Genetic Analysis of the Bacteriophage T4-Encoded Cochaperonin Gp31

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ABSTRACT

Previous genetic and biochemical analyses have established that the bacteriophage T4-encoded Gp31 is a cochaperonin that interacts with *Escherichia coli*'s GroEL to ensure the timely and accurate folding of Gp23, the bacteriophage-encoded major capsid protein. The heptameric Gp31 cochaperonin, like the *E. coli* GroES cochaperonin, interacts with GroEL primarily through its unstructured mobile loop segment. Upon binding to GroEL, the mobile loop adopts a structured, β-hairpin turn. In this article, we present extensive genetic data that strongly substantiate and extend these biochemical studies. These studies begin with the isolation of mutations in gene *31* based on the ability to plaque on *groEL*44 mutant bacteria, whose mutant product interacts weakly with Gp31. Our genetic system is unique because it also allows for the direct selection of revertants of such gene *31* mutations, based on their ability to plaque on *groEL*515 mutant bacteria. Interestingly, all of these revertants are pseudorevertants because the original *31* mutation is maintained. In addition, we show that the classical *ts*A70 mutation in gene *31* changes a conserved hydrophobic residue in the mobile loop to a hydrophilic one. Pseudorevertants of *ts*A70, which enable growth at the restrictive temperatures, acquire the same mutation previously shown to allow plaque formation on *groEL*44 mutant bacteria. Our genetic analyses highlight the crucial importance of all three highly conserved hydrophobic residues of the mobile loop of Gp31 in the productive interaction with GroEL.

THE *groES* and *groEL* genes of *Escherichia coli* were first identified as host factors required for bacteriophage morphogenesis (Georgopoulos *et al.* 1972, 1973; Takano and Kakefuda 1972; Coppo *et al.* 1973; Sternberg 1973; Revel *et al.* 1980). Subsequent analysis revealed that they are linked to many different functions in the cell and that their defective alleles produce pleiotropic phenotypes (reviewed in Zeilstra-Ryalls *et al.* 1991). The essential function of their GroEL and GroES products is to facilitate folding of a subclass of *E. coli* polypeptides (Horwich *et al.* 1993; Ewalt *et al.* 1997). The GroEL/GroES chaperone machine carries out its function via two modes of action, not necessarily separable (Netzer and Hartl 1998; Richardson *et al.* 1998; Sigler *et al.* 1998). First, GroEL not only prevents aggregation, but also allows the partial unfolding of kinetically trapped intermediates by interacting with the hydrophobic surfaces of polypeptides. Second, GroEL, with the assistance of GroES, provides a shielding and sequestering environment in which the polypeptide may find its proper folding pathway undisturbed. Thus, because GroEL and GroES are necessary to chaperone a subclass of proteins by either preventing their aggregation, facilitating their folding, or "guiding" folding incompetent intermediates to degradation pathways (Kandror *et al.* 1995), *groEL* or *groES* mutations produce multiple defects in *E. coli*. Hence, it is not surprising that GroEL and GroES are essential at all temperatures, but their function is in higher demand at elevated temperatures (Fayet *et al.* 1989).

While bacteriophage lambda growth is affected by mutant alleles in either *groEL* or *groES*, bacteriophage T4 growth is impaired only by certain *groEL* mutant alleles and its morphogenesis is seemingly independent of *groES* (Tilly *et al.* 1981). This original genetic analysis led to the realization that bacteriophage T4 encodes its own GroES homologue, Gp31. Gp31's role as GroEL's cohort was originally suspected because T4 suppressors overcoming the *groEL*44-imposed block mapped to gene *31* (Georgopoulos *et al.* 1972) and because mutations in either *groEL* or gene *31* led to the same phenotype, namely massive intracellular aggregation of Gp23, T4's major capsid protein (Laemmli *et al.* 1970). Subsequent biochemical studies showed that Gp31 can completely substitute for GroES in the GroEL-mediated *in vitro* refolding of prokaryotic Rubisco (van der Vies *et al.* 1994) and citrate synthase (Richardson *et al.* 1999). Furthermore, gene *31* can complement a *groES* temperature-sensitive allele for growth at the nonpermissive temperature (van der Vies *et al.* 1994) or even completely substitute for *groES*, thus allowing *groES*'s deletion (F. Keppel and C. Georgopoulos, unpublished results).

The recently solved structures of GroES and Gp31 by X-ray crystallography have permitted the recognition of structural features that explain the similarity of the two

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cochaperonins, while also highlighting essential differences. For example, although GroES and Gp31 share only 14% amino acid sequence identity (Koonin and van der Vies 1995), their overall tertiary structures are very similar (Hunt *et al.* 1996, 1997). Perhaps the most important common feature is the GroEL-binding mobile loop, first identified in both proteins by nuclear magnetic resonance (NMR) experiments and by limited proteolysis (Landry *et al.* 1993, 1996). This flexible polypeptide contains the highly conserved hydrophobic tripeptide sequence (I25-V26-L27 in GroES and L35-I36-I37 in Gp31; see Figure 1), which interacts directly with GroEL, as demonstrated by NMR studies and highlighted again in the GroEL/ADP/GroES crystal structure (Xu *et al.* 1997). Landry and co-workers (Landry *et al.* 1993, 1996; Richardson *et al.* 1999) have hypothesized that the mobile loop plays a key regulatory role in the mechanism of action of the GroEL/GroES chaperone machine.

