Reports

Fine Structure of RNA Codewords Recognized by Bacterial, Amphibian, and Mammalian Transfer RNA

Abstract. *Nucleotide sequences of 50 RNA codons recognized by amphibian and mammalian liver transfer RNA preparations were determined and compared with those recognized by* Escherichia coli *transfer RNA. Almost identical translations were obtained with transfer RNA from guinea pig liver,* Xenopus laevis *liver (South African clawed toad), and* E. coli. *However, guinea pig and* Xenopus *transfer RNA differ markedly from* E. coli *transfer RNA in relative response to certain trinucleotides. Transfer RNA from mammalian liver, amphibian liver, and amphibian muscle respond similarly to trinucleotide codons. Thus the genetic code is essentially universal, but transfer RNA from one organism may differ from that of another in relative response to some codons.*

Many studies indicate that the genetic code is largely universal. However, the fidelity of translation can be altered in vivo by extragenic suppressors (*1*), and in vitro, by altering components or conditions required for protein synthesis (for reviews, see *1–3*). Thus, cells sometimes differ in specificity of RNA codon translation.

Nucleotide sequences of RNA codons can be determined by stimulating AA-tRNA (*4*) binding to ribosomes with trinucleotide templates of known sequence (*5*). This approach has been used to determine nucleotide sequences of RNA codons recognized by *Escherichia coli* AA-tRNA (*5–11*). We now describe the nucleotide sequences and relative template activities of trinucleotide codons recognized by AA-tRNA from the South African clawed toad (*Xenopus laevis*) and guinea pig tissues and compare them to sequences recognized by *E. coli* AA-tRNA. Almost identical nucleotide sequence–amino-acid translations were obtained with bacterial, amphibian, and mammalian AA-tRNA. However, *Xenopus* and guinea pig AA-tRNA differ markedly from *E. coli* AA-tRNA in relative response to certain trinucleotides. Thus, the genetic code is essentially universal, but AA-tRNA from one organism may differ from that of another in relative response to degenerate codons.

Methods. Transfer RNA was prepared from livers of adult guinea pigs (250 g, average) of mixed sex, Hartley strain, and from liver and skeletal muscle of *Xenopus laevis* (50 g, average) of mixed sex (*12*) by a modification of the method of Brunngraber (*13*) as follows. Transfer RNA was eluted from a DEAE column as described, and then precipitated with one-tenth volume of 20 percent potassium acetate and three volumes of absolute ethanol. The suspension was stored overnight at −20°C; the precipitate was then collected by centrifugation, subsequently dissolved in H_2O, and stored in a liquid-nitrogen refrigerator.

Approximately 40 mg (960 A^{260} units) of tRNA were obtained from 100 g of guinea pig or *Xenopus* liver and 8 mg (192 A^{260} units) of tRNA from 100 g of *Xenopus* muscle. A solution containing 1.0 mg RNA per milliliter of H_2O was assumed to be equivalent to 24 A^{260} units in a cell with a 1-cm light path, with the use of a Zeiss spectrophotometer.

Supernatant solutions (150,000*g*) were prepared from homogenates of guinea pig and *Xenopus* liver and *Xenopus* muscle (*14*). Aminoacyl-tRNA synthetases were precipitated by the addition of solid ammonium sulfate until the solution was 70 percent saturated; *p*H was maintained at approximately 7.0 with solid ammonium carbonate (added to the ammonium sulfate, 1:50, by weight). Precipitates were collected by centrifugation, dissolved in medium A (0.35*M* sucrose; 0.035*M* potassium bicarbonate; 0.025*M* potassium chloride; 0.004*M* magnesium chloride; and 0.02*M* potassium phosphate, *p*H 7.4), so that the final protein concentration was 25 to 30 mg/ml, and stored in a liquid-nitrogen refrigerator. Dithiothreitol was added to *Xenopus* AA-tRNA synthetase preparations 15 minutes before use so that the final concentration was $6 \times 10^{-3}M$.

Transfer RNA was acylated in reactions containing 0.05*M* tris chloride, *p*H 7.5; 0.01*M* magnesium chloride; 0.005*M* ATP (as the sodium salt); $1 \times 10^{-4}M$ H^3- or C^{14}-L-amino acid; $1 \times 10^{-4}M$ each of 19 other C^{12}-L-amino acids; 1 to 2 mg of tRNA per milliliter of reaction; and 1 to 2 mg of homologous (species and tissue) aminoacyl-tRNA synthetase protein per milliliter of reaction. Reaction mixtures were incubated for 20 minutes at 37°C and were deproteinized with one-tenth their volume of 20 potassium acetate, *p*H 5.5, and one volume of phenol saturated with water. After a second phenol extraction, the combined aqueous phases were passed through a Sephadex G-25 column equilibrated with $5 \times 10^{-4}M$ potassium cacodylate, *p*H 5.5. Fractions containing H^3- or C^{14}-AA-tRNA were lyophilized and stored at −20°C.

Trinucleotide synthesis, isolation, purity, and nucleotide sequence analyses have been described (*5–8, 10, 11, 15*). The presence of an ultraviolet-absorbing contaminant comprising 2 percent of the total would be detected by the methods employed. The trinucleotide GGU contained a contaminant (10 percent). No contaminants were observed in other trinucleotide preparations.

