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Bacteriophage T4 Transfer RNA

I. Isolation and Characterization of Two Phage-coded Nonsense Suppressors

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Two phage-coded nonsense suppressors, psu_a^+ and psu_b^+, have been isolated and characterized. Both were isolated as pseudo-wild type revertants of phage strains which carry multiple amber mutations. psu_a^+ is an amber suppressor which occurs at a frequency of 10^{-11} to 10^{-12} and is indistinguishable from wild type phage in its growth on both B and K strains of *Escherichia coli* bacteria. psu_b^+ may be either an amber or an ochre suppressor, which occurs at a frequency of 10^{-7} to 10^{-10} and makes small plaques on B strains, but grows very poorly or not at all on K strains. Phage with the characteristics of psu_a^+ occur in populations of psu_b^+ phage at a frequency of 10^{-4}. Both suppressors insert serine in response to the amber codon at an efficiency of about 45%.

psu_a^+ and psu_b^+ map less than 0·3 map units apart and are located between genes *e* and *57* about 8 map units from gene *e*. That psu_a^+ and psu_b^+ insert the same amino acid and map so close together suggests that they result from different mutations within the same gene. On the basis of their initial frequencies of appearance and the frequency of psu_b^+ mutation to psu_a^+, we speculate that psu_a^+ is derived from wild type by two base changes and that psu_b^+ is a one-base-change intermediate.

1. Introduction

Upon infection of its host bacterium, the bacteriophage T4 directs the synthesis of several phage specific transfer RNA's (Weiss, Hsu, Foft & Scherberg, 1968; Daniel, Sarid & Littauer, 1968; Tillack & Smith, 1968). Fingerprint analysis of the individual low-molecular weight RNA bands from polyacrylamide gel fractionation of the post-infection RNA suggests that T4 produces 6 to 10 tRNA's (W. H. McClain, personal communication; J. N. Abelson, personal communication). tRNA's specific for arginine, glycine, isoleucine, leucine, proline and serine have been identified among the phage-coded species (Scherberg & Weiss, 1970; W. H. McClain, personal communication).

Why does the virus synthesize its own species of tRNA in apparent partial duplication of the host tRNA species? Though there has been much speculation no reason for their presence has been demonstrated (Sueoka & Kano-Sueoka, 1970; Daniel, Sarid & Littauer, 1970). Are these tRNA's capable of reading codons which are commonly used by the virus, but never or infrequently by some of its hosts? Are they involved in

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the turn-off of host protein synthesis, or the turn-on or turn-off of any viral protein? Do they in some manner regulate the relative amounts of viral proteins? Are they essential in some hosts T4 encounters naturally, but not in the common laboratory strains of *Escherichia coli*?

We have begun to investigate the function of the T4 tRNA's by the isolation and characterization of genetically altered phage strains which differ from wild type phage in the number or nature of their tRNA's. The first report describes the properties of two phage-coded nonsense suppressors. One of these, psu_a^+, appears to be identical to psu_1^+, a phage-coded amber suppressor described by McClain (1970).

2. Materials and Methods

(a) *Phage strains*

Phage strains derived from the wild type T4D were obtained from the collection of Dr R. S. Edgar. Most of the amber (*am*) and temperature-sensitive (*ts*) mutants used have been described elsewhere (Epstein *et al.*, 1963; Edgar & Lielausis, 1965; Wood & Henninger, 1969). Those *am* mutants designated NG were isolated by Edgar & Lielausis (unpublished results) from wild type T4D after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. K6 is an *am* mutant selected to grow on H12R8a (*su-3*$^+$) but not CR63 or S/6/5 (Edgar & Lielausis, unpublished observations). The *rII* mutant *rdf41* is a deletion in T4D which encompasses both the A and B cistrons. The *rII* ambers, ochres (*oc*) and opals (*op*) derived from the wild type T4B were obtained from Dr S. Champe. The gene *e* ochres and opals and the deletion eG223 derived from the standard type T4B were obtained from the collection of Dr G. Streisinger *via* Dr W. Salser. Phage stocks of gene *e* mutants were prepared on citrate lysozyme plates by the confluent lysis technique, as described by Epstein *et al.* (1963). Multiply-mutant phage strains are described in Table 1.

(b) Escherichia coli *host strains*

CR63 was used as the permissive host for *am* mutants and as the non-permissive host for psu_b^+ strains. S/6/5 was used as the non-permissive host for *am* mutants and the permissive host for psu_b^+ strains. The *am* suppressor strains S26R1e (*su-1*$^+$), S26R1d (*su-1*$^+$), S26R1d (*su-2*$^+$), H12R8A (*su-3*$^+$) which insert serine, glutamine and tyrosine respectively, in response to the *am* codon, and their parent strain S26 (*su*$^-$) (Garen, Garen & Wilhelm, 1965) were obtained from Dr A. Garen. 594 is a streptomycin-resistant K strain. CAJ70, obtained from Dr S. Brenner, is permissive for *op* mutants. The B strains of *E. coli*, B/5, G(λ) and Bb, which are non-permissive for *am* mutants have been described by Wilson, Luftig & Wood (1970). BB from the Caltech collection is a B strain which is permissive for *am* mutants, it was originally obtained from Berkeley as a non-permissive strain. The origin of the difference is unclear (Russell, 1967).

(c) *Media and buffers*

H broth used for phage and bacterial growth, and EHA top and bottom agar used for plating assays, were prepared as described by Steinberg & Edgar (1962). Synthetic growth medium contained per l., 7 g Na_2HPO_4, 3 g KH_2PO_4, 6 g NaCl, 1 g NH_4Cl, 0·12 g $MgSO_4$, 0·01 g $CaCl_2$, 4 g glucose and 0·6 g Casamino acids. Dilution buffer was prepared as described by King (1968). Synthetic medium minus Casamino acids was used for preparation of radioactively labeled infected-cell lysates.

(d) *Radioactively labeled lysates of phage-infected cells*

Strain Bb was grown to a concentration of 5×10^7 cells/ml. in synthetic medium lacking Casamino acids. The cells were collected by centrifugation at 10,000 *g* for 10 min and resuspended at 2 to 4×10^8 cells/ml. One-ml. fractions of this suspension were warmed to 37°C, infected with phage at a multiplicity of 5 to 10, and aerated. 15 min after infection, 2·5 to 10 μCi of a uniformly ^{14}C-labeled amino-acid mixture (New England Nuclear) were added. For pulse-labeled lysates an equal vol. of 10% trichloroacetic acid was added 2·5 min after addition of the ^{14}C-labeled amino acids. Non-pulse-labeled cultures were lysed by

TABLE 1

T4 multiple mutants

Strain designation	Mutations	Defective genes
X4E	*am*B25, *am*A455, *am*B252, *am*N52, *am*B280, *am*B262	*34, 35, 37, 38*
X74	*am*B20, *am*N133	*14, 15*
SX4	*am*E18, *am*B17, *am*N120 *am*B252, *rdf41*	*18, 23, 27, 35, rII*
X174	*am*B255, *am*N128	*10, 11*
X372	*am*N52, *am*B280, *am*M69	*37, 63*

addition of $CHCl_3$ (Mallinckrodt) 45 min after infection. All lysates were dialyzed against 0·065 M-Tris, pH 6·8 1% sodium dodecyl sulfate.

