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Genetics

An *Escherichia coli* mutant with a temperature-sensitive function affecting bacteriophage Qβ RNA replication

(Qβ replicase/elongation factor-T/ribosomal protein S1/host factor)

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ABSTRACT **We report the isolation of an *E. coli* mutant capable of supporting replication of bacteriophage Qβ at 33°, but not at 40°. Coliphages f2, R23, fd, and λ formed plaques on mutant cells at both temperatures. Temperature-shift experiments showed that bacteriophage Qβ replication was blocked in the mutant within the first 20–30 min of infection. The defect did not prevent translation of the Qβ polymerase gene or assembly of catalytically active Qβ replicase molecules. In fact, mutant cells infected at 40° hyperinduced replicase active both *in vivo* and *in vitro*. However, zone sedimentation of the *in vivo* RNA product showed it to consist of partially double-stranded material sedimenting at 9 S, with little or no viral 32S RNA. The 9S RNA was also found, along with a predominant peak of 32S RNA, in parental cells infected at 40°, but not in cells infected at 33°. It thus appears that the temperature-sensitive component is required for viral RNA replication, but not for other RNA synthesis catalyzed by the replicase. Uninfected mutant cells grew normally at 40° in nutrient broth, but not in glucose- or glycerol-minimal media. Revertants selected for their ability to grow in minimal medium at 40° also supported bacteriophage Qβ replication at 40°.**

Bacterial proteins participating in the replication of bacteriophage Qβ RNA *in vitro* as subunits of the Qβ RNA replicase have been identified as ribosomal protein S1 (1) and protein synthesis elongation factors EF-Tu and EF-Ts (2). Another bacterial protein (host factor) active in the reaction has been purified from uninfected cells (3). This heat-stable protein is required for the enzyme to initiate synthesis with Qβ RNA as template (3, 4) and functions separately from the enzyme.

The function of these four bacterial proteins can be assigned to different stages of the overall Qβ RNA replication reaction. Host factor protein and S1, both RNA-binding proteins (3, 5–7), are required for the first step, synthesis of the Qβ complementary strand when Qβ RNA is the template for the replicase; neither protein is required for the second stage, synthesis of Qβ RNA when the complementary strand is template, or for RNA synthesis catalyzed by the replicase with other naturally-occurring or synthetic RNA templates (8, 9). The elongation factors are required for initiation of RNA synthesis even with synthetic RNA templates, but need not be present in stoichiometric amounts during RNA chain elongation, which is evidently catalyzed by the phage-specified subunit of the enzyme (10).

The relation between the activities of these four bacterial proteins in Qβ RNA replication and in uninfected cells is not clear. Treatment of the Qβ replicase with reagents that abolish or alter its EF-T activities in protein synthesis leaves its RNA polymerase activity intact (8, 11, 12), and indicates that the elongation factors function differently in protein and bacteriophage RNA synthesis. Travers (13) has suggested that EF-T has a common function in Qβ RNA replication and as a transcriptional control factor in uninfected cells. S1 is active as a ribosomal protein in the translation of naturally occurring mRNA (14), but inhibits translation when present as a free molecule (5, 14). As a subunit of the Qβ replicase, S1 may be required not only for recognition of Qβ RNA as template (9), but also for the activity of the enzyme as a translational repressor (15). It is not clear which, if either, of these activities is analogous to its function as a ribosomal protein. The function of host factor protein in uninfected cells is not known. A role in RNA metabolism is suggested by its ability to bind a variety of single-stranded RNAs (3, 5) and in protein synthesis by the finding that the bulk of the host-factor protein in crude extracts is associated with ribosomes, though not as a unit ribosomal protein (16).

Bacterial mutants conditionally defective in each of the proteins active in Qβ RNA replication would clearly be a valuable adjunct to further biochemical studies of their functions in infected and uninfected cells. We report here the isolation and properties of a temperature-sensitive *Escherichia coli* mutant defective in Qβ RNA replication. The altered component in this mutant is required for an early step of Qβ RNA replication *in vivo*. It is either still active at the nonpermissive temperature or not required for translation of the Qβ polymerase gene in Qβ-infected cells, for replication of other bacteriophage RNAs or for the growth of uninfected cells in nutrient broth.