The assay for AA-tRNA-ribosome-codon binding has been described (*5*) as well as the preparation of *E. coli* W3100 ribosomes (*16*). Each reaction mixture was incubated for 15 minutes at 24°C and contained the following in a final volume of 55 μl: 0.05*M* tris-acetate, *p*H 7.2; 0.05*M* potassium acetate; 0.02*M* magnesium acetate (unless otherwise indicated); *E. coli* W3100 ribosomes, washed three times; and H^3- or C^{14}-AA-tRNA, as indicated in Table 1; and oligonucleotide as specified. Ribosomes were washed on Millipore filters (*17*) and dried, and radioactivity was determined in a liquid-scintillation counter (*18*) with a counting efficiency of 70 to 80 percent for C^{14} and 10 to 15 percent for H^3. All assays were performed in duplicate.

Data relating to C^{14}- and H^3-amino acids are listed in Table 1. The purities of guinea pig liver C^{14}-Ser-, C^{14}-Cys-, and C^{14}-Met-tRNA preparations were assessed by deacylating the tRNA in

ammonium carbonate (0.1*M*, *p*H 8.8) for 1 hour at 37°C, followed by paper electrophoresis with 6.8 percent formic acid to separate the free amino acids. A 1-percent contaminant would be detected under the conditions used. None was found.

Results. The amounts of AA-tRNA and ribosomes added to reactions are shown in Table 1. Acylation of the tRNA was catalyzed in all cases by aminoacyl-tRNA synthetases from corresponding organisms and tissues. Ribosomes of *E. coli* were used for binding studies so that codon recognition by tRNA from different organisms could be investigated under uniform conditions. *Xenopus* and guinea pig AA-tRNA preparations bind to *E. coli* ribosomes with approximately the same efficiency as *E. coli* AA-tRNA.

The specificity and activity of trinucleotides in stimulating binding to ribosomes of AA-tRNA from guinea pig liver, *Xenopus* liver, and *Xenopus* skeletal muscle are shown in Table 2. For most amino acids, three or four *Xenopus* liver and guinea pig liver AA-tRNA preparations were studied; however, only representative experiments are presented here. For comparative purposes, responses of *E. coli* AA-tRNA preparations to trinucleotides (*5–11*) also are included. Almost identical translations of nucleotide sequences to amino acids were found with bacterial, amphibian, and mammalian AA-tRNA. Similar sets of degenerate trinucleotides usually were recognized by AA-tRNA from each organism. However, *Xenopus* and guinea pig liver AA-tRNA often differ quite markedly from *E. coli* AA-tRNA in relative response to synonym trinucleotides; for example, Ala-tRNA from each organism binds to ribosomes in response to GCU, GCC, GCA, and GCG. However, mammalian and amphibian Ala-tRNA responds only slightly to GCG, whereas *E. coli* Ala-tRNA responds best to GCG.

The trinucleotides CGU, CGC, CGA, and CGG stimulate binding to ribosomes of Arg-tRNA from guinea pig liver, *Xenopus* liver, *Xenopus* muscle, and *E. coli;* amphibian and mammalian Arg-tRNA responded well to AGG; *E. coli* Arg-tRNA did not. Relative responses of amphibian and mammalian Arg-tRNA to AGG and CGG are high compared to that of *E. coli* Arg-tRNA. As reported previously, the trinucleotide AGG had little or no template activity with *E. coli* Arg-tRNA or with unfractionated *E. coli* tRNA corresponding to each of the 19 other amino acids (*9*, *11*). AGA does not stimulate binding of Arg-tRNA appreciably, except possibly in the case of *Xenopus* muscle Arg-tRNA. However, Söll *et al.* (*19*) have shown that one fraction of yeast Arg-tRNA responds well to AGA and AGG, whereas another fraction responds to CGU, CGC, CGA, and possibly also, to CGG.

Preparations of Cys-tRNA from guinea pig liver and *E. coli* recognize UGU, UGC, and UGA; however, the relative response of mammalian Cys-tRNA to UGA is higher than that of *E. coli* Cys-tRNA. Both AGU and AGC stimulate binding of Cys- and of Ser-tRNA. The results are discussed below with other Ser-tRNA findings.

Isoleucine-tRNA from guinea pig liver, *Xenopus laevis* liver, and *E. coli* recognize AUU and AUC. Guinea pig and *Xenopus* Ile-tRNA preparations also respond to AUA. At 0.02*M* Mg^{++} AUU, AUC, and AUA are recognized, in order of decreasing template activity, by guinea pig and *Xenopus laevis* Ile-tRNA; whereas *E. coli* Ile-tRNA responds almost equally well to AUU and AUC, but does not respond detectably to AUA. In previous studies AUA had little effect upon the binding of unfractionated *E. coli* AA-tRNA corresponding to any of the other common amino acids (*11*). However, Wahba *et al.* (*20*) have reported that oligonucleotides containing the sequence $AAAAU(A)_N$ direct isoleucine incorporation into peptide linkages in reactions containing supernatant from *L. arabinosus* homogenates centrifuged at 100,000*g* and *E. coli* ribosomes. Attention should also be called to a universal error; that is, Ile-tRNA from each species responds to polyU.

In reactions containing 0.01*M* Mg^{++},

Table 1. AA-tRNA and ribosomes added to reactions. GPL, guinea pig liver; XL, *Xenopus* liver; XM, *Xenopus* muscle. All isotopes were uniformly labeled except where specified.

