Elongation factor Ts (EF-Ts) is the guanine-nucleotide exchange factor of elongation factor Tu (EF-Tu), which promotes the binding of aminoacyl-tRNA to the mRNA-programmed ribosome in prokaryotes. The EF-Tu-EF-Ts complex, one of the EF-Tu complexes during protein synthesis, is also a component of RNA-dependent RNA polymerases like the polymerase from coliphage Qβ. The present study shows that the Escherichia coli mutant GRd.tsf lacking the coiled-coil motif of EF-Ts is completely resistant to phage Qβ and that Qβ-polymerase complex formation is not observed. GRd.tsf is the first E. coli mutant ever described that is unable to form a Qβ-polymerase complex while still maintaining an almost normal growth behavior. The phage resistance correlates with an observed instability of the mutant EF-Tu-EF-Ts complex in the presence of guanine nucleotides. Thus, the mutant EF-Tu-EF-Ts is the first EF-Tu-EF-Ts complex ever described that is completely inactive in the Qβ-polymerase complex despite its almost full activity in protein synthesis. We propose that the role of EF-Ts in the Qβ-polymerase complex is to control and trap EF-Tu in a stable conformation with affinity for RNA templates while unable to bind aminoacyl-tRNA.

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Elongation factor Tu (EF-Tu) is the guanine-nucleotide exchange factor of elongation factor Tu (EF-Tu), which promotes the binding of aminoacyl-tRNA to the mRNA-programmed ribosome during the elongation step of protein synthesis in prokaryotes. EF-Tu is a G protein and thus cycles between an active GTP-bound form and an inactive GDP-bound form. The active EF-Tu-GTP binds aa-tRNA to form the ternary complex EF-Tu-GTP-aa-tRNA. Binding of a cognate ternary complex to the ribosome induces the hydrolysis of the EF-Tu-bound GTP, causing the dissociation of EF-Tu-GDP from the ribosome (1). The inactive EF-Tu-GDP is catalytically recycled to EF-Tu-GTP by elongation factor Ts (EF-Ts). Binding of EF-Ts to EF-Tu-GDP stimulates the exchange of GDP with GTP via formation of the EF-Tu-EF-Ts complex.

Besides being an intermediate in the translation machinery, EF-Tu-EF-Ts is a component of the Qβ-polymerase complex, which is responsible for the replication of the single-stranded RNA genome of coliphage Qβ (2, 3). Qβ belongs to the family of Leviviridae that consists of plus-stranded RNA phages and is genetically among the simplest phages known. The Qβ genome (4217 nucleotides) has four genes encoding the replication protein (44 kDa), the coat protein (14 kDa), the read-through coat protein (34 kDa) produced by occasional read-through of the coat-protein stop codon (4), and the Qβ-replicase subunit (65 kDa) (5–7).

Upon entry into the bacterial cytoplasm after phage attachment to the F pilus, the plus-stranded viral RNA genome is translated and then replicated by the resulting Qβ-polymerase complex (2, 9). The phage RNA is exponentially amplified since both the plus strand and the minus strand of the phage serve as templates. At the end of the infection, the coat, read-through, and maturation protein molecules assemble with plus strand RNA to form phage particles, which are released by phage-induced lysis of the host cell (10).

The holoenzyme of the Qβ-polymerase complex is a heterotetramer comprising the Qβ-replicase subunit and the three host-encoded proteins EF-Tu (43 kDa), EF-Ts (30 kDa), and ribosomal protein S1 (61 kDa) (Ref. 9 and references therein). Ribosomal protein S1 is normally associated with the 30 S ribosomal subunit, where it participates in the binding of mRNA (11, 12). The host-encoded subunits are required for initiation of RNA synthesis, whereas the Qβ-replicase subunit is responsible for the RNA polymerization activity (13). Two of the host subunits are involved in template binding. One is protein S1, which is required for polymerase binding to the plus strand RNA. The other is EF-Tu, which could be cross-linked to simple templates that are not dependent on S1 for replication, e.g. MDV-1 RNA and RQ RNA (14, 15). All the templates of the Qβ-polymerase complex contain CCCA3, at their 3’ end, which is used as the initiation site for replication. Poly(C) is therefore often used as a template in replication assays (16).

When the Qβ plus strand RNA is the template, the holoenzyme (EF-Tu, EF-Ts, S1, and replicase subunit) needs the presence of an additional host factor, protein HF (6 × 11 kDa) (17, 18). HF is an RNA-binding protein required for an efficient translation of the rpoS-encoded σ54 factor and for induction of the stationary phase in the uninfected cell (19, 20). When using Qβ minus strand RNA, MDV-1 RNA, or poly(C) as a template, replication can be accomplished by a polymerase core complex containing only EF-Tu, EF-Ts, and the replicase subunit (21). EF-Ts is the only subunit in the polymerase complex that has been associated neither with RNA binding nor with polymerization activity. Thus, the role of EF-Ts in the Qβ-polymerase complex remains unknown.

