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Genetic Properties of Temperature-Sensitive Folding Mutants of the Coat Protein of Phage P22

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ABSTRACT

Temperature-sensitive mutations fall into two general classes: those generating thermolabile proteins; and those generating defects in protein synthesis, folding or assembly. Temperature-sensitive mutations at 17 sites in the gene for the coat protein of Phage P22 are of the latter class, preventing the productive folding of the polypeptide chain at restrictive temperature. We show here that, though the coat subunits interact intimately to form the viral shell, these temperature-sensitive folding (TSF) mutations were all recessive to wild type. The mutant polypeptide chains were not rescued by the presence of wild-type polypeptide chains. Missense substitutions in multimeric proteins frequently exhibit intragenic complementation; however, all pairs of coat protein TSF mutants tested failed to complement. The recessive phenotypes, absence of rescue and absence of intragenic complementation are all accounted for by the TSF defect, in which destabilization of a folding intermediate at restrictive temperature prevents the mutant chain from reaching the conformation required for subunit/subunit recognition. We suggest that absence of intragenic complementation should be a general property of TSF mutations in genes encoding multimeric proteins. The spectra of new loci identified by isolating second-site suppressors and synthetic lethals of temperature sensitive mutants will also differ depending on the nature of the defect. In the case of TSF mutations, where folding intermediates are defective rather than the native molecule, the spectra of other genes identified should shift from those whose products interact with the native molecule to those whose products influence the folding process.

TEMPERATURE-sensitive mutations were originally isolated by HOROWITZ and LEUPOLD (1951) to estimate the fraction of essential genes in *Escherichia coli*. Subsequently R. S. EDGAR and coworkers recognized the potential of TS alleles as conditional lethal mutations for identifying and characterizing genes for essential proteins in bacteriophage (EDGAR *et al.* 1964). Temperature-sensitive mutations have since proved invaluable for the dissection of such diverse processes as the yeast cell cycle (HARTWELL *et al.* 1970), Chlamydomonas flagellar assembly (ADAMS *et al.* 1982) and protein folding in the cell (GOLDENBERG *et al.* 1983).

Temperature-sensitive mutations were initially divided into two classes by SADLER and NOVICK (1965). The first class, which was labeled TL (thermolabile), represented mutants that showed phenotypic defects when grown at high temperature, or when grown at low temperature and transferred subsequently to restrictive temperature. Such mutants are often considered to represent the destabilization of the native protein by the TS amino acid substitution (GOLDENBERG 1988). For example, TS mutants of lambda repressor (HECHT *et al.* 1984), yeast mitochondrial hsp60 (MARTIN *et al.* 1992) and LAR (TSAI *et al.* 1991) have melting temperatures below the wild-type melting temperature.

The second class of mutations were labeled TSS (temperature-sensitive synthesis). These mutations, like the TL class, were defective when grown at restrictive temperature. However, if grown at permissive temperature, and then shifted to restrictive temperature, they did not display phenotypic defects. The defect only occurred if the mutant protein was expressed and permitted to assemble at restrictive temperature. Mutants of this class include TS mutants in more than 30 genes of bacteriophage T4 (EDGAR and LIELAUSIS 1964), TS mutants of T4 DNA Polymerase (DEWAARD *et al.* 1965) and TS alleles at more than 30 sites in the P22 tailspike endorhamnosidase (SMITH and KING 1981). Mutants of this class may be expected to be defective at restrictive temperature in protein synthesis, protein folding or, in the case of multimeric proteins, some aspect of the protein assembly process.

A systematic examination of the TSS mutations in the tailspike of Phage P22 revealed that the primary defect was in the folding of the polypeptide chain (GOLDENBERG and KING 1981; GOLDENBERG *et al.* 1983; STURTEVANT *et al.* 1989; MITRAKI *et al.* 1991). The TS amino acid substitutions had no effect on the stability or activity of the thermostable tailspike protein, once matured at permissive temperature. However, if expressed at restrictive temperature, a folding

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intermediate was unable to continue productive folding. These mutations were subsequently called "temperature-sensitive folding" (TSF) mutations (YU and KING 1984; KING *et al.* 1989). Similar TSF phenotypes have also been reported for mutants of D-lactate dehydrogenase (TRUONG *et al.* 1991) and bacterial luciferase (SUGIHARA and BALDWIN 1988).

Missense mutations in genes encoding multimeric proteins often display intragenic complementation (FINCHAM 1966). Studies with alkaline phosphatase, beta-galactosidase, glutamate dehydrogenase and T4 tail fibers indicated that the mechanism of this phenomenon was correction of mutant defects by subunit/subunit interaction in the multimeric protein (SCHLESINGER and LEVINTHAL 1963; GAREN and GAREN 1963; CODDINGTON and FINCHAM 1965; BERNSTEIN *et al.* 1965; ZABIN and VILLAREJO 1975).

This model predicts that in order for intragenic complementation to occur, mutant chains must attain a conformation capable of interaction with other chains. In support of this, intragenic complementation by nonsense mutations is extremely rare (EPSTEIN *et al.* 1963; EDGAR *et al.* 1964). These fragments are often degraded within cells and generally express no functions of the complete polypeptide chain. An exception is beta-galactosidase where chain fragments can complement certain mutations (ZABIN and VILLAREJO 1975).

Intragenic complementation may be expected to potentially correct defects in association-competent (folded) protein conformations. Complete polypeptide chains that were blocked in the folding process would be expected to fail to display intragenic complementation. The group of TSF mutations in the P22 tailspike gene failed to display intragenic complementation between any pairs tested (SMITH *et al.* 1980), unlike for example, TS mutants in the tailspike and tailfiber genes of Phage T4 (BERNSTEIN *et al.* 1965). Unfortunately, the absence of the positive control, a pair of tailspike mutants that did display intragenic complementation, made it difficult to conclude that the negative results were due directly to the folding defects.

The distinction between TSF mutations and mutations that perturb the function of the folded molecule (TL or assembly-defective TSS) is likely to be particularly important in the isolation of second-site suppressor mutations. Selection of second-site suppressors of TS mutants has provided a general approach to identifying genes whose products interact with an initial protein of interest (JARVIK and BOTSTEIN 1975; MOIR and BOTSTEIN 1982; LUX and DUTCHER 1991; HAN *et al.* 1990; NELSON *et al.* 1992). If the initial mutation destabilizes a folding intermediate rather than impairing the function of the active intracellular conformation, this approach may be less productive for the isolation of gene products interacting with the mature form of the protein. On the other hand, the selection may yield sites influencing folding and maturation pathways (FANE and KING 1991; FANE *et al.* 1991; MITRAKI *et al.* 1991). Selection of second-site suppressors of the tailspike TSF mutations yielded global intragenic suppressors (FANE *et al.* 1991; MITRAKI *et al.* 1991). These substitutions improved the folding efficiency of different TSF mutations, as well as of the wild-type tailspike protein, by suppressing the pathway to inclusion body formation (MITRAKI *et al.* 1991).