MATERIALS AND METHODS

Bacteriophage and Bacterial Strains. RNA bacteriophage Qβ, R23, and f2 were obtained from J. T. August; DNA bacteriophage fd from R. C. Valentine, and phage λ_c from J. Hurwitz. *E. coli* K35 (HfrC, *met, his, rel*) was obtained from N. D. Zinder. *E. coli* K35 ($Q\beta^{ts}$) is the temperature-sensitive, Qβ-resistant mutant of K35 described below.

Growth Media. The nutrient broth used has been described (17). Minimal medium contained, per liter: 7 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 1 mM $CaCl_2$, 0.25 mM $MgSO_4$, 3 μM $FeCl_3$, 0.2% glucose, and 40 mg each of methionine and histidine. Agar plates were prepared by addition of 10 g of agar per liter.

Chemicals. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from the Aldrich Chemical Co., Milwaukee, Wisc.; actinomycin D from the Sigma Chemical Co., St. Louis, Mo.; and rifampicin, 2-phospho*enol*pyruvate, and pyruvate kinase from Calbiochem. Labeled compounds were obtained from Schwarz/Mann Laboratories, Orangeburg, N.Y. Other chemicals were purchased from standard commercial sources.

Mutagenesis and Selection of Temperature-Sensitive Qβ-Resistant Mutants. The procedure was essentially as described for the isolation of f2-resistant mutants (17). *E. coli* K35

Abbreviations: EF, elongation factor; S1, ribosomal protein S1; PFU, plaque-forming units.

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Table 1. Efficiencies of plating of different bacteriophage on parental and temperature-sensitive cells

Bacteriophage	Efficiency of plating*	
	Parental cells	Temperature-sensitive cells
Qβ	1.0	10^{-4}
f2	1.0	1.1
R23	1.6	0.8
fd	1.5	1.4
λc	1.7	1.5

* Values are the ratio of PFU/ml at 40° to PFU/ml at 33°; PFU were determined as described in *Materials and Methods*.

($Q\beta^{ts}$) was one of several-hundred mutants initially selected for resistance to Qβ at 40°.

Phage Titers. Plaque-forming units (PFU) were determined by the agar overlay method. Infected cells were lysed with chloroform (0.2%, vol/vol, final concentration) before being titered. Unless otherwise stated, *E. coli* strain Q13 was used as plating bacterium and incubation was at 37° for 18 hr.

Bacterial Growth. Bacterial mass was estimated by measuring the optical density (660 nm) of cultures. Viable cell concentrations were determined by plating a suitable dilution of cells on nutrient agar plates. Colonies were counted after incubation for 24 hr at 37°. With parental strain K35 growing exponentially at 40° in minimal medium (doubling time of 43 min), one optical density unit corresponded to 4.8×10^8 cells per ml. Protein, RNA, and DNA accumulation were measured by the Lowry *et al.* (18), orcinol (19), and diphenylamine (20) reactions, respectively, with serum albumin, yeast RNA, and salmon testes DNA as standards.

RESULTS

Isolation of Temperature-Sensitive Qβ-Resistant Mutant. Of several hundred clones resistant to Qβ at 40°, one was sensitive at 33°. This mutant, designated $Q\beta^{ts}$, retained the histidine and methionine growth requirements of the parental strain and supported the growth of bacteriophage f2, R23, fd, and λ at both 33° and 40° (Table 1).

Temperature-Shift Experiments. These experiments showed that the temperature-sensitive step of Qβ replication in mutant cells occurred within the first 20–30 min of infection (Fig. 1). The data also indicate that the temperature-sensitive component functions for a short time at 40°, because cells grown and maintained at 40° throughout infection produced less than 1 PFU per cell, whereas cells grown at 33° and shifted to 40° at the time of infection still produced 10–20 PFU per cell (Fig. 1). Conversely, once activity was lost at 40°, it was not immediately restored at 33°, because cells grown at 40° and shifted to 33° at the time of infection yielded fewer PFU than cells grown and infected at 33° (Fig. 1).