(e) *Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate*

Procedures for the preparation and running of discontinuous polyacrylamide gels containing sodium dodecyl sulfate were as described by Laemmli (1970). Dialyzed lysates were made 1 to 2% in 2-mercaptoethanol. Proteins were dissociated by heating the sample for 2 min in boiling water. Samples containing 300,000 cts/min in a vol. of 0·3 ml. were run on 7·5% polyacrylamide gels containing 0·1% sodium dodecyl sulfate. Gels were stained by the method of Weber & Osborn (1969) with Coomassie brilliant blue R250 (Mann) and destained by the method of Ward (1970). Gels were sliced vertically, dried on filter paper and autoradiographed for 72 hr on Kodak no-screen X-ray film (Fairbanks, Levinthal & Reeder, 1965). Resulting autoradiographs were traced with a Joyce/Loebl densitometer and the area under curves determined with a planimeter. Over the range of exposure times used the area under all measured bands was a linear function of the exposure time, indicating that the area under the tracing of a band is a measure of the amount of radioactivity in the band.

(f) *Other methods*

Standard phage crosses were a modification of the procedure of Steinberg & Edgar (1962). A stationary-phase culture of CR63 was diluted 1/1000 in H broth and grown for 3 hr at 30°C. Cells were collected by centrifugation and resuspended at 4×10^8 cells/ml. Parent strains at a multiplicity of 7·5 each were added to the cells in the presence of 0·004 M-KCN at 30°C. After 5 min, anti-T4 serum was added to kill all unadsorbed phage. 10 min after infection the cells were diluted 10^4 into H broth at 30°C. 90 min after infection $CHCl_3$ was added.

Serum blocking assays were as described by Ward *et al.* (1970). All assays were done with hyperimmune rabbit antisera prepared directly by injection of a purified antigenic component. Tail fiberless particles were prepared from B/5 cells infected with the multiple *am* mutant X4E as described by Edgar & Wood (1966).

3. Results

(a) *Isolation of* psu_a^+ *and* psu_b^+

Our initial discovery of a phage-coded nonsense suppressor resulted from an unrelated attempt to find variants of T4 which could adsorb solely by their baseplates. We anticipated that such mutants, if they exist, would appear as pseudo-wild type revertants of the multiple *am* mutant strain X4E, which carries six *am* mutations in four tail fiber genes. In such a strain, revertants to true wild type were expected to occur at a frequency of less than 10^{-30}. No phenotypic am^+ revertants ($<10^{-10}$)

could be detected in phage stocks of X4E. In order to detect revertants at frequencies as low as 10^{-13} on a single plate, we prepared tail fiberless particles using the X4E strain and plated them directly on an *su*$^-$ host, S/6/5. Plaques appeared at a frequency of about 10^{-11} relative to the titer of the tail fiberless particles. Revertants from two different preparations of tail fiberless particles each divided into two groups. Neither group contained the type of revertant in which we were originally interested.

None of the five revertants in the first group yielded *am* progeny when crossed to wild type phage. In addition, they appeared to produce normal tail fibers; the phage particles contained the products of genes *34* and *37* as analyzed by sodium dodecyl sulfate gel electrophoresis and blocked both A- and BC'-specific sera (Ward *et al.*, 1970) equally as well as wild type phage. These observations suggest that all the original *am* lesions have been repaired. Since these apparent revertants to true wild type are present at least 10^{19} times more frequently than expected, they may contain an additional mutation which drastically alters the frequency of reversion. In line with this suggestion is the observation that several of these revertants gave rise to a high proportion of progeny with altered plaque morphology. This group has not been investigated further.

All six of the revertants in the second group plated like wild type phage, but when crossed to wild type phage yielded progeny carrying *am* mutations. By complementation tests it was shown that these progeny carried, in various recombinant combinations, all the *am* mutations present in X4E. Even though these revertants still retained all six *am* mutations, they have normal tail fibers by the criteria applied above. Thus these phage must contain an additional mutation, which in some manner suppresses the X4E *am* mutations. One of the revertants, X4ER2, was designated as carrying phage suppressor a (psu_a^+) and renamed psu_a^+: X4E. psu_a^+: X4E was then crossed to the other five revertants in this group. If any of the other X4E revertants contained a suppressor at a site other than psu_a^+, *am* mutants would appear in the progeny of such a cross at a frequency related to the distance between it and psu_a^+. None of the progeny examined, approximately 200 for each cross, was of the *am* phenotype. Thus, all these revertants of X4E must contain suppressor mutations which map very close to psu_a^+ and may be identical to it.

Two additional pseudo-wild type revertants which contain suppressor mutations have been isolated from phage stocks of the multiple *am* mutants X74 and SX4. Though not tested extensively, both these revertants show suppression characteristics in common with psu_a^+, suggesting they may also contain psu_a^+. In summary, we find that pseudo-wild type revertants which carry a suppressor mutation, possibly psu_a^+ in all cases, occur in a population of tail fiberless particles or phage at a frequency of 10^{-11} to 10^{-12}.

Among the phenotypically *am*$^+$ revertants of the multiply-mutant strains X74, X174 and X372 and the single mutant *am*N133 (gene *15*), we have found an additional class of revertants distinct from either of the above two classes. These revertants arise at a frequency of 10^{-7} to 10^{-10}. All make very small plaques on S/6/5 and no plaques on CR63. When crossed to wild type, they yield progeny which carry the *am* mutations present in the original strains, suggesting that they carry a second-site suppressor mutation. One of the revertants, X74R7, was designated as carrying phage suppressor b, psu_b^+, and renamed psu_b^+:X74. To investigate the plating characteristics of phage carrying psu_b^+, we plated psu_b^+:X74 and the single mutant psu_b^+ (see the legend to Table 2) on many of the K and B strains in common use in our laboratory. The results

TABLE 2

Plating characteristics of psu_b^+*:X74 and* psu_b^+

Bacterial strains		*am* suppression	psu_b^+:X74	psu_b^+
K strains	S26	su^-	±	±
	S26R1e	su^+	±	±
	H12R8A	su^+	±	±
	594	su^-	0	
	CR63	su^+	0	0
	CAJ70	su^-	0	
B strains	B/5	su^-	+	
	S/6/5	su^-	+	+
	G(λ)	su^-	+	
	BB	su^+	+	+
	Bb	su^-	+	

psu_b^+ was isolated from the progeny of a cross of psu_b^+:X74 by wild type phage. That it contains none of the *am* mutations present in X74 is based on two criteria: (1) no *am* mutants, to a level of 1%, are found among the progeny of a cross of psu_b^+ by wild type. (2) Greater than 10% wild type phage are present in the progeny of a cross of psu_b^+ by X74. psu_b^+ makes a slightly larger plaque on S/6/5 than does psu_b^+:X74, though still much smaller than wild-type phage. In the Table the plaque size of psu_b^+:X74 and psu_b^+ on S/6/5 is represented by +. ± indicates that plaques are just barely visible. 0 indicates that no plaques can be seen.

are shown in Table 2. It appears that the peculiar plating phenotype of strains which carry psu_b^+ reflects a general difference in the ability of B and K bacterial strains to support psu_b^+ growth.

