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[7] When bacterial DNA is isolated by other standard procedures (e.g., Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961)) or by density-gradient centrifugation of bacterial lysates[2] the satellite band is not observed. Our isolation procedure utilizes deproteinization with phenol (Kirby, K. S., *Biochem. J.*, **66**, 495 (1957)) and ammonium sulfate-isopropanol precipitation of the nucleic acids. It will be described in detail elsewhere. The heavy satellite band is not an artifact of preparation, as it has been verified experimentally that native DNA is not denatured by our isolation procedure.

[8] With saturated cultures, or cultures in which DNA synthesis has run to completion after a shift to a medium lacking an essential amino acid (or after a shift from a medium containing amino acids to a minimal medium),[9] the satellite band is absent or greatly reduced in amount. However, if DNA synthesis in *E. coli* 15 T-A-U is stopped by shifting an exponentially growing culture to a thymineless medium the satellite band is observed to be undiminished (Fig. 1).

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*THE EFFECT OF SECONDARY STRUCTURE ON THE TEMPLATE ACTIVITY OF POLYRIBONUCLEOTIDES**

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Current experiments on the synthesis of polynucleotides and proteins seem to us to emphasize the functional importance of polynucleotide secondary structure. Thus, for example, the highly ordered double-helical structure of DNA[1] readily leads to ideas concerning DNA replication.[2, 3] Although our notions concerning the

secondary structure of the various types of RNA are much less precise, it does appear that RNA molecules are primarily single stranded and contain varying degrees of helical content.[4, 5] Recently, investigators from two different laboratories have proposed similar helical structures for transfer-RNA[6, 7] and both groups have discussed the functional significance of the suggested configurations. It is therefore of interest to consider the secondary structure of template RNA. Previous reports from this laboratory[8, 9] described the template RNA dependent incorporation of amino acids into proteins in a stable cell-free system from *E. coli*. Using this system and randomly mixed polyribonucleotides composed of various combinations of the four common ribonucleotides, nucleotide codewords corresponding to almost all of the protein amino acids have been determined.[10-15] Observations made with this system suggested that the secondary structure of polymers influenced template efficiency. For example, the ability of poly U to direct polyphenylalanine synthesis is lost when poly A-poly U double or triple helices are formed.[9, 10]

The present report describes certain physical properties of a series of poly UG preparations as well as the efficiency of these polymers in directing amino acid incorporation in the cell-free system. The data indicate that secondary structure in a polyribonucleotide limits its efficiency as a template. This finding is discussed and a specific functional role for polynucleotide secondary structure in the coding mechanism is proposed.

A preliminary account of some of these data has been published.[10]

Materials and Methods.—The procedures for the synthesis of polymers, determination of base ratios in the polymers, and measurement of amino acid incorporation have been described previously.[9, 14] The UG polymers are isolated by a modified procedure[16] designed to concentrate the longer chain length polymer and eliminate some of the shorter chain material. At the end of the polymerization the reaction mixture is deproteinized by the method of Sevag.[17] The aqueous solution of polymer is made 2 *M* in KCl by addition of an appropriate amount of solid KCl. Polymer is precipitated by the addition of 0.2 volume of cold absolute alcohol, and collected by centrifugation. The precipitate is dissolved in a small amount of water. In several cases the polymers did not dissolve readily in water unless a small amount of EDTA (final concentration about 5 mM) was added. Precipitation with KCl and alcohol is repeated two more times. The final precipitate is washed successively with 80%, 95%, and 100% alcohol and finally with ether and dried over paraffin. Polymers were subsequently dissolved and dialyzed against distilled water before use.

The phosphorolysis of the polymers by polynucleotide phosphorylase was determined by measuring the formation of P^{32}-labeled nucleoside diphosphate in the presence of P_i^{32}. The procedure referred to as Assay A by Singer and Guss[18] was used; the pertinent polymer was substituted for the poly A of that method. For the phosphorolysis studies, *Micrococcus lysodeikticus* polynucleotide phosphorylase, purified approximately 250-fold (Fraction VIII[19]) was used.

