*TRANSFER RNA, I. ISOLATION AND CHARACTERIZATION OF A NEW YEAST ALANINE TRANSFER RNA**

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During attempts to prepare homogeneous tRNA $^{ala}_{yeast}$, we obtained material with very high alanine acceptor activity whose T1 RNase digest differed markedly from that reported by Penswick and Holley.[1] This material proved to be a mixture of tRNA$^{ala\ Iab}$ (see ref. 2) described by Holley *et al.*[3] and a new tRNA^{ala}, tRNA$^{ala\ II}$. By a combination of techniques described in this paper, we have been able to separate tRNA$^{ala\ Iab}$ from tRNA$^{ala\ II}$ and to prepare material with close to theoretical acceptor activity.

Examination of the T1 RNase digest of tRNA$^{ala\ II}$ indicates that its primary sequence is quite different from that of tRNA$^{ala\ Iab}$. The most interesting change appears to be in the anticodon, since the ribosome binding response of tRNA$^{ala\ Iab}$ is different from that of tRNA$^{ala\ II}$. This is of particular interest because the genetic code, as it now stands,[4] has four alanine codons $\left(\begin{matrix}\text{U}\\\text{C}\\\text{A}\\\text{G}\end{matrix}\right)$, and according to the wobble hypothesis,[5] *at least* two RNA species with different anticodons are required to recognize all of these codons. To our knowledge, no prior evidence for the existence of a second tRNA^{ala} in yeast has been reported, perhaps because the work on codon response by tRNA$^{ala}_{yeast}$ has been done with highly purified tRNA$^{ala\ Iab}$.[6, 7]

Materials and Methods.—(a) *Preparation of activating enzyme:* This was done by the method of Hoskinson and Khorana[8] from fresh bakers' yeast (Fleishmann's) with the following modifications. After the streptomycin sulfate precipitation the supernatant was treated with ammonium sulfate, and the protein precipitating between 40 and 80% saturation was collected and dissolved in 60 ml 0.05 *M* Tris (HCl), pH 7.5, + 0.005 *M* $MgCl_2$ + 0.02 *M* mercaptoethanol. The solution was brought to 50% (v/v) glycerol, and 2-ml aliquots were stored at −20°C in separate tubes. Just before use an aliquot of the crude enzyme was freed of low-molecular-weight material by gel filtration on a 2 × 15-cm Sephadex G-25 column, equilibrated at 5° with 0.05 *M* Tris (HCl), pH 7.0, + 0.001 *M* sodium EDTA.

(b) *Assay of tRNA^{ala}:* The reaction was carried out at 25°C in a mixture of 50 m*M* Tris (HCl), pH 7.0; 1 m*M* sodium EDTA; 30 m*M* $MgCl_2$; 5 m*M* sodium ATP; 0.04 m*M* L-alanine-C^{14} (Schwarz BioResearch, Inc.), 41.5 cpm/pmole; 0.04 A_{260} units tRNA^{ala} (80 pmole); and 300 μg enzyme protein in a final volume of 0.25 ml. At 10 min, 100-μl aliquots were applied to 2.5-cm Whatman 3 MM filter disks[9] and washed for 10 min each (10 ml/filter) with ice-cold 10% TCA, ice-cold 5% TCA (two times), ethanol:ether 50:50 (v/v), and ether. The filters were dried for 15 min under infrared lamps and placed in scintillation vials with 5 ml of 4% Liquifluor (Nuclear-Chicago) in toluene. Radioactivity was counted with a Nuclear-Chicago Mark I scintillation spectrometer.

(c) *T1 digestion and DEAE-cellulose chromatography:* Purified tRNA^{ala} was digested with T1 RNase and the nucleotide fragments were separated on DEAE-cellulose by a modified procedure of Penswick and Holley.[1] Before use, T1 RNase (5000 units, Calbio-

chem) in 1 ml 0.02 *M* Tris (HCl), pH 7.5, was heated at 85°C for 5 min to inactivate a contaminating phosphatase activity.