Recently, we have characterized a set of TS mutants in the major coat protein of phage P22 (GORDON and KING 1993). The coat protein, encoded by gene *5*, has 430 amino acids and is not cleaved or otherwise covalently modified during its life cycle, except for removal of its N-terminal methionine residue (KING *et al.* 1973; EPPLER *et al.* 1991). The 17 sites of TS mutations were distributed throughout the gene (Figure 1A).

Viral capsids are composed of several hundred protein molecules that are tightly associated in an icosahedral lattice. In the double-stranded DNA bacteriophages (HENDRIX 1985), as well as the eukaryotic viruses herpesvirus (NEWCOMB and BROWN 1991) and adenovirus (HORWITZ 1991), the viral DNA is packaged into a preformed shell. These precursor shells, which are referred to as procapsids, are the direct products of the polymerization of the coat and scaffolding subunits (Figure 1B).The scaffolding molecules exit the procapsid as DNA is pumped in, and the coat protein shell undergoes a structural transformation to give rise to the mature virion (EARNSHAW and CASJENS 1980).

In bacteriophage P22, approximately 420 molecules of coat protein, 300 molecules of scaffolding protein and other minor proteins polymerize into the procapsid particle (KING *et al.* 1973; KING and CASJENS 1974; PREVELIGE *et al.* 1988). The capsid subunits exhibit multiple bonding contacts in their icosahedral lattices (PRASAD *et al.* 1993).

At permissive temperature the TS coat protein molecules folded and assembled into virions that remained stable and functional at higher restrictive temperatures (GORDON and KING 1993). At restrictive temperature complete chains were synthesized and were stable in the cell, but the chains did not form subunits competent to polymerize into capsid structures. The TS chains synthesized at restrictive temperature polymerized into inclusion bodies, which generally derive from folding intermediates rather than native-like states (MITRAKI and KING 1989). The mutations appeared to destabilize a folding intermediate, preventing productive folding to the functional subunit at high temperature, and resulting in irreversible aggregation at restrictive temperature. This interpretation is also supported by *in vitro* studies of

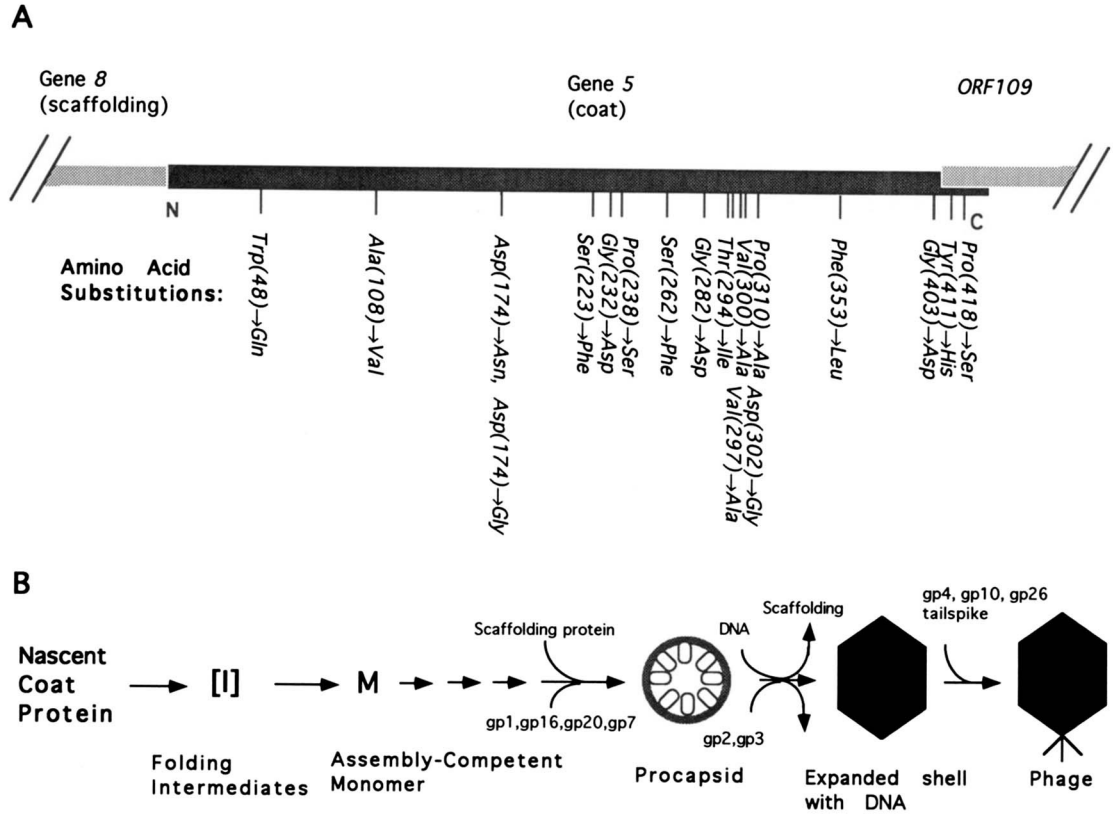

FIGURE 1.—(A) Genetic map of coat protein TSF mutations; (B) folding and assembly pathway for the P22 coat protein.

purified mutant coat protein, which indicated that folded mutant coat protein had near wild-type stability (M. L. GALISTEO, C. L. GORDON, and J. KING, unpublished data). Thus, these capsid protein mutants appear to be temperature sensitive folding mutants similar to those described for the P22 tailspike (GOLDENBERG *et al.* 1983; HAASE-PETTINGELL and KING 1988; KING *et al.* 1989).

We report here a series of experiments that genetically characterize the set of TSF coat protein mutants. The TSF phenotype, in which a destabilized folding intermediate prevents the chain from reaching the association-competent conformation, accounts for a number of genetic properties of the mutations. These are their recessive character, their lack of intragenic complementation and their inability to be rescued by wild-type chains. Such TSF phenotypes may be common among existing sets of TS mutations in genes of other organisms. We also report the sequences of two intragenic amino acid substitutions, which appear to function as second site suppressors, presumably by improving capsid protein folding efficiency. The results reported bear on the use of TS mutations generally as tools for studying gene function.

MATERIALS AND METHODS

Bacteria: All bacterial strains were derivatives of *Salmonella typhimurium* LT2. The suppressor minus host DB7136 [(*leu A414* (Am)*his C525* (Am)] was used for propagation of phage not carrying amber mutations. Its suppressor plus derivative DB7155 [(*leuA414* (Am)*hisC525* (Am)], which inserts glutamine, was used for propagation of strains carrying amber mutations (WINSTON *et al.* 1979).

Bacteriophage: P22 is a temperate phage. All strains used in these experiments carried the *c1-7* allele, which prevents lysogeny. The strains carrying TS mutations in the coat protein gene were isolated originally from a variety of selections and chemical mutageneses followed by screens for temperature sensitivity (JARVIK and BOTSTEIN 1975; CASJENS *et al.* 1991; GORDON and KING 1993). CASJENS *et al.* (1991) mapped the TS coat protein mutations discussed here by plasmid rescue experiments to intervals of gene *5* (encoding the coat protein). The CS mutations were described by JARVIK and BOTSTEIN (1973, 1975). Other mutations carried by some phage used in these experiments were gene *13 H101* (Am), which delays lysis, and gene *3 N6* (Am), which prevents DNA packaging (BOTSTEIN *et al.* 1973). Multiple mutant strains carrying the coat protein TS mutations as well as other extragenic alleles were constructed by genetic crosses (GORDON and KING 1993).