Radioactive amino acid	Origin of tRNA	Components added to each reaction: C^{14}– or H^3–AA–tRNA: A^{260} units	C^{14}– or H^3–AA–tRNA: Radioactivity (C^{14}– or H^3–AA) ($\mu\mu$mole)	*E. coli* ribosomes (A^{260} units)
DL–Ala–H^3*	GPL	0.15	3.2	2.0
L–Ala–C^{14}*	XL	.49	8.7	2.0
L–Arg–H^3*	GPL	.15	4.0	2.0
	XL	.17	4.1	1.25
	XM	.19	4.7	1.5
L–Asp–C^{14}†	GPL	.26	7.2	2.0
	XL	.35	6.0	2.0
L–Cys–C^{14}†	GPL	.31	7.0	2.5
DL–Glu–3–H^3†	GPL	.18	1.7	2.0
	XL	.20	2.2	2.0
2–Gly–H^3*	GPL	.18	12.8	2.0
	XL	.07	11.0	2.0
L–His–2, 5–H^3*	GPL	.02	0.3	1.0
L–His–C^{14}†	XL	.42	4.5	1.5
L–Ile–C^{14}†	GPL	.34	6.1	2.0
	XL	.78	2.7	2.5
DL–Lys–4,5–H^3†	GPL	.07	2.0	0.5
	XM	.14	2.4	1.5
L–Lys–C^{14}†	XL	.26	5.3	0.5
L–Met–C^{14}*	GPL	.31	4.5	2.0
	XL	.50	5.2	2.0
	XM	.24	2.1	2.0
L–Phe–4–H^3*	GPL	.10	0.9	1.5
	XL	.04	1.4	1.0
L–Pro–3,4–H^3†	GPL	.12	1.9	1.0
	XL	.15	1.3	1.25
L–Ser–H^3‡	GPL	.21	8.4	2.0
	XL	.37	8.3	2.0
	XM	.25	7.8	2.0
L–Thr–C^{14}*	GPL	.04	12.9	2.0
	XL	.37	9.9	1.5
L–Trp–H^3*	GPL	.14	1.8	2.5
	XL	.32	1.8	2.0
L–Tyr–3,5–H^3†	GPL	.17	2.7	2.0
	XL	.60	2.1	2.5
L–Val–C^{14}*	GPL	.21	5.6	2.0
	XL	.32	7.2	1.5

* Nuclear-Chicago Corporation. † New England Nuclear Corporation. ‡ Schwarz BioResearch Corporation.

AAA and AAG stimulate binding to ribosomes of Lys-tRNA from guinea pig liver, *Xenopus* liver, and *Xenopus* muscle. Under identical conditions, *E. coli* C^{14}-Lys-tRNA responds to AAA, but not to AAG. However, at 0.02*M* Mg^{++}, *E. coli* C^{14}-Lys-tRNA responds to both AAA and AAG (data not shown here). This difference is probably due to the presence of an additional species of Lys-tRNA in tissues from higher organisms, for Carbon and Hung have shown that one fraction of Lys-tRNA from rat liver binds to ribosomes in response to AAG but not to polyA, whereas unfractionated Lys-tRNA responds to both AAG and polyA (*21*).

Codon recognition by Met-tRNA is of particular interest, for *N*-formyl-Met-tRNA (*22*) from *E. coli* may initiate protein synthesis by selecting the first codon and thereby phasing subsequent reading (*23*). Two fractions of Met-tRNA from *E. coli* have been separated by countercurrent distribution. The major peak of Met-tRNA can be converted to *N*-formyl-Met-tRNA and responds to the codons, UUG, AUG, and, to a lesser extent, GUG; whereas, the smaller peak of Met-tRNA does not accept formyl moieties and responds primarily to AUG (*24, 25*). At 0.01*M* Mg^{++}, mammalian and amphibian Met-tRNA responds well only to AUG. At 0.02*M* Mg^{++} relatively small responses

Table 2. The effects of trinucleotides and polynucleotides upon the binding of AA-tRNA from *Xenopus* liver, *Xenopus* skeletal muscle, and guinea pig liver to *E. coli* ribosomes. For comparative purposes, previous results with *E. coli* AA-tRNA are shown also (*5–11*). Reactions contained the components described; and ribosomes and C^{14}– or H^3–AA–tRNA as reported in Table 1. Reactions contained 0.150 ± 0.010 A^{260} units of trinucleotide or 0.250 A^{260} units of polynucleotide except where indicated. Unless otherwise indicated, reactions for guinea pig and *Xenopus* AA-tRNA contained 0.02*M* Mg^{++}, and reactions for *E. coli* AA-tRNA contained 0.03*M* Mg^{++}.