A remarkable feature of the T4-encoded Gp31 cochaperonin is that its structure dictates, in an as yet unsolved manner, a functional specificity that differentiates it from GroES. Various genetic analyses have shown that only the Gp31 cochaperonin can assist GroEL in the intracellular folding of the bacteriophage T4 capsid protein Gp23 in a timely fashion (Andreadis and Black 1998). Yet, our genetic studies have also shown that Gp31 is able to substitute for GroES in *E. coli* growth, suggesting that Gp31 is able to assist in the folding of diverse, essential *E. coli* proteins. Hunt *et al.* (1997) have pointed out the following features, which may functionally distinguish Gp31 from GroES: (1) Gp31 has a longer mobile loop than GroES, which may result in a taller Gp31 "dome" structure over GroEL compared to that formed by GroES. (2) Gp31 lacks the roof loop, an eight-residue peptide that caps the dome of GroES. Again, this feature may allow for the accommodation of a larger polypeptide under the Gp31 cochaperonin dome. (3) Gp31 lacks the amino acid corresponding to tyrosine 71 in GroES, highly conserved among all other cochaperonins (see Figure 1). In the crystal structure of GroES, the tyrosine 71 residue projects out in the interior wall of the dome, thus limiting available space in the GroEL/GroES cavity (Hunt *et al.* 1996, 1997). These combined differences, as well as others (such as charge and hydrophobicity of the interior lining of the two cochaperonin dome structures), may contribute to a cochaperonin that is more tolerant to some specific, large substrates, such as the Gp23 capsid protein, whose molecular mass is ~56 kD, close to the mass limit of GroEL's substrates (Ewalt *et al.* 1997).

Our interest lies in a structure/function dissection of Gp31. Over the years, we have accumulated a number of interesting mutations and their intragenic suppressors. The isolation and identification of these mutants are discussed in detail in this article.

MATERIALS AND METHODS

Bacteria: *E. coli* B178 (a *galE* derivative of W3110) is *sup*$^+$, *i.e.*, nonpermissive for bacteriophage T4 amber (*am*) mutants (Georgopoulos 1971). CR63 *supD* is permissive for bacteriophage T4 amber mutants and has been described by Epstein *et al.* (1964). *groEL*44, *groEL*515, *groEL*140, and *groEL*673 are isogenic to B178 and the *groEL* mutant alleles encode the following amino acid changes: *groEL*44(E191G), *groEL*515(A383T), *groEL*140(S201F), and *groEL*673(G173D, G337D) (Zeilstra-Ryalls *et al.* 1993). DH5α *supE* and CJ236 *supE* strains, both permissive for T4 amber mutants, were used for cloning and site-directed mutagenesis purposes (Hanahan 1983; Kunkel *et al.* 1991).

Bacteriophage: T4 Do, T4 *31am*NG71 (carries an amber allele in gene *31*; Keppel *et al.* 1990), and T4 *31ts*A70 are from our collection or that of R. H. Epstein at the University of Geneva.

Spontaneous selections: Twenty independent T4 wild-type lysates were prepared from single plaques and plated on *groEL*44 mutant bacteria at 37°. Plaque formers, occurring at a frequency of $\sim 10^{-7}$, called ε, were purified by restreaking and were further characterized on B178 and *groEL*44, *groEL*515, *groEL*673, and *groEL*140 mutant bacteria. Forty independent lysates of the original T4 *31*ε1 mutant were prepared from single plaques and plated on a lawn of *groEL*515 bacteria. Plaque formers, occurring at a frequency of $\sim 10^{-6}$, were isolated, restreaked, and characterized for plating ability on various *groEL* mutant hosts.

DNA sequencing: Primers were constructed corresponding to sequences centered ~30 bases 5′ to the start ATG codon and just 3′ to the stop codon of gene *31* (5′ primer sequence: ggggtacctaaatgctttaagaactatttgtt; 3′ primer sequence, gctctagaac ttattattccgacacccaattc). The minimal *31* gene was amplified by PCR (30 cycles) using Dynazyme *Taq* polymerase directly from a plaque grown on the appropriate bacterial lawn following the method described by Repoila *et al.* (1994). The PCR product was sequenced directly using the Amersham Pharmacia Biotech (Uppsala, Sweden) Delta *Taq* sequencing kit and the same primers as those used for PCR amplification.

Site-directed mutagenesis: Gp31(I36W), Gp31(G34D), Gp31(G34I), and Gp31(T31A) were created by the method of Kunkel *et al.* (1991) or by using the Stratagene (La Jolla, CA) QuickChange site-directed mutagenesis kit. The mutations were placed in plasmid pALEX1 (Richardson *et al.* 1999).