Phage nomenclature is as follows. *H* in a strain name indicates that hydroxylamine was the mutagen used; *N* indicates that N-methyl-N′-nitro-N-nitrosoguanidine was the mutagen; *r* indicates that the strain was isolated as an apparent revertant (BOTSTEIN *et al.* 1973; JARVIK and BOTSTEIN 1973).

Media: Luria broth was used to support bacterial growth for plating experiments and preparation of phage stocks. M9 media was 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.01% NH_4Cl. M9/Mg^{++} media was M9 plus 0.002 M $MgSO_4$. Minimal media was M9/Mg^{++} plus 0.4% glucose, 0.003% histidine, 0.008% leucine, 10^{-6} M $FeCl_3$, 10^{-6} M $CaCl_2$. Dilution fluid (DF), used for serial dilutions of phage for titering, was 0.1% tryptone, 0.7% NaCl, 2 mM $MgSO_4$.

Preparation of phage stocks: Young plaques picked with

a glass capillary tube were added to 30 ml LB with approximately 5×10^9 log-phase cells, and aerated by shaking at 30° until lysis (except for the exceptionally temperature-sensitive strain *Pro(310)→Ala*, which was grown at 24°). Cell debris was pelleted (10,000 rev/min for 10 min in a Sorvall SS34). Phage in the supernatant were pelleted by a 90-min centrifugation at 15,000 rev/min in the SS34 rotor. The pellets were drained and resuspended in 4 ml M9/Mg^{++}. Residual debris was pelleted by a second 10-min centrifugation at 10,000 rev/min.

DNA sequencing: Eppler *et al.* (1991) determined the sequence of wild-type gene *5*. Gordon and King (1993) determined the substitutions of the TS coat protein mutations by sequencing at least the interval of gene *5* to which the mutation had been mapped and comparing this sequence with the known wild-type sequence. The sequencing of apparent revertants reported here was performed as in Gordon and King (1993). Briefly, we performed a symmetric PCR reaction, using apparent revertant phage DNA as template, with primers spanning gene *5*. The resultant double-stranded DNA was gel-purified and used as template in a second asymmetric PCR reaction, in which only one primer was present. The resultant single-stranded DNA was precipitated and sequenced (Sequenase). The primers used for PCR amplification reactions and sequencing of the apparent revertants were the same as those utilized for sequencing the parent TS mutants, as described in Gordon and King (1993).

Single burst experiment and complementation experiment: A 1/100 dilution of an overnight culture of DB7136 cells was grown at 30° in LB to a concentration of about 10^8/ml. Cells were pelleted, resuspended at a concentration of 4×10^8/ml and put on ice. For each infection, 0.2 ml of phage at a total titer of 4×10^9/ml was placed at either 30° or 39°. In the co-infections, each of the two phage strains contributed half of this titer; 0.2 ml of cells were added to the phage to begin the infection; 90 min after infection initiation at 30° and 60 min after infection initiation at 39°, infections were terminated by dilution into DF saturated with $CHCl_3$. Resultant bursts were determining by plating at 24°.

In several cases where the level of cell killing was examined by plating the infection for viable cells, less than 1% of the cells were uninfected. In addition, titering for phage particles 12 min after infection, during the eclipse phase of the phage life cycle, demonstrated that greater than 99% of input phage particles had infected cells.

These data are representative of at least two experiments.

Radiolabeling experiment: The phage strains used in this experiment were *3N6* (Am)/*13*H101 (Am)/*c1-7* and *Trp(48)→Gln/3N6* (Am)/*13*H101 (Am)/*c1-7*. An overnight culture of DB7136 cells growing in minimal media was diluted 1/100 in fresh media and grown to a concentration of 1×10^8/ml at 32°. Cells were pelleted, resuspended in fresh media to a concentration of 4×10^8/ml and placed on ice. For each infection 0.2 ml of phage at a total titer of 4×10^9/ml, derived equally from each of the above phage strains in the co-infection, was placed at either 29° or 39°. Infections were initiated by addition of 0.2 ml of cells to the phage. The 39° cultures were labeled with ^{14}C-amino acids at a final concentration of 2 μCi/ml 40 min after infection, chased with casamino acids at a final concentration of 2% 45 min after infection, and placed on ice 70 min after infection. The 29° cultures were labeled 55 min after infection, chased 60 min after infection, and placed on ice 90 min after infection.

To lyse the infected cells, samples were frozen at −20°, thawed, frozen again in a dry ice/ethanol bath and thawed. A pellet/supernatant separation was then performed as follows: 25 μl of each sample were centrifuged for 3 min in a microfuge. The supernatants were removed and the pellets were washed in 25 μl M9/Mg^{++} and recentrifuged. These supernatants were removed and combined with the corresponding supernatants from the first centrifugation. The pellets were resuspended in 50 μl M9/Mg^{++}. Samples were mixed 1:2 with 3× SDS sample buffer (0.1875 M Tris, 6% SDS, 15% β-mercaptoethanol, 30% glycerol) and electrophoresed through a 10% SDS gel; 10 μl of unfractionated lysate, 20 μl of the pellet fraction and 20 μl of the supernatant fraction were loaded. Samples of the infections with one phage strain (Figure 3A, lanes 1–12) were electrophoresed through a 10% SDS gel until the Bromphenyl Blue dye had migrated ~15 cm. Samples of the co-infections (Figure 3A, lanes 13–18) were electrophoresed through a longer SDS gel until the Bromphenyl Blue marker dye had migrated ~40 cm, in order to resolve the two different coat protein species.

Coat protein bands were quantified by exposing gels to phosphorimager screens for 5 days and using the ImageQuant software (Molecular Dynamics). All coat protein bands were clearly visible and distinct. Bands were circled at "4×" magnification using the region tool, and radioactivity outside the gels was defined as background. The data presented in Figure 3B are the percentage of coat protein found in the pellet, using the sum of coat protein in the pellet and coat protein in the supernatant as total coat protein. The results were not significantly different if the total coat protein was defined as that present in the unfractionated lysate samples.

RESULTS

Efficiency of TSF mutant phage growth as a function of temperature: Most of the gene *5* TSF mutants display more than five orders of magnitude decrease in plating efficiency between growth at 30° and at 39°. The mutants are very "tight." The capsids assembled from the mutant proteins at low temperature are stable and functional at restrictive temperature (Gordon and King 1993). Thus, the growth defect appears to reflect thermosensitive properties or conformations of folding intermediates in the coat protein folding and assembly pathway, rather than properties of the assembled shells.