Several studies have shown that some of the properties of the EF-Tu-EF-Ts complex as a component of the Qβ-polymerase

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1 The abbreviations used are: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; aa-tRNA, aminoacyl-tRNA; HF, host factor; poly(C), poly(30-mer, poly(C), poly cytidylic acid; tef; the EF-Tu gene; d.tsf, genotype of mutant strain lacking the region encoding the coiled-coil motif of EF-Ts, pfu, plaque-forming units; Ni2+-NTA, nickel-nitrilotriacetic acid.
complex are different from those observed during protein synthesis. Thus, reagents such as N-losyl phenylalanine chloromethyl ketone, N-ethyl maleimide (13, 22), and dimethyl sulfoxide (23), which abolish or alter the activities of the elongation factors in protein synthesis, leave the activity of the EF-Tu:EF-Ts complex in the Qβ-polymerase complex intact. The crystal structure of the *Escherichia coli* EF-Tu:EF-Ts complex (24) reveals a protruding antiparallel coiled-coil motif of EF-Ts composed of helices 10 and 11 (residues 187–226). In the crystal structure, a tetramer of two molecules of EF-Tu and two molecules of EF-Ts is observed. The coiled-coil motifs of each of the two EF-Ts molecules form intimate contacts with each other, and the tetramer is therefore best described as (EF-Ts)2:EF-Tu. This quaternary structure does probably not exist in solution, where thus far only the heterodimer, EF-Tu:EF-Ts, has been detected (25, 26). According to alignment analysis, the coiled-coil motif of EF-Ts is preserved in all prokaryotes but completely absent in mammalian mitochondrial EF-Ts (27, 28).

In another work (29), the function of the coiled-coil motif of EF-Ts in translation was studied by deletion of the region encoding residues 185–224 in *tsf*, the single EF-Ts gene. The resulting *E. coli* mutant (GRd.tsf) has a slightly reduced growth rate when compared with the wild-type strain, and it was also found that deletion of the coiled-coil motif only slightly reduces the ability of EF-Ts to stimulate the nucleotide exchange in EF-Tu. However, the mutant EF-Tu:EF-Ts complex is very unstable in the presence of guanine nucleotides in comparison with the wild-type complex. Thus, the concentration of guanine nucleotides required to dissociate the EF-Tu:EF-Ts complex is reduced at least 2 orders of magnitude by the deletion of the coiled-coil motif in EF-Ts.

In the present study, the ability of the coiled-coil deletion mutant of EF-Ts to support Qβ-phage replication was examined both at the cellular level and at the molecular level. The results show that the *E. coli* mutant GRd.tsf is completely Qβ-phage-resistant because the mutant EF-Ts does not allow the formation of a stable Qβ-polymerase complex.

**EXPERIMENTAL PROCEDURES**

*E. coli Strains—* *E. coli* strain UV211 (ara, Δlac-pro), nalA, thi) (30) and mutant strain GRd.tsf (ara, Δlac-pro), thi, Δtsf) lacking the coiled-coil motif of EF-Ts (d.tsf) (29) were converted into *F*− strains by conjugation with strain JC7822 (tolA, met, hisD, lacY) *F*′ (*proAB−, lacF−, lacZAM15, Tn10(tec−)). The F transduction of both strains was verified by infection with M13 phages. Strain GM1 (KA797) was used as an indicator for phage titers to assay the number of plaque-forming units (pfu).

**Phage Titrations**—Culture samples were clarified by centrifugation, and the supernatants were serially diluted in LB medium or glucose-M9 medium (31). Indicator strain GM1 was infected by adding 100 μl of overnight culture prepared in glucose-M9 medium and 10 μl of diluted phage-containing supernatant (or 250 μl of undiluted supernatant from cultured GRd.tsf cells) to 5 ml of 0.75% (w/v) agar in LB medium at 42 °C. After gently mixing, the agar was poured onto LB medium plates with 250 μl/million pfu. The F transduction of both strains was verified by infection with M13 phages. Strain GM1 (KA797) was used as an indicator for phage titers to assay the number of plaque-forming units (pfu).

