Isomeric Specificity of Aminoacylation of Wheat Germ Transfer Ribonucleic Acid and the Specificity of Interaction of Elongation Factor Tu with Aminoacyl Transfer Ribonucleic Acid†

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ABSTRACT: The initial site of aminoacylation of wheat germ transfer RNA has been determined for all 20 amino acids. The method employed involves the use of an analogue of tRNA (Fraser, T. H., and Rich, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2671–2675; (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3044–3048) in which either the 2′- or 3′-hydroxyl group at the 3′ end of the molecule is replaced with an amino group. By comparison of these results with earlier findings for *Escherichia coli* tRNA (Fraser, T. H., & Rich, A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3044–3048) it appears that the specificity of this reaction has been maintained during the evolutionary process. However, it has been found that some *E. coli* aminoacyl-tRNA synthetases differ from those in wheat germ in their ability to catalyze amide bond formation. We have also examined the interaction of both the 2′- and 3′-amino isomers of *E. coli* tRNA with elongation factor Tu and GTP. Both of these positional isomers are capable of participating in ternary complex formation, even though such a complex appears to be weaker than that formed with normal tRNA. In light of the finding that EF-Tu can accept either the 2′- or 3′-amino-tRNA analogue for complex formation, it seems that the positional specificity of the initial aminoacylation plays no role in determining the specificity of subsequent steps in ribosomal protein synthesis.

It has recently been determined that aminoacylation of tRNA involves the specific attachment of an amino acid to either the 2′- or 3′-hydroxyl of the 3′ terminal adenosine (Sprinzl & Cramer, 1975; Fraser & Rich, 1975; Hecht & Chinault, 1976). The hydroxyl group to which an amino acid is initially attached appears to be determined by the particular aminoacyl-tRNA synthetase which catalyzes the reaction. Due to rapid, spontaneous migration of the amino acid between the 2′- and 3′-hydroxyl groups at the 3′ terminus, the initial attachment site of an amino acid cannot be directly ascertained using normal aminoacyl-tRNA; this site may be determined using analogues of tRNA which prevent acyl migration, thereby allowing the initial site of aminoacylation to be deduced. Thus, analogues which have either a 2′-amino-2′-deoxy-AMP or 3′-amino-3′-deoxy-AMP substituted for the 3′-terminal AMP (Fraser & Rich, 1975) or analogues which have a 2′-deoxy-AMP or 3′-deoxy-AMP (Sprinzl & Cramer, 1975; Hecht et al., 1977) substituted at the 3′ terminus have been employed to determine the initial site of aminoacylation on *E. coli*, yeast, and calf liver tRNAs. There appears to be a striking conservation of aminoacylation specificities for a given amino acid among the organisms that have been investigated. We have now extended these studies to include the higher plant, wheat. The experiments that we report here employ the 2′- and 3′-amino-tRNA analogues. As we will show, the specificities of aminoacylation determined using these analogues appear to be essentially the same in both *E. coli* and wheat germ.

These modified tRNAs have also been used to study the interaction between aminoacyl-tRNA and elongation factor Tu. It is known that once a tRNA is aminoacylated it must form a ternary complex with EF-Tu and GTP in order to bind to the ribosome A site and take part in protein synthesis. Determination of the isomeric specificity of aminoacyl-tRNA binding to EF-Tu–GTP may thus allow one to determine whether or not specific tRNA aminoacylation plays a role in the ribosomal protein synthetic mechanism. Some results bearing on this question have recently been published (Chinali et al., 1974; Hecht et al., 1977; Sprinzl et al., 1977). The results which we present here extend these observations and support the conclusion that ternary complexes may be formed with both 2′ and 3′ analogues of aminoacyl-tRNA.

Experimental Section

Materials. Purified *E. coli* tRNA-nucleotidyl transferase was a gift of Dr. Georg Philipps. Purified EF-Tu–GDP was a gift of Dr. Elliot Jekowsky and was purified according to the method of Miller & Weissbach (1970). [^{3}H]Glutamate, [^{3}H]tyrosine, [^{14}C]phenylalanine, and [^{35}S]methionine were obtained from Amersham Searle Co. All other labeled (^{3}H or ^{14}C) amino acids were obtained from New England Nuclear Co. Ribonuclease T_1 was obtained from Sigma.

Preparation of 3′-Amino-3′-deoxy-ATP and 2′-Amino-2′-deoxy-ATP. These compounds were prepared as previously described by Fraser & Rich (1973, 1975).