Generation of a *31* gene PCR-mutagenized library: Gene *31* was mutagenized following the PCR methods of Spee *et al.* (1993) and Zhou *et al.* (1991). Mutagenized gene *31* pools were obtained with both methods and combined. Specifically, we used the pALEX1 plasmid as a template and the following primers: a 5′ primer, which introduces an *Eco*RI site and 23 bases into the start site of gene gene *31* (sequence: ggaattca tatgtctgaagtacaacagctacc), and the 3′ primer described above, which introduces a *Xba*I site. Either limiting dATP with addition of dITP (20 μm dATP, 200 μm dITP, dCTP, dGTP, dTTP, and 0.1 mm $MnCl_2$; Spee *et al.* 1993) or the presence of $MnCl_2$ (200 μm dNTPs and 0.5 mm $MnCl_2$; Zhou *et al.* 1991) was used to increase mutation frequency during PCR. *Taq* Polymerase was purchased from QIAGEN (Basel, Switzerland). The PCR fragment was cloned into the *Eco*RI and *Xba*I sites of the high-copy pBAD vector pMPM201 (ColE1 *ori*, ampicillin resistance; Mayer 1995). DH5α *supE* was transformed with the ligation mixture and plasmid-carrying transformants were selected at 37° on LB plates containing ampicillin (100 μg/ml) and 0.05% glucose. The resulting 10^6 colonies were pooled and grown in LB broth for 2 hr. Aliquots were kept frozen at −80°. We randomly selected 30 individual clones to verify the

frequency of the correct-size insertion: 85% of the clones had the correct size. Of these clones, 19 were sequenced using automated sequencing (Li-Cor, Inc., Lincoln, NE). It turned out that 3/19 clones carried no mutations in gene *31*, 10/19 carried a single mutation, 2/19 carried two mutations, 1/19 carried three mutations, 1/19 carried four mutations, 1/19 carried five mutations, and 1/19 carried six mutations.

Recombination of mutant gene *31* alleles from plasmid onto the T4 chromosome: DH5α *supE* bacteria transformed with the PCR-mutagenized gene *31* library or with a plasmid bearing a specific gene *31* mutant allele were infected with T4 *31am*NG71 at a multiplicity of infection of 0.01 bacteriophage per bacterium. Lysis was observed 4 hr later, at which point chloroform was added. Tenfold dilutions were made on B178 *sup*$^+$ (nonpermissive for T4*31am*NG71) and CR63 *supD* (permissive for T4*31am*NG71) bacterial lawns and the plates were incubated overnight at 37°. From the bacteriophage growth results, we calculate an appropriate recombination frequency of 10^{-3} with the plasmid *am*$^+$ allele per bacteriophage progeny. Lysates from the gene *31* mutagenized library were diluted 10-fold and aliquots were plated on *groEL*44 bacterial lawns. Wild-type T4 plates with an efficiency of $\sim 10^{-7}$ on this strain, while the PCR-mutagenized T4 library plated with an efficiency of 5×10^{-6}. Six individual plaques were purified by restreaking on the same *groEL*44 bacterial lawn from which they were isolated, and then they were tested for plating ability on various *groEL* mutant strains. Six mutant gene *31* alleles thus isolated were PCR amplified from single plaques and the PCR product was sequenced using the automated sequencing scheme described above.

RESULTS

Spontaneously arising T4 mutants are able to overcome the *groEL*44-imposed block: To ensure the isolation of independent mutants, 20 stocks of T4 wild type were prepared, each from a single plaque. When these bacteriophage stocks were plated on *groEL*44, plaque formers (termed ε) appeared at a frequency of $\sim 10^{-7}$ compared to the B178 isogenic wild-type bacterial host. A single plaque former from each lysate was purified and tested further. It turned out that all 20 independently isolated T4ε mutants plated well on *groEL*44 and B178 but did not plate on the *groEL*515 mutant host. As seen in Table 1, *groEL*515 is permissive for T4 wild type. Thus, with respect to plating, the 20 independently and spontaneously isolated T4ε mutants behaved like T4*31*ε1, the spontaneously occurring mutant previously characterized in detail in our laboratory (Table 1; Georgopoulos *et al.* 1972; Keppel *et al.* 1990; Richardson *et al.* 1999).

The *31* gene of 12 of the newly isolated T4ε mutants was amplified by PCR (see materials and methods) and sequenced. All 12 new T4ε mutants possessed the same C → A transversion at codon 35, resulting in the substitution of leucine for isoleucine at that position in Gp31 (Table 2). This is the identical mutation found in *31*ε1, previously identified and sequenced in our laboratory (Keppel *et al.* 1990). Thus, it appears that the observed C → A transversion at codon 35 of gene *31* is the most frequent, spontaneously occurring mutation that enables bacteriophage T4 to efficiently bypass the *groEL*44-imposed block. In a recent biochemical study, we showed that wild-type Gp31 does not stably interact with GroEL44 at 25° and that the leucine 35 → isoleucine substitution restores interaction with GroEL44 (Richardson *et al.* 1999).

PCR-generated T4 mutants are able to overcome the *groEL*-imposed block: Because we repeatedly isolated the same ε1 type of mutation from the nonmutagenized bacteriophage T4 stocks, we wondered whether it is solely due to a mutational hotspot in gene *31*, or whether the leucine 35 → isoleucine substitution represents the only mechanism by which Gp31 can effectively bypass the *groEL*44-imposed block. To help answer this question, we designed a system using PCR mutagenesis to specifically mutagenize gene *31* of T4, as described in detail in materials and methods. In a nutshell, gene *31* was PCR mutagenized and cloned onto a plasmid vector. The mutagenized *31* gene sequences were introduced into DH5α *supE* bacteria (permissive for amber mutations) and infected *en masse* with bacteriophage T4 *31am*NG71. The resulting T4 *am*$^+$ recombinants were