**Cloning of EF-Ts and His6-tagged Qβ-Replicase Subunit—**Wild-type *E. coli* EF-Ts was cloned by PCR into the *Neol* and HindIII restriction sites of the low copy number plasmid pTrc99A (Amersham Biosciences) (32). The resulting plasmid pTrc99A-tsf was used for complementation experiments. A DNA fragment encoding an N-terminal His6-tagged form of the Qβ- replicase subunit in plasmid pYES2-HisQβ (a generous gift from Søren Mondrup, Department of Molecular Biology, University of Aarhus) was subcloned into plasmid pTrc99A by the use of *Neol* and XbaI restriction sites. The resulting plasmid pTrc99A-HisQβ was used for expression and purification of the replicate subunit.

**Qβ-Phage Production from Infectious Plasmid pQM100—**Strains UV211 *F*− and GRd.tsf *F*− were transformed with the infectious plasmid pQM100 containing the β-lactamase gene as an ampicillin resistance marker and the entire Qβ cDNA-genome in reverse orientation relative to the *trp* promoter (33). From each transformation, four colonies were tested for harboring pQM100 by plasmid purification followed by restriction enzyme digestions. The colonies were cultured at 37 °C in LB medium containing 200 μg/ml ampicillin, and at mid-log phase (A600 of ~1.0), culture samples of 1 ml were withdrawn and used for phage titers.

**Purification of Qβ-Phages—** *E. coli* strain JM101 *F*− transformed with pQM100 was grown overnight at 37 °C in 40 ml of LB medium containing 100 μg/ml ampicillin. The culture was clarified by centrifugation at 12,000 × g for 30 min, and the supernatant was sterilized by filtration through a 0.2-μm filter. The phage particles were concentrated using a YM30 Amicon ultrafilter (Amicon). Amino acids and other nutrients were removed from the concentrated phage particles by dialysis overnight against a 500-fold excess of Qβ buffer (20 mM Tris–HCl, pH 7.6, 10 mM MgCl₂). Finally, the Qβ phages were further concentrated using a Centricon YM-100 (Amicon) and stored in 33% (v/v) glycerol at −20 °C. The concentration of the purified phage particles was determined by phage titration.

**Qβ Infection of UV211 F− and GRd.tsf F− and Complementation with Wild-type EF-Ts—** Overnight cultures of UV211 *F*− and GRd.tsf *F*− prepared in glucose-M9 medium were inoculated into 15 ml of the same medium to an initial A600 of about 0.1 (~10⁶ cells/ml). UV211 *F*− and GRd.tsf *F*− transformed with either empty plasmid pTrc99A or pTrc99A-tsf were incubated in the same medium containing 1 mM isopropyl-1-thio-β-D-galactopyranoside to examine the effects of overexpression of wild-type EF-Ts. At t = 0, purified Qβ-phages were added to all cultures at a multiplicity of infection of 0.02, and the cultures were incubated at 37 °C under shaking. At appropriate time intervals, 0.5 ml culture samples were withdrawn. After A600 measurement, the samples were used for the determination of phage titers.

The phage infection experiments with UV211 *F*− and GRd.tsf *F*− in liquid cultures were supported by infection experiments on LB medium plates with UV211 *F*− and GRd.tsf *F*− as indicator strains. The experiments were performed as described for the phage titrations except that 10 μl of a solution containing ~200 of purified Qβ-phages was used for the infection.

**In Vivo Translation of the Qβ Genome—** Infection with Qβ-phages, protein labeling, and electrophoresis was basically done as described previously for MS2 phages (34) except that cells of strain UV211 *F*− and GRd.tsf *F*− were grown at 37 °C in glucose-M9 medium and that 14C-labeled amino acid mix (Amersham Biosciences) was used for the protein labeling.

**Purification of the Qβ-Polymerase Complex—** Partial purification of the Qβ-polymerase complexes with His-tagged EF-Ts was basically done as described elsewhere (35). The wild-type Qβ-replicase subunit, cells transformed from plasmid pRep (36), was a C-terminal His-tagged EF-Ts. EF-Tu was expressed from plasmid pSM1 (35). After co-expression of the native replicase subunit and the His-tagged EF-Tu in strains UV211 and GRd.tsf, cells were harvested by centrifugation (4,300 × g, 20 min), resuspended in Qβ-replicase buffer (50 mM Tris–HCl, pH 7.8, 60 mM NaCl, 7 mM MgCl₂ and 10% glycerol), and lysed by sonication. The cell extract was clarified by centrifugation at 30,000 × g for 20 min. The resulting cell-free S30 extract was applied to a column with 1 ml of Ni²⁺-NTA material (Qiagen). Non-adsorbed material was washed with the column with Qβ-replicase buffer, and Ni²⁺-NTA-bound material was subsequently eluted by a 0–500 mM imidazole gradient in the same buffer.

