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Qβ RNA Bacteriophage: Mapping *cis*-Acting Elements within an RNA Genome

DONALD R. MILLS,* CHRISTINE PRIANO, PATRICIA A. MERZ,† AND BRUCE D. BINDEROW

Department of Microbiology & Immunology, Box 44, State University of New York, Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, New York 11203

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We have identified, for the first time, regions of *cis*-acting RNA elements within the bacteriophage Qβ replicase cistron by analyzing the infectivities of 76 replicase gene mutant phages in the presence of a helper replicase. Two separate classes of mutant Qβ phage genomes (35 different insertion mutants, each containing an insertion of 3 to 15 nucleotides within the replicase gene, and 41 deletion genomes, each having from 15 to 935 nucleotides deleted from different regions of the gene) were constructed, and their corresponding RNAs were tested for the ability to direct the formation of progeny virus particles. Each mutant phage was tested for plaque formation in an *Escherichia coli* (F^+) host strain that supplied helper Qβ replicase in *trans* from a plasmid DNA. Of the 76 mutant genomes, 34% were able to direct virus production at or close to wild-type levels (with plaque yield ratios of >0.5), another 36% also produced virus particles, but at much lower levels than those of wild-type virus (with plaque yield ratios of <0.5), and the remaining 30% produced no virus at all. From these data, we have been able to define regions within the Qβ replicase gene that contain functional *cis*-acting RNA elements and further correlate them with regions of RNA that are solely required to code for functional RNA polymerase.

The small, linear genome of the single-stranded RNA bacteriophages contain an abundance of vital information ranging from the simple coding of viral proteins (1, 10, 27, 29) to a variety of more complex structure-function interactions that govern the overall process of viral reproduction. Aside from its coding properties, the RNA chromosome directs its own autocatalytic replication by a phage-induced, RNA-directed RNA replicase (8) and further provides information for the control of protein synthesis (15, 25, 28), for the specific recognition and binding of the RNA replicase (18, 22), for the production of single-stranded RNA copies (21, 24), and for the final assembly of progeny phage particles (14). Many of these *cis*-acting RNA properties include functional surfaces for protein recognition and binding (3, 15, 18, 22, 26, 28) as well as sequences that define long-range RNA interactions implicated in genome conformation (13) and in translational regulation (16). Since 90% of the RNA phage chromosome comprises the four genes that code for functional proteins (7, 11, 12; P. Mekler, Ph.D. thesis, University of Zurich, Zurich, Switzerland, 1981), these *cis*-acting RNA properties must overlap to a large degree with coding domains. Our current research focuses on how such diverse functional properties are integrated and packaged into the structural organization of the bacteriophage RNA.

Recently, we described a means by which we can separately isolate and study the coding function of the RNA phage Qβ replicase gene from the functional *cis*-acting ribonucleotides contained within the same RNA sequences (20). By supplying Qβ replicase in *trans* from a plasmid source, we can introduce replicase gene mutations into a cloned DNA copy of Qβ RNA, express the RNA by transcription in vivo, and assay for the production of infectious virus. Using this method, we have been able to examine the effects of a variety of replicase gene mutations on the production of functional replicase in vivo (20). In this paper, we describe how we have used the same method to probe for functional *cis*-acting RNA sequence elements that are contained within the coding structure of the Qβ replicase cistron.

* Corresponding author.
† Permanent address: New York State Institute for Basic Research and Developmental Disabilities, Staten Island, NY 10314.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass. *Escherichia coli* NP20 [*endA1 hsdR17* (r_k^- mk^+ *supE44 thi-1* λ^- *recA1 gyrA96 relA1* φ80 Δ*lacZ* M15 F'150*cps*::Tn*10*] was a generous gift from Pierre Jelenc, Columbia University, New York, N.Y. *E. coli* HB101 (17) was used for growing and maintaining all plasmids. A full-length DNA copy of Qβ RNA having *Hin*dIII-*Pst*I cohesive ends and inserted into pBR322 was generously provided by M. A. Billeter, University of Zurich. The replicase plasmid pRep211(3936), a derivative of plasmid pRep201 (20), was used in the experiments described below as a source of helper replicase. This plasmid contained the 3936.15/r linker insertion mutation in the Qβ replicase gene, conveyed resistance to kanamycin, and produced a replicase protein with wild-type activity.

General procedures. The methods employed for plasmid constructions, bacterial transformations, and plasmid preparations were standard techniques and have been described in detail elsewhere (17). The orientation and nucleotide sequence that resulted from each step of a plasmid construction were routinely confirmed by restriction enzyme analysis and by Maxam-Gilbert DNA sequence analysis. Transformed bacterial cultures were selected by growth in N broth (20) containing 50 μg of chloramphenicol per ml (to select for pRep201), 50 μg of ampicillin per ml (to select for pQβm100), and/or 50 μg of kanamycin per ml [to select for pRep211(3936)].

Construction of pQβm100. A full-length cDNA copy of Qβ

RNA obtained from the Qβ cDNA plasmid (see Materials) was inserted into a 2.7-kilobase *Hin*dIII-*Bgl*I fragment (containing the E1a origin of replication) from the plasmid pDM1000 (20) such that the modified *trp* promoter directs Qβ minus-strand RNA synthesis. This plasmid could be grown and maintained in *E. coli* HB101, a nonhost (F^-) strain, at 80 to 100 copies per cell and conferred resistance to 50 μg of ampicillin per ml.

Construction of mutant genomes. Construction of *Bgl*II linker insertion Qβ replicase gene mutants has been described previously (20). Deletion mutants were constructed by mixing and matching *Bgl*II-digested DNA from the different linker insertion mutant replicases in pRep201 clones (20). Each pRep201 mutant plasmid was then digested with either *Nsi*I and *Bss*HII or *Nsi*I and *Bgl*I to release the desired mutant fragment within the replicase gene. Each mutant element was then used to replace the corresponding wild-type element in the pQβm100 plasmid (see Fig. 1 and 2).

Obtaining mutant virus particles. *E. coli* NP20 cells were transformed with the helper replicase plasmid pRep211 (3936) (see Materials) in the presence of 50 μg of kanamycin per ml. These cells were made competent and then transformed again with each mutant pQβm100 plasmid in the presence of 50 μg of kanamycin per ml and 50 μg of ampicillin per ml. Transformants were prepared as soft-agar bacterial lawns (20, 23), and virus particles were obtained from individual plaques by removing soft-agar plugs with 100-μl capillary pipettes. The plugs were expelled into 2 ml of N broth (20) and then allowed to stand for 2 h at room temperature to enable the bacteriophage particles to diffuse out of the agar.