A solution containing 5.0 A_{260} units of tRNA was dialyzed three times for 2 hr against 4 liters H_2O and then reduced to a volume of 0.1 ml (30°C, 18 mm Hg). The tRNA was incubated at 37°C with 0.25 ml 0.1 *M* Tris (HCl), pH 7.5, and 60 μl (750 units[10]) T1 RNase solution (see above). After 3 hr, 5.0 ml of 0.2 *M* Tris (HCl), pH 8.0, in 7 *M* urea was added and the digested tRNA pumped onto a 0.5 × 120-cm DEAE-cellulose column. The fragments were eluted with a 600-ml linear gradient from 0 to 0.45 *M* NaCl in 0.02 *M* Tris (HCl), pH 8.0, + 7 *M* urea[1, 11] at a flow rate of 15 ml/hr. The effluent was passed through a Gilford flow cell (1-cm light path) and monitored with a Gilford model 2000 absorbance recorder at 260 nm with a full scale of 0.270. It should be noted that these data were obtained with $^1/_8$ the sample size used by Penswick and Holley.[1]

(d) *Base analysis:* Oligonucleotides from a T1 RNase digest (16 A_{260} of $tRNA^{ala\ II}$), fractionated as above, were freed of urea by passage through a 1 × 100-cm column of Biogel P2 (Bio-Rad) with water as an eluent. The solutions were evaporated to dryness (30°C, 18 mm Hg), dissolved in 0.2 ml of 0.05 *M* NaOAc buffer, pH 4.5, and treated first with 20 μl of T2 RNase solution[12] (20 units) at 37° for 15 hr. Then 0.1 ml of 0.5 *M* Tris (HCl), pH 8.2, and 10 μl of alkaline phosphatase (Worthington Biochemicals, BAPC grade, 3 units) were added, and the incubation was continued for 3 hr at room temperature. The nucleoside composition of each digest was determined with a rapid micromethod of ion exchange on Dowex 50W, X4, "minus 400" mesh. The details of this procedure will be published elsewhere.

(e) *Ribosome binding:* Ribosome binding of alanyl-$tRNA^{ala}$ was carried out as described by Nirenberg and Leder[13] except that 0.44 *M* NH_4Cl was used in place of 0.05 *M* KCl in the reaction mixture. For our initial experiments the triplets GpCpX (X = U, C, A, G) were supplied by Dr. H. G. Khorana; for later experiments they were synthesized by the method of Sekiya *et al.*,[14] from guanosine 2′:3′ cyclic phosphate and the appropriate dinucleoside monophosphates by means of the synthetic reaction of T1 RNase. Purified ribosomes were supplied by Drs. A. J. Wahba and K. Iwasaki of this department.

(f) *Purification of $tRNA^{ala}$:* Crude transfer RNA from *Saccharomyces cerevisiae* (Schwarz BioResearch, Inc.) was enriched tenfold in $tRNA^{ala}$ by counter-double-current distribution (CDCD)[15] using a 100-tube all-glass apparatus (H. O. Post Scientific Instrument Co.). The distribution was carried out at 24 ± 0.5°C by means of the redistribution solvent system of Apgar *et al.*[16] with 22 ml upper phase and 10 ml lower phase per tube. The results of this fractionation will not be described here (to be published elsewhere) since special equipment is necessary for this step and this method is not a prerequisite for the procedure that follows.