Preparation of Wheat Germ tRNA and Wheat Germ RNA-Free S-100. tRNA from wheat germ was isolated according to the method of Dudock et al. (1969). An RNA-free S-100 was prepared from 25 g of raw wheat germ. This material was homogenized in 10 mM Tris-HCl, pH 7.8, 10 mM NH_4Cl, 10 mM $Mg(OAc)_2$, and 6 mM β-mercapoethanol. The homogenate was centrifuged for 20 min at 30000*g*. The resulting supernatant was recentrifuged for 20 h at 60000 rpm in a type 65 rotor. The supernatant was removed and dialyzed against 2 × 1 L of buffer III (20 mM Tris-HCl, pH 7.8, 2 mM β-mercaptoethanol, 10% glycerol) overnight. In order to remove RNA, the dialyzed solution was brought to 200 mM in KCl and passed over a 1 × 20 cm Whatman DE-52 ion-exchange column equilibrated with buffer III made 200 mM in KCl. The desired fractions were collected and dialyzed against 2 × 1 L of buffer III. The RNA-free S-100 was then divided into aliquots, frozen in liquid

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nitrogen, and stored at −80 °C.

Preparation of Venom Phosphodiesterase-Treated tRNA. Wheat germ tRNA deprived of all or part of its C-C-A end was prepared from intact wheat germ tRNA (tRNA-C-C-A) by a limited digestion of the 3′ end with snake venom phosphodiesterase (VPD). Wheat germ tRNA 25.5 mg (previously deacylated by incubation at pH 9.5 and 37 °C), 0.2 mg of VPD (Worthington), 0.4 mM glycine, pH 9.0, and 10 mM $Mg(OAc)_2$ were incubated in a volume of 2 mL for 2 h at room temperature. The tRNA was precipitated with 125 μmol of cetyltrimethylammonium bromide (CTAB) and centrifuged at 27000*g* for 15 min. The pellet was redissolved in 2 mL of 1.0 M NaCl and precipitated with 4 mL of 100% EtOH for 30 min at −20 °C. After several ethanol washes the tRNA was dried by vacuum desiccation and dissolved in distilled water.

Preparation of tRNA-C-C-2′-A_N and tRNA-C-C-3′A_N. The 3′ end of the partially digested wheat germ tRNA was reconstituted using tRNA nucleotidyltransferase from *E. coli*. In this reaction either 2′-amino-2′-deoxy-AMP or 3′-amino-3′-deoxy-AMP was incorporated in place of normal AMP. Because several products result from the limited VPD digestion (i.e., tRNA-, tRNA-C, and tRNA-C-C), CTP is also included in this reaction mixture in order to ensure that the tRNA nucleotidyltransferase can add the ATP analogue to those tRNAs which are missing more than AMP. The reaction mixture contained: 26 mM reduced glutathione, 0.30 mg/mL bovine serum albumin, 15 mM $Mg(OAc)_2$, 50 mM glycine, 60 mM KCl, 0.50 mM CTP, 10 μg/mL of purified *E. coli* tRNA nucleotidyltransferase, 1.44 mg/mL of VPD-treated wheat germ tRNA, 1.2 μmol/mL of 3′-amino-3′-deoxy-ATP or 2′-amino-2′-deoxy-ATP. The reaction mixture was incubated for 75 min at 37 °C, after which deprotonization was effected by the addition of 7.5 μmol of CTAB (where the total amount of tRNA was 0.72 mg). The precipitate was dissolved in 1.0 M NaCl and precipitated with twice the volume of 100% EtOH. After several such washes the tRNA was dried under vacuum and dissolved in distilled water.

Aminoacylation of Modified and Unmodified Wheat Germ tRNA. Aminoacylation was accomplished as previously described for *E. coli* tRNA (Fraser & Rich, 1975) with the exception that RNA-free S-100 from wheat germ was used (rather than from *E. coli*). The S-100 preparation, 33.5 μg, was used in a 40-μL reaction mixture. Also, the reaction mixture for aminoacylation of cysteine was 10 mM in dithiothreitol (DTT).

Ribonuclease Digestion of Aminoacyl-tRNA in Ternary Complex. EF-Tu–GDP was first converted to EF-Tu–GTP by adding 730 pmol of the former species to a 75–90 μL reaction mixture containing: 5 μmol of Tris-HCl, pH 7.2, 1 μmol of $MgCl_2$, 0.5 μmol of DTT, 10 μg of pyruvate kinase, 0.6 μmol of phosphoenolpyruvate, 20 nmol of GTP. This reaction mixture was incubated at 37 °C for 10 min and then cooled to 0 °C. Radioactive aminoacyl-tRNA, aminoacyl-2′N-tRNA, or aminoacyl-3′N-tRNA was subsequently added to this reaction mixture and incubated for 5 min at 0 °C. Twenty microliters of pancreatic ribonuclease (0.01 mg/mL) was added to the reaction mixture, incubation was continued at 0 °C, and aliquots were taken at designated times. The samples were precipitated in Cl_3CCOOH and filtered through Whatman glass fiber filters. These filters were dried and then counted in a nonpolar scintillant.

