QUALITATIVE SURVEY OF RNA CODEWORDS*

BY OLIVER W. JONES, JR., AND MARSHALL W. NIRENBERG

NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

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A degenerate genetic code was suggested a number of years ago by Gamow[1] and by Crick.[2] In such a code, an amino acid may be directed into protein by two or more codewords. Previous work demonstrated that C^{14}-amino acids were directed into protein by synthetic polynucleotides in cell-free *E. coli* extracts,[3] and that leucine incorporation was stimulated by either poly UG,† UC, or UA.[4–6] Thus the code was shown to be degenerate with respect to leucine.[4–6]

Initially, all of the codewords found contained U. However, assuming a triplet code, the proportion of U compared with other nucleotides in codewords seemed unusually high, for natural template RNA, such as viral RNA, did not contain such a preponderance of U. To resolve this paradox, a more degenerate code was proposed with both non-U and U containing codewords.[5] An alternative hypothesis was advanced by Roberts, who suggested a doublet code, for in such a code the proportions of nucleotides would be within the range found in viral RNA.[7, 8]

The existence of non-U codewords was suggested when poly AC preparations were found to direct small amounts of proline and threonine into protein.[9, 22] Recently, in a careful study, Bretscher and Grunberg-Manago clearly demonstrated coding by non-U words.[10] Several poly AC preparations were reported to code well for proline, threonine, histidine, and glutamine. This work indicated that other

non-U polynucleotides might have template activities. In this communication, further qualitative analysis of coding by such polynucleotides will be reported.

Materials and Methods.—The preparation of *E. coli* extracts (preincubated, DNAase-treated S-30 fractions), and the techniques used in washing, plating, and counting protein precipitates have been reported.[3, 5] Polyribonucleotides were synthesized according to the method of Singer and Guss.[11]

The base-ratio of each polynucleotide preparation was determined by incubation in 0.4 *N* KOH at 25° for 18 hours. Under these conditions, little deamination occurred.[12] Such mild conditions were not sufficient to hydrolyze certain polymers; however, in such cases incubation in 0.3 *N* KOH at 37° for 18 hours resulted in complete hydrolysis.[13] Mononucleotide products were separated either by paper electrophoresis (Whatman No. 3 MM paper, 0.05 *M* ammonium formate, pH 3.7) or by descending paper chromatography (Whatman No. 3 MM paper and a solvent system containing 0.1 *M* sodium phosphate, pH 7.0, and 3 *M* ammonium sulfate). Two per cent or greater contamination of polynucleotides by U would have been detected. No contamination by U was found.

Reaction mixtures used to determine C^{14}-amino acid incorporation into protein contained the following components: 0.1 *M* Tris (hydroxymethylaminoethane) pH 7.8; 0.01 *M* magnesium acetate; 0.05 *M* KCl; 6×10^{-3} *M* mercaptoethanol; 1×10^{-3} *M* ATP; 5×10^{-3} *M* potassium phosphoenolpyruvate; 20 μg/ml of crystalline phosphoenolpyruvate kinase (California Biochem. Corporation); 0.8×10^{-4} *M* C^{14}-amino acid (approximately 60,000 counts/minute/reaction mixture); 3.2×10^{-4} *M* each of 19 C^{12}-L-amino acids minus the C^{14}-amino acid; 10 μg of polynucleotide/reaction mixture where specified; and *E. coli* preincubated S-30 extracts (1–2 mg protein/reaction mixture). Total volume of each reaction mixture was 0.3 ml. Reaction mixtures were incubated at 37° for 30 minutes; thus total incorporation rather than rate was measured.