TABLE 1

Plating properties of various bacteriophage T4 mutants

	B178	*groEL44* (E191G)	*groEL*515 (A383T)	*groEL*140 (S201F)	*groEL*673 (G173D, G337D)
T4	+	−	+	+	+
T4 *31*ε1 (L351)	+	+	−	+	−
T4 *31*ε2, *31*ε3, *31*ε4, *31*ε5 (I36F)	+	+	±	+	−
T4 *31*ε6 (E29V)	+	+(s)	±	+	−

B178 is the wild-type bacterial strain from which all other *groEL* mutant strains are derived. The designation in parentheses under each bacterial mutant strain represents the amino acid substitution in GroEL resulting from the particular mutation; *e.g.*, E191G represents the glutamic → glycine substitution at codon 191. All platings were done at 37°. +, an efficiency of plating of ~1.0 and good-size plaque, comparable to that on the permissive B178 bacterial host; +(s), an efficiency of plating of ~1.0, but a smaller plaque size compared to that on the permissive B178 bacterial host; ±, an efficiency of plating of ~0.1–1.0 and a much smaller plaque size compared to that on B178; −, an efficiency of plating of $<10^{-5}$ compared to that on B178.

selected on the basis of their ability to plaque on B178 *sup*$^+$ bacteria and pooled. This pool of *31am*$^+$ mutagenized bacteriophage T4 recombinants was plated on *groEL*44 *sup*$^+$, and ε mutants were isolated at a frequency of $\sim 5 \times 10^{-6}$, ~50-fold higher than that of spontaneously occurring ε mutants. Six of these new T4ε mutants were purified and tested further by spot tests on various bacterial hosts. It turned out that a new spectrum of plating phenotypes, distinct from that observed with the nonmutagenized T4 stocks, was encountered with the PCR mutagenesis-derived T4 mutants (Table 1). For example, unlike T4ε1, all six newly isolated T4ε mutants were able to grow, albeit to a limited extent, on *groEL*515 mutant bacteria. The gene *31* of these six isolates was amplified by PCR and sequenced; the results are given in Table 1. Three of these mutants, T4 *31*ε2, T4 *31*ε3, and T4 *31*ε4 were shown to carry an A → T transversion at codon 36 resulting in the isoleucine 36 → phenylalanine substitution at the corresponding position in Gp31. Another mutant, T4 *31*ε5, carried the same A → T transversion at codon 36 as mutants T4 *31*ε2, T4 *31*ε3, and T4 *31*ε4, but, in addition, carried a silent T → A transversion at codon 35 (Table 2). The fifth mutant, T4 *31*ε6, carried an A → T transversion at codon 29, resulting in a glutamate 29 → valine substitution at that position, and two silent mutations, namely a T → A transversion at codon 46 and a C → T transition at codon 54 (Table 2). The sixth mutant, T4 *31*ε7, was not studied in detail because its *31* gene sequence was identical to that of wild type. Likely, the ε7 mutation represents an unmapped, extragenic suppressor, which may either influence the folding of Gp23 (Andreadis and Black 1998) or affect the intracellular levels of Gp31.

We found a potential explanation for the overwhelming spontaneous occurrence of the C to A transversion at codon 35 of gene *31*. It has been suggested that some mutations in bacteriophage T4 may be induced by a sequence conversion mechanism (Shinedling *et al.* 1987). A sequence conversion event was first invoked as a possible mechanism for a frequently occurring insertion at the FC47 site, a dispensable region of the T4 *rIIB* gene. More analogous to our *31*ε1 observed mutation is the *lacI* mutation 022 that is caused by a transversion far more frequent than any other transversion in that gene. A six-nucleotide sequence, located 41 bp downstream from the mutational hotspot, may serve as a template for this sequence conversion (Shinedling *et al.* 1987). Encouraged by these results, we searched the bacteriophage T4 DNA and found a nine-nucleotide sequence (CAGGAATTA) located ~1800 bp upstream in the deoxycitidylate deaminase (*cd*) gene that could serve as a template causing the mutation CAGGAC TTA → CAGGAATTA at codon 35 of gene *31*.

The classical *31ts*A70 temperature-sensitive mutation affects the Gp31 mobile loop: The bacteriophage T4 *31ts*A70 mutation was originally described by Epstein *et al.* (1964). In our hands, bacteriophage T4 *31ts*A70 forms small plaques at the permissive temperature of 30° and does not form plaques at 39° on wild-type B178 bacteria. The gene *31* DNA of the T4 *31ts*A70 mutant was amplified by PCR and sequenced. A single T → C transition mutation was found at codon 37, which results in an isoleucine 37 → threonine substitution at that position in Gp31. Because this particular amino acid position is always occupied by a hydrophobic member in all cochaperonins sequenced (Figure 1), the substitu-

TABLE 2

DNA sequence analysis

Mutant	DNA sequence changes	Amino acid changes
T4 *31*ε1	CTT-ATT	L35I
T4 *31*ε1, ε4, ε4	ATT-TTT	I36F
T4 *31*ε5	CTT-CTA, ATT-TTT	L35L, I36F
T4 *31*ε6	GAA-GTA, CCT-CCA, GTC-GTT	E29V, P46P, V54V
T4 *31ts*A70	ATC-ACC	I37T
T4 *31ts*A70-R1	CTT-ATT, ATC-ACC	L35I, I37T
T4 *31ts*A70-R2	ACC-ATC	T37I
T4 *31*ε1-T31A	ACA-GCA, CTT-ATT	T31A, L35I
T4 *31*ε1-T31I	ACA-ATA, CTT-ATT	T31I, L35I
T4 *31*ε1-A23V	GCA-GTA, CTT-ATT	A23V, L35I
T4 *31*ε1-G26S	GGT-AGT, CTT-ATT	G26S, L351
T4 *31*ε1-E28G	GAA-GGA, CTT-ATT	E28G, L351
T4 *31*ε1-G38D	CTT-ATT, GGT-GAT	L351, G38D
T4 *31*ε1-R40H	CTT-ATT, CGT-CAT	L351, R40H
T4 *31*ε1-R40C	CTT-ATT, CGT-TGT	L35I, R40C