A 2 × 19-cm column of BD-cellulose[17] (Schwarz BioResearch, Inc.) was washed with 2 *M* NaCl + 25% ethanol, equilibrated with 0.3 *M* NaCl + 0.01 *M* $MgSO_4$ + 0.01 *M* NaOAc, pH 4.5 (solution I), and repacked. The partially purified $tRNA^{ala}$ (440 A_{260} containing 172 nmoles $tRNA^{ala}$) in 10 ml of solution I was pumped onto the column at a flow rate of 1.3 ml/min. The column was washed with 90 ml of solution I and then eluted with 0.8 *M* NaCl + 0.01 *M* $MgSO_4$ + 0.01 *M* NaOAc, pH 4.5 (solution II), until the A_{260} dropped to about 0.1. The material eluted with solution II was pooled, concentrated to 12 ml (18 mm Hg, 30°C), and precipitated with 24 ml ethanol. The tRNA was dissolved in 3 ml of H_2O and reprecipitated with 6 ml of ethanol. This procedure was repeated and the final precipitate was washed with 5 ml ethanol followed by 5 ml ether before it was allowed to air-dry overnight.[18]

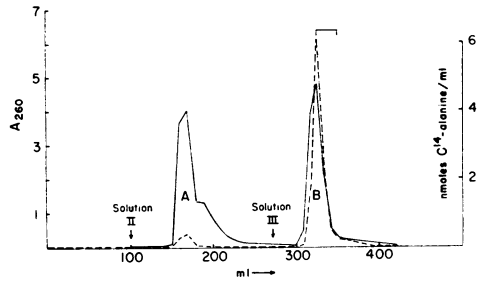
This tRNA (285 A_{260} units containing 160 nmoles $tRNA^{ala}$) was esterified with alanine in three 10-ml incubation mixtures of the following composition: 50 m*M* Tris (HCl), pH 7.0; 1 m*M* sodium EDTA; 30 m*M* $MgCl_2$; 5 m*M* sodium ATP; 1 m*M* sodium CTP; 0.11 m*M* L-alanine-C^{14} (3.77 cpm/pmole); and 20 mg enzyme protein. After 30 min at 25°C each reaction mixture was brought to 0°C, and 0.2 ml 1 *M* NaOAc, pH 5.0, was added. The three solutions were pooled and applied to a 1 × 5-cm DEAE-cellulose

column equilibrated with 0.2 *M* NaCl + 0.1 *M* NaOAc, pH 5.0. The column was washed with 20 ml of equilibrating buffer to remove protein, ATP, and alanine. The tRNA was eluted with 10 ml 2 *M* NaCl + 0.1 *M* NaOAc, pH 5.0, and precipitated with 20 ml ethanol. The centrifuged precipitate was washed once with 5 ml ethanol and stored overnight at −20°C.

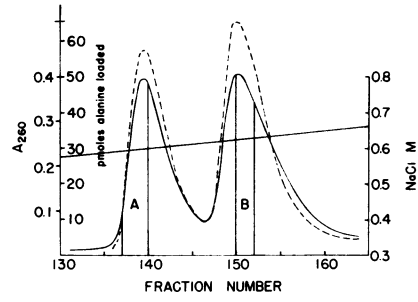
This material was converted to N-phenoxyacetyl-L-alanyl-tRNA^{ala} according to the procedure of Tener *et al.*[19] Since the details of this method are still unpublished, the conditions we used will be described here.

The esterified tRNA preparation (above) was dissolved in 5 ml of 1.0 *M* triethanolamine (HCl) + 0.01 *M* $MgSO_4$, pH 4.0, cooled to 0°C, and a solution containing 12.5 mg of the N-hydroxysuccinamide ester of phenoxyacetic acid (Schwarz BioResearch, Inc.) in 0.5 ml of tetrahydrofuran was added. The pH was adjusted quickly to 8.0 with 1 *M* NaOH. After 10 min the pH was brought to 4.5 with 30 μl glacial acetic acid. The tRNA was precipitated with 1.0 ml solution II (see above) and 3 vol ethanol. The centrifuged precipitate was washed with 5.0 ml of ethanol and dissolved in 10 ml solution I.

