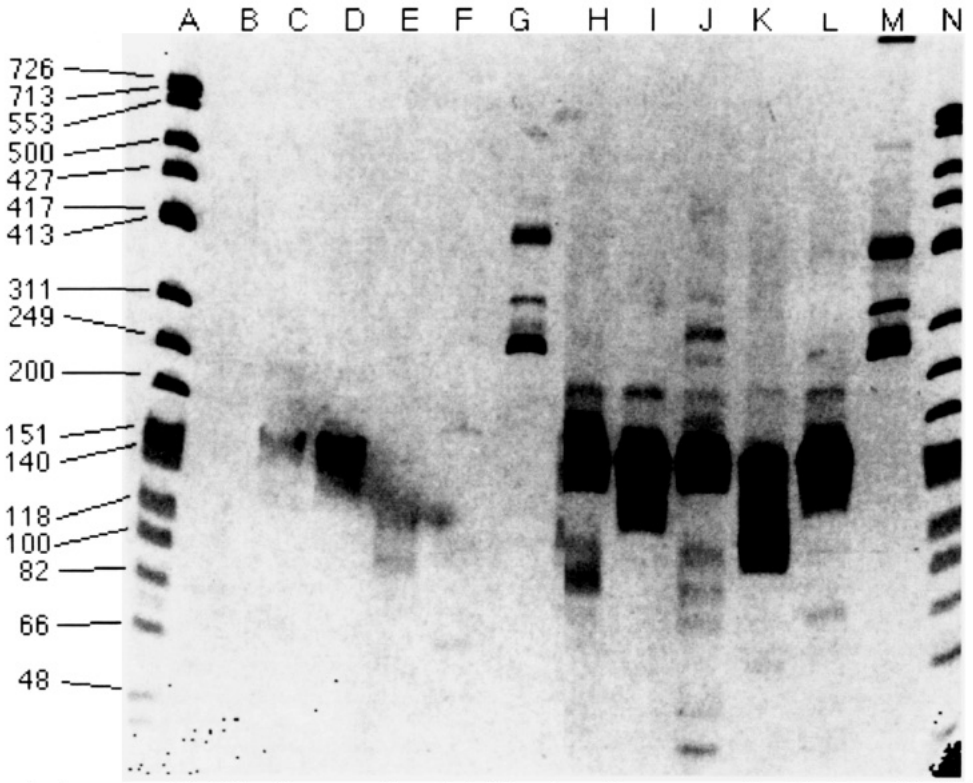

FIGURE 7: RNA products produced in Qβ replicase reactions that did not contain exogenously added template RNA. Reactions were performed under standard conditions, except that the $MgCl_2$ concentration was 11.25 mM and the concentration of Qβ replicase was either 30 or 100 nM. No RNA was added to the reactions except where noted. Lanes A and N, molecular weight markers; lanes B−G, samples from reactions that employed 30 nM Qβ replicase; lanes F−M, samples from reactions that employed 100 nM Qβ replicase; lanes G and M, samples from reactions that included 10^7 copies of C29 RNA as positive controls. The image shown is a DeskScan black and white photo scan of the stained gel.

```
MAR3      GGGCTTCCTCCCCTACAAGGAGGGGGTCAGCGGCTCGAACCCGTCATCACCCGAAGGCTTTCCTCCCTT
MAR57     ............................................................-........
MAR58     ...........T................................................-........
MAR59     ...........T................................................-........
2TRNAVAL       .....T.............G....T....T.............-.C--.A--........

MAR3      ACAAGGAGGGGGTCAACGG------CCCGT--TC-CCCA
MAR57     ..................------.....--..-....
MAR58     ..................------.....--..-....
MAR59     ..................------.....--..-....
2TRNAVAL ..............GG...TTCGAT.....CA..A....CCA
```