The exact procedures used in the DNA sequence analysis of the bacteriophage T4-encoded mutations studied in this work are described in materials and methods. The particular T4 mutant sequenced is indicated in the left column. The nucleotide changes that were found are shown in the middle column. The corresponding amino acid changes in Gp31 are given in the right column.

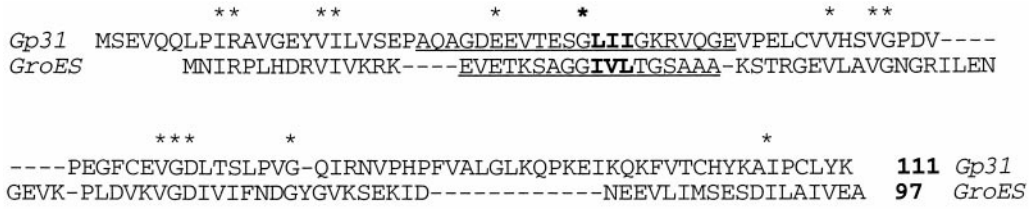

Figure 1.—Amino acid sequence alignment of the Gp31 and GroES cochaperonins. Identity is indicated by an asterisk. The bold asterisk indicates the glycine residue in the mobile loop conserved in all cochaperonins. The mobile loop sequences of each cochaperonin are underlined. The hydrophobic tripeptide, which in the case of GroES has been shown to directly interact with GroEL in the crystal structure, is shown in boldface. The amino acid sequence alignment is essentially taken from Hunt *et al.* (1997).

tion of a hydrophilic amino acid at this position most likely weakens interaction with GroEL. Thus, it is likely that the Gp31tsA70 mutant protein makes an unstable complex with the GroEL chaperone (this explains its small plaque phenotype at all permissive temperatures) and that the instability of the Gp31tsA70/GroEL complex increases as a function of temperature.

Suppressor analysis of the T4 *31ts*A70 temperature-sensitive mutation: To get some insight into the mechanism resulting in the temperature-sensitive phenotype of the T4 *31ts*A70 mutation, we isolated 11 temperature-resistant, plaque-forming revertants at 39° on B178 bacteria at a frequency of $\sim 10^{-6}$. The plating characteristics of these temperature-resistant revertants are shown in Table 3. Based on their plating phenotype on various bacterial mutant hosts, two types of revertants were encountered. The first class, exemplified by *31ts*A70-R2, behaved like wild type on all bacterial hosts tested. The second class of revertants, exemplified by *31ts*A70-R1, was more susceptible to the effects of some of the *groEL* mutations, *e.g.*, unlike T4 wild type, these revertants did not form plaques on the *groEL*44 mutant host at 25° or on the *groEL*140 mutant host at 37° (Table 3). The gene *31* DNA sequence of all 11 temperature-resistant revertants was determined. It turned out that 4 of them, all belonging to one class (R2), had regained the wild-type DNA sequence at codon 37, *i.e.*, the C → T transition restoring the wild-type sequence. The remaining 7 isolates, all belonging to the second class (R1), retained the original T → C transition mutation at codon 37 and had acquired an additional C → A transversion mutation at codon 35 (Table 2). This *31ts*A70 intragenic suppressor mutation at codon 35 is identical to the *31*ε1 mutation discussed above, resulting in the leucine 35 → isoleucine change at that position in Gp31. The strengthening effect of the leucine → isoleucine substitution at codon 35 may compensate for the weakening of the Gp31/GroEL interaction that may be caused by the isoleucine → threonine substitution at codon 37. However, although the compensatory mutation at codon 35 allows growth on the B178 wild-type strain at 39°, it does not fully restore the wild-type bacteriophage T4 growth pattern on all *groEL* mutant hosts, as discussed above and shown in Table 3.

TABLE 3

Plating properties of the bacteriophage T4*t31s*A70 temperature-sensitive mutant and its Ts$^+$ revertants

	B178 39°	*groEL*44 (E191G) 25°	*groEL*515 (A383T) 37°	*groEL*140 (S201F) 37°
T4 Do	+	+(s)	+	+
T4 *31ts*A70 (I37T)	−	−	−	−
T4 *31ts*A70-R1	+	−	+	−
T4 *31ts*A70-R2	+	+(s)	+	+

Revertants of bacteriophage T4*31ts*A70 were isolated as plaque formers on B178 at 39°. See text for details and Table 1 for an explanation of the symbols. The temperature of incubation varied and is indicated under each bacterial strain.