During these preliminary characterizations of psu_b^+, it was noted that crosses involving psu_b^+ strains showed a significantly reduced yield of progeny. Since all crosses were done in CR63 on which psu_b^+ does not make a plaque, this observation suggested that the poor growth properties of psu_b^+ might be partially dominant. To investigate this dominance effect we measured the burst size of CR63 cells mixedly infected with psu_b^+ and wild type, as a function of the ratio of psu_b^+ to total input phage as shown in Figure 1. The burst size is almost directly proportional to the fraction of wild type phage in the input. One interpretation of these results is that the wild-type product of the gene corresponding to psu_b^+ is required, approximately stoichiometrically, for phage growth in CR63. However, since strains which carry deletions encompassing the entire gene grow like wild type on CR63 (Wilson, Kim & Abelson, 1972) we interpret the partial dominance of psu_b^+ to indicate that the altered product of the gene defined by psu_b^+ has a deleterious effect on phage growth in CR63. The basis for this effect remains unclear. One possibility is considered in the discussion.

Because of the poor growth of strains which carry psu_b^+ we have found this suppressor difficult to analyze. Consequently, its characterization is not as extensive as that of psu_a^+. However, exactly these peculiar growth properties have made it invaluable in the studies described in the following paper by Wilson & Abelson (1972).

(b) *Suppression pattern of* psu_a^+ *and* psu_b^+

The isolation of phage suppressors apparently identical to psu_a^+ and psu_b^+ from different multiply mutant strains suggests that psu_a^+ and psu_b^+ can suppress a number of *am* mutations in several different late genes. Because of clear differences in the quality of psu_a^+ suppression of *am* mutations in early and late genes, their suppression patterns are discussed separately.

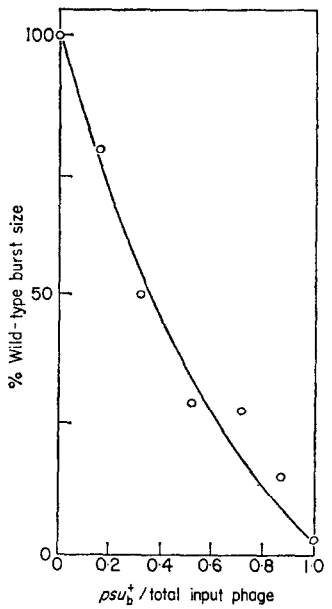

FIG. 1. Gene dosage effects of psu_b^+. CR63 bacteria were grown to 5×10^7 cells/ml. in H broth, collected by centrifugation, and resuspended at a concentration of 4×10^8 cells/ml. Phage at a total multiplicity of 13 to 15 were added in the ratios indicated in the presence of 0·004 M-KCN at 30°C. After 10 min anti-T4 serum was added to kill all unadsorbed phage. 15 min after infection the cells were diluted 10^4 into H broth at 30°C. Infective centers were assayed on S/6/5 at 30°C 5 min after dilution. Total progeny were assayed on CR63 65 min after dilution. The burst size for each was calculated as total progeny/infective center.

(i) psu_a^+ *and* psu_b^+ *suppression of nonsense mutations in late genes*

To determine the pattern of psu_a^+ suppression, we devised a method which would allow us to easily test a large number of mutations. It is described in detail below for mutations in gene *37*. First we constructed strains which carry psu_a^+ and a *ts* mutation in gene *37* (*ts*B78) as follows: the progeny of a cross of psu_a^+:X4E by the triple mutant *am*B25:*ts*B78:*rdf41* were plated on S/6/5 at 25°C. Since all the progeny must contain *am*B25 (X4E contains *am*B25), only those that also contain psu_a^+ will make plaques on S/6/5. By testing *r* plaques for temperature-sensitivity we isolated psu_a^+ strains which have the following genotype:

$$psu_a^+ : ts\text{B78} : rdf41 : am\text{B25} + \ldots$$

where + . . . indicates there may be present additional *am* mutations from X4E. *rdf41* was included for reasons not relevant to the experiment described and is an unselected marker in the crosses described in Table 3. To determine whether a particular mutation in gene *37* is suppressed by psu_a^+, we crossed it to the psu_a^+ strain just described and measured the percentage of the progeny which plated under conditions that are non-permissive for both parents (S/6/5 at 42°C). There are two types of ts^+ recombinants which may plate under these conditions: (1) those ts^+am^+ or ts^+op^+ recombinants which have arisen from a cross-over in the interval between *ts*B78 and the mutation being tested (Fig. 2(a)), and (2) those which arise in the interval between *ts*B78 and psu_a^+, if the test mutation is suppressed at 42°C (Fig. 2(b)).

The presence of other *am* mutations in the psu_a^+ strain tends to decrease the

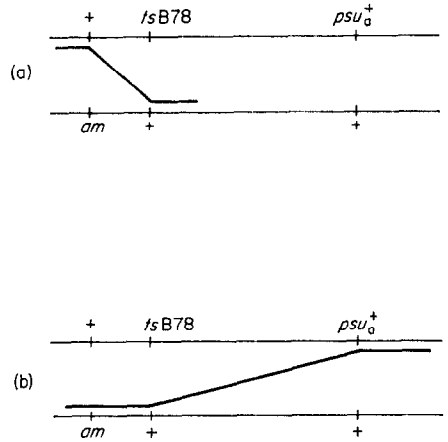

FIG. 2. Diagrammatic representation of a cross between psu_a^+:*ts*B78 and an *am* in gene *37*. (a) The heavy line indicates the interval in which cross-overs can generate ts^+am^+ recombinants. (b) The heavy line indicates the interval in which cross-overs can generate psu_a^+:ts^+:*am* recombinants.

observed recombination in the first interval, but does not affect recombination in the second interval. If the test mutation is not suppressed by psu_a^+, the percentage of progeny which plate under non-permissive conditions will be less than that expected for the control cross of the test mutation by *ts*B78 alone. If the test mutation is suppressed, the percentage of progeny which plate under non-permissive conditions will be greater in the test cross against the psu_a^+ strain than in the control cross against *ts*B78 alone. The difference in percentage recombination between the psu_a^+ strain and *ts*B78 is an indication of the degree of linkage between psu_a^+ and gene *37*.

