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The Effects of *supB* and *supE* on Thy- Suppression in *Escherichia coli*

Annie Woo

A Thesis

in

The Department

of

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ABSTRACT

The Effects of *supB* and *supE* on Thy- Suppression in *Escherichia coli*

Annie Woo

In this study, I examined the possible involvement of transfer RNA in nonsense and frameshift suppression by thymine requiring strains of *Escherichia coli*. Strain N4316 is auxotrophic for thymidine at 37 C, due to a temperature sensitive mutation in the *thyA* gene. This strain suppresses nonsense and frameshift mutants of T4 phage at 37 C.

Strains were constructed in which glutamine suppressor tRNAs (supB and supE) were combined with the *thyA* ts mutation to determine if they affect nonsense and frameshift suppression. Examination of suppression patterns may thus provide some information as to how suppression is related to the *thyA* mutation.

It was found that Thy- suppression does occur at the translational level. The presence of a suppressor tRNA (supB/ supE) effectively eliminated suppression of UGA and reduced suppression of UAA and frameshift mutants. These results suggest a competition effect between suppressor tRNAs and wild type or noncognate tRNAs (cysteinyl or tryptophanyl tRNA) for the UGA site. Competition may occur as a result of a conformational change in suppressor tRNAs.

Upon binding however, suppressor tRNAs do not appear to translate the UGA codon.

It was also found that the genetic background of the strain plays an important role in determining whether suppression will occur or not.

The base composition of tRNAs from the suppressing strain N4316 and a related strain D10 was analyzed to see if there were any differences in amounts and types of modified bases which may provide evidence to support a previously proposed model that suppression may be due to differentially modified tRNAs. Results reveal no major differences although there may be slightly decreased amounts of dihydrouridine in tRNAs of strain N4316.

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Dedicated

to

My Parents

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INTRODUCTION

Mutations which affect the function of a protein can be missense, nonsense, frameshift, deletions or insertions. A missense mutation is usually due to a single base change which results in a different amino acid in the protein. The function of the protein may thus be altered. Nonsense mutations occur when a sense codon which codes for an amino acid mutates to a nonsense codon which codes for a translational stop. This results in premature termination and thus a shortened inactive protein. There are three nonsense codons: UAG (amber), UAA (ochre) and UGA (opal). Frameshift mutations occur when there is an addition or a deletion of either one or two bases. Since the genetic code is read in triplets, frameshift mutations give rise to garbled proteins with amino acid sequences which are nonfunctional. The protein may be either longer or shorter than the wild type, depending on where a nonsense codon occurs in the new reading frame, but frequently a termination codon is uncovered in the new reading frame after about twenty one amino acids (Kurland, 1979).

The wild type phenotype of mutants can be restored in two ways: either by true reversion in which the original wild

type is restored, or by pseudoreversion in which the mutation is still present, but a secondary mutation has occurred. This secondary mutation is a suppressor.

Suppressors can be further divided into two categories; intragenic and intergenic (Hartman and Roth, 1973). An intragenic suppressor mutation affects the same gene as the initial lesion. For example, a mutation resulting in an amino acid substitution in a protein could be suppressed if a second event caused another amino acid substitution which compensates for the first. The final gene product, which differs by two amino acids from the original wild type protein, has a wild type or pseudo wild type conformation and function. A frameshift mutation can be suppressed by the addition or deletion of one or two base pairs which shifts the reading frame back to the wild type.

An intergenic suppressor is located in a gene other than that where the initial lesion is located. Intergenic suppression can be functional or informational (Hawthorne and Leupold, 1974). A functional suppressor bypasses the original mutation without restoring the gene function. For example, if the primary mutation resulted in a less active enzyme, a secondary mutation affecting another biosynthetic pathway could make this pathway more active and this could now compensate for the less active enzyme which resulted from the initial mutation. Such indirect suppressors usually suppress mutations affecting only a single gene or a group

of related genes.

A direct or informational suppressor (Hawthorne and Leupold, 1974) functions at the translational level correcting the mutation by substituting a functional amino acid for a nonfunctional one, by inserting an amino acid in response to a premature termination codon, or by correcting the reading frame. Direct suppressors are allele specific, but can act on a variety of genes.

Informational suppression may also be caused by "altered" ribosomes, but this is one of the least well characterized possibilities. Gorini (1970) has shown with streptomycin-resistant strains of Escherichia coli that ribosomal proteins play a role in translational fidelity. A mutation affecting ribosome protein S12 confers both streptomycin resistance and stricter misreading control thus increasing translational fidelity. More recent findings in yeast indicate that mutations affecting mitochondrial 15S ribosomal RNAs suppress ochre and frame shift mutations and those affecting the 23S mitochondrial rRNA suppress +1 frameshift and missense mutations (Gorini, 1970).

Many informational suppressor mutations affect tRNA genes. Most of the well characterized suppressor tRNAs have a base change in the anticodon region so that they read nonsense or missense codons. Changes in regions other than the anticodon and in tRNA processing have also been shown to confer suppressor ability (Hirsch, 1971; Smith, 1979).

There are seventeen mapped suppressor tRNA genes in E. coli (Bachmann, 1983). These tRNA suppressors are listed in Table 1. Of these 17 tRNA suppressors, 6 require further investigation but the majority have a base change in the anticodon loop of the tRNA.

Two genes in E. coli which mutate to yield nonsense suppressors are supB and supE (Brenner and Beckwith, 1965; Inokuchi et al ., 1979). The supB locus which maps at 16 minutes on the E. coli genome codes for a glutamine tRNA. The mutant tRNA inserts glutamine when it encounters a UAA or UAG nonsense codon. The supE (alias su+2) locus also maps at 16 minutes and codes for another glutamine tRNA but the mutant tRNA only recognizes UAG nonsense codons (Bachmann, 1983; Ozeki et al ., 1980).

Although the primary change in many of the transfer RNA suppressor mutants is a change in the anticodon region, this change may affect the properties of the transfer RNA in unexpected ways. Studies done by Murgola et al . (1984) on glycine tRNAs show that a base substitution in an anticodon may affect several properties of the tRNA. In some cases there was a loss of a specific base modification in the immediate area of the base substitution. In others, wobble pairing involves the first or second position of the codon. In others, aminoacylation was decreased or misacylation was increased.

Some frameshift tRNA suppressors have been characterized

Table 1: List of Mapped Transfer RNA Suppressors in *E. coli* K-12

tRNA suppressor	Nonsense codon recognized	Map Location	Amino acid substitution	Location of sequence change
sup B	UAA / UAG	16	glutamine	anticodon
sup C (Su+4)	UAA / UAG	27	tyrosine	anticodon
sup D (Su+1)	UAG	43	serine	nd
sup E (Su+2)	UAG	16	glutamine	anticodon
sup F (Su+3)	UAG	27	tyrosine	anticodon
sup G (Su+5)	UAA / UAG	17	nd	nd
sup H	nd	43	nd	nd
sup L (Su+beta)	UAA / UAG	17	lysine	anticodon
sup M	UAA / UAG	90	tyrosine	nd
sup N	UAA / UAG	52	nd	nd
sup O (Sup C?)	UAA / UAG	27	nd	nd
sup P (Su+6)	UAG	97	leucine	nd
sup Q	nd	13	nd	nd
sup T	nd	62	glycine	nd
sup U (Su+7)	UAG	85	tryptophan	anticodon
sup V (Su+8)	UAA / UAG	85	tryptophan	anticodon
Su+9	UGA	83	tryptophan	D-stem

nd= not determined
Ref: Bachmann, 1983; Glass, 1982

and shown to have an extra nucleotide in the anticodon loop. This extra nucleotide could correct a frameshift mutation by allowing four base reading, or by preventing normal translocation. An example of such a suppressor is the sufD suppressor in Salmonella typhimurium (Riddle and Carbon, 1973). The anticodon in the mutated glycyl suppressor tRNA has four C residues instead of the three C residues as in the wild type tRNA. The mechanism of suppression postulated is quadruplet base pairing with the mRNA (Riddle and Carbon, 1973). Alternatively, the extra nucleotide might occupy a larger space on the messenger RNA acting as a spacer so that one base in the message does not act as part of a codon (Atkins, 1980; Yourno and Tanemura, 1970; Riddle and Carbon, 1973; Kurland, 1979).

Suppressor transfer RNAs may result from changes in other regions of the tRNA besides the anticodon loop. In 1971, Hirsch discovered that a tryptophan suppressor tRNA (Su +9) can suppress UGA mutants. This tRNA had the same anticodon loop as the wild type tRNA, but a G residue in the D stem of the tRNA was replaced by an A residue. This was the first evidence that an alteration elsewhere in the tRNA molecule can influence the reading of a triplet code, possibly through a change in conformation.

The suppression phenotype may occur as a result of a lack of modification in a tRNA. SupK mutants of S. typhimurium which suppress UGA as well as frameshift mutations (Atkins

and Ryce, 1974) lack a tRNA methylase enzyme.

In this thesis, I study the E.coli suppressor strain N4316. This strain was isolated by Phillips et al. 1969(b), while looking for ribosomal mutants. The D1Ø Thy- parent strain was subjected to nitrosoguanosine mutagenesis and mutants (starvation temperature sensitive or sts) that were unable to recover from starvation at 43 C were isolated. They discovered that strain N4316 was able to suppress UAA and UGA nonsense mutations in the lysozyme gene of bacteriophage T4, while its grandparent strain, D1Ø, did not suppress these mutants. They did not however, test suppression on the D1Ø Thy- parent strain. Strain N4316 was later shown to suppress UAG and frameshift mutants both in the lysozyme gene and other genes of T4 phage (Cheung and Herrington, 1982; Herrington, Kohli and Faraci, 1986). Suppression by strain N4316 is temperature dependent. Suppression was observed only at 37 C and not at 31 C. The thymine auxotrophy of strain N4316 is also temperature dependent; it requires thymine at 43 C and 37 C but does not require thymine at 31 C. In addition, suppression is inhibited upon supplementation of high levels of thymine or thymidine in the culture medium. This suggested that suppression was probably due to low intracellular levels of thymidylate. When strain N4316 was converted to a non thymidine requiring strain, either by reversion or by recombination, the suppression phenotype was no longer

observed (Cheung and Herrington, 1982; Herrington, Kohli and Lapchak, 1984). These observations indicate that the suppression by N4316 results from the thyA mutation present in its parent, the D10 thy- strain, rather than the sts mutation. Further studies reveal that all Thy- strains of E.coli have similar suppressor activity (Herrington, Kohli and Faraci, 1986; M.B. Herrington and M. Faraci, unpublished). The suppression by strain N4316 is allele specific rather than gene specific. That is, it suppresses certain types of mutations in a variety of genes. It was concluded the suppressor activity of N4316 is likely to be an informational suppressor since it suppresses many T4 mutants (Cheung and Herrington, 1982). When N4316 was made streptomycin resistant, suppression of nonsense codons was greatly reduced although frameshift suppression was not affected (Herrington, Kohli and Lapchak, 1984; Herrington, Kohli and Faraci, 1986), suggesting that suppression occurs at the level of translation.

No other suppressors have been described that suppress frameshift mutations and all three nonsense mutations. Thus, N4316 is of interest due to its unusual suppressor ability. Also the temperature dependency of the suppression by strain N4316 is unusual. Of the known temperature sensitive suppressor strains, suppression usually occurs at the lower temperature of 31 C and not at 37 C or higher. The temperature sensitive suppressors su+1 and su+4, (Table 1)

(Oeschger and Berlyn, 1973; Nagata and Horuichi, 1973; Gallucci, Pacchetti and Zangrossi, 1970), have been demonstrated to have suppressor ability at 31 C with decreasing efficiency of suppression with increasing temperatures up to 42 C. At 42 C, there was no detectable suppressor activity.

There are three models which have been proposed to account for the suppression demonstrated by strain N4316. The first model was proposed by Phillips, Schlessinger and Apirion, 1969(a)). This model suggested that N4316 suppresses UGA and UAA nonsense mutations when the ribosome pauses at the nonsense codons because it is unable to terminate properly. This pausing would allow non cognate tRNA to misread the nonsense codon. This model was based on in vitro protein synthesis studies which showed that in extracts of strain N4316 incubated at high temperatures, polypeptides accumulated on ribosomes rather than being released. A protein factor was identified from wild type cells that could restore termination. The Z or rescue factor restored protein synthesis and termination at 43 C (Ganoza et al., 1973; Philips, Schlessinger and Apirion, 1969(a)). It was not determined whether the in vitro termination defect was due to the thyA mutation or to the sts (starvation temperature sensitive) mutation in strain N4316. However, we now know that the suppression by the strain N4316, is a result of the thy A mutation which this model does not

take into account.

The second model is based upon the discovery by Belfort et al. (1983) that there may be a gene which overlaps thyA. A translational stop, UGA, overlaps the ribosome binding site of the thyA gene and is preceded by an open reading frame which extends upstream to the 5' end of the published sequence. The sequence analysis also reveals a stretch of GC rich direct and inverted repeats followed by poly T tracts located within the thyA structural gene. This sequence resembles a transcriptional stop. These observations suggest that a 3' end of an unknown gene overlaps the 5' end of the thyA gene. Belfort and Pedersen- Lane (1984) have mapped the thyA (ts) mutation of the strain N4316. It is located in the region of the gene which contains the putative transcriptional terminator for the overlapping gene. Based on these findings, they proposed that the suppression might result from an effect of the thyA mutation on the overlapping gene. Unfortunately, the function of this overlapping gene product remains unknown and it is thus difficult to formulate a detailed model which relates the overlapping gene with suppression. More recently, M.B.Herrington, M. Faraci and C. Autexier, (unpublished results) have shown that all thyA alleles including those which are not located within the region of overlap, confer a suppression phenotype. However, it is still possible that any thyA mutation affects the

overlapping gene or its expression.

The third model was proposed by Cheung and Herrington, 1982, and states that suppression by N4316 is an indirect result of altered tetrahydrofolate pools in the cell caused by the thyA ts mutation. Thymidylate synthase (TS) is the main user of 5, 10- methylenetetrahydrofolate (THF). The reaction catalyzed by thymidylate synthase is the only reaction in the cell in which THF is oxidized to dihydrofolate (DHF) (O'Donovan, and Neuhard, 1970). The DHF is then converted to THF by dihydrofolate reductase. The lack of TS activity in Thy- strains would interrupt the constant cycling of THF and this might lead to an alteration of THF pools. If any tRNA modifying enzymes use THF as the cofactor, several species of tRNA could then have altered modification. These altered tRNAs would then be able to recognize the nonsense and frameshift mutants thus accounting for the observed suppression patterns.

The latter model is not exclusive of the other two models. If ribosome pausing occurs, then there is a greater chance that a noncognate tRNA, possessing differential modification misreads the nonsense codon. It is possible the overlapping gene may code for the release factor, or a product involved in the metabolism of THF or for a tRNA modifying enzyme and if thyA mutations affects expression of this overlapping gene, then it is possible suppression may result.

The purpose of this study is to determine whether tRNA is

involved in suppression by Thy+ strains. Two experimental approaches were taken. The first was to examine whether combining the thyA ts mutation from N4316 with known tRNA suppressor mutations had any effect on frameshift suppression. Of the frameshift mutants tested, all generate barriers or nonsense codons downstream of the frameshift (Herrington, Kohli and Faraci, 1986). We think that frameshift suppression by the thyA ts mutant occurs via a two step mechanism. The initial step is the recognition of the barrier sequence by an aberrantly modified tRNA and the second step is the actual frameshifting event promoted by this tRNA recognition. In nonsuppressible frameshift mutants, barrier sequences may not be recognized possibly due to context effects and thus no frameshifting event occurs. The experiments were designed in such a way so that it was possible to combine the thyA mutation with supB or supE suppressors which recognize UAA/ UAG and UAG respectively. In this way, we now have a tRNA in the cell with an anticodon which can pair accurately with a nonsense codon or the barrier. The question we will ask is whether this will affect suppression of frameshift mutants.