Results

In order to study the isomeric specificity of certain steps in protein synthesis, unfractionated wheat germ or *E. coli* tRNA was modified at the 3′ end, replacing the terminal AMP moiety with 2′-amino-2′deoxy-AMP or 3′-amino-3′-deoxy-AMP. This was accomplished, as previously described (Fraser & Rich, 1973, 1975), by first partially digesting tRNA with snake venom phosphodiesterase (VPD), thereby removing all or part of the C-C-A terminal sequence. In order to assess the extent of digestion, the C-C-A end of the exonuclease treated tRNA was reconstituted using [^{3}H]ATP, unlabeled CTP, and the enzyme CTP(ATP):tRNA nucleotidyltransferase. from *E. coli*. For the wheat germ tRNA this incorporation was approximately 66% of the theoretical maximum. Since the VPD-treated wheat germ tRNA was aminoacylated to an extent less than 1% that of the undigested tRNA, this indicates that in some cases more than three nucleotides were removed from the 3′ terminus, inactivating the tRNA as a substrate for the nucleotidyltransferase.

Table I: The First Four Columns Show the Extent of Aminoacylation of Normal tRNA and Amino tRNA Analogues from Wheat Germ, Expressed as Picomoles of Amino Acid/A_{260} and the Aminoacylation Class. The specificity of *E. coli* tRNA (Fraser & Rich, 1975) is shown in the last column

amino acid	tRNA-C-C-A	tRNA-C-C-2′A_N	tRNA-C-C-3′A_N	wheat germ class	*E. coli* class
Ala	11.57	3.51	0.00	3′	3′
Arg	76.70	7.28	16.40	2′,3′	2′,3′
Asn	16.40	2.52	0.10	3′	3′
Asp	29.20	6.68	0.29	3′	3′
Cys	9.72	7.42	0.00	3′	
Glu	20.70	1.86	1.16	2′,3′	2′
Gln	23.10	0.00	2.46	2′	2′
Gly	96.20	5.67	0.00	3′	3′
His	29.90	2.02	0.02	3′	3′
Ile	6.90	0.00	0.74	2′	2′,3′
Leu	45.50	0.00	6.37	2′	2′
Lys	66.30	5.96	2.61	2′,3′	3′
Met	84.20	6.35	12.00	2′,3′	2′,3′
Phe	29.00	3.34	1.83	2′,3′	2′
Pro	22.90	5.81	0.65	3′	2′,3′
Ser	33.20	9.50	0.67	3′	2′,3′
Thr	39.30	7.62	0.00	3′	3′
Trp	25.50	0.67	0.32	2′,3′	2′,3′
Tyr	2.14	0.00	0.25	2′	2′
Val	24.20	0.00	0.97	2′	2′

Addition of the modified AMP was accomplished by incubating the partially digested tRNA with either 2′-amino-2′-deoxy-ATP or 3′-amino-3′-deoxy-ATP, CTP, and CTP(ATP):tRNA nucleotidyltransferase (from *E. coli*).

One important property of the amino tRNA analogues is that the amide bond formed between such a modified tRNA and an amino acid is stable to base-catalyzed hydrolysis, unlike the ester bond which results from the aminoacylation of unmodified tRNA (Fraser & Rich, 1973). Successful incorporation of the modified AMP onto the 3′ terminus of exonuclease treated tRNA was therefore estimated by measuring the amount of base-stable aminoacyl-tRNA formed after charging with a radioactively labeled amino acid mixture.

Specificity of Wheat Germ Aminoacylation. Aminoacylation was carried out for wheat germ tRNAs using homologous crude synthetase preparations (RNA-free S-100) as previously described for *E. coli* tRNAs (Fraser & Rich, 1975). The results of these assays for all 20 amino acids are presented in Table I. The first column shows the extent of aminoacylation for normal wheat germ tRNA. A significant difference in the amount of charging among the various amino acids is obvious. One or more factors may be responsible for the nonuniform charging observed. In addition to the fact that the unfractionated wheat germ tRNA used would not be

expected to have equal amounts of the different tRNAs, the crude synthetase preparation may have differing amounts of the various enzymes. In addition, no attempt was made to optimize reaction conditions for any charging assay of the individual amino acids.

The initial site of enzymatic attachment of an amino acid to tRNA is deduced by determining whether or not a base-stable amide bond can be formed between that amino acid and either tRNA-C-C-2′A_N or tRNA-C-C-3′A_N. For example, if a certain amino acid is found to be stably attached to tRNA-C-C-2′A_N but not to tRNA-C-C3′A_N after pH 9.5 incubation, then one concludes that the initial site of aminoacylation was the 3′-hydroxyl group (Fraser & Rich, 1975). The synthetase has placed the amino acid onto the 3′-hydroxyl from where it has undergone a rapid, spontaneous migration to the 2′-amino group. Any further isomerization is precluded, resulting in a stable aminoacyl-tRNA-C-C-2′A_N species. The aminoacyl-tRNA is stable since the amino acid is attached to the tRNA via an alkali-stable amide bond instead of the normal alkali-labile ester bond. The failure to isolate an aminoacyl-tRNA-C-C-3′A_N species with this amino acid indicates that charging cannot occur at the 2′-hydroxyl group, nor can the synthetase directly catalyze amide bond formation to the 3′-amino group. Amino acids which fall into this category will be referred to as class 3′, according to the initial site of aminoacylation. The mechanism ascribed to class 2′ charging is, of course, the converse of class 3′.