Measuring plaque yield ratios. Titers of isolated mutant virus particles (see above) were determined on *E. coli* NP20 cells in the presence or absence of the pRep211(3936) helper replicase plasmid. The ratio of the number of mutant plaques obtained to the number of wild-type plaques obtained in a parallel control experiment was used as a quantitative measure of fitness for each mutant virus. All experiments were done in triplicate, and an average plaque yield ratio was determined (Tables 1 and 2).

Purification of viral RNA. Mutant phage was grown in *E. coli* NP20 cells that contained the pRep211(3936) helper replicase plasmid. Cultures were grown to 3×10^8 cells per ml in N broth (20) and infected with mutant phage particles at a multiplicity of infection of 10. Growth was continued for 3 h, and the phage particles were collected as described elsewhere (30). Phage RNA was isolated as described previously (23).

Sequencing of deletion mutant RNA. RNA that was isolated from virions was sequenced by using the GemSeq sequencing system according to the methods outlined by Promega Biotec (Madison, Wisc.). The 22 nucleotide primer sequence that was used, 5′-AAAGGGAGTAACATCTA CGCCA-3′, was synthesized by using a Biosearch 8600 DNA synthesizer and was complementary to nucleotides 3560 to 3581 in Qβ RNA.

RESULTS

Experimental design. In a previous report, we described the use of oligonucleotide linker insertion mutagenesis (9) to generate mutations in a cloned DNA copy of the Qβ replicase gene (20). We collected a library of 37 different mutant pRep201 clones, each containing only one in-frame insertion of 3 to 15 nucleotides within the coding region (Table 1 and Fig. 1). Each mutant replicase was tested for its ability to generate functional Qβ replicase in vivo. The results indicated that mutations within a central domain of the replicase gene are all lethal to replicase function, whereas five of nine insertions within the amino terminus and five of six insertions within the carboxy terminus allow the production of functional replicase.

This report describes how we tested 35 of these same Qβ replicase gene mutants for the ability to function as part of the intact Qβ phage genome during infection. In addition, we tested 41 deletion gene mutants, each having from 15 to 935 nucleotides deleted from different regions of the replicase cistron. To carry out these studies, we designed a plasmid, pQβm100, that expressed full-length copies of Qβ minus-strand RNA in vivo from a modified tryptophan promoter (Fig. 2). This plasmid could be grown and maintained in *E. coli* HB101, a nonhost (F^-) strain. When pQβm100 was used to transform an *E. coli* host (F^+) strain carrying helper Qβ replicase, phage plaques formed on bacterial lawns.

TABLE 1. Plaque yield ratios of insertion mutant phages

Qβ phage mutant[a]	Plaque yield ratio[b]		Replicase helper function[c]
	− pRep211 (3936)	+ pRep211 (3936)	
2372.12	—	0.01	+
2413.15/r	L	0.14	−
2430.15/r	0.21	0.58	+
2445.9	L	0.99	−
2469.12	L	0.22	−
2488.15/r	—	0.38	−
2533.15/r	1.05	0.54	+
2687.12	0.03	0.69	+
2692.15/r	—	0.35	+
2732.6/t	L	0.87	−
2847.12	L	0.54	−
2888.12	L	0.18	−
2986.12	L	0.03	−
3073.12	L	1.19	−
3251.15/r	L	0.79	−
3264.15/r	L	1.69	−
3342.12/r	L	1.11	−
3471.3/t	L	1.37	−
3524.12	L	0.62	−
3649.12	—	0.24	−
3669.12/r	—	0.10	−
3690.12	L	1.03	−
3702.15/r	L	0.49	−
3722.12	L	1.11	−
3795.12	L	1.28	−
3811.12/r	L	0.56	−
3819.12	L	0.69	−
3823.12	L	1.10	−
3836.15/r	L	0.64	−
3896.12	0.16	0.64	+
3897.15/r	0.01	1.64	+
3936.15/r	L	L	+
3937.12/r	L	L	+
4047.12	—	0.08	−
4105.6	—	0.03	+

[a] Designations are taken from those used for each mutant Qβ replicase gene described by Mills et al. (20). The number preceding the decimal point indicates the nucleotide position at which the *Bgl*II linker element was inserted.

[b] Calculated on the basis of the phage titer for host cells carrying wild-type Qβ phage. A total of 17,000 plaques were counted on control plates. These were diluted to 100 to 200 phage particles per plate. —, A plaque yield ratio of <0.01; L, lethal (indicates a genome that could not produce infectious virus).

[c] Indicates which mutant replicase genes produce replicases that can (+) or cannot (−) complement a replicase-defective Qβ phage in vivo (20).

TABLE 2. Plaque yield ratios of deletion mutant phages

Deletion[a]	Nucleotides		Plaque yield ratio[c]
	Coordinates[b]	No. deleted	
1	2430–2445	15	0.36
2	2430–2469	39	0.39
3	2430–2488	58	0.16
4	2430–2533	103	0.44
5	2446–2469	23	0.62
6	2469–2488	19	0.40
7	2488–2533	45	0.65
8	2533–2687	154	0.11
9	2533–2847	314	—
10	2533–2888	355	—
11	2687–2847	160	0.42
12	2780–3264	484	—
13	2847–2888	41	0.48
14	2888–3524	636	—
15	2888–3823	935	—
16	2986–3342	356	0.78
17	2986–3471	485	0.24
18	2986–3524	538	0.83
19	2986–3649	663	—
20	2986–3669	683	—
21	2986–3690	704	—
22	2986–3722	736	—
23	2986–3823	837	—
24	3073–3342	269	0.95
25	3073–3471	398	0.27
26	3073–3524	451	0.38
27	3251–3823	572	—
28	3471–3823	352	—
29	3795–3936	141	—
30	3936–4105	169	—
31	3936–4216	280	—
32	4105–4216	111	—
a	2888–2986	129	—
b	2888–3073	185	—
c	2986–3073	87	0.30
d	3342–3471	129	0.11
e	3342–3524	182	0.10
f	3342–3649	307	—
g	3471–3524	53	0.25
h	3471–3649	178	—
i	3524–3649	125	—

[a] Deletions 1 through 32 are numbered according to the location of the deletion in the genome and its size. Deletions *a* through *i* involve RNA sequences in the 5′ and 3′ sides of the region encoding the conserved amino acid sequence.