The second approach was to compare the modified nucleotides of tRNAs from strains D10 and N4316. One distinguishing characteristic of tRNAs is the presence of many unique modified nucleotides. Approximately ten percent of nucleotides in all tRNAs are modified (Gauss, Gruter and

Sprinzl, 1979). Areas in which modifications occur frequently are in the anticodon loop and at position 37, the residue adjacent to the 3' end of the anticodon. Many different modified bases have been isolated and identified from E.coli (Schimmel, Soll and Abelson (eds.), 1980; Buck and Ames, 1983; Nishimura, 1972) . The more common modifications are dihydrouridine, ribothymidine, pseudouridine and 4- thiouridine which are found in every tRNA molecule in amounts of 1- 3 base residues per tRNA molecule. Among the less common modified bases are 2-thio- 5 methylaminomethyluridine which is found in the anticodon region of 10% of tRNAs and 7- methyl- guanosine which is found in the middle of the extra loop in approximately 20% of tRNAs (Nishimura, 1972). The hypermodified bases, methyl-2-thio-6-isopentenyladenosine (ms2i6A) and N6-threonylcarbamoyladenosine (t6A) may be located in wild type tRNAs at position 37. The tRNAs that translate codons beginning with U and A have ms2i6A and t6A respectively at position 37. Likewise, tRNAs which read codons ending in A or G often have, a modified derivative of U at position 34 of the tRNA (Nishimura, 1972; Murgola, 1985; Celis and Smith, 1979; Altman, 1978). However, studies with glycyl transfer RNAs, in which the base in position 37 was completely unmodified revealed that it can be as efficient a suppressor as those with the modification (Murgola, 1985).

Although much work has been directed towards understanding

the function of modified bases, the role of many modifications is not yet known. Some evidence for possible roles include stabilizing codon-anticodon interactions (Elseviers, Petrullo and Gallagher, 1984), amino acid acceptor activity (Murgola, 1985) and in determining how some adjacent tRNAs fit on the ribosome during protein synthesis (Kurland, 1979).

Some evidence indicate that undermodification of tRNAs can allow "slippage" of the reading frame, resulting in frameshift suppression. In S. typhimurium , SupK mutants which lack a tRNA methylase, are able to suppress UGA and some frameshift mutants (Atkins and Ryce, 1974; Pope, Brown and Reeves, 1978).

The codon-anticodon pairing is destabilized in derivatives of UAG suppressor mutants which are deficient in the biosynthesis of 5-methylaminomethyl-2-thiouridine (m5am2U), (Elseviers, Petrullo and Gallagher, 1984). These mutants have 2-thiouridine instead of m5am2U in the anticodon loop. It is found that these mutants have difficulty reading through UAG nonsense codons, therefore suggesting that m5am2U stabilizes base pairing between the modified base and the G residue in UAG.

The pyrimidine analogs, 5-fluorouracil and 5-flubrouridine can replace uridine and be incorporated into tRNA of growing bacterial cultures (Frendewey and Kaiser, 1979). With increasing 5-fluorouracil incorporation, there is an

exponential decrease in modified nucleosides in tRNA, particularly of pseudouridine, ribothymidine and dihydrouridine; others, which showed a lesser decrease, include uridine- 5- oxyacetate, 5- methylamino methyl-2 thiouridine and 2- thiocytidine. 4- thiouridine decreased linearly with increasing 5-fluorouracil incorporation. The large exponential decrease in tRNA modification could not simply be explained by the incorporation of fluorouracil which could not be modified in place of a uracil which could be modified. To explain this, Frendewey and Kaiser, (1979) suggested that inhibition of modification was due to either 5-fluorouracil or a byproduct of 5-fluorouracil that binds irreversibly to the modifying enzyme.

Fluorouracil is also converted to FdUMP in the cell. Since FdUMP is a potent inhibitor of thymidylate synthase, wild type cells growing in the presence of fluorouracil might contain very little active thymidylate synthase and thus would be phenotypically like Thy- cells. Thus, the exponential decrease in tRNA modification observed by Frendewey and Kaiser might be an effect of a deficiency of thymidylate synthase activity. If so, we might see a decrease in modification with Thy- cells which would support the hypothesis that suppression is a result of altered tRNA modification.

In this study, I labelled bulk tRNA with 14C- uracil. In this way, all uracil and cytosine residues and their

modified derivatives are radiolabelled and we can examine their relative amounts to see if there are any differences between the strains D10 and N4316. Cytosine derivatives are labelled also because cytosine initially shares a common biosynthetic pathway with uridine. ^{14}C Uracil labelled tRNA was digested with ribonucleases and the resulting nucleotides were separated using the thin layer chromatography method of Nishimura (1978).

MATERIALS AND METHODS

Bacterial strains:

The genotypes and sources of the *E. coli* K-12 strains used in this study are listed in Table 2. *E.coli* B was obtained from S. Champe.

Bacteriophage Strains:

T4 bacteriophage mutant strains used are listed in Table 3. P1CM phage was used for transductions.

Storage and Maintainance of Bacterial Cells:

The bacterial strains were restreaked on agar plates once a month. For long term storage, two ml cultures of newly constructed strains were grown overnight in either AB medium or appropriately supplemented minimal medium. An equal volume of 80% glycerol was added to the cultures and this mixture was transferred to sterile vials which were stored at -70 C.

Media:

AB medium (Apirion, 1966) contains 10 g Difco Nutrient broth, and 10 g Difco Vitamin Free casamino acids in one liter distilled water. AB agar plates contain 15 g agar and

Table 2: *E. coli* K-12 Strains

Strain	Genotype	Source/ Reference *
D10	metB rna-10 (lambda)	M.C. Ganoza[1]
N4316	metB rna-10 thyA ts sts (lambda)	M.C. Ganoza[2]
CA165	supB lacI lacZ permissive host of UAA mutants	M.C. Ganoza[3]
CAJ64	lac supUGA permissive host of UGA mutants	M.C. Ganoza[4]
CR63	supD60 F+ lambda sensitive permissive host of UAG mutants	M.C. Ganoza[5]
SP238	his purB proA thi galK lacY mtl xyl str supE rodA52 ts zbe::Tn10	B.G. Spratt[6]
MH420	lysA24 Hfr C lacZ	
MH473	thy supB lacI lacZ	this study
MH474	lysA supB lacI lacZ	this study
MH476	thyA ts supB lacI lacZ	this study
MH508	thi leu his arg thr relA2 lacZ 813(UGA)	J. Basso
MH516	thyA ts supB lacI lacZ zbe::Tn10 rodA52 ts	this study
MH518	supB lacI lacZ zbe::Tn10 rodA52 ts	this study
MH520	thyA ts supB zbe::Tn10 lacI lacZ rodA52 ts	this study
MH525	supB lacI lacZ zbe::Tn10 rodA52 ts	this study
MH522	thyA ts supE lacI lacZ zbe::Tn10 rodA52 ts	this study

Table 2: *E. coli* K-12 Strains (continued)

MH523	supE lacI lacZ zbe::Tn10 rodA52 ts		this study

* Ref: 1) Gesteland, 1966
2) Phillips, Schlessinger and Apirion, 1969
3) Hirsh, 1971
4) Sambrook, Fan and Brenner, 1967
5) Bachmann, 1972
6) Spratt *et al.*, 1980

Table 3: Bacteriophage T4 Strains

Strain	Permissive Host	Type of Mutant and Gene Affected	Source/ Reference *
eL1P12	N4316	UGA ; (e) lysozyme	
N21	CA165	UAA ; (rII) membrane protein	Benzer and Champe, 1961
M103	CR63	UAG ; (e) lysozyme	Stresinger et al., 1961
FC0	B	(+) frameshift; rII membrane protein	J. Gallant[1]
FC47	B	(+) frameshift; rIIB	J. Gallant[1]
370	B	(+) frameshift; rIIB	J. Gallant[1]
FC151	B	(-) frameshift; rIIB	J. Gallant[1]
221	CR63	deletion rII membrane	Benzer, 1959

*Ref: 1) Barnett et al., 1967
Pribnow et al., 1981

AB top agar contains 6.5 g agar per liter AB medium. 50 µg/ml thymidine was added to AB medium for growth of Thy- strains.

Minimal medium A (Miller, 1972) consists of 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g sodium citrate.$2H_2O$ per liter medium. 1 ml/ liter minimal medium of 20% $MgSO_4$ and 10 ml/ liter 20% glucose was added prior to use. Required supplements (amino acids, thymidine) were added at concentrations of 40 µg/ ml media. Minimal Medium A bottom agar plates contain 15 g agar/ liter. Trimethoprim plates used for the selection of Thy- bacterial mutants, consists of Minimal Medium A with the addition of methionine (40 µg/ml), thymidine (50 µg/ml) and trimethoprim (5.0 µg/ ml) (Herrington, Lapchak and Kohli, 1983).

Superbroth was used in the preparation of P1 lysates used in the transductions. This broth consists of 32 g tryptone, 20 g yeast extract, 5 g NaCl and 5 ml of 1N NaOH per liter media. (Howe, MM, 1973)

Antibiotic Containing Culture Plates:

Tetracycline Plates: (Maniatis, p. 72)

These plates consists of 10 g tryptone, 5 g yeast extract, 5 g sodium citrate, 1 g glucose, and 15 g agar per

liter of plates. 6.2 ml of a tetracycline stock was added per liter prior to pouring. The tetracycline stock was made up of 4 mg tetracycline/ml of methanol and was stored at -20 C in a foil wrapped bottle because of its light sensitivity.

Chloramphenicol Plates: (Miller, p. 226)

One liter of plates consists of 10 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2 ml of 1 M NaOH and was adjusted to pH7.0 with NaOH prior to adding 15 g agar. After autoclaving, 10 ml of 20% glucose and 0.5 ml chloramphenicol stock solution was added. The chloramphenicol stock solution contained 25 mg chloramphenicol/ml absolute ethanol and was stored at -20 C.

T4 Bacteriophage Assays:

To obtain lysates or to determine accurate titers, dilutions of T4 phage mutants were plated on appropriate permissive hosts using the overlay method (Adams, 1959) on AB plates with soft agar. When host strains were Thy-, the AB plates were supplemented with 20 µg/ml thymidine. Plates were incubated at 37 C.

Lysates were prepared from single plaques which were picked with a sterile pasteur pipette and were suspended in 1 ml aliquots of AB medium. These phage suspensions were diluted and plated. The plates were incubated overnight.

Lysates were prepared from confluently lysed plates. Approximately three ml of AB media was added to plates and swirled. Plates were left for 2 to 3 hours at room temperature with occassional swirling. The medium containing extracted phage was collected in screwcap tubes and extracted three times with an equal volume of chloroform to ensure all bacterial cells were lysed. The final lysate is stored with one third volume chloroform in tightly capped tubes at 4 C. Fresh lysates were assayed and reversion frequency determined by plating diluted phage with permissive and nonpermissive hosts respectively. Titers of 1×10^{8} to 1×10^{11} pfu/ml and reversion frequencies of 1×10^{3} to 1×10^{4} pfu/ml lysate were usually obtained.

Suppression Assays:

Phage mutants were titered on different strains. Suppression indices (S.I.) were calculated with the following equation: S.I.= titer of bacteriophage on the test strain divided by the titer of phage on the nonpermissive host. If a value of 100 or greater was obtained, the test strain is said to suppress that T4 bacteriophage mutant. The value of 100 is a convenient but arbitrarily chosen number (Herrington, Kohli and Lapchak, 1984).

Fast assays were developed by Cheung and Herrington for the quick screening of strains to see if they have suppressor ability or not. Two drops (approximately 0.1 ml) of diluted phage and test bacteria were added into wells of 24 well cluster dishes. One ml AB soft agar containing 20 µg/ml thymidine, was added to each well and plates were incubated overnight at 37 C. If more plaques appear in the test strain than in the control strain, then it is said to suppress the T4 mutation.

Transductions:

Preparation of P1 Lysates:

P1CM lysogens were prepared by streaking 0.05 ml P1CM lysate on a chloramphenicol plate and cross streaking with a heavy inoculum of the desired donor strain. Plates were incubated either at 31 C or at room temperature for one to two days. One ml superbroth was inoculated with the lysogenic bacteria and grown overnight at 31 C. This was diluted 50 fold with superbroth, either in a 250 ml erlenmeyer flask or in a bubbler tube containing 0.1 ml of 3% antifoam solution. Cultures were aerated by shaking or by passing air through the culture. Cultures were incubated at 31 C until very cloudy, upon which they were heat shocked at 42 C for 30 min (aeration continued) and transferred to 31 C until the cells lysed as indicated by the culture clearing.

The phage lysates were collected in small screw cap tubes, extracted three times with chloroform and stored at 4 C.

Transductions:

P1 transductions (Miller, 1972) were used to construct the different strains required for the present study. A 5 ml culture of the recipient strain in AB or AB thy was grown overnight, then was centrifuged for 10 min at 3200 x g, suspended in 5 ml MC buffer (0.1 M MgSO4 and 0.005 M CaCl2) and incubated with aeration for 15 minutes at 37 C. This cell suspension (0.1 ml) and 0.1 ml of undiluted or diluted phage lysate was mixed and incubated with shaking at 37 C for 20 minutes. The adsorption reaction was stopped by addition of 0.2 ml of 1 M Na Citrate. One ml AB or AB thy was added to each tube and further incubated an hour with aeration. F top agar (8 g NaCl and 8 g agar per liter of H2O) (2.5 ml) was added to each tube and the contents were plated onto selective plates which were then incubated at 37 C or 31 C. Individual transductants were picked onto selective plates and screened for the desired phenotype. Strains were purified twice and the phenotype checked before further characterization.

RNase 1 Assay:

The plate assay (Gesteland,1966) was used to determine the presence of RNase 1 activity in a strain. Cells were

grown overnight on an AB plate. A suspension containing 3% w/v yeast RNA (BDH chemicals) and 3% w/v molten agar was poured onto the plate and allowed to solidify. The plates were incubated at 42 C for 3-5 hours. Approximately 4 ml of 1N HCl was poured onto the plate to precipitate the RNA in the overlay. A clear halo around a strain indicates that the strain contains RNase 1 while the absence of a halo indicates no RNase 1 activity.

Rod A ts Assay:

This assay determined whether the strains carried the rod A ts mutation. The rodA gene product is involved in cell shape determination (Spratt, Boyd and Stoker,1980). Cells were grown overnight on AB plates at 31 C, 37 C and 42 C, suspended in water on a microscope slide and were fixed and stained with methylene blue. Stained cells were examined microscopically. Cells that appear spherical rather than rod shaped at 37 C and 42 C have the mutant phenotype.

Growth and Labelling of Cells for tRNA Extraction:

Overnight cultures of strains were grown in either AB or AB thy (50 µg/ml) medium at 31 C or 37 C. Cells (0.2 ml) were subcultured into 20 ml of medium in a sidearm flask. Growth of cells was monitored turbidimetrically. When cells reached an OD660 of approximately 0.15, 4 µCi of [2-14C]-uracil (ICN, specific activity of 53.2 mCi/ mmol) was added

to the bacterial culture (Frendewey and Kaiser, 1979) and the incubation continued until cultures reached OD660 of 0.9 to 1.0. Cells were harvested by centrifuging at 3200 x g for 10 min. Pellet was washed three times with 10mM Tris- 10mM MgCl2 pH7.4, to remove unincorporated 14-C-uracil. The pellet of cells was weighed and then frozen at -70 C for overnight storage.

tRNA Extraction:

Transfer RNA was extracted essentially by the method of Last and Laskin, 1971. The frozen cells were suspended in cold sterile 10mM Tris-10mM MgCl2 buffer (4 to 5 ml buffer per gram cells) and were transferred to a sterile 50 ml erlenmeyer flask containing a stirring bar. Water saturated phenol (1.2 volumes) was slowly added while stirring. The flask was vigorously shaken for one minute and vigorous stirring was continued for at least two hours at 4 C. Contents were centrifuged in 15 ml corex tubes at 18,400 x g for 20 minutes in an IEC centrifuge. The aqueous phase was collected and the phenol phase re- extracted with a smaller volume of Tris-Mg buffer. The combined aqueous phase was extracted twice with a 1:1 mixture of phenol:chloroform. If the aqueous layer appeared cloudy at this point, it was centrifuged at 30,000 x g for 30 min. The rotor was allowed to coast to a stop and the supernatant was collected. 0.1

volume cold 20% potassium acetate and 2.5 volumes cold 95% ethanol were added to precipitate the nucleic acids. After at least 4 hours at -20 C, the precipitate was collected and the pellet suspended in sterile water. Aliquots (200 µl) were transferred to Eppendorf tubes. The nucleic acids were precipitated with 95% ethanol and 2.0% potassium acetate, pelleted as before and dried under vacuum in the Speedivac (Savant). The pellet was dissolved in 100 µl DNase buffer (0.02 M Tris pH8.0, and 1 X10 -4 M CaCl2) and 10 µl of a DNase 1 solution (0.2 mg/ml, grade 1 from bovine pancreas, RNase free, Boehringer) was added. DNase digestion was carried out at 37 C for 30 to 45 minutes. DNase 1 was removed by a phenol: chloroform extraction. The aqueous phase was precipitated with 95% ethanol and the pellet was dried as before. The RNA containing pellets were suspended in 500 µl 2M LiCl- 0.1M Potassium Acetate pH5.0, vortexed for 2 min at room temperature, chilled on ice for 10 min and centrifuged for 20 min at 4 C. The supernatant containing the tRNA was transferred to a fresh Eppendorf and precipitated as before. The tiny pellet containing the larger RNAs was discarded. The tRNA pellet was washed twice with 80% ethanol-2% Potassium Acetate, dried and suspended in 200 µl H2O. The purity of tRNA was estimated by measuring the A260/A280 ratio. A sample of tRNA was run on an agarose gel (Figure 1) to see if any large nucleic acids were present. The yield was calculated as follows: 50 µg/ml x

A260 x dilution factor x total volume = yield in μg. The yields obtained were approximately 1mg/g of cells. To determine the amount of radioactivity incorporated into the tRNA, a one μl aliquot was spotted onto a $1cm^2$ piece of cellulose TLC plate which was put into a glass vial containing 7 ml Bray's scintillation fluid. The samples were counted in a LKB Liquid Scintillation Counter.