The second and third columns in Table I show the extent of aminoacylation for both of the tRNA analogues. Charging seen here is significantly less than that observed with normal tRNA. This is perhaps due to the incomplete conversion of tRNA to amino tRNA but it may also reflect a possible decreased interaction of the analogue with synthetase as compared with normal tRNA.

The data presented in Table I have been used to assign each amino acid to one of three classes, which are indicated in the fourth column of the table. Two of these, class 2′ and class 3′, include those amino acids which are stably bound only to tRNA-C-C-3′A_N or only to tRNA-C-C-2′A_N, respectively. The third group, class 2′,3′, includes those amino acids which are found to be covalently bound to both the 2′- and 3′-amino-tRNA analogues. Two possible mechanisms exist which could describe class 2′,3′ (Fraser & Rich, 1975). The synthetase may be acting in a site-specific manner, in which case it has the ability to catalyze directly the formation of an amide bond. Alternatively, the mechanism may be an hydroxyl-specific one in which the site of charging is variable and the amino acid is only attached to a hydroxyl group initially. Results obtained using these amino-tRNA analogues are unable to distinguish between these mechanisms. Some information, however, may be gained by a kinetic analysis of aminoacylation for those amino acids in class 2′,3′.

An example which is interesting to consider is lysine, which is class 3′ in *E. coli* and class 2′,3′ in wheat germ. The kinetic data given in Figure 1 show that lysyl-tRNA-C-C-2′A_N formation occurs more rapidly than formation of lysyl-tRNA-C-C-3′A_N. Charging of the 2′ analogue more closely parallels the aminoacylation kinetics of normal tRNA. This suggests that normal ester bond formation is occurring on the 3′-hydroxyl. Thus, if the reaction is site specific, it is almost certainly specific for the 3′ site. These kinetic data, however, cannot distinguish between the site-specific and hydroxyl-specific mechanisms.

Interaction with EF-Tu. When the role of isomeric specificity in protein synthesis is studied, the class 2′,3′ amino acids

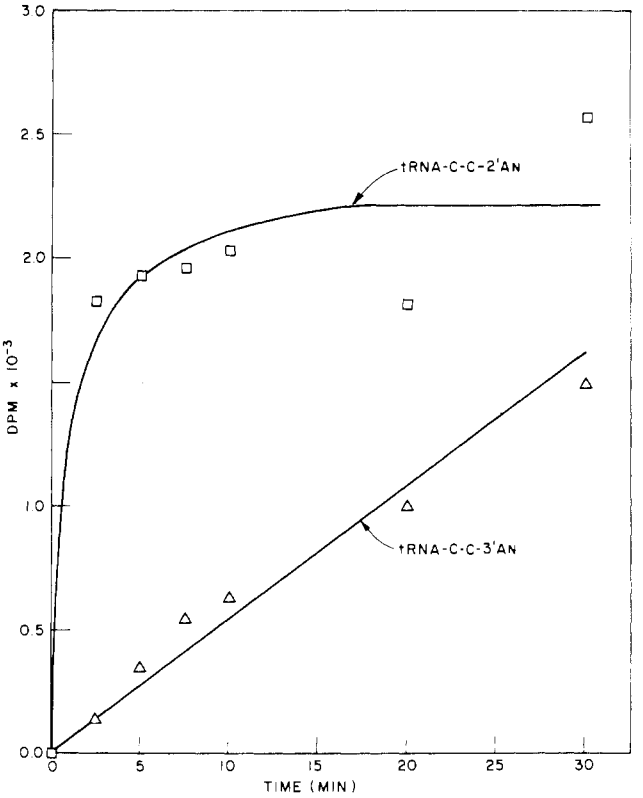

FIGURE 1: Kinetics of wheat germ lysyl-tRNA analogue formation with wheat germ tRNA-C-C-3′A_N and tRNA-C-C-2′A_N. The Cl_3CCOOH-precipitable dpm of [^{3}H]lysine are plotted vs. the time of incubation of the aminoacylation reaction mixture at 37 °C. Incubation conditions are described in Materials and Methods and in Fraser & Rich (1975).

are the most useful because they can be enzymatically attached to both the 2′- and 3′-amino tRNA analogues. For this reason arginine and proline were employed to investigate the interaction of *E. coli* tRNA with elongation factor Tu (EF-Tu) and GTP. A ternary complex composed of these three elements is formed prior to the binding of aminoacyl-tRNA to the ribosomal acceptor site. Ternary complex formation in vivo is apparently necessary for binding of aminoacyl-tRNA to the ribosomal acceptor site (Lucas-Lenard & Lipmann, 1971).