This solution (254 A_{260} units containing ~120 nmoles phenoxyacetylalanyl-tRNA) was applied to a 2 × 19-cm column of BD-cellulose that had been washed and equilibrated as before. Elution was carried out as before. Phenoxyacetylalanyl-tRNA was eluted with solution III. Fractions containing radioactivity (Fig. 1) were pooled and concen-



(*Left*) FIG. 1.—Isolation of N-phenoxyacetyl-L-alanyl-tRNA^{ala} on BD-cellulose. Absorbance, ——; radioactivity (100-μl aliquots dried on Whatman 3 MM disks), - - - -. For the composition of solutions II and III, see text and ref. 18.



(*Right*) FIG. 2.—Separation of tRNA$^{ala\ Iab}$ and tRNA$^{ala\ II}$ by chromatography on BD-cellulose using gradient elution. Absorbance, ——. Radioactivity, - - - -. The relative peak areas do *not* represent the amounts of each tRNA present in yeast because fractions from CDCD rich in tRNA$^{ala\ II}$ were used in the purification. The T1 RNase patterns from pool *A* and from pooled fractions 140–146 were identical, which indicates the homogeneity of the entire peak.

trated. The tRNA was precipitated as before. The precipitate was dissolved in 5.0 ml of 1 *M* Tris (HCl), pH 9.0, and incubated at room temperature for 2 hr to remove the phenoxyacetylalanyl group. The pH was adjusted to pH 7.0 with acetic acid and the solution was applied to a 1 × 100-cm column of Sephadex G-100 (Pharmacia) which had been equilibrated with 0.01 *M* $MgCl_2$. The tRNA was eluted with 0.01 *M* $MgCl_2$ at room temperature as a symmetrical peak. The pooled fractions contained highly purified tRNA^{ala} (39 A_{260} units containing 66 nmoles tRNA^{ala} sp. act. 1700 pmole/A_{260}) in 38% yield. This material is a mixture of tRNA$^{ala\ Iab}$ and tRNA$^{ala\ II}$. These were separated as follows.

(g) *Separation of tRNA$^{ala\ Iab}$ and tRNA$^{ala\ II}$*: The tRNA^{ala} (§*f* above) was fractionated on a 2 × 19-cm column of BD-cellulose with a linear gradient elution[17] with 500 ml of solution I in the mixer and 500 ml of solution II in the reservoir. Five-ml fractions were collected at a flow rate of 0.5 ml/minute. The results are shown in Figure 2. The appropriate fractions were pooled as shown in Figure 2 and dialyzed against water. The

specific activity of $tRNA^{ala\ Iab}$ (peak *B*) was 1600 pmoles/A_{260}; and that of $tRNA^{ala\ II}$ (peak *A*), 1450 pmoles/A_{260}.

Results and Discussion.—(a) *Isolation and characterization of* $tRNA^{ala\ II}$: Preparation of biologically pure $tRNA^{ala}$ was achieved in two steps. First, crude $tRNA_{yeast}$ was fractionated by CDCD. Then the partially purified $tRNA^{ala}$ was converted to its N-phenoxyacetyl-L-alanyl derivative as shown in Chart 1 and fractionated on BD-cellulose.[19] The $tRNA^{ala}$ prepared in this way (for details see *Materials and Methods*, § *f*) had a specific activity of 1700 pmoles/A_{260} after removal of the phenoxyacetylalanyl group. The biological activity of this material was much higher than the *best* sample we have ever prepared by CCD or CDCD (~1200 pmoles/A_{260}, <68% of theory) and by phosphate analysis has a specific activity within 5 per cent of the theoretical value.