Bray's Scintillation Counting Fluid:

The scintillation fluid (Bray, 1960) used in ^{14}C counting was made up of 60g napthalene, 4g PPO, 0.2g POPOP, 100 ml methanol, 20 ml ethylene glycol dissolved in p-dioxane and made up to a volume of one liter in a foil covered volumetric flask. The fluid was stirred overnight in a fumehood and was transferred to a brown bottle for storage.

Agarose Gels:

One percent agarose gels were made by dissolving 1 g ultrapure agarose (IBI) in 100 ml Tris acetate buffer (Maniatis, et al., p 156). This buffer contains 4.84g Tris, 1.56g NaOAc3H2O and 0.74 g EDTA per one liter buffer. Gels were run at 100 mV. After staining with ethidium bromide (approximately 0.5 mg/ml), nucleic acids were detected by viewing the gel on a transilluminator (Ultra Violet Products Inc.). Photographs were taken using a

Polaroid camera with Polaroid Coaterless Land Pack film Type 667.

TLC Analysis of Labelled Nucleotides in tRNA:

The isolated tRNA was digested to nucleotides or small nucleotide fragments in two ways. In the first method (Sanger, Brownlee and Barrell, 1965), 100 µg tRNA was mixed with 0.05 mg/ml RNase A, and 5 units RNase T1 in 0.02M Tris HCl pH7.4 and 0.002M EDTA. The digestion was carried out for 6 to 8 hours at 37 C. In the second method, (Frendewey and Kaiser, 1979) 100 µg tRNA was digested with 0.05 mg/ml RNase A, 10 units/ ml RNase T2 and 5 units RNase T1 in 0.06M Ammonium acetate pH4.5 for 6 to 8 hours at 37 C. The reaction volume was kept between 50 to 100 µl.

The digested tRNA was spotted onto a cellulose TLC plate (DC-Plastikfolien Cellulose from Merck). Two µl of the reaction mixture was applied at a time and dried between each application. If the volume of the reaction mix was greater than 75 µl, the volume was reduced in the speedivac before application. Two dimensional chromatography (Nishimura, 1972) was performed using the following solvent systems: 1) isobutyric acid: 0.5M NH4Cl in a 5:3 ratio (V/V) for the first dimension and 2) isopropanol: HCl: H2O in a 70:15:15 ratio (V/V/V) for the second dimension. The TLC plates were dried thoroughly in the fumehood after each

dimension. The plates were examined under UV light at 254 nm to locate the major nucleotides. The plates were wrapped up in Saran wrap and exposed on film (Kodak X-Omat AR, XAR-5) for 10 to 14 days. The spots, which correspond to CMP and UMP and modified derivatives, seen on the autoradiograph were located on the original TLC plate. The corresponding areas on the TLC plate were cut out and the amount radioactivity in each was measured using liquid scintillation counting. Similarly the background of the TLC plate was counted to see if there were other areas with high radioactivity. Percent radioactivity is defined as the amount radioactivity in that spot relative to total radioactivity on the TLC plate multiplied by 100%.

RESULTS

I) THE EFFECTS OF SupB AND SupE ON SUPPRESSION BY Thy A ts

N4316 suppresses nonsense and frameshift mutations in bacteriophage T4 mutants. The purpose of this study is to examine whether known tRNA suppressors of nonsense mutations affect frameshift suppression by Thy- strains. To examine this question, strains were constructed which had a thyA mutation combined with a supB or supE mutation. The supB gene codes for glutamine tRNA1 and is able to read UAA as well as UAG nonsense codons. The supE gene codes for a glutamine tRNA2 and can read UAG nonsense codons only.

a) Suppression by SupB- Thy- and SupB+ Thy+ strains:

The conventions used for describing the phenotypes of suppressing and nonsuppressing strains vary in the literature. A Sup+ and Su- strain indicates the wildtype nonsuppressing phenotype, whereas Sup- and Su+ indicate the suppression phenotype (Glass, 1982). In my work, I have chosen the convention that SupE+ and SupB+ refer to the nonsuppressing phenotypes of wild type alleles of supE and

supB respectively, and SupE+ and SupB+ refer to the suppressing phenotypes of mutant alleles of supE and supB .

Strain CA165 has a supB mutation which affects the anticodon region of the tRNA so that it recognizes UAA and UAG nonsense codons. Thy- derivatives of strain CA165 were selected on trimethoprim plates (Herrington, Lapchak and Kohli, 1983). Trimethoprim is used to select for such mutants because this drug interferes with the regeneration of 5,10 methylenetetrahydrofolate (THF) from dihydrofolate (DHF). THF is the primary methyl donor and is directly used in the synthesis of thymidylate. Since mutations affecting thymidylate synthase do not deplete the THF in cells, the colonies that grow on these plates are frequently Thy- mutants. Twenty colonies were isolated and tested for the thymine requirement at 31,37 and 42 C. A non temperature sensitive Thy- strain (MH473) was used for further strain constructions.

P1 generalized transductions were used to construct the desired strains, and are summarized in Table 4. Strain MH474, (Thy+ Lys+) was constructed (Table 4, cross #1). Since the lysA gene is cotransducible with thyA it facilitates the transfer of the thyA alleles from characterized strains. The thyA ts allele from N4316 was transduced into MH474, (Table 4, cross #2).

Suppression assays were done with strains MH474 (Lys+

Table 4: Summary of P1CM Mediated Transductions Used in Strain Constructions

Cross no.	Donor	Recipient	Selected Phenotype	Relevent Genotype	Constructed Strain
1	MH420	MH473	thy+	lys- supB-	MH474
2	N4316	MH474	lys+	thy- supB-	MH476
3	SP238	D10	tet R	thy+ supE-	#44
				thy+ supE+	#28
4	#28	MH476	tet R	thy- supB+	MH516
				thy- supB-	MH520
5	#44	MH476	tet R	supE+ supB+	MH522
6	MH508	MH516	thy+	supB+ thy+	MH518
7	MH508	MH522	thy+	supE- thy+	MH523
8	MH508	MH520	thy+	supB- thy+	MH525

the co-transduction frequencies observed were as follows:

Cross # 1,2: thy- lys 100%

Cross #3: Tn10- supE 90%
Tn10- rna 80%
Tn10- rod 100%

Cross #4: Tn10- supB 98%
Tn10- rna 67%
Tn10- rod 100%

Cross #5: supB- supE 98%

Thy+ SupB-), MH476 (Lys+ Thy- SupB-), N4316 (Lys+ Thy- SupB+) and CA165 (Lys+ Thy+ SupB-). Results of the assays are shown in Table 5. Data are expressed as suppression indices. Values of 100 or less are arbitrarily taken as indicating lack of suppression (Herrington, Kohli and Lapchak, 1984).

The two T4 nonsense mutants, eL1P12 (UGA) and N21 (UAA) were used to differentiate between the suppression due to the supB allele (N21 is suppressible while eL1P12 is not) and that due to the thyA allele (N21 should not be suppressible and eL1P12 should be suppressible) (Cheung and Herrington, 1982). Four frameshift mutants were also tested. These mutants are not suppressible by strain N4316 (Herrington, Kohli and Faraci, 1986).

Strain N4316 suppressed eL1P12 (UGA) but did not suppress N21 (UAA) or any of the frameshift mutants tested. These results were consistent with results previously observed (Cheung and Herrington, 1982; Herrington, Kohli and Faraci, 1986). Strain CA165 did not suppress eL1P12, but did suppress N21 as expected. The frameshift mutants were not tested on this strain.

Strain MH474 (Lys- Thy+ SupB-) suppressed N21 (UAA) and did not suppress the UGA mutant eL1P12. This is the same suppression pattern seen for its parent strain CA165, thus indicating that the supB has not been affected by the strain construction. None of the frameshift mutants were

Table 5: Suppression by SupB Derivative Strains of CA165

Bacterial Strains	Average Suppression Indices[*][1] on T4 Bacteriophage Strains[2]					
	eL1P12 (UGA)	N21 (UAA)	FC0 (+)	FC47 (+)	FC151 (-)	370 (+)
N4316 (thyA ts)	$2x10^3$	1.0	1.0	1.8	1.3	1.8
CA165 (supB)	1.0	$3.0x10^5$	nd	nd	nd	nd
MH474 (supB lysA)	<1.0	$2.9x10^6$	4.7	1.3	6.2	3.5
MH476 (supB thyA) (20 ug/ml thy)	1.5	$3.7x10^6$	$3.0x10^4$	$1.2x10^4$	$1.1x10^4$	$4.1x10^4$
MH476 (supB thyA) (50 ug/ml thy)	1.3	$3.7x10^7$	1.0	1.3	1.5	50

(*) the average suppression index was based on at least two to three separate determinations

(1) Suppression Index= pfu on test strain/ pfu on nonpermissive host (D10)

(2) The same phage lysates were used for the suppression assays above and in Table 6.

nd = not determined

suppressed.

Strain MH476 (Lys+ Thy- SupB-) did not suppress eL1P12. This was unexpected, but may result from strain differences (Herrington, Kohli, and Lapchak, 1984). Strain MH476 did suppress N21 (UAA) confirming that the strain was SupB-. Strain MH476 suppressed the frameshift mutants. The plaques made by the frameshift mutants were smaller than those made by the N21 (UAA) mutant. All these assays were performed in the presence of thymidine (20 µg/ml), since bacterial lawns were not very healthy otherwise.

Suppression by Thy- strains is inhibited by higher concentrations of thymidine (Cheung and Herrington, 1982; Herrington, Kohli and Faraci, 1986). I tested suppression by MH476 in the presence of 50 µg/ml thymidine. Frameshift suppression was completely eliminated, while suppression of T4 phage mutant N21 was unaffected.

This preliminary experiment showed that suppression of frameshift mutants that are not suppressible by strain N4316 or by MH474 (Lys- Thy+ SupB-) alone were rendered suppressible when there is a combination of the thyA and supB mutations. These results suggest two possiblities: either the thyA directly influenced the way in which the UAA tRNA suppressor behaves or the frameshift suppression is due to the thyA mutation in a different strain background. It has been observed that all Thy- strains suppress and that strain differences other than the thyA allele can affect

the spectrum of suppression (Herrington, Kohli and Lapchak, 1984; M.B. Herrington, and M. Faraci, unpublished results). To distinguish between the two possibilities, and to examine if *supE* affects the suppression by Thy- strains, a set of isogenic strains having either the wild type or *thyA* ts allele and either a *supB* or *supE* mutation or no tRNA suppressor mutation present was constructed.

b) Suppression by SupB- and SupE- strains:

Strain SP238 carries Tn10 located within one minute of the *supE* gene (Spratt *et al*., 1980). Thus, the tetracycline resistance coded by Tn10 and *supE* are highly cotransducible. Tetracycline resistance allows easy selection of recombinant strains. The *supB*, *rna-10* and *rodA* genes are also located near this Tn10 and since the strains used carried different alleles of these genes, their phenotypes had to be monitored in the strain constructions.

The Tn10 of strain SP238 was transferred into strain D10 (Table 4, cross #3). Fast assays with the UAG mutant phage M103 were used to screen the colonies for the supE- characteristic. The Tn10 from strain #28 (SupB+ SupE+ Thy+) was transduced into strain MH476 (SupB- SupE+ Thy-), (Table 4, cross #4). Tetracycline resistant recombinants were screened for their ability to suppress the UAA mutant N21. Both SupB+ and SupB- derivatives were obtained. Since the

rodA ts allele from SP238 and the rna-10 allele from D10 might have been transferred along with the Tn10, the recombinants were screened for their rna and rodA phenotypes. Strain MH520 (SupB- SupE+ Thy- Rna+ RodA ts) and strain MH516 (SupB+ SupE+ Thy- Rna+ RodA ts) were used for further study.

The Tn10 from strain #44 (SupE- SupB+ Thy+) was transduced into strain MH476 (SupB- SupE+ Thy-) and tetracycline resistant colonies were screened for their ability to suppress UAG, (cross# 5 in Table 4). Strain MH522 (SupE- SupB+ Thy- Rna+ RodA ts) was isolated from this cross.

The wild type thyA allele was transduced into strains MH516, MH520 and MH522 to obtain Thy+ derivatives of these strains. The resultant Thy+ strains are MH518, MH525 and MH523 respectively. (Table 4, crosses 6,7,and 8)

The T4 frameshift mutants used here to assay suppression are rII mutants. The rII mutants do not grow on E.coli strains which are lambda lysogens but will grow if a strain loses the prophage or if the rII mutation is suppressed (Benzer and Champe, 1961). To ensure that my strains were lambda lysogens, I tested the T4 rII deletion mutant 221 on the strains. The rII deletion mutant did not grow on any of the strains indicating they were still lambda lysogens.

Three nonsense and four frameshift phage mutants were assayed on the six isogenic strains which had the various combinations of supB , supE and thyA alleles. The

results of the assays are shown in Table 6.

Strain MH516 (Sup+ Thy-) suppressed both the UGA and UAA mutants and did not suppress the UAG mutant. Since this strain has no tRNA suppressor but does have the thyA ts allele, we expected it to behave as strain N4316 which suppressed the UGA and the UAG mutants but does not suppress the UAA mutant (Cheung and Herrington, 1982; Herrington, Kohli, and Lapchak, 1984) thus the suppression of the UAA mutant and lack of suppression of the UAG mutant by strain MH516 was unexpected. Conversion to a Thy+ strain eliminated both UGA and UAA suppression indicating that the suppression by strain MH516 is due to the Thy- phenotype.

The strain MH520 (SupB- SupE+ Thy-) suppressed UAA and UAG nonsense mutants as expected since the supB suppressor can read both UAA and UAG codons. This strain however did not suppress UGA. Since this strain has the thyA ts allele it was expected to suppress the UGA mutant as does strain N4316 and strain MH516. This inability of the newly constructed SupB- Thy- strain to suppress UGA was consistent with the observation of lack of suppression by strain MH476 in the preliminary study. The isogenic Thy+ strain behaved similarly to its Thy- counterpart indicating that the suppression of UAA and UAG mutant was due to the supB mutation and not the thyA ts mutation. It should be noted that the UAG mutant phage M103, used in this study, was very leaky so that the Suppression Index is relatively low even

Table 6: Suppression by SupB- and SupE- Strains Derived From CA165

Bacterial Strains	Average Suppression Indices[1] of T4 Bacteriophage Strains[2]						
	M103 (UAG)	eL1P12 (UGA)	N21 (UAA)	FC0 (FS+)	FC47 (FS+)	FC151 (FS-)	370 (FS+)
MH516 (sup+ thy-)	40	2.7×10^4	5.6×10^5	7.6×10^3	1.1×10^4	1.0×10^4	9.2×10^4
MH518 (sup+ thy+)	6.0	15	5.5	<1.0	<1.0	1.4	8.0
MH520 (supB- thy-)	140	31	5.6×10^6	1.7×10^4	2.9×10^4	2.2×10^4	1.1×10^5
MH525 (supB- thy+)	200	4.9	8.4×10^6	<13	<1.3	<16	8.3
MH522 (supE- thy-)	158	12	1.1×10^4	1.0×10^4	2.7×10^3	3.0×10^3	1.7×10^3
MH523 (supE- thy+)	143	12	<10	<1.0	1.2	<16	6.3

(1) Suppression Index calculated as in Table 5
(2) The same phage lysates were used as in Table 5

in suppressing strains.