Tri- or poly-nucleotides	Δμμmole C^{14}– or H^3–AA–tRNA bound to ribosomes*			
	Guinea pig liver	*Xenopus laevis* Liver	*Xenopus laevis* Muscle	*E. coli*
Ala–tRNA (cpm/μμmole, 412 guinea pig, 150 *Xenopus*)				
GCU	0.93	2.39		1.55
GCC	0.67	1.96		0.70
GCA	1.05	2.43		2.66
GCG	0.40	0.14		2.84
UUU	−0.03	−0.20		
None (μμmole)	(0.44)	(1.89)		(0.38)
Arg–tRNA (404 cpm/μμmole)		0.01*M* Mg^{++}		
CGU		0.62	0.49	0.64
CGC		0.27	0.26	0.05
CGA		1.10	0.70	0.26
CGG		0.50	0.65	0.11
AGA		0.02	0.09	0.01
AGG		1.27	1.37	0.04
None (μμmole)		(1.70)	(1.26)	(0.43)
		0.02*M* Mg^{++}		
CGU	0.81	0.38	0.81	0.90
CGC	0.67	0.32	1.00	0.47
CGA	1.28	1.09		1.09
CGG	0.97	0.98	2.09	0.20
AGA	0.12	0.17	0.40	0.10
AGG	0.63	0.98	1.35	0.12
AGU, AGC		<0.02		0
UAA, UAG, UUU	0	0		
None (μμmole)	(1.23)	(1.41)	(1.61)	(1.27)
Cys–tRNA (193 cpm/μμmole)				
UGU	0.49			0.93
UGC	0.62			0.74
UGA	0.34			0.10
AGU				0.39
AGC	0.27			0.53
PolyUG*	0.50			0.57
UUU, UCC, PolyUC†	0			
None (μμmole)	(0.41)			(0.57)
Ile–tRNA (407 cpm/μμmole)		0.01*M* Mg^{++}		
AUU	1.06	0.33		0.04
AUC	0.05	0.11		0.02
AUA	0.05	0.08		0
PolyU	0.80	0.44		0.88
None (μμmole)	(0.46)	(0.29)		(0.04)
		0.02*M* Mg^{++}		
AUU	1.78	1.15		0.64
AUC	0.60	0.51		0.73
AUA	0.38	0.34		−0.01
UUA, UUU	0	<0.04		
None (μμmole)	(0.81)	(0.59)		(0.15)

Tri- or poly-nucleotides	Δμμmole C^{14}– or H^3–AA–tRNA bound to ribosomes*			
	Guinea pig liver	*Xenopus laevis* Liver	*Xenopus laevis* Muscle	*E. coli*
Lys–tRNA (cpm/μμmole, 1342 guinea pig, 374 *Xenopus*)				
		0.01*M* Mg^{++}		
AAA	0.33‡	0.87‡	0.46	1.00‡
AAG	0.50‡	0.62‡	0.57	0.07‡
None (μμmole)	(0.69)	(1.20)	(1.31)	(0.70)
Met–tRNA (335 cpm/μμmole)		0.01*M* Mg^{++}		
UUG	0.04			0.39
CUG	0			
AUG	1.92			0.69
None (μμmole)	(0.16)			(0.25)
		0.02*M* Mg^{++}		
UUG	0.17	0.23	0.12	0.41
CUG	0.08	0.09	0.13	0.15
AUG	1.67	2.24	0.73	1.00
GUG	0.47	0.81	0.26	0.65
PolyUG†	0.10	0.30	0.09	0.10
UUU, UAG, UGA, UUA, AUA, AGG, PolyUC†	0	0	0	
None (μμmole)	(0.15)	(0.26)	(0.05)	(0.41)
Ser–tRNA (225 cpm/μμmole)				
UCU	1.21	2.46	1.79	1.27
UCC	0.13	0.48	0.38	0.54
UCA	0.77	2.27	1.54	1.56
UCG	0.50	0.36	0.14	1.09
AGU	0.77	1.41	0.97	0.21
AGC	0.83	1.23	0.96	0.26
AGA	0.17	0.19	0.09	0.03
PolyUC†	2.79	4.42		
PolyUG†	−0.02	0.42		
UGU, UGC, UGA, AGG	0.10	0.07		
Nohe (μμmole)	(0.68)	(1.75)	(1.39)	(0.43)
Thr–tRNA (238 cpm/μμmole)				
ACU	1.13	2.98		0.91
ACC	0.98	2.07		0.50
ACA	0.94	2.67		0.45
ACG	0.46	1.36		1.10
UUU	0.01	−0.05		
None (μμmole)	(0.37)	(0.66)		(0.63)
Asp–tRNA (237 cpm/μμmole)				
GAU	1.22	1.62		1.29
GAC	1.22	1.52		1.32
GAA	0.09	0.11		0.01
GAG	0.02	0.03		0.02
None (μμmole)	(0.23)	(0.28)		(0.18)

to UUG and GUG are observed compared to those observed with *E. coli* Met-tRNA. Noll has reported the formation of *N*-formyl-Met-tRNA in reticulocyte extracts (*26*). Acceptance of formyl groups by guinea pig liver Met-tRNA has been studied (*27*). Only one Met-tRNA fraction was formylated in the presence of a highly purified *E. coli* formylase preparation.

Ser-tRNA prepared from guinea pig liver, *Xenopus* liver, *Xenopus* muscle, and *E. coli* respond to UCU, UCC, UCA, UCG, AGU, and AGC. However, AGU and AGC are relatively more effective templates for guinea pig and *Xenopus* Ser-tRNA than *E. coli* Ser-tRNA. Responses of amphibian and mammalian Ser-tRNA to UCG are low, compared to that of *E. coli* Ser-tRNA.