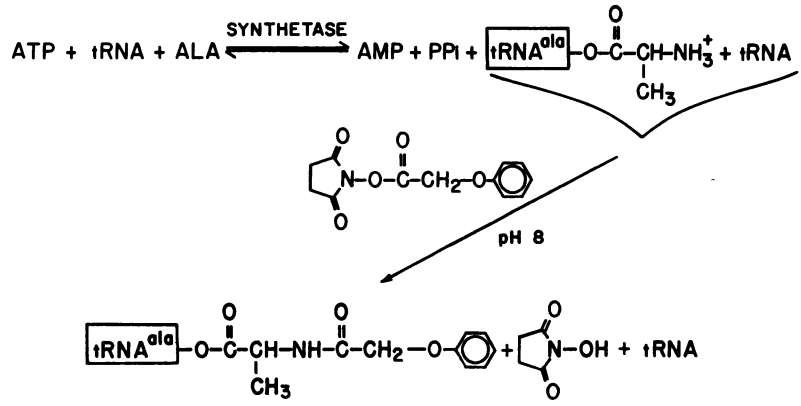


CHART I

This highly active $tRNA^{ala}$ was digested with T1 RNase, and the resulting oligonucleotides were fractionated by DEAE chromatography in the presence of 7 *M* urea[1, 11] (results shown in Fig. 3*a*). This material was shown to be a mixture by comparison with the pattern obtained from $tRNA^{ala\ Iab}$ (Fig. 3*b*). With a specific activity of 1700 pmoles/A_{260}, this was clearly a mixture of $tRNA^{ala\ Iab}$ and a new species of tRNA with alanine acceptor activity, $tRNA^{ala\ II}$. These two alanine tRNA's were separated by chromatography on BD-cellulose (see §*g* of *Materials and Methods*). As shown in Figure 2, the two peaks account for more than 90 per cent of the material applied to the column, and the specific activity of the peak tubes was *A* = 1450; *B* = 1600 pmoles/A_{260}. These represent minimal values since the assays were done directly on aliquots from each peak and the salt present in these solutions is known to inhibit the enzymatic attachment of alanine to tRNA to some extent.

Samples were taken from the areas indicated in Figure 2 and their T1 RNase digests were examined in the usual way. The pattern for peak *B* material (Fig. 2) is shown in Figure 3*b*. Comparison of this pattern with that of Penswick and Holley[1] shows that this material is $tRNA^{ala\ Iab}$. Figure 3*c* is clearly different

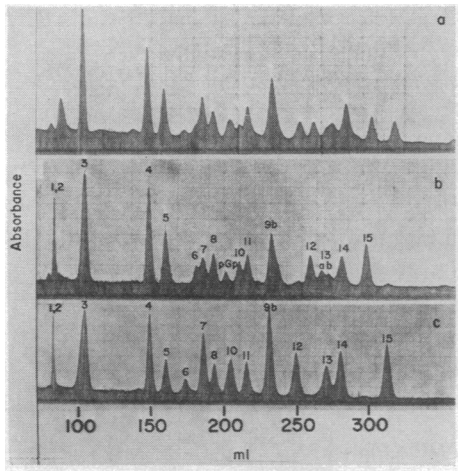


FIG. 3.—Fractionation of T1 RNase digests of highly purified $tRNA^{ala}$ by chromatography on DEAE-cellulose.[1] (*a*) Material from peak *B*, Fig. 1; (*b*) $tRNA^{ala\ Iab}$ from peak *B* of Fig. 2; (*c*) $tRNA^{ala\ II}$ from peak *A* of Fig. 2.

from 3*b*, which shows that peak *A* (Fig. 2) contains a new $tRNA^{ala}$, $tRNA^{ala\ II}$. The summation of these two patterns (Fig. 3*b* and *c*) is consistent with the pattern of the mixture (Fig. 3*a*) from which they were obtained.

A number of differences are apparent between the tRNA's. The pattern of $tRNA^{ala\ II}$ (Fig. 3*c*) has displaced peaks 6, 10, 12, and 15, compared to the peaks in $tRNA^{ala\ Iab}$. In addition, peak 7 has about twice the area in $tRNA^{ala\ II}$. The base compositions of T1 RNase-produced oligonucleotides from $tRNA^{ala\ Iab}$ and $tRNA^{ala\ II}$ are compared in Table 1. It is clear from these data that the primary structure of $tRNA^{ala\ II}$ differs in several respects from that of $tRNA^{ala\ Iab}$.