The results of test crosses of the kind described for gene *37* are shown in Table 3. psu_a^+ suppressed all except one *am* mutation, but does not suppress any *op* mutation in gene *37*. Due to the high recombination in gene *34*, the test crosses with various gene *34 am* and *op* mutations are not so clear-cut as with gene *37* mutations and only the results with *am*A455 (closest to *ts*A44 of mutations tested) have been included in Table 3. The results for the crosses against *am*H26 in gene *e* suggest that psu_a^+ suppresses the *am* mutation and is closely linked to gene *e*. The linkage to gene *e* is verified in the mapping crosses described in section (d) below.

Since the only *oc* mutations in T4 late genes are in gene *e* and psu_a^+ is closely linked to gene *e*, we used an alternative method for testing psu_a^+ suppression of these mutations. We constructed a strain containing psu_a^+ and a deletion of gene *e*, eG223, which overlaps the sites of the nonsense mutants tested. In a cross of psu_a^+:eG223 by a gene *e* nonsense mutant only those mutations which can be suppressed by psu_a^+ will give rise to recombinant progeny capable of making a plaque on S/6/5 in the absence of added egg white lysozyme (Fig. 3). Eight nonsense mutants (*am*H26, *oc*eL1, *oc*eL2, *oc*eL3, *oc*eL4, *oc*eL5, *op*eL1P12 derived from *oc*eL1 and *op*eL4P41 derived from *oc*eL4) were tested. *am*H26 yielded about 3% viable recombinant progeny, while the others yielded no detectable recombinants (less than 0·1%). In an analogous manner we had hoped to define the suppressor capabilities of psu_b^+. However, we have not been able to isolate the double mutant psu_b^+:eG223, presumably because the combined growth limiting properties of the two mutations prevent the double mutant from making a plaque.

TABLE 3

psu$_a^+$ *suppression of nonsense mutations in late genes*

Gene	Mutant	% R in crosses against		Suppression of mutation at 42°C
		*ts*B78	*psu*$_a^+$:*ts*B78:*rdf41*:*am*B25 + ...	
37	*am*A481	†	32	+
	*am*NG182	†	42	+
	*am*N52	†	31	+
	*am*NG220	†	40	+
	*am*NG187	†	3·3	−
	*am*NG475	†	41	+
	*op*C23	†	2·4	−
	*op*C91	3·4	2·4	−
	*op*C121	†	3·9	−
		*ts*A44	*psu*$_a^+$:*ts*A44:*rdf41*:*am*N52 + ...	
34	*am*A455	6·0	36	+
		*ts*eC3	*psu*$_a^+$:*ts*eC3:*rdf41*:*am*B25 + ...	
e	*am*H26	2·9	7·5	+

Crosses are of the standard type. The total progeny were measured by plaque assay on CR63 at 25°C and recombinant progeny were titered by plaque assay on S/6/5 at 42°C. The percentage recombination (% R) is calculated as 200% × (recombinant progeny/total progeny).

† *ts*B78 is located near the middle of gene *37* and is expected to give less than 6% recombination with any of the mutants listed.

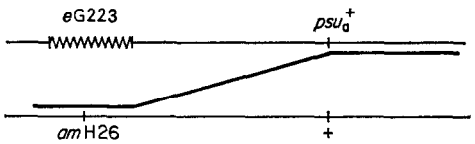

FIG. 3. Diagrammatic representation of a cross between *psu*$_a^+$:eG223 and *am*H26. The heavy line indicates the interval in which cross-overs can generate recombinant progeny which will make a plaque on S/6/5 bacteria in the absence of added egg white lysozyme.

To compare the pattern of *am* mutant suppression by *psu*$_a^+$ and *psu*$_b^+$ with that of bacterial suppressors, we isolated several *psu*$_a^+$:*am*, *psu*$_a^+$:*op*, and *psu*$_b^+$:*am* double mutants as described in the legend of Table 4. In Table 4 the growth of these double mutants on S/6/5 is compared with that of the nonsense mutant alone on the bacterial suppressors, *su-1*$^+$, *su-2*$^+$ and *su-3*$^+$. It should be noted that the occurrence of an *am* mutation which is temperature-sensitive on a particular suppressor host seems to be relatively rare. Of the 20 gene *34* and *37* *am* mutations tested only one, *am*NG187, is temperature-sensitive on *su-1*$^+$. To find another, *am*NG197, we screened 71 *am* mutations in genes *6*, *7*, *18* and *23*. The identity of the suppression patterns of *psu*$_a^+$ and *su-1*$^+$ indicates that *psu*$_a^+$ probably inserts serine in response to the *am* codon. The results with *psu*$_b^+$:*am* double mutants, though not compelling, suggest that *psu*$_b^+$ also inserts serine.

Taken together, the results on suppression of nonsense mutations in late genes suggest that *psu*$_a^+$ is a classical amber suppressor. The results do not define the range of mutations which *psu*$_b^+$ can suppress, but only extend the observation that *psu*$_b^+$ can suppress a variety of *am* mutations.

TABLE 4

Suppression patterns of bacterial and phage nonsense suppressors

Gene	Mutant	S26R1e *su-1*$^+$	S26R1d *su-2*$^+$	H12R8A *su-3*$^+$	psu_a^+ :mutant S/6/5 su^-	psu_b^+ :mutant S/6/5 su^-
37	*am*A481	+	+	+	+	+
	*am*NG182	+	+	+	+	+
	*am*N52	+	+	+	+	+
	*am*NG187	*ts*	0	+	*ts*	*ts*
	*am*NG475	+	+	0	+	
	*am*B280	+			+	
	*op*C121	0	0	0	0	
34	*am*H34	+	+	+	+	+
	*am*B25	+	+	+	+	+
	*am*B265	+	+	0	+	+
	*am*N58	+	+	0	+	
	*am*A459	+	+	0	+	+
	*am*B258	+	0	+	+	
	*am*K6	0	0	*ts*	0	
18	*am*NG197	*ts*	0	+	*ts*	

All the psu_a^+ double mutants except psu_a^+ :*am*NG197 were isolated from the crosses described in Table 3. With the exception of psu_a^+ :*am*NG187, psu_a^+ :*am*K6, and psu_a^+ : *op*C121 the doubles were isolated from the progeny plated on S/6/5 at 42°C. psu_a^+ :*am*NG187 was isolated from progeny plated on S/6/5 at 30°C. psu_a^+ :*am*K6 was isolated from progeny plated on H12R8A at 30°C. psu_a^+ :*op*C121 was isolated from progeny plated on CAJ70 at 42°C. psu_a^+ :*am*NG197 was isolated from the progeny of a cross of psu_a^+ by *am*NG197, which were plated on S/6/5 at 30°C. Those listed as double mutants yield only the indicated mutant, as judged by complementation, in the progeny of a cross to wild-type phage. The presence of psu_a^+ in psu_a^+ :*am*K6 and psu_a^+ :*op*C121 was verified by crossing them to *am*NG187 and testing for progeny which are temperature-sensitive on S/6/5.

psu_b^+ :*am* doubles were generated in crosses of psu_b^+ or psu_b^+ :*am*N133 by the respective *am*. The criteria for the double mutants are that they have the psu_b^+ plating phenotype and that the suppressor-negative revertants of them (see Results section (e)) contain only the *am* of interest. The psu_b^+ strain which contains *am*N52 also carries *am*N133.

ts indicates plaque formation at 30°C but not at 42°C. Plaque formation at both temperatures is designated as +. 0 means no plaques can be seen.

