Amber Suppression: a Nucleotide Change in the Anticodon of a Tyrosine Transfer RNA

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In certain mutants a single base change alters the meaning of a messenger codon in such a way that, instead of spelling out an amino-acid, it spells out chain termination. Mutants in a quite different gene, called a suppression gene, allow the chain-terminating triplet to be read as an amino-acid. Experiments have shown that this is caused by a mutated *t*RNA which carries a single base change in its anticodon. This allows it to read the chain-terminating codon as if it spelt tyrosine.

In *Escherichia coli* the amber codon UAG is one of the three "nonsense" triplets (reviewed in ref. 1) that code as signals for termination of messenger RNA translation. Strains of *E. coli*, which allow translation of UAG as an amino-acid and subsequent propagation of the polypeptide chain, result from mutation in one of several suppressor genes. There are two classes of UAG suppressors: first, ochre suppressors which permit both UAG and the related chain terminating triplet UAA to be read with low efficiency, and, second, the relatively efficient amber suppressors which are specific for UAG. It has been shown for three amber suppressors that suppression is caused by the presence of an altered *t*RNA in the su^+ cell[2-5]. Although there was no doubt that the suppressor itself was a *t*RNA molecule, these experiments did not show how the suppressor gene acts. It could, for example, control the synthesis of a *t*RNA not present in su^- cells, or of an enzyme which modifies *t*RNA. More simply it could specify the sequence of a *t*RNA. We will show that the amber suppressor su_{III} gene is the structural gene for one of the tyrosine *t*RNAs of *E. coli* and that the mutation su^-_{III} to su^+_{III} results in a base change in the anticodon of this *t*RNA.

The nature of the mutational change is shown by comparison of the nucleotide sequences of the su^+_{III} tyrosine *t*RNA and the corresponding *t*RNA specified by the su^-_{III} allele. The su_{III} suppressor *t*RNA is a small part of the total tyrosine *t*RNA in the cell. The rest of the tyrosine *t*RNA consists of two species (I and II). To overcome this difficulty we used a technique designed greatly to increase the amount of su_{III} gene product in the cell by increasing the number of copies of the gene. The su_{III} gene, which maps very near to the attachment site of phage φ80, was placed on a defective transducing φ80. Infection of *E. coli* with the transducing phage resulted in a phage DNA pool containing many copies of the su_{III} gene. In this way, the amount/cell of the su_{III} gene product, su_{III} tyrosine *t*RNA, was greatly increased. This technique also allows selective isotopic labelling of the *t*RNA for sequence determination.

Transducing Phage φ80 carrying the su_{III} Gene

In the experiments described here, a particular defective transducing phage φ80 dsu^+_{III} (V) was used[4]. The su^+_{III} gene was isolated as a spontaneous mutant from an su^-_{III} strain. The homologous phage, transducing the wild type allele, φ80 dsu^-_{III}, was derived as a recombinant from heterozygous strains lysogenic for φ80 dsu^+_{III}. Thus φ80 dsu^-_{III} and φ80 dsu^+_{III} are identical except for the single spontaneous mutation which produced the su^+_{III} allele.

Synthesis of su_{III} Tyrosine *t*RNA after Infection by φ80 dsu_{III}

Infection of *E. coli CA*274 (su^- lac^- amber) with φ80 dsu^+_{III} resulted in the appearance of suppressor activity as measured by the suppression of an amber mutation in the β galactosidase gene. This was closely paralleled by an increase in the amount of tyrosine *t*RNA in the cell (Fig. 1). No increase occurs in the relative proportion of any other *t*RNA.

Ribosome binding experiments with the *t*RNA synthesized after infection with φ80 dsu^+_{III} showed that the su^+_{III} strains contain a tyrosine *t*RNA which recognizes the amber codon. The corresponding *t*RNA in cells infected with φ80 dsu^-_{III} recognizes the normal tyrosine codons UAU and UAC[4]. Although these experiments do not prove that the suppressor gene specifies a tyrosine *t*RNA, this, however, is likely because φ80 dsu^+_{III} does contain a structural gene for tyrosine *t*RNA as shown by specific hybridization of tyrosine *t*RNA with the DNA from this phage[6].

Nucleotide Sequences of su^+_{III} and su^-_{III} Tyrosine *t*RNAs

If the su_{III} gene is the structural gene for a tyrosine *t*RNA, we expect that the mutation from the su^-_{III} to su^+_{III} allele will have produced a nucleotide change in the su_{III} *t*RNA. To test this idea we have determined the nucleotide sequence of the su^+_{III} tyrosine *t*RNA and compared it with that of the su^-_{III} *t*RNA.

