Intragenic Suppressors of Folding Defects in the P22 Tailspike Protein

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ABSTRACT

Within the amino acid sequences of polypeptide chains little is known of the distribution of sites and sequences critical for directing chain folding and assembly. Temperature-sensitive folding (*tsf*) mutations identifying such sites have been previously isolated and characterized in gene *9* of phage P22 encoding the tailspike endorhamnosidase. We report here the isolation of a set of second-site conformational suppressors which alleviate the defect in such folding mutants. The suppressors were selected for their ability to correct the defects of missense tailspike polypeptide chains, generated by growth of gene *9* amber mutants on Salmonella host strains inserting either tyrosine, serine, glutamine or leucine at the nonsense codons. Second-site suppressors were recovered for 13 of 22 starting sites. The suppressors of defects at six sites mapped within gene *9*. (Suppressors for seven other sites were extragenic and distant from gene *9*.) The missense polypeptide chains generated from all six suppressible sites displayed *ts* phenotypes. Temperature-sensitive alleles were isolated at these amber sites by pseudoreversion. The intragenic suppressors restored growth at the restrictive temperature of these presumptive *tsf* alleles. Characterization of protein maturation in cells infected with mutant phages carrying the intragenic suppressors indicates that the suppression is acting at the level of polypeptide chain folding and assembly.

THE cytoplasmic folding of polypeptide chains off ribosomes has until recently been viewed as a spontaneous process (ANFINSEN 1973) posing few problems in the physiology of cells. Current developments have altered this view: the inability to interpret rapidly accumulating gene sequence information in terms of three-dimensional structure; emerging practical problems in obtaining correctly folded proteins rather than misfolded inclusion body forms of the protein products of cloned genes (MARSTON 1986; MITRAKI and KING 1989); and the recognition of auxiliary genetic information needed for folding, localization and transport (PELHAM 1986; BECKWITH and FERRO-NOVICK 1986; GOLOUBINOFF *et al.* 1989; OSTERMANN *et al.* 1989).

Although the amino acid sequence of a polypeptide chain may contain all the information to determine its spatial conformation when in its proper environment, not all of the local sequence participates in determining the chain fold (BASHFORD, CHOTHIA, and LESK 1987; FANE and KING 1987; REIDHAAR-OLSON and SAUER 1989). Comparisons of the sequences of proteins with homologous structures identify many sites within the chains that can tolerate substantial variations without affecting either function or folding (DICKERSON and GEIS 1983; KREBS, SCHMID and JAENICKE 1983; BASHFORD, CHOTHIA and LESK 1987).

Certain residues, however, tolerate little or no variation. Some of these conserved residues are likely to play critical roles in catalysis or other mature functions, such as the conserved histidines of hemoglobin (BASHFORD, CHOTHIA and LESK 1987). Other residues may be critical for polypeptide chain folding and assembly (SMITH and KING 1981; KREBS, SCHMID and JAENICKE 1983; YU and KING 1984; VILLAFANE and KING 1988; REIDHAAR-OLSON and SAUER 1989; SCHWARZ and BERGET 1989a; STURTEVANT *et al.* 1989).

A variety of evidence indicates that the sites and sequences needed for the correct folding of the chain into the native state of the protein are different from the sites and sequences needed for the function of the native state. For example, substitutions at the active site of many proteins can sharply alter catalytic activity, without affecting the ability of the polypeptide chain to fold into the native conformation (FERSHT *et al.* 1985). On the other hand, temperature-sensitive folding mutations of the P22 tailspike alter the folding process without altering the function or stability of the mutant protein when folded into the native state (GOLDENBERG and KING 1981; STURTEVANT *et al.* 1989; KING *et al.* 1989).

In order to reach their native conformation, polypeptide chains pass through intermediate conformations which are critical for the success of the folding process (KIM and BALDWIN 1982; CREIGHTON and GOLDENBERG 1984). These conformations must be

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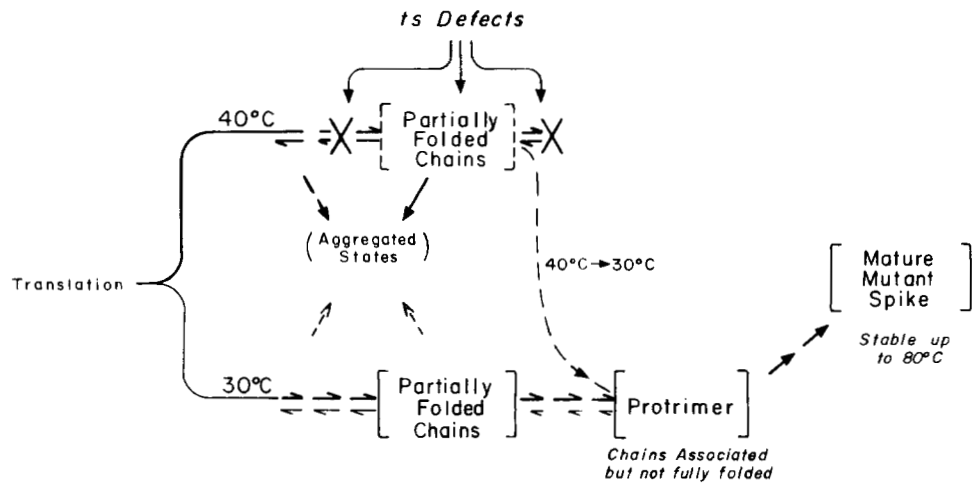

FIGURE 1.—*In vivo* folding and subunit assembly pathway for the P22 tailspike protein and its *tsf* mutants. At permissive temperature newly released polypeptide chains form a partially folded single-chain intermediate. This species is thermolabile and aggregates at higher temperatures (HAASE-PETTINGELL and KING 1988). In the productive pathway these single chains fold further into a state competent for chain/chain recognition. The product of the chain association reaction, the protrimer, is metastable and can be trapped in the cold (GOLDENBERG and KING 1982). The stability toward heat and chemical denaturants is achieved in the transformation of the protrimer to the native trimer. Temperature-sensitive folding mutants further destabilize the early intermediate (SMITH and KING 1981; GOLDENBERG, SMITH and KING 1983; YU and KING 1984; YU and KING 1988). These are kinetically trapped in an aggregated inclusion body state (HAASE-PETTINGELL and KING 1988).

encoded in the amino acid sequence of the gene and polypeptide chain. We have been interested in identifying those amino acid residues and local sequences which direct the conformation of folding intermediates, as distinct from those that stabilize the mature conformation (GOLDENBERG, SMITH and KING 1983; YU and KING 1984; VILLAFANE and KING 1988; STURTEVANT *et al.* 1989).

One of the few prokaryotic proteins in which folding defects can be readily distinguished from both functional and stability defects is the P22 tailspike protein. The tailspike polypeptide chain is encoded by gene *9* of P22 (BOTSTEIN, WADDELL, and KING 1973; BERGET and POTEETE 1980). The native tailspike is an elongated trimer (GOLDENBERG, BERGET and KING 1982). Each monomer contains 666 amino acids (SAUER *et al.* 1982) The proximal end of the tailspike binds noncovalently, but irreversibly, to mature phage heads. The distal end has a glycosidic activity which hydrolyses the O-antigen of *Salmonella typhimurium* (IWASHITA and KANEGASAKI 1976).

Although its three-dimensional structure has not yet been solved, Raman spectroscopy reveals that the secondary structure consists of more than 50% beta sheet structure (SARGENT *et al.* 1988). The native protein is thermostable with a melting temperature of 88° (GOLDENBERG and KING 1981; STURTEVANT *et al.* 1989). Associated with this high stability is resistance to cleavage by proteases and to denaturation by sodium dodecyl sulfate (SDS) (GOLDENBERG, BERGET and KING 1982).

The intracellular chain folding and subunit assembly pathway is depicted in Figure 1. Newly synthesized chains form partially folded intermediates which are thermolabile (GOLDENBERG, BERGET and KING 1982) and prone to intracellular aggregation and inclusion body formation (HAASE-PETTINGELL and KING 1988). These intermediates, which are capable of specific chain recognition, associate to form the protrimer, a partially folded three-chain intermediate. The protrimer then transforms to the native tailspike (GOLDENBERG and KING 1982). The early single-chain-folding intermediate and the protrimer folding intermediate are distinguishable from the native structure by a variety of criteria, including resistance to proteases and detergents (GOLDENBERG, BERGET and KING 1982; GOLDENBERG and KING 1982; HAASE-PETTINGELL and KING 1988).