For purification of the Qβ-polymerase complex containing a His-tagged Qβ-replicase subunit, cells of UV211 and GRd.tsf harboring plasmid pTrc99A-HisQβ were grown at 37 °C in LB medium containing 200 μg/ml ampicillin. At A600 of ~0.8, expression of the His-tagged Qβ-replicase subunit was induced by adding 0.25 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were grown at 30 °C for 2 h, harvested by centrifugation, and resuspended in Qβ-buffer (50 mM Tris–HCl, pH 7.8, 60 mM NaCl, 5 mM MgCl₂, and 100 μM phenylmethylsulfonyl fluoride, 10 mM MgCl₂, and 5% (v/v) glycerol) before lysis by passage through a French press twice. The lysate was supplemented with 0.3% (v/v) of polyethyleneimine and centrifuged at 20,200 × g for 20 min. Purification of the Qβ-polymerase complex containing the His-tagged Qβ-replicase subunit was basically performed as described in Ref. 38 except that a 5-mL HiTrap-Q column (Amersham Biosciences) and a 1-mL HiTrap-SP column (Amersham Biosciences) were utilized. The Qβ-polymerase complex was eluted from the SP column with 50 mM Tris–HCl, pH 7.6, 250 mM NaCl, and 5 mM MgCl₂ and stored in the same buffer at −80 °C.
Enhanced Chemiluminescence Immunodetection Assay—The SDS-polyacrylamide gels were blotted on polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with buffered low fat milk solution and incubated with a dilution of polyclonal rabbit anti-bodies raised against two synthetic peptides (SktASSRNSLSAQLR and DGQLPRGSGCDSAD) from the sequence of the Qβ-replicase subunit (Davids Biotechnologie, Regensburg, Germany), followed by incubation with peroxidase-conjugated anti-rabbit immunoglobulins (Sigma). The ECL detection was performed according to the Sigma protocol.

In Vitro Replication Assays—The Qβ-polymerase activity was measured in vitro as described earlier (35), with polyC (Amersham Biosciences) or Qβ minus strand RNA (39) as template and 8-[3H]GTP (−αs) (Amersham Biosciences) as labeled substrate. The assay was performed by incubating 10 µl of the indicated sample with 18 µl of polymerase activity mixture at 30 °C for 20 min. Thereafter, 25 µl was taken for the determination of the RNA replication activity, as measured by the incorporation of [3H]GMP and as detected by trichloroacetic acid precipitation, filtration, and liquid scintillation counting. The assays were done in duplicate unless otherwise indicated, and the reported values were corrected for background activity.

Purification of the Host-encoded Qβ-Polymerase Subunits—The wild-type E. coli EF-Tu, EF-Ts, and mutant EF-Ts were purified as described elsewhere (29, 40). The purification procedure for ribosomal protein S1 was performed as described for E. coli EF-Ts (29) except that S1 was further purified by hydrophobic interaction chromatography on a phenyl-substituted high pressure liquid chromatography column (TSK Phenyl-5PW, Tossek).

Pull-down Assay of the Qβ-replicase Subunit by Matrix-bound GST-tagged EF-Tu—Plasmid pGDKT7-Qβ (a generous gift from Dr. Francesco M. Castano, Department of Molecular Biology, University of Aarhus) was used as a template in the Tn7-T7-coupled reticulocyte-lysate system (Promega) to synthesize the Qβ-replicase subunit according to the manufacturer's instructions. Aliquots of ~10 µl of glutathione resin (Amersham Biosciences) equilibrated in buffer B (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween 20) were incubated in 500 µl of the same buffer, supplemented with 100 µM phenylmethylsulfonyl fluoride, 0.1 mg/ml bovine serum albumin, Complete EDTA-free protease inhibitor (Roche Applied Science), and 40 pmol of purified GST-tagged EF-Tu (40) under rotation at 4 °C for 1 h. The resin was spun down and incubated under rotation at 4 °C with 40 pmol of wild-type or mutant EF-Ts and freshly prepared translation products in 5 µl of TnT lysate in another 500 µl of this buffer for 2 h. The glutathione resin was washed five times for 10 min in 500 µl of TBST buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween 20, and 0.1 mg/ml bovine serum albumin) at 4 °C under rotation. The resin was spun down and resuspended in 20 µl of 2-fold concentrated SDS-sample buffer and loaded on a 12% (w/v) SDS-polyacrylamide gel. The dried gel was exposed and developed by phosphorimaging, and the intensities of the radioactive bands of the Qβ-replicase subunit were quantified. The effect of guanine nucleotides on the stability of the polymerase core complex was investigated by including either 0.1 mM GDP or 1 mM GTP with 80 µM phospho-endo-pyruvate and 0.16 µg/ml pyruvate kinase in the TBST buffer used for washing.