The su^+_{III} *t*RNA labelled with phosphorus-32 was prepared as previously described[6] or by a slightly modified procedure (see legend of Fig. 1). The selective labelling of su_{III} tyrosine *t*RNA after infection was evident from the high proportion of *t*RNA labelled with phosphorus-32 eluting with tyrosine *t*RNA during purification on the DEAE 'Sephadex' column. Pooled fractions from this region often contained about 70 per cent radiochemically pure tyrosine *t*RNA and were sufficiently homogeneous

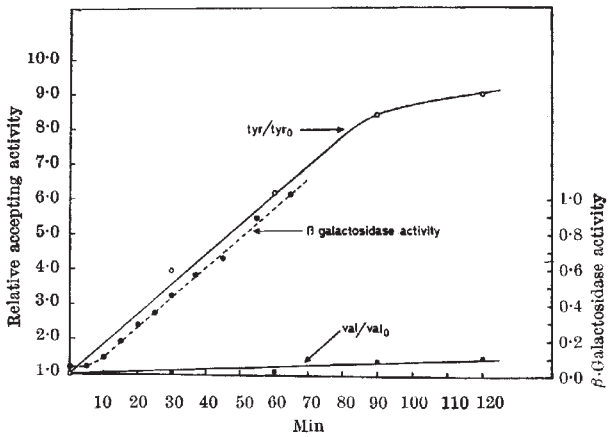

Fig. 1. Increase of tyrosine *t*RNA and suppressor activity in *E. coli* *CA274* *su⁻ lac⁻* amber following infection by φ80 dsu^{+}_{III}. *E. coli* *CA274* was grown to 2×10^{8} cells/ml. in LP medium[6]. The cells were collected by centrifugation and concentrated ten-fold in preconditioned LP medium. $MgSO_4$ was added to 0·02 M and the cell suspension was irradiated with ultraviolet light for 90 s. This dose kills 70 per cent of the cells. The culture was then infected with 30 φ80 dsu^{+}_{III} particles/bacterium. After adsorption for 7 min the cell suspension was diluted ten-fold in preconditioned media. Two separate experiments are shown. In one experiment, the suppression of an amber mutation in the β-galactosidase gene was measured by assaying enzyme activity at various times after dilution into the growth medium. One mM of isopropylthio-galactoside was added to induce the β-galactosidase. In the second experiment, aliquots of the infected culture were removed, *t*RNA was extracted, and tested for its ability to be acylated with several different amino-acids. Fifty μg/ml. of chloramphenicol added at 25 min prevented lysis which normally occurs at 60 min after dilution. In experiments where the *t*RNA is labelled with ^{32}P, 0·05 m Ci/ml. is added 5 min after dilution of the infected cells and the culture is collected after 2 h.

Table 1. END-PRODUCTS OF RIBONUCLEASE DIGESTION OF su^{+}_{III} TYROSINE-*t*RNA

T_1 ribonuclease	Pancreatic ribonuclease
7 G	20 C
2 AG	8 U
UG	ψ
pGp	AoH
CCG	2 AC
CAG	GC
AAG	AU
$CG_{2'OMe}G$	GAC
CCAAAG	AGAC
TψCG	$G_{2'OMe}GC$
U*UCCG	AA*Aψ
UCAUCG	GAGC
ACUUCG	GU
AAUCCUUCCCCCACCACCA–OH	GAAU
ACUCUAA*AψCUG	AAAGGGAGC
	GAAGGT
	GGGGU*
	pGGU

for some of the nucleotide sequence work. In other cases, these fractions were further purified by either of two chemical methods which depend on the ability of the *t*RNA to be acylated with tyrosine[7,8].

The sequence of the su^{+}_{III} tyrosine *t*RNA labelled with phosphorus-32 was determined using the methods of Sanger *et al.*[9,10]. The end-products of complete digestion with T_1 ribonuclease or pancreatic ribonuclease were separated on a two-dimensional electrophoretic system and their sequences determined by further enzymatic digestion. These are listed in Table 1. The combination of products from the two enzyme digests gave only two significant overlaps—GAAGGTψCG and CCAAAGGGAGC.