Stimulation of amino acid incorporation by polynucleotides containing four bases: The data of Table 1 demonstrate that synthetic polynucleotides containing four bases stimulate the incorporation of a large number of amino acids into protein. In

TABLE 1

STIMULATION OF AMINO ACID INCORPORATION BY POLY UGAC

Polynucleotide	UGAC	UGAC	UGAC	UGAC	UGAC	UGAC	
Base-ratio moles per cent	U 55 G 32 A 5 C 8	U 56 G 25 A 5 C 13	U 3 G 45 A 9 C 43	U 23 G 21 A 4 C 52	U 27 G 22 A 22 C 29	U 27 G 43 A 21 C 9	Minus polynucleotide control
Designation	Ap231	Ap232	Ap233	Ap234	Ju258	Ju2510	
C^{14}-amino acid	Incorporation above control ($\Delta\mu\mu$moles)*						
Alanine	110	127	62	152	31	5	10
Arginine	69	270	68	212	99	57	11
Aspartic acid[26]	10	40	9	10	25	12	12
Glutamic acid[26]	16	52	12	9	14	...	23
Glycine	62	663	25	40	12	6	13
Histidine	20	8	11	24	13	0	4
Isoleucine	68	301	60	90	0	0	22
Leucine	168	1,243	125	418	12	0	41
Lysine	10	21	0	3	25	...	4
Methionine	9	64	0	9	15	4	12
Phenylalanine	152	606	86	64	14	0	10
Proline	50	140	125	1,007	121	10	7
Serine	179	807	181	445	37	0	47
Threonine	15	54	19	78	44	0	7
Tryptophan	23	8	16	8	1	...	45
Tyrosine	14	80	11	12	6	0	17
Valine	100	602	57	70	43	13	7
Total	1,075	5,086	867	2,651	512	107	292

* $\Delta\mu\mu$moles represents the difference between C^{14}-amino acid incorporation into protein in the presence and absence of polynucleotides.

The components of the reaction mixtures are presented under *Methods and Materials*. The specific radioactivies of C^{14}-amino acids varied from 2.0 to 9.4 mcuries/mmole.

100 $\mu\mu$moles of C^{14}-amino acid is equivalent to 420 counts/minute obtained with a Nuclear-Chicago thin-window, gas flow counter. Basal incorporations obtained when polynucleotides were omitted are presented in the last column (Minus polynucleotide).

the last column is given the basal level of C^{14}-amino acid incorporation obtained in the absence of polynucleotide; other figures refer to the net increase above basal incorporation due to addition of polynucleotide. The base-ratios of the polynucleotides vary widely. The fifth polynucleotide (Ju-258) contains approximately equal proportions of U, G, A, and C, whereas the other polynucleotides contain predominant amounts of two or three nucleotides. All of the polynucleotides were active in directing amino acid incorporation except polynucleotide Ju-2510. Although 10 μg of polynucleotide were added to each reaction mixture, the total amount of C^{14}-amino acid directed into protein by each polynucleotide varied more than 50-fold. As we have shown previously, the template activity of polynucleotides is dependent upon factors other than nucleotide sequence. For example, large polymers of chain length greater than 100 units are considerably more active than shorter ones.[14] Single-stranded polynucleotides are active, whereas double or triple stranded polymers are not.[3] In addition, randomly mixed copolymers which have a high degree of secondary structure are inactive in coding.[15] In particular, polymers containing much G have little activity, possibly because of G–G interactions. Thus, the relative inactivity of the last poly UGAC preparation (Ju-2510) should not be ascribed necessarily to the presence of a high proportion of nonsense nucleotide sequences. Such considerations make it difficult to compare with validity the relative abilities of different polynucleotides to code for the same amino acid; thus, *such comparisons should be made with caution.* The fact that polynucleotides containing four bases coded so well for so many amino acids strongly suggests that most nucleotide sequences can be read. In addition, a high proportion of U clearly was not required for messenger RNA activity.