That both these suppressors appear to insert serine raises a question as to their origin. The original stocks of the multiple *am* mutants from which psu_a^+ and psu_b^+ were isolated were grown on CR63, which carries *su-1*$^+$ (Stretton & Brenner, 1965). Though there is no precedent for the incorporation of host DNA into the T4 genome, it is theoretically possible that either psu_a^+ or psu_b^+ or both were picked up from the host bacterium and are not alterations of phage genes. To eliminate this possibility, we isolated suppressor mutations with the plating characteristics of psu_a^+ and psu_b^+ from a stock of X74 phage which had been grown from a single plaque on H12R8A (*su-3*$^+$). The revertant with psu_a^+ plating characteristics, when crossed to *am*NG187 yielded progeny which were temperature-sensitive on S/6/5. Isolation of suppressors with properties like psu_a^+ and psu_b^+ from stocks grown on an *su-3*$^+$ host argues that psu_a^+ and psu_b^+ were not picked up from the host bacterium.

(ii) psu_a^+ *suppression of nonsense mutations in early genes*

psu_a^+ suppression of nonsense mutations in early genes was tested in crosses with

TABLE 5

psu$_a^+$ *suppression of nonsense mutations in early genes*

Gene	Mutant	% R in crosses against (1)	(2)	Difference in % R (2) − (1)	% R in psu_a^+ crosses (2) wild type	small	Size of smaller plaque	psu_a^+ suppression of mutation
		*ts*L66 (*42*)	psu_a^+ : *ts*L66 : *am*N52 + . . .					
56	*am*E114	14·7	22	+ 7·3	16·4	5·3	2	+†
41	*am*N81	13·9	36	+22	9·8	26	2	+
42	*am*N122	2·8	2·8	0			—	—
	*am*N55	2·1	1·7	− 0·4	1·5	0·2	1	+†
	*am*E117	0·96	7·1	+ 6·1	1·2	5·9	1	+†
	*am*NG352	3·7	31	+27	3·4	28	2	+
	*am*NG411	3·4	12·9	+ 9·5	2·5	10·4	1	+†
43	*am*B22	7·0	32	+25	2·8	29	2	+
	*am*NG305	7·2	31	+24	4·2	27	2	+
	*am*NG493	7·5	33	+26	4·9	28	3	+
	*am*NG562	8·7	43	+34	8·4	35	3	+
	*am*E4305	7·9	23	+15·1	5·0	18	2	+
		rdf41	psu_a^+ : *rdf41* : *am*N52 + . . .					
52	*am*E38	6·3	28	+22	4·3	24	1	+
	*am*E118	7·5	28	+20	3·7	24	1	+
	*am*E663	7·8	2·0	− 5·8			—	—
39	*am*N116	6·6	21	+14·4	3·1	17·8	1	+
	*am*C237	5·0	37	+32	2·7	34	1	+
	*am*E29	5·9	28	+22	3·5	25	1	+
	*am*E142	5·9	19·7	+13·8	2·6	17·1	2	+
rIIA	*am*HB118	‡	<0·1					—
	*am*HB122		<0·1					—
	*am*EM640		<0·1					—
	*am*HB35		<0·1					—
	*am*N11		<0·1					—
	*am*C204		<0·1					—
	*am*HB32		<0·1					—
	*oc*N21		<0·1					—
	*op*X665		<0·1					—
rIIB	*am*HB232	‡	15·6	+15·6	0	15·6	1	+
	*am*EM84		4·2	+4·2	0	4·2	1	+

	*am*NT332		7·3	+ 7·3	0	7·3	1	+
	*oc*UV375		<0·1					−
	*op*X655		<0·1					−
		*ts*CB53 (*45*)	psu_a^+:*ts*CB53:*am*N52+. . .					
44	*am*N82	2·4	3·6	+ 1·2			−	−
45	*am*NG18	1·2	1·0	− 0·2			−	−
46	*am*B3	2·8	24	+21			2	+
	*am*N130	8·0	31	+23			3	+
	*am*NG209	3·6	31	+27			2	+
	*am*NG371	4·9	27	+22			2	+
	*am*NG454	4·7	27	+22			2	+
47	*am*A456	12·1	10·4	− 1·7			−	−
	*am*NG106	6·3	34	+28			2	+
	*am*NG163	8·3	32	+24	6·2	26	2	+
	*am*NG277	10·2	26	+15·8	3·6	22	2	+
	*am*NG583	7·1	6·9	− 0·2			−	−
		*ts*CB106 (*31*)	psu_a^+:*ts*CB106:*am*B25+. . .					
55	*am*B1292	16·3	26	+ 9·7			2	+
	*am*NG372	13·0	28	+15			2	+
30	*am*H39	16·3	18·6	+ 2·3			2	(±)
	*am*C104	16·1	5·6	−10·5			−	−
	*am*NG83	9·7	15·7	+ 6			3	+
31	*am*N54	1·3	9·2	+ 7·9	0·45	8·8	2	+
	*am*S54	0·9	8·5	+ 7·6	0·77	7·7	1	+
	*am*NG71	2·9	35	+32			−	+
		*ts*P7 (*32*)	psu_a^+:*rdf41*:*ts*P7:*am*N52+. . .					
32	*am*E315	1·9	18·7	+16·6			1	+
	*am*NG461	1·5	18	+16·5			1	+

Crosses are of the standard type. The percentage recombination (% R) is determined as in the legend to Table 3. Crosses involving genes *52*, *39* and *rII* were plaque assayed on CR63 at 30°C for total progeny and on G(λ) at 30°C for recombinant progeny. Two additional % R's are calculated for crosses against psu_a^+ strains which yield two easily distinguishable plaque types among the recombinant progeny. The larger plaques are assumed to be wild type and the % R is tabulated under that heading. The % R for the smaller class of plaques is listed under small. The size of the smaller class of plaques when the progeny are plated on S26 (su^-) relative to the *am* mutant alone on S26R1e($su\text{-}1^+$) is denoted by a number from 1 to 3. 3 indicates the plaques are approximately the same size. 1 and 2 denote two arbitrary classes of intermediate sized plaques. − indicates that a class of smaller plaques is not detectable. All test *am* mutants are suppressed in bacterial hosts which carry $su\text{-}1^+$. psu_a^+ suppression or non-suppression of the test mutant is designated by + and − respectively.