Peak 15 (Fig. 3) is of particular interest since it contains the "wobble base" of the anticodon of $tRNA^{ala\ Iab}$.[3, 20] The position and the base composition of peak

TABLE 1. *Comparison of the composition of T1 RNase-produced oligonucleotides from $tRNA^{ala\ Iab}$ and $tRNA^{ala\ II}$.*

Peak no.[a]	$tRNA^{ala\ Iab}$[b]	$tRNA^{ala\ II}$[c]
1 + 2	$Um^1G + Cm_2^2G$	$Um^1G + Cm_2^2G$
3	9G	6G
4	UG + 4CG	4CG
5	2AG	1.5AG
6	hUCG	hUhUG
7	hUAG	(hUA)G + UUG
8	UAG	(CA)G
pGp	G	—
10	$CmI\psi G$	AAG + G from pGp
11	$T\psi CG$	$(T\psi C)G$
9*b*	UC_4A_2	$(UC_4A)A + (ACN)G$[d]
12	AC_2UG	(ACUN)G[d]
13*a*	$hUUC_3G$[e]	—
13*b*	U_2C_3G[f]	$(UAC_3)G$
14	AU_2C_2G	$(AU_2C_2)G$
15	U_3C_4I	$(U_4C_4)G$

[a] Peaks are numbered as in Fig. 3.
[b] From Penswick and Holley.[1]
[c] These data are from a single analysis and are tentative.
[d] N = unknown base.
[e] $tRNA^{ala\ Ia}$ only.
[f] $tRNA^{ala\ Ib}$ only.

15 from $tRNA^{ala\ II}$ are consistent with replacement of the wobble base, I, in the octanucleotide CUCCCUU*I* in $tRNA^{ala\ Iab}$ with U in $tRNA^{ala\ II}$ to give the nonanucleotide sequence CUCCCUU*U*G. This would give the anticodon ugc in $tRNA^{ala\ II}$.

(b) *Ribosome binding:* If the anticodon in $tRNA^{ala\ II}$ is ugc, as suggested above, then ala-$tRNA^{ala\ II}$ should respond to the triplets GCA and GCG in the binding assay.[5] As shown in Table 2, ala-$tRNA^{ala\ II}$ does respond to these codons at 0.02 *M* Mg^{2+}. Furthermore, ala-$tRNA^{ala\ Iab}$ does not respond to GCG at all under these conditions as expected from the wobble hypothesis and in agreement with previous work.[6, 7] It should also be noted that the response of ala-$tRNA^{ala\ II}$ to the codon GCA is much greater than that of ala-$tRNA^{ala\ Iab}$ to the same codon, as expected for the anticodons ugc and igc, respectively. However, ala-$tRNA^{ala\ II}$ responds to the codons GCU and GCC. This does not agree with the expectation for a tRNA with an anticodon ugc. Furthermore, the response to GCU is much too large to ignore.

TABLE 2. *Triplet-stimulated binding of ala-$tRNA^{ala}_{yeast}$ to E. coli ribosomes.*

	Aminoacyl-tRNA Bound to Ribosomes (pmoles)*			
	Alanyl-C^{14}-$tRNA^{ala\ Iab}$ (13 pmoles)		Alanyl-C^{14}-$tRNA^{ala\ II}$ (16 pmoles)	
Triplet added (0.1 A_{260})	0.01 *M* Mg^{2+}	0.02 *M* Mg^{2+}	0.01 *M* Mg^{2+}	0.02 *M* Mg^{2+}
GCU	0.32	2.18	2.85	7.78
GCC	0	0.78	0	0.93
GCA	0.14	1.59	2.82	7.73
GCG	0	0	0	2.16
Anticodon:	igc		ugc (?) + ?	

* Two A_{260} units of ribosomes were used. These values represent the binding of alanyl-C^{14}-tRNA's to *E. coli* ribosomes in the presence of triplet minus binding in the absence of triplet. These blank values, from left to right, were 0.49, 1.12, 0.57, 1.32.