Escherichia coli and guinea pig liver C^{14}-Cys-tRNA also bind to ribosomes in response to AGC and AGU. Guinea pig liver Cys-tRNA, however, responds less well to these codons than *E. coli* Cys-tRNA. The possibility that Ser-tRNA preparations were contaminated with Cys-tRNA, and vice versa, was negated, for Ser-tRNA did not respond to Cys-codons (UGU and UGC) and Cys-tRNA did not respond to Ser-codons (polyUC). C^{14}-Ser-tRNA was deacylated, and the C^{14} product was identified by paper electrophoresis. C^{14}-cysteine was not detected. Similarly, C^{14}-Cys-tRNA was deacylated, and the product was then subjected to paper electrophoresis. C^{14}-serine was not detected.

ACU, ACC, ACA, and ACG stimulate bacterial, amphibian, and mammalian Thr-tRNA binding to ribosomes; however, ACG is relatively less active with *Xenopus* and guinea pig Thr-tRNA than with *E. coli* Thr-tRNA.

Although Val-tRNA from each species recognizes GUU, GUC, GUA, and GUG, possible differences in relative response to GUG can be seen. Similarly, His-tRNA from each species recognizes CAU and CAC, but relative responses possibly differ. Preparations of Trp-tRNA from guinea pig and *Xenopus* liver were poorly acylated and did not respond well to any trinucleotide tested. A slight response to polyUG may be seen. Although the response to polyUG is in accord with the assignment of UGG as an *E. coli* Trp- codon, further work is needed to clarify the base sequences recognized by amphibian and mammalian Trp-tRNA.

The AA-tRNA from each organism responds similarly to the following trinucleotides; GAU and GAC, aspartic acid; GAA and GAG, glutamic acid; GGU and GGC, glycine (other Gly-codons, GGA and GGG, were not tested); UUU and UUC, phenylalanine; CCA, CCG, CCU, and CCC, proline (CCC had little template activity for Pro-tRNA from each organism); and UAU and UAC, tyrosine.

UAA and UAG do not appreciably stimulate binding of unfractionated *E. coli* AA-tRNA corresponding to the 20 common amino acids (*10*); *Xenopus* liver Arg-, Phe-, Ser-, or Tyr-tRNA; or guinea-pig-liver Ala-, Arg-, Asp-, His-, Ile-, Lys-, Met-, Pro-, Ser-, or Thr-tRNA (data not shown here).

Studies in the laboratories of Brenner and Garen on extragenic suppressor mutations indicate that UAA and UAG may serve as terminator codons in *E. coli* (*28*). The lack of template activity of UAA and UAG for the prep-

Table 2 (continued).

Tri-or poly-nucleotides	Δμμ Mole C^{14}- or H^3-AA-tRNA bound to ribosomes*			
	Guinea pig liver	*Xenopus laevis* Liver	*Xenopus laevis* Muscle	*E. coli*
Glu-tRNA (1170 cpm/μμmole)				
GAU	0.03	0.09		0.05
GAC	0.03	0.03		0
GAA	0.08	0.69		0.38
GAG	0.15	0.56		0.46
UUU	0	0		
UUA, UAG		0.01		
None (μμmole)	(0.05)	(0.07)		(0.12)
Gly-tRNA (240 cpm/μμmole)				
GGU	1.10	0.33		1.28
GGC	2.10	0.83		2.46
AGG	0	0		
None (μμmole)	(1.12)	(0.42)		(3.20)
His-tRNA (cpm/μμmole, 10,980 guinea pig, 375 *Xenopus*)				
CAU	0.15	1.25		0.88
CAC	0.09	0.69		0.78
UUU		0		
PolyAC†	0.11			
None (μμmole)	(0.03)	(0.51)		(0.18)
Phe-tRNA (2250 cpm/μμmole)				
UUU	0.41	0.83		1.29
UUC	0.37	0.75		1.59
UUA	0.02	0.06		
PolyU	0.99	1.62		
UAA, UAG		0.03		
None (μμmole)	(0.26)	(0.32)		(0.48)

Tri-or poly-nucleotides	Δμμ Mole C^{14}- or H^3-AA-tRNA bound to ribosomes*			
	Guinea pig liver	*Xenopus laevis* Liver	*Xenopus laevis* Muscle	*E. coli*
Pro-tRNA (1265 cpm/μμmole)				
CCU	0.13	0.01		0.15
CCC	−0.02	−0.02		0.08
CCA	0.14	0.10		0.40
CCG	0.28	0.07		0.75
PolyUC†	0.91	0.68		
CUU, CUC, UAA, UAG	0.04			
None (μμmole)	(0.89)	(0.48)		(0.63)
Trp-tRNA (792 cpm/μμmole)				
UGG	0.08			1.95
PolyUG†	0.17	0.18		4.22
None (μμmole)	(0.05)	(0.13)		(0.53)
Tyr-tRNA (1025 cpm/μμmole)				
UAU	0.40	0.18		0.81
UAC	0.25	0.13		0.56
UAA, UAG	0	0		
None (μμmole)	(0.33)	(0.27)		(0.23)
Val-tRNA (306 cpm/μμmole)				
GUU	0.46	0.89		1.00
GUC	0.12	0.40		0.75
GUA	0.42	1.10		1.33
GUG	0.32	0.96		1.08
UUU	0	0.01		
None (μμmole)	(0.13)	(0.23)		(0.30)

* The change in the number of micromicromoles, Δμμmole, was obtained by subtracting C^{14}- or H^3-AA-tRNA bound to ribosomes without trinucleotides from that bound with trinucleotides. The number of micromicromoles of C^{14}- or H^3-AA-tRNA bound to ribosomes in the absence of trinucleotides is enclosed within parentheses. † PolyAC (designation S 182, No. 1087) was synthesized with an input ADP : CDP ratio of 1 : 1. PolyUC (designation Jul. 271, No. 845) by analysis contained 0.57 U/ 0.43 C. PolyUG (designation Jul. 242, No. 1395) was synthesized with an input UDP : GDP ratio of 3 : 1. ‡ 0.075 A^{260} units of trinucleotide.

arations of guinea pig and *Xenopus* AA-tRNA tested suggests that UAA or UAG also may serve as terminator codons in mammals and amphibians. Further studies are needed to clarify this question.