Gene *9* is the locus of over 30 sites of temperature-sensitive folding mutants (*tsf*) (SMITH, BERGET and KING 1980; YU and KING 1984, 1988; KING *et al.* 1986; VILLAFANE and KING, 1988) which are a class of *tss* (temperature-sensitive synthesis) mutants (SADLER and NOVICK 1965). This is a consequence of the existence of a very thermolabile intermediate in the folding pathway for a very thermostable protein (GOLDENBERG and KING 1981; GOLDENBERG, SMITH and KING 1983). The *tsf* mutations further destabilize the single-chain thermolabile folding intermediate but do not alter the function or the stability of the native protein once correctly folded and assembled at permissive temperature (SMITH and KING 1981; GOLDENBERG and KING 1981; GOLDENBERG, SMITH and KING 1983). Thus these residues cannot be playing an im-

portant role in the native protein. The sites of the *tsf* mutations are probably associated with beta turns (YU and KING 1988; VILLAFANE and KING 1988). It is likely that the mutations disrupt the formation of the correct beta sheet conformation needed for the productive folding and chain association pathway.

In addition to the *tsf* mutations, two other sets of mutations have been isolated in gene *9*. Amber mutations have been mapped to more than 60 sites (FANE and KING 1987). Missense chains were generated with serine, glutamine, tyrosine or leucine inserted at the nonsense codon by growth on the appropriate Salmonella host strains (MILLER *et al.* 1979; WINSTON, BOTSTEIN and MILLER 1979) at various temperatures. At about half of the 60 sites the missense chains generated by insertion of non-wild-type amino acids at the nonsense codons were defective (FANE and KING 1987). Preliminary characterization of the defects indicated that many of the missense polypeptide chains were defective in the folding or assembly into the native tailspike trimer (FANE and KING 1987; FANE 1988).

Absolute defective mutations have also been isolated in gene *9* (BERGET and CHIDAMBARAN 1989). The tailspikes assemble efficiently onto phage heads *in vitro* (ISRAEL, ANDERSON and LEVINE 1967) and this reaction proceeds efficiently in Petri dishes (BERGET and POTEETE 1980). This permitted rescue of mutants carrying absolute lethal mutations in the tailspike gene by exogenous tailspike protein. SCHWARZ and BERGET (1989a,b) have characterized a set of these and shown that they also affect steps in maturation, though not necessarily the same steps as the *tsf* mutations. Unlike many soluble enzymes, mutations affecting the mature functions without causing major conformational disruptions are rare in the tailspike. Only three such sites have been identified (BERGET and POTEETE 1980; SCHWARZ and BERGET 1989b; MAURIDES, SCHWARZ and BERGET 1990).

In order to determine which amino acids were interacting within the mature α subunit of tryptophan synthase, YANOFSKY and co-workers isolated intragenic, second site revertants (YANOFSKY 1966, 1971; KLIG, OXENDER and YANOFSKY 1988). Since then, this rationale has been used to investigate the function, structure and stability of many other proteins. These investigations have generally focused on interactions occurring within the native forms of these proteins (HECHT and SAUER 1985; SHORTLE and LIN 1985). Recently MAURIDIES, SCHWARZ and BERGET (1990) have isolated second site suppressors of a functionally defective mutation in the N-terminal region of the tailspike. The suppressors mapped to a single site in the N terminus of the chain identifying a putative salt bridge stabilizing the mature structure.

Second-site suppressors have also provided a means to probe the character of precursor states such as signal sequences (BENSON, HALL and SILHAVY 1985; BECKWITH and FERRO-NOVICK 1986). By starting with mutations which affected transient folding intermediates, we hoped to isolate second-site mutations influencing the structure of the intermediates rather than the native protein. Since inclusion bodies derive from folding intermediates and not from the native state, such suppressors might be of practical importance in the recovery of cloned proteins.

Distinguishing second-site suppressors from true and pseudorevertants can be laborious. In order to conveniently distinguish second-site suppressors from same site events, we utilized the missense proteins enerated by amber mutants of gene *9* growing on host strains inserting various amino acids at the amber codons (FLOOR 1970; FANE and KING 1987). Phage retaining the amber mutation are unable to grow on the su^- host.

A distinctive feature of the tailspike system is that all of the temperature-sensitive mutations within gene *9* have *tsf* phenotypes; thermolabile mutants have not been recovered. Single amino acid substitutions presumably cannot lower the melting temperature of the native protein from 88° to 40° needed to generate a thermolabile phenotype. The *ts* phenotypes associated with the missense polypeptide chains generated with amber mutants were therefore also likely to represent chain folding or assembly defects. In addition these sites were concentrated in the central third of the polypeptide chain, which we believe is the critical region in the early chain-folding pathway (VILLAFANE and KING 1988). These criteria indicated that missense proteins with *ts* and lethal phenotypes generated from amber mutants were appropriate starting alleles for selection of second-site suppressors affecting polypeptide chain folding.

In standard microbial genetic terminology, the term "suppressor" is used to denote both host informational suppressors–altered tRNAs–which insert an amino acid at a stop codon, and other suppressors, for example within the phage genome which act through macromolecular interactions. This causes a terminology problem in this work since we start with missense proteins generated by informational suppression and isolate phage second site suppressors which correct the defect in these missense proteins. To avoid this confusion we will refer to the process of tRNA mediated information suppression as "amino acid insertion" wherever possible. Where helpful we also use the term "conformational suppression" to distinguish the phage suppressors from the host tRNA informational suppressors.

MATERIALS AND METHODS

Phage and bacterial strains and terminology (SUSSKIND and BOTSTEIN 1978): The P22 amber mutants in gene *9*

TABLE 1

Amber mutants used to isolate the second-site suppressors

Mutant (map order)	Fragment	Missense Phenotype				Suppressors recovered?
		Ser	Gln	Tyr	Leu	
amUS11		ts	++	ts	++	Yes
amUT48		ts	−−	ts	−−	Yes
amUT93		ts	ts	++	ts	No
amUT34	11.8 K	++	−−	tyr	ts	Yes
amUT66		++	−−	ts	ts	Yes
amUT44		ts	ts	ts	ts	Yes
amH1291	22 K	ts	ts	++	ts	Yes
amUT105	23.1 K	−−	−−	ts	−−	No
amUT57	24.5 K	ts	ts	ts	ts	Yes
amUS13		++	−−	ts	−−	Yes
amUT71	25 K	++	−−	++	−−	Yes
amUT51	25.3 K	ts	−−	++	−−	Yes
amUT111	27.6 K	ts	ts	++	−−	No
amUT52		ts	−−	ts	ts	No
amUS14		ts	++	ts	++	No
amUT86		ts	−−	ts	−−	Yes
amUT64		−−	ts	++	ts	No
amUT83	54 K	−−	−−	ts	ts	No
amH1014	54.3 K	−−	++	ts	++	Yes
amUG4	54.2 K	++	++	−−	++	No
amH703	54.8 K	ts	++	−−	++	No
amUG2		cs/ts	++	ts	++	Yes

The characterization of the amber fragments is reported in SMITH, BERGET and KING (1980) and FANE and KING (1987). The amber mutations are listed in the approximate N-terminal to C-terminal order in the gene by crosses and amber fragment size. The "++" signifies growth, and "−−" failure to grow at all temperatures tested 25°, 30°, 37° and 39°.

used in these experiments are listed in Table 1, and are described in detail in FANE and KING (1987). The "H" mutants were isolated using hydroxylamine mutagenesis (POTEETE and KING 1977) while the "U" series was isolated after ultraviolet irradiation of phage particles (FANE and KING 1987). The second letter refers to the amino acid inserted by the permissive host used for recovery of the mutants–thus "UT" mutants were recovered after UV irradiation on tyrosine inserting Salmonella hosts. They all carry the *c1–7* clear plaque mutation ensuring entry into the lytic cycle of growth. The five Salmonella hosts strains are derivatives of Salmonella LT2; DB7136 *su*$^-$, DB7154 *supD* inserting serine; DB7155, *supE* inserting glutamine; DB7156 *supF* inserting tyrosine; DB7157 *supJ* inserting leucine (WINSTON, BOTSTEIN and MILLER 1979).

Media and chemicals: LB Broth contains 1% tryptone; 0.5% yeast extract; 0.5% NaCl. LB agar contains LB broth with 1% agar. Broth and agar are brought to pH 7 with 1 N NaOH. Soft top agar contains 0.8% nutrient broth; 0.5% NaCl; 0.65% agar. Dilution fluid contains 0.1% tryptone; 0.7% NaCl. M9 salts contains 0.5% Na_2HPO_4; 0.3% KH_2PO_4; 0.05% NaCl; 0.1% NH_4Cl. Minimal media contains M9 with the addition of presterilized, 1 mM $MgSO_4$, 10^{-6} M $FeCl_2$, 10^{-6} M $CaCl_2$, 0.4% glucose, 0.0015% histidine and 0.004% leucine.