Ternary complex formation was assayed in three different ways. The first method involved binding to nitrocellulose filters (Millipore); whereas the binary complex of EF-Tu and GTP sticks to these filters, a ternary complex with aminoacyl-tRNA falls through, allowing an indirect measurement of complex formation (Gordon, 1968). The second method is based on the finding that aminoacyl-tRNA which is involved in a ternary complex with EF-Tu is more resistant to pancreatic ribonuclease digestion at the 3′ end than when it is not bound to EF-Tu (Jekowsky, 1976).

In the filter binding assay, the release of α-^{32}P-labeled GTP bound to EF-Tu could be detected when normal arginyl- and prolyl-tRNA were added to a binary complex. However, no ternary complex could be detected with either arginyl- or prolyl-2′N- or -3′N-tRNA. The fact that the filter binding assay was able to easily detect ternary complex formation using normal arginyl- or prolyl-tRNAs as substrates suggests that, if a ternary complex does form with either amino-tRNA analogue, then it is weaker than normal. This assay does not allow one to determine whether the analogue complex falls apart as it is filtered or whether the aminoacyl-tRNA analogue's interaction with EF-Tu is unable to induce the con-

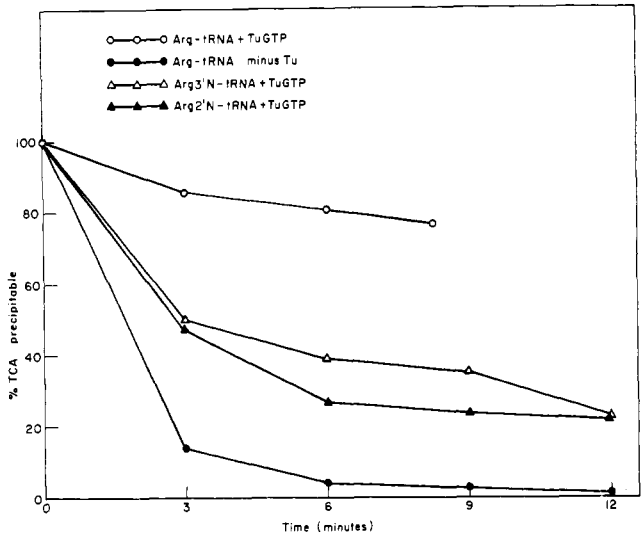

FIGURE 2: Results of the ribonuclease protection assay for ternary complex formation with Arg-tRNA and Arg-amino-tRNA analogues. The percentage of Cl_3CCOOH-precipitable [^{3}H]arginine (compared with zero time) is plotted vs. the length of the 0 °C incubation with pancreatic ribonuclease. The experimental procedure is fully described in Materials and Methods.

formational change which allows the normal ternary complex to pass through a nitrocellulose filter.

Results with the ribonuclease protection assay are shown in Figure 2 for arginyl-tRNA. Normal aminoacyl-tRNA is protected against complete digestion so that a Cl_3CCOOH-precipitable fragment including the 3′ end with its labeled amino acid attached remains intact. This is shown in contrast to a control sample lacking EF-Tu, where digestion fails to leave a labeled acid precipitable fragment. The results with arginyl-3′N-tRNA and arginyl-2′N-tRNA indicate that both isomers are partially protected from digestion. When proline was the amino acid used, the Pro-3′N-tRNA was well protected, while Pro-2′N-tRNA was not protected at all. Several other modified aminoacyl-tRNAs were examined, including Leu-3′N-tRNA, His-2′N-tRNA, and Phe-3′N-tRNA. In these cases there appears to be little, if any, complex formation as assayed by ribonuclease protection. A comparison of these results with those found for unmodified aminoacyl-tRNA also suggests that the ternary complex formed with the amino-tRNA analogues is weaker than that formed with normal aminoacyl-tRNA.

In the third, indirect method, poly(U)-dependent EF-Tu catalyzed binding of the Phe-3′N-tRNA to ribosomes at low magnesium concentrations has been measured. As can be seen in Table II, the binding of normal Phe-tRNA is enhanced fourfold, while that of Phe-3′N-tRNA is increased twofold in the presence of EF-Tu. It is possible that a weak ternary complex is formed as an intermediate in this binding, although these results do not prove that the Phe-3′N-tRNA interacts directly with EF-Tu.