[b] Positions of *Bgl*II linker elements in the replicase gene that were joined together to create a deletion.

[c] Calculated for mutant phage particles in the presence of helper Qβ replicase (as described in Table 1, footnote *b*. —, A genome that could not produce infectious virus.

Figure 3 illustrates our experimental design. The wild-type Qβ replicase gene sequence in pQβm100 was replaced with one of the 76 mutant replicase genes. *E. coli* NP20, a RecA$^-$, F$^+$ host cell strain, was then transformed with the altered plasmid, and we observed whether plaques formed. Because many of the replicase gene mutants generate nonfunctional replicases (20), these experiments were also carried out in the presence of a helper plasmid, pRep211(3936), that constitutively supplies Qβ replicase. Thus, phage plaque formation indicates that a particular mutant phage genome can direct reproduction of infectious phage particles even though its replicase might be defective.

Note that for these experiments, it was necessary to use a recombination-negative (RecA$^-$) bacterial host to minimize the possibility of DNA recombination occurring between the pQβm100 and the pRep211(3936) plasmids. It was further necessary to perform control experiments to eliminate the possibility of some of the mutant replicases interfering with the wild-type replicase present in the NP20 host cells (see below).

Rebuilding insertion mutant genomes. To replace the wild-type Qβ replicase gene in pQβm100 with a mutant replicase gene, we first removed either an *Nsi*I-*Bgl*I or an *Nsi*I-*Bss*HII restriction fragment that contained the mutant nucleotide sequence from each replicase gene clone (20). In each case, the isolated mutant fragment was then incorporated into the corresponding restriction sites within pQβm100 (Fig. 2). Mutant pQβm100 plasmids were constructed for 35 different insertion mutant replicase genes (Table 1 and Fig. 1).

Obtaining insertion mutant virus particles. Mutant virus particles were generated by transforming host cells with each mutant pQβm100 plasmid and then isolating progeny phage from resulting plaques. *E. coli* NP20 (F$^+$ RecA$^-$) host cells were first transformed with the helper replicase plasmid pRep211(3936) to ensure the presence of functional Qβ replicase within the host. Upon transformation of these host cells with each of the 35 mutant pQβm100 clones, all but two of the transformants generated infectious centers, indicating that infectious virus was being produced. We isolated virus particles from each transformant by removing defined cross sections of the resulting plaques. In this way, we collected a library of intact mutant phages, each putatively containing a specific replicase gene mutation. Although we could isolate phage particles from most of the transformed host cells, two transformants repeatedly resulted in no plaque formation. The mutant genomes that were putatively contained within these cells (genomes 3936.15/r and 3937.12/r) were considered to be lethal to plaque formation (Table 1).

Redetermining titers of insertion mutant virus particles. To obtain a quantitative measure of mutant phage infectivity, we measured the plaque yield ratios of the isolated virus particles contained within each plaque cross section (see above). The isolated phage particles were used to reinfect *E. coli* NP20 host cells in the presence or absence of the replicase helper plasmid, pRep211(3936). In each case, the number of plaques generated from each reinfection was compared with that of a wild-type phage control to obtain a plaque yield ratio value. These experiments were repeated several times, yielding consistent results (Table 1 and Fig. 4B). When no helper replicase was present in the infected cells, only one mutant phage, 2533.15/r, generated virus particles with a high plaque yield ratio of 1.05 (Table 1). This result was surprising, since this mutant phage is only 1 of 10 that contain a mutant replicase gene capable of producing functional replicase (helper activity) in vivo (20) (Table 1). Of the remaining nine phage mutants that can produce functional replicase, two had plaque yield ratios of 0.21 (2430.15/r) and 0.16 (3896.12), and the remainder had very low plaque yield ratios (less than 0.03). These results suggest that even though these mutant phages can putatively produce functional replicase, they cannot generate infectious progeny virus particles.

When helper replicase was present in infected cells, 33 of 35 infections generated plaques with variable plaque yield ratios (Table 1). These results indicate that nearly all of the defective phage genomes could be complemented to some degree by the helper replicase (Fig. 4B). Ten of the mutant viruses had very high plaque yield ratios (greater than 0.90), indicating that their mutant genomes could be complemented by the helper replicase. Seven of these (3073.12, 3264.15/r, 3342.12/r, 3471.3/t, 3690.12, 3722.12, and 3795.12) contain