Isolation of second site suppressors of missense suppressed amber mutants: We examined those amber mutants that had tightly restricted phenotypes on *su*$^+$ hosts at temperatures ranging from 21° to 39°. For those amber mutants which displayed a lethal missense phenotype, the plates were incubated at 30°. For those amber mutants which displayed a *ts* phenotype, the plates were incubated at 37° or 39° depending on the severity of the temperature restriction. Phage (4×10^8) were plated on the restrictive *su*$^+$ host(s) at restrictive temperature and incubated for 6–8 hr. Fifty revertant plaques were then stabbed into a set of indicator lawns: *su*$^-$, permissive *su*$^+$ and restrictive *su*$^+$. We identified the putative second site suppressors as those revertants that now grew on the restrictive su$^+$ host but maintained the amber phenotype (no growth on the *su*$^-$ host). In general second site events were as frequent as same site events. Full details are given in FANE (1988).

Isolation of *am*$^+$*ts* pseudorevertants: Reversion events at the actual amber site could result in a wild-type phenotype or the reversion event could lead to a *ts* phenotype due to a non-wild-type residue at the amber site (JARVIK and BOTSTEIN 1975). To obtain *am*$^+$/*ts* pseudo revertants, we plated 4×10^8 phage on the *su*$^-$ host at either 30° or 21° depending on the severity of the *ts* phenotypes observed on the four *su*$^+$ host (FANE and KING 1987). Temperature of 21° was chosen for all amber mutants that displayed a lethal missense phenotype. The plates were incubated for 8 or 14 hr, depending on the temperature. At least 48 revertants were stabbed into sets of four plates with a *su*$^-$ lawns which were then incubated at 21°, 30°, 37° and 39°. We identified putative *ts* revertants as those phage that only grew at lower temperatures.

Nomenclature of the newly isolate phage mutants: The names of the phage suppressors indicate the original mutation for which they were selected. The name *su(UT51)-2* indicates that this was the second putative suppressor isolated that suppressed a missense defective phenotype of the parent mutant *amUT51*. Similarly the name *tsR(am51)-a* indicates that this mutant was isolated as a revertant of the amber phenotype of *amUT51* to a *ts* phenotype.

Genetic crosses: Crosses were performed as described in FANE and KING (1987). The *5*$^-$*amN114/13*$^-$*amH101/tsR(am)* triple mutants used in the radiolabeled lysates were made by crossing a *5*$^-$*am/13*$^-$*am* double mutant with the various *tsR(am)* mutants. The triple recombinants were identified by the suppression pattern of the triple mutants: (1) the host *supJ* mutation (DB7157) inserting leucine is *cs* (WINSTON, BOTSTEIN and MILLER 1979) and as unable to suppress the *5*$^-$ *amN114* mutation at 21°; (2) DB7156, the tyrosine inserting host, does not suppress the *13*$^-$*amH101* allele and (3) the *tsR(am)* mutations confers a *ts* phenotype. The triple mutant phage cannot grow at elevated temperatures on the *su*$^+$ host inserting glutamine that suppresses both amber mutations.

The *5*$^-$*am/13*$^-$*am/su(UT51)-2/htsR(am51)-e* quadruple mutant was identified in the same manner; the *su(UT51)-2/htsR(am51)-e* double mutant is still *ts* at 35°. The *htsR(am51)-e* mutation by itself is restricted for growth at temperatures above 28° and is referred to as *hyper-ts* (*hts*). The remaining *5*$^-$*am/13*$^-$*am/su/tsR(am)* quadruple mutants were made by crossing the *5*$^-$*am/13*$^-$*am/tsR(am)* triple mutants with their respective *su/tsR(am)* double mutants. The progeny was then screened for recombinants that retained the *5*$^-$*am* and *13*$^-$*am* alleles as described above but had lost the *ts* phenotype associated with the gene *9* allele.

In the mapping cross between the *su(UT51)-2/amUT51* and *tsR(am51)-e*, mutants, it became apparent that the putative *su/am* double mutant was actually a triple mutant carrying both a tightly linked intragenic suppressor and an extragenic suppressor. When the progeny was plated on the *su*$^-$ host at 30° to select for the recombinants, two distinct plaque morphologies were present, large and small. Although both classes were restricted for growth above 35°, only the large recombinant was rescued by exogenously added tailspikes at elevated temperatures indicating that this genotype was associated with the gene *9* locus. On further

backcrosses, the gene *9* allele was separated from the extragenic suppressor. (It will be described in a further report along with the other extragenic suppressors mapping within the late operon.) The results presented in the tables and elsewhere in this report, reflect only those of the intragenic suppressor.

When both of the suppressors are present in *cis* with the original *amUT51* mutation, phage grow with 100% efficiency on the glutamine inserting host at 21°. The intragenic suppressor alone results in an eop of 10^{-2} on the glutamine inserting host at 21° (see Table 3). The original mutant is completely restricted with an eop below 10^{-6}.

Complementation assays: For these experiments, *su*$^-$ cells, at a concentration of 4×10^8, were coinfected at an moi of 10 at 30° in broth. Ten minutes after infection, the infections were diluted 1/200 and partitioned between 30° and 39°. To compensate for the reduced synthesis of tailspikes due to the presence of the *9*$^-$*am* mutation in one of the parent phage, the titer of each lysate was determined in the presence of exogenously added native tailspikes.

Radiolabeled lysates and SDS-gel electrophoresis: Tailspike synthesis and maturation was following by SDS-gel electrophoresis of samples labeled with ^{14}C-amino acids. The samples were not heated prior to application to the gel, allowing discrimination between mature native tailspikes and intracellular folding and assembly intermediates (GOLDENBERG, BERGET and KING 1982). The protocols for lysate preparation and analysis are described in SMITH, BERGET and KING (1980) and SMITH and KING (1981).

RESULTS

All of the previously characterized *tsf* mutations isolated in gene *9* blocked the folding pathway at the single-chain stage, before the formation of the protrimer intermediate, in which the three monomer chains are associated but not yet native (GOLDENBERG, SMITH and KING 1983). These mutations all had a recessive phenotype and failed to exhibit intragenic complementation (SMITH, BERGET and KING 1980). We presume that this reflects the inability of the *tsf* mutant chains to productively interact with each other at restrictive temperature. Preliminary characterization of the *ts* phenotypes exhibited by the missense chains of the amber mutants indicated that they also represented folding defects (FANE 1988; C. HAASE-PETTINGELL, A. MITRAKI and J. KING, unpublished results).

Less is known about the behavior of the missense proteins exhibiting lethal–but not *ts*–defects (Table 1). Some of the absolute lethal mutations isolated by SCHWARZ and BERGET (1989a,b) were defective late in the maturation pathway. Before proceeding with the selection for second site revertants we tested the amber mutants exhibiting absolute lethal defects to determine if the defective missense chains were dominant or recessive to wild-type chains. Defects occurring later in the maturation pathway, after chain-chain interactions, might have a dominant phenotype.

Dominance tests with missense chains of gene *9* amber mutations: Amber mutants at 11 sites produced missense polypeptide chains which were defective independent of temperature, with at least one of four amino acids–serine, glutamine, tyrosine and leucine–inserted at the amber site. Ten of these missense chains were defective in maturation (FANE and KING 1987). One, *amH1014,* forms a mature tailspike which is defective in the cell attachment function (BERGET and POTEETE 1980).

TABLE 2

Dominance assays

Restricted parent	Phenotype[a]	Restricted parent[b] P[c]	Restricted parent[b] R[d]	*amUT102* P	*amUT102* R	Coinfection P	Coinfection R	Inserted amino acid P	Inserted amino acid R
amH703	Lethal	270	6.1	97	208	124	185	Gln	Tyr
amUG4	Lethal	244	9.2	70	138	128	142	Gln	Tyr
amUT51	Hyper-ts	116	0.4	185	195	140	61	Tyr	Ser
	Lethal		0.3	185	94	140	26	Tyr	Gln
amUS13	Lethal	56	0.2	178	55	200	19	Ser	Gln
amUT66	Lethal	78	2.2	160	76	98	6.8	Ser	Gln
amH1014	Lethal	270	0.8	70	138	145	180	Gln	Tyr

[a] The resulting phenotype of the restrictive parent when the restrictive amino acid was inserted at the amber site.

[b] The results are presented as the average burst of phage per infectious center.

[c] "P" signifies an infection conducted in the restricted parent's permissive host.