Strain MH522 (SupE- SupB+ Thy-) suppressed both the UAG and UAA phage mutants and did not suppress the UGA mutant. The suppression of the UAG mutant is presumably due to the SupE mutation and is not affected when the strain is made Thy+ (MH523). The suppression by MH522 of the UAA phage mutant was thyA dependent since addition of thymidine (50µg/ml) inhibits it and the Thy+ derivative does not suppress.

The most noteworthy observation from these suppression assays was that the presence of either glutamine tRNA suppressor (SupE-, or SupB-) eliminates the suppression of eL1P12 (UGA).

The frameshift mutants were suppressed by strain MH516 (Sup+ Thy-). Frameshift suppression was not seen in strain MH518, the isogenic Thy+ strain, and thus is presumably due to the thyA mutation. These frameshift mutants were not suppressible by strain N4316 (Herrington, Kohli & Faraci, 1986), and so there may be undefined differences between strains N4316 and CA165 which interact with the thyA mutant.

Strains MH520 (SupB- SupE+ Thy-) and MH522 (SupE- SupB+ Thy-) suppressed all the frameshift mutants, and suppression was no longer seen if the strain was converted to Thy+ strains, MH525 and MH523. It is clearly shown by the data in Table 6 that suppression of frameshifts is due to the

Thy- mutation.

To determine the effects of supB and supE on suppression by Thy+ strains, the relative suppression indices for the nonsense and frameshift mutants was calculated by dividing the SupB+ and SupE+ suppression indices by those of the Sup+ strain (Table 7). The supB and supE mutation eliminates the Thy+ dependent suppression of the eL1P12 (UGA) mutant, (Table 6,7). Sup B may increase slightly the suppression of three of the four frameshift mutants, and supE decreases the suppression of the UAA mutant N21 and two of the frameshift mutants (Table 7).

II) COMPOSITION OF tRNA FROM STRAINS N4316 AND D10

Since strain N4316 suppresses many nonsense and frameshift mutations in a variety of T4 genes, and nonsense suppression is restricted by an rpsL mutation, the suppressor appears to act during translation. The model proposed (Cheung and Herrington, 1982) for suppression by Thy+ strains suggests that suppression results from altered modification of tRNA. The tRNAs of strains N4316 and D10 were examined to see if there were any differences in their nucleotide compositions.

Table 7: Effect of SupB and SupE on Suppression by Thy- Strains

Bacterial Strain and Genotype	Relative Suppression Indices of T4 Bacteriophage Strain					
	eL1P12 (UGA)	N21 (UAA)	FC0 (FS+)	FC47 (FS+)	FC151 (FS-)	370 (FS+)
MH516 (sup+ thy+)	1.0	1.0	1.0	1.0	1.0	1.0
MH520 (supB thy+)	$1.2 \times 10^{+3}$	nd*	2.2	2.6	2.2	1.2
MH522 (supE thy+)	4.4×10^{-4}	0.02	1.3	0.25	0.03	0.03

nd* - The relative Suppression Index was not determined because this mutant is suppressed equally well by SupB+ Thy- and SupB+ Thy+ strains (Table 6)

a) Extraction and Purification of 14 C labelled Transfer RNA:

Exponentially growing cells were labelled with 14 C uracil. The cells were harvested in late exponential phase. Nucleic acids were phenol extracted from whole cells, and DNA was digested with DNase. Transfer RNA was separated from larger RNAs with lithium chloride extraction. This method effectively removed ribosomal RNA. The smaller 5S rRNA was more difficult to remove and sometimes remained in the tRNA preparation. The results of a typical tRNA purification is shown in Figure I.

The yields obtained using this method ranged from 0.8 to 1 mg/g cells (wet weight). The purity of the tRNA preparations were estimated spectrophotometrically using the A260/ A280 ratio. Values of 1.7 to 1.9 were obtained, which indicated the samples were not significantly contaminated with phenol or protein. Approximately 1% of the added 14 C uracil was incorporated into the tRNA, giving a specific activity of 500 to 2000 cpm/ µg of tRNA.

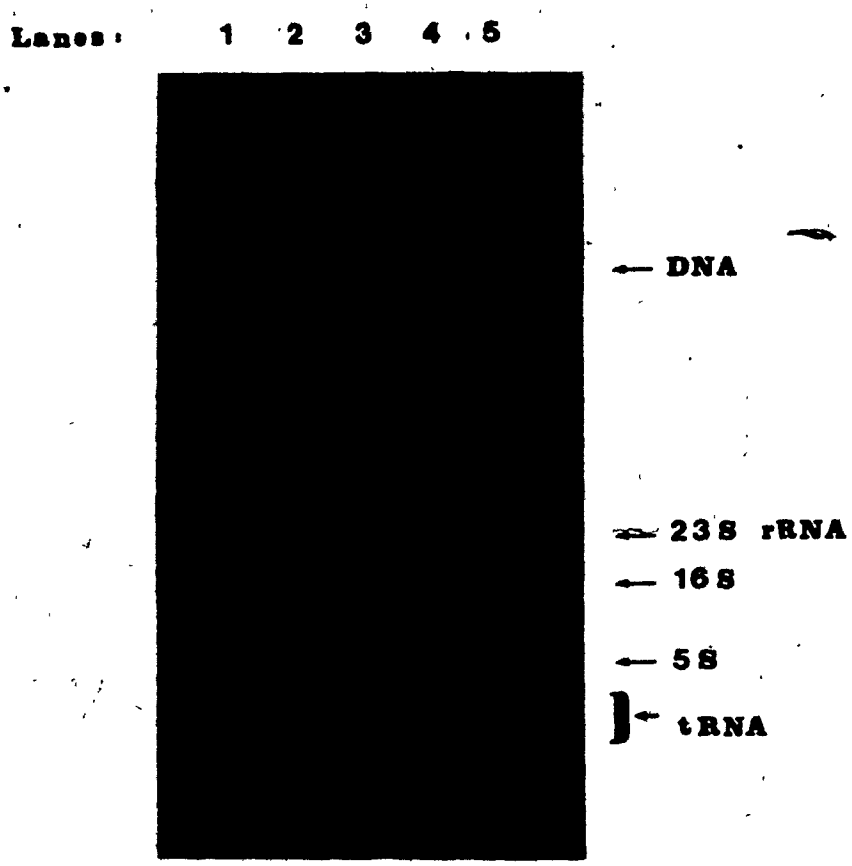
b) Digestion of Transfer RNA

(i) Preparation of tRNA for Thin Layer Chromatography

The bulk tRNA was subjected to RNase treatment to obtain mononucleotides. RNase digestion is preferred over alkaline hydrolysis because many modified nucleotides are labile

Figure 1: Transfer RNA Isolation Using the Lithium Chloride Fractionation Method

Transfer RNA from three steps in the extraction procedure were run on a 1% agarose gel. Approximately 5 to 7 µg of sample was applied in each lane. Gels were stained with ethidium bromide after running at 100 mV.
Lanes 1,2 and 4: whole cell extracts, (lane 1 (courtesy of Pantelis Simantirakis)); Lane 3: tRNA sample after lithium chloride treatment; Lane 5: tRNA sample after DNase treatment.


Lanes: 1 2 3 4 5
DNA
23S rRNA
16S
5S
tRNA

under alkaline conditions (Sanger, Brownlee, and Barrell, 1965; Nishimura, 1972).

The RNase enzymes used were RNase A, RNase T1 and RNase T2. These are endoribonucleases with different specificities and generate 3' nucleotide monophosphates. RNase A is pyrimidine specific, RNase T1 is specific for guanine residues and RNase T2 has no base specificity. Two procedures were used for tRNA digestion. The first procedure used RNase T1 and A (Brownlee, Sanger and Barrell, 1965) which from their specifities could not be expected to give complete digestion but to yield small oligonucleotides. Since this method was used successfully in previous studies, I tried it to see how good the method was for my purposes. The second method involved a combination of all three enzymes and thus should give a complete digest to yield mononucleotides (Nishimura, 1972; Frendewey and Kaiser, 1979). This method is widely used to detect minor nucleotides.

RNase treatments were incubated 6 to 8 hours to ensure complete digestion. The progress of the tRNA digestion was monitored by measuring the acid precipitable radioactivity at different times after the addition the RNases. Results are shown in Table 8. Transfer RNA containing approximately 3500 to 4000 cpm were digested until 250 cpm remained after which the acid precipitable counts did not decrease any further upon increasing incubation time. Most of the tRNA

was digested in the first 60 minutes of the reaction. The shortest time in which the least amount of 14 C label was left was the time used for the digests. The RNase A and T1 treatment required three hours and the RNase A, T1, and T2 treatment required about eight hours.

(c) Comparison of Digestion Procedures

The digested tRNA samples were fractionated by two dimensional thin layer chromatography. Chromatograms were examined under ultraviolet light and were autoradiographed. After autoradiography, the radioactive areas were cut out and the radioactivity measured by scintillation counting.

Samples of AMP, GMP, CMP and UMP were also fractionated by the same method to determine their mobilities. In the first dimension, the order in which the standards ran are AMP, CMP, UMP, and GMP, with AMP travelling the furthest. Chromatography in the second dimension showed UMP travelling the furthest followed by CMP, AMP and GMP.

Under UV illumination, the nucleotides AMP, CMP, and UMP appear as dark fluorescing spots, while GMP was light in color and was generally more smeared than the other three major nucleotides.

After running chromatograms in the first dimension, either three or four major bands were visualized under UV

Table 8: Determination of the Time Required For tRNA Digestion

Time (min)	Acid Insoluble Radioactive Material (cpm)	
	RNase A, T1	RNase A, T1, and T2
0	4008	3508
60	458	854
120	437	557
180	232	---
480	230	237

illumination depending on which digestion procedure was used. Transfer RNA digested with RNase A and T1, showed only three fluorescing bands and some smearing in the area between the origin and the GMP spot. The band corresponding to AMP was not observed as expected from the RNase specificities. Some UV absorbing material was seen at the origin of the two enzyme digests. After the second dimension, three distinct spots with similar mobilities to the standards were seen.

Transfer RNA digested with RNase A, T1 and T2 showed four bands after running in the first dimension and four distinct spots corresponding to the four major nucleotides, after the second dimension.

Since unfractionated tRNA labelled with 14 C uracil was used, we expect to detect only those modified nucleotides of uracil and cytosine that are present in relatively large amounts. The major modified nucleotides derived from uridine are ribothymidine, pseudouridine, dihydrouridine, 5-methylaminomethyl-2-thiouridine, uridin-5-oxyacetic acid and 4-thiouridine; and from cytidine are 2-thiocytidine, 3-methylcytidine and N4 acetylcytidine (Frendewey and Kaiser, 1979; Nishimura, 1972; Lipsett, and Doctor, 1967; Carbon, David and Studier, 1968; Nishimura, 1972).

Autoradiography of chromatograms indicated that the separation of modified nucleotides was good and comparable to those of Nishimura (1972). In general, the two enzyme

digests (RNase A and T1) gave autoradiographs with slight smearing of radioactivity in the background and many more spots were seen as compared to three enzyme digests. This was expected since RNase A and T1 hydrolyzes tRNA into oligonucleotides. The smearing and the many spots may be accounted for by the varying sizes and the base compositions of oligonucleotides. Some oligonucleotides did not travel at all in the second dimension. The three enzyme digests showed fewer spots and much cleaner separation. A schematic representation of the separation is seen in Figure 2. To facilitate comparison of different chromatograms, nucleotides were numbered (Figure 2). Since the two enzyme digestion was not giving a complete digest, further analysis used only the three enzyme digests.

The Rf values of the common radioactive spots were determined. These values are shown in Table 9. The Rf values in the first dimension correspond better to the published values (Nishimura, 1972; Marinus et al., 1975). Rf2 values were generally more variable.

Nucleotides #1 and 2 were identified as CMP and UMP respectively. Identification was based on running standards simultaneously. Modified nucleotides 3B, 4 and 6A may be tentatively identified as ribothymidine(rT), dihydrouridine (DHU) and pseudouridine (PSU) respectively. Identification was based upon Rf values given by Nishimura, (1972) and Marinus, (1975) and the relatively high radioactivity in

Figure 2: Transfer RNA Fingerprint Pattern After RNase Treatment

Schematic representation of an autoradiogram of a TLC plate after 10 to 14 days exposure on X-ray film. 100 µg of tRNA was digested with RNase A, T1 and T2. Samples were applied on a cellulose chromatography plate and run in two dimensions using the following solvent system: 1) isobutyric acid: 0.5M NH_4Cl, 5:3 (V/V); 2) isopropanol: HCl: H_2O, 70:15:15 (V/V/V).

Dotted lines represent the areas seen under UV light. Solid lines represent the radiolabelled areas seen after exposure on film. Areas are assigned numbers for identification.

(Ori) is the origin where sample was applied.
(↑) indicates direction of the first dimension.
(←) indicates the direction of the second dimension.

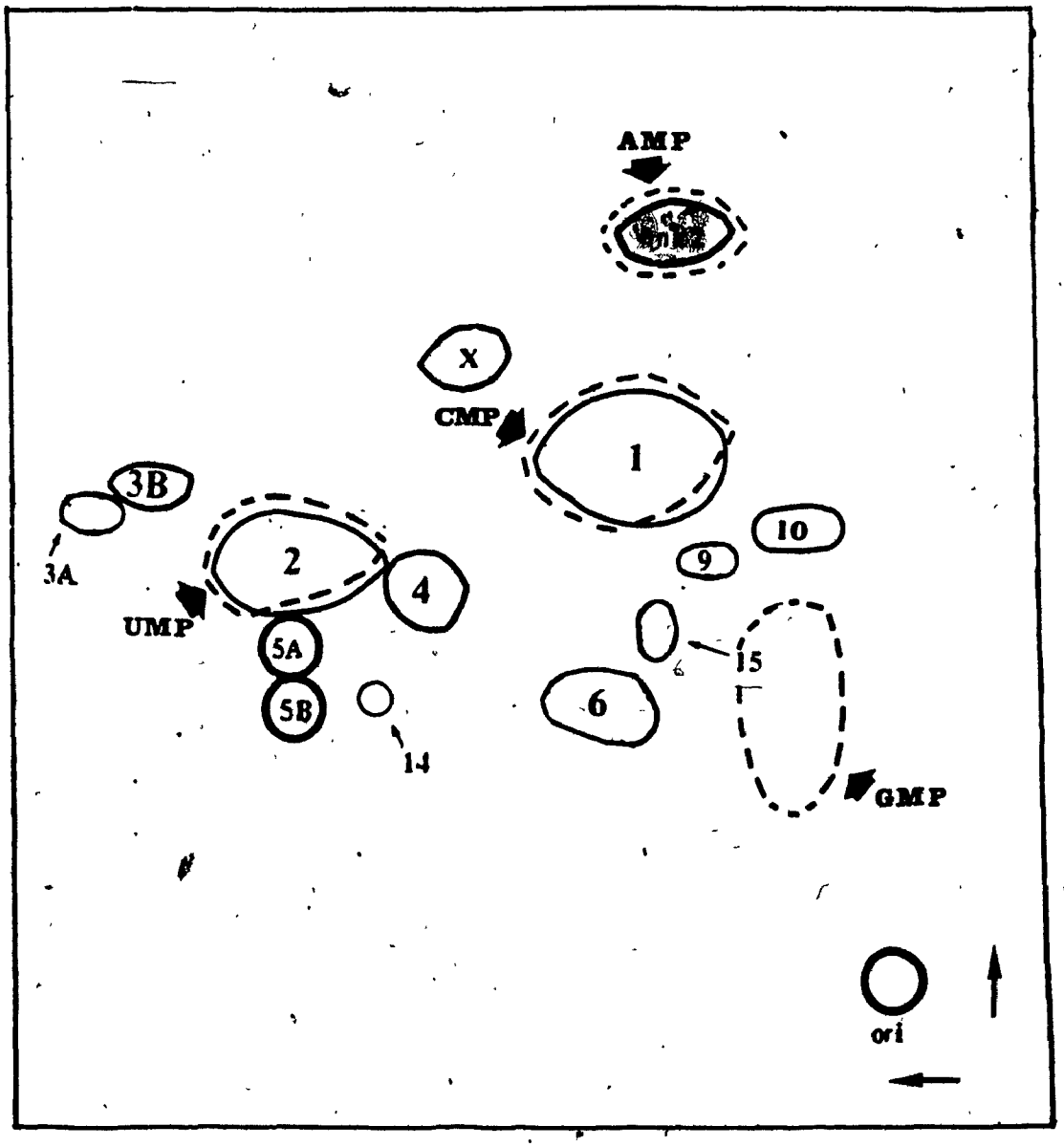

AMP
X
CMP
1
3B
3A
2
4
UMP
5A
5B
14
10
9
15
6
GMP
ori

Table 9: Rf values[1] of the Common Modified Nucleotides

Nucleotide No.	Ratio of Fronts (Rf) Rf 1	Rf2	Nucleotide
1	.54	.40	CMP*
2	.46	.61	UMP*
3B	.50	.67	rT
4	.47	.55	DHU
5	.35	.59	?
6	.37	.44	PSU
X	.60	.50	?