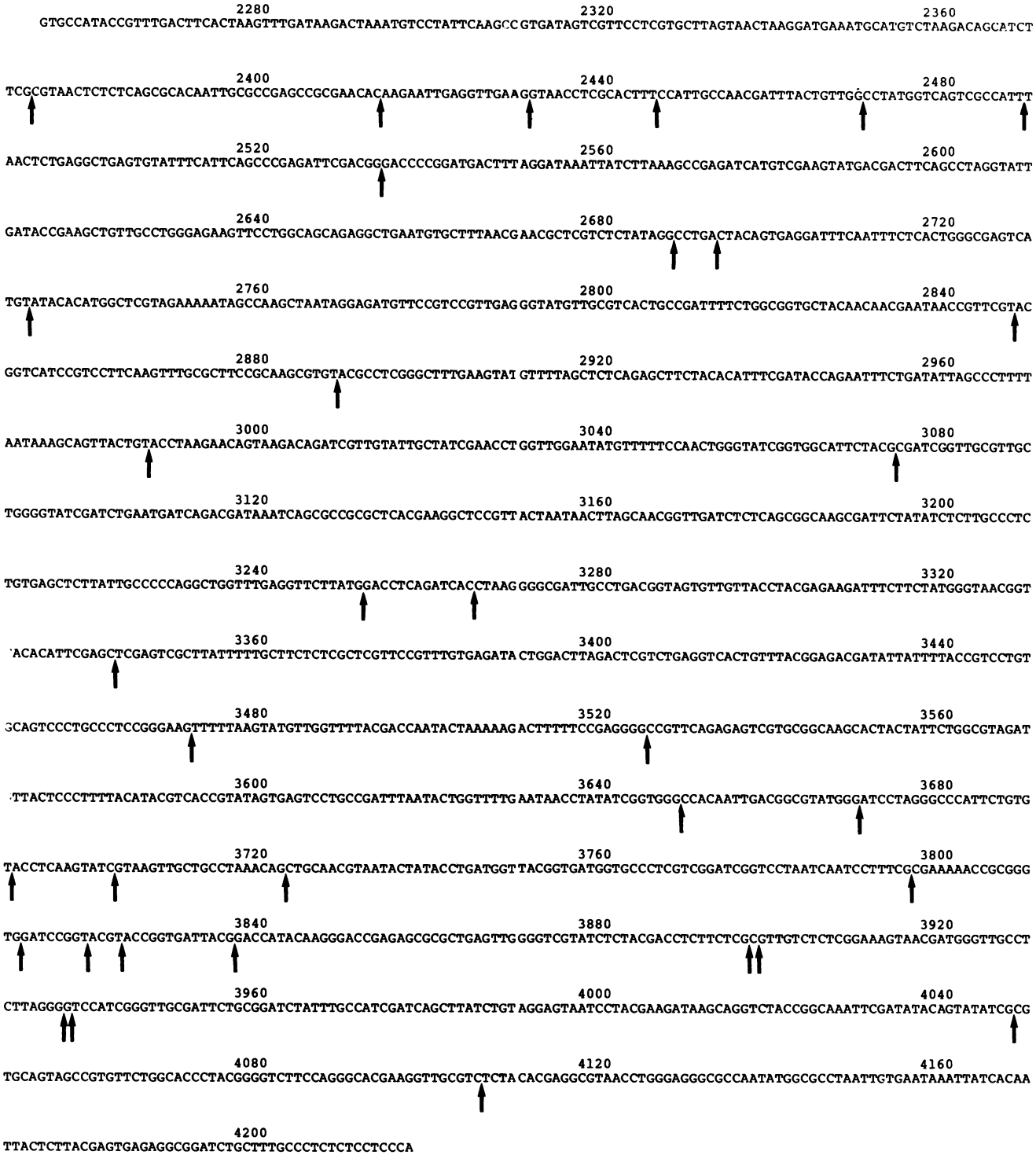

FIG. 1. Sites of *Bgl*II linker insertions within the cDNA of the Qβ replicase gene. Nucleotides 2251 to 4217 of Qβ RNA are numbered in the 5′-to-3′ direction above the nucleotide sequence (Mekler, Ph.D. thesis, amended in nine single nucleotide positions; A. B. Jacobson, personal communication). Arrows indicate the locations at which a *Bgl*II linker element was inserted into the replicase gene (20). For the construction of deletion genomes, restriction endonuclease *Nsi*I was used to cleave the cDNA between nucleotides 2352 and 2353 and *Bgl*I was used to cleave the cDNA between nucleotides 4139 and 4140. The initiation codon (ATG) of the replicase gene is located at position 2352 and the termination codon (TAA) is located at position 4119.

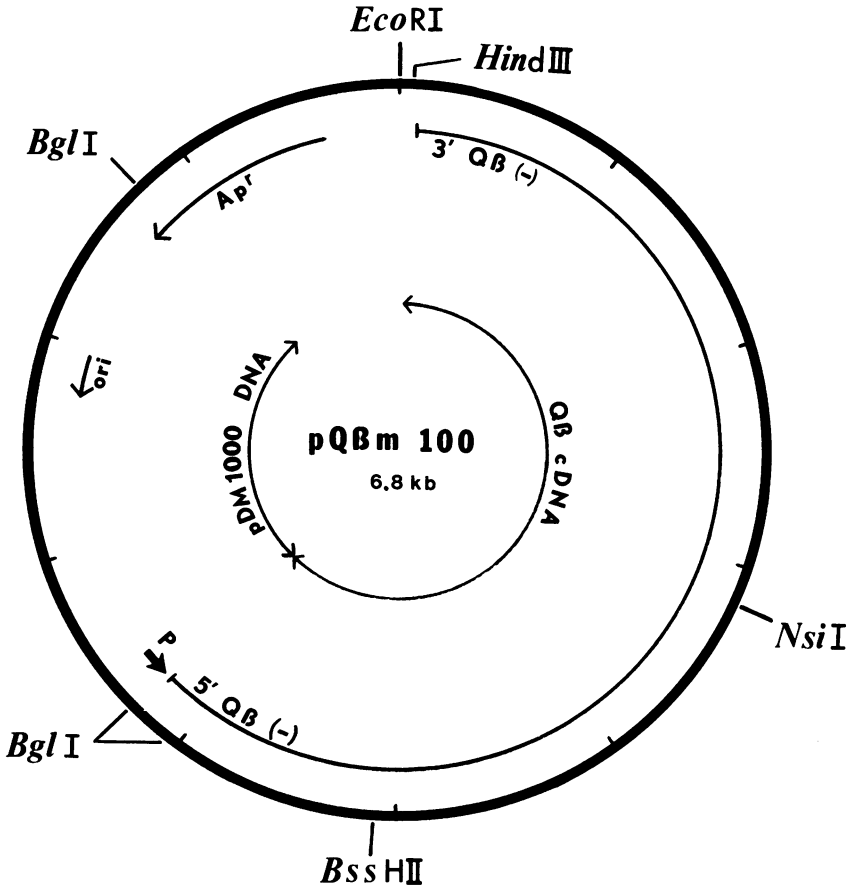

FIG. 2. Plasmid pQβm100. Details concerning the construction of this plasmid are described in Materials and Methods. Transcription in vivo from the modified *trp* promoter resulted in full-length copies of Qβ minus-strand RNA. This plasmid carried an E1a replicon and an ampicillin resistance gene. kb, Kilobases.

replicase gene mutations in a region where the coding sequences are critical for replicase function (20). In contrast, six of the mutant viruses had very low plaque yield ratios (less than 0.15), indicating that their mutant genomes could not be sufficiently complemented by the helper replicase. Four of these mutants (2372.12, 2413.15/r, 4047.12, and 4105.6) contained genome mutations in either the 5′ or the 3′ end of the replicase gene, where coding sequences appear to be less important for replicase function (20). Furthermore, two of these mutant phages (2372.12 and 4105.6) contained replicase genes that were fully capable of producing functional replicase. This result is especially interesting since the 3936.15/r and 3937.12/r mutants, which generated no infectious virus particles in transformed cells (see above), also contain mutations at the 3′ end of the replicase cistron that support the production of functional replicase (20). These results suggest that the two N-terminal and four C-terminal genome mutations that cannot be complemented by Qβ replicase in *trans* are *cis*-dominant mutations that affect the function of the viral RNA and are lethal to virus production.