Stimulation of amino acid incorporation by poly ACG: The coding activities of polymers which did not contain U are given in Table 2. Base-ratio analysis of each poly ACG preparation failed to detect contamination by U. Poly ACG preparations

TABLE 2

STIMULATION OF AMINO ACID INCORPORATION BY POLY ACG

Polynucleotide	ACG	ACG	ACG	ACG	
Base-ratio moles per cent	A 46 C 32 G 22	A 2 C 89 G 9	A 4 C 77 G 19	A 16 C 72 G 12	Minus polynucleotide control
Designation	J251	M 76	M 75	M 74	
C^{14}-amino acid	Incorporation above control ($\lambda\mu\mu$moles)*				
Alanine	123	45	56	85	8
Arginine	128	30	40	74	9
Aspartic acid[26]	167	0	0	24	13
Glutamic acid[26]	326	0	0	33	21
Glycine	5	0	0	0	13
Histidine	71	6	9	95	5
Isoleucine	0	0	0	0	20
Leucine	0	10	0	0	40
Lysine	820	5	0	23	6
Methionine	1	4	0	0	10
Phenylalanine	0	0	1	6	9
Proline	147	320	185	41	8
Serine	182	24	30	55	45
Threonine	250	11	13	11	8
Tryptophan	1	0	0	0	43
Tyrosine	0	4	0	0	18
Valine	1	5	5	7	6
Total	2,222	464	339	454	282

* $\lambda\mu\mu$Moles represents the difference between C^{14}-amino acid incorporation into protein in the presence and absence of polynucleotides. Assay procedures are described under *Materials and Methods.*

stimulated the incorporation of many amino acids tested, including alanine, arginine, glutamic acid, lysine, proline, and threonine. Such high incorporations of glutamic acid, lysine, and threonine were not observed previously.[36] A number of amino acids did not appear to be coded by any ACG preparations, which suggested that U may be an absolute requirement in coding for some amino acids.

Stimulation of amino acid incorporation by polynucleotides containing two bases: The polynucleotides in Table 3 are listed in order of decreasing C content. In accord with the findings of Bretscher and Grunberg-Manago,[10] poly AC stimulated incorporation of proline, threonine, and histidine. In addition, poly AC was found to direct aspartic acid, glutamic acid, and lysine into protein.[36] Poly AC (J-104) contained 97 per cent C, yet actively directed proline into protein. Thus, it appears that one codeword for proline may contain only C. Relatively large amounts of lysine were directed into protein by AC J-109 and J-108 which contained 67 and 80 per cent A respectively. Thus, the lysine codeword may contain only A.

The data of Table 4 demonstrate the effects of poly CG and AG preparations in directing amino acids into protein. The first three CG polymers contained high proportions of C and directed alanine, arginine, and proline into protein. The last poly CG preparation (F-135) contained 91 per cent G, and was inactive as template RNA. Poly AG directed incorporation of glutamic acid and lysine into protein.

Quantitative aspects of data: A comparative study of polynucleotides of varying base-ratios is helpful in evaluating amino acid incorporation data, for relative amino acid incorporations can be easily correlated with changes in base-ratio. Occasional inconsistencies and the significance of minor incorporations become apparent.

Isotope dilution experiments were performed routinely to detect the possible presence of radioactive impurities in C^{14}-amino acids. The presence of C^{14}-impurities seemed unlikely, for incorporation of a C^{14}-amino acid was reduced sharply if the reaction mixture contained both a C^{14}-amino acid (0.05 μmole) and the same

TABLE 3

STIMULATION OF AMINO ACID INCORPORATION BY POLY AC

Polynucleotide	AC	AC	AC	AC	AC	AC	Minus
Base-ratio	A 3	A 6	A 12	A 30	A 67	A 80	polynucleotide
moles per cent	C 97	C 94	C 88	C 70	C 33	C 20	control
Designation	J104	J103	J102	J101	J109	J108	
C^{14}-amino acid	Incorporation above control ($\Delta\mu\mu$moles)*						
Alanine	0	1	0	0	0	0	11
Arginine	0	4	1	1	0	0	12
Aspartic acid**	0	0	9	51	157	53	24
Glutamic acid**	4	19	24	53	135	53	15
Glycine	5	16	0	2	0	0	6
Histidine	0	0	5	198	85	17	23
Isoleucine	6	0	0	0	0	0	42
Leucine	0	0	...	...	...	1	3
Lysine	5	10	14	47	909	441	5
Methionine	0	10	0	0	0	0	10
Phenylalanine	0	0	1	0	4	0	11
Proline	625	1,132	643	1,102	140	20	9
Serine	11	19	18	16	9	8	46
Threonine	30	65	75	170	176	105	9
Tryptophan	23	0	1	1	1	9	44
Tyrosine	14	2	2	0	0	0	19
Valine	0	0	0	0	0	0	5
Total	723	1,278	793	1,641	1,616	707	294