Discussion. In this study, nucleotide sequences of 50 RNA codons recognized by preparation of amphibian and mammalian liver AA-tRNA are established and compared with nucleotide sequences recognized by *E. coli* AA-tRNA (*5–11*). Almost identical translations were obtained with tRNA from each organism (Table 3). Thus an essentially universal genetic code is demonstrated.

Nucleotide sequences recognized by *Xenopus* skeletal muscle Arg-, Lys-, Met-, and Ser-tRNA also were determined and compared with sequences recognized by corresponding *Xenopus* liver AA-tRNA preparations. No differences between liver and muscle AA-tRNA were detected, either in nucleotide sequences recognized or in relative response to synonym codons.

However, marked differences were observed in the relative responses of AA-tRNA from various organisms to certain trinucleotides. Species-dependent differences are summarized in Table 4. Trinucleotides with high template activity for *Xenopus* or guinea pig liver AA-tRNA, but with relatively low activity for unfractionated *E. coli* AA-tRNA are: AGG, CGG, arginine; AUA, isoleucine; AAG, lysine; AGU, AGC, serine; and UGA, cysteine. Those trinucleotides with high activity for *E. coli* AA-tRNA, but low activity for *Xenopus* or guinea pig liver AA-tRNA are: UUG, methionine (*N*-formylmethionine in the case of *E. coli*); GCG, alanine; and UCG, serine. Additional, less striking differences also are indicated. Thus, the results show that some degenerate trinucleotides are active templates with tRNA from each species studied; whereas, other trinucleotides are active with tRNA from one or two, but not from every organism studied. Most of the major differences are between *E. coli* tRNA and tRNA from higher organisms. Amphibian and mammalian tRNA respond similarly to trinucleotide codons.

Table 3. Nucleotide sequences of RNA codons recognized by AA-tRNA from bacteria, amphiban liver, and mammalian liver were determined by stimulating, with trinucleotide codons, the binding of AA-tRNA to *E. coli* ribosomes. The key is as follows. *Boxed areas,* relative response of AA-tRNA from one organism to degenerate trinucleotides differs from that of another organism. *Capital letters,* AA-tRNA from guinea pig liver, *Xenopus laevis* liver, and *E. coli (5–11)* assayed with trinucleotides. *Lower case letters,* AA-tRNA only from *E. coli* assayed *(5–11)* with the exception of Cys-codons which were assayed with guinea pig liver and *E. coli* AA-tRNA.

1st Base	2nd Base				3rd Base
	U	C	A	G	
U	PHE	SER	TYR	Cys	U
	PHE	SER	TYR	Cys	C
	(leu,phe?)	SER	term ?	[Cys]	A
	leu, [F-MET]	[SER]	term ?	TRP	G
C	leu	PRO	HIS	ARG	U
	leu	PRO	HIS	ARG	C
	leu	PRO	gln	ARG	A
	leu	PRO	gln	[ARG]	G
A	ILE	THR	asn	[SER]	U
	ILE	THR	asn	[SER]	C
	[ILE]	THR	LYS	[ARG]	A
	MET, [F-MET?]	THR	[LYS]	[ARG]	G
G	VAL	ALA	ASP	GLY	U
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	gly	A
	VAL [F-MET?]	[ALA]	GLU	gly	G

The tRNA content of metazoan tissues and *E. coli* may differ qualitatively and quantitatively. Previous studies have shown that some species of tRNA recognize only one codon, other tRNA species respond either to two, three, or possibly four synonym codons (*19, 25, 29*). Cells often contain multiple species of tRNA for the same amino acid which may recognize different codons or overlapping groups of codons. Thus relative responses of AA-tRNA to each trinucleotide for the same amino acid may be due to the recognition of two or more codons by one species of tRNA or, when multiple species of tRNA for the same amino acid are present, to the sum of codons recognized by each tRNA species.

Codon recognition is a function of three or more tRNA interactions; with mRNA codons, with ribosomes, and with aminoacyl-tRNA synthetases. Transfer RNA acylation was catalyzed in all cases by an AA-tRNA synthetase preparation from the same organism and tissue; however, *E. coli* ribosomes were used in all binding reactions so that codon recognition could be investigated under uniform conditions. Thus, differences could be due to tRNA interactions with heterologous ribosomes rather than with mRNA codons. Amphibian and mammalian AA-tRNA apparently bind to *E. coli* ribosomes with approximately the same efficiency as *E. coli* AA-tRNA; thus, tRNA and ribosomes from each species must contain many similar structural features. It seems unlikely that many of the low responses of tRNA to trinucleotides can be attributed to faulty interactions of tRNA with heterologous ribosomes, for low responses often were observed with

homologous *E. coli* AA-tRNA and ribosomes.