When progeny mutant virus particles were used to reinfect host cells, the results paralleled the original data (Table 1) both in the presence and in the absence of helper replicase. Moreover, these results were repeated for several subsequent generations in which progeny virus particles were retitered: i.e., progeny of defective phage particles always required the presence of the wild-type helper replicase, and progeny of viable phage particles faithfully reproduced the same discernable phenotypes. These observations clearly indicate that wild-type genome revertants were not being produced during reinfection.

The results of these experiments suggest that the nucleotide sequences present within the replicase cistron at the 5′ and 3′ ends of the Qβ replicase gene contain information that is required for productive viral infection as well as for coding function, while the RNA sequences present within the central domain of the gene are required primarily to ensure replicase function (20).

Rebuilding deletion mutant genomes. We further explored the *cis*-acting RNA properties contained within the Qβ replicase cistron by examining a number of deletion mutant genomes. Whereas the insertion element studies focused on small local mutations, the availability of deletion mutants enabled us to examine the effects of omitting relatively large sequences within the replicase gene and to approximate boundaries of *cis*-acting areas. Deletion genomes were prepared by mixing *Bgl*II-digested fragments from several of the linker insertion mutant replicase (pRep201) clones described above (20). Since the *Bgl*II linker element in each mutant clone was unique to the pRep201 plasmid, we were able to mix and match *Bgl*II restriction digests of these clones to construct several sets of deletion genomes. Each of 41 different deletion mutant replicase genes was rebuilt into the whole viral cDNA genome in pQβm100 as described above.

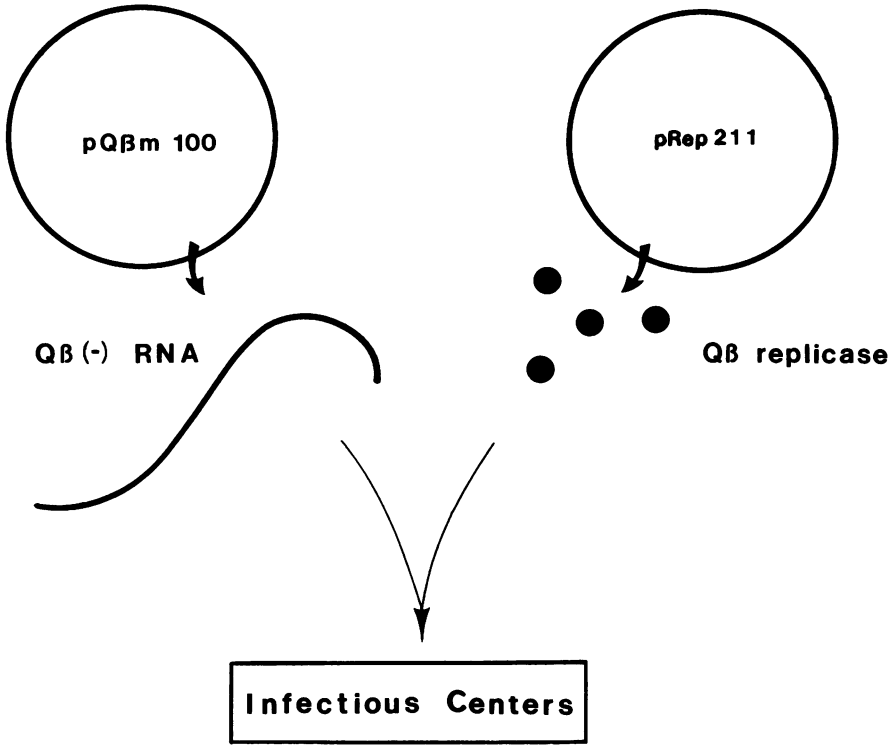

FIG. 3. Assay for the production of infectious virus particles. In *E. coli* NP20 host cells, the replicase plasmid, pRep211(3936), contained a p15a replicon, carried a kanamycin resistance gene, and produced active Qβ replicase by transcription. Simultaneously, the compatible plasmid pQβm100 produced full-length Qβ minus-strand RNA by transcription. The presence of infectous centers on bacterial lawns prepared from transformed host cells indicates that viral reproduction occurred within these cells to produce infectious Qβ phage particles.

Figure 4C illustrates the sizes and locations of the putative ribonucleotide deletions contained within each mutant genome. These deletions spanned four major regions of interest within the replicase gene: (i) 5′-terminal sequences containing the translational operator region (26, 28), (ii) the M-binding region for Qβ replicase (18), (iii) the region coding for the conserved amino acid sequence, and (iv) 3′-terminal sequences. The largest active deletion genome, the 538-nucleotide deletion mutant (deletion 18), was characterized by oligonucleotide sequence analysis (Fig. 5).

Obtaining deletion mutant virus particles. To generate mutant phage particles, *E. coli* NP20 host cells were first transformed with the helper replicase plasmid, pRep211 (3936), and then with each of the 41 deletion mutant pQβm 100 plasmids. As expected, mutant virus was produced only in the presence of helper replicase. When plaques formed, mutant phage particles were isolated from each transformation as described above.

Redetermining titers of deletion mutant virus particles. Upon reinfection, each deletion mutant phage required the presence of helper replicase to form plaques. Plaque yield ratio values were therefore determined for each mutant virus in the presence of the pRep211(3936) helper replicase plasmid. In Fig. 4C, deletions shown above the line indicate those genomes that were active in the presence of helper replicase. These gave variable plaque yield ratios ranging from 0.11 to 0.95 when compared with a wild-type Qβ virus control (Table 2). Deletions shown below the line indicate those genomes that did not support viral infection and were thus considered to be lethal to viral reproduction. These experiments were repeated several times, yielding consistent results (Table 2). The data suggest that RNA sequences in certain regions of the Qβ genome can be deleted without any effect on genome function, while other sequences comprise regions that are indispensable to virus formation (Fig. 4C).

Deletions in the 5′ end of the replicase gene, between the translational operator region and the M-binding site for Qβ replicase (deletions 1 through 7), resulted in functional genomes that generated infectious virus particles. All of these deletions, however, were relatively small (Table 2).

Within the M-binding region (nucleotides 2545 through 2872), it appears that any one of three M-binding elements can be deleted and infectious virus is still produced: M5 (deletion 8), M2b (deletion 11), or M11 (deletion 13). However, if M5 plus M2b (deletion 9) or all three M-binding elements (deletion 10) were deleted, no plaques were produced. Apparently, all three binding elements are not simultaneously required for the generation of infectious virus.