FIGURE 8: Tandem repeat of nucleotides 27−73 of *E. coli* tRNA val (2TRNAVAL) aligned with MAR 3, MAR57, MAR58, and MAR59. Dots mark positions of identity with MAR3. Gaps are indicated by lines. MAR58 and MAR59, separate isolates, have identical nucleotide sequences. Of the 98 nucleotides in MAR57, 80 appear to have arisen from the tRNA val sequence.

```
MAR72       GGGAAGGCCCGGCTAAATCCGGGCGTGTCCTCTCTAACTTTCGAGTAGAGAGGTGAGAAAACCCTAT
MAR75    'GG...TT.........G.....................................................
WS1         ...TT.A........G.......T.........................................CG.
WS3         ...TT.A........G.......T.........................................C..
WS2         ...TT.A........G.......T.........................................C..

MAR72     TGTCCTGGTAACAGGATTTCCCCA
MAR75     ........................
WS1       .A......................
WS3       .A...C......G...........
WS2       ....TC......GA.....T....
```

FIGURE 9: Nucleotide sequences of WS1, WS2, and WS3 (Schaffner et al., 1977) aligned with those of MAR72 and MAR75 isolated in this study. Dots mark positions of identity with MAR72. MAR75 has five G residues at the 5′ end, where the others have only three G residues.

conserved than that at the 3′ end, which appears mutated near its 3′ end.

MAR72 Replication. Transcripts of *Sma*I-cut plasmids encoding MAR72 RNA were used as templates for Qβ replicase reactions. Replication of these molecules under restrictive conditions (21 mM $MgCl_2$) shows a considerably faster replication rate than that of MAR57, with 10 molecules giving a response of only 8 min.

DISCUSSION

Qβ replicase can amplify RNA by 1 billion-fold within 15 min. This amplification of RNA requires only one enzyme and does not require thermocycling. Because of these attributes, Qβ replicase has been used to amplify RNA containing hybridization probes (Lizardi et al., 1988; Lomeli et al., 1989; Cahill et al., 1991; Lane & Collins, 1991; Pritchard & Stefano, 1991). Although the template specific-

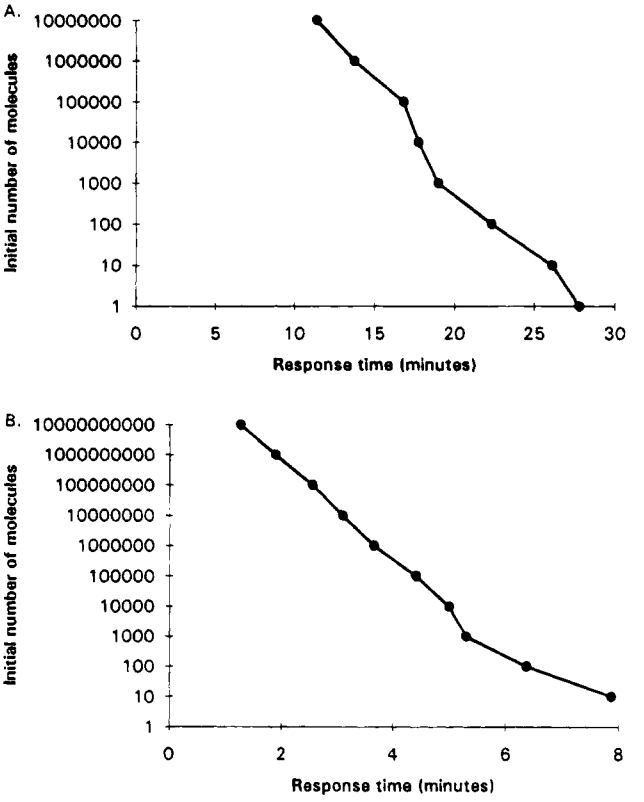

FIGURE 10: (A) MAR57 replication. The figure shows the response time for reactions initiated with various numbers of MAR57 molecules ranging from 1 to 10^7. Each point is the average response time of four simultaneous reactions carried out under standard conditions. (B) MAR72 replication. The figure shows the response time for reactions initiated with various numbers of MAR72 molecules ranging from 10 to 10^{10}. Each point is the average response time of four simultaneous reactions carried out under standard conditions.