To examine more carefully the temperature dependence of the TSF defect conferred by the different amino acid substitutions, the efficiency of plating (eop) of the gene *5* mutants was examined as a function of temperature. Figure 2 shows the eop at five temperatures for reference phage and each of the TSF mutants expressed as the percentage of the yield at 24°. The control phage had a uniform eop over the temperature range.

The most restricted mutants, *Pro(310)→Ala* and *Thr(294)→Ile*, had sharply reduced yields even at 33° (Figure 2A). *Val(297)→Ala*, *Asp(302)→Gly*, *Gly(232)→Asp* and *Gly(282)→Asp* were all partially restricted for growth at 33° and completely restricted for growth at 37°. Six of the mutants propagated with good efficiency at 33° but were severely restricted by 37° (Figure 2B).These are *Asp(174)→Asn*, *Ser(223)→Phe*,

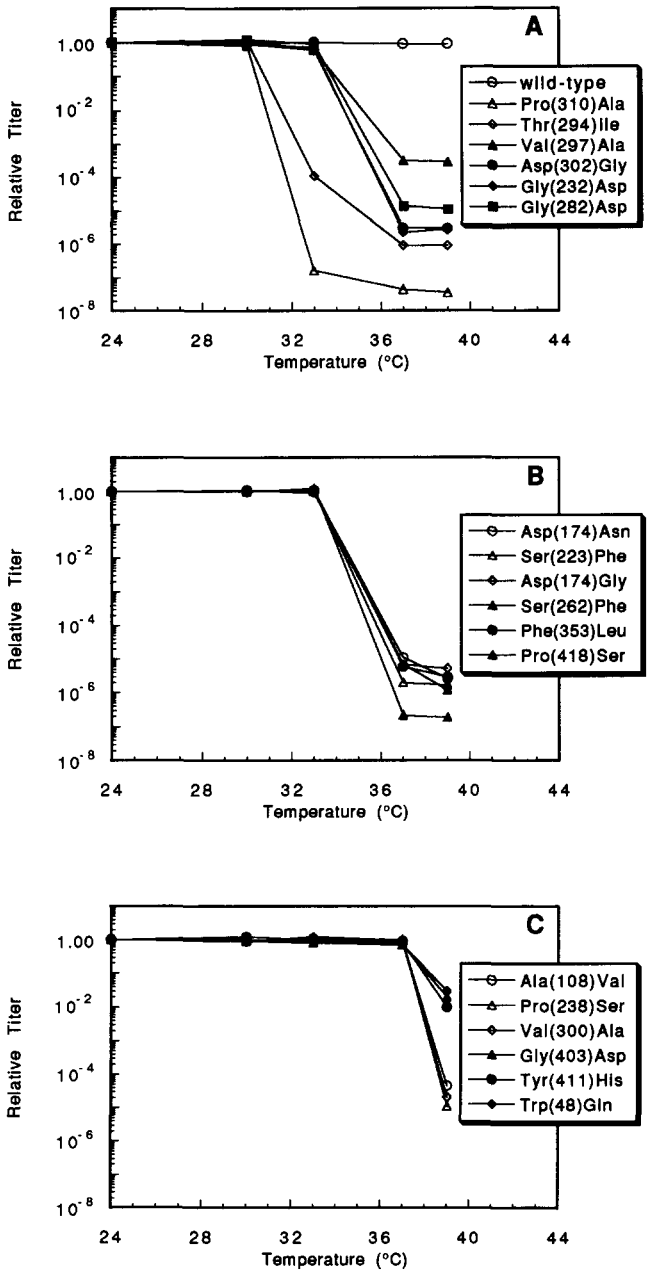

FIGURE 2.—Efficiency of plating of TSF coat protein mutants as a function of temperature. Phage, all carrying the *c1-7* allele, were plated and incubated at 24°, 30°, 33°, 37° and 39° for at least 16 hr. The ratio of plaques obtained at the indicated temperatures to plaques obtained at 24° is plotted. *Pro(310)→Ala* gave small plaques at 30°; *Val(297)→Ala* and *Gly(282)→Asp* gave small plaques at 30° and 33°; *Thr(294)→Ile*, *Asp(302)→Gly* and *Gly(232)→Asp* gave small plaques at 33°; *Ala(108)→Val* gave small plaques at 37°; *Val(300)→Ala* gave small plaques at 30°, 33° and 37°; *Gly(403)→Asp*, *Tyr(411)→His* and *Trp(48)→Gln* gave tiny plaques at 39° at the frequencies shown as well as large plaques at frequencies of 10^{-6}.

Asp(174)→Gly, *Ser(262)→Phe*, *Phe(353)→Leu* and *Pro(418)→Ser*.

The mutants *Ala(108)→Val*, *Pro(238)→Ser* and *Val(300)→Ala* were restricted for growth only at the higher temperature of 39°. The three mutants that were least restricted for growth as a function of temperature are *Gly(403)→Asp*, *Tyr(411)→His* and *Trp(48)→Gln*. These mutants gave small plaques at 39° at a high frequency, as well as larger plaques at frequencies typical of reversion events.

TABLE 1

Phage bursts produced by TSF mutants in solitary infections or co-infections with wild-type phage

	Phage per infected cell			
	30°		39°	
Strain	Mutant	Mutant/ wild-type	Mutant	Mutant/ wild-type
Wild type	141	NA[a]	131	NA[a]
Gene 5 (Am)	<0.1	109	<0.1	176
Pro(310)→Ala	16	185	<0.1	118
Thr(294)→Ile	56	143	<0.1	138
Val(297)→Ala	27	116	0.4	123
Asp(302)→Gly	62	158	<0.1	114
Gly(232)→Asp	75	120	<0.1	116
Asp(174)→Asn	44	25	<0.1	38
Ser(223)→Phe	78	107	<0.1	96
Asp(174)→Gly	55	201	0.2	74
Ser(262)→Phe	76	141	<0.1	155
Gly(282)→Asp	16	75	<0.1	98
Phe(353)→Leu	123	128	0.1	128
Pro(418)→Ser	61	187	<0.1	92
Ala(108)→Val	102	183	0.2	132
Pro(238)→Ser	57	101	0.1	92
Val(300)→Ala	20	104	0.9	75
Gly(403)→Asp	103	170	2.2	119
Try(411)→His	71	90	7.5	130
Trp(48)→Gln	117	176	6.7	159

[a] NA, not applicable.

We believe the tightness of the mutant defects reflects two aspects of the system: first, more than 400 molecules of coat protein are needed to build one particle; and second, the TSF chains accumulate as inclusion bodies, which are kinetically trapped. The differences in eop *vs.* temperature for the mutants examined presumably represent differential effects of the amino acid substitutions on the stability of critical folding intermediates of the coat protein, rather than any of their mature states (STURTEVANT *et al.* 1989; GORDON and KING 1993).

We examined the propagation efficiency of these mutants more quantitatively by measuring the phage yield from single bursts of infected cells at 30° and 39°. Exponentially growing cells were infected in broth with phage at a multiplicity of infection (moi) of 10. Infections were terminated with addition of $CHCl_3$ and the number of phage particles produced determined by titering. At 39° most of the mutants had bursts of less than one phage per cell. At 30° the mutant bursts ranged from 10–90% of the wild-type bursts (Table 1, columns 2 and 4).