The 538-nucleotide deletion between nucleotides 2986 and 3524 (deletion 18) removed almost the entire region of conserved amino acid sequence that is present in four different RNA phage replicase genes (11, 12, 27). Figure 5 shows an electrophoretic characterization of this mutant genome. It is striking that this entire 538-nucleotide region, as well as all subsets of this region (deletions 16, 17, 24, 25, 26, *c*, *d*, *e*, and *g*), can be deleted without the loss of infectious virus production in the presence of helper replicase (Fig. 4C). This region of the replicase gene has been shown to be required for the synthesis of functional Qβ replicase (20). It is also interesting that any of the deletions extending outside this conserved region, on either the 5′ or 3′ side, are lethal to virus propagation (Fig. 4C). Apparently,

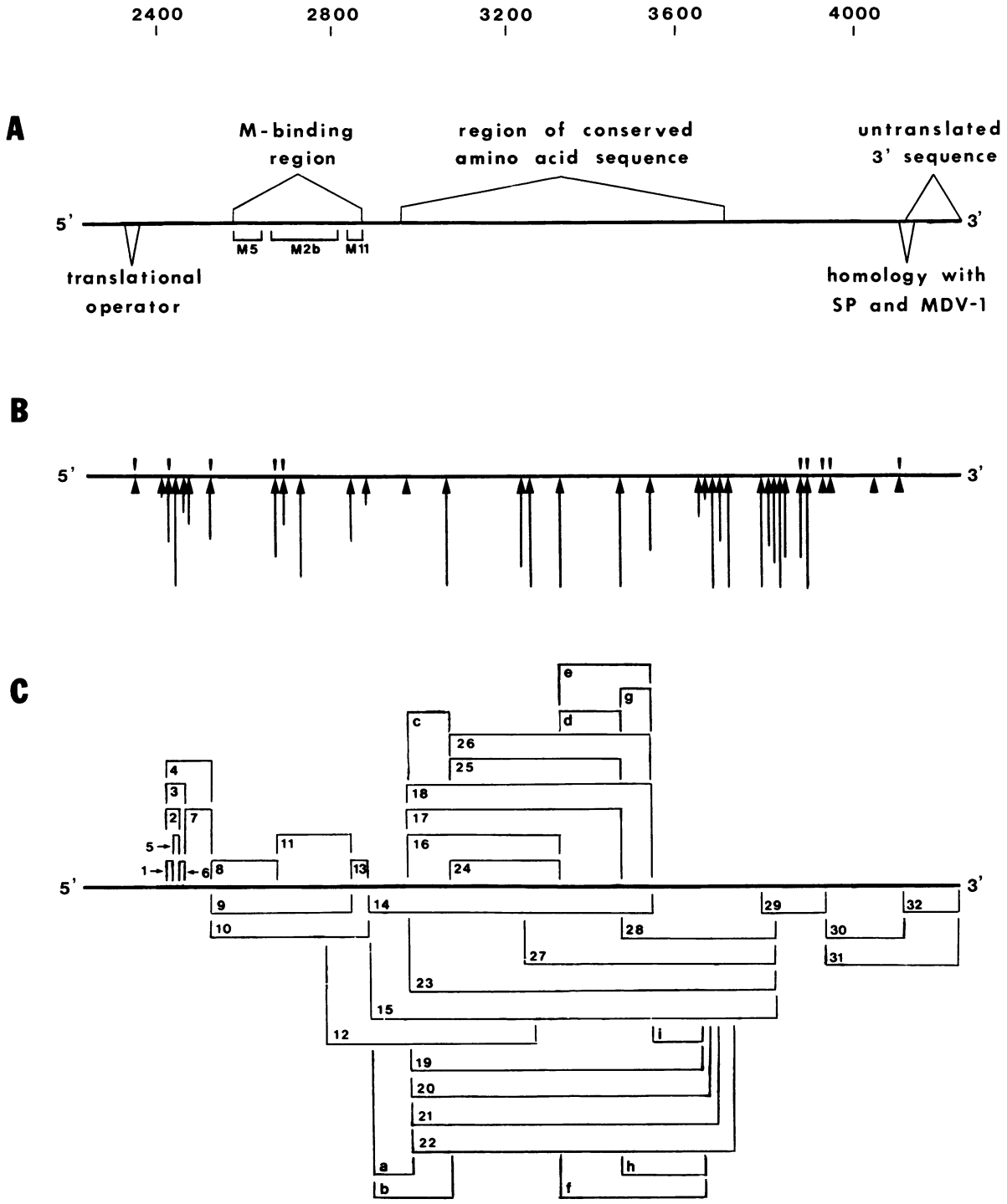

FIG. 4. Summary of mutation analysis. The three maps illustrate the Qβ RNA sequence from nucleotides 2251 through 4217 (3′ terminus). (A) Known functional regions within the replicase gene; (B) results of the insertion mutant genome analysis; (C) results of the deletion mutant genome analysis. All three maps are aligned to illustrate how the mutated regions correspond to functional domains. From left to right, the regions shown in panel A encode nucleotides 2349 to 2368 (26, 28), 2545 to 2872 (18), 2970 to 3715 (11, 12, 27), 4096 to 4120, (11, 19), and nucleotides 4117 to 4217. Panel B shows the results of the linker mutation analysis. Arrows indicate points of linker insertions (Table 1); the sizes of the arrows reflect relative plaque yield values, with the longest arrow representing a plaque yield ratio of 1.0; marks above the line indicate that genomes containing mutations at these sites produce active Qβ replicase (20). Deletions in panel C are indicated by number or letter (Table 2). Deletion genomes that could produce virus in the presence of helper replicase are shown above the line; deletion genomes that did not result in infectious virus particles are shown below the line.