RESULTS

No Qβ-Phage Production in E. coli Mutant GRd.tsf—The Qβ production in strain GRd.tsf was examined by the use of the infectious plasmid pQM1000. Use of a plasmid system in E. coli F- host cells allows phage production independently of the initial steps of normal phage infection and replication (41). No plasmid could be detected in transformants of our wild-type strain UY211 by agarose gel electrophoresis, which probably reflects the toxicity of the infectious plasmid for this strain. However, the level of pQM1000 in the tested colonies of transformants of GRd.tsf was equal to that of a normal plasmid. The Qβ-phage yield of UY211 was about 2 × 10⁶ pfu/ml × A₆₆₀₀, whereas essentially no phages were produced in GRd.tsf.

Qβ Production in E. coli Mutant GRd.tsf Can Be Restored by Complementation with Wild-type EF-Ts—UY211 wild-type and GRd.tsf mutant cells were converted into F- strains, which was verified by the resulting susceptibility to infection by M13 phages. The F- strains were infected with Qβ-phages and grown in liquid culture to examine their efficiency as hosts for production of Qβ-phages (Fig. 1A). Although the absorbancy of the culture of the wild-type host reaches a plateau due to lysis, the mutant strain shows exponential growth. During growth of strain UY211 F-, the number of pfu increases exponentially until it finally reaches a plateau when the culture is completely lysed. For the mutant strain, the number of pfu in the culture decreases exponentially, probably by inactivation of the inoculated phages upon infection of the cells.

To ascertain that resistance of GRd.tsf F- to Qβ-phage is an effect of the EF-Ts mutation and not of any other fortuitous difference between the wild-type and the mutant strain, phage production was assayed in the mutant strain complemented with wild-type tsf behind the trc promoter on plasmid pTrc99A-tsf. The plasmid was able to efficiently complement the coiled-coil deletion mutant of EF-Ts for phage production up to wild-type level, even without induction (Fig. 1B). The trc promoter is very strong but leaky (see pTrc99A description, Amersham Biosciences catalog 1999, catalog number 275007-01). As controls for the complementation experiment, Qβ production in the strains with an empty plasmid pTrc99A was assayed in parallel (data not shown). The empty plasmid had no effect on the Qβ resistance of the mutant strain and only slightly reduced the phage production in the wild-type strain. Overexpression of wild-type EF-Ts obtained by induction of pTrc99A-tsf with 1 mM isopropyl-1-thio-β-D-galactopyranoside had no additional effect on the production of Qβ-phages (data not shown).

As a supplementary experiment, strains UY211 F- and GRd.tsf F- were infected with serial dilutions of purified Qβ-phages on plates. As expected from the previous results, the
In Vitro Complementation with Wild-type EF-Ts — A possible complementation by wild-type EF-Ts of the abolished Qβ-polymerase activity in an S30 extract with mutant EF-Ts was investigated with again poly(C) as a template (Table I, experiments III—V). An S30 extract from cells of the wild-type strain not expressing the Qβ-replicase subunit was added as a source of wild-type EF-Ts to a replicase-containing S30 extract from strain GRd.tsf (Table I, VI). In contrast to the results obtained by in vivo complementation (Fig. 1B), the wild-type EF-Ts was unable to complement the mutant EF-Ts with the coiled-coil deletion in vitro.

**Purification of the Qβ-Polymerase Complex** — To understand the abolished replication activity in the mutant strain, partial purification of the Qβ-polymerase complexes with His-tagged EF-Tu subunits by means of Ni2+-affinity chromatography was performed. The purification from strain UY211 resulted in fractions that were active in replication, with either poly(C) (217.5 pmol of incorporated [3H]GMP) or Qβ minus strand RNA (68 pmol of incorporated [3H]GMP) as a template (Table I). In accordance with the absence of Qβ-polymerase activity in the S30 extract from strain GRd.tsf (Table I), essentially no replication activity was detected in the purified fractions from the mutant strain neither with poly(C) (0.89 pmol of incorporated [3H]GMP) nor with Qβ minus strand RNA (0.54 pmol of incorporated [3H]GMP) as templates (Table II). When both for strains the Ni2+-affinity eluates were analyzed by immunoblotting, the replicase subunit was present in the fraction from the wild-type strain (Fig. 3, lane 4) as expected but was not detectable in the fraction from the mutant strain (Fig. 3, lane 3). This result indicates that the formation of a Qβ-polymerase complex is hampered in strain GRd.tsf.