Possibly, tRNA was lost selectively during deproteinization or unlabeled amino acids were still present on tRNA before incubation with labeled amino acids. However, results obtained with different AA-tRNA preparations for the same amino acid were fully reproducible [for most amino acids, three or four AA-tRNA preparations from *Xenopus* liver, three or four from guinea pig liver, and 6 to 20 from *E. coli (5–11)*].

A trinucleotide with little or no template activity in the binding assay may still serve as a recognizable sequence during protein synthesis, perhaps at an internal position of mRNA (*3, 8, 30*). For example, polyUC actively stimulates Leu-tRNA binding to ribosomes (*8*) and leucine incorporation into protein (*31*). In contrast, trinucleotides containing U and C have little effect upon Leu-tRNA binding to ribosomes (*8, 9*). Similarly, the trinucleotides, AUA and AGA, do not appreciably stimulate the binding to ribosomes of unfractionated *E. coli* AA-tRNA (*9–11*); yet results obtained by directing protein synthesis in *E. coli* extracts with synthetic polynucleotides indicate that AUA and AGA serve, to some extent, as codons for isoleucine and arginine, respectively (*31, 32*). It should be emphasized that one molecule of tRNA may function repetitively when protein synthesis is assayed; whereas, the binding assay depends upon the amount of AA-tRNA forming stable codon-ribosome-AA-tRNA complexes. Other possible explanations for low template activities of trinucleotides in binding assays include the following: appropriate species of tRNA absent or in low concentration; competition for codons or ribosomal sites by additional species of tRNA; high ratio of acylated to deacylated tRNA; special codon function; aberrant concentration of Mg^{++} or other reaction component; or the temperature or length of incubation.

Table 4. Species-dependent differences in response of AA-tRNA to trinucleotide codons. The following scale indicates the approximate response of AA–tRNA to a trinucleotide relative to the responses of the same AA–tRNA preparation to every other trinucleotide for that amino acid (with the exception of Gly–tRNA which was assayed only with GGU and GGC). ++++, 70 to 100 percent; +++, 50 to 70 percent; ++, 20 to 50 percent; ±, 0 to 20 percent.

Codon		tRNA		
		Bacteria (*E. coli*)	Amphibia (*Xenopus laevis* liver)	Mammalia (Guinea pig liver)
Arg	AGG	±	++++	++
	CGG	±	++++	++++
Met	UUG	++	±	±
Ala	GCG	++++	±	++
Ile	AUA	±	++	++
Lys	AAG	±	++++	++++
Ser	UCG	++++	±	++
	AGU	±	+++	+++
	AGC	±	+++	+++
Cys	UGA	±		+++

Possible differences: AGC, Thr; AUC, Ile; CAC, His; GUC, Val; and GCC, Ala.

No differences found: Other degeneracies for the amino acids cited above and codons for Asp, Gly, Glu, Phe, Pro, and Tyr.

Evolution of the Code. Fossil records of bacteria 3.1 billion years old have been reported (*33*). The first vertebrates appeared approximately 510 million years ago, and amphibians and mammals, 355 and 181 million years ago, respectively (*34*). The genetic code may have been functional 3 billion years ago; almost surely the code is more than 600 million years old. Hinegardner and Engelberg (*35*) and Sonneborn (*36*) have suggested that the code became frozen by the time that organisms as complex as bacteria had evolved. However, as Woese has noted (*37*), certain extragenic suppressors are capable of changing codon translation to some extent. For example, the specificity of codon recognition can be altered, in vivo and in vitro, by factors which modify components required for codon translation, such as tRNA, ribosomes, AA-tRNA synthetases, and so forth. Some modifications also may influence the rates with which specific proteins are synthesized. Control mechanisms sometimes may be based upon nonrandom codon frequency and distribution in mRNA, coupled with differential recognition of degenerate codons by multiple sets of tRNA (*38*).

We find that bacterial, amphibian, and mammalian AA-tRNA use essentially the same genetic language, but that relative responses of AA-tRNA from higher organisms to trinucleotides sometimes differs from that of *E. coli* AA-tRNA. The possible relation between such differences and changes in codon translation due to extragenic suppressor mutations should be considered. The species-dependent differences observed may reflect changes in the codon recognition apparatus, acquired after cells had evolved, which may enable cells to store additional genetic information and become more highly differentiated. Certain aspects of embryonic differentiation may even be dependent upon changes in codon recognition. Further studies are needed to assess this hypothesis.

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4. The following abbreviations and symbols are used: tRNA, transfer RNA; AA-tRNA, aminoacyl-tRNA, mRNA, messenger RNA; U, uracil; C, cytidine; G, guanine; A, adenine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CDP, cytidine diphosphate; the term trinucleotide refers to trinucleoside diphosphate; UCA represents a trinucleoside diphosphate with 5′-terminal uridine at hydroxyls attached to U and 2′, 3′-terminal hydroxyls attached to A (U_pC_pA); codon, codeword; Ala-, alanine; Arg-, arginine; Asp-, aspartic acid; Cys-, cysteine; Glu-, glutamic acid; Gly-, glycine; His-, histidine; Ile-, isoleucine; Lys-, lysine; Met-, methionine; Phe-, phenylalanine; Pro-, proline; Ser-, serine; Trp-, tryptophan; Thr-, threonine; Tyr-, tyrosine; Val-, valine-tRNA; polyU, polyuridylic acid; polyUC, randomly ordered copolymer of uridylic and cytidylic acid; polyAC, copolymer of adenylic and cytidylic acid; polyUG, copolymer of uridylic and guanilic acid; A^{260}, absorbancy at 260 mμ; DEAE, *O*-diethylaminoethyl-cellulose; MAK, methylated albumin kieselguhr.
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Milk-Like Fluid in a Mammary Adenocarcinoma: Biochemical Characterization