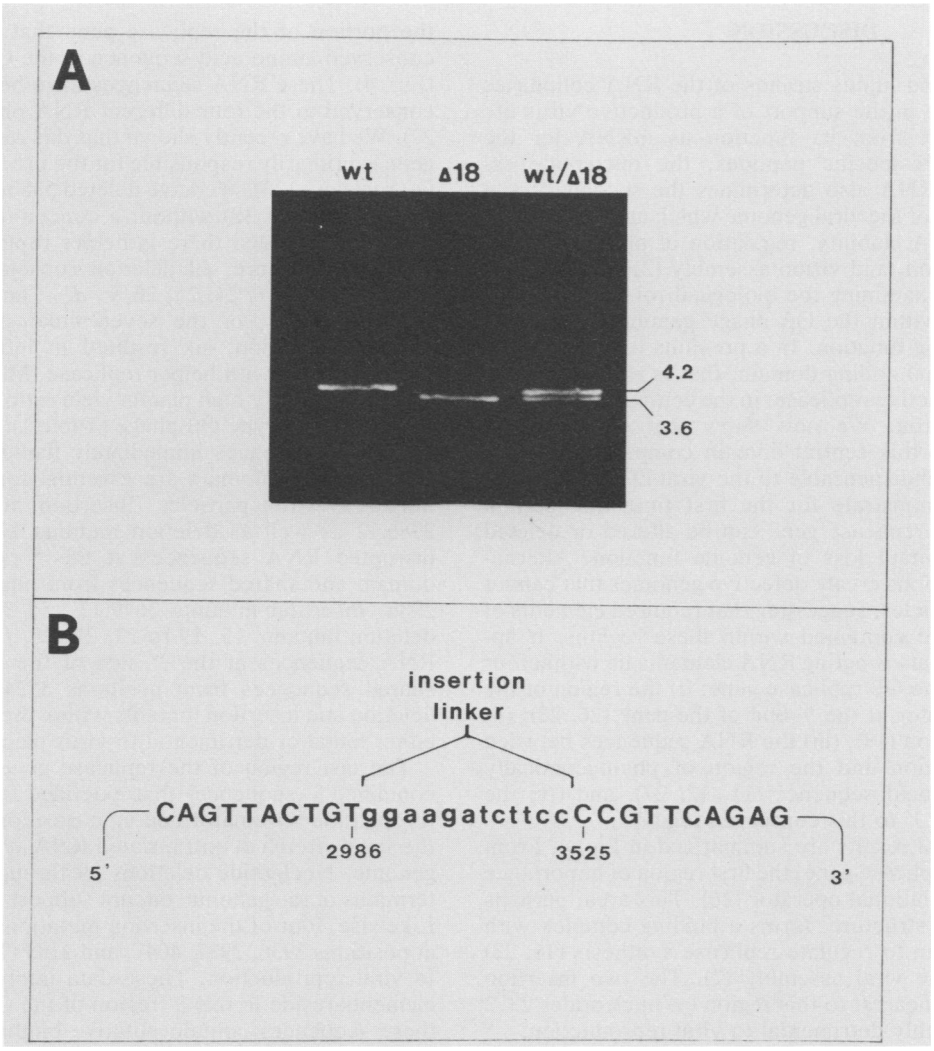

FIG. 5. Characterization of Qβ deletion 18 virion RNA. (A) RNA isolated from virus particles was analyzed on a 1.8% agarose gel that was cast and run in 50 mM Tris acetate (pH 7.5)–3 mM EDTA–0.5% ethidium bromide. The gel was run at 20 V for 2 h and then photographed with an FCR-10 camera system from Fotodyne with Polaroid 667 instant-pack film. The gel shows the relative mobilities of wild-type (wt) Qβ RNA, Qβ deletion 18 mutant RNA, and a mixture of the two. Sizes (in kilobases) are given on the right. (B) The nucleotide sequence of Qβ Δ18 RNA was confirmed by sequence analysis. Shown is the local nucleotide sequence in the region of the *Bgl*II linker insertion element, where 538 nucleotides were deleted from the wild-type Qβ RNA sequence between positions 2986 and 3524.

there are elements of RNA sequence to either side of this phylogenetically conserved region that are required for virus production.

Sequences deleted between position 3669 and the 3′ end of Qβ RNA rendered the genomes incapable of producing virus. These sequences include the 3′ untranslated region (deletion 32), the region of the terminator codon, the region of sequence homology between SP RNA and midivariant RNA (11), and any deletions that flank or extend into the 3′ side of the central conserved region of the gene.

As with the insertion mutants, when deletion mutant phages were used to reinfect host cells, the results were consistent with the original data (summarized in Table 2). These results were also repeated for several subsequent generations in which titers of progeny virus particles were redetermined, indicating that wild-type genome revertants were not being produced upon reinfection.

Control for defective interfering replicases. Other investigators have reported that certain defective Qβ replicases can interfere with Qβ phage infection (11). To eliminate the possibility that some of our mutant genomes were producing replicases that interfered with the plasmid-derived helper replicase, we incorporated an amber mutation into the replicase genes of several of our insertion and deletion mutant pQβm100 plasmids at position 2732 (20). Protein gel analysis confirmed that these plasmids do not synthesize Qβ replicase. Thus, when these plasmids were introduced into host cells that contained the pRep211(3936) plasmid, the only replicase protein present was the wild-type helper. In each case, experimental plaque yield ratios were determined (as described above), and the results paralleled those summarized in Tables 1 and 2. These data are consistent with our experimental results, suggesting that interfering replicases were not present in our experiments and that our results were not affected by altered translation of the mutant genomes.

DISCUSSION

Both the plus and minus strands of the RNA coliphages play an active role in the support of a productive virus life cycle. Quite apart from its function as mRNA for the synthesis of phage-specific peptides, the nucleotide sequence of phage RNA also determines the secondary and tertiary structures of the viral genome which are required for bacteriophage RNA stability, regulation of protein synthesis, RNA replication, and virion assembly (21, 24, 27).

We have been examining the biological role of *cis*-acting RNA sequences within the Qβ phage genome apart from their protein coding function. In a previous report (20), we mapped a functional coding domain, that is responsible for the production of active replicase, in the central region of the Qβ replicase cistron. We now show that several RNA sequences outside this central domain comprise *cis*-acting elements that are indispensable to the viral life cycle.

Our results demonstrate for the first time that certain regions of the Qβ replicase gene can be altered or deleted without a concomitant loss of genome function. Alternatively, some mutations create defective genomes that cannot generate virus particles, suggesting that required elements of RNA sequence are contained within these regions. It appears that functional *cis*-acting RNA elements lie within four major regions of the Qβ replicase gene: (i) the region of the translational operator at the 5′ end of the gene (26, 28), (ii) the M-binding region (18), (iii) the RNA sequences between the M-binding region and the region of phylogenetically conserved amino acid sequence (11, 12, 27), and (iv) the sequences located 3′ to this conserved region.