[d] "R" signifies an infection conducted in the restricted parent's restrictive host.

Mixed infections between each of these mutants and a reference phage were done in the appropriate amino acid inserting hosts to determine dominance. In all four of the amino acid inserting host strains, a substantial fraction of initiated chains terminate at the amber codon. This reduces the intracellular concentration of completed tailspike chains. To control for this, the reference phage used as the productive parent in the mixed infections carried a tolerant amber mutation of gene *9, amUT102.* The missense proteins generated by *UT102* on the four amino acid inserting Salmonella *su*$^+$ strains are functional (FANE and KING 1987). Use of a tolerant amber rather than wild type as the reference phage ensured that the intracellular concentration of the functional chains was close to that of the defective chains.

Restrictive amino acid inserting hosts were coinfected at a multiplicity of infection (m.o.i.) total of 10 with the amber mutant of interest and with *amUT102.* Table 2 presents the results of mixed infections with a subset of these mutants. The defects of the missense tailspike chains generated from *amH703, amUT51,* and *amUG4* were recessive to the functional chains. The *amH1014* allele whose missense chain forms a mature tailspike also displayed a recessive phenotype.

The defect of only one missense chain, that of *amUT66,* was clearly dominant to the functional protein. The dominant phenotype suggests that the mutant chains generated by glutamine insertion at the *amUT66* site are interacting with the functional *UT102* chains in the coinfections, blocking their normally

productive maturation. The bursts from the mixed infections with *amUS13* and *amUT51* were somewhat reduced, perhaps representing partial dominance. The dominant and partial dominant alleles may be defective at a later stage of the maturation pathway.

The other amber mutants at sites conferring defects in tailspike maturation–*amUT64, amUT52, amUT71, amUT83, amUT86* and *amUT105*–also exhibited recessive phenotypes. The recessive phenotype of these alleles, together with their failure to form mature tailspikes, resembles the *tsf* mutants.

Isolation of second site suppressors: Salmonella host strains inserting four different amino acids–serine, glutamine, tyrosine and leucine–were used to generate missense proteins from the amber mutants. Twenty-two of the gene *9* amber mutants were tightly restricted for growth, with an e.o.p. (efficiency of plating) less than 10^{-6}, on at least one amino acid inserting host of the four (Tables 1 and 3). These include all the strains tested for dominance. Some of the growth restrictions occurred only at high temperatures (*ts*), while others were independent of temperature and depended only on the mutant site and inserted amino acid (absolute lethal).

For each restrictive condition, we plated for apparent revertant phage which could form a plaque; the apparent revertant plaques were tested for the inability to grow on the su^- host.

The apparent revertants fell into three classes: (1) true or pseudorevertants back to an am^+ phenotype; (2) the original mutant itself, presumably arising through leakage; and (3) phage containing the amber allele and presumably carrying a second site suppressor. When "leakers" of the original mutant occurred, we did not include them into the total revertant tally; we either repeated the screen or elevated its temperature.

Each putative second site suppressor was back crossed to the original amber mutant and scored for am^+ recombinants. In all crosses the am^+ frequencies were similar to the am^+ reversion frequencies obtained in the control infections of each parent alone, indicating that the suppressor phage strain retained the original mutation. These values were typically between 10^{-6} and 10^{-7}.

Putative second site suppressors were found among the revertants of the missense phenotypes of 13 of the 22 amber mutants. Amber mutants *amUG2, amUS11, amUS13, amUT34, amUT44, amUT48, amUT51, amUT57, amUT71, amUT66, amUT86, amH1014* and *amH1291,* yielded second site suppressors. Amber mutants *amUG4, amUS14, amUT52, amUT64, amUT83, amUT93, amUT105, amUT111* and *amH703* did not. The frequency of suppressors among the am^+ revertants ranged from 1/48 for *amH1291* on DB7155 inserting glutamine at 37° to 3/4 for *amUT71* on DB7155 at 30°.

The phenotypes of these 13 amber mutants with and without their suppressors are presented in Table 3. The table summarizes the patterns of the mutant's growth as a function of the amino acid tRNA inserting host and temperature. The condition under which the suppressor was isolated is starred in bold. For the remaining nine amber mutants assayed, all the revertants had lost the amber phenotype and were presumably am^+ revertants of the original mutant.

Of the 13 mutant sites that yielded second site suppressors, ten displayed phenotypes resembling the *tsf* class, defective early in chain folding; two displayed absolute lethal defects in chain folding or maturation, and one (*amH1014*) affected the function of the mature protein (BERGET and POTEETE 1980). A variety of trivial factors could underlie the failure to recover second site suppressors of the nine mutants which did not yield them. However, it is also possible that those amber sites which failed to yield second site suppressors of their missense defects represent a different classes of defects in chain folding, association, or function than the sites which yielded second site revertants.

To identify phenotypically different suppressors, we measured the efficiencies of plating of the double mutants carrying both the starting amber mutation and the second site suppressor of its missense chain over an 18° range with four different amino acid inserting Salmonella hosts (Table 3). If two *su/am* double mutants behaved identically under these conditions, only one was selected for further experimentation. For four of the 13 sites which yielded suppressors—*amUT34, amUT57, amUT71* and *amUT86*–more than one suppressor phenotype was recovered (Table 3).

The comparisons of the suppressed phenotypes of the *su/am* double mutants with the parent mutant also served as the preliminary indicator as to whether the suppressor mutation had a phenotype in itself. In general the second site suppressors corrected the defect for which they were selected, without introducing any additional restrictions on phage growth.

Isolation of *ts* pseudorevertants at the amber sites: To assist in the mapping of the suppressors we attempted to obtain *ts* pseudo revertants at the amber site in which the stop codon had mutated to a *ts* missense phenotype. Given the frequency of *tsf* phenotypes in gene *9* (VILLAFANE and KING 1988) and the missense *ts* phenotypes among the ambers, we thought recovery of such pseudorevertants was likely. Putative *ts* pseudorevertants were identified as those phage that grew on the su^- host but only at lower temperatures. Such am^+ts revertants, *tsR(am)*, were isolated for nine of the original mutants: *amUS11,*

TABLE 3

Patterns of mutant and suppressor/mutant growth as a function of inserted amino acid and temperature

	Inserted amino acid at the amber site:														
	Serine				Glutamine				Tyrosine				Leucine		
Mutant	21°	30°	37°	39°	21°	30°	37°	39°	21°	30°	37°	39°	30°	37°	39°
amUG2	−	+	+	−	+ +	+ +	+ +	+	+ −	+ −	− −*	− −	+ −	− −	− −
su(UG2)-2/amUG2	+	+	+	−	+ +	+ +	+ +	−	−	+ +	+	−			
amUS11		+ +	− −*	− −		+ +	+ +	+ +		+ +	− −	− −	+	+	+ −
su(US11)-2/amUS11		+ +	+ +	+ −		+ +	+ +	+ −		+ +	+ −	−	+	+	+ −
amUS13		+ +	+ +	+		−	− −*	− −		+ +	−	−	−	−	−
su(US13)-1/amUS13		+ +	+ +	+ +		+ +	+	−		+ +	+ +	−	+ +	+ +	−
amUT34		+ +	+ +	+ +		− −*	− −	− −		+ +	+	−	+ −	−	−
su(UT34)-2/amUT34		+ +	+ +	+ +		+ +	+	−		+ +	+ +	+ +	+ +	+ +	+
su(UT34)-5/amUT34		+ +	− −	− −		+ +	− −	− −		+ +	− −	− −	+ +	− −	− −
su(UT34)-6/amUT34		+ +	+	− −		+ +	−	− −		+ +	+	− −	+ +	−	− −
amUT44		+ +	− −*	− −		+ +	+	−		+ +	+ +	−	+ +	+ +	−
su(UT44)-1/amUT44		+ +	+ +	−		+ +	+ +	+ +		+ +	+ +	+ +	+ +	+ +	+
amUT48		+ +	+ +	−		−	− −*	− −		+ +	+ +	−	−	−	−
su(UT48)-3/amUT48		+ +	+ +	+ +		+ +	+ +	+ −		+ +	+ +	+ +	+ +	+ +	+ −
amUT51	+ +	− −*	− −	− −	− −	− −	− −	− −	+ +	+ +	+ +	+	− −	− −	− −
su(UT51)-2/amUT51	+ +	+ +	− −	− −	+ −	− −	− −	− −	+ +	+ +	+ +	+	− −	− −	− −
amUT57		+	− −	− −		+ +	− −*	− −		+ +	+ +	− −*	+ +	− −	− −
su(UT57)-1/amUT57		+ +	−	−		+ +	+ +	+ −		+ +	+ +	+ +	+ +	+ +	−
su(UT57)-4/amUT57		+ +	−	−		+ +	−	−		+ +	+ +	+	+ +	−	−
amUT66		+ +	+ +	+		−	− −*	− −		+ +	+ +	+ −	+	− −	−
su(UT66)-1/amUT66		+ +	+ +	+ +		+ +	+ +	−		+ +	+ +	+ +	+ +	+ +	+
amUT71		+ +	+ +	+ +		− −*	− −	− −		+ +	+ +	+ +	−	−	−
su(UT71)-1/amUT71		+ +	− −	− −		+ +	− −	− −		+	− −	− −	−	− −	− −
su(UT71)-3/amUT71		+ +	− −	− −		+ +	− −	− −		+ +	− −	− −	+ −	− −	− −
su(UT71)-4/amUT71		+ +	+ +	+ +		+ +	−	−		+ +	+ +	−	+ −	+ −	−
amUT86	+ +	+ +	−	− −	− −	− −	− −	− −	+ +	+ +	+	− −*	−	− −	− −
su(UT86)-3/amUT86		+ +	+ +	+ +		− −	− −	− −		+ +	+ +	+ +	+	+ −	−
su(UT86)-5/amUT86	− −	− −	− −	− −	− −	− −	− −	− −	+ +	+ +	+ +	+ +	− −	− −	− −
amH1014		− −*	− −	− −		+ +	+ +	+ +		− −	− −	− −	+ +	+ +	+ +
su(amH1014)-1/amH1014		+ +	+ +	+ +		+ +	+ +	+ +		− −	− −	− −	+ +	+ +	+ +
amH1291		+ +	− −	− −		+ +	− −*	− −		+ +	+ +	+ +	+	− −	− −
su(amH1291)-2/amH1291		+ +	+ +	−		+ +	+ +	−		+ +	+ +	+ +	+	+	−