For further verification that a Qβ-polymerase complex is not formed in strain GRd.tsf, another purification approach was performed using an N-terminally His-tagged Qβ-replicase subunit that was overexpressed in the wild-type and mutant strains. The His-tagged Qβ-replicase subunit expressed in the wild-type strain was unable to bind to the Ni2+-NTA resin, but fortunately, the His-tagged wild-type Qβ-polymerase complex could be successfully purified by the method described earlier (38) (data not shown). The yield of the purified Qβ-polymerase complex from strain UY211 was 45 μg/g of wet cell paste. The His-tagged replicase subunit in the purified complex showed a high level of RNA replication activity with poly(C) as a template. Contrary to the wild-type strain, no Qβ-polymerase complex from GRd.tsf overexpressing the His-tagged Qβ-replicase subunit could be purified by the method of Moody et al. (38). This confirms our previous observation that the formation of a Qβ-polymerase complex is hampered in strain GRd.tsf.

**Pull-down Assays of the Qβ-replicase Subunit** — Pull-down experiments with N-terminally GST-tagged EF-Tu and 35S-labeled Qβ-replicase subunit were performed to verify by a direct method that the coiled-coil deletion in EF-Ts abolishes the formation of a Qβ-polymerase complex. The Qβ-replicase subunit was synthesized in a translation system from rabbit reticulocyte lysate to avoid the presence of procaryotic elongation factors. Ribosomal protein S1 was not included in the pull-down experiments as it turned out to reduce the level of pull-down of the Qβ-replicase subunit (data not shown), probably by interference with the GST tag of EF-Tu. The level of formation of the Qβ-polymerase core complex was determined by the ability of EF-Ts to stimulate the pull-down level of the Qβ-replicase subunit by GST-EF-Tu bound to glutathione-Sepharose (Fig. 4, A and B). Pull-down of the Qβ-replicase subunit by GST-EF-Tu alone was used as a negative control. Although stimulation of the pull-down level of the Qβ-replicase subunit by wild-type EF-Ts was 7–8-fold higher than the negative control, no stimulation was observed with the mutant EF-Ts (Fig. 4B).

Additional pull-down experiments with either GDP or GTP in the TBST wash buffer were performed to evaluate the effect of guanine nucleotides on the stability of the Qβ-polymerase core complexes (Fig. 4B). In the presence of wild-type EF-Ts, the Qβ-replicase subunit was less efficiently pulled down by GST-EF-Tu.
The indicated amounts of cell extracts from strains containing plasmids pRep and/or pSM1 were used to measure the Qβ-polymerase activity given as pmol of incorporated [3H]GMP. Plasmid pRep overexpresses the Qβ-replicase subunit, whereas plasmid pSM1 overexpresses His-tagged EF-Tu. Experiments I and II show the Qβ-polymerase activity in extracts of the wild-type and mutant strain, respectively. Experiments III–VI show the in-vitro complementation with wild-type EF-Ts from UY211 pSM1. The indicated mixtures were prepared and kept on ice for 1 hour before their addition (together with 5 μl H2O) to the assay mixtures. Expression from the indicated plasmids was in all experiments induced during growth prior to the preparation of the S30 extracts. The subtracted background activity was derived from a blank containing only Qβ-replicase buffer.

### Table I

| Strains and plasmids | Qβ-replicase buffer | Incorporated [3H]GMP (pmol) |
|----------------------|---------------------|---------------------------|
| Exp. I 10 μl         |                     |                           |
| Exp. II 10 μl        |                     |                           |
| Exp. III 5 μl        |                     |                           |
| Exp. IV 2.5 μl       |                     |                           |
| Exp. V 2.5 μl        |                     |                           |
| Exp. VI 2.5 μl       |                     |                           |

* Exp., experiment.

![Figure 3](image)

**FIG. 3.** Partial purification of the Qβ-polymerase complex with His-tagged EF-Tu from pSM1. ECL immunodetection of the replicase subunit in S30 extracts and Ni2+ affinity-purified samples from strains UY211 pSM1 pRep and GRd.tsf pSM1 pRep, with antibodies raised against synthetic replicase-peptides, is shown. Lane 1, reference sample containing about 0.4 μg of purified wild-type Qβ-polymerase complex; lane 2, S30 extract from GRd.tsf pSM1 pRep; lane 3, Ni2+ affinity-purified sample from GRd.tsf pSM1 pRep; lane 4, Ni2+ affinity-purified sample from UY211 pSM1 pRep. Lanes were loaded with 10 μl of S30 extract or Ni2+ affinity eluates.