Dominance tests: Many of the TS mutants were initially assigned to gene 5 by recombinational deletion mapping (BOTSTEIN *et al.* 1972; JARVIK and BOTSTEIN 1973, 1975; GORDON and KING 1993). More recently, these TS mutants were mapped by recom-

TABLE 2

Phage bursts produced by cells co-infected with TSF mutants

Infecting phage	Phage per infected cell 30°	39°
Wild type	141	165
Wild type/gene 5 (Am)	109	176
Ser(223)→Phe/Pro(418)→Ser	75	0.8
Pro(310)→Ala/Tyr(411)→His	103	5.5
Asp(174)→Asn/Ser(262)→Phe	70	<0.1
Gly(232)→Asp/Ser(262)→Phe	83	<0.1
Gly(232)→Asp/Phe(353)→Leu	92	0.2
Asp(302)→Gly/Pro(418)→Ser	60	0.3
Asp(302)→Gly/Ser(262)→Phe	93	0.3
Asp(302)→Gly/Phe(353)→Leu	110	0.1
Pro(418)→Ser/Ser(262)→Phe	73	0.9
Ser(262)→Phe/Phe(353)→Leu	105	1.0
Trp(48)→Gln/Val(297)→Ala	94	5.2
Try(411)→His/Ala(108)→Val	121	5.3
Gly(282)→Asp/Ser(262)→Phe	69	0.9
Gly(282)→Asp/Asp(302)→Gly	54	0.1
Ser(262)→Phe/Ser(223)→Phe	120	<0.1
Ser(223)→Phe/Gly(232)→Asp	80	0.1
Ser(223)→Phe/Asp(302)→Gly	75	1.8
Ser(223)→Phe/Phe(353)→Leu	73	1.1
Ala(108)→Val/Gly(232)→Asp	95	0.2
Ala(108)→Val/Asp(302)→Gly	140	0.5
Ala(108)→Val/Pro(418)→Ser	70	0.6
Ala(108)→Val/Ser(262)→Phe	64	0.3
Ala(108)→Val/Phe(353)→Leu	85	0.3
Gly(232)→Asp/Asp(302)→Gly	100	<0.1
Gly(232)→Asp/Pro(418)→Ser	63	0.4
Gly(403)→Asp/Asp(174)→Gly	96	1.9
Val(300)→Ala/Asp(302)→Gly	57	0.5
Pro(418)→Ser/Phe(353)→Leu	101	0.1
Thr(294)→Ile/Gly(232)→Asp	56	<0.1
Pro(238)→Ser/Ala(108)→Val	101	0.8
Asp(302)→Gly/Gly(232)→Asp	57	<0.1
Thr(294)→Ile/Ser(223)→Phe	100	<0.1
Thr(294)→Ile/Ala(108)→Val	70	<0.1
Thr(294)→Ile/Asp(302)→Gly	53	<0.1
Thr(294)→Ile/Ser(262)→Phe	60	<0.1
Thr(294)→Ile/Phe(353)→Leu	96	0.1
Ser(223)→Phe/Ala(108)→Val	100	0.3
Thr(294)→Ile/Pro(418)→Ser	53	0.4

binational marker rescue with a series of plasmids (CASJENS *et al.* 1991). Thus, whether or not these gene 5 TS mutants were generally dominant or recessive to wild-type was not known. Given the multimeric nature of phage capsids, it was reasonable to expect that some of the mutants would be dominant, with the mutant protein interacting with and poisoning the productive pathway (WHITAKER-DOWLING and YOUNGNER 1987). To determine the recessive or dominant character of the coat TSF mutants, cells in liquid culture were mixedly infected with a TSF mutant and wild-type phage, each at a moi of 5.

As shown in Table 1, the bursts obtained in the mixed infections with wild-type and TSF mutant phage were close to the wild-type bursts and were much larger than the bursts of the TSF mutants alone.

TABLE 3

Phage bursts produced by cells infected with cold-sensitive coat protein mutants

Infecting phage	Phage per infected cell 30°	17°
Wild type	141	58
CsH126	173	3
CsrrH58G1	92	2
CsH126/CsrrH58G1	133	30
Gene 5 (Am)	<0.1	<0.1
Wild type/gene 5 (Am)	109	56

Therefore, with the possible exception of *Asp(174)→Asn*, all of the mutants were recessive to wild type. The recessive character of these mutations is consistent with the evidence that the mutant polypeptide chains destabilize folding intermediates that have not attained a conformation permitting interaction with wild-type subunits.

Intragenic complementation: Many sets of missense mutants, including TS mutants, exhibit intragenic complementation (BERNSTEIN *et al.* 1965; ZABIN and VILLAREJO 1975). To determine whether the TSF mutant chains could complement each other, we coinfected cells with pairs of two coat TSF mutants and determined the bursts derived from infections at 30° and 39°. As shown in Table 2, the bursts obtained at 39° from the mixed infection were similar to the restrictive bursts of the mutants alone. None of the mixed infections gave yields significantly higher than those obtained in the single infections, indicating that all pairs failed to exhibit intragenic complementation. The absence of intragenic complementation is understandable if the block in the folding pathway occurs prior to the conformation needed for subunit/subunit interaction.

Bursts obtained at both temperatures for these pairs of mutants were near the burst obtained with the less restricted mutant. Just as the recessive character of these mutations indicated that the mutant coat protein did not substantially perturb the folding of the wild-type coat protein, mutations that severely reduce the efficiency of coat protein folding do so without substantially perturbing the folding of mutants whose coat protein folding efficiency is higher.

Two cold-sensitive mutants in the coat protein have been isolated (JARVIK and BOTSTEIN 1973, 1975) and characterized. Cold-sensitive mutant phage particles are stable and functional at low temperature. However, in infections performed at low temperature, newly synthesized coat protein chains fold into assembly-competent conformations capable of subunit/subunit recognition, but which are defective in later aspects of the particle maturation process (C. L. GORDON, H. LEE, J. REINER and J. KING, unpublished data). These CS mutants can be classified as assembly-

defective CSS mutants. In contrast to the TSF coat protein mutants, these CS mutants exhibited intragenic complementation (Table 3). The yield of the restrictive mixed infection was more than 10-fold higher than each allele alone and represented 50% of the control yield. Presumably, interactions between the folded chains corrected the CS defects.

Can wild-type coat protein rescue TSF mutant coat protein? While the above experiments indicated that the wild-type coat protein could attain its correct folded conformation in the presence of the TSF mutant coat protein, these experiments did not provide information as to the fate of the TSF mutant coat protein in the mixed infection. At restrictive temperature and in the presence of wild-type coat protein, did the TSF mutant coat protein fold correctly and assemble into shells or did it still misfold?