The starred symbols mark the condition at which the suppressor was found. A "++" symbol indicates that neither the plaque morphology nor the eop of the mutant is significantly affected. The "+" symbol signifies that the mutant is still permissive for growth but either the plaque morphology is reduced and/or the eop drops into the 10^{-1} to 10^{-2} range. A "+ −" indicates that the eop drops below 10^{-2} but remains above 10^{-3}; the plaque size is considerably reduced. A "−" designates an eop somewhere between 10^{-3} and 10^{-5}. These phenotypes are typically leaky. And finally, a "− −" denotes where growth is severely restricted with an eop less than 10^{-6}. We searched for second site suppressors for the original amber mutant on any tRNA suppressor on which the original mutant was tightly restricted for growth, an eop less than 10^{-6}. Phage mutants were not assayed on DB7157 at 21° due to the *cs* phenotype of the host *supJ* mutation (WINSTON, BOTSTEIN and MILLER 1979).

amUT44, amUT51, amUT57, amUT64, amUT86, amUT105, amH703 and *amH1291.*

After the initial characterization of the *ts* phenotypes, one phage of each phenotype was crossed with its original amber parent to ensure that recombination could not separate the *ts* and *amber* phenotypes. No wild-type recombinants were detected among the progeny above the background levels of revertants, indicating that the *ts* mutations were indeed at the *amber* sites.

As expected, the *tsR(am)* revertants had phenotypes similar to the missense phenotypes of the amber mutants. *AmUT57,* for example, produces a missense protein which was *ts* at 37° when glutamine was inserted at the amber site and a missense protein which was *ts* at 39° when tyrosine was inserted at the amber site. *TsR(am)* revertants were recovered with both these phenotypes. *AmUT51* does not grow with serine inserted at the amber site at 30° but grew well with this insertion at 21°. This phenotype was also observed amongst the am^+/ts revertants. At 30° the e.o.p. of this revertant, *tsR(am51)-e,* was below 10^{-6}.

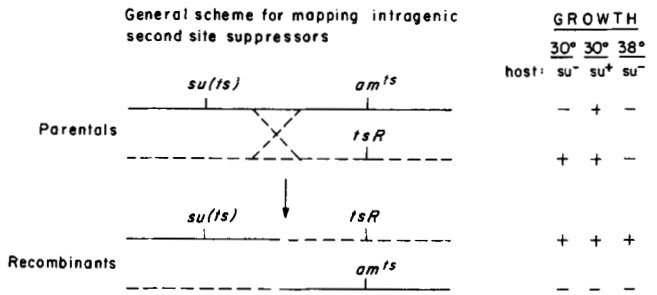

FIGURE 2.—General scheme for mapping the intragenic suppressors. The *tsR(am)* and *su/am* double mutants were crossed at an m.o.i. of 10 in permissive Salmonella hosts at low temperatures. The progeny were then screened for am^+/ts^+ recombinants which would represent the phage with the *su/tsR* genotype.

With the exception of *amUT93,* the amber mutants which did not yield *ts* pseudorevertants did not display tight *ts* phenotypes on any of the su^+ hosts. Most of them yielded either fully functional or absolute defective polypeptides (FANE and KING 1987).

Dominance tests were carried out with the *tsR(am)* pseudorevertants of *amUS11, amUT44, amUT51, amUT57, amUT86* and *amH1291.* They exhibited recessive phenotypes like the previously characterized *tsf* mutants. These were also the six sites which yielded intragenic second-site suppressors (see below).

The e.o.p. for the newly isolated *tsR(am)* mutants were also examined as a function of temperature. Again, if two *tsR(am)* mutants displayed the same patterns of growth, only one was used for further experimentation.

Genetic mapping of the second-site suppressors: To determine the extent of linkage between the original amber mutations and the second-site suppressors, the *su/am* double mutants were crossed with wild-type P22 (*cl-7*) and the progeny were scored for the segregation of the original amber mutant. The second-site suppressors of seven mutants were readily separable from their respective parent mutations. These extragenic suppressors will be described in a later manuscript. The second-site suppressors of at least six mutants, *amUS11, amUT44, amUT51, amUT57, amUT86* and *amH1291,* were not readily separable from their respective original mutations indicating that they might be intragenic.

Ts pseudorevertants had been isolated at all of these amber sites. To map the intragenic suppressors more carefully, we crossed the second-site *suppressor/amber* double mutants with the *ts* pseudo revertants with the hopes that the suppressor would also suppress the *ts* allele at the amber site. The progeny of these crosses were scored for phage which had neither an amber nor a *ts* phenotype. Figure 2 presents the general scheme for these experiments.

The nature of the assay required the suppressor to suppress the defects associated with an amino acid at the amber site for which it may not have been selected.

TABLE 4

Fine structure mapping of intragenic suppressors

Parents	$\frac{am^+/ts^+}{\text{Total progeny}}$
amUS11	0.00092×10^{-3}
amN20	0.00083×10^{-3}
su(US11)/amUS11	0.0015×10^{-3}
tsR(amUS11)	0.00016×10^{-3}
Su(amUS11)/amUS11 × *tsR(amUS11)*	0.41×10^{-3}
Su(amUS11)/amUS11 × *amN20*	24×10^{-3}
amUT57	0.00059×10^{-3}
amN20	0.00082×10^{-3}
Su(UT57)-1/amUT57	0.00026×10^{-3}
tsR(am57)-a	0.0014×10^{-3}
Su(UT57)-1/amUT57 × *tsR(am57)-a*	1.68×10^{-3}
Su(UT57)-1/amUT57 × *amN20*	34×10^{-3}
amUT86	0.0002×10^{-3}
amN20	0.001×10^{-3}
Su(UT86)-5/amUT86	0.0002×10^{-3}
tsR(am86)	0.0013×10^{-3}
Su(UT86)-5/amUT86 × *tsR(am86)*	2.2×10^{-3}
Su(UT86)-5/amUT86 × *amN20*	78×10^{-3}

We therefore crossed every phenotypically distinct *tsR* with its corresponding *su/am* counterpart. In crosses involving derivatives of *amUT57,* for example, the *su(UT57)-1/amUT57* mutant was crossed with the *tsR(am57)-b* mutant in the permissive tyrosine inserting host (DB7156) at 30°. We then screened the progeny at 39° on the su^- host (DB7136). Since these are restrictive conditions for both parents, those phage that grow should be present well above the reversion background of either parent under these conditions and will have a genotype of *su(UT57)-1/tsR(am57)-b.* For this particular experiment the background reversion of both parents was 10^{-7} and the frequency of the recombinants was above 10^{-4}.

Table 4 contains the results of such mapping experiments for suppressors of three sites. Crosses with a distant mutation, *amN20* in gene *20* are included as controls. The small genetic distances between the original mutations and their respective suppressors are consistent with values expected for intragenic recombination.