### Table II

| Poly (C) | Qβ minus-strand |
|----------|-----------------|
| Strains and plasmids | UY211 pSM1 | GRd.tsf pSM1 | UY211 pSM1 | GRd.tsf pSM1 |
| Incorporated [3H]GMP (pmol) | 217.5 | 0.89 | 68 | 0.54 |

both nucleotides were found to reduce the pull-down of the Qβ-replicase subunit. With 0.1 mM GDP or 1 mM GTP in the wash buffer, the pull-down was reduced to 2.5- or 3.2-fold of the negative control, respectively. As expected, the pull-down of the Qβ-replicase subunit in the presence of the mutant EF-Ts remained at the level of the negative control, independent of the presence or absence of either GDP or GTP.

**DISCUSSION**

**Qβ-Phage Resistance**—The present study demonstrates that the *E. coli* mutant GRd.tsf is completely resistant to Qβ-phage due to the inability of mutant EF-Ts without the coiled-coil motif to participate in the formation of a Qβ-polymerase complex. The absence of pfu for the mutant strain harboring the infectious plasmid pQM100 indicates that the mutant EF-Ts does not support the production of Qβ-phages. In addition, when a culture of the mutant strain was infected with Qβ-phages, bacterial growth was not affected, whereas the number of pfu in the medium decreased exponentially with time. Together, these observations indicate that phage propagation in the mutant cells was completely abolished and that the inoculated phage particles became inactivated by adsorption to the growing number of cells. The Qβ-resistant phenotype of GRd.tsf was eliminated by complementation with plasmid-borne wild-type *tsf*, which shows that exclusively the deletion of the coiled-coil motif in EF-Ts is responsible for the phage resistance. The fact that the EF-Ts mutant was fully complemented, even at a low level of wild-type EF-Ts expression in uninduced cells, indicates that deletion of the coiled-coil motif in EF-Ts results in a recessive phenotype for resistance to Qβ-phage infection. In contrast to the wild-type strain, no synthesis of phage-specific proteins was detected in infected cells of GRd.tsf, indicating that an early step in the Qβ life cycle is defective.

**Abolished Formation of the Qβ-Polymerase Complex**—
cell-free extract from mutant cells expressing the Qβ-replicase subunit showed no RNA polymerization activity, which indicates that the mutant EF-Ts is unable to form an active polymerase complex. The inability of the wild-type EF-Ts to complement the mutant EF-Ts in vitro could mean that the mutant EF-Ts in the polymerase complex was not replaced by wild-type EF-Ts or that the replicase subunit in the cell-free extract from strain GRd.tsf was not in an active conformation. The failure to detect the Qβ-replicase subunit in the preparation from the mutant strain after Ni²⁺-affinity chromatography utilizing His-tagged EF-Tu as bait for the replicase subunit indicated that no stable Qβ-polymerase complex was formed in strain GRd.tsf. This result was confirmed by another approach of purification using the method of Ref. 38. The inability of the mutant EF-Ts to form a Qβ-polymerase core complex was also shown in vitro by pull-down experiments. Apparently, a Qβ-polymerase complex containing the mutant EF-Ts subunit without the coiled-coil motif is very unstable or simply not formed, which makes GRd.tsf unable to sustain any Qβ-polymerase activity and explains the Qβ resistance of this mutant strain.

**Stability of the Qβ-Polymerase Complex**—The present results, combined with additional knowledge about the functioning of the mutant EF-Tu-EF-Ts complex during protein synthesis (29), suggest that the very low stability of the mutant EF-Tu-EF-Ts complex in the presence of guanine nucleotides hampers the assembly of a Qβ-polymerase complex but is still sufficient to catalyze guanine-nucleotide exchange during translation. Thus, the inability of the mutant EF-Ts to form a polymerase complex under in vivo conditions (with estimated concentrations of 1 mM GTP, 0.1 mM GDP (44)) or under the conditions of the pull-down experiment can be explained by the presence of guanine nucleotides. In the pull-down experiments, about 0.6 μM GTP and 0.02 μM GDP (31, 45) may derive from the reticulocyte lysate used for the synthesis of the Qβ-replicase subunit. Since complex formation of mutant Qβ-polymerase could by no means be detected, the effect of guanine nucleotides on the complex stability was analyzed in pull-down experiments with the wild-type polymerase complex. The presence of either 0.1 mM GDP or 1 mM GTP in the wash buffer indeed reduced the pull-down effect, in accordance with previous studies (13). This demonstrates that, despite the stabilization of the EF-Tu-EF-Ts complex by association with the Qβ-replicase subunit, both GDP and GTP are able to abolish formation of the Qβ-polymerase core complex, presumably by dissociation of EF-Tu-EF-Ts.