To overlap the remaining end-products, larger fragments were obtained by partial enzyme digestion and separated as described before[11]. The sequences of these partial fragments were determined from the complete products of their digestion with T_1 ribonuclease and pancreatic ribonuclease. About one hundred large fragments were obtained by partial digestion with various enzymes and their sequences were determined. Fig. 2 shows a selected set of fragments from which the complete sequence was deducted. Fig. 3*a* shows the su^{+}_{III} tyrosine *t*RNA sequence arranged in the "clover leaf" pattern characteristic of the yeast *t*RNA structures. For comparison, the sequence of yeast tyrosine *t*RNA as determined by Madison, Everett and Kung[12] is shown in Fig. 3*b*. Note that in su^{+}_{III} tyrosine *t*RNA, CUA is in the position expected for the anticodon.

There is only a single nucleotide difference between the su^{+}_{III} and su^{-}_{III} tyrosine *t*RNAs and this is best illustrated by comparison of their T_1 ribonuclease digestion products. Fig. 4 compares the fingerprints of the T_1 ribonuclease digestion products of the two *t*RNAs.

Comparison of the T_1 products shows that oligonucleotide 17, ACUCUAA*AψCUG, in the su^{+}_{III} *t*RNA is replaced in the su^{-}_{III} *t*RNA by the two oligonucleotides ACUG and UAA*AψCUG. For the moment we will ignore the presence in the su^{+}_{III} fingerprint of oligonucleotide 17a (which is related to 17); its significance will be discussed later. There are no differences in any other T_1 ribonuclease digestion products. In the su^{-}_{III} *t*RNA the sequence corresponding to oligonucleotide 17 (su^{+}_{III}) must therefore be ACUGUAA*AψCUG which is split by T_1 ribonuclease to give ACUG and UAA*AψCUG. The sequence GUA in su^{-}_{III} *t*RNA is thus changed to CUA in the su^{+}_{III} *t*RNA. GUA is the expected anticodon for the dual recognition of the tyrosine codons UAU and UAC (ref. 13) and in fact GψA is found in the expected anticodon position of yeast tyrosine *t*RNA (Fig. 3*b*) which recognizes both these codons[14]. CUA is the anticodon expected for the unique recognition of the amber codon UAG. In the complete sequence (Fig. 3*a*) of su^{+}_{III} *t*RNA, CUA occupies the anticodon position. These results show that the mutational event giving rise to the su^{+}_{III} allele can be accounted for by a G to C change in the structural gene for a tyrosine *t*RNA.

G in the Anticodon is Normally Modified

In these experiments, the su^{-}_{III} *t*RNA had an unmodified G in the first position of the anticodon. Normally, however, the G in the anticodon of the su^{-}_{III} tyrosine *t*RNA is

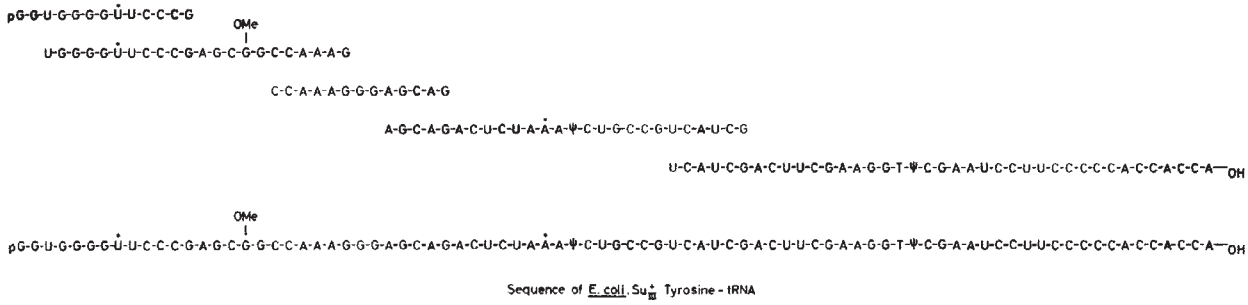

Fig. 2. Examples of overlapping oligonucleotides used to determine the sequence of su^{+}_{III} tyrosine *t*RNA. The five oligonucleotides were separated after partial T_1 ribonuclease digestion and their sequences determined as described in the text.