Since we were unable to detect any phenotype of the suppressors by themselves, or evidence for segregating markers in addition to the parentals, we were unable in these experiments to isolate the gene *9* suppressors without the parental ambers.

The specificity of the intragenic second-site suppressors: Many of the starting amber mutants generated defective missense proteins with more than one amino acid inserted at the stop codon (Tables 1 and 3). Some second-site suppressors restored function only for the primary amino acid substitutions for which they were selected. Others suppressed a variety of restrictive amino acid substitutions at the amber site. Figure 3 presents the e.o.p. as a function of amino

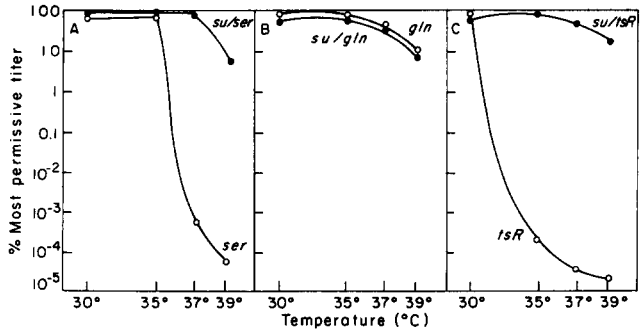

FIGURE 3.—Suppression of growth defects in *amUS11* strains. The data shows the e.o.p. as a function of temperature. The e.o.p. values are expressed as the percentages of the most permissive titer as a function of amino acid insertion and temperature for the *amUS11* amber mutant and its *ts* pseudorevertants with and without its second site suppressors. The individual curves are labeled as to inserted amino acid at the amber site. The second site suppressor is indicated by closed circles (●); parental amber mutant and *tsR(am)* allele (○). For these experiments the most permissive titer was obtained on the glutamine inserting host (DB7155) at 30°.

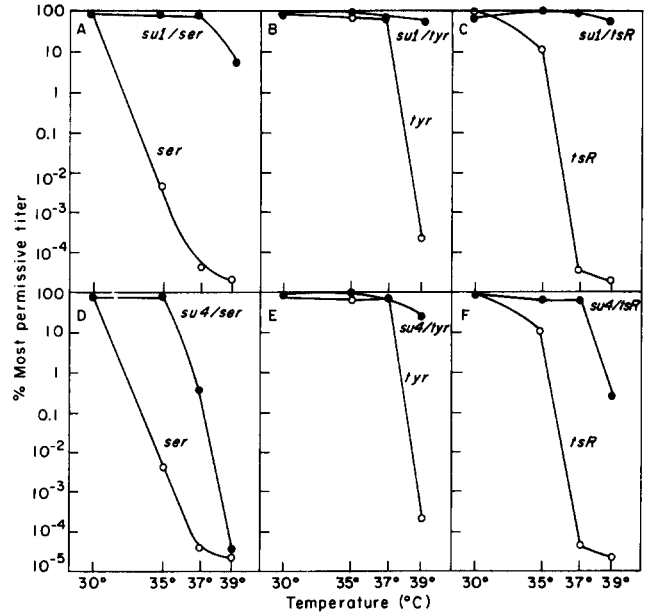

FIGURE 4.—Suppression of *ts* missense growth defects in *amUT57* by two suppressors. The e.o.p. at different temperatures are expressed with respect to the most permissive condition as 100%. Phage which carry the suppressor mutations in addition to the starting *amber* or *ts* mutation (●); parental amber mutant and the *tsR(am57)-b* allele (○). The individual curves are labeled as to inserted amino acid at the amber site. The tyrosine inserting host (DB7156) was the most permissive host for this amber mutant.

acid insertion and temperature for the *amUS11* amber mutant and its am^+/ts pseudorevertants with and without its second-site suppressors. The individual curves are labeled as to the genotype of the produced gene *9* polypeptide and the presence of the suppressor is further indicated by closed circles.

Serine insertion at the *US11* site resulted in a sharp drop in plating efficiency between the temperatures of 30° and 35°. The presence of the suppressor relieved this defect and did not inhibit the maturation of the tailspike when the most tolerated amino acid, glutamine, was inserted at the site. The suppressor also relieved maturation defects associated with tyrosine insertion (data not shown). In panel C, one can see that the suppressor mutation also acts on the *tsR(am)* pseudorevertant of the *amUS11* mutant.

Figure 4 presents the efficiency of plating as a function of temperature associated with *amUT57* and two phenotypically different suppressors. The *su(UT57)-1* suppressor relieves the maturation defects associated with a number of *ts* insertions at the *UT57* site without inhibiting growth with permissive insertions. The tyrosine inserting host (DB7156) is the most permissive host for this amber mutant.

The *su(UT57)-4* suppressor was somewhat less effective in restoring growth at high temperature. It was initially isolated for its ability to suppress the *ts* defect associated with tyrosine insertion at 39°. Although it suppressed tyrosine insertion well at this temperature, it suppressed the *ts* defects associated with the mutant *tsR(am57)-b* residue and serine insertion less efficiently (panels F and D, respectively). Another *ts* revertant of *amUT57, tsR(am57)-a,* had an e.o.p. profile similar to the tyrosine-suppressed amber mutant. Unlike *tsR(am57)-b,* this mutant is well suppressed by *su(UT57)-4,* (data not shown).

Physiology of the second-site suppressors: Figure 5 depicts the production of viable phage as a function of time after infection, for *tsR(am57)-b* with and without its two second-site suppressors. At temperatures above 37°, where the production of native tailspikes becomes the rate-limiting step in phage assembly, these curves serve as an indirect measure of the kinetics of native tailspike maturation. At permissive temperatures all the strains produce more than 200 phage/cell.

At restrictive temperature *tsR(am57)-b* did not produce viable phage; the products of these nonpermissive infections are phage heads which can be efficiently rescued by adding wild-type native tailspikes *in vitro* (data not shown). The presence of the suppressor *su(UT57)-1* brought the yield up to the control level. The *su(UT57)-4* suppressor increased the yield 20-fold over the *ts* strain alone, but did not reach the permissive temperature yield or the wild-type elevated temperature yield. The efficiency of this suppressor was higher when the experiments were conducted with the double mutants that contained the *tsR(am57)-a* allele (data not shown).

The intragenic suppressors correct the missense defects at the level of polypeptide chain maturation: The suppressors could act on a variety of different levels. They could, for example, increase the rate of polypeptide chain translation or the efficiency of protein maturation. In order to investigate the mecha-

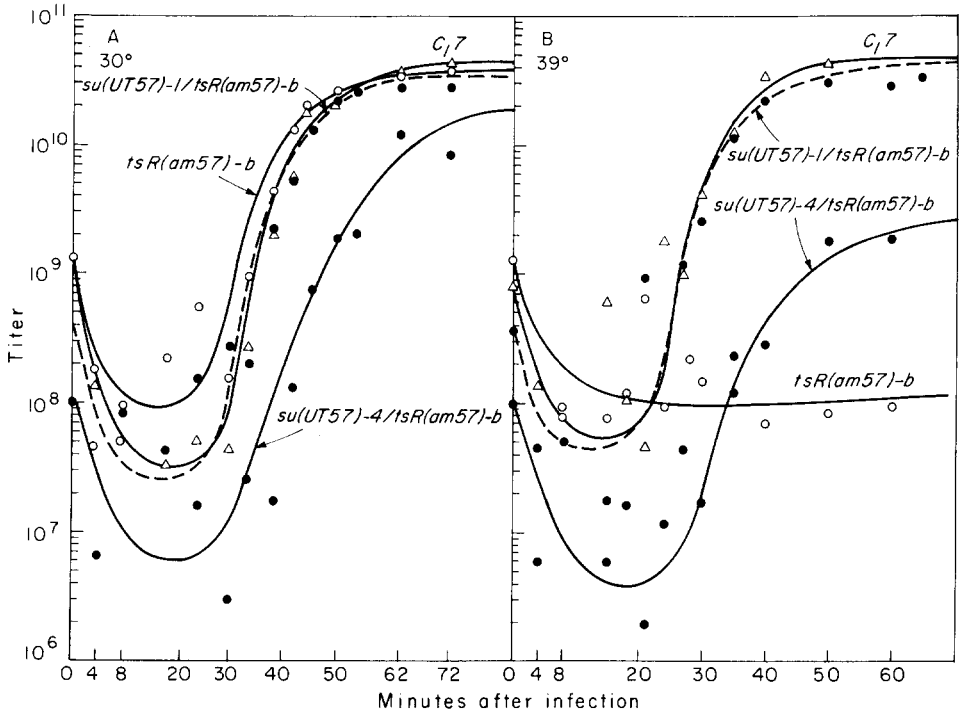

FIGURE 5.—Growth curves of phage carrying *tsR(am57)-b* with and without its second site suppressors *su(UT57)-1* and *su(UT57)-4*. Infection with *c1–7* serves as the wild-type reference. Salmonella DB7136 host cells were infected with the mutants of interest at an m.o.i. of 10. After a 12-min incubation at 39° to allow for phage absorption, the cultures were diluted 1/10,000 and samples incubated at 30° and 39°. At designated times after infection, samples were lysed with chloroform and plated permissively.

nism of suppression we directly examined protein maturation *in vivo*. For these experiments we constructed multiple mutant strains carrying in addition to the starting mutant and second site suppressor, amber mutations in the genes for coat (*5*) and lysis control (*13*) proteins. This ensures that the tailspike will accumulate as a soluble species within the cell (GOLDENBERG and KING 1981). These experiments were conducted with the assistance of C. HAASE-PETTINGELL and A. MITRAKI.