† Suppression of these mutations was determined directly by isolation of the double mutant (see text).

‡ The *rdf41* crosses for the *rII* test mutants were not done since *rdf41* encompasses the entire *rIIA* and *rIIB* cistrons.

strains carrying psu_a^+ and a *ts* mutation as described above for late genes (Table 3), and the results are shown in Table 5.

Since psu_a^+ is unlinked to all the genes tested (see section (d) below) we were surprised to find such generally low recombination values in these crosses. As shown in Table 3, analogous crosses involving *am* mutations in unlinked late genes yield a much higher percentage recombination. The reason for these reduced recombination values in crosses against *am* mutations in early genes is not clear.

In most of the crosses between *am* mutants and strains carrying psu_a^+ there were two plaque types among the recombinant progeny. From the relative frequency of the large (wild-type) and small plaques we anticipated that the small plaques represented phage carrying psu_a^+ and the test *am* mutation. To test this idea we picked small plaques from among the recombinant progeny of crosses involving *am*E114, *am*N55, *am*E117 and *am*NG411. Each of these small plaques, when crossed to wild-type phage, yielded the test *am* as anticipated. Thus, in addition to the criterion of an increase in recombination frequency as described in Table 3, we have used the criterion of a smaller plaque type among the recombinant progeny to decide whether a particular mutation is suppressed by psu_a^+. Using these two criteria, we conclude that most *am* mutations in early genes are suppressed by psu_a^+. However, psu_a^+ suppression of *am* mutations in early genes is qualitatively different from its suppression of *am* mutations in late genes, since strains which carry psu_a^+ and an *am* mutation in a late gene make plaques on S26 (su^-) which are indistinguishable from the *am* mutant alone on S26R1e.

One striking result is the lack of suppression of all seven *am* mutations tested in the *rIIA* cistron. psu_a^+ shows suppression of at least one *am* mutation in all other early genes for which more than one *am* mutation has been tested. The meaning of this result is unclear.

(c) *Efficiency of* psu_a^+ *and* psu_b^+ *suppression*

(i) *Burst size of* psu_a^+:am *double mutants*

We had assumed from the reduced plaque size (and reduced number of phage in the plaque) of strains which carry psu_a^+ and an *am* mutation in an early gene that psu_a^+ suppressed these mutations very poorly. To get a rough measure of the efficiency of psu_a^+ suppression and an idea of the relationship of plaque size to degree of suppression, we compared the burst sizes of various psu_a^+:*am* double mutants on S26 (su^-) with that of the *am* alone on S26R1e ($su\text{-}1^+$). The results are shown in Table 6. To our surprise the burst sizes on S26 of the psu_a^+:*am* double mutants defective in early or late genes are indistinguishable from, and equal to about 60% of the burst of wild-type phage. The reason for the small plaque phenotype of psu_a^+:*am* doubles in early genes is not clear, but may simply reflect the difference in physiological state of bacteria in liquid culture and in agar.

(ii) *Gene product synthesis in* psu_a^+:am *and* psu_b^+:am *double mutants*

The above results suggest that psu_a^+ suppresses *am* mutations with reasonable efficiency. The poor growth characteristics of the single mutant psu_b^+ preclude any such simple assessment of its efficiency. To determine accurately the efficiency of suppression of psu_a^+ and psu_b^+ we measured the amount of gene *34* or *37* product (P34 or P37) produced in doubly mutant strains which carried an *am* mutation in one of those genes as well as psu_a^+ or psu_b^+. The bands corresponding to P34 and P37 have

TABLE 6

Burst sizes of psu_a^+ : am *double mutants*

Gene	Mutant	% wild-type burst size: Single mutant S26R1e(*su-1*$^+$)	% wild-type burst size: psu_a^+ : *am* double mutant S26(*su*$^-$)
56	*am*E114	119	55
42	*am*N55	98	55
	*am*E117	90	49
	*am*NG411	95	51
31	*am*NG71	116	95
	average (early genes)	104	61
34	*am*H34	110	71
	*am*B25	109	73
	*am*B265	131	44
	*am*A459	125	78
37	*am*A481	100	62
	*am*NG182	115	48
	*am*N52	126	55
	*am*NG187		60
	average (late genes)	117	61
	psu_a^+	117	101

S26 and S26R1e bacteria were grown to 5×10^7 cells/ml. in H broth, collected by centrifugation, and resuspended at 4×10^8 cells/ml.

Phage at a multiplicity of 0·3 were added to the cells in the presence of 0·004 M-KCN at 30°C. After 5 min anti-T4 serum was added to kill all unadsorbed phage. 10 min after infection the cells were diluted 10^4 into H broth at 30°C. Infective centers were assayed on CR63 5 min after dilution. Total progeny were assayed on CR63 60 min after dilution. The burst size for each was calculated as total progeny/infective center.

been identified on sodium dodecyl sulfate polyacrylamide gels (Laemmli, 1970; Ward & Dickson, 1971). P7 has been identified as the band located between P34 and P37 (King & Laemmli, manuscript in preparation). As a measure of the absolute amount of these gene products present in individual ^{14}C-labeled lysates, we used P7 as an internal standard and normalized the amount of P34 or P37 to the amount of P7 on the same gel. The percentage of P34 or P37 present in the suppressor strain, as compared with wild type, is taken as the efficiency of suppression. The results are shown in Table 7, and typical densitometer tracings from which the data were derived are shown in Figure 4. Both suppressors show an efficiency of about 45%.

(d) *Map positions of* psu_a^+ *and* psu_b^+

The experiments on suppression of a gene *e am* mutation (shown in Table 3) suggested that psu_a^+ is closely linked to gene *e*. To determine its map position more accurately, we constructed the strain psu_a^+ : *ts*eC3 : *am*N52 and crossed it to *r*48 : *am*N52. Since both parental strains contain *am*N52, only those progeny which have undergone a cross-over, as indicated in Figure 5 and carry psu_a^+ but not *ts*eC3, will plate on S/6/5 at 42°C. The ratio of *r*48 to r^+ plaques among these recombinant progeny is 2·8, indicating that psu_a^+ and *r*48 are on opposite sides of *ts*eC3. If the progeny of this cross are assayed at 25°C on S/6/5 so that *ts*eC3 is an unselected marker, the titer of *r*48

TABLE 7

Efficiency of psu_a^+ *and* psu_b^+ *suppression*

Gene	Strain	% wild type P34	% wild type P37
34	psu_a^+:*am*H34	45	
	psu_a^+:*am*B25	42	
	psu_a^+:*am*B265	52	
	psu_b^+:*am*B265	34	
37	psu_a^+:*am*A481		48
	psu_a^+:*am*NG182		52
	psu_a^+:*am*N52		42
	psu_a^+:*op*C121		0
	psu_b^+:*am*A481		56

Preparation of lysates, electrophoresis, autoradiography and analysis of the densitometer tracings of the autoradiographs are described in Materials and Methods. All values for P34 or P37 are normalized to the amount of P7 present in the same gel. The values shown represent a single determination for psu_b^+ strains and an average of two determinations for the psu_a^+ strains.

plaques becomes a measure of the distance from psu_a^+ to *r*48. The results of this and other crosses between markers in the same region are summarized in Figure 6.