One could argue that the enhanced instability of the mutant EF-Tu-EF-Ts complex in the presence of guanine nucleotides does not much affect its role in translation and would not exclude that the mutant EF-Ts could support the formation of a transient but potentially active Qβ-polymerase complex. However, this would dramatically reduce the probability of initiation of RNA synthesis and thereby effectively prevent polymerization activity as indicated by the absence of Qβ-phage progeny.

Results of previous studies (13, 46) indicate that the Qβ-replicase subunit is very unstable when not bound to the EF-Tu-EF-Ts complex. Thus, the failure to complement the mutant EF-Ts with wild-type EF-Ts in a replicase-containing cell-free extract from strain GRd.tsf may indicate that the replicase subunit is inactivated or misfolded in the presence of the unstable mutant EF-Tu-EF-Ts complex. Most likely, a considerable amount of misfolded replicase subunit in strain GRd.tsf becomes degraded, as suggested by the observed cross-reactivity of smaller protein products in the mutant S30 extract (Fig. 3, lane 2) with the replicase antiserum. This view supports observations (13, 23, 47), that the presence of a native and stable EF-Tu-EF-Ts complex is required for the refolding of an active replicase subunit. Thus, a stable EF-Tu-EF-Ts complex may function as a folding template or chaperone for the proper folding of the nascent replicase-subunit chain as well as for maintaining the subunit in an active conformation. In previous studies, both EF-Tu and EF-Ts have been shown to have chaperone-like activities (48, 49). In this context, it should be noted that the formation of wild-type Qβ-polymerase core complex in the pull-down experiments is probably due to a high input of labeled Qβ-replicase subunit and that the subunit was synthesized freshly before use in an in vitro reticulocyte system that may less rapidly degrade misfolded E. coli proteins.

In the presence of guanine nucleotides, the EF-Tu-EF-Ts complex appears to be more stable as a component of the Qβ-polymerase complex than as a free complex (13). This observation, combined with those of the present study, suggests that the Qβ-replicase subunit and the EF-Tu-EF-Ts complex stabilize each other, thereby reducing the dissociation of the polymerase complex by guanine nucleotides.

**The Role of EF-Ts in the Qβ-Polymerase Complex**—In contrast to the roles of EF-Tu and EF-Ts in the translation machinery, little is known about their roles in the Qβ-polymerase complex. According to the model of Qβ-polymerase functioning proposed by Brown and Gold (15), RNA binding is an important function of EF-Tu in the Qβ-polymerase complex. In contrast, the function of EF-Ts in the polymerase complex remains completely unknown.

It was shown in a complementary study (29) that deletion of the coiled-coil motif in E. coli EF-Ts only slightly reduces the ability of EF-Ts to promote the nucleotide exchange in EF-Tu, although the stability of the mutant EF-Tu-EF-Ts complex is strongly reduced in the presence of guanine nucleotides. Since the polymerization activity of the Qβ-polymerase complex is very sensitive to the dissociation of the EF-Tu-EF-Ts complex (13, 22, 23), we think that the role of EF-Ts in the Qβ-polymerase complex is related to stable complex formation rather than to nucleotide-exchange activity.

Results by Brown and Gold (15) have shown that their Site II replicons can be bound to free EF-Tu-GTP with an affinity similar to that of the Qβ-polymerase complex. This may indicate that EF-Ts in the polymerase complex is not necessarily used as inducer of a conformational change in EF-Tu required for binding of RNA templates. However, based on the present results, we propose that the role of EF-Ts in the polymerase complex is to control and trap EF-Tu in a stable conformation with an affinity for RNA templates but not for aa-tRNA. Thus, the competition between aa-tRNA and template for binding to EF-Tu in the polymerase complex would be won by the template.

**Perspectives**—To our knowledge, E. coli GRd.tsf is the first Qβ-phage-resistant mutant ever characterized that maintains a good vitality and at the same time blocks the formation of the Qβ-polymerase complex by virtue of an altered host component (8, 50–52). This makes GRd.tsf a perfect strain for future investigations of mutant EF-Ts species in the Qβ-polymerase complex because its endogenous EF-Ts is not incorporated into the Qβ-polymerase complex when the EF-Ts species of interest is expressed from a plasmid.

Using an approach like that of Schuppli et al. (37), who performed Qβ-mutant selection in E. coli mutants without an active gene for HP subunit production, it would be interesting to select for Qβ-phages adapted to the EF-Ts deletion mutant and to analyze the Qβ mutations responsible for such an adaptation. However, despite many efforts with Qβ as well as MS2 phage, no adapted plaques appeared. Such a result
strongly suggests that phage RNA replication has virtually come to a standstill. This opens possibilities for creating bacterial resistance against all coliphages of the family of Leviriridae.

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