Infected cell cultures were exposed to a short pulse of ^{14}C-amino acids to label newly synthesized polypeptide chains. At various times after the chase samples were withdrawn and mixed with SDS sample buffer. These samples were not heated. Under these conditions folding intermediates dissociate to SDS/polypeptide chain complexes, whereas mature trimeric tailspikes remain native binding very little SDS. Upon SDS gel electrophoresis the native tailspikes migrate very slowly in the SDS gel and are very well separated from the SDS/polypeptide complexes (GOLDENBERG, BERGET and KING 1982).

Figure 6 contains the results of these experiments. The lanes of the autoradiograph labeled "a" through "f" correspond to the time of the sampling after the chase. In the control *5⁻/13⁻* infections the newly synthesized tailspike polypeptides chase from a partially folded, SDS-sensitive state to the mature, SDS-resistant tailspike at both permissive and restrictive temperatures. The *tsR(am57)-b* polypeptide chains are synthesized at the normal rate (upper panels). At permissive temperature a fraction of the chains reach the mature conformation, but at restrictive temperature all of the chains fail to reach the native SDS-resistant conformation. The chains are not degraded but remain in an SDS-sensitive state. This is the phenotype associated with *tsf* mutations (SMITH and KING 1981; GOLDENBERG, SMITH and KING 1983).

The presence of the suppressor mutation (middle panels) together with the *tsf* mutation results in the formation of mature SDS-resistant tailspikes at the restrictive temperature. The suppressor also increases the efficiency of maturation of the *tsR* polypeptide chain at permissive temperatures.

These results suggest that the intragenic suppressors act by alleviating the folding defects conferred by the original mutation. Similar results have been obtained with *su(UT86)-5/tsR(am86)-a* and *tsR(am86)-a*, and *su(UT51)-2/tsR(am51)-e* and *tsR(am51)-e* (data not shown). Although the *su/tsR* double mutants for the *amH1291*, *amUS11* and *amUT44* have not been assayed, the *tsR(am)* mutants at these sites have been assayed. They also display a temperature-sensitive folding phenotype (data not shown).

DISCUSSION

Consideration of mutational and comparative sequence data on the genetic control of polypeptide chain folding indicate that the residues controlling chain folding and assembly are dispersed through the polypeptide chain (KING 1986; BASHFORD, LESK and CHOTHIA 1987; PRESTA and ROSE 1988). If only some residues in the chain control the folding process, it is not surprising that algorithms and models which as-

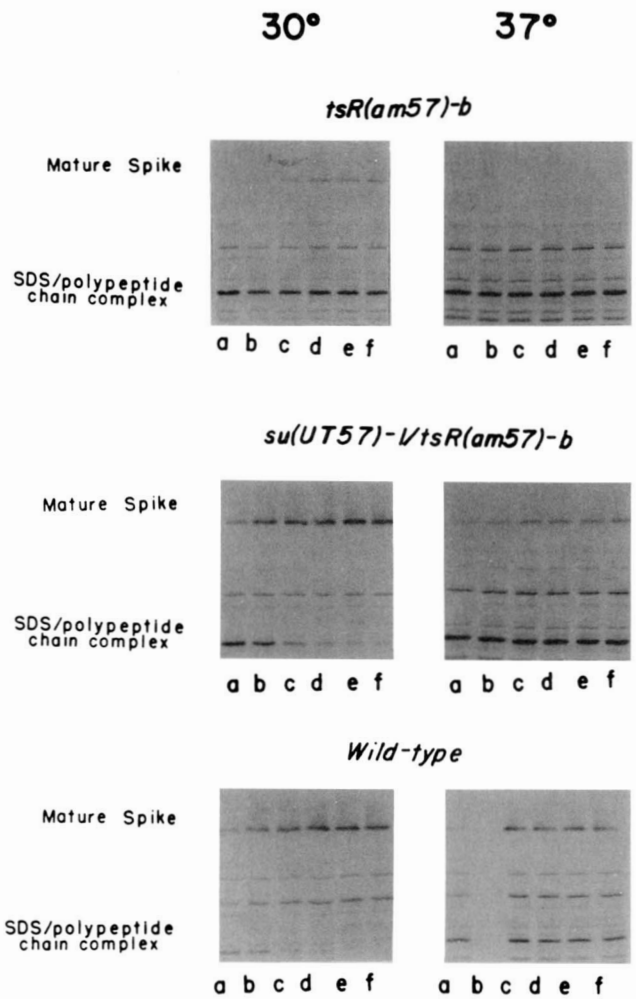

FIGURE 6.—Intracellular maturation of newly synthesized native tailspike polypeptide chains. *Su*$^-$ host cells were infected at 37° with the *tsR(am57)-b* and *su(UT57)-1/tsR(am57)-b* phage strains carrying in addition amber mutations in genes 5 and *13*. The *5*$^-$*am/13*$^-$*am* derivatives of and reference (*5*$^-$*am/13*$^-$*am*) strains at 37°. The cultures were divided and incubated at 37° and 30°. At 40 min after infection after infection, they were exposed to a 1.5-min pulse with ^{14}C-labeled amino acids. At various times after the chase, the cells were harvested and prepared for SDS-gel electrophoresis without heating. The lanes of the autoradiograph "a" through "f" and correspond to the time of the sampling after the chase–4, 7.5, 13.5, 19.5, 28.5 and 38.5 min, respectively.

sume that every residue in the chain contribute to determining conformation have had limited success. The systematic isolation and characterization of temperature-sensitive folding mutations in the tailspike gene have provided one approach for identifying residues critical for controlling the folding process (KING *et al.* 1989). The isolation and characterization of suppressors of such folding defects provides a route for further identification of related sequence information.

Tailspike conformation and mutation: Tailspike structure is dominated by beta sheet structure (SARGENT *et al.* 1988), probably in a rodlike cross-beta conformation (GREEN *et al.* 1983). The *tsf* mutations affect the formation or stability of a thermolabile single-chain folding intermediate in the folding pathway (GOLDENBERG, SMITH and KING 1983;). A variety of evidence indicates that the sites of the *tsf* mutations are involved in the formation of the correctly located turns (YU and KING 1988; VILLAFANE and KING 1988), suggesting that the thermolabile intermediate is forming the core of the cross beta sheet. At high temperatures the *tsf* mutant chains aggregate into inclusion bodies (HAASE-PETTINGELL and KING 1988). This state is a kinetic trap for the chains accounting for their tight phenotypes at restrictive temperatures.

Selection for suppressors of polypeptide chain-folding defects: The second site conformational suppressors were isolated by selecting for growth of amber mutants on Salmonella host strains which inserted a missense amino acid at the amber codon. Thus the selection was for alleviation of the defect in the complete polypeptide chain, rather than of the translational stop. The selection was designed to pick up any phage mutations that could suppress the starting mutants (JARVIK and BOTSTEIN 1975). The second site suppressors might represent sites within the gene *9* polypeptide chain, or sites in other phage specified macromolecules. Mutants of the Salmonella host strain, such as in the genes for ribosomal proteins or chaperonin proteins, would not have been recovered. VAN DYK, GATENBY and LAROSSA (1989) have reported that some P22 *tsf* mutations can be rescued by growth on Salmonella strains overproducing the *GroEL* and *GroES* subunits.

Since the defects of the missense chains generated at the 22 starting amber sites have not been fully characterized, suppressor selection was not in all cases for alleviation of a folding defect. However, many of these missense defects are in the folding pathway (FANE and KING 1987) so that we expected to recover suppressors of folding defects.