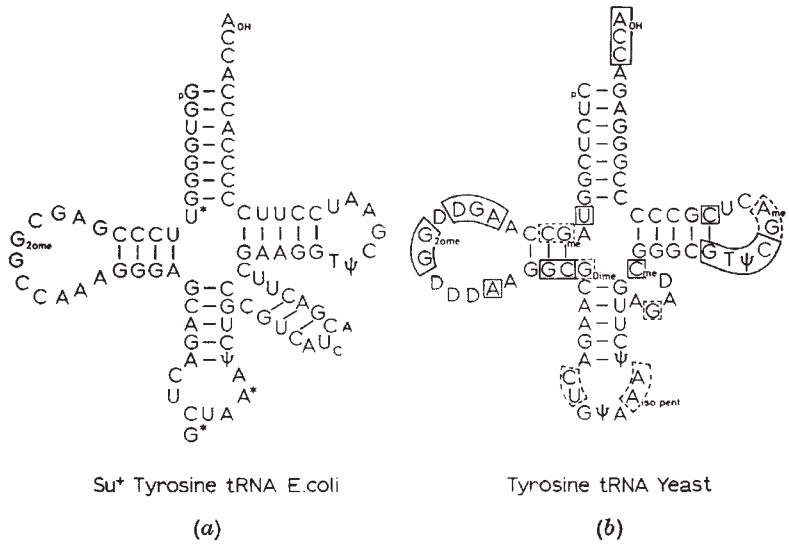

Fig. 3. Clover leaf models of *E. coli* and yeast tyrosine *t*RNAs. The sequence of the su^{+}_{III} tyrosine *t*RNA is shown with the differences that occur in su^{-}_{III}, and species I and II (*a*). For identification of the minor bases see Fig. 4 and the text. The su^{-}_{III} and species I tyrosine *t*RNAs have identical sequences and differ from su^{+}_{III} in having a G* in the anticodon. Species II *tyr t*RNA has the same anticodon sequence containing G* but is different in two positions where the T_1 ribonuclease product UCAUCG is replaced by UCACAG. The latter oligonucleotide has been independently identified by B. P. Doctor and S. Nishimura. The sequence of the yeast tyrosine *t*RNA is taken from Madison *et al.*[12] (*b*). The sequences enclosed in solid boxes are common to all the five yeast *t*RNA structures so far determined (neglecting differences in modification), while those enclosed in dotted boxes are common to most of the sequences.

a modified guanine derivative (G*). In su_{III} tyrosine *t*RNA synthesized in conditions where the cells were starved before infection[6] we have observed that A* and $G_{2'OMe}$ remain largely unmodified. The same is true of the G residue in the anticodon. In su^{-}_{III} *t*RNA prepared from cells not starved before infection (legend to Fig. 1), the G residue in the anticodon is partly or completely modified to G*. This is not split by T_1 ribonuclease so that the T_1 ribonuclease product containing the anticodon is ACUG*UAA*AψCUG. The same anticodon G*UA also occurs in the two principal tyrosine *t*RNA components of *E. coli* (Fig. 3*a*, I and II), and therefore it was possible to isolate small quantities of non-radioactive G^{*}_{p} to study its properties. G^{*}_{p} has an ultraviolet absorption spectrum very similar to that of G_p. It differs from G_p in having an additional basic group with a pK_a between 5 and 6. Neither T_1 nor pancreatic ribonuclease splits the anticodon at the position of G^{*}_{p} and hence in a pancreatic ribonuclease digest it is obtained as the dinucleotide $G^{*}_{p}U_p$. From ribosome binding studies on *E. coli* tyrosine *t*RNAs it is clear that G* has the same codon recognition properties as G. The nature of the modification (which we suspect is on the imidazole part of the purine ring) and its biological significance remain unknown.

Heterogeneity of su^{+}_{III} Tyrosine *t*RNA

In the su^{+}_{III} *t*RNA T_1 ribonuclease fingerprint we noted the presence of an additional oligonucleotide 17a (Fig. 4). This has the sequence ACUG*UAA*AψCUG and is present in amounts varying between 20 per cent and 40 per cent of the su^{+}_{III} anticodon-containing T_1 product ACUCUAA*AψCUG. The sum of the two is in molar yield in the *t*RNA. Thus the tyrosine *t*RNA synthesized after infection by φ80 dsu^{+}_{III} contains in addition to su^{+}_{III} *t*RNA a smaller proportion of a tyrosine *t*RNA with the *su*⁻ anticodon sequence. As will be shown later, the tyrosine *t*RNA in *E. coli* can be separated into two kinds of molecules. One of these, tyrosine *t*RNA I, has the same sequence as the *su*⁻ *t*RNA. The other, tyrosine *t*RNA II, differs by two nucleotides. Fingerprints of the su^{+}_{III} tyrosine *t*RNA exclude the presence of tyrosine *t*RNA II as the source of the contaminating *su*⁻ anticodon sequence. We have considered four possible explanations for this. (i) The φ80 dsu^{+}_{III} carries an additional tyrosine *t*RNA gene determining the structure of tyrosine *t*RNA species I. This is not supported by hybridization experiments between φ80 dsu^{+}_{III} DNA and tyrosine *t*RNA, which show that the transducing phage carries a single *t*RNA gene[6]. (ii) The φ80 dsu^{+}_{III} phage preparations always contain a proportion of φ80 dsu_{III} transducing phage. Such phages could be produced by abnormal excisions occurring in the induction of the heterogenote. We have no good method of assessing the frequency of such events although the general phenomenon is known to occur[15]. (iii) The transcription of genes near the φ80 attachment site is specifically promoted on infection by the φ80 transducing phage. (iv) The presence of many copies of the structural gene for the su_{III} tyrosine *t*RNA specifically induces the expression of one class of chromosomal tyrosine *t*RNA genes.