Measurements of the temperature dependence of the spectra of polymers ("melting curves") were carried out either in a Cary recording, model 14M, or Beckman, model DU, spectrophotometer. The Cary instrument was equipped with a thermostatted cell holder and the temperature was measured inside the sealed quartz cuvette by means of a hypodermic needle type thermistor. The Beckman instrument was equipped with thermospacers and the temperature was estimated in a water-filled, unsealed, blank cuvette. All solutions were gassed with helium just before filling the cuvettes and the cuvettes were sealed with a coating of General Electric Company RTV-60, silicone rubber compound.[20]

The chain lengths of polymers were determined by measuring the ratio of total organic phosphate to phosphate removable by *E. coli* alkaline phosphatase (Worthington Biochemical Corporation). The procedure outlined by Heppel and co-workers[21] was followed.

Sedimentation velocity studies were performed at 56,100 rpm and 20°C using the Spinco

Model E Ultracentrifuge equipped with UV absorption optics. The solutions contained approximately 0.03 mg of polymer per ml of 0.05 *M* cacodylate and were 0.1 *M* in NaCl. For measurements at neutral pH the cacodylate served as a buffer at pH 7.2; for alkaline measurements the solutions were made 0.01 *M* in KOH (pH 12). Tracings were obtained from photographic images of the cell using the Joyce-Loeble Recording Microdensitometer. The sedimentation coefficient was calculated[22] from the rate of change of position of the 50% point of the boundary.

Optical rotation was measured on a Rudolph Polarimeter using the mercury line at 365 mμ and a 1 decimeter light path.

Results.—Dependence of amino acid incorporation on the base ratio in poly UG: Table 1 shows the incorporation of phenylalanine, valine, leucine, and tryptophan into protein, measured with a series of poly UG preparations of increasing G content. The relative amounts of the four amino acids incorporated with any particular polymer are clearly related to the proportion of U and G. The data show that efficient incorporation of tryptophan requires a greater proportion of G in the polymer than is necessary for incorporation of valine or leucine. These data, therefore, confirm the earlier assignment of codewords UUG, UUG, and UGG to valine, leucine, and tryptophan, respectively.[11, 12, 14]

It is evident that the efficiency of a polymer as template RNA (Table 1) for any of the amino acids shown decreases sharply when the U/G ratio becomes less than 1. For example, the incorporation of phenylalanine with the polymer of U/G ratio 6.7/1 is about 60-fold greater than that obtained with the polymer of U/G ratio, 0.58/1. Although the decreased ability of the latter polymer to direct phenylalanine incorporation is expected, the lack of activity in coding for leucine, valine, and tryptophan is surprising. In an attempt to understand this observation various properties of the polymers were investigated and the results of these studies are presented below.

Chain length of polymers: Each of the polymers shown in Table 1 had a chain length in excess of 300 nucleotide units but probably not greater than 500 units. Because of the large amounts of polymer required for the chain length determination, more accurate measurements were not made.

Sedimentation characteristics of polymers: The sedimentation coefficients of the polymers described in Table 1 are given in Table 2. At pH 7.2 the S_{20} of the poly-

TABLE 1

STIMULATION OF C^{14} AMINO ACID INCORPORATION BY POLY UG

Polymer base ratio (U/G)*	6.7/1	3.3/1	1.6/1	0.58/1	
Nucleoside diphosphate ratio (U/G)†	8/1	5/1	3/1	1/1	Control minus polynucleotide
		Incorporation above Control‡ μμmoles			
C^{14} amino acid					
Phenylalanine	901	1708	1067	15	45
Valine	287	1036	1050	61	13
Leucine	267	834	856	35	64
Tryptophan	38	210	276	10	60

* Base ratio of polymer determined as previously described.[14]

† Ratio of UDP to GDP used in polymer synthesis.

‡ Figures represent incorporation of C^{14} amino acid above the basal incorporation obtained in the absence of added polymer. Basal incorporation is given in the last column.

Reaction mixtures (0.5 ml) contained 0.1 *M* Tris buffer, pH 7.8, 0.01 *M* magnesium acetate; 0.05 *M* KCl; 6×10^{-3} *M* β-mercaptoethanol; 1×10^{-3} *M* ATP; 5×10^{-3} *M* potassium phosphoenolpyruvate; 10 μg crystalline phosphoenolpyruvate kinase (CalBiochem); 2×10^{-4} *M* of each of 19 L-amino acids lacking the C^{14} amino acid; 0.8×10^{-4} *M* C^{14} amino acid; 5 μg of polynucleotide; and preincubated, dialyzed S–30 *E. coli* extract.[9] Each assay was performed in duplicate. The specific radioactivities of the amino acids varied 2–6 millicuries per millimole. All reaction mixtures were incubated at 37° for 90 min. These incorporation data therefore represent total incorporation of C^{14}-amino acids into protein rather than rate of incorporation.