Table II: Poly(U)-Directed Binding of *E. coli* Phe-tRNA and Phe-3′N-tRNA to Ribosomes at 6 mM Mg^{2+} [a]

	pmol bound/A_{260} ribosomes	
	Phe-tRNA	Phe-3′N-tRNA
complete system	1.62	1.20
minus EF-Tu	0.43	0.62
minus ribosomes	0.15	0.13

[a] The assay incubations contained per mL: either 20.0A_{260} units of salt washed ribosomes or no ribosomes as indicated, 80 μg of poly(U), either 880 pmol of purified EF-Tu or no EF-Tu as indicated, 0.2 μmol of GTP, 60 μmol of PEP, and 80 μg of pyruvate kinase (400 units/mg). The mixture was buffered with 50 mM Tris-HCl, pH 7.8, and contained 80 mM NH_4Cl, 6 mM β-mercaptoethanol and 6 mM $Mg(OAc)_2$. After an initial incubation for 5 min at 37 °C, the reaction mixtures were cooled on ice and either 242 pmol of [^{14}C]Phe-tRNA (specific activity, 466 μCi/uM) or 258 pmol of [^{3}H]Phe-3′N-tRNA (specific activity, 1.1 mCi/μM) was added per mL. Incubation was continued on ice for 10 min, at which time the reaction mixture was filtered through a nitrocellulose filter. The filters were washed with 10 mL of wash buffer containing 5 mM Tris-HCl, pH 7.8, 80 mM NH_4Cl, 6 mM β-mercaptoethanol, and 6 mM $Mg(OAc)_2$. After washing, the filters were dried and radioactivity was determined in a liquid scintillation counter.

Discussion

Aminoacyl-tRNA exists as a mixture of two species in solution; these two forms differ with regard to the position of the amino acid on the 3′-terminal ribose ring. Their interconversion is mediated by a 2′-hydroxyl → 3′-hydroxyl acyl migration which has a half-time of equilibration of 0.2 ms (Griffin et al., 1966). This obviously makes any investigation involving positional specificity of the amino acid difficult, if not impossible, to pursue. For this reason several analogues of tRNA which preclude equilibration of the amino acid between the 2′ and the 3′ positions have been utilized. One way in which this has been accomplished is by replacing the terminal adenosine on the C-C-A end of the tRNA with either 2′- or 3′-deoxyadenosine (Sprinzl & Cramer, 1973; Chinali et al., 1974; Hecht & Chinault, 1976; Chinault et al., 1977) or 2′- or 3′-*O*-methyladenosine (Hecht et al., 1973). A second approach has been to similarly use an adenosine analogue in which an amino group replaces the 2′- or 3′-hydroxyl group (Fraser & Rich, 1973, 1975). Aminoacylation of this latter species yields a charged tRNA which is stable to base-catalyzed hydrolysis. An advantage conferred by the amino tRNA analogue as compared with the deoxy-tRNA analogues is that contamination with unmodified tRNA is not a problem; normal aminoacyl-tRNA which may contaminate the analogues after a charging reaction can be eliminated by incubation at pH 9.5, leaving only alkali-stable aminoacyl-2′N-tRNA or aminoacyl-3′N-tRNA.

The results presented here offer an opportunity to compare the specificities of aminoacylation for two organisms, both determined with the amino tRNA analogues. We have found a conservation of aminoacylation specificity between *E. coli* and wheat germ (Table I). From the results obtained using our tRNA analogues, we note that ten amino acids are unambiguously attached to their respective tRNAs with the same specificity in both *E. coli* and wheat germ. Furthermore, in no case has a switch in specificity been observed.

We may also compare the aminoacylation specificities obtained with the amino-tRNA analogues with those found by others using the deoxy-tRNA analogues (Table III). In *E. coli* there is excellent agreement of specificities using these two different analogues. The deoxy-tRNA results further indicate that in most cases in *E. coli* the mechanism of aminoacylation for class 2′,3′ amino acids is site specific and not hydroxyl specific. For example, serine can be base stably bound to both *E. coli* tRNA-C-C-2′A_N and tRNA-C-C-3′A_N (Fraser & Rich, 1975), but it is found by Hecht & Chinault (1976) and Sprinzl & Cramer (1975) to charge only tRNA-C-C-2′dA. When given a free 2′-hydroxyl (tRNA-C-C-3′dA), we observe no charging. It is therefore likely that tRNA-C-C-3′A_N is aminoacylated with serine by direct amide bond formation involving the 3′-amino. Thus, assuming that they are valid analogues, we may use the deoxy-tRNA results to resolve ambiguity of aminoacylation specificity for the class