There was no obvious correlation between map location of the starting sites and the overall recovery of second-site suppressors. Mutations mapping from the amino terminus (*amUS11*) to the most carboxy terminus mutant (*amUG2*) yielded second site suppressors. Conversely, starting sites that did not yield suppressors also map throughout the gene.

With respect to the intragenic suppressors, five of the six sites for which we recovered intragenic suppressors mapped in the central region of the gene where the *tsf* mutations cluster (VILLAFANE and KING 1988). We believe that this is the region of the chain which is critical in the formation of the early thermolabile folding intermediate. The five mutations mapped throughout the central region (FANE and KING 1987). Since the starting mutations probably exert their affects in partially folded states of the newly synthesized polypeptide chain, we did not ex-

pect any simple correlation between linear position in the gene and recovery of suppressors.

From their isolation and characterization of gene *9* mutations with absolute defects, SCHWARZ and BERGET (1989b) suggested that the tailspike chain was differentiated into three regions; an N-terminal region involved in head binding, the central region controlling chain folding, and a C-terminal region involved in the endorhamnosidase activity. MAURIDES, SCHWARZ and BERGET (1990) isolated second site suppressors to one of the N-terminal sites Asp-100>Asn and found that they were substitutions of Arg-13. The amino acid substitutions were consistent with the existence of Asp-100-Arg-13 salt bridges stabilizing the N-terminal region of the mature tailspike.

Isolation of *tsf* alleles at amber sites: For those amber sites which displayed a *ts* missense phenotype on one or more of the Salmonella amino acid inserting hosts, we were able to recover *ts* alleles as pseudorevertants (JARVIK and BOTSTEIN 1975). Analysis of the *in vivo* synthesized *ts* chains revealed that they failed to fold or assemble into mature tailspike trimers. All of the *ts* pseudorevertants exhibited a recessive phenotype as do the previously isolated *tsf* mutants.

The degradation of improperly folded polypeptide chains is a well documented phenomenon (GOLDBERG and ST. JOHN 1976). The defects associated with these mutations could increase the mutant chains' sensitivity to degradation. We observed no overall loss of material of the partially folded species in the experiments that follow the *in vivo* synthesis and maturation of the mutant polypeptide chains. HAASE-PETTINGELL and KING (1988) also report that the improperly folded chains which form intracellular aggregates do not have a modified molecular weight.

Further experiments examining the *in vivo* maturation of the mutant chains, indicate that the *ts* alleles of amber codons are not fundamentally different from the previously isolated *tsf* strains (C. HAASE-PETTINGELL and A. MITRAKI, personal communication). The mutations at all the *ts* sites in gene *9* previously studied–more than 30–have all turned out to be temperature-sensitive folding mutations (SMITH and KING 1981; YU and KING 1988). We conclude that the *ts* mutations isolated as pseudorevertants at the amber sites also cause folding or maturation defects.

Recovery of extragenic second-site suppressors: The suppressors isolated for seven of the starting ambers were extragenic. In general the suppressible sites yielded either intragenic or extragenic suppressors, but not both. Extragenic suppressors were only found for missense polypeptide chains exhibiting lethal (temperature-independent) phenotypes. In addition these sites did not yield *ts* pseudorevertants. We presume the sites suppressed by extragenic suppressors represent a different class of polypeptide chain defects than the sites suppressed by intragenic suppressors. Mapping and complementation experiments indicate that the extragenic second site suppressors are in the genes for the procapsid and DNA packaging proteins (FANE 1988; B. FANE and J. KING, unpublished results).

An unusual mode of interaction between the tailspike gene and the head genes has been recently identified (BAZINET, VILLAFANE and KING 1990). Certain *tsf* mutations in gene *9* suppress a cold sensitive defect in the gene *1* portal protein, which sits at the initiating vertex of the procapsid shell. BAZINET, VILLAFANE and KING (1990) present evidence that this suppression is not acting directing through protein/protein interactions but rather through a structural role of the late messenger RNA (WEINSTOCK, RIGGS and BOTSTEIN 1980). In this model the gene *9* mutations generate sequences in the gene *9* messenger RNA which mimic an RNA locus in the head region messenger used for initation of procapsid assembly.

Tailspike intragenic suppressors: Second site suppressors of six amber sites were identified as intragenic due to the failure to recover the parental amber mutants among the progeny of backcrosses with wild-type P22 (*cl-7*). Further mapping of the intragenic suppressors relied on their abilities to suppress *ts* mutations at the amber site. At the onset of these experiments, we did not know if a conformational suppressor would correct the defects associated with a *ts* substitution for which it had not been selected. Therefore, all *ts* alleles recovered were tested. In fact there was no case in which the second site suppressor did not alleviate the *ts* defect, though the efficiencies varied where there were different *ts* alleles at the same site.

Subsequent sequencing of DNA from phage carrying the amber and suppressor mutations has confirmed the intragenic location. Phage strains carrying *Su(UT51)-2* and *su(UT57)-4* both display substitutions of valine to alanine at position 331, and strains carrying suppressors *su(UT57)-1* and *su(amH1291)-2* displayed substitutions of alanine to valine at position 334 (B. FANE, R. VILLAFANE and J. KING, unpublished results). Strains carrying *amUT51* contained a nonsense codon at tyrosine 232, strains carrying *amUT57* contained a nonsense codon at tryptophan 207, and strains carrying *amH1291* contained a nonsense codon at tryptophan 202 (B. FANE, R. VILLAFANE and J. KING, unpublished results).

The intragenic suppressors act at the level of protein folding or conformation: The suppressors could act on a variety of levels, transcription, translation or maturation (GRODZICKER and ZIPSER 1968; MORSE and YANOFSKY 1969; JARVIK and BOTSTEIN 1975; KLIG, OXENDER and YANOFSKY 1988). Tran-

scription of gene *9* is influenced both by the late P22 promotor and by a transcription terminator adjoining the gene (BERGET, POTEETE and SAUER 1983). However for the *ts/su* strains, we measured the rate of tailspike synthesis directly, which was not altered with respect to the controls. The defective phenotypes of gene *9 tsf* mutations are in fact quite insensitive to level of protein synthesis. ADAMS, BROWN and CASJENS (1985) demonstrated that blocks in P22 head assembly lead to an over production of tailspike polypeptides. Double mutants with amber mutations in gene *5*, encoding the major coat protein produce 10-fold more tailspike chains than wild-type (*cl-7*) strains. Yet the *5⁻am/13⁻am/tsR(am)* strains constructed for the *in vivo* maturation experiments exhibited similar *ts* phenotypes as their *tsR(am)* parents, indicating the dosage insensitivity of the mutants yielding intragenic suppressors.

The *in vivo* maturation of tailspike proteins was followed for *ts* revertants at six sites, with and without their second site suppressors. In the *ts* infected cells the mutant gene *9* chains failed to mature into native tailspikes. The suppressors alleviated these defects and the double mutant *su/tsR* chains matured into native tailspikes at restrictive temperatures. This result suggests that the intragenic suppressors act directly in correcting folding defects.

SHORTLE and LIN (1985) have reported the isolation of global, second site suppressors in staphylococcal nuclease which increase the stability of the native protein. Similarly, ALBER and WOZNIAK (1985) have reported on the isolation of T4 lysozyme mutants with increased, native thermostability. The intragenic second-site suppressors of the P22 tailspike seem to be different from these mutations in that they act during the folding pathway. Purified proteins carrying the suppressors have been examined by differential scanning calorimetry and do not show altered thermostability (A. MITRAKI, C. HAASE-PETTINGELL, J. KING and J. STURTEVANT, unpublished results).

Recovery of intragenic second site suppressors was strongly correlated with the existence of a strong *ts* phenotypes on at least one amino acid inserting host. All the *tsf* mutations appear to affect the same stage, a partially folded single-chain intermediate. It is possible that all of the intragenic suppressors are acting at this stage of the pathway.

The *tsf* mutant polypeptide chains synthesized at high temperature can be rescued by shift down to permissive temperature (SMITH and KING 1981). However, with increasing time of incubation at high temperature this reversability is lost due to intracellular aggregation (HAASE-PETTINGELL and KING 1988). We do not know if the tailspike conformational suppressors might identify pairs of directly interacting residues, or act more generally in the folding pathway. They could do this by stabilizing a common structure within an intermediate or blocking nonproductive, off pathway reactions. Detailed investigations with purified proteins carrying the suppressor mutations are currently underway to determine whether this operates through the tailspike polypeptide chain itself or through interaction with host factors such as the cellular chaperonins (BOCHKAREVA, LISSIN and GIRSHOVICH 1988; GOLOUBINOFF *et al.* 1989; VAN DYK, GATENBY and LAROSSA 1989).

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