(1) calculated based on data obtained by strains D10 and N4316 at 37C in AB medium and Minimal medium.

(*) positively identified.

these spots. We expected these modified bases to be labelled and present in fair amounts (1 to 3 mol% each) since these three modified nucleotides are found in all species of tRNA. Two other nucleotides (5B and X) were seen consistently but have not been identified.

d) Comparison of Nucleotide Content of tRNA from Strains N4316 and D10

It has been previously suggested the suppression of strain N4316 may be due to altered tRNA modification (Cheung and Herrington, 1982). The nucleotide compositions of tRNA from strains N4316 and D10 were compared to determine whether there were any differences. Labelled nucleotides were cut out of the chromatograms and amounts were determined by liquid scintillation counting. Approximately 70 to 98% of the counts applied were recovered from the TLC plates.

The average limit of detection was equal to 0.05% of the total counts, which coincides with the lower limit of detection of a minor nucleotide present in one species of tRNA in unfractionated tRNA. (Nishimura, 1972).

The amount of the modified nucleotides were quantitated by measuring the amount of radioactivity in a spot relative to the total amount radiolabel present on the TLC plate. Since the background of the TLC plate did contain some radioactivity, the total percentage of radioactivity in the

spots do not add up to 100%. In order to compare values obtained between strains as well as between growth conditions, an N/D ratio (the percent radiolabel in strain N4316 divided by the percent radiolabel in strain D10) was calculated. (Table 10 and 11)

(i) AB Medium at 37 C

When tRNA was isolated from cells grown in AB medium at 37 C, a total of seven spots were seen consistently in both strains D10 and N4316, (Table 10 (a)). Although variation did occur as seen in the values of experiment 1 and experiment 2, the average N/D ratio values (Table 11) indicate no very large differences in amounts of nucleotides in strains N4316 and D10. There may be slightly lower amounts of nucleotides #4 and X in strain N4316.

(ii) AB Medium at 31 C

There are nine nucleotides found in both strains D10 and N4316 (Table 10 (b)) when grown in AB medium at 31 C. Two additional nucleotides (#5A and 14) were present in strain D10. The N/D ratio values show that tRNAs of strain N4316 contain less amounts of spots #3B, 5B and 6, and much higher amounts of nucleotide #9 and slightly higher of nucleotides

#15 and X. Since this experiment was not duplicated, these values may not be reproducible.

(iii) Minimal Medium at 31 C

When tRNA was isolated from cells grown in minimal medium at 31 C, there were ten nucleotides found in common between strains (Table 10 (c)). Transfer RNA of strain D10 did not contain nucleotides #9 and 10. The results of experiment 2 varied more as smearing was a problem due to a higher salt concentration in the D10 sample. There was more of nucleotides #1, X and unk2 in tRNAs of strain N4316. All other nucleotides were present in similar quantities.

(e) Comparison of Nucleotide Pools of Strains N4316 and D10 Under Different Growth Conditions

To facilitate comparisons of transfer RNA nucleotide composition in the two strains, the observations described above are summarized (Table 11). There were five nucleotides (#1, 2, 3B, 6 and X) that were seen in both strains under all the conditions. The relative amount of #2 (UMP) remained fairly constant under all conditions. The quantity of #1 (CMP) was relatively constant in tRNA of strains grown in AB medium but was higher in strain N4316 when strains were grown in minimal medium at 31 C. The reason for this is not

known.

Component #3B, which is assumed to be ribothymidine, was found in reduced amounts in strain N4316 at 31 C in AB medium but was present in roughly the same amounts in both strains in the other growth condition.

Nucleotide X was present in low amounts in AB at 37 C and in high amounts in Minimal medium at 31 C. Its presence at 31 C is about two fold higher than when grown at 37 C in both media.

At the lower temperature, more modified nucleotides are detected. This is more prominently seen in tRNAs of cells grown in AB medium.

There seem to be differences in nucleotide content between strains under the different growth conditions. However we cannot say at this point whether these are directly involved in the suppression mechanism of strain N4316.

Table 10: Percentage of Radioactivity in Areas Corresponding to Modified Nucleotides in Transfer RNA of Strains D10 and N4316 Under Different Growth Conditions

(a) AB Thy Medium at 37 C

Spot #	% Radioactivity				Ratio N/D	
	Experiment 1		Experiment 2			
	N4316	D10	N4316	D10	Exp 1	Exp 2
1	47.9	56.0	47.9	52.1	0.86	0.92
2	32	28.0	25.4	24.0	1.14	1.06
3B	0.45	0.36	0.35	0.36	1.25	0.97
4	2.47	4.81	2.17	2.93	0.51	0.74
6	1.77	1.71	1.26	0.93	1.04	1.35
10	0.28	0.30	0.23	0.17	0.93	1.35
X	0.34	0.55	0.11	0.25	0.62	0.44

Table 10: Percentage of Radioactivity (continued)

(b) AB Medium at 31 C

Spot #	% Radioactivity				Ratio	N/D
	Experiment 1		Experiment 2		Exp 1	Exp2
	N4316	D10	N4316	D10		
1	20.6	20.7	---	---	1.00	---
2	21.6	24.6	---	---	0.88	---
3B	0.46	1.94	---	---	0.24	---
4	3.26	4.14	---	---	0.79	---
5A	---	8.76	---	---	----	---
5B	2.11	8.52	---	---	0.25	---
6	1.90	3.09	---	---	0.61	---
9	12.0	2.50	---	---	4.80	---
14	---	4.2	---	---	---	---
15	11.5	7.88	---	---	1.46	---
X	0.34	0.26	---	---	1.31	---

Table 10: Percentage of Radioactivity (continued)

(c) Minimal Medium at 31 C

Spot #	% Radioactivity				Ratio N/D	
	Experiment 1		Experiment 2		Exp 1	Exp2
	N4316	D10	N4316	D10		
1	31.6	16.3	45.7	19.1	1.94	2.39
2	28.0	25.7	22.1	20.3	1.09	1.09
3A	0.18	0.26	0.25	0.15	0.69	1.67
3B	1.41	1.23	1.24	0.62	1.15	2.0
4	2.44	2.42	4.36	1.13	1.10	3.86
5A	6.50	6.61	4.74	8.41	0.98	0.56
5B	2.87	3.18	2.29	0.86	0.90	2.66
6	0.98	2.40	1.77	3.26	0.41	0.54
9	4.87	---	2.52	---	---	---
10	0.50	---	0.52	---	---	---
X	0.75	0.38	0.45	0.24	1.97	1.88
unk2	0.53	0.14	0.36	0.07	3.79	5.14

Table 11: The Average (N4316/D10) Ratio of Labelled Modified Components in Transfer RNA (RNase A, T1 and T2 digests)

Spot No.	Component Identification	Growth Condition AB medium 37 C	AB medium 31 C	Minimal medium 31 C
1	CMP	0.89	1.0	2.17
2	UMP	1.10	0.88	1.09
3A		---	---	1.18
3B	rT	1.11	0.24	1.58
4	DHU	0.63	0.79	2.48
5A		---	D	0.77
5B		---	0.25	1.78
6	PSU	1.20	0.61	0.48
9		---	4.80	N
10		1.14	---	N
14		---	D	---
15		---	1.46	---
X		0.53	1.31	1.93
unk2		---	---	4.47

--- not present
D present in strain D10 only
N present in strain N4316 only

DISCUSSION

The primary objective of this project was to see whether transfer RNA was involved in Thy- suppression. The first experimental approach was to construct double mutant strains of *E. coli* which have a *thyA* mutation and a known tRNA suppressor mutation to see if they would interact. Results indicate that there is a definite interaction, as the tRNA suppressors (supB and supE) alter the way in which *thy A* mutants suppress. The presence of either tRNA suppressor eliminated Thy- dependent suppression of the opal mutant eL1P12, and reduced the suppression of two of the frameshift mutants. This provides further evidence that the suppression mechanism does indeed occur at the translational level.

(A) Glutamine tRNA suppressors interfere with Thy- dependent suppression of the opal (eL1P12) mutant

The most striking observation is the interference between suppressor tRNAs and Thy- suppression. Either mutant glutamine tRNA (supB or supE) drastically decreased eL1P12 (UGA) suppression (Table 7).

The mutant eL1P12 is a UGA mutant of the lysozyme gene. Its origin is a bit obscure, however we believe it is a

result of a double mutation in the lysozyme gene. eL1 is an ochre mutant of lysozyme, (Remington et al .,1978) and we think eL1P12 was probably derived from eL1 (Figure 3). This eL1P12 mutation is presumed to be at or near the eL1 site. The eL1 site in wild type phage codes for the amino acid glutamine. However, tyrosine, serine and tryptophan inserted in this position are acceptable in the lysozyme protein (Remington et al ., 1978).

For suppression to occur, in this case UGA suppression by strain MH516 (Sup+ Thy-), there are certain requirements the transfer RNA must possess. The tRNA must be able to bind to the UGA codon in the ribosomal A site, undergo any conformational changes required in order to line up the amino acid with the peptidyl tRNA and be able to transfer the amino acid to the peptide chain and release itself from the ribosome site.

To determine how the suppressor glutamine tRNAs might interfere with read through of UGA nonsense codon, we must first examine the difference in the structure of these tRNAs in comparison to wild type (Figure 4) and their possible codon- anticodon pairing interactions, (Figure 5(a) and (b)). Wild type glutamine tRNA1 has the anticodon NUG and the glutamine tRNA2 has CUG which reads codons CAA and CAG respectively. Figure 5(a) depicts the codon- anticodon pairing that the wild type glutamine tRNA1 and tRNA2 have with UGA. Reading of UGA by these glutamine tRNAs would

Figure 3: The origin of the Lysozyme Mutant eL1P12

This diagram illustrates the amino acid and nucleotide sequence of the T4 lysozyme protein. The underlined codon codes for the amino acid glutamine at position 124. The eL1 mutant is the result of a mutation in which the C residue is replaced by a U residue. The mutant eL1P12 may be the result of a secondary mutation in the same codon; the A residue is replaced by a G residue. (↓) indicates the residue that is substituted.

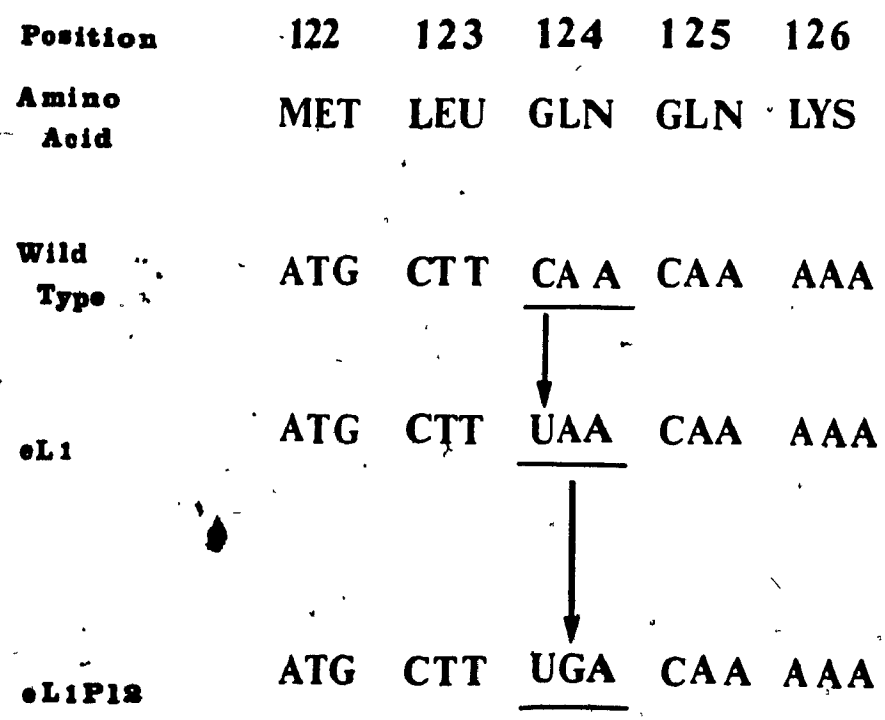
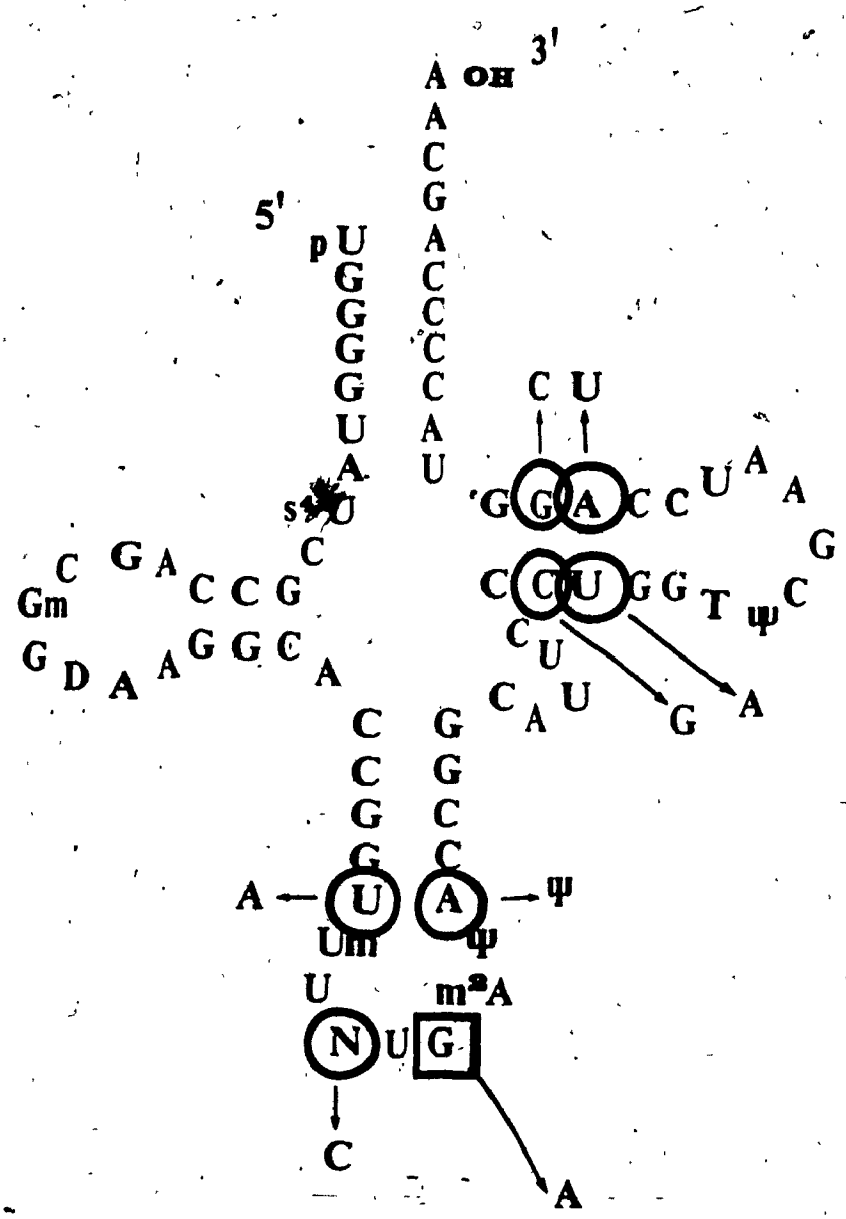

Position
122 123 124 125 126
Amino Acid
MET LEU GLN GLN LYS
Wild Type
ATG CTT CAA CAA AAA
oL1
ATG CTT UAA CAA AAA
oL1P12
ATG CTT UGA CAA AAA

Figure 4: The Cloverleaf Structure of Wild Type and Suppressor Glutamine Transfer RNA

The cloverleaf structure of wild type glutamine transfer RNA1 is represented in bold characters. Encircled are the residues in glutamine tRNA2 that are different from glutamine tRNA1.