Site-directed mutagenesis of gene *31*: Because previous studies had highlighted the importance of the highly conserved glycine amino acid residue at position 34 of Gp31 (see Figure 1), its corresponding codon was altered by site-directed mutagenesis as described in materials and methods. Specifically, the GGA wild-type codon (coding for glycine) was altered to either GAC (coding for aspartate) or ATC (coding for isoleucine). Both of these mutations proved to be lethal for bacteriophage T4 growth, because none of the mutant Gp31 proteins, even when expressed at high levels from an appropriate plasmid construct, was capable of restoring growth to the bacteriophage T4 *31am*NG71 mutant on the B178 *sup*$^+$ host. However, neither of the mutant Gp31 proteins exerted a dominant negative effect, because they did not inhibit growth of T4 wild type under the same conditions. In separate experiments, we showed that both mutant proteins are expressed to comparable levels, similar to those of wild-type Gp31 from the same plasmid vector, and they can properly oligomerize to form heptamers (data not shown).

In additional site-directed mutagenesis experiments, codon 36 was changed from ATT to TGG, resulting in an isoleucine 36 → tryptophan substitution at the corresponding amino acid position of Gp31. The corresponding T4 *31*I36W bacteriophage mutant plated like wild type on all bacterial strains tested, with the notable exception that it formed very small plaques, with an approximate efficiency of 10^{-1}–10^{-2}, on the *groEL*44 mutant host at 37°. Thus, it appears that the isoleucine

TABLE 4

Plating properties of the T4 *31*ε1 revertants

	B178	*groEL*44 (E191G)	*groEL*515 (A383T)	*groEL*140 (S201F)	*groEL*673 (G173D, G337D)
T4	+	−	+	+	+
T4 *31*ε1 (L35I)	+	+	−	+	−
T4 *31*ε1-T31A	+	−	+	+	+
T4 *31*ε1-T31I	+	−	+	±	−
T4 *31*ε1-A23V	+	−	+	+	−
T4 *31*ε1-G26S	+	−	+	+	−
T4 *31*ε1-E28G	+	−	+	−	−
T4 *31*ε1-G38D	+	−	±	±	−
T4 *31*ε1-R40C	+	−	+	+	−
T4 *31*ε1-R40H	+	−	+	+	±
T4 *31*T31A	+	−	+	−	−

Revertants of T4 *31*ε1 were isolated as plaque formers on the *groEL*515 nonpermissive host. The independently isolated bacteriophage revertants are designated as ε1 to signify the fact that they all retain the original ε1 mutation, followed by a designation indicative of the effect of the suppressor/compensatory mutation in each case, *e.g.*, T4 *31*ε1-T31A is a revertant that retained the original ε1 mutation (L35I) and its particular suppressor mutation results in the threonine → alanine substitution at codon 31. All platings were done at 37°. Other symbols used are described in the legend to Table 1.

36 → tryptophan amino acid substitution in Gp31 strengthens its effective interaction with GroEL44, but not to the same extent as the isoleucine 36 → phenylalanine substitution, which allows the bacteriophage T4 *31*ε2 mutant to grow well on *groEL*44 at 37° (Table 1).

Suppressor analysis of the *31*ε1 mutation: As stated above, one of the characteristic phenotypes of the T4 *31*ε1 mutant is its failure to plaque on the *groEL*515 mutant host (Georgopoulos *et al.* 1972; Keppel *et al.* 1990). Although the restrictive phenotype is quite tight on *groEL*515 bacteria, nevertheless, at a frequency of 10^{-6}, spontaneous mutants of T4 *31*ε1 capable of plaque formation can be readily isolated. Accordingly, we prepared 40 independent stocks of T4 *31*ε1 on *groEL*44 bacteria, each initiated from a single plaque. These 40 independent T4 *31*ε1 lysates were plated on the *groEL*515 mutant bacteria. The spontaneously occurring plaque formers were purified, grown up, and tested on various *groEL* mutant hosts. This preliminary classification scheme enabled the identification of different plating phenotypes among the T4 *31*ε1 plaque formers on *groEL*515, thus ensuring the presence of different suppressor mutations (Table 4). Although many different plating phenotypes were encountered among the T4 *31*ε1 revertants on *groEL*515 bacteria, a notable communal phenotype was the simultaneous loss of ability to plaque on the *groEL*44 mutant host at 37° (Table 4).

Twenty of the T4 *31*ε1 suppressor mutants were selected and their gene *31* DNA was amplified by PCR and sequenced. All 20 suppressors retained the original ε1 mutation at codon 35. Strikingly, all 20 had acquired a second site suppressor mutation, all of which mapped in the DNA region encoding for the mobile loop (Figure 2). Fourteen of the 20 independently isolated suppressors had a change at codon 31, resulting in the substitution of a threonine to either alanine (12 independent isolates) or to isoleucine (2 independent isolates) in Gp31. Two of the suppressors had a change at codon 40, resulting in the substitution of arginine to either cysteine or histidine in Gp31. The 4 remaining suppressors mapped in codons 23, 26, 28, and 38, respectively (Table 4; Figure 2). Thus, it appears that the inability of the T4 *31*ε1 mutant to plaque on *groEL*515 bacteria can be overcome by a variety of intragenic suppressor mutations, each altering one of six different amino acid residues in the mobile loop. We believe that the most likely explanation for the seeming "randomness" of mutational events that can lead to this common phenotype is that each of the suppressor mutations results in a relative weakening of the otherwise very strong Gp31ε1/GroEL515 protein-protein interaction.