Abstract. *The milk-like accumulation in the R3230AC mammary adenocarcinoma that follows treatment with estrogen contains lactose, fatty acids, and proteins with electrophoretic properties similar to those of casein and whey of rat milk. This mammary tumor retains the biochemical capacity of the mammary gland in its lactational response to administration of hormone.*

Studies in our laboratory of the hormone-responsive, transplantable, R3230AC mammary adenocarcinoma have demonstrated that estrogen treatment of the tumor-bearing host results in many striking morphologic and biochemical changes. Microscopic examination revealed extensive secretory activity and vacuolization, and histochemical procedures employing oil red O clearly demonstrated the presence of sizable quantities of lipids. It has been reported that administration of estrogen increased: glucose-6-phosphate dehydrogenase, malate dehydrogenase (decarboxylating), and phosphoglucomutase activities; concentrations of free fatty acids and triglycerides; and the RNA:DNA ratio (*1*). The fact that these changes can be prevented by concomitant administration of actinomycin D or cycloheximide suggests that these hormone-induced alterations occur by way of *de novo* synthesis of protein (*2*). Following excision and section of the tumors, a white fluid was readily expressed from the neoplasm. It was of interest, therefore, to examine this milk-like fluid for lactose, fatty acids, and casein, substances that characterize milk.

Tumor fluid was obtained by direct aspiration with a hypodermic needle and syringe from neoplasms of animals that had received subcutaneously estradiol valerate at 10 mg kg^{-1} $week^{-1}$ for 3 weeks; the yield was usually 3 to 5 ml of a whitish, viscous fluid. Rat milk was obtained from actively lactating Fischer rats (*3*). Fluid from tumors and milk were kept at −20°C pending analysis; both fluids were always treated identically.

Lactose was determined on the deproteinized filtrate by paper chromatography by the procedure of Roberts *et al.* (*4*). Under these conditions the tumor fluid contained a sugar identical in R_F with the lactose present in rat milk, as well as with a crystalline lactose standard. However, the content of lactose in the tumor fluid was approximately 0.06 percent, considerably below the 3 percent reported for rat milk (*5*). Shatten *et al.* also investigated the amount of lactose in this neoplastic fluid by a colorimetric procedure, finding 0.05 percent (*6*). It is of interest that Shatten, in her studies of galactose-synthesizing enzymes, observed that the activities of uridine diphosphate glucose pyrophosphorylase and uridine diphosphate glucose epimerase were lower in the neoplasm than in the lactating mammary gland, an observation that would account for the low level of lactose found in the tumor fluid of these estrogen-treated animals.

The fatty-acid composition of this tumor fluid was examined and compared with that of rat milk. Samples were extracted with Folch reagent and

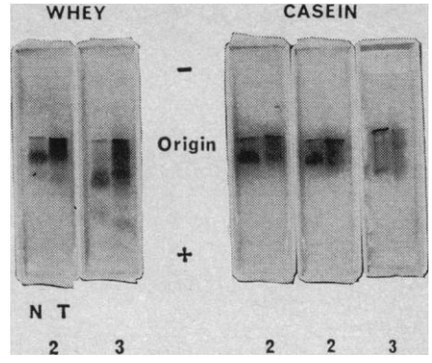

Fig. 1. Starch-gel electrophoresis of casein and whey proteins from rat milk and R3230AC mammary-tumor fluid. Samples were electrophoresed for 2 or 3 hours as indicated. Origin, anode, and cathode are indicated. Normal (*N*) rat milk is located at the left of each cup; tumor fluid (*T*), at the right.

the lipids were methylated directly, before separation by gas-liquid chromatography (*7*). The areas under the peaks were calculated by triangulation, and the data are presented as percentages, the total area being set at 100 (Table 1). The major components of rat milk analyzed in this manner were palmitic (16:0), lauric (12:0), oleic (18:1), myristic (14:0), linoleic (18:2), and stearic (18:0), in descending order of magnitude; they comprised 87 percent of the total. (The fatty acid ratios are of the numbers of carbons to numbers of unsaturated bonds.)

Analyses of fatty acids in the tumor fluid by this procedure revealed the following, in descending order of

Table 1. Fatty acids (percentages of totals) in rat milk and in tumor fluid from R3230AC mammary adenocarcinomas of rats being treated with estrogen. The fatty acids were extracted, methylated directly, and separated by gas-liquid chromatography. Areas under peaks were calculated by triangulation; total area was set at 100.

Fatty acids*	Content (%)	
	Rat milk	Tumor fluid
10:0	0.9	0.5
12:0	16.3	20.9
13:0	0.3	0.3
14:0	14.5	21.1
14:1	1.2	0.3
16:0	27.0	21.3
16:1	3.6	2.0
18:0	5.3	3.8
18:1	15.8	15.2
18:2	8.2	8.4
18:3	0.9	0.4
20:0	.3	.6
20:4	5.0	5.3
21:0	0.9	
22:0	.5	

* Expressed as the ratio of carbon atoms to the number of unsaturated bonds.