The suppressor tRNAs differ from their corresponding wild type sequence only in the G residue located in the anticodon loop, (□). An A residue replaces the G residue in suppressor tRNAs. N is a thio containing derivative of U.


A OH 3'
5'
pU
s²U
Gm
D
Um
ψ
m²A
N
U
G
C
A

involve weak nonstandard base pairing in the first two positions of the codon which are particularly important positions in the decoding process. Thus, it is unlikely that wild type tRNAs are reading the UGA but possibly other noncognate tRNAs are involved.

Examination of the suppressor tRNAs (supB and supE), Figure 5(b), show that the first position of the codon pairs properly with the third position in the supB or supE tRNA anticodon; whereas the second position still pairs in a nonstandard fashion (G=U). Thus, the glutamine suppressor tRNAs have slightly stronger base pairing than wild type glutamine tRNAs with UGA codons.

If Thy- suppression is due to an altered tRNA, then it is probable that the tRNA which reads the UGA does not have an anticodon to complement UGA. Possible candidates are those that are able to read UGC, UGG and UGU codons, since the third position is less important in decoding. These codons correspond to the amino acids cysteine (UGC and UGU) and tryptophan (UGG). As mentioned before, tryptophan is an acceptable amino acid in the lysozyme protein at this position (Remington et al ., 1978). In the Thy+ strain, cysteinyl or tryptophanyl tRNAs do not recognize the UGA nonsense codon and therefore no translation occurs.

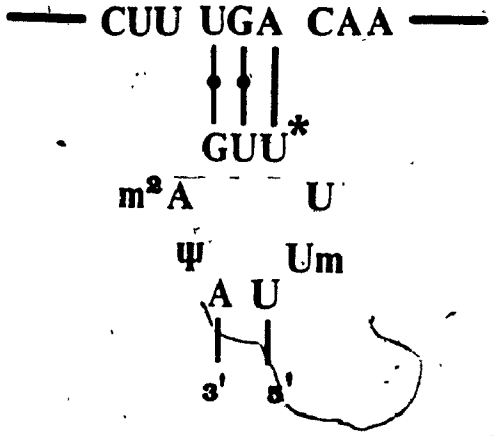
The presence of the SupB or SupE suppressor greatly interferes with Thy- suppression. This suggests that the suppressor tRNA (either SupB or SupE) may be able to

Figure 5: Base Pairing of the UGA codon with Glutamine Transfer RNA

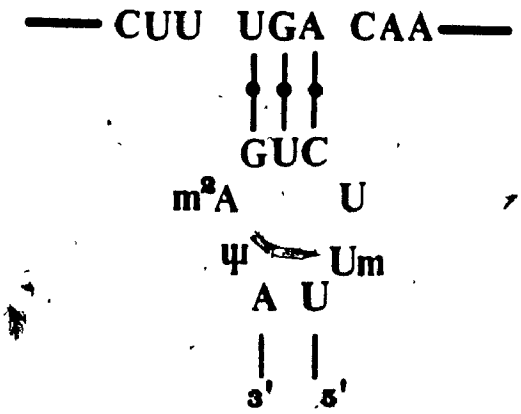
The possible base pairing of wild type and suppressor glutamine tRNAs are examined.
(A) The wild type species of glutamine tRNA1 and tRNA2 base pair very weakly with the UGA codon. tRNA1 involves two weak nonstandard G=U pairs in the first two positions of the codon and one stable A=U* base pair. tRNA2 base pairs weakly in all three positions.
(B) A mutation leading to suppressor tRNAs enhance base pairing slightly. supB tRNAs base pair better than supE tRNAs with UGA.

(⧫) represents nonstandard base pairing. Such possible base pairs are: G=U and A=C. G=U is the most frequently occurring nonstandard base pair. A=C pairing occurs rarely.

[A]

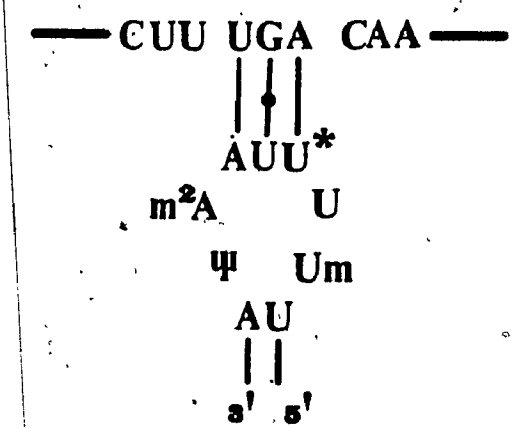


wild type $tRNA_1^{gln}$

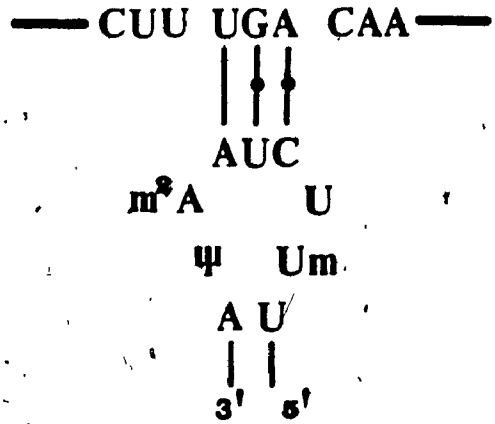


wild type $tRNA_2^{gln}$

[B]



sup B



sup E

recognize and compete with cysteinyl or tryptophanyl tRNAs for the UGA site. Since the wild type lysozyme sequence of eL1 has the amino acid glutamine at the mutation site, then glutamine inserted by SupB or SupE would be expected to give a functional protein. Thus, the transfer of the amino acid glutamine to the growing peptide chain probably does not occur. One would expect if corrective tRNAs (cys or trp tRNAs) bind to the site first, then a functional protein product results. If the glutamine suppressor tRNA binds to the site, then further translation is blocked. Results of the suppression assays indicate that it is the suppressor tRNAs that are outcompeting corrective tRNAs for the UGA site, possibly due to a preferred tRNA conformation, which is assumed by suppressor glutamine tRNAs, as a result of specific changes induced by the Thy- mutation. This preferred conformation ultimately outcompetes the stable base pairing property of noncognate tRNAs for the UGA site. Upon binding to UGA, the site is blocked and further translation is prevented.

There are several ways in which this blocking effect by suppressor tRNAs, may prevent suppression or corrective reading. Protein termination factors may play a role in the competition process. If suppressor tRNAs block the UGA site from pairing with noncognate tRNAs (or wild type glutamine tRNAs), then because supB or E tRNAs do not base pair very well with UGA, will prevent other tRNAs but not termination

factors from attacking the UGA site. In this case perhaps it is a matter of the greater affinity of termination factors for the UGA site.

Alternatively, if the glutamine suppressor tRNAs have an altered conformation that prevents transfer of an amino acid to the growing peptide chain or somehow the nonfunctional conformation locks the tRNA into the ribosomal A site thereby blocking release and preventing competition. Either way suppression will not be observed. We are therefore assuming the suppressors B and E prevent suppression of eL1P12 because of competition for the UGA codon at the ribosomal A site, and because of possible defective modification which prevents normal translocation on the ribosome and thus protein synthesis.

If the Thy- mutation induces an improper codon- anticodon interaction possibly via specific base modifications , then perhaps context effects play a role, ie. if a modification occurs only when a particular sequence is present therefore inducing a conformational change and subsequent blocking and termination at the UGA site. The question arises as to why only the suppressor tRNAs have these changed conformations. Context effects have been shown to play major roles in stabilizing certain base interactions as well as in more accurate recognition of specific sequences (Elseviers, Petrullo and Gallagher, 1984). The base composition of cys and trp tRNAs are clearly different from suppressor

glutamine tRNAs and thus it is difficult to analyze which sequences might be recognized in order to induce a conformational change. If we look at the wild type glutamine tRNAs and suppressor glutamine tRNAs, the only difference in sequence (Figure 4) found is in the anticodon loop in which the third position G residue in wild type glutamine tRNAs is mutated to an A residue. If we assume this residue substitution can change the modification of the adjacent m2A, thereby allowing a change in shape.

Modified nucleotides adjacent to the anticodon region usually are known to be involved in stabilization. From this I speculate that the bases adjacent to the anticodon of supB and supE tRNAs are modified so that not only is there a conformational change in the tRNA molecule itself but this shape change allows the modified adjacent base to pair better with the neighboring bases of the UGA site. In this way, the suppressor tRNAs have the preferred conformation as well as enhancement of base pairing with the aid of neighboring nucleotides.

The possibility of modification in areas other than the anticodon region is entirely possible. The CAJ64 UGA suppressor, otherwise known as Hirsh's UGA tRNA suppressor, possessed an alteration in the D- arm that eventually led to a conformational change. Although it maintained its ability to read UGG, it also reads UGA nonsense codons efficiently. Kurland (1975), proposed that tRNA conformational selection

exists and only when at least two out of three proper base pairings occur, that a shape change takes place. The theory put forth here is that a modification induces a shape change in the tRNA molecule which then goes to the UGA site and since base pairing is possibly enhanced by neighboring modified nucleotides, blocks the site from other UGA translating tRNAs from entering thus preventing suppression.

(B) Interference by SupE on Thy- Suppression of UAA and Frameshift Mutants

The presence of supE decreased UAA Thy- suppression fifty fold and decreased the frameshift mutants FC151 and 370, thirty fold. To account for the interference of supE tRNA with Thy dependent suppression of ochre (UAA) mutants, the same argument as for eL1P12 can be used (Figure 6). There is more stable base pairing between supE and UAA than between wild type glutamine tRNA1 and UAA, in terms of two stable base pairs formed as compared to one stable base pair, thus supE tRNA could out compete wild type glutamine tRNA2 for the UAA site.

This model can also be used to account for the decrease in frameshift suppression in Sup- strains. SupE strains decreased suppression by Thy- strains of the rIIB frameshift mutants, FC151 and 370 thirty fold. Sequence examination of the frameshift mutants indicate that immediately downstream

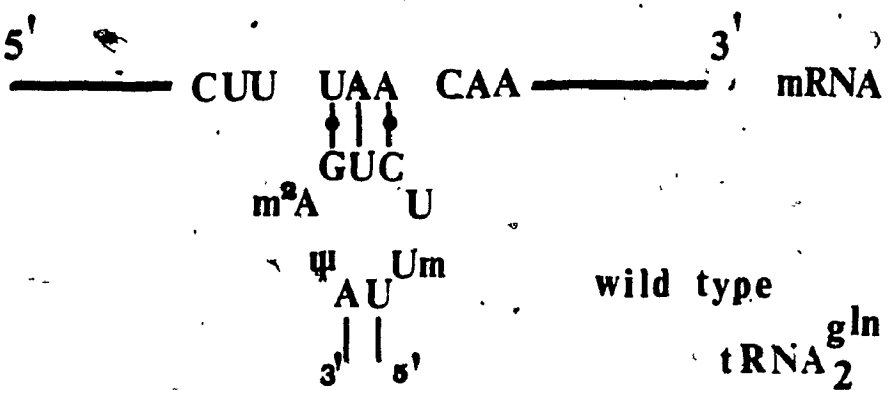
of the frameshift region, there are out of frame barrier sequences. These barrier sequences are nonsense codons. Both frameshift mutants FC151 and FCØ generate a TAA barrier, whereas FC47 and 37Ø generate TGA and TAG barriers respectively. It was suggested that these barrier sequences are involved in frameshift suppression by Thy- strains (Herrington, Kohli and Faraci, 1986). A tRNA with altered modification recognizes the barrier and shifts in the direction that will correct the frameshift mutation. Putting this hypothesis together with the results of the suppression assays, we see that, since the presence of supE decreases suppression of both UGA and UAA mutants possibly by blocking other tRNAs, then this suppressor glutamine tRNA could recognize the barrier and prevent the corrective tRNA from entering the UGA site. If no corrective frameshift occurs, then synthesis is either terminated or a resultant nonfunctional protein is produced, neither of which would allow plaque formation.

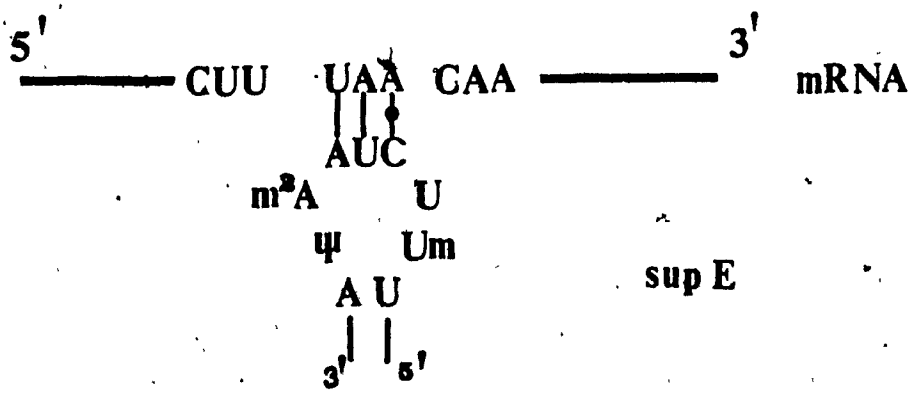
The suppression of frameshift mutant FCØ is not affected by supE which seems to indicate that if the glutamine tRNA binds to the barrier codon, it does not compete with the corrective tRNA as we would have expected on the basis of our observations in the nonsense suppression assays. Alternatively, it can be explained if the SupE tRNA allows for four base reading to occur (ie a + frameshift). In contrast, frameshift mutant FC151 suppression was decreased,

Figure 6: Base Pairing of the UAA codon with Wild Type and Suppressor Glutamine tRNA2

This figure depicts the possible interactions between the UAA codon of the T4 phage mutant, N21, with the wild type and suppressor glutamine tRNA2.

The suppressor tRNA has more stable base pairing than wild type tRNA. Two stable A=U pairs are present in the first two positions of the codon. The proposed model suggests that suppressor glutamine tRNA2 out competes wild type tRNAs for the UAA site due to enhanced stability of codon- anticodon interactions.


5'
CUU UAA CAA
3'
mRNA
GUC
m²A U
ψ Um
A U
3' 5'
wild type
tRNA gln 2


5'
CUU UAA CAA
3'
mRNA
AUC
m²A U
ψ Um
A U
3' 5'
sup E

which indicates that supE competes effectively with corrective tRNA but does not allow two base pair reading (ie -frameshift).

(C) Possible background differences that can affect suppression by Thy- strains

These experiments also show further that the suppression by Thy- strains is affected by the genetic background of the strain. Strain N4316 (thy A ts) can suppress the T4 phage mutants M103 (UAG) and eL1P12 (UGA) but cannot suppress N21 (UAA) or any of the four frameshift mutants used in this study (Herrington, Kohli and Lapchak, 1984). In contrast, strain MH516 (Sup+ Thy-) suppressed T4 mutants eL1P12 (UGA), N21 (UAA) and all four frameshift mutants tested, but did not suppress M103 (UAG). Suppression of these T4 mutants by strain N4316 and MH516 was Thy- dependent as conversion of these strains to a Thy+ derivative (MH518) or addition of relatively high concentrations of thymidine eliminated suppression.

Since both strains N4316 and MH516 both possess the same thyA mutation, the difference in suppression pattern we see is probably due to background differences. Background differences have been observed in other studies on Thy- derivatives of strains D10 and DS4680A (Herrington, Kohli and Lapchak, 1984; M.B. Herrington and M. Paraci,

unpublished). Strains D10 and DS4680A are both E. coli K12 strains. Thy- derivatives of D10 suppress the T4 phage mutants M103 (UAG), L3 (UAA) and eL1P12 (UGA). In contrast, DS4680A thy- derivatives suppressed M103 (UAG) mutant only (Herrington, Kohli and Lapchak, 1984). The T4 mutants used in this study are rII mutants and thus cannot be assayed on DS4680A Thy+ strains which are not Lambda lysogens.

The genetic background of the strain is important in determining whether suppression occurs or not. There is a clear difference in the spectrum of suppression demonstrated by strain D10 and CA165 derivatives. Both these strains are derived from the same ancestral strain 58-161 (Bachmann, 1987; pers. comm. to Dr. Herrington). The strain N4316 is a result of six mutagenic steps, of which at least two involved nitrosoguanidine mutagenesis and one UV mutagenesis step. It is well known that both treatments, especially nitrosoguanidine induces multiple mutations. The strain CA165 is the result of thirteen mutagenic steps on the 58-161 ancestral strain. The CA165 derivatives used in this study involve four additional steps. Clearly there is much induced mutagenesis involved in deriving these strains and thus, it is quite possible one strain has an otherwise cryptic mutation affecting suppression. We can only speculate as to what this background difference could be.