The inability of psu_b^+ phage to form plaques on CR63 makes this suppressor much simpler to map. Recombinant progeny from a cross between psu_b^+ and a *ts* mutant can be assayed by plating on CR63 at 42°C. psu_b^+ was mapped with respect to *ts* mutations in genes *e*, *31*, *42* and *45* as shown in Table 8.

The results suggest that psu_b^+ also is linked to gene *e*. Control crosses between *am*A455 in gene *34* and each of the *ts* mutants show an average of 35% recombination. The low recombination in psu_b^+ crosses may result from reduced burst size due to the partial dominance of the psu_b^+ growth properties.

That psu_b^+ is linked to gene *e* is confirmed by the cross of psu_b^+ to psu_a^+:*am*N52 (Table 8). *am*N52 single mutants should appear in the progeny of this cross at a frequency dependent on the distance between psu_a^+ and psu_b^+. Since no suppressor-negative recombinants were found, psu_b^+ must map very near psu_a^+, and is therefore linked to gene *e*.

(e) *Are* psu_a^+ *and* psu_b^+ *in the same gene?*

Several observations suggest that psu_a^+ and psu_b^+ may result from different mutations within the same gene. As already described, they are located within a few tenths of a map unit of each other and both appear to insert serine in response to the *am* codon. A third piece of suggestive evidence comes from an analysis of the pseudo-wild type revertants of psu_b^+. When psu_b^+:X74 is plated at high concentration on CR63 two distinctive plaque types appear. At a frequency of about 10^{-3} of the psu_b^+:X74 titer is a class of small heterogeneous plaques, which when picked and replated make normal plaques on CR63 and no plaques on S/6/5. The basis for the change in the nature of the plaques upon replating is not known. These phage are suppressor-negative revertants to the *am* phenotype and are characterized in detail by Wilson & Abelson (1972). At a frequency of about 10^{-4} is a class of normal plaques. Nine independent plaques of this type have been picked, characterized and shown to have properties in common with psu_a^+. They make normal plaques on both CR63 and S/6/5,

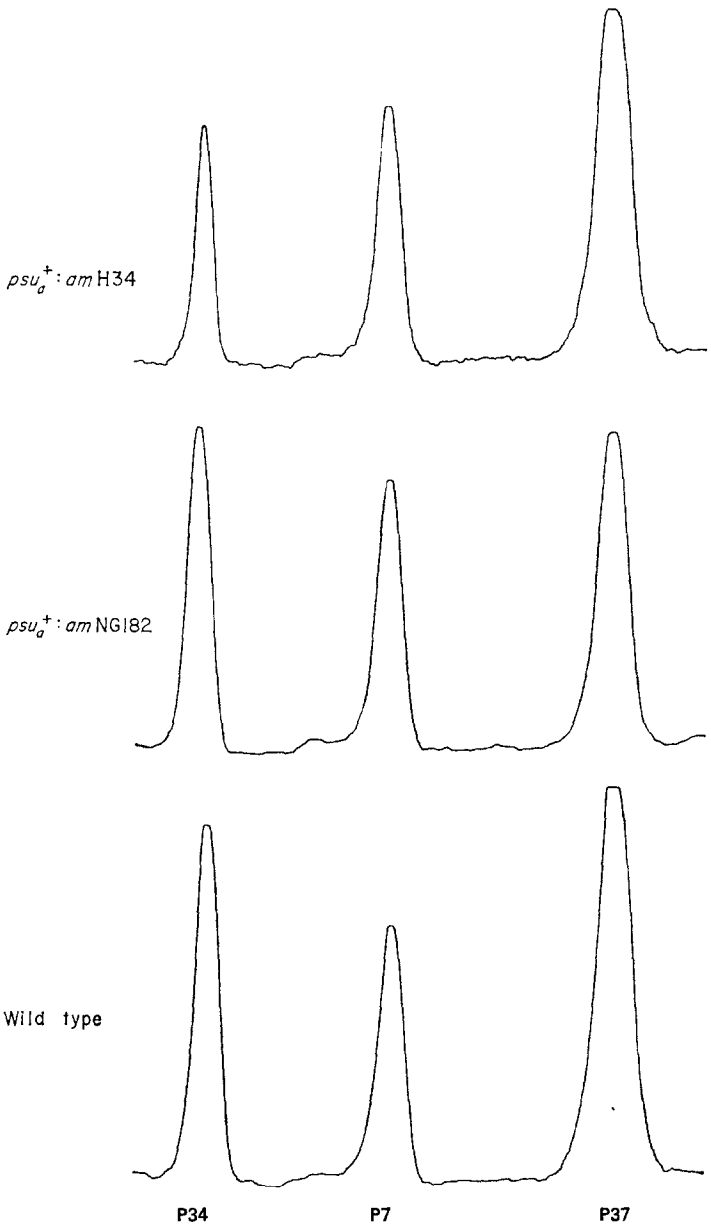

FIG. 4. Densitometer tracings of autoradiographs.

Preparation of lysates, electrophoresis and autoradiography are as described in Materials and Methods. The 2-cm of the autoradiograph containing the bands which correspond to P34, P7 and P37 were traced with a Joyce/Loebl densitometer at an expansion ratio of 20:1. Typical tracings are shown.

but when crossed to wild-type phage yield the *am* mutations contained in X74. One of the nine has been crossed to *am*NG187. The progeny of this cross contain phage which are temperature-sensitive on S/6/5, suggesting that these pseudo-wild type phage insert serine at the site of the *am* codon. The high frequency of appearance of these pseudo-wild type phage suggests they are a single step change from psu_b^+. To deter-

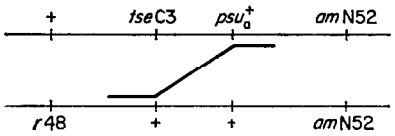

FIG. 5. Diagrammatic representation of a cross between psu_a^+:*tse*C3:*am*N52 and *r*48:*am*N52. The heavy line indicates the interval in which cross-overs can generate recombinant progeny which will make a plaque on S/6/5 bacteria at 42°C.