At present we cannot say which of these mechanisms produces the effect we have observed. Experiments are in progress which should distinguish between them.

Tyrosine tRNAs in Uninfected *E. coli*

There are two principal tyrosine *t*RNAs in *E. coli* which can be separated by column chromatography on DEAE 'Sephadex'[16]. These are referred to as I and II in the order they elute from the column. They are present in proportions of approximately 40 per cent (I) and 60 per cent (II). We have determined the sequences of I and II from *CA*265 su^{+}_{III} uniformly labelled with phosphorus-32. The labelled tyrosine *t*RNAs were isolated by a combination of chromatography on DEAE 'Sephadex', and either reversed phase partition chromatography[17] or chromatography on benzoylated DEAE cellulose[18]. The sequences were derived in the same way as for su^{+}_{III} *t*RNA. Certain overlaps of the complete T_1 and pancreatic digestion

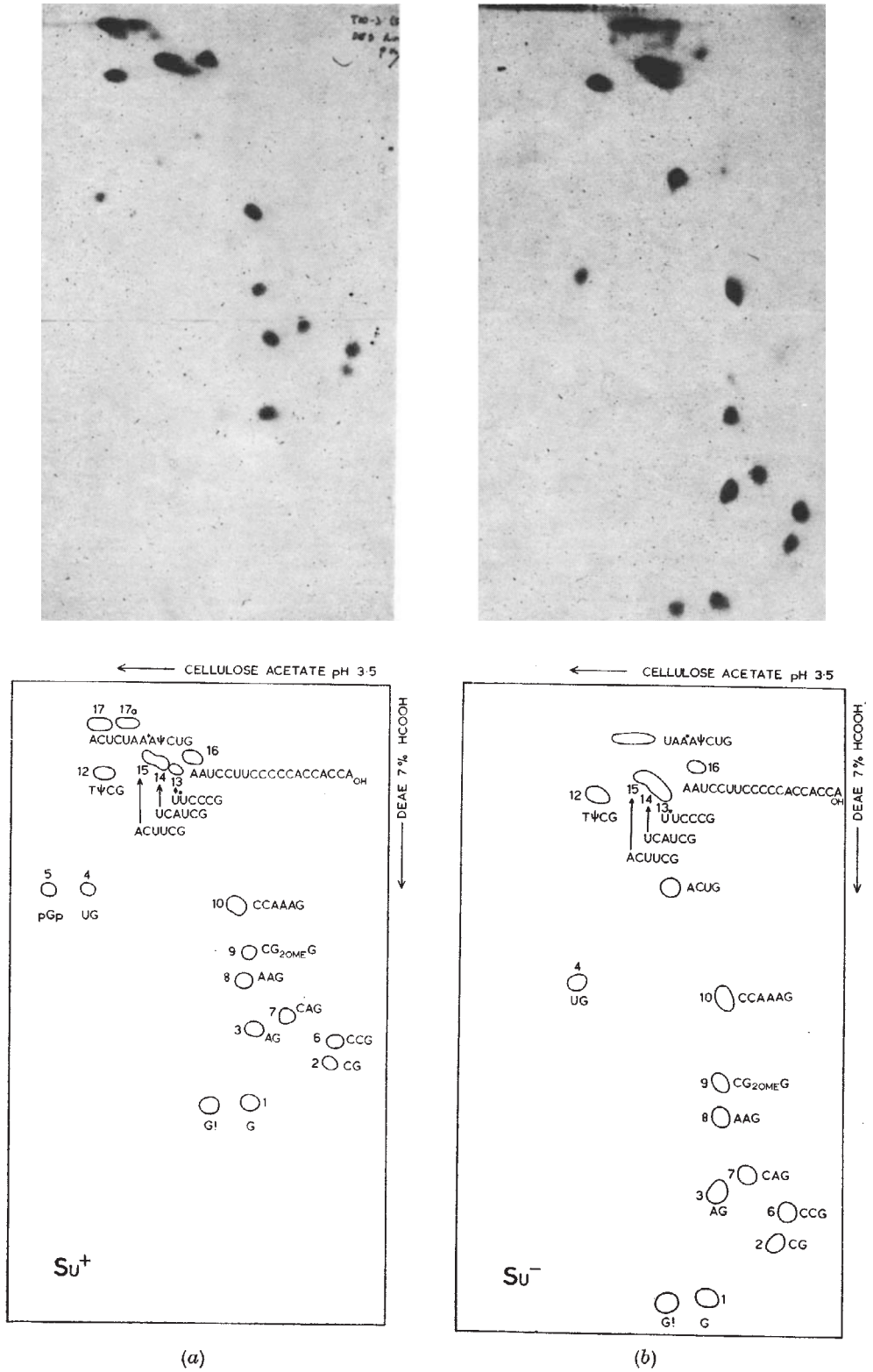

Fig. 4. Two dimensional electrophoretic separation of a ribonuclease T_1 digest of su^+_{III} tyrosine *t*RNA labelled with ^{32}P. *a*, The end-products of ribonuclease T_1 digestion were separated by two dimensional electrophoresis first on cellulose acetate in pyridine–acetate buffer, *p*H 3·5, and then on DEAE–cellulose paper in 7 per cent formic acid (v/v). The lower diagram shows the identification of the nucleotides. The three hexanucleotides 13, 14 and 15 can be resolved after removal of their terminal 3′-phosphates with phosphomonoesterase. The appearance of CG is a consequence of incomplete 2′-*O*-methylation of $CG_{2'OMe}G$ giving rise to CG plus G after digestion with ribonuclease T_1. The hexanucleotide, U*UCCCG, always occurs in low yield, a fact that has not yet been adequately explained. pGp streaks badly and has partially migrated off the paper, making it difficult to detect. The two spots to the right and slightly above UG are minor contaminants which disappear on further purification of the *t*RNA. U* is a sulphur-containing nucleotide, tentatively identified as 4-thiouridylic acid. A* is derived from adenylic acid and, because it can be labelled with (^{35}S) sulphate and (^{14}C)-methyl methionine, it presumably contains both sulphur and a methyl group. *b*, Two dimensional electrophoretic separation of a T_1 ribonuclease digest of su^-_{III} tyrosine *t*RNA labelled with ^{32}P produced after infection with φ80 dsu^-_{III}. In this separation spot 16 has trailed but is normally present in molar yield. The time of electrophoresis on DEAE paper was longer than in *a*.