TABLE 2

SEDIMENTATION COEFFICIENTS OF POLY UG PREPARATIONS

Polymer base ratio (U/G)	S_{20}*	
	pH 7.2	pH 12
6.7/1	5.9	5.2
3.3/1	3.1	2.3
1.6/1	5.7	3.3
0.58/1	11.0	2.1

* Inspection of the densitometer tracings indicated relatively little or no breakdown of the polymers during sedimentation at pH 12. Nonsedimenting material amounted to approximately 5%, at pH 7 and 12, with all the polymers except the last (U/G, 0.58/1), when it was about 20% at pH 12. Furthermore, in each case the sedimenting material was more homogeneous at pH 12 than at pH 7 and this was most striking with the last polymer (U/G, 0.58/1).

mer containing 70 per cent G is considerably higher than the S_{20} values of the others. However, the decrease in S_{20} noted for all polymers at pH 12 is much greater for this high G containing polymer.

Phosphorolysis of polymers by polynucleotide phosphorylase: The susceptibility of the poly UG preparations to phosphorolytic cleavage by *M. lysodeikticus* polynucleotide phosphorylase was studied and the results are presented in Table 3.

TABLE 3

PHOSPHOROLYSIS OF POLY UG BY POLYNUCLEOTIDE PHOSPHORYLASE

Base Ratio (U/G)	Phosphorolysis Rate (mμmoles/15 min.)	Phosphorolysis Rate† / Rate with poly A
poly U*	...	6.4
6.7/1	22.6	3.2
3.3/1	25.9	3.7
1.6/1	11.6	1.7
0.58/1	1.1	0.16

* Experiment carried out at a separate time.
† The numbers in the last column represent the rate of phosphorolysis of the given polymer relative to that of poly A.
The incubation mixtures contained approximately one μmole of polymer phosphate per ml (see section on *Materials and Methods* for details).

The rate of phosphorolysis (estimated with a measurement at a single time when the reaction rate is known to be linear with homopolymers) depends on the relative content of uracil and guanine. In particular, when the polymer has more guanine than uracil the rate falls off very sharply. This effect is not the result of the decreasing uracil content since the members of an analogous series of poly UC preparations were all phosphorolyzed at similar rates. Thus, when the ratio of uracil to cytosine was 4/1, 3.3/1, 1.7/1, and 0.6/1, the rates of phosphorolysis relative to poly A were 3.3, 4.0, 3.7, and 2.2, respectively. The slow phosphorolysis of poly UG containing more guanine than uracil appears to result from the high guanine content.

Effect of temperature on the ultraviolet absorption spectra of poly UG preparations: Thomas[23] and Doty and co-workers[4, 5] have discussed the use of hypochromicity as an indicator of secondary structure in polynucleotides. We have determined so-called "melting curves" for polymers described in Table 1. Polymers having ratios of uracil to guanine greater than 2/1 showed essentially no change in absorption between 200 and 300 mμ between room temperature and 85°. With poly UG having a uracil to guanine ratio of 1.6 to 1, we observed a slow increase in the absorption at 250, 260, 270, and 280 mμ upon increasing the temperature from 22° to 82°; however, the total increase at 270 mμ was only about 5 per cent (Fig. 1).