Table III: AComparison among *E. coli*, Wheat Germ, Yeast, and Calf Liver tRNAs with Regard to the Site(s) at Which a Given Amino Acid is Found to Aminoacylate[a]

amino acid	*E. coli* amino-tRNA[b]	*E. coli* deoxy-tRNA[c]	deoxy-tRNA[d]	yeast deoxy-tRNA[d]	wheat germ amino-tRNA[e]	calf liver deoxy-tRNA[f]
Ala	3′	U	3′	3′	3′	3′
Arg	(2′, 3′)*	2′	2′	2′	(2′,3′)*	2′
Asn	3′	2′	2′, 3′	2′, 3′	3′	2′, 3′
Asp	3′	U	U	2′, 3′	3′	3′
Cys	U	2′, 3′	2′, 3′	2′, 3′	3′	3′
Glu	2′	U	2′	U	(2′, 3′)*	U
Gln	2′	U	U	3′	2′	U
Gly	3′	3′	3′	3′	3′	3′
His	3′	3′	3′	3′	3′	3′
Ileu	(2′, 3′)*	2′	2′	2′	2′	2′
Leu	2′	2′	2′	2′	2′	2′
Lys	3′	3′	3′	3′	(2′, 3′)*	3′
Met	(2′, 3′)*	2′	2′	2′	(2′, 3′)*	U
Phe	2′	2′	2′	2′	(2′, 3′)*	2′
Pro	(2′, 3′)*	U	3′	3′	3′	U
Ser	(2′, 3′)*	3′	3′	3′	3′	3′
Thr	3′	3′	3′	3′	3′	3′
Trp	(2′, 3′)*	2′	2′	3′	(2′, 3′)*	3′
Tyr	2′	2′, 3′	2′, 3′	2′, 3′	2′	2′, 3′
Val	2′	2′	2′	2′	2′	2′

[a] A number of different 2′- and 3′-tRNA analogues are used as indicated. U designates uncertainty; * designates the initial site of aminoacylation for these amino acids cannot be deduced from the amino-tRNA charging data (see text). [b] Fraser & Rich, 1975. [c] Sprinzl & Cramer, 1975. [d] Hecht & Chinault, 1976. [e] This work. [f] Chinault et al., 1977.

2′,3′ amino acids. In *E. coli* it is found that arginine, isoleucine, methionine, and tryptophan are aminoacylated at the 2′ position, while proline and serine are aminoacylated at the 3′ position. In wheat germ, employing the amino tRNAs, we have found that isoleucine is unambiguously aminoacylated at the 2′ position and both serine and proline are aminoacylated at the 3′ position. Kinetic data previously reported (Fraser & Rich, 1975) for aminoacylation of *E. coli* amino-tRNA analogues also support this comparison.

If the mechanism of aminoacylation for the class 2′,3′ synthetase is indeed a site-specific one, then the kinetics shown in Figure 1 for wheat germ lysyl-tRNA formation indicate that the observed shift for lysine from class 3′ in *E. coli* to class 2′,3′ in wheat germ represents a change in the ability of the synthetase to catalyze directly amide bond formation, rather than a change in the positional specificity of aminoacylation. Faster aminoacylation of tRNA-C-C-2′A_N than of tRNA-C-C-3′A_N indicates that the cognate synthetase is specific for the 3′ site, the same specificity as the *E. coli* enzyme.

In summary, the site specificity of aminoacylation for wheat germ tRNA has been found to be identical with that for *E. coli* tRNA for 13 amino acids. In the remaining cases including lysyl-tRNA, we have been able to observe no differences in aminoacylation specificities between *E. coli* and wheat germ.

In addition, the evidence suggests that during evolution the capability of aminoacyl-tRNA synthetase to catalyze the direct formation of amide bonds has changed considerably, some having lost the ability while others have gained it. There does not appear to be any discernible pattern in these changes and it seems likely that the ability of a synthetase to catalyze the formation of amide bonds is not directly selected for or against in evolution.

When the conservation of aminoacylation specificities determined using the amino tRNA analogues in *E. coli* and wheat germ are considered along with those determined using the deoxy-tRNA analogues in *E. coli*, yeast, and calf liver, it is clear that there must be a strong selective advantage for the evolutionary preservation of this specificity. Recent findings suggest that there may be a relationship between the isomeric specificity of aminoacylation and the checking mechanism which guards against misacylation (von der Haar & Cramer, 1976; Fersht & Kaethner, 1976; Igloi et al., 1977). Thus, the initial sites of aminoacylation may remain invariant throughout evolution due to the pressure to continually maintain an error-free aminoacylation checking mechanism.

Aminoacylation occurs prior to the interaction of tRNA with the ribosome. After charging occurs, the resultant aminoacyl-tRNA is positioned in the ribosomal acceptor site by EF-Tu and the GTP complexed with EF-Tu is hydrolyzed in the process (Lucas-Lenard & Lipmann, 1971). We have attempted to determine whether the interaction of aminoacyl-tRNA with EF-Tu is specific for either the 2′ or 3′ isomer of aminoacyl-tRNA.

The results reported here for *E. coli* arginyl-tRNA show that the protein molecule interacts with both the 2′- and the 3′-amino tRNA analogues. Hecht et al. (1977) have recently studied ternary complex formation using the deoxy analogues of various aminoacyl-tRNAs. Their results and conclusions are consistent with ours, showing interaction of both isomeric species with EF-Tu and GTP. It should be noted that this finding does not a priori rule out the possibility that one isomeric species may normally be preferred over the other during ternary complex formation; however, since aminoacylation can occur at either the 2′ or 3′ positions, depending upon the particlar amino acid, it is not surprising that EF-Tu is able to form a ternary complex with both isomers.