ity of Qβ replicase is relatively specific, there have been numerous reports that Qβ replicase has produced RNA in reactions that did not contain added template molecules. Because the spontaneous production of replicatable RNA would limit the usefulness of the Qβ replicase amplification reaction, it is important to understand the origin of spontaneously replicated RNA and control its replication. Three possible sources of spontaneously arising replicatable RNA molecules have been suggested: (1) they arise from the replication of preexisting replicatable RNA contaminating the enzyme preparations (Chetverin et al., 1991; Hill & Blumenthal, 1983); (2) they arise from preexisting, but poorly replicating RNAs, which evolve *in vitro* during the course of Qβ amplification reactions into efficiently replicating RNA species (Chetverin et al., 1991); or (3) they arise by *de novo* synthesis of replicatable RNA from the nucleotides present in the Qβ replicase assay (Sumper & Luce, 1975; Biebricher, 1987; Biebricher et al., 1986; Biebricher & Luce 1993).

It is difficult to prove any of these hypotheses because Qβ replicase has a prodigious ability to evolve highly replicatable RNAs from poorly replicating RNA, in response to a variety of selection pressures (Bauer et al., 1989; Kramer et al., 1974; Mills et al., 1967; Levisohn & Spiegelman, 1968a,b; Saffhill et al., 1970). This evolution of improved templates derives in part from a relatively high misincorporation rate (Domingo et. al., 1978), a high frequency of deletions for undesirable sequences (Mills et al., 1967; L. Burg, unpublished observations), the ability to add nucleotides to the 3′ end of a sequence (Biebricher & Luce, 1992), and a capacity for recombining separate RNA molecules (L. Burg, unpublished observations; Biebricher & Luce, 1992; Munishkin et al., 1991). Consequently, the products of Qβ replicase-driven evolution are often very different from their progenitor RNA, and thus, the sequence of a replication product often provides little clue as to the identity of the starting material. Efforts to identify the source of spontaneous RNA are further complicated by the ability of Qβ replicase to amplify a single contaminating replicating RNA molecule (Lizardi et al., 1988). Thus, the prevention of assay contamination by the products of previous assays requires considerable attention.

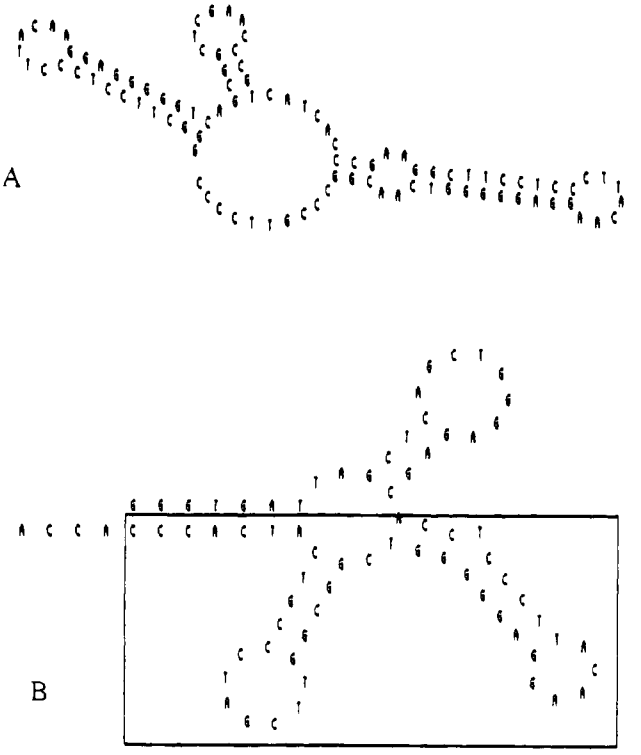

FIGURE 11: (A) Nucleotide sequence and computer-generated secondary structure of MAR58 RNA (Jaeger et al., 1990). (B) This shows the unmodified nucleotide sequence and similarly calculated secondary structure for *E. coli* tRNA val (ECTRNAVAL, GenBank accession number X02682). The box encloses part of the tRNA val nucleotide sequence, a duplication of which occurs in MAR58.