Our experimental results are summarized in Fig. 4. From the 5′ end of the replicase gene, the first region of importance is that of the translational operator (26). This area, perhaps through its folded structure, forms a binding complex with the Qβ coat protein to regulate replicase synthesis (16, 28) and acts to initiate viral assembly (2). The two insertion mutations located nearest to this region (at nucleotides 2372 and 2413) were highly detrimental to viral reproduction.

Upon examining mutants of the M-binding region, we observed some very interesting results. This region, spanning from nucleotides 2545 to 2872, is composed of three elements: M5, M2b, and M11 (18). Insertion mutations within this region had little or no effect on viral infectivity (Fig. 4). Similarly, small deletions that eliminated any one of the three binding elements (deletions 8, 11, and 13) were not lethal to the viral life cycle. These results were unexpected since a hairpin stem present in the M2b region is conserved in both Qβ RNA and SP RNA and has been implicated in the recognition of ribosomal protein S1 (6), a subunit of the Qβ replicase holoenzyme (5). We further observed that the removal of both the M5 and the M2b elements (deletion 9) or all three of the binding elements simultaneously (deletion 10) resulted in noninfectious virus. Since the M-binding region is quite large, extending across some 320 nucleotides (18), it is possible that relatively small deletions or insertions within these sequences do not completely destroy all binding elements, whereas larger deletions begin to interfere more drastically with the replicase RNA binding process.

Several mutations were made immediately upstream from the M-binding sequences. All of these (insertion mutations 2430.15/r, 2445.9, 2469.12, 2488.15/r, and 2533.15/r and deletions 1 through 7) resulted in infectious virus particles (Tables 1 and 2). These data suggest that this region of Qβ RNA is expendable with respect to viral reproduction.

The most striking results were observed upon examining the portion of the replicase gene that encodes the highly conserved amino acid sequence in the Qβ replicase peptide (Fig. 4). These RNA sequences have been phylogenetically conserved in the four different RNA phage groups (11, 12, 27). We have recently shown that this region of the replicase gene is primarily responsible for the production of functional Qβ replicase (20). We have deleted 538 nucleotides from this region (deletion 18) without a concomitant loss of genome function, provided there is helper replicase present in the host. Furthermore, all deletion subsets within this region (deletions 16, 17, 24, 25, 26, *c*, *d*, *e*, and *g*) yielded similar results. Finally, of the seven linker insertion mutations within this region, six resulted in infectious virus when complemented with helper replicase. Most of these mutants yielded relatively high plaque yield ratio values, comparable to that of wild-type Qβ phage (Table 1). On the other hand, the RNA sequences immediately flanking this highly conserved central domain are essential for the production of infectious virus particles. Insertion mutants 2888.12 and 2986.12 as well as deletion mutants 12, 14, 15, *a*, and *b* disrupted RNA sequences at the 5′ side of this central domain and shared sequences from positions 2888 through 2986. Insertion mutants 3649.12 and 3669.12/r as well as deletion mutants 15, 19 to 23, 27, 28, *f*, *h*, and *i* disrupted RNA sequences at the 3′ side of the central domain and shared sequences from positions 3524 through 3669. All deletion and insertion mutants within these two regions were either lethal or detrimental to virus plaque formation.

The last region of the replicase gene that we examined contained 3′ sequences that extended from nucleotide 3669 through the terminator codon at position 4117 and included the small stretch of untranslated RNA at the 3′ end of the Qβ genome. Nucleotide deletions 29 through 32, within the 3′ terminus of the genome, did not support viral infection at all. Likewise, four of the insertion mutations within this region, at positions 3936, 3937, 4047, and 4105 (Table 1), were lethal to viral reproduction. These data imply that critical RNA elements reside in this 3′ region of the Qβ genome. Indeed, these sequences contain putative binding elements for Qβ replicase that extend from the 3′ terminus of the RNA into the 3′ coding region of the replicase gene (22). These elements include a region of sequence homology among Qβ RNA, SP RNA, and midivariant RNA (12, 19, 22) in the region of the replicase gene termination codon. Note that although deletion 29, which removed a portion of this 3′ region, did not yield infectious virus, many of the insertion sequences located 5′ to position 3936 resulted in viable phenotypes with relatively high plaque yield ratio values (Table 1). A more detailed analysis of the region between nucleotides 3669 and 3936 will be needed to clarify these ambiguous results.

The results of our mutation analyses indicate that there are multiple *cis*-acting RNA sequences within the Qβ replicase gene that are required for viral reproduction. It is likely that many of the lethal genome mutations in these *cis*-dominant regions affect local RNA folding patterns or long-range base pair interactions or both (13). Such conformational changes could easily destroy surfaces for protein interactions, especially in the region of the translational operator (16, 26, 28), the M-binding region (18), and the 3′ terminus of the replicase gene (22). Furthermore, the replication mechanism of the bacteriophage RNA during infection depends upon the conformational fluidity of RNA secondary structure: as a template RNA strand is copied into a complement by the RNA replicase, the product strand is displaced from base-pairing interactions with the template by the intermittent

formation of local, intramolecular pairings (21, 24; V. Axelrod and D. R. Mills, unpublished data). This process prevents the formation of an extended RNA-RNA duplex which is nonfunctional during RNA replication (4, 22). Insertions or deletions of the RNA sequence that interfere with this folding process could therefore be quite detrimental to the replication of the RNA. It is also possible that the *cis*-dominant mutations directly or indirectly affect viral assembly or lysis of host cells during infection or both. Finally, it should be considered that although we have discussed genome alterations as mutations of the plus strand, it is possible that complementary mutations in the minus-strand RNA counterparts also affect the viral life cycle.

Our data clearly indicate that the organization and packaging of information into the RNA phage genome is much more complicated and intricate than originally thought. As such, it had not been possible in the past to examine isolated gene function in the RNA bacteriophage genomes. Since we now have a system in which we can supply a phage-coded protein in *trans* from a plasmid, we can test many of these genome mutants for a variety of *cis*-acting RNA functions, including binding to replicase, initiation and completion of RNA synthesis, packaging, and RNA stability. Moreover, it will be possible to explore the functional RNA properties that lie within the RNA sequences of the coat, readthrough, and maturation cistrons by supplying one or more of the phage proteins in *trans*. We can then determine how functional coding domains are organized with respect to elements of RNA sequence throughout the remainder of the RNA bacteriophage genome.

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