In addition to the results for arginyl-tRNA, however, we also found that, with prolyl-tRNA, the 3′ isomer formed a ternary complex with EF-Tu–GTP, while the 2′ isomer did not. A number of other aminoacyl-tRNA analogues were also unable to form complexes. One of those unable to form a complex, Phe-3′N-tRNA, was, however, able to bind to the ribosome at higher levels when EF-Tu was added to the assay mixture. It is clear that the presence of an amide bond linking the amino acid and tRNA, rather than the normal ester bond, markedly weakens the interaction of the aminoacyl-tRNA analogue with EF-Tu as has been stated by Sprinzl et al. (1977). Due to the variable results of the nuclease protection test, we conclude that the amino acid side chain does play some role in the interaction of the analogues with EF-Tu. This may or may not be the case with normal aminoacyl-tRNA.

In light of the finding that both the 2′- and the 3′-positional isomers of aminoacyl-tRNA are able to interact with EF-Tu–GTP, it seems likely that the isomeric specificity of aminoacylation plays no obvious role in ribosomal protein synthesis. This isomeric specificity could, however, play an important part in the synthetase mechanism preventing mischarging.

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Simple Isolation of DNA Hydrophobically Complexed with Presumed Gene Regulatory Proteins (M_3)†

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ABSTRACT: Chromatin from chicken reticulocytes and mouse Ehrlich ascites tumor cells has been extracted with 2 M NaCl, leaving a portion of the DNA still complexed with a fraction of nonhistones (designated M_3, since it can be dissociated from DNA in solutions of 3 M NaCl containing 5 M urea). The DNA complexed with M_3, separated from the bulk DNA by centrifugation, was found to contain sequences poorly represented in bulk DNA. Specifically we found that DNA-M_3 complexes isolated from chicken reticulocyte chromatin were enriched in globin gene sequences by 20-fold relative to unfractionated DNA and by over 1000-fold relative to DNA rendered free of protein following the extraction of chromatin with 2 M NaCl. We have therefore isolated DNA fractions complexed with M_3 which are enriched in specific sequences as may be determined by M_3.

In this laboratory we have performed numerous experiments in order to classify the nonhistone chromosomal proteins into groups possessing distinguishable properties (see review by Bekhor, 1978). In the following experiments we have continued to study the characteristics of those nonhistones which are tightly bound to DNA. Those tightly bound proteins, designated M_3, require 3 M NaCl–5 M urea for dissociation and constitute about 5% of the total nonhistones.

The discovery of nucleosomes in chromatin (Olins & Olins, 1974; Kornberg, 1974; Sahasrabuddhe & Van Holde, 1974) has advanced our understanding of how the DNA is packaged with histones (Felsenfeld, 1978). The finding (Lacey & Axel, 1975; Kuo et al., 1976; Garel & Axel, 1976) that active genes may be clustered in nucleosomes suggested that both active and inactive chromatin may contain the general subunit structures with a repeating DNA chain length of 140 to 200 base pairs (Woodcock, 1973; Olins & Olins, 1973; Finch et al., 1975; Oudet et al., 1975; Johnson et al., 1976). Additional findings by Weintraub & Groudine (1976) and Levy & Dixon (1977) suggested that active genes may be more susceptible to digestion with DNase I than inactive genes. These findings supported the hypothesis that nucleosomes containing active genes are less rigid than DNA–histone complexes (Pyhtila et al., 1976). Such data led to the conclusion that nucleosomes are essential to the function of the genome, leaving unresolved the role of nonhistones in chromatin.

We have reported (Bekhor & Samal, 1977) that the tightly bound nonhistones showed dramatic effects on the transcription of DNA complexed with histones. Gadski & Chae (1978) reported that specific genetic activity might be determined by the DNA binding nonhistone fraction which reassociates prior to histones during reconstitution. Paulson & Laemmli (1977) and Adolph et al. (1977) have shown that HeLa cell metaphase chromosomes possessed a highly organized structure retaining the familiar metaphase morphology even after removal of the histones and most of the nonhistones. This structure was stabilized by a small number of nonhistones, which the authors (Adolph et al., 1977) called scaffolding proteins. The "scaffolding proteins", the tightly bound nonhistones of Gadski & Chae (1978), and our M_3 (Bekhor & Samal, 1977) probably are of the same class of proteins. Therefore, it is apparent that M_3 may contain proteins responsible for particular effects on the genome in general.

For these various reasons we have postulated that the removal of histones from chromatin into 2 M NaCl results in a fraction of DNA that is complexed with M_3 and enriched in specific sequences as determined by M_3. In previous communications (Samal & Bekhor, 1977; Bekhor & Samal,

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