Synthesis of Phage-Specific RNA *In Vivo*. The intracellular events in RNA phage replication occurring primarily within the first 20–30 min of infection are (*i*) translation of the viral polymerase gene (21, 22), (*ii*) assembly of phage RNA replicase (23–25), and (*iii*) synthesis of phage complementary RNA (26, 27). Experiments were carried out to determine which of these events was blocked by the mutation.

Infected mutant cells synthesized phage-specific RNA at the nonpermissive temperature. In the experiment shown (Fig. 2), the amount of radioactivity incorporated into RNA from equivalent numbers of mutant cells infected at 40° or at 33°

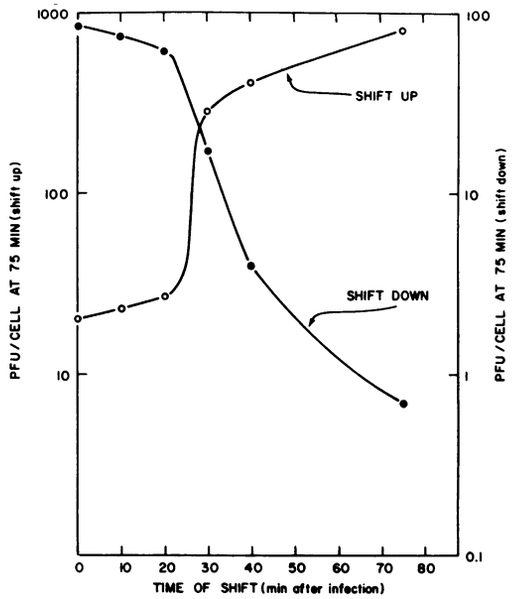

FIG. 1. Effect of temperature shifts on Qβ replication in mutant cells. In a shift-up experiment, cells were grown and infected at 33°. At different times after infection an aliquot was shifted to 40°. The yield of phage after a total of 75 min was then determined as a function of the time at which the infected cells were shifted. In a shift-down experiment, cells were grown and infected at 40°, and aliquots shifted to 33° at different times after infection. Phage titers were determined as described in *Materials and Methods*.

or from parental cells infected at 40° were 2.5×10^5, 1.6×10^5, and 3.9×10^5 cpm, respectively, whereas incorporation in uninfected cells was about 4×10^3 cpm. The phage-specific RNA synthesized in mutant cells infected at 33° consisted primarily of viral 32S RNA (Fig. 2*A*), whereas RNA synthesized in mutant cells infected at 40° consisted mostly of material sedimenting at about 9 S (Fig. 2*B*). This RNA was approximately 50% resistant to pancreatic ribonuclease in 0.15 M NaCl/0.015 M sodium citrate buffer, but was completely digested by this enzyme at lower ionic strength. Its sedimentation rate was not appreciably altered after denaturation in 1 M formaldehyde at 70°. Low-molecular-weight RNA was also found, along with viral 32S RNA, in parental cells incubated at 40° (Fig. 2*C*), indicating that its synthesis is related to infection at 40° rather than to the mutation.

These incorporation experiments show that mutant cells infected with Qβ RNA at 40° translate the Qβ polymerase gene and assemble active Qβ replicase molecules. As they accumulate little, if any, viral 32S RNA at 40°, the temperature-sensitive component appears to be required for Qβ RNA replication, but not for synthesis of the 9S RNA.

Induction of the Qβ RNA Polymerase. Furthermore, phage-specific RNA-dependent RNA polymerase activity could be assayed in extracts of mutant cells infected at 40° (Fig. 3). In fact, the specific activity of mutant cell extracts exceeded that of parental cell extracts after 20 min infection. As both extracts already contained saturating amounts of endogenous template, it appears that mutant cells synthesize more enzyme than parental cells. This could be a direct result of the small amount of Qβ RNA and, consequently, the reduced synthesis of coat protein in mutant cells. RNA bacteriophage coat protein represses translation of the phage RNA polymerase gene *in vitro* (30), and infection of nonpermissive cells with certain coat protein amber mutants of RNA bacteriophage results in more extensive than normal translation of the phage polymerase gene *in vivo* (22, 23, 31–34). Induction in mutant cells at 33° ap-