products in the first sixteen residues have not yet been obtained, and the sequence in this region is assigned by analogy with that of the su^{+}_{III} *t*RNA.

Both tyrosine *t*RNAs I and II contain the anticodon G*UA and differ only in a sequence of two nucleotides in the loop between the anticodon and TψCG arms (Fig. 3*a*). Tyrosine I has a sequence apparently identical with that of su^{-}_{III} tyrosine *t*RNA so that in *E. coli* there are only two basic types of tyrosine *t*RNA sequences.

Thus it is clear that the two different sequences (I and II) must be specified by separate genes. If tyrosine *t*RNA I and su^{-}_{III} were determined by the same gene, then we would expect that in an su^{+}_{III} strain all of tyrosine *t*RNA I would be converted to su^{+}_{III} *t*RNA. When tyrosine *t*RNA from *E. coli CA*275 su^{+}_{III} is fractionated on a DEAE 'Sephadex' column, assays for su^{+}_{III} *t*RNA show that it is located between the peaks of tyrosine *t*RNAs I and II (Fig. 5). This is confirmed by fingerprinting. We conclude that there is a separate gene for su^{-}_{III} *t*RNA even though it has the same sequence as tyrosine I.

Comparison between Yeast and *E. coli* Tyrosine tRNAs

It is interesting to compare the sequences of the yeast and *E. coli* tyrosine *t*RNAs. The shape and hydrogen bonding pattern of the clover leaf model are common to the two organisms. As indicated on the diagram, most of the specific sequence similarities between the two tyrosine *t*RNAs are also shared by all the other yeast *t*RNA structures[19–22], so that when these are taken into account the sequence differences between the two tyrosine *t*RNAs are very striking. An interesting feature is the complete absence of dihydro-uridylic acid (D) from the *E. coli* tyrosine *t*RNA. The greatest similarity is in the anticodon loop and the lower three base pairs of the anticodon arm, which, except for modified bases, are identical in the two *t*RNAs.