Chains that pass productively through the folding and assembly pathway form procapsids and subsequently mature phage, both soluble species. The TSF mutant chains at high temperature accumulate as aggregated inclusion bodies, which sediment at at low speed (GORDON and KING 1993). The mutant *Trp(48)→Gln* has a slightly lower electrophoretic mobility in SDS gels than wild-type coat protein. This permits differentiation between wild-type coat protein and *Trp(48)→Gln* coat protein in a co-infection (Figure 3A). To determine whether or not *Trp(48)→Gln* could be rescued and incorporated into procapsids in the presence of wild-type coat protein, we co-infected cells with phage carrying a wild-type coat protein allele and phage carrying the *Trp(48)→Gln* allele. In this experiment both phage also carried a gene *13* (Am) mutation, which prevents premature cell lysis, and a gene *3* (Am) mutation, which blocks productive phage assembly at the procapsid stage (BOTSTEIN *et al.* 1973).

Cells were infected at 29° and at 39°, with phage carrying a wild-type coat protein allele, with phage carrying the *Trp(48)→Gln* allele, or with both phage strains. Infected cells were incubated with a short pulse of ^{14}C-amino acids, chased with cold amino acids and then lysed. A pellet/supernatant separation was performed, and samples were electrophoresed through SDS gels. An autoradiograph developed from such a gel is shown in Figure 3A, and quantification of the distribution of coat protein between pellet and supernatant is presented in Figure 3B.

As expected, in the infection with phage carrying the wild-type coat protein allele, the coat protein was recovered predominantly in the supernatant, at both temperatures, presumably assembled into procapsid particles. In the infection with the *Trp(48)→Gln* phage, at 29° the mutant coat protein was found largely in the supernatant. At 39° the *Trp(48)→Gln* protein was largely pelleted, as expected from the prior work that had indicated that the TS mutant coat protein formed inclusion body aggregates at restrictive temperature (GORDON and KING 1993).

In the co-infections, wild-type coat protein remained largely in the supernatant at both temperatures, congruent with the results of the complementation experiments. The *Trp(48)→Gln* coat protein synthesized in the co-infection remained in the supernatant at 29°. At 39° the *Trp(48)→Gln* chains in the mixed infection were still largely pelleted.

These results illustrate that the presence of the *Trp(48)→Gln* coat protein chains did not perturb wild-type coat protein folding, and that the folding of the *Trp(48)→Gln* coat protein was still significantly defective in the presence of wild-type coat protein. These results are consistent with the TSF mutation destabilizing a conformation which occurs before chain/chain recognition.

Revertants and second site suppressors: Starting with a mutant in a known gene, the isolation of second-site suppressors has been used to find new genes encoding interacting proteins (JARVIK and BOTSTEIN 1975; MOIR and BOTSTEIN 1982; LUX and DUTCHER 1991; HAN *et al.* 1990; NELSON *et al.* 1992). Since TSF mutations act by destabilizing folding intermediates, while TL and assembly-defective TSS mutations act by destabilizing or impairing the function of folded molecules, one might expect the spectrum of second-site suppressors to differ for TSF mutations. We expect that proteins which interact with the native state of TSF alleles would not be recovered at high frequency, as these potential interactions would not be expected to correct the folding defect. In fact FANE and KING (1991), starting with TSF mutations in the tailspike of P22, reported the efficient isolation of intragenic suppressors of these mutants.

As part of verifying that the TS phentoypes of the gene *5* mutants were associated with the reported amino acid substitutions, we carried out a limited analysis of second-site revertants. For nine of the TSF alleles, several spontaneous revertants forming healthy plaques at restrictive temperature were selected and grown into stocks at low temperature. Revertants of the extremely TS *Pro(310)→Ala* and *Thr(294)→Ile* mutants were selected at 33°, and revertants of the other mutants mentioned below were selected at 39°. Using the PCR methodology, the sequence of several hundred nucleotides was determined in the region of the coat protein gene to which the particular TSF mutation had been originally mapped (CASJENS *et al.* 1991; GORDON and KING 1993).

True revertants were obtained from *Gly(282)→Asp*, *Asp(174)→Gly* (twice), *Val(300)→Ala* (twice), *Pro(310)→Ala*, *Thr(294)→Ile* (three times) and *Pro(418)→Ser*. This confirmed that these TSF phenotypes were caused by these single amino acid substitutions. In three of the apparent revertants of the TSF alleles, an additional nucleotide substitution was