The binding results and the large number of structural differences that seem to exist between $tRNA^{ala\ Iab}$ and $tRNA^{ala\ II}$ were unexpected. Recent studies on a suppressor tRNA from *E. coli* mutants indicate that a single base change in the anticodon can promote recognition of a new codon.[21] Since the major alanine tRNA in the yeast cell is $tRNA^{ala\ Iab}$ with the anticodon igc, [3, 20] which can recognize three of the four alanine codons, all that is *necessary* to recognize the fourth alanine codon, GCG, is replacement of the i with c. Our results so far indicate that this does *not* occur. The binding data suggest that $tRNA^{ala\ II}$ may still be a mixture of two species containing the anticodons ugc and ggc. In fact, the codon response of $tRNA^{ala\ II}_{yeast}$ resembles that of $tRNA^{ala\ I,II}_{E.\ coli}$.[7] It is possible, therefore, that both yeast and *E. coli* contain $tRNA^{ala}$ species with the same anticodons (ugc and ggc), but that yeast has an additional $tRNA^{ala}$ with the anticodon igc.

Summary and Conclusions.—We wish to make three major points. First, chromatography of phenoxyacetylaminoacyl-tRNA's on BD-cellulose[19] represents a powerful tool for isolating tRNA species with full acceptor activity. Obtaining such material has been a serious technical problem with many tRNA's in the past. With this technique, a new level of sophistication is possible in structure-action studies on tRNA.

Second, we have isolated a new species of $tRNA^{ala}$, $tRNA^{ala\ II}$, the codon response of which differs from that of $tRNA^{ala\ Iab}$. To the best of our knowledge, this is the first time that tRNA's with different anticodons have been isolated in sufficient purity for structural studies from normal, wild-type cells.

Finally, the response of $tRNA^{ala\ II}$ to *all four* alanine codons in the ribosome binding assay represents a critical test of the wobble hypothesis. This hypothesis predicts that *at least two* different anticodons are required to recognize $GC\begin{matrix}U\\C\\A\\G\end{matrix}$. So far, our data show no evidence of heterogeneity in $tRNA^{ala\ II}$. The very high response to $GC\begin{matrix}U\\A\end{matrix}$ that persists even at 10 m*M* Mg^{2+} is also puzzling. The wobble hypothesis predicts that inosine must be the wobble base in order to recognize *both* of these codons. We have been unable to detect inosine in $tRNA^{ala\ II}$. Further structural and biochemical studies are in progress in an effort to explain these results and to gain further insight into structure-action relationships in transfer RNA.

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[2] In order to distinguish closely related species of tRNA, we shall use the following nomenclature. Replacement of a major base by a *minor* one is designated with lower-case letters, e.g., $tRNA^{ala\ Ia}$ and $tRNA^{ala\ Ib}$. Replacement of a major base with another *major* one is designated with Roman numerals, e.g., $tRNA^{ala\ I}$ and $tRNA^{ala\ II}$. If the preparation is a mixture, this is designated by a combination of the above nomenclature, e.g., $tRNA^{ala\ Iab}$.

Abbreviations: $GC\begin{matrix}U\\C\\A\\G\end{matrix}$ stands for the alanine codons, GCU, GCC, GCA, and GCG, capital letters stand for codons, and lower-case letters (as in ugc) for anticodons; both codons and anticodons are written in the 5′ → 3′ direction; m^1G, 1-methylguanine; m_2^2G, 2,2-dimethylguanine; hU, 5,6-dihydrouracil; BD-cellulose, benzoylated DEAE-cellulose; CDCD, counter-double-current distribution; nm, 10^{-9} meters; nmole, 10^{-9} moles; pmoles, 10^{-12} moles; Tris, tris (hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; ATP, adenosine 5′-triphosphate; TCA, trichloroacetic acid; DEAE, *O*-(diethylaminoethyl)cellulose; CTP, cytidine 5′-triphosphate.

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