At this stage it is not useful to speculate which part of the structures serve as the amino-acyl synthetase recognition site. Neither yeast nor *E. coli* tyrosine *t*RNA can be acylated by the heterologous synthetase[23], so the two transfer RNAs may have quite different recognition sites.

Discussion

The mutation giving rise to one su^{+}_{III} gene changes the anticodon of a minor tyrosine *t*RNA species from GUA to CUA. This change enables the *t*RNA to recognize the amber codon UAG instead of the tyrosine codons UAU and UAC. It seems likely that the mutations leading to the su^{+}_{I} and su^{+}_{II} amber suppressor genes have similarly affected structural genes for serine and glutamine *t*RNA. These *t*RNAs could have anticodons related to the amber anticodon by a single base change.

The ochre suppressor gene su_c maps in the same position as the su_{III} gene[24] and also causes the insertion of tyrosine[25]. The su_c gene may therefore be another allele of the su_{III} gene. Amber suppressor genes may only arise where there are multiple genes for a particular *t*RNA species. In the case of tyrosine *t*RNA we have provided evidence that there are at least three structural genes. If there are no more than three genes our results suggest that the genes are expressed at different rates. We estimate that tyrosine *t*RNAs I, II and *su* are present in the approximate ratios 25 : 60 : 15. The need for this differential control is obscure because the three *t*RNAs have nearly identical sequences.

One might predict that multiple amber suppressor genes could arise corresponding to the multiple tyrosine *t*RNA genes. Perhaps not all of these could be obtained as the large amount of tyrosine II *t*RNA in the cell might make an amber suppressor mutant of this gene lethal. We have examined three independently isolated amber suppressor strains which insert tyrosine. All these map very close to the original su^{+}_{III}. In addition, Garen's *su*3 (ref. 26) and Yanofsky's *suYmel* (personal communication from G. Dennert and U. Henning) insert tyrosine and map in this region. These results suggest either that only one of the tyrosine *t*RNA genes can mutate to su^{+}_{III} or that at least two of the genes are located close to each other.

The G to C change in the nucleotide sequence of the *su* tyrosine *t*RNA specifically changes its codon recognition but does not affect any of the other functional properties of the *t*RNA. This formally designates this position as part of the anticodon and also shows that at least the first position of the anticodon is not necessary for recogni-