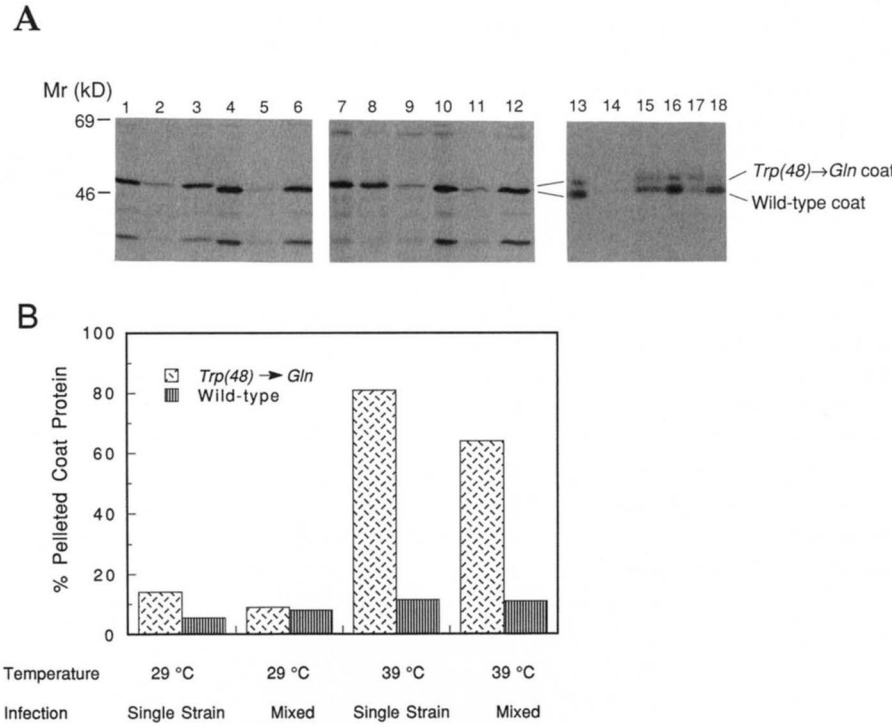

FIGURE 3.—Interaction of TSF mutant coat protein with wild-type coat protein *in vivo*. Infected cells were pulsed with ^{14}C-amino acids, lysed by freeze/thaw and separated into pellet and supernatant fractions. Samples were electrophoresed through SDS gels. (A) Films exposed to SDS gels of infected cell lysates at low or high temperature, before or after pellet/supernatant fractionation. Samples in lanes 1–12 derive from cells infected with either *Trp(48)→Gln* phage or phage carrying a wild-type coat protein allele. lane 1, *Trp(48)→Gln* lysate at 29°; lane 2, *Trp(48)→Gln* pellet at 29°; lane 3, *Trp(48)→Gln* supernatant at 29°; lane 4, wild-type coat lysate at 29°; lane 5, wild-type coat pellet at 29°; lane 6, wild-type coat supernatant at 29°; lane 7, *Trp(48)→Gln* lysate at 39°; lane 8, *Trp(48)→Gln* pellet at 39°; lane 9, *Trp(48)→Gln* supernatant at 39°; lane 10, wild-type coat lysate at 39°; lane 11, wild-type coat pellet at 39°; lane 12, wild-type coat supernatant at 39°. Samples in lanes 13–18 derive from cells co-infected with phage carrying a wild-type coat protein allele and *Trp(48)→Gln* phage. These samples were electrophoresed through a long SDS gel to resolve the two coat protein species; lane 13, co-infection lysate at 29°; lane 14, co-infection pellet at 29°; lane 15, co-infection supernatant at 29°; lane 16, co-infection lysate at 39°; lane 17, co-infection pellet at 39°; lane 18, co-infection supernatant at 39°. (B) Phosphorimager quantification of pelleting of coat protein. The gels of Figure 3A were exposed to a phosphorimager screen, and the distribution of coat protein between pellet and supernatant was quantified, as described in MATERIALS AND METHODS. Results from the infections with phage carrying wild-type coat protein are depicted by bars 2 and 6 (counting from left). Results from the infections with phage carrying *Trp(48)→Gln* coat protein are depicted by bars 1 and 5. Results from the co-infections are depicted by bars 3, 4, 7 and 8.

detected, representing intragenic second-site suppression: (1) *Ser(223)→Phe* was suppressed by the additional amino acid substitution Phe(170)→Leu (resulting from cytosine 510 → adenine); (2) *Pro(310)→Ala* was suppressed by the additional amino acid substitution Ala(263)→Ser (resulting from guanine 787 → thymine); (3) *Gly(282)→Asp* was suppressed by an intra-codon nucleotide substitution leading to the substitution of this aspartate at position 282 with asparagine (resulting from guanine 844 → adenine). Four of the apparent revertants (of TSF strains *Ser(223)→Phe*, *Pro(418)→Ser*, *Phe(353)→Leu* and *Ala(108)→Val*) retained their original substitution and did not have another mutation in the region sequenced. These four apparent revertant strains presumably carry either intragenic second-site suppressors outside the region sequenced or extragenic second-site suppressors.

These preliminary results are consistent with the findings obtained for the gene *9* TSF mutants that intragenic suppressors are recovered at a significant frequency (FANE and KING 1991; FANE *et al.* 1991).

DISCUSSION

Temperature-sensitive mutations have been particularly valuable in identifying genes coding for essential proteins (EDGAR and LIELAUSIS 1964; SUZUKI 1970; HARTWELL *et al.* 1970; ADAMS *et al.* 1982). As the functions and interactions of identified proteins come under closer scrutiny, it becomes more important to understand which properties of the mutant proteins are the loci of the TS defects.

Intragenic complementation: Intragenic complementation provides a window into polypeptide chain domains and subunit interactions (FINCHAM 1966). Two general models have been proposed to explain this phenomenon. First, if each allele has knocked out a different enzymatic function of the protein, the presence of both alleles may restore full protein activity (MCGAVIN 1968). Second, if the protein is oligomeric, the two alleles may help each other to correctly fold and assemble (CRICK and ORGEL 1964). Interaction of missense polypeptide chains, following this

second model, has been demonstrated for many different oligomeric proteins (SCHLESINGER and LEVINTHAL 1963; ZABIN and VILLAREJO 1975). Significant intragenic complementation has been reported for temperature sensitive mutations within the genes for a number of phage T4 structural proteins (BERNSTEIN *et al.* 1965) and temperature-sensitive mutations in the SV40 major coat protein (BEHM *et al.* 1988). For structural proteins such as the P22 coat protein, we assume that direct interaction would provide the mechanism of intragenic complementation.

The two cold-sensitive alleles of the P22 coat protein gene generate mutant coat proteins that assembled into defective shell structures at low temperature (C. L. GORDON, H. LEE, J. REINER and J. KING, unpublished data). The intragenic complementation observed between these two mutants presumably represents correction of the mutant defects by subunit interaction in the shell structures.

In contrast, despite the intimate interactions required between coat protein molecules during the subunit assembly process, the TSF mutants in the coat protein did not display intragenic complementation. They were also recessive. These genetic properties presumably reflect the nature of the intracellular defect: defective folding of the polypeptide chain. The same recessive character and lack of intragenic complementation has been found with the TSF mutants of the trimeric P22 tailspike protein (SMITH *et al.* 1980; GOLDENBERG *et al.* 1983). These properties are consistent with the idea that these mutant coat proteins molecules are not defective in the stability (or function) of the folded molecule, but of an assembly-incompetent precursor. Subunit/subunit interaction between different alleles would be expected to offer the possibility of improving the stability or function of folded assembly-competent molecules, but not of these destabilized TSF folding intermediates. This is also consistent with the inability of wild-type coat protein to rescue the *Trp(48)→Gln* coat protein. These would be expected to be general properties of TSF mutations.

Location of TSF amino acid substitutions: A group of well-characterized TS mutations in phage T4 lysozyme have been shown to destabilize the native state of the protein (HAWKES *et al.* 1984). ALBER *et al.* (1987) found that these mutants were associated with the most buried sites in the three-dimensional structure of the native protein. This result was consistent with the notion that these TL mutations act by destabilizing native states (GOLDENBERG 1988).

Since the TSF mutations act on folding intermediates and do not destabilize the native state, we would not necessarily expect similar patterns of sites. YU and KING (1988) reported that the TSF sites of the tailspike protein were preferentially at the protein surface, in contrast to the lysozyme TL mutations. The surface location accounted for the ability of the native protein to tolerate extreme substitutions, such as arginines for glycines (VILLAFANE and KING 1988).

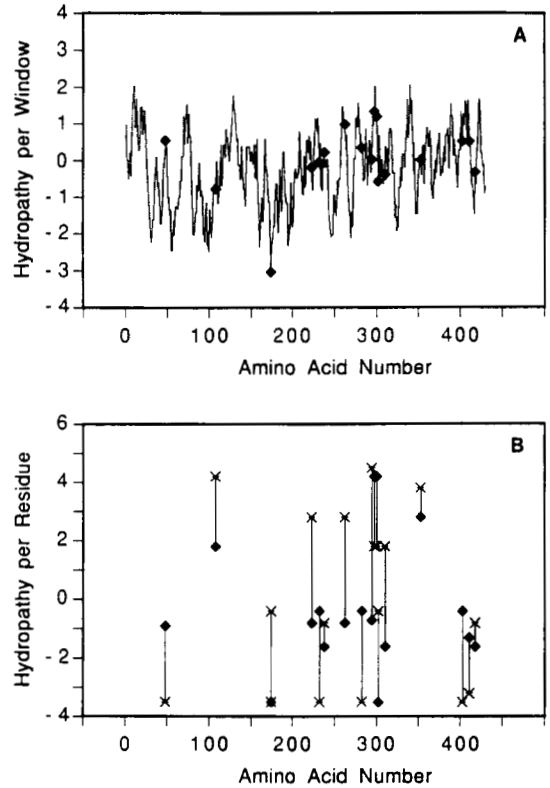

FIGURE 4.—Mutant amino acid substitutions in relation to coat protein hydropathy. (A) KYTE/DOOLITTLE hydropathy plot for wild-type coat protein. Values were computed according to the scale of KYTE and DOOLITTLE (KYTE and DOOLITTLE 1982) with a window size of 7. Larger numbers indicate greater hydropathy. ♦, the site of a temperature-sensitive mutation. (B) KYTE/DOOLITTLE hydropathy values for wild-type amino acids that are substituted and their mutant counterparts. ♦, hydropathy of wild-type amino acid which is replaced in a temperature-sensitive mutant. X, hydropathy of new amino acid introduced by a temperature-sensitive mutation.