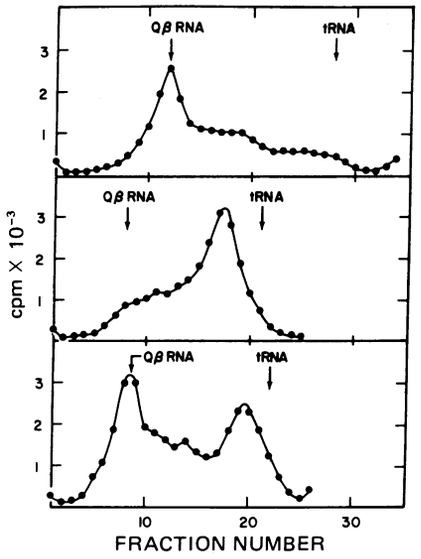

FIG. 2. Synthesis of phage-specific RNA *in vivo*. Cells were grown in nutrient broth (5 ml) to an optical density of 0.5 and infected with Qβ at a multiplicity of 5 PFU per cell. Five min after addition of phage, rifampicin was added to a final concentration of 60 μg/ml. After an additional 25 min at 40° or 35 min at 33°, [2-^{14}C]uracil (1 μCi/ml, 50 mCi/mmol) was added and 10 min later the cultures were chilled, the cells collected by centrifugation, and washed once with ice-cold buffer (contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The washed cells were suspended in 2.5 ml of the same buffer containing 1 mg of washed bentonite (28) and 2% sodium dodecyl sulfate. Lysis occurred within 15 min at 25°. Protein was removed from the lysed-cell suspension by three extractions with freshly distilled phenol saturated with buffer. Nucleic acids were precipitated from the aqueous phase by addition of two volumes of 95% ethanol and storage at −20° for at least 60 min. The precipitate was collected by centrifugation, dissolved in 0.1 M potassium acetate (pH 5.5) and precipitated again with ethanol. After a third ethanol precipitation, nucleic acids were dissolved in 1 mM neutralized EDTA. An aliquot of each sample containing approximately 50,000 cpm was mixed with Qβ [^{3}H]RNA, *E. coli* [^{3}H]tRNA (each containing 100,000 cpm) and 0.1 mM EDTA to give a final volume of 0.2 ml. Each sample was layered on top of a 5 to 20% (vol/vol) glycerol gradient (4.4 ml) containing 50 mM Tris·HCl (pH 7.6) and 0.01% sodium dodecyl sulfate. Different RNA species were separated by centrifugation at 55,000 rpm and 4° for 150 min in the Spinco SW65 rotor. Fractions (3 drops) were collected from the bottom of each tube and the total radioactivity in each fraction determined in a liquid scintillation spectrometer. Only the ^{14}C radioactivity is shown; the positions in each gradient of the Qβ RNA and tRNA markers are indicated by the arrows. (*A*) mutant infected at 33°; (*B*) mutant infected at 40°; (*C*) wild type infected at 40°.