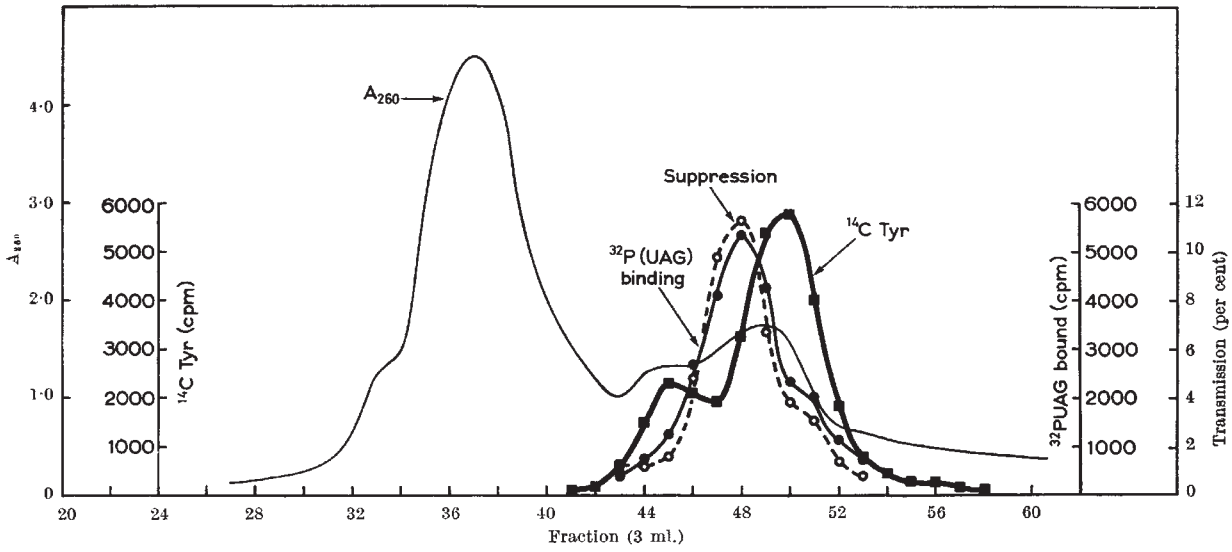

Fig. 5. Separation of the tyrosine *t*RNA components from *E. coli CA*275 su^{+}_{III} and identification of the su^{+}_{III} tyrosine *t*RNA. Tyrosine *t*RNA (from *CA*265 su^{+}_{II}) was purified approximately five-fold by chromatography on benzoylated DEAE cellulose. The figure shows the separation of this material by chromatography on a DEAE 'Sephadex' column (0·5 × 200 cm). Tyrosine *t*RNA elutes in two peaks, I and II (in order of elution). The suppressor *t*RNA was located by two methods: (i) fractions were tested for their ability to bind (^{32}P)pUpApG to ribosomes at 30 mM Mg^{++} using the Nirenberg and Leder assay[27]. (ii) Fractions were also tested for their ability to suppress *in vitro* the *sus* 3 amber mutation of the coat protein of the phage *f*2. This assay was performed by Dr Harvey Lodish using a modification[28] of the method of Engelhardt *et al.*[3]. The measure of suppression is the percentage of transmission, defined as the ratio of completed coat protein molecules : the total number of polypeptide chains initiated, at a concentration of 8 μg/ml. of suppressor *t*RNA added.

tion of amino-acyl synthetase. To identify the function of other parts of the molecule, different mutants of this gene must be studied. These can be obtained by isolating su^- revertants of the su^+ gene. Any mutation which gives rise to a non-functional *t*RNA will have the su^- phenotype. Garen, Garen and Wilhelm[26] have isolated su^- revertants of the su_I^+ gene. These map in five closely linked sites indicating that different parts of the su_I^+ gene may be mutated to give the su^- phenotype.

With L. Barnett we have isolated a number of su^- revertants of the su_{III}^+ gene. We are in the process of characterizing the structural and functional changes in the su^- tyrosine *t*RNA caused by these mutations. This should provide insight into the structural basis for function in *t*RNA molecules.

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Erythropoietin and the Development of Erythrocytes

During the differentiation of erythrocytes some cells develop which contain a high concentration of haemoglobin and no cytoplasmic basophilia. Erythropoietin has the effect of increasing the proportion of these cells in preparations of marrow. Two of the communications which follow discuss the effect on these cells of erythropoietin and of ageing and haemorrhage. The third communication describes a factor in the serum of a polycythaemic calf which enhances the activity of erythropoietin.

Effect of Erythropoietin *in vitro* which simulates that of a Massive Dose *in vivo*

In smears of normal rabbit marrow a few erythroblasts can be seen which are of the same size as the haemocytoblasts, pronormoblasts and basophilic erythroblasts, with almost the same large ratio of nucleus to cytoplasm. They differ, however, in that they have no cytoplasmic basophilia. The concentration of haemoglobin seems to be greater in these than in their basophilic counterparts. The high ratio of nucleus to cytoplasm as well as the larger size distinguishes them from polychromatic and orthochromatic erythroblasts. For convenience they are referred to as line 2 cells and the typical early basophilic erythroblasts are referred to as line 1 cells.

It is well known that in the marrow of a severely anaemic animal there is, with the erythroid hyperplasia, a shift of the distribution towards the less mature erythroblasts. In such animals the number of early line 1 cells and all line 2 cells are increased. In the rabbit made severely anaemic by injections of phenylhydrazine, the ratio of line 2 : line 1 is increased several times. Early line 2 cells are seen more often in some stage of cell division than any other class of erythroblasts. Often their nuclei became pyknotic, indicating their early conversion to large non-nucleated erythroid cells—reticulocytes and erythrocytes.

There are three possibilities regarding the origin of line 2 cells: first, that they arise directly from the stem cell compartment; second, from early line 1 cells; or, third, by both routes. In the search for ways of obtaining direct evidence it was found that rabbit erythroblasts kept overnight in normal rabbit plasma at 5°–15° C seemed from their staining characteristics to be well preserved. At higher temperatures many had degenerated. When 10 u/ml. of erythropoietin was added to the plasma, there was a significant increase in the number of early line 2 cells on the following day. Without the erythropoietin there was no increase.

The following procedure was adopted to investigate the effect of erythropoietin in these conditions. Rabbit marrow erythroblasts were fractionated in a buoyant