Lacking a high resolution structure for the P22 capsid protein, we used a KYTE/DOOLITTLE hydropathy calculation (KYTE and DOOLITTLE 1982) to determine whether the capsid TSF mutations are found preferentially in local sequences of high or low hydropathy (Figure 4A). In this plot, peaks correspond to local hydrophobic regions, which may be expected to be buried in the folded molecule, and troughs correspond to local hydrophilic regions, which may be expected to be on the surface of the folded protein. The sites of the mutations are marked with diamonds. The mutations occur in regions of both high and low local hydropathy.

Figure 4B illustrates the change in hydropathy for each individual amino acid substitution. Some substitutions are replacing hydrophobic amino acids with hydrophilic ones, while other substitutions are replacing hydrophilic amino acids with hydrophobic ones. No simple trend predicting the hydrophobicity of regions prone to TSF amino acid substitutions of this

sort or the nature of the substitutions themselves is apparent.

The TSF phenotype and inclusion body formation: Temperature-sensitive folding mutations appear to act by destabilizing intracellular folding intermediates (HAASE-PETTINGELL and KING 1988; MITRAKI *et al.* 1991). Loss of intracellular folding intermediates to irreversible aggregation (inclusion bodies) is a problem in both biomedical research and in the biotechnology industry, particularly in the expression of cloned genes in foreign hosts (KANE and HARTLEY 1988; WILLIAMS *et al.* 1982; MITRAKI and KING 1989). It also occurs physiologically during heat shock and other stressful situations (SCHLESINGER 1990). *In vitro*, irreversible aggregation when refolding is a common problem, especially for larger and oligomeric proteins (GOLDBERG *et al.* 1991; MITRAKI and KING 1989; LONDON *et al.* 1974; RUDOLPH *et al.* 1979).

Inclusion bodies presumably grow by accretion of cytoplasmic folding intermediates at their surfaces. The presence of aggregation-prone folding intermediates of the TSF coat subunits might have been expected to draw wild-type folding intermediates into the inclusion bodies, generating a dominant phenotype for the TSF mutants. However, the wild-type coat protein folded productively even in the presence of the aggregating TS chains. This suggests that wild-type folding intermediates have little tendency to form inclusion bodies, even when the inclusion bodies are seeded by the mutant coat protein. In cells co-infected with two different TSF mutants, we do not know if the different mutant chains segregate into different inclusion bodies or if they co-aggregate.

HAASE-PETTINGELL and KING (1988) showed that once TSF tailspike chains entered the inclusion body state, they could not reenter the productive pathway after shifting to permissive temperature. Similarly, coat TSF chains, once aggregated into inclusion bodies, could not be recovered by shifting down the temperature (GORDON and KING 1993). This explains in part why the TSF mutants, though functional at permissive temperature, are very tight at restrictive temperature: the TSF chains become kinetically trapped in the inactive inclusion body state.

Isolation of second site suppressors: One mechanism of second-site suppression is via direct interaction between protein molecules encoded by different genes (HARTMAN and ROTH 1973; JARVIK and BOTSTEIN 1975; MOIR and BOTSTEIN 1982; LUX and DUTCHER 1991; HAN *et al.* 1990; NELSON *et al.* 1992). Analogously, isolation of mutations that are synthetically lethal with a starting mutation of interest is often performed to find interacting proteins (DESHAIES *et al.* 1991; SANDERS and SCHEKMAN 1992). If the initial mutation impairs the function of a folding intermediate rather than the folded protein, isolation of second-site suppressors and synthetic lethals may yield genes encoding for proteins that interact with folding intermediates rather than with the mature form of the protein.

In the preliminary characterization reported here, second-site suppressors were recovered for TSF mutants at six sites. Three of the TSF mutants were shown to be suppressed by intragenic second-site supressors, consistent with FANE and KING's (1991) results with the TSF tailspike mutants. A caveat is that these selections only detected mutations in phage genes. One might expect that in other systems, isolation of second-site suppressors of TSF mutants would identify genes coding for cellular factors influencing polypeptide chain folding (GETHING and SAMBROOK 1992).

The TSF coat protein mutant *Ser(223)→Phe* was suppressed by the intragenic amino acid substitution Phe(170)→Leu, and the mutant *Pro(310)→Ala* was suppressed by the intragenic amino acid substitution Ala(263)→Ser. These results support the notion that intragenic amino acid replacements may be a general tool for improving protein folding efficiency, without impairing the function of the folded molecule (MITRAKI *et al.* 1991).

Three of the TS mutants in this study, *Val(297)→Ala*, *Val(300)→Ala* and *Pro(310)→Ala*, were themelves isolated as second-site suppressors of the cold-sensitive mutant *CsH137* in gene *1* of P22 (JARVIK and BOTSTEIN 1975). Gene *1* encodes the dodecameric portal protein that is the site of DNA packaging (BAZINET and KING 1985). The *CsH137* allele prevents incorporation of the portal protein into procapsid particles at low temperature (BAZINET *et al.* 1990). The suppression occurred at 18°, a permissive temperature for the TS mutants.

Three possible models can be proposed for such suppression: (1) The suppressing substitutions might alter the properties of the native coat subunit to permit interaction with the *CsH137* portal protein, while at the same time making the coat polypeptide chain temperature-sensitive for folding. The clustering of these three mutations around amino acid 300 would appear to support this model and suggests that this region of the coat protein interacts with the portal. (2) The suppression might be due to altering the ratio of active coat protein to portal protein due to a decreased level of correctly folded TS subunits even at the permissive temperature (FLOOR 1970; GORDON and KING 1993). (3) The suppression might result from an altered mRNA sequence that can efficiently serve as an initiator for particle assembly (BAZINET *et al.* 1990). With respect to the latter model, JARVIK and BOTSTEIN (1975) also isolated second site suppressors of the *CsH137* gene *1* allele that were temperature-sensitive folding mutants of the P22 tailspike. BAZINET *et al.* (1990) noticed sequence similarities between nucleotides near these tailspike suppres-

sor mutations and the scaffolding gene translational initiation region, which is a putative site of assembly initiation. They proposed a model in which these suppressor mutations generated RNA sites which could initiate assembly, thereby incorporating the *CsH137* portal protein. We noticed no similarities between the DNA sequence near the three coat TS mutations that were isolated as suppressors of *CsH137* and the scaffolding translation initiation region.

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