(D) Base Composition of tRNAs

Several models for suppression by Thy- strains have been proposed (Phillips, Schlessinger and Apirion, 1969(a); Ganoza et al., 1973; Belfort et al., 1983). One of these models (Cheung and Herrington, 1982; Herrington, Kohli and Faraci, 1986) suggest that THF pools are altered in a Thy- cell so that THF dependent tRNA modification is altered. This would affect many tRNA species thus accounting for the wide spectrum of suppression. I compared tRNA modification on strains N4316 and D10 by extracting tRNA, digesting it to nucleotides and separating the nucleotides by thin layer chromatography. The variability using this method was unfortunately quite high so it is difficult with the data available to judge significant differences. Since suppression by N4316 is usually only observed in AB medium at 37 C, differences between the modifications of tRNA from D10 and N4316 grown under these conditions are most likely to be involved in suppression. The tRNA from strain N4316 grown under suppressing conditions contained slightly less of nucleotides #4 and X. Nucleotide number 4 has been tentatively identified as dihydrouridine (DHU) but X remains unidentified.

Preliminary experiments using a spectrophotometric assay (Molinaro et al., 1968) for estimation of DHU in bulk tRNA suggested that DHU was not as abundant in tRNA from

strain N4316 as from D1Ø (M.B. Herrington, A. Kohli, unpublished). The other differences observed between strains D1Ø and N4316, in AB at 31 C and in Minimal Medium may result from the thy A mutation but are unlikely to be involved in suppression.

The differences in the quantities and types of modified nucleotides found in the three different growth conditions may result from media differences or different responses to temperature. These differences could reflect actual differences in the extent or types of modification or the relative abundance of particular tRNA species in different strains grown under different conditions. There was in general a higher overall amount of unmodified CMP (#1) and UMP (#2) at the non permissive temperature of 37 C, irrespective of the medium type, which reflects in the fewer types of different modifications. Many more different modified nucleotides were present at the lower temperature (Table 1Ø and 11).

Nucleotides present under all growth conditions are: # 1,2,3B,6 and X. Nucleotides #1 and 2 have been positively identified as unmodified CMP and UMP respectively. Nucleotides #3B and 6 have been tentatively identified as ribothymidine and pseudouridine. Nucleotide X has not been identified (Table 9). The UMP content was consistent under all conditions. CMP had elevated levels in Minimal Medium at 31 C for unknown reasons.

Although the separation patterns of the tRNAs were highly reproducible, the values of percentage of radioactivity in the separated nucleotides were not. This irreproducibilty in values may be due to the small radioactive areas which were difficult to cut out accurately. This separation method was not sensitive and reproducible enough to detect small differences in tRNA base composition of unfractionated tRNA. Thus, differential nucleotide modifications are still candidates for suppression.

Further experimentation is required and what is needed is to use purified tRNA species and subject them to TLC analysis. Purified tRNA species may be obtained either by HPLC or two dimensional electrophoresis. In this way, specific modifications or base substitutions can be more readily visualized, isolated and examined. With the tRNA sequencing method of Gupta and Randerath (1979), the position of the modification may be found. Analysis of the location and type of modification may be associated with mechanisms which may be responsible for the Thy- suppression of N4316.

(E) Possible Involvement of Folate Imbalance in Thy- Suppression

The proposed Thy- suppression model (Cheung and Herrington, 1982; Herrington, Kohli and Faraci, 1986)

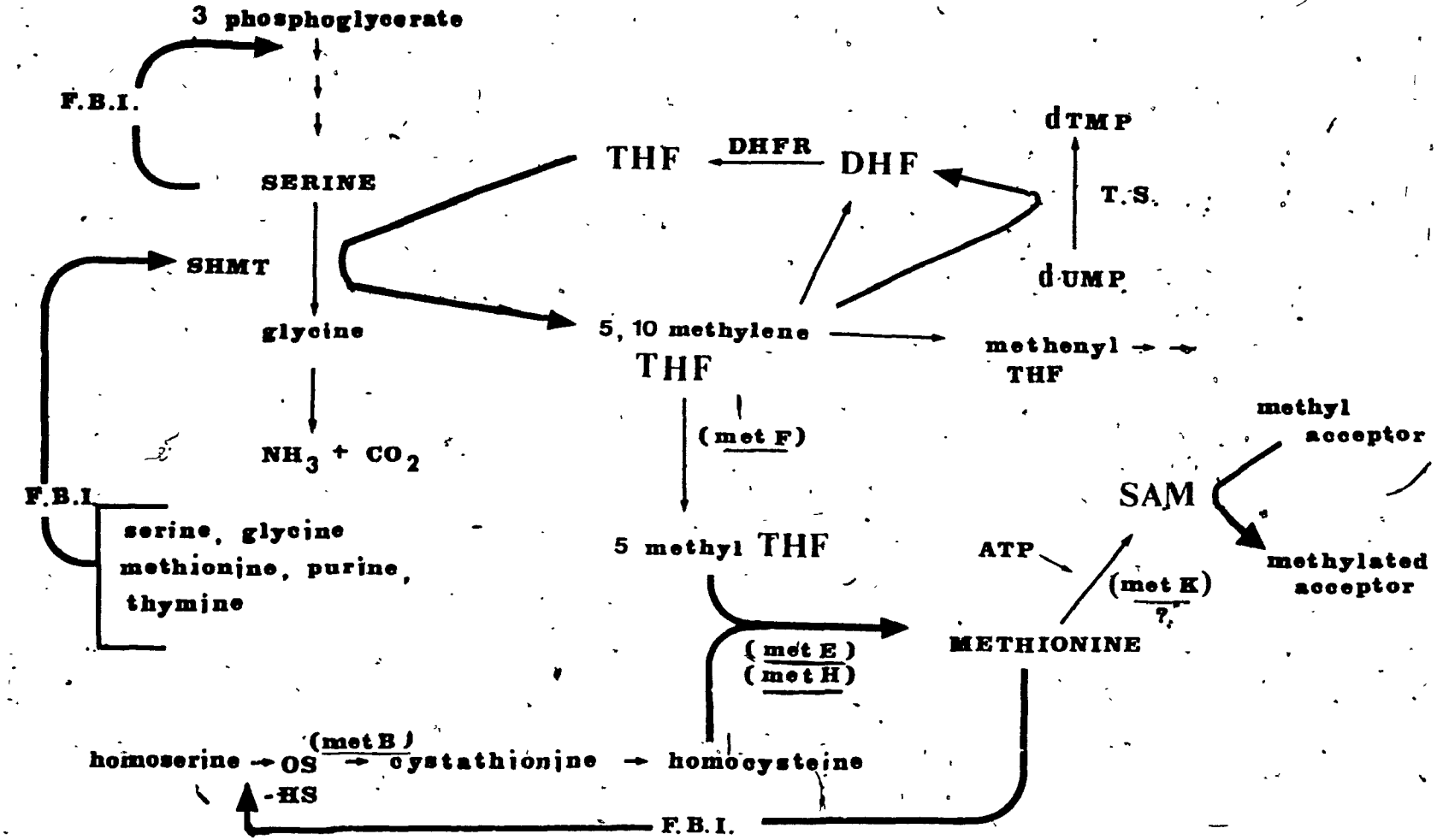
suggests the involvement of folates. It is necessary to examine folate metabolism (Figure 7) in relation to the many different pathways which might link the thyA mutation and the observed suppression. In Figure 7, the only reaction which uses 5-10 methylene THF (Me-THF) is the reaction in which dUMP is converted to dTMP using thymidylate synthase (TS) (O'Donovan and Neuhard, 1970). THF gets its one carbon unit from serine via the serine hydroxymethyl transferase (SHMT) reaction. The SHMT reaction accounts for at least 60% of one carbon units in E. coli . This pathway is feedback inhibited by metabolites such as serine, glycine, methionine, purines and thymine. The drug trimethoprim (which prevents DHF from converting to THF) derepresses the SHMT pathway. Since THF is primarily used in the TS reaction, it may be that mutations inactivating TS can derepress the pathway (Cohen and Saint-Girons, 1987). This would result in an increase in Me-THF pools; since TS is nonfunctional, the excess Me-THF gets shunted to the methionine biosynthetic pathway and may result in an increase in pools of methionine and S-adenosylmethionine (SAM). The presence of high amounts of methionine represses methionine biosynthesis as well as the enzyme, encoded by the metK gene, which is involved in SAM biosynthesis. It does not however, affect metH which is responsible for methylating homocysteine to methionine.

There are many factors which could cause an imbalance in

THF and SAM pools in the E. coli cell. Any alterations in the sensitivities of the reactions in Figure 7, to repression or end product inhibition (FBI) could therefore alter pool sizes of THF which would in turn alter SAM pools. SAM is a known methyl donor involved in tRNA modification in E coli , whereas THF is known only to be involved in ribothymidihe synthesis in gram positive organisms. (Romeo, Delk and Rabinowitz, 1974). The pathway depicted in Figure 7 suggest that a mutation in the methionine biosynthethic pathway might affect suppression due to its requirement in SAM synthesis. Strains CA165 and D10 differ in their methionine requirement. Strain D10 and its derivative N4316 both have the metB mutation, whereas CA165 and its derivatives do not. The metB encodes for an enzyme involved in the conversion of O- Succinyl- homoserine (OS-HS) to cystathionine, the step immediately prior to methionine synthesis. From the data obtained in the suppression assays, CA165 derivatives suppress UAA and frameshift mutants that N4316 cannot (Table 6). In the Met-Thy- strain, the pools of Me-THF increase due to possible derepression of SHMT and lack of TS activity thus pushes methionine biosynthesis. However, due to the metB mutation, no homocysteine is available to pick up the methyl group thus no methionine and no SAM is made. In this model, we are assuming that both THF and SAM are required by tRNA modifying enzymes. The result would be no SAM modifications

Figure 7: The Relation of Metabolic Pathways in *E coli* K-12 and Their Possible Roles in Thy- Suppression

The pathways of S- adenosylmethionine and THF synthesis are shown interconnected with possible users of these cofactors. It is suggested that Thy- suppression may be the result of an imbalance in pools of these cofactors in the cell.


3 phosphoglycerate
F.B.I.
SERINE
SHMT
glycine
NH3 + CO2
F.B.I
serine, glycine
methionine, purine,
thymine
THF
DHFR
DHF
dTMP
T.S.
dUMP
5, 10 methylene
THF
methenyl
THF
(met F)
5 methyl THF
methyl
acceptor
SAM
methylated
acceptor
ATP
(met K)
?
METHIONINE
(met E)
(met H)
homoserine
OS
-HS
(met B)
cystathionine
homocysteine
F.B.I.

thus undermodified tRNAs. Whereas, in a Thy- strain the build up of 5-methyl THF would increase synthesis of methionine and SAM therfore modifications are 'normal'. Although this may explain why derivatives of D10 suppress and those of DS4680A do not. It does not explain why CA165 derivaives suppress (Table 6) what N4316 does not, since CA165 derivatives do not possess the metB mutation. However, it can be argued that since the metB mutation was induced in strain 58-161 by X-rays, it is possible that the metB mutation is not a point mutation and that remnants of perhaps a regulatory mutation might be present in CA165 and not DS4680A.

(F) Background Mutations and Their Possible Roles in Thy- Suppression

The fact that suppression has been seen only in AB medium suggests possibly that another background mutation may be involved. This mutation is the relA-. All three strains D10, DS4680A and CA165 and their derivatives are relA mutants. It has been reported that starvation for an amino acid causes the occurrence of a stringent response, i.e. no de novo synthesis of RNA. However RelA- strains continue to make RNA. Starvation for methionine results in undermethylated tRNAs in E. coli (Mandel and Borek, 1963) as well as in B. subtilis (Keisel and Vold, 1976).

Undermodification of tRNA has been shown by Elseviers (1984), to cause suppression. It is unlikely that AB medium contains limiting amounts of methionine or other amino acids. However, there may be an imbalance (not necessarily a limiting amount) in methionine metabolism or the metabolism of another amino acid even in AB medium and this is responsible for the different suppression spectra.

Conversion of Met- strains to Met+ does not seem to affect suppression (M.B. Herrington, pers. comm). We do not know, however, if the thyA mutation has an effect on the relaxed response therefore we cannot ignore the fact that amino acid starvation may be involved.

Kitchingman and Fournier (1977) have reported there are a variety of conditions that can induce changes in the chromatographic profiles of phenylalanine and leucine tRNAs. They claim that amino acid starvation in Rel- strains deplete certain modifications in particular, DHU and PSU. The end product is a mixture of mature and modification deficient tRNAs. From the results of the relative base composition of tRNAs from N4316 and D10, it seems to suggest that N4316 has less DHU content in its tRNA. It is possible that the Thy- mutation affects this relaxed mutation. If DHU is reduced in tRNA from strain N4316, the D loop of the tryptophan tRNA is probably affected (trp tRNA has three DHU in the D loop), thus the suppression might result from increased misreading by tryptophanyl tRNA.

The suppression phenotype demonstrated by the Thy- mutant strain N4316 has been shown to occur at the translational level. Current experiments indicate that the glutamine suppressor tRNAs affect Thy- suppression. The results of these preliminary experiments reveal several interesting aspects of the involvement of suppressor and wild type transfer RNAs in Thy- suppression. Many questions remain unanswered - however, and we should investigate specific purified tRNA species such as cysteinyl and tryptophanyl tRNA. Extraction of these tRNAs from strains D10 and N4316 at the two growth temperatures, as well as from the newly constructed MH516 and MH518 strains. SupB and SupE tRNAs from Thy+ and Thy- strains also need to be investigated in order to look for differential modification. The TLC method may be used with the purified tRNA species. A tRNA sequencing method (Gupta and Randerath, 1979) may be used to locate the position of these modifications. The specific base modifications and their location in the tRNA might give a better understanding of what base interactions can occur, whether adjacent nucleotides play a role or if there is a preferred conformation that the tRNA assumes due to specific modifications.

Another aspect to investigate is the relationship between the metB , thyA and relA genes in the E.coli cell. An assay to measure the intracellular pools of THF and SAM under methionine excess and limiting conditions, in both Thy-

and Thy+ strains need to be developed. Answers to these questions will reveal whether the hypothesis or model proposed does indeed occur.

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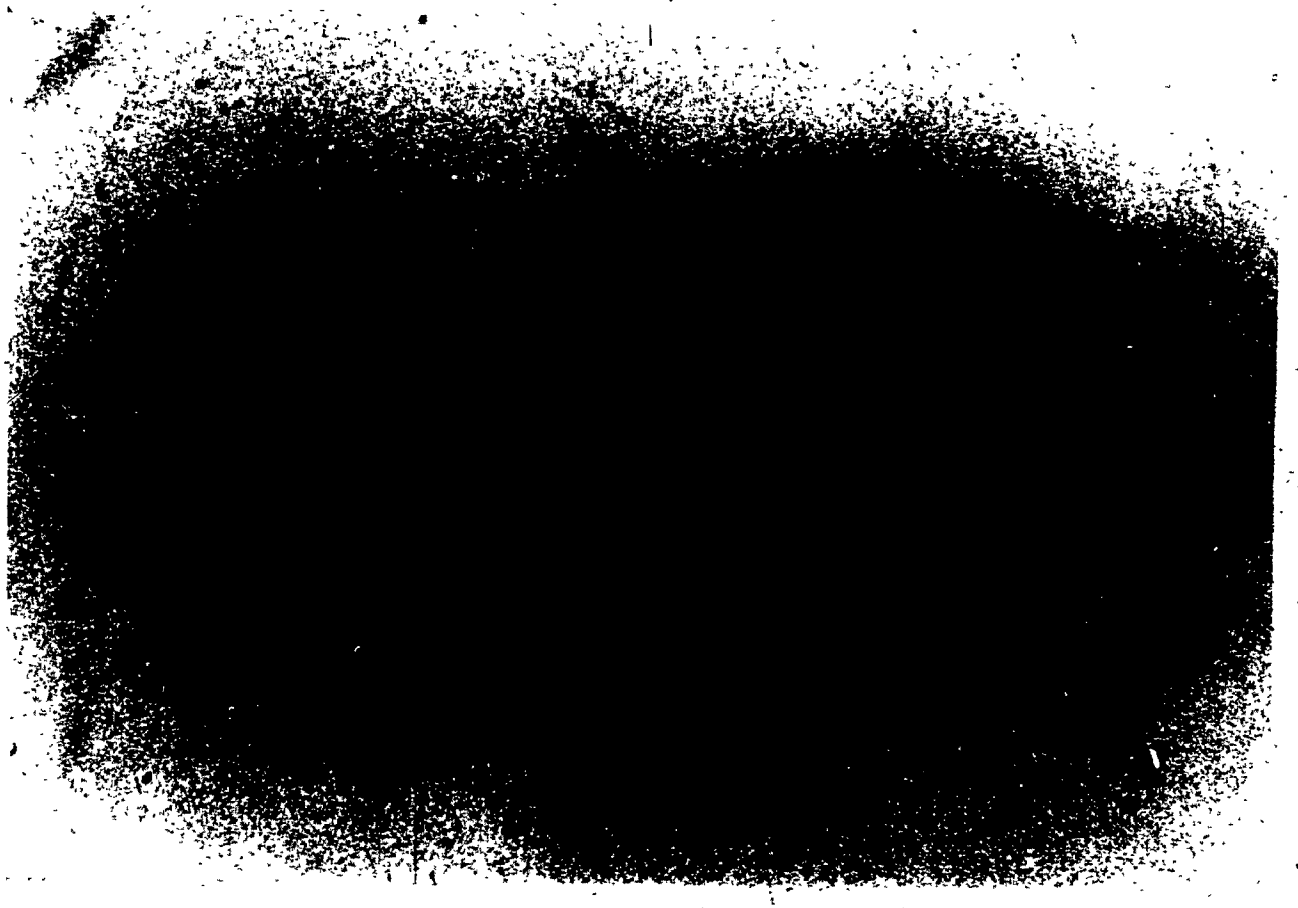
APPENDIX

This section of the thesis contains photographs of the autoradiograms of the TLC plates. The modified nucleotides are numbered as in Figure 2. The growth conditions of the strains are indicated.