Phenotype of the *31*T31A suppressor in the absence

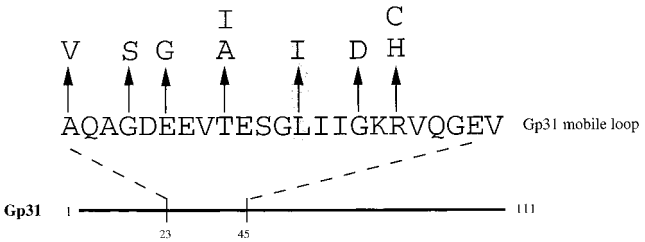

Figure 2.—All T4 *31*ε1 suppressor mutations affect the Gp31 mobile loop. All amino acid changes found in this work as capable of reverting the effect of the *31*ε1 mutation and thus permitting growth on *groEL*515 mutant bacteria are summarized. Highlighted in the shaded area is the leucine 35 to isoleucine substitution resulting from the original *31*ε1 mutation. See text for details.

of ε1: One of the intragenic suppressors of the ε1 mutation, *31*T31A, was chosen for further study. The rationale for choosing this particular suppressor mutation was based on (1) the fact that it represents by far the majority type of all spontaneously isolated suppressors of ε1, and (2) we had already purified and studied the *in vitro* properties of the Gp31ε1T31A protein (Richardson *et al.* 1999). The A → G transition at codon 31 of gene *31* was introduced by site-directed mutagenesis into the minimal gene *31* cloned in an appropriate plasmid (see materials and methods for details). The introduced mutation was verified by sequencing gene *31* from the resulting plasmid. The *31*T31A mutation was crossed back onto the T4 genome in the manner previously described for the PCR-induced gene *31* mutagenized pool. Twelve T4 am^+ recombinant plaques were purified on B178 sup^+ bacteria and subsequently tested for growth on various *groEL* mutant hosts. It turned out that eight of these am^+ recombinant bacteriophages behaved like wild type, whereas the remaining four am^+ recombinants did not propagate on either *groEL*140 or *groEL*673 mutant bacteria, both of which allow wild-type T4 growth (Table 4). We verified that the latter class of bacteriophage recombinants carried the *31*T31A mutation and no other mutation in *31* gene by amplifying gene *31* from two members of this class and sequencing it. The fact that the T4 *31*T31A mutant is more sensitive than wild type to the effects of the *groEL*140 and *groEL*673 mutations suggests that the Gp31T31A protein interacts more weakly with the GroEL140 and GroEL673 mutant proteins than does wild-type Gp31. This explanation is based primarily on the ability of the *31*T31A mutation to significantly lower the affinity of Gp31ε1 for $GroEL^+$ (Richardson *et al.* 1999).

DISCUSSION

The genetic data presented here complement and extend the structural observation that the mobile loop of Gp31 is the key mediator of its interaction with GroEL (Landry *et al.* 1996; Xu *et al.* 1997). All 15 different mutant gene *31* alleles reported here that affect GroEL/Gp31 interaction encode an amino acid change in the mobile loop segment. The allele-specific analysis of Gp31 and GroEL also contributes evidence to a recently proposed hypothesis by our group, in collaboration with the laboratory of Sam Landry. The hypothesis states that the mutations that we isolated in either *groEL* or *31* act primarily by altering the affinity of their products for each other (Richardson *et al.* 1999). Obviously, Gp31 must interact with GroEL to ensure proper and timely substrate folding, but, to permit the GroEL/Gp31 chaperone machine to recycle itself in a timely fashion, this interaction cannot be too strong (Landry *et al.* 1996; Richardson *et al.* 1999). Below, we summarize the primary findings reported in this article and attempt to explain their structural significance.

Structural studies indicate that the Gp31 mobile loop forms a β-hairpin turn upon binding to GroEL (Landry *et al.* 1996). A key residue in the formation of this β-hairpin turn is the universally conserved glycine (at position 24 for GroES and 34 for Gp31; see Figure 3), most likely because only glycine can assume the positive dihedral phi angle required for such a turn (Landry *et al.* 1993, 1996). In this work we showed that this glycine residue of Gp31 is most likely essential for the biological function of the cochaperonin because mutating it to either an isoleucine or an aspartate abolishes Gp31's biological function. We chose to mutate this