Other workers have described methods for producing highly purified Qβ replicase. However, these methods required, in addition to ion-exchange chromatography, procedures such as liquid polymer phase partitioning, $(NH_4)_2SO_4$ precipitation (Eoyang & August, 1971; Kamen et al., 1972; Sumper & Luce, 1975), and glycerol (Kamen, 1972) or CsCl gradient centrifugation (Sumper & Luce, 1975). Extraction and centrifugation procedures are cumbersome for gram-scale enzyme production, and we set out to develop a simple purification method that could be directly scaled up to produce hundreds of grams of Qβ replicase if needed.

In this paper, we have described an expression and purification system that produces gram quantities of Qβ replicase; the yield of Qβ replicase from 3 kg of *E. coli* cells (wet weight) is 2.2 g of Qβ replicase that is 85% pure. We have developed assay conditions that allow the Qβ replicase to generate 10^{11} molecules from a single molecule of detector probe in 15 min. Furthermore, the Qβ replicase produced by this system is free from both Qβ phage RNA (except for

the RNA encoding the replicase) and 6S RNA (Banerjee et al., 1969) and functions under our assay conditions without the false positive responses caused by RNA replication in the absence of added template.

We make no claim that our Qβ replicase preparation is devoid of all RNA impurities, but we can control assay conditions to eliminate the replication of any such RNA. Others claim to have proven that their Qβ replicase and nucleotide preparations are free from contaminating RNA templates (Biebricher & Luce, 1993). This argument is based upon the observation that these reagents do not spontaneously produce RNA under conditions of low enzyme and low nucleotide concentrations. Replicatable RNA was spontaneously produced by these reagents when used at greater concentrations. We offer the alternative interpretation that this is not proof of template-free reagents, but is the description of reaction conditions that do not allow detectable amplification of contaminating template RNA over the time of the assay. As shown in Table 2, the production of false positive RNA by our Qβ replicase preparation was dependent on assay conditions. Changes in either the concentration of Mg^{2+} and/or the concentration of Qβ replicase can change the false positive rate from 0% to 100%. We generally employ 21.5 mM $MgCl_2$ in assays to suppress false positive replication. Because Mg^{2+} could interact with both Qβ replicase and template RNA, Mg^{2+} may influence replication by more than one mechanism. Lowering the Mg^{2+} concentration to 11.25 mM increases the slope and decreases the response time (Table 2), indicating that decreasing the Mg^{2+} concentration from 21.5 to 11.2 mM increases the replication rate in both the exponential and linear phases of the reaction. Since the replication rate is decreased in 21.5 mM $MgCl_2$, the elimination of false positives may simply be due to the delay of their replication to detectable levels during the 45 min assay. It is also possible that the structure of some RNAs in high $MgCl_2$ may be incompatible with template activity. Increasing the $MgCl_2$ concentration to 21.5 mM may also reduce spontaneous RNA replication by directly affecting the structure of Qβ replicase.

The response time of the false positive RNA could be increased from approximately 28 min to several hours by decreasing the Qβ replicase concentration from 100 to 30 nM (Table 2). Other workers (Sumper & Luce, 1975; Biebricher et al., 1981b, 1986) have interpreted the observation that reduced Qβ replicase concentrations increase the lag time for spontaneous RNA synthesis as support for *de novo* synthesis. While we agree that the enzyme concentration sensitivity of spontaneous RNA replication is very different from the Qβ replicase concentration independence of MDV-1-based templates (Figure 6), we offer an alternative hypothesis. Biebricher and co-workers (Biebricher et al., 1983, 1985) have developed kinetic models that describe the replication of RNA molecules under a variety of conditions. These models predict that the rate of exponential growth is independent of enzyme concentration as long as the Qβ replicase concentration is above a threshold concentration. The threshold concentration is determined by the rate constants that describe the ability of template RNA to form an initiation complex with Qβ replicase. If these rate constants are different for a good replicating template (such as C29) and the poorly replicating contaminant RNA species, then it is possible that the 30 nM Qβ replicase concentration is near or above the threshold concentration for C29 RNA (Figure 6), but below the threshold concentration for the contaminating RNA species. Thus, the replication of the contaminating template RNA molecules (with poor initiation characteristics) is greatly delayed at the 30 nM Qβ replicase concentration. We propose that during the long lag time before detection, RNA mutants with improved initiation and replication characteristics evolve and that the progeny RNA replicate efficiently to become the most abundant species in the reaction (Kramer et al., 1974; Mills et al., 1967; Levisohn & Spiegelman, 1968a,b; Saffhill et al., 1970).