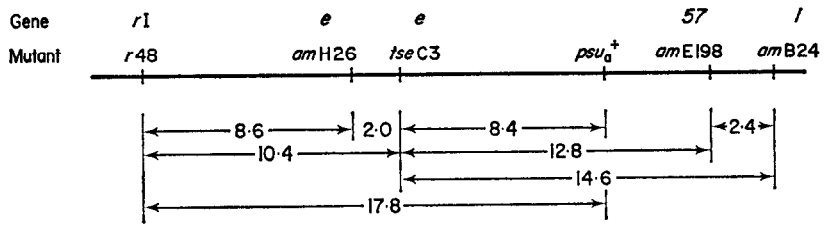

FIG. 6. Map position of psu_a^+.

Crosses are of the standard type. The crosses involving psu_a^+ are described in the text. Total progeny for all crosses were plaque assayed on CR63 at 25°C. Except as noted in the text recombinant progeny were plaque assayed on S/6/5 at 42°C. The numbers below the map are %R and were calculated as 200% × (recombinant progeny/total progeny). Recombinant progeny from crosses involving *r*48 were pre-adsorbed on S/6/5 at room temperature for 5 min before plating to insure uniform plaque morphology. A three-factor cross between *r*48:*am*H26 and *tse*C3 shows a ratio of *r*48 to r^+ in the recombinant progeny of 0·5, indicating that *tse*C3 maps to the right of *am*H26. The strain psu_a^+:*tse*C3:*am*N52, used to position psu_a^+, was isolated as a temperature-sensitive plaque from the progeny of the cross psu_a^+:*am*N52 by *tse*C3:*am*N52 plated at 25°C on S/6/5. The map is drawn so that left to right corresponds to the clockwise direction on the circular T4 map as it is customarily represented (Edgar, 1968). The order shown for genes *1* and *57* is currently accepted (Edgar, 1968; S. K. Beckendorf, R. D. K. Josslin & R. L. Russell, personal communications).

mine the distance from psu_b^+ to the site of the change, we crossed each of the pseudo-wild type phage to wild-type phage and screened more than 53,000 total progeny for the psu_b^+ plating phenotype. Three of the progeny made small plaques on S/6/5 and no plaques on CR63. These plaques have not been characterized further to determine whether they are psu_b^+. Nevertheless this result indicates a maximum recombination frequency between psu_b^+ and the second site of 0·01%. If the pseudo-wild type derivatives of psu_b^+ are in fact psu_a^+, then these results suggest that both suppressors do affect the same gene.

4. Discussion

We have isolated and characterized two phage-coded nonsense suppressors, psu_a^+ and psu_b^+. Both suppressors seem to insert serine in response to the *am* codon and map no more than a few tenths of a map unit apart. On the basis of their initial frequencies of appearance (10^{-7} to 10^{-10} for psu_b^+ and 10^{-11} to 10^{-12} for psu_a^+) and the frequency of psu_b^+ reversion to what seems to be psu_a^+ (10^{-4}), we speculate that (1) psu_a^+ is an amber suppressor with the anticodon -CUA-, which is derived by two mutations from a serine tRNA with the anticodon -UGA-, and (2) psu_b^+ is a one-base-change intermediate, an ochre suppressor with the anticodon -UUA-. This interpretation of the relationship of psu_a^+ and psu_b^+ is consistent with all the observations we have made and in addition suggests a reason for the partial dominance of the poor growth of psu_b^+.

TABLE 8

Mapping data for psu_b^+

Strain	% R in crosses against psu_b^+
*ts*eC3 (*e*)	7·2
*ts*CB106 (*31*)	17·3
*ts*L66 (*42*)	25
*ts*CB53 (*45*)	19·1
psu_a^+ : *am*N52	<0·3

Crosses are of the standard type. Total progeny from the crosses of the temperature-sensitive mutants and psu_b^+ were measured by plaque assay on S/6/5 at 25°C. Recombinant progeny were titered by plaque assay on CR63 at 42°C. % R is calculated as 200% × (recombinant progeny/total progeny). The total progeny from the cross of psu_b^+ by psu_a^+ : *am*N52 were measured by plaque assay on CR63. None of 664 plaques tested were *am* in phenotype. Since only about one-half the suppressor-negative recombinants would be expected to contain *am*N52 and only one of the two parents plate on CR63, the % R is calculated as <0·3.

Bacteria which carry an ochre suppressor generally grow very poorly. One explanation for this, as yet unproven, is that the ochre codon is normally used in the chain termination signal for protein synthesis and that the ochre suppressor tRNA by reading that codon interferes with normal termination, causing the poor growth phenotype (Lu & Rich, 1971). By analogy we might expect that a phage-coded ochre suppressor would lead to a reduced phage burst size. We might also expect that the amount of the reduction would depend upon the concentration of the ochre suppressor within the cell. As shown in Figure 1, psu_b^+ meets both expectations.

A relationship analogous to that predicted for psu_a^+ and psu_b^+ in T4 has been demonstrated for two nonsense suppressors carried by another bacteriophage, ϕ80. Plaque-forming derivatives of the temperate bacteriophage ϕ80 have been isolated which carry a bacterial tyrosine tRNA gene (Russell *et al.*, 1970). The presence of the bacterial gene as the wild type or as the amber suppressor su_{III}, does not affect the growth properties of the phage. However, if the bacterial gene is present as the ochre suppressor, phage growth is considerably reduced (Altman, Brenner & Smith, 1971). The amber- and ochre-suppressing tyrosine tRNA's were shown by nucleotide sequence analysis to differ from the wild-type tRNA, respectively, by a one and a two base change in the anticodon (Altman *et al.*, 1971; Goodman, Abelson, Landy, Brenner & Smith, 1968).

McClain (1970) has isolated and characterized another T4-coded nonsense suppressor psu_1^+. psu_1^+ and psu_a^+ appear to be identical (J. Wilson, unpublished experiments). McClain has demonstrated that psu_1^+ is an amber suppressor which inserts serine in response to the *am* codon with an efficiency of about 50% and is located about eight map units from gene *e*. The nucleotide sequences of the suppressor and wild-type tRNA's have been determined (W. H. McClain & B. G. Barrell, personal communication). The suppressor tRNA has the anticodon -CUA- and was derived from the wild type by two base changes in the anticodon. As is shown by Wilson & Abelson (1972), the mutation which results in psu_b^+ activity maps at or near the anticodon as defined by psu_1^+.

Though the circumstantial evidence is quite strong that psu_b^+ is a one-base-change intermediate between the wild type and the amber suppressor, we have been unable

to demonstrate the expected ochre suppressor phenotype. Consequently, the relationship between psu_a^+ and psu_b^+ remains unclear, and its final elucidation may require nucleotide sequence analysis of the psu_b^+ suppressor tRNA.

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Note added in proof: In support of the proposed relationship of psu_a^+ and psu_b^+ is the observation made by Christine Guthrie & William H. McClain (personal communication) that an ochre-suppressing derivative of psu_1^+ has plating characteristics like those of psu_b^+.