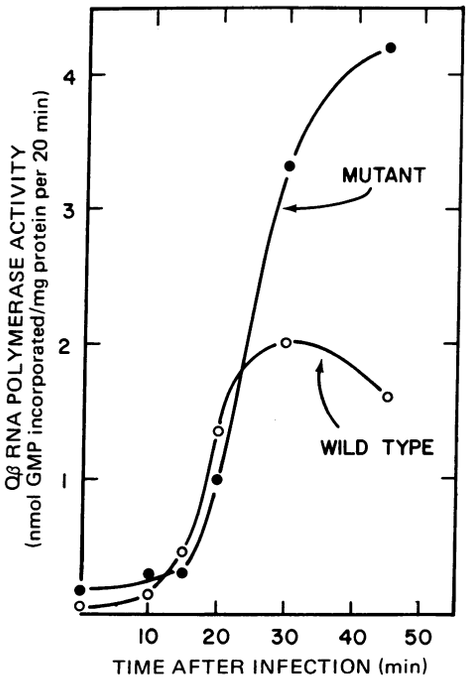

FIG. 3. Induction of Qβ RNA replicase in infected cells. Cells were grown in nutrient broth to an optical density of 0.7 and infected with Qβ at a multiplicity of 5 PFU per cell. At different times after infection, an aliquot containing approximately 4 × 10^{8} cells was withdrawn and assayed for Qβ replicase activity. The assay measures incorporation of [^{14}C]GMP from GTP into acid-insoluble material. Cells were collected by centrifugation and suspended in 0.04 ml of buffer containing 0.1 M Tris·HCl (pH 8.5), 10% (wt/vol) of sucrose, 2 μg of lysozyme, and 5 mM EDTA. After 30 min at 4°, the cells were lysed by addition of the nonionic detergent Brij-58 [0.1%, (wt/vol), final concentration] and 10 mM $MgCl_2$. The RNA synthesis reaction mixture (0.3 ml) contained 100 mM Tris·HCl (pH 7.6), 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 1 mM each of ATP, UTP, CTP, and [8-^{14}C]GTP (2000 cpm/nmol), 4 mM phospho*enol*pyruvate, 1 μg of pyruvate kinase, 1 μg of rifampicin, 1 μg of actinomycin D, and 0.03 ml of Brij extract (0.1–0.3 mg of protein). Incubation was for 20 min at 33°, after which acid-insoluble radioactivity was measured as described (29). Addition of exogenous template [poly(C)] did not alter the amount of incorporation, and indicates that sufficient template to saturate the enzyme was already present in the extracts. Incorporation catalyzed by extracts of uninfected cells was less than 0.2 nmol of GMP/mg of protein per 20 min.

peared normal whether the enzyme activity was assayed with endogenous template at 33° or at 40° (not shown).

Properties of Uninfected Cells. Both mutant and parental cells grew well in nutrient broth at 40° (doubling time of 30 min; data not shown) but in minimal media, with either glucose or glycerol as carbon source, mutant cells exhibited temperature-sensitive growth (Fig. 4). Four spontaneous revertants were selected that grew as well at 40° as parental cells. All of these revertants required histidine and methionine for growth and supported Qβ replication at 40° (efficiencies of plating were 0.2–0.6), suggesting that the two temperature-sensitive phenotypes are genetically related.

We have not been able to identify the primary defect preventing growth in minimal media. Mutant cells accumulated protein, RNA, and DNA at 40° for at least 180 min after they ceased division, albeit at a lower rate than parental cells. The mutant cells appeared normal from the sedimentation pattern of their stable RNA synthesized at 40° (measured by [2-^{14}C] uracil incorporation) and in DNA replication at 40°, as analyzed by a density shift experiment (35). Even after their growth had ceased at 40° in glycerol-minimal medium, mutant cells were able to induce β-galactosidase (36) to the same differential rate as wild-type cells, although it took 60 min for mutant cells to achieve this rate and only about 30 min for wild-type cells. When cultures of mutant cells were shifted from 40° to 33°, the number of viable cells increased 2- to 3-fold within 10 min, and normal exponential growth resumed after 20 min. This rapid increase in cell number, which also occurred in the presence of chloramphenicol, 100 μg/ml, has been reported to occur with temperature-sensitive cell-division mutants (35). Mutant cells incubated in minimal medium at 40° appeared somewhat longer than parental cells grown under the same conditions, though very long filaments or cells lacking DNA (37) were rarely observed.

DISCUSSION

Mutants such as K35 (Qβ^{ts}) should aid in understanding the functions of bacterial proteins active in bacteriophage RNA