The observation that the products of spontaneous replication are a mixture of different RNA species (based upon size as revealed by gel electrophoresis) has been interpreted by proponents of *de novo* synthesis to be evidence of a lack of contaminating template RNA. We believe that the diversity of spontaneous RNA products is the result of the Qβ replicase preparation containing a small mixed population of poorly replicating progenitor template RNAs. The evolution of a particular RNA species into the final replicating RNA species is the outcome of mutation and selective pressure for the generation of RNA molecules, both strands of which are good replication templates.

If we accept the view that replicatable RNAs do not arise by *de novo* synthesis from nucleotides in the assay, but instead evolve from preexisting RNA species, then important questions are where and when do they evolve? It is possible that they evolve in phage-infected cells and in the amplification reactions. Banerjee and co-workers (1969) found 6S RNA preexisting in Qβ phage-infected cells. Schaffner and co-workers (1977) described the isolation and sequencing of three nanovariant RNAs, which appeared to have arisen from a common ancestor and may have evolved in the infected cells and preexisted as a replicatable RNA in the Qβ replicase preparation. Schaffner and co-workers suggested that if the RNA were packaged in the phage particles, the RNA could be transferred as a molecular parasite from host to host. This possibility was confirmed when Chetverin and co-workers (Chetverin et al., 1991) detected replicatable RNA tightly associated with Qβ phage particles and found that this RNA was propagated through serial transfers of the Qβ phage. In addition, striking similarities between replicatable RNAs isolated by different laboratories provide evidence that replicatable RNAs may have spread as molecular parasites of Qβ phage or as contaminants in shared reagents (Chetverin et al., 1991). However, convergent evolution resulting from a small repertoire of progenitor RNAs and constraints of the exponential amplification reaction could also explain the isolation of similar molecules in different laboratories.

The Qβ replicase in our studies was produced without Qβ phage, but instead used a plasmid that expressed Qβ replicase under tight regulation. Because we do not rely on Qβ phage as a source of the Qβ replicase gene, we have avoided introducing both Qβ RNA and other replicatable RNAs such as MDV-1 or RQ87, which might be spread as molecular parasites. The elimination of this source of replicatable RNA has been very important in our ability to produce Qβ replicase that can function without false positive amplification. To control false positive results, considerable effort was expended to prevent contamination with replicating RNA in the laboratory environment. The preparation of reagents was physically isolated from Qβ replicase template RNA amplifications, and assay components were assembled in

biological safety cabinets equipped with HEPA filters. Disposable equipment was used whenever possible, and nondisposable equipment was carefully cleaned. These efforts eliminated the false positives that can be produced by contamination of the laboratory with template RNA (Chetverin et al., 1991). However, when the Mg^{2+} concentration is lowered to 11.2 mM, or when amplification reactions are carried out for extended times, we observe highly elevated rates of spontaneous RNA production in untemplated assays—approaching 100% under some conditions (Table 2). The source of these false positives could be environmental contamination of the assays, but because the frequency in 11.2 mM Mg^{2+} can be as high as 100% (and this is seen in all Qβ preparations—even those produced in new facilities, completely isolated from previous operations), it is likely that the replicating molecules or their progenitors already existed in the Qβ replicase preparation.