* $\Delta\mu\mu$Moles represents the difference between C^{14}-amino acid incorporation into protein in the presence and absence of polynucleotides. Assay procedures are described under *Materials and Methods*.

TABLE 4

STIMULATION OF AMINO ACID INCORPORATION BY POLY CG AND AG

Polynucleotide	CG	CG	CG	CG	AG	AG	Minus
Base-ratio moles per cent	C 90 G 10	C 87 G 13	C 82 G 18	C 9 G 91	A 73 G 27	A 48 G 52	polynucleotide control
Designation	M141	M 71	F120	F135	J106	J107	
C^{14}-amino acid	Incorporation above control ($\lambda\mu\mu$moles)*						
Alanine	30	20	63	0	0	0	14
Arginine	39	16	86	1	10	8	13
Aspartic acid[36]	0	0	6	3	12	10	26
Glutamic acid[36]	0	0	0	0	44	5	11
Glycine	5	0	8	0	2	0	4
Histidine	0	0	0	0	0	0	26
Isoleucine	0	0	0	0	1	0	39
Leucine	0	0	0	5	0	11	7
Lysine	2	0	0	0	110	8	3
Methionine	0	0	0	0	0	0	12
Phenylalanine	5	5	4	8	0	0	14
Proline	144	202	356	2	1	1	8
Serine	18	0	6	0	0	0	42
Threonine	0	0	1	0	1	0	5
Tryptophan	0	1	17	1	0	0	40
Tyrosine	2	6	2	0	0	0	14
Valine	1	1	0	0	0	0	4
Total	246	251	549	20	181	43	282

* $\lambda\mu\mu$Moles represents the difference between C^{14}-amino acid incorporation into protein in the presence and absence of polynucleotides. Details of the assay procedures are described under *Materials and Methods*.

C^{12}-amino acid (1.0 μmole). The purity of each C^{14}-amino acid also was determined by paper electrophoresis followed by radioautography as described previously.[5]

Limiting amounts of polynucleotides were added to reaction mixtures and *total* amino acid incorporations were measured rather than rates of amino acid incorporations. *E. coli* extracts contain nucleases which rapidly degrade synthetic polynucleotides and the nuclease content may vary from one preincubated S-30 preparation to another. Since many different enzyme extracts were used in this study, *the data are not useful for quantitative analyses.* Comparisons between theoretical frequencies of triplets, etc., in polynucleotides and relative amino acid incorporations have not been presented because the data do not permit such calculations to be made with accuracy. The data demonstrate only qualitative aspects of the code; that is, nucleotide compositions of words and the degree of code degeneracy.

Summary of Incorporation Data.—Table 5 summarizes all of the coding data previously published[3-6, 16-18] and those obtained in this study. Only polynucleo-

TABLE 5

SUMMARY OF CODING DATA

C^{14}-amino acid	Stimulated by poly-*				C^{14}-amino acid stimulated by poly-*	
Phenylalanine	U(98)†				Isoleucine	UA(8)
Proline	C(?)	CA(87)	CU(60)	CG(80)	Tryptophan	UG(6)
Lysine	A(?)	AC(53)	AG(60)	AU(?)	Tyrosine	UA(9)
Threonine	AC(15)				Arginine	CG(15)
Serine	UC(23)	UCG(23)?			Methionine	UAG(1)
Valine	UG(15)				Histidine	AC(10)
Leucine	UG(14)	UC(13)	UA(?)		Alanine	CG(11)
Glycine	UG(5)				Aspartic acid[36]	AC(8)
Cysteine	UG(8-15)					
Glutamic acid[36]	AC(7)	AG(20)				

* Polymers used for these calculations contain the minimal number of bases necessary to direct the C^{14}-amino acids into protein.