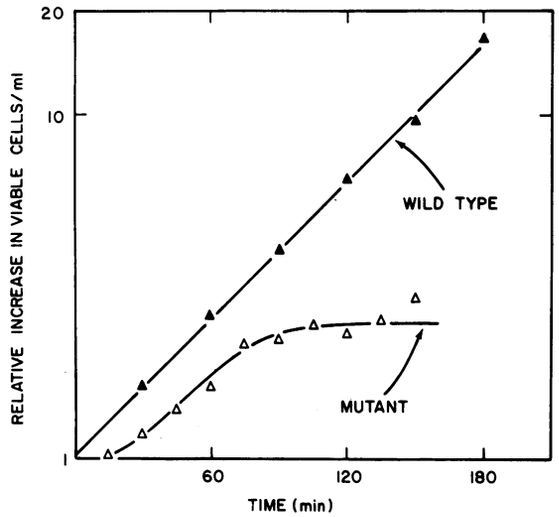

FIG. 4. Growth of mutant and parental cells in glucose minimal medium at 40°. Cells were grown to stationary phase in glucose minimal medium at 30°. An aliquot was then used to inoculate a flask containing the same medium equilibrated at 40°. At different times aliquots were withdrawn for viable count determination, as described in *Materials and Methods*. Initial cell concentrations were about 10^7 cells per ml.

replication, as well as their functions in uninfected cells. We have now shown that such mutants can be isolated. The temperature-sensitive component in K35 ($Q\beta^{ts}$) is specifically required for an early step in Qβ RNA replication *in vivo*, but it is not required or still active at the nonpermissive temperature in the replication of f2 and R23 RNA, and for the synthesis of 9S RNA in Qβ-infected mutant cells. Of the bacterial proteins required for Qβ RNA replication *in vitro*, host factor and S1 are most likely to manifest these properties *in vivo*. Both proteins are required for RNA synthesis catalyzed by the Qβ replicase only when Qβ RNA is a template (8, 9). This occurs early in infection (26, 27), when a temperature-sensitive defect in either protein would be expected to manifest itself in temperature-shift experiments (Fig. 1). For the same reason, defective host factor or S1 would not be expected to prevent synthesis of the 9S RNA. In one respect, S1 is a more attractive candidate than host factor, because the host factor is a heat-stable protein at least in regard to its activity in Qβ RNA replication (3). The temperature-sensitive component in K35 ($Q\beta^{ts}$) is also not required or still active at the nonpermissive temperature for translation of the Qβ RNA polymerase gene and for the growth of uninfected cells in a rich growth medium. An important implication of this phenotype is that it may be possible to alter, by mutation, proteins required for Qβ RNA replication, so that they no longer can function in that reaction but can still carry out major cellular functions required for growth.

It is difficult to predict how defects in bacterial proteins required for Qβ RNA replication should affect replication of RNA from bacteriophage other than Qβ, because these replication reactions have not been studied in as much detail. K35 ($Q\beta^{ts}$) supports replication of RNA bacteriophages f2 and R23 at 40° as measured by the plaque assay, though we cannot exclude a reduction in phage yield at this temperature relative to K35 parental cells. Federoff and Zinder (38) have isolated an RNA-binding factor from f2-infected cells that was required for f2 RNA polymerase activity with either f2 RNA or the f2 complementary strand as template, and which could not be replaced by purified host factor required for Qβ RNA replication (38). However, it is not clear whether the f2 factor was required instead of the Qβ host factor, in which case a Qβ host factor mutant should be permissive for f2 RNA replication, or in addition to it. The Qβ and f2 replicases are thought to contain the same bacterial subunits (39). If all of these proteins are essential for f2 RNA replication, a mutant carrying a defective S1 should be resistant to both Qβ and f2. However, S1 could be an essential subunit of both enzymes in K35 ($Q\beta^{ts}$) if its thermal stability depended on the phage-specified subunit of the enzyme, which is known to be different in the Qβ and f2 replicases (39). In fact, crosslinking experiments indicate that S1 is in close association with the phage-specified subunit of the Qβ replicase (40). The temperature-sensitive component in K35 ($Q\beta^{ts}$) could also affect Qβ RNA replication *in vivo* as part of a cellular replication site (41) or as an altered ribonuclease that degrades Qβ RNA at 40°. Analysis of additional mutants, now in progress‡, should clarify the relation between Qβ and f2 RNA replication and, more importantly, between the functions of bacterial proteins in bacteriophage RNA replication and in uninfected cells.

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‡ An abstract describing some of the properties of K35 ($Q\beta^{ts}$) was published at the time the mutant was isolated (42). Since then, this mutant has been lost. We have recently isolated a variety of other Qβ-resistant mutants which are now being genetically and biochemically characterized.

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