Sequence analysis of a number of spontaneously generated replicatable RNAs has shown that some of them share considerable homology with known RNA molecules. MDV-1 is a 221-nucleotide RNA that is not a direct fragment of Qβ RNA, yet the nucleotide sequences of both a 35-nucleotide sequence at the 3′ end of MDV-1 and a 46-nucleotide sequence near the center of the MDV-1 sequence are almost identical to sequences of Qβ RNA (Nishihara et al., 1983). Sequence analysis of the template RNA, RQ120, shows that it is composed of 80 nucleotides of Qβ coat protein RNA and a 33-nucleotide-long fragment of *E. coli* $tRNA^{Asp}$ (Munishkin et al., 1988). Another replicatable RNA, RQ135, appears to have evolved from a recombination of ribosomal 23S RNA and nucleic acid encoding the phage λ origin of replication (Munishkin et al., 1991). These studies support the idea that spontaneous RNA replication is due to the evolution of poorly replicating contaminant RNA into efficient template RNA. There have also been reports that the substrate specificity of Qβ replicase can be overcome and that the enzyme can recognize cellular RNA as a template for the production of a complementary strand (but does not amplify the RNA) (Haruna & Spiegelman, 1965; Palmenberg & Kaesberg 1974; Feix & Haake, 1975; Blumenthal, 1980).

We hypothesize that spontaneous production of RNA in our untemplated reactions derives from the evolution and amplification of low levels of *E. coli* RNA contaminating the enzyme preparation. This hypothesis is best supported by the sequence analysis of a false positive RNA produced in an assay that contained no exogenously added template. Homology with preexisting host-cell RNA would not be expected for templates generated by *de novo* synthesis.

The MAR57 RNA clearly arose from an *E. coli* tRNA val molecule (Figures 8 and 11). Since one molecule of MAR57 gives a signal in 25 min, it would be expected to produce a signal prior to 200 min if it had been present at the beginning of the reaction. MAR57 must have evolved from a poorly replicating substrate during the 200 min lag phase. Whether this progenitor substrate was tRNA val, a degradation product, or an evolutionary intermediate remains unknown. It is possible that the evolution of MAR57 began in the cell from which the enzyme was purified and continued *in vitro*.

Several templates [e.g., WS1 and microvariant (Schaffner et al., 1977; Mills et al., 1975)] are known, for which likely ancestor molecules have not been identified by nucleotide sequence homology. Although it is formally possible that such molecules arose by the *de novo* assembly of nucleotides by Qβ replicase, we consider this explanation unlikely.

We have identified two variants of WS1 in our reactions. These arose in reactions after more than 200 min of incubation. In the same reaction conditions that were used in the initial isolation, a single molecule of MAR72 gives a signal after only 10 min. Presumably, therefore, these WS1-like molecules were not present at the start of the reaction, but arose during it. Since MAR72 and MAR75 each differ from WS1 by only one or two point mutations (Figure 9), their *de novo* synthesis by Schaffner in 1977 and by us in 1992 would imply a very stringent sequence requirement by the Qβ replicase. An examination of the known sequences of replicatable molecules shows no such stringent sequence requirement, but instead shows a wide variety of replicatable RNA sequences. In the face of evidence that a Qβ replicase amplifiable RNA has evolved from host-cell RNA, it appears likely that WS1, MAR72, and MAR75 arose from a common preexisting nucleic acid that has yet to be identified.

Despite the recent redefinition of *de novo* synthesis to include the possibility of template-instructed synthesis, the claim is still made that there is no evidence for such synthesis (Biebricher et al., 1993). However, as the nucleotide sequences of MAR57, RQ135 (Munishkin et al., 1991), and RQ120 (Munishkin et al., 1988) demonstrate, such evidence does indeed exist. In contrast, there is no evidence for the template-free synthesis hypothesis, which also does not support template-instructed RNA synthesis as an explanation for the origin of RNA in Qβ replicase-catalyzed reactions that contain no exogenously added template.

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