† Numbers in parentheses refer to: μmoles of amino acid incorporated $\times$ 100/Sum of incorporation of all amino acids at the optimal base ratio assayed.

tides containing the minimum number of bases capable of stimulating an amino acid into protein are given in Table 5. The coding of proline by poly C and lysine by poly A was suggested by the poly AC experiments presented in Table 2. The fact that poly C and poly A code so weakly may be due to inhibitory effects of secondary structure. In solution at acid pH, poly A is double-stranded,[19, 20] and at neutral pH, both poly A and poly C have partially ordered structure.[21]

A surprising conclusion drawn from this summary is that almost every amino acid tested could be coded by a polymer containing only two bases. Methionine could be coded only by poly UGA as previously reported,[5, 18] but since the amount of methionine directed into protein was small, this codeword remains questionable.

Assuming a triplet code, a summary of codewords estimated thus far is presented in Table 6. Previously, poly UCG was found to direct alanine and arginine into

TABLE 6

TENTATIVE SUMMARY OF CODE WORDS *

C^{14}-amino acid			M-codewords	
Alanine	CCG			
Arginine	CGC			
Aspartic acid[36]	ACA			
Asparagine[36]	UAC or UAA†			
Cysteine	UUG or UGG‡			
Glutamic acid[36]	ACA	AGA	AGU§	
Glycine	UGG			
Histidine	ACC			
Isoleucine	UCA			
Leucine	GUU	CUU	AUU†	(UUU)
Lysine	AAA	AAC	AAG	AAU
Methionine	UGA§			
Phenylalanine	UUU			
Proline	CCC	CCU	CCA	CCG
Serine	UCG	UCU		
Threonine	CAC	CAA		
Tryptophan	UGG			
Tyrosine	UAU			
Valine	UGU			

* Nucleotide sequence in codewords is arbitrary.
† Proposed by Speyer *et al.*[18]
‡ We cannot now differentiate between these possibilities.
§ It is not entirely clear whether these codewords require U.

protein and codewords containing U, C, and G were proposed for these amino acids.[4, 5, 17, 18] The observed frequencies of incorporations[5] suggest coding of alanine and arginine by *either* UCG or CCG, but not by both codewords. In addition, the data of Table 4 show that poly CG codes for alanine and arginine; thus, codewords corresponding to these amino acids do not appear to contain U.

Since it is not possible at this time to distinguish between triplet and doublet codes, etc., the assignments in Table 6 represent current approximations of codewords. It seems probable that additional codewords will be found.

Discussion.—Codeword specificity in protein synthesis: The term *degeneracy* refers to the phenomenon whereby one amino acid is coded by two or more codewords. This term is inadequate when applied to the mechanism of coding, for it does not indicate codeword *specificity*. A degenerate code may have high or low specificity depending upon the fidelity of protein synthesis. In most cases the fidelity of protein synthesis *in vivo* appears to be high, and amino acid replacements other than those due to mutation, have not been found. However, although the amino acid sequence analyses would reveal mistakes at one site occurring with a

frequency higher than 1 or 2 per cent, they would not reveal occasional mistakes occurring at different sites. Thus, occasional coding errors of 1 or 2 per cent, distributed at random over entire protein molecules, might not be detected.

In the *in vitro* system, coding during protein synthesis displays striking specificity.[22] In Table 3, for example, poly AC preparations do not direct the incorporation into protein of alanine, arginine, glycine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, or valine. The specificity of coding by poly CG and AG preparations in Table 4 is equally apparent. Such negative data clearly demonstrate the very high fidelity of codeword recognition during protein synthesis.