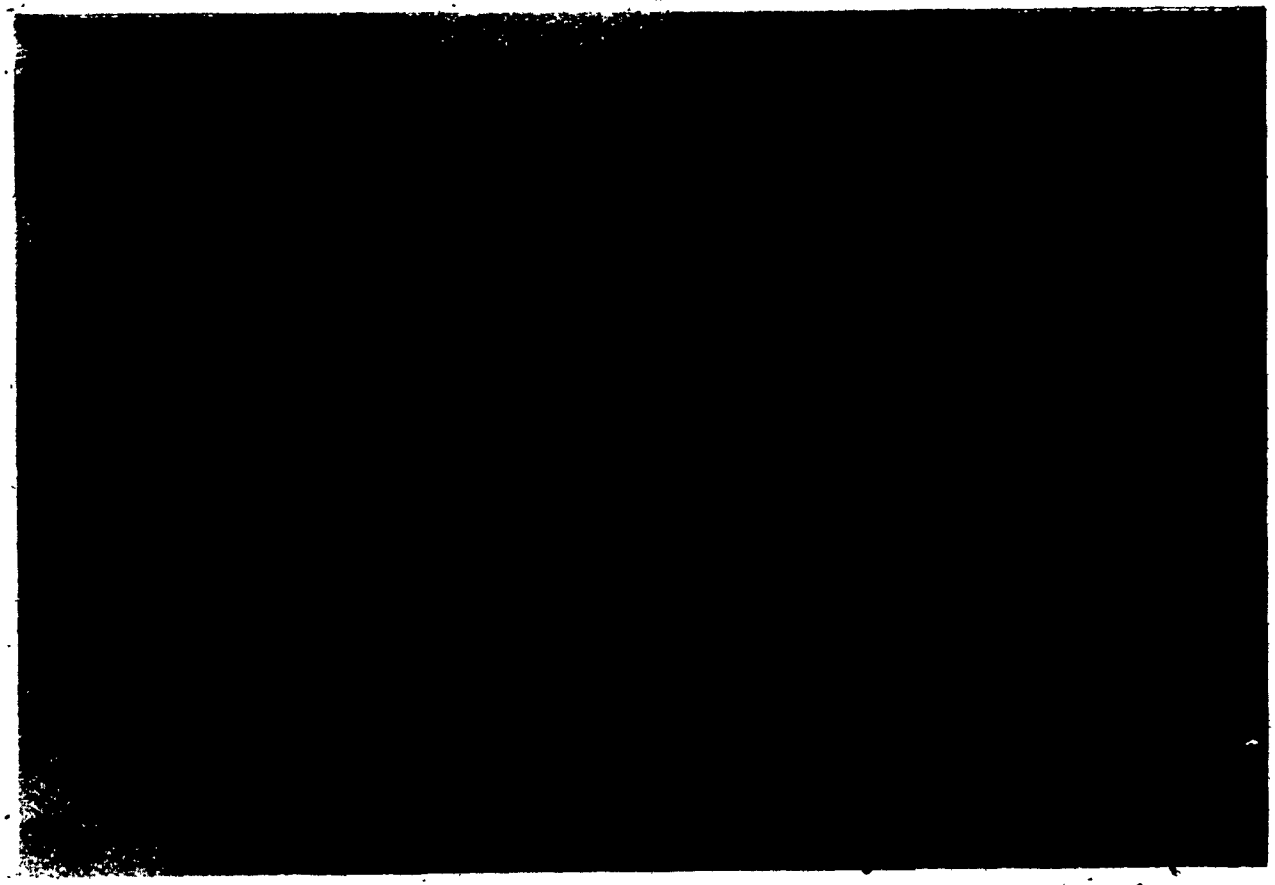


D10 grown in AB medium at 37°C

First Dimension

Second Dimension

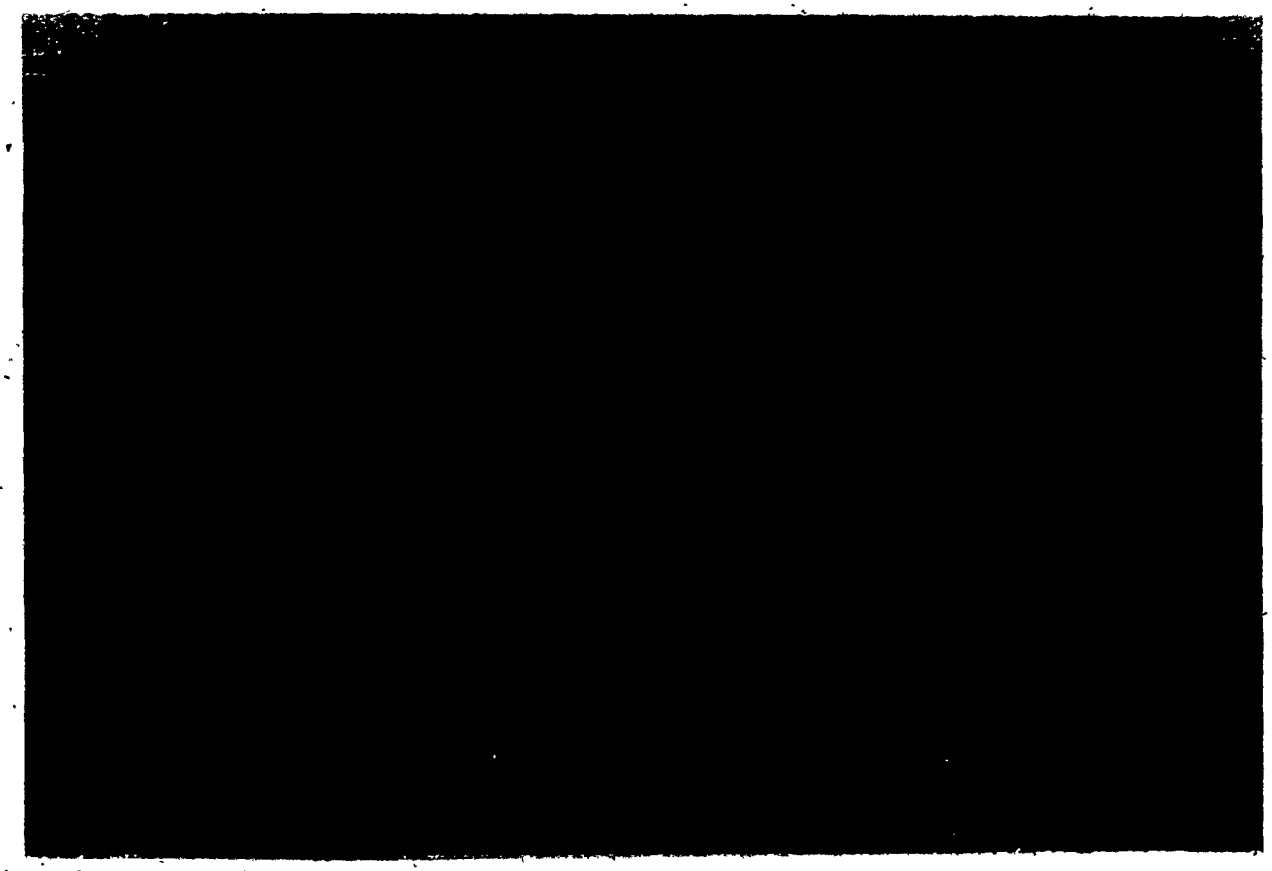
N4316
grown in
AB medium
at 37°C



First Dimension

Second Dimension

D10 grown in AB medium at 37°C



First Dimension

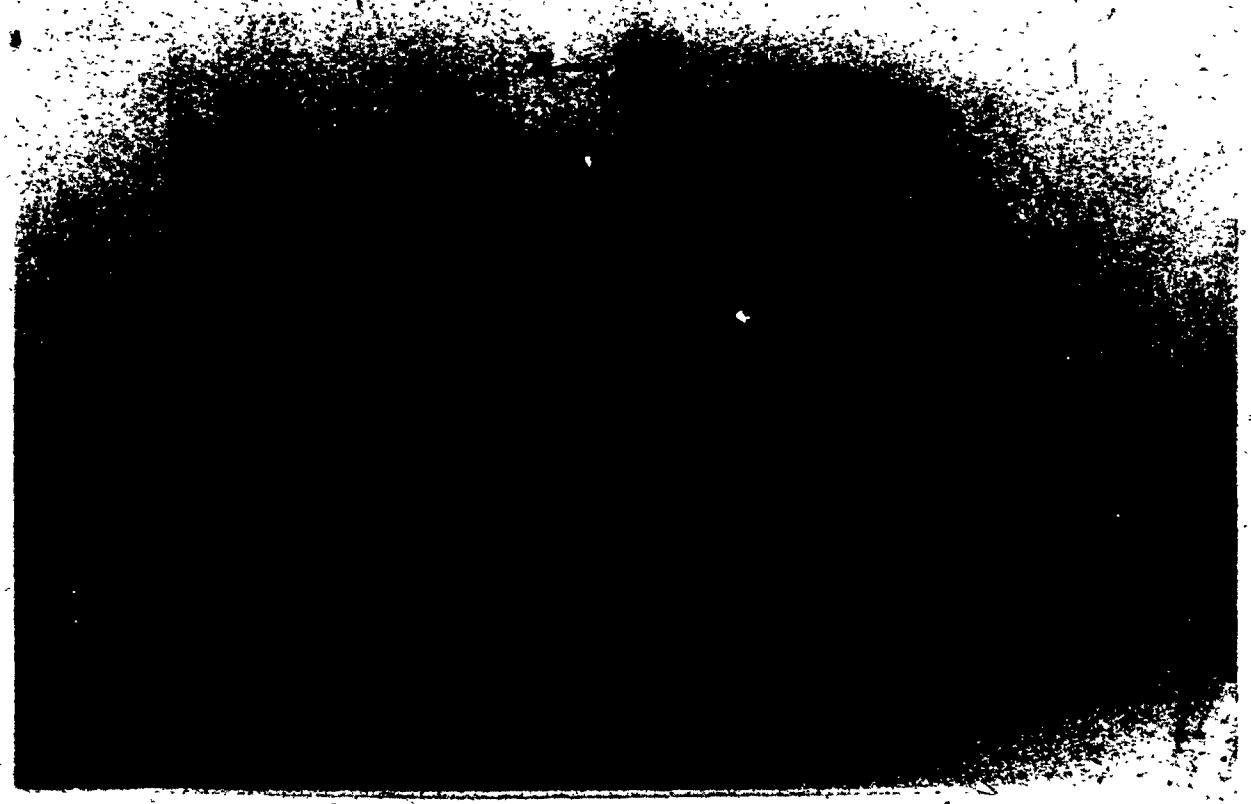
Second Dimension

N4316
grown in
AB medium
at 37°C

First Dimension

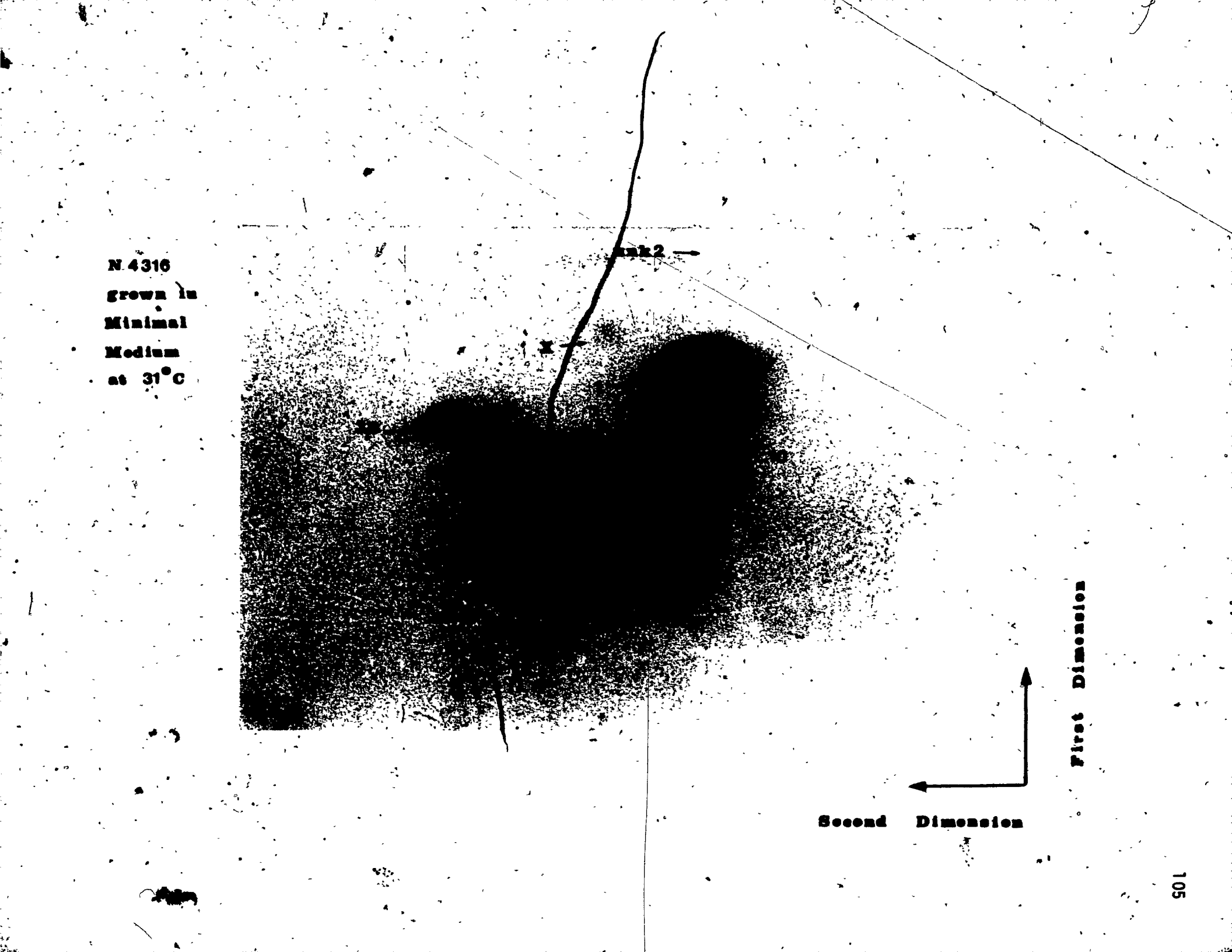
Second Dimension

D10
grown in
Minimal
Medium
at 31°C



First Dimension

Second Dimension


N.4316
grown in
Minimal
Medium
at 31°C
Ink2
X
First Dimension
Second Dimension