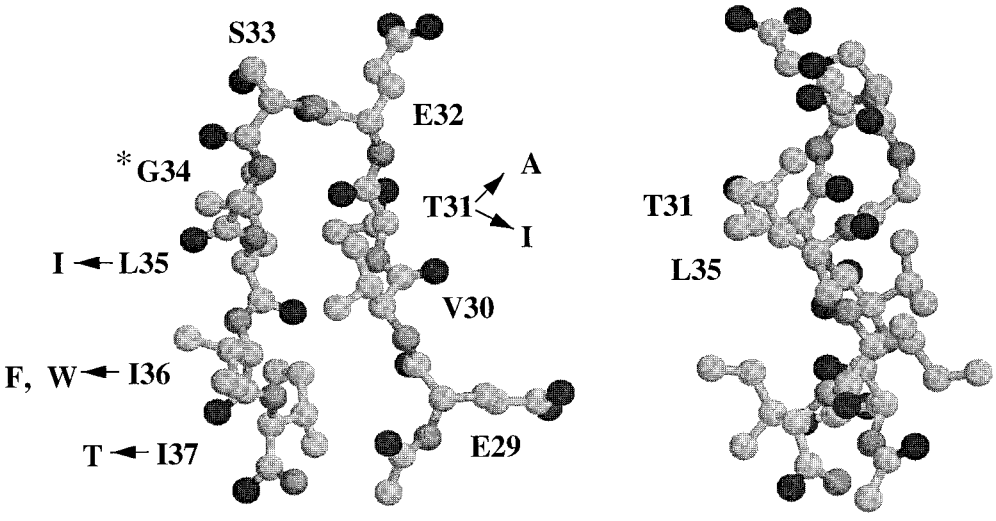

Figure 3.—Gp31 mobile loop peptide structure. The model was created in Rasmol by Sam Landry based on the NMR structure of the GroES mobile loop peptide (Landry *et al.* 1993). The mobile loop forms a β-hairpin turn upon binding to GroEL (all mutations isolated in this study affect mobile loop residues). A universally conserved glycine (G34, indicated by an asterisk), found in all existing sequences of cochaperonins, occurs at the β-hairpin turn. A highly conserved hydrophobic tripeptide (L35-I36-I37), found adjacent to the glycine, makes the primary contact between Gp31 and GroEL. Altering any of these three hydrophobic residues leads to aberrant chaperone function depending on the particular GroEL mutant partner. Altering the first two residues of the hydrophobic tripeptide in Gp31 (L35I, I36F, and I36W) results in total or partial rescue of interaction with GroEL44. However, alteration of the third residue from I37T results in poor growth, even on a wild-type host. Several other residues in the mobile loop create intramobile loop contacts that may be important for regulating the propensity of β-hairpin formation (*e.g.*, T31A and T31I).

position to an aspartate because the analogous mutation in the *E. coli groES* cochaperonin gene, *groES*619, has already been isolated and studied. Specifically, *groES*619 mutant bacteria do not form colonies at high temperatures and are restrictive for bacteriophage lambda and T5 growth at all temperatures (Georgopoulos *et al.* 1973; Tilly *et al.* 1981). It could be that the *groES*619 mutation is not lethal for *E. coli* growth because the neighboring glycine, G23, may substitute, at least partially, to ensure the β-hairpin formation. Because this neighboring glycine is not available in the Gp31 sequence, it may not be surprising that glycine 34 is essential for Gp31 function. Overproduction of either Gp31(G34D) or Gp31(G34I) does not interfere with growth of wild-type bacteriophage T4, suggesting that the mutant proteins do not bind effectively to GroEL.

Our results also highlight the importance of the highly conserved hydrophobic tripeptide (L35-I36-I37) in Gp31 (Figure 3). Specifically, we repeatedly isolated suppressors on *groEL*44 mutant bacteria that affected residue 35 in Gp31. We showed here that mutating isoleucine 36 to phenylalanine (T4 mutants *31*ε2, *31*ε3, *31*ε4, *31*ε5) also enables T4 to form plaques on *groEL*44 mutant bacteria. Similarly, the mutant Gp31(I36W), created by site-directed mutagenesis, has both *in vivo* and *in vitro* characteristics that suggest that it partially restores interaction with GroEL44 by increasing Gp31 affinity.

Contrary to the effects of these GroEL44/Gp31 interaction-strengthening mutations that map to the hydrophobic tripeptide, we found that the classical bacteriophage T4 *31*tsA70 temperature-sensitive mutant does not grow well on wild-type bacteria, even at permissive temperatures, and is very susceptible to various mutations in the *groEL* gene to which wild-type T4 is refractory. Sequencing of the *31*tsA70 allele showed that it results in a change in the hydrophobic tripeptide from the conserved hydrophobic residue (isoleucine) at position 36 to a hydrophilic residue (threonine). Spontaneous reversion of the *31ts*A70 temperature sensitivity results from either restoration of the wild-type amino acid sequence or a second site change, resulting in the leucine to isoleucine substitution at codon 35. Since the leucine 35 to isoleucine substitution alone has been shown to enhance Gp31's affinity for GroEL, it is reasonable to propose that the leucine 35 to isoleucine substitution also rescues Gp31(I37T)'s weak affinity for GroEL by a similar mechanism.

Our systematic suppressor analyses identified many different mutations that led to the same phenotype, *i.e.*, restoration of bacteriophage T4ε1 growth on *groEL*515 and simultaneous loss of ability to plaque on *groEL*44. The genotypes of these suppressors show that six different amino acids can be changed to eight other residues in the mobile loop of Gp31(L35I), resulting in similar plating phenotypes (Figure 2; Table 4). The most frequent suppressor target was the threonine codon at position 31 (14 out of 20; Figure 3). Structural studies with a synthetic peptide of the mobile loop of Gp31 have highlighted the physical interaction between the leucine 35 and threonine 31 residues (Landry *et al.* 1996; Figure 3). Substituting threonine 31 with alanine or isoleucine at this position should weaken the β-sheet propensity according to Minor and Kim (1994). Another change that reduces Gp31's affinity affects amino acid residue 28 (from glutamate to glycine), which, according to the NMR structure, interacts with isoleucine 36. This glutamate to glycine substitution should result in a weakening of β-sheet propensity. Finally, according to this same paradigm, the leucine 35 to isoleucine change, observed in the T4ε1 mutant, should result in increased β-hairpin stability because isoleucine is more favorable for β-sheet formation (Minor and Kim 1994).

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