The codewords corresponding to both leucine and valine contain U and G.[4-6] Although the nucleotide content of these codewords is identical, each word was shown to code only for the appropriate amino acid.[22] Thus, nucleotide sequence as well as chemical structure confers specificity upon codewords.

However, one unclarified example of ambiguity has been found. Poly U directs about 3–5 per cent as much leucine into protein as phenylalanine.[5] Bretscher and Grunberg-Manago also have reported this phenomenon.[10] In the absence of phenylalanine poly U coded for leucine about 50 per cent as well as it would code for phenylalanine.[23]

Efficiency of synthetic RNA in coding: In spite of the previously mentioned difficulties in comparing template activities of RNA preparations with different chain lengths and degrees of secondary structure, it seems clear that synthetic polynucleotides containing 4, 3, or 2 bases code as well in this system as natural template RNA obtained from viruses.[3, 24-26] The efficiency in coding displayed by synthetic polynucleotides suggests that most nucleotide sequences direct amino acids into protein, and that few nonsense nucleotide sequences are present.

Although alternative explanations of coding efficiency, such as nonrandom polynucleotides or nonsequential reading of template RNA, may be considered, such efficiency cannot be ascribed simply to random error in directing amino acids into protein, for amino acids are coded with marked specificity.

Considerations such as these may be used to approximate the coding ratio. In a doublet code only 16 base permutations are possible. Thus, the information content would be insufficient to code specifically for all amino acids. Triplet and quadruplet codes would contain 64 and 256 codewords respectively. Since almost every amino acid tested was found to be coded by polynucleotides containing only 2 bases, specific and efficient coding by quadruplet words would not seem likely. The data suggest either coding of all amino acids by triplets or coding of some by triplets and others by doublets (mixed doublet-triplet code).

Recently Weisblum, Benzer, and Holley[27] have established a molecular basis of degeneracy by demonstrating that multiple species of transfer RNA recognize different codewords with specificity. Multiple peaks of transfer RNA corresponding to at least four amino acids have been found independently by Holley *et al.*,[28] Sueoka *et al.*,[29] and Doctor *et al.*[30]

If a triplet code is assumed, each cell would require almost 64 transfer RNA species. Alternatives which do not require so many transfer RNA species deserve consideration. For example, Donohue and others have described many models other than Watson-Crick pairing.[31] The demonstrated interaction between poly A and poly I,[32] and the type of base-pairing suggested by Hoogsteen[33] also might be

cited. Theories which require recognition of either the 2- or 6-substituents of bases[34] are not supported by the demonstration that hypoxanthine functions in codewords like G.[15, 35] The 2-amino group of G does not appear to be required.

A triplet code may be constructed wherein correct hydrogen bonding between two out of three nucleotide pairs may, in some cases, suffice for coding. Correct pairing of a base at one position in the triplet sometimes may be optional. It should be noted that a triplet code of this type in some respects would bear a superficial resemblance to a doublet code and would be in accord with all of the data.

Any theory concerning the physical basis of the code must attempt to explain the following experimentally obtained data: (1) high coding efficiency by synthetic polynucleotides; (2) marked codeword specificity; (3) degenerate codewords; (4) the 2-amino group of G is not essential for proper coding; (5) RNA with a high degree of secondary structure has little ability to code; and (6) almost all amino acids tested can be coded by polynucleotides containing only two bases.

Summary.—Synthetic polynucleotides containing 4, 3, or 2 bases have been found to direct amino acids into protein with high efficiency and specificity. Many additional RNA codewords which do not contain uridylic acid have been found. Almost all amino acids could be coded by polynucleotides containing only 2 bases. These results have been discussed in terms of the general nature of the code.

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† The following abbreviations are used: A, adenylic acid; G, guanylic acid; C, cytidylic acid; U, uridylic acid.

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[36] Poly AC is reported to code for glutamine.[11] We have been unable to obtain C^{14}-glutamine and C^{14}-asparagine. Therefore, we cannot differentiate between codewords corresponding to each free acid and its respective amide.