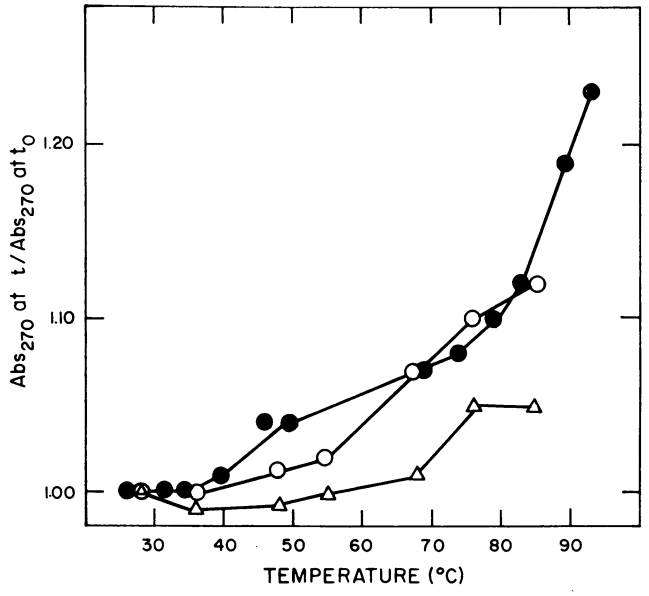

FIG. 1.—Changes in ultraviolet absorption of poly UG as a function of temperature. The ordinate is the ratio between the absorption at 270 mμ at the indicated temperature and the absorption at 270 mμ at the first temperature measured. —●—, poly UG, U/G ratio equal to 0.58/1, in 0.1 *M* KCl, pH 7.8; —○— poly UG, U/G ratio equal to 0.58/1, in 0.01 *M* potassium phosphate, pH 7.0; —△—, poly UG, U/G ratio equal to 1.6/1 in 0.01 *M* potassium phosphate, pH 7.0.

Under the same conditions (0.01 *M* phosphate buffer) poly UG having a uracil to guanine ratio of 0.58 to 1 gave a total increase in absorption at 270 mμ of 12 per cent on raising the temperature from 30° to 85° (Fig. 1). The data are given for 270 mμ since the hypochromicity of G containing structures appears to be optimal at that wavelength.[24] Figure 1 shows two curves for poly UG (U/G, 0.58/1); one was obtained in 0.1 *M* KCl, pH 7.8, the other in 0.01 *M* phosphate buffer, pH 7.0. The curves are similar although the start in the increase in absorption is somewhat delayed in 0.01 *M* phosphate buffer, pH 7.0. This observation is consistent with the finding[25] that the "T_m" of d-pGpGpGpG is lower in 0.2 *M* NaCl than in phosphate buffer alone. The experiment in 0.1 *M* KCl was carried out in the Cary instrument and a temperature dependent shift in the wavelength of maximum absorption from 255.5 mμ to 253 mμ was also observed. This shift took place at about 90°.

Optical rotation of poly UG preparations: As an independent measure of polynucleotide helical content[4] the optical rotations of several polymers shown in Table 1 were measured. Determinations were carried out at room temperature in 0.01 *M* potassium phosphate buffer, pH 7.0, using the mercury line at 365 mμ. The specific rotations of the polymers having U/G ratios of 6.7/1, 3.3/1, and 0.58/1, were +99, +74, and +399, respectively.

Discussion.—Amino acid incorporation into protein: The relative amounts of phenylalanine, valine, leucine, and tryptophan directed into protein by the poly UG preparations (Table 1) is clearly related to the base ratio of the polymer and

these data provide strong support for earlier codeword assignments.[11, 12, 14] However, several properties of the poly UG preparations used indicate that meaningful comparisons between theoretical frequencies of doublets or triplets and relative amino acid incorporations cannot be made. One such factor is the secondary structure of the polymers and is discussed in detail below. Another factor is the possibility that the polymers may be nonrandom. Since G is incorporated into polymer preferentially (Table 1) the ratio of UDP to GDP changes during polymer synthesis. Thus, the base ratio determined by analysis could represent the average base ratio rather than that of any particular polynucleotide region or molecule. Preferential incorporation of G into polymers has been noted previously,[14, 15] and similar observations have been made by Bretscher and Grunberg-Manago,[26] using *A. agilis* polynucleotide phosphorylase. The mechanism of this concentration phenomenon is currently under investigation.[27]

Effect of secondary structure on the template activity of messenger RNA: Several lines of evidence presented above indicate that copolymers of U and G contain a high degree of secondary structure when the relative content of G is high, and this interpretation is consistent with recent reports on the secondary structure of poly G itself.[24, 25, 28–30] (1) Although the four UG preparations described have approximately the same chain lengths the sedimentation coefficient of poly UG (0.58/1) at neutral pH is markedly higher than that of the others. This increase in S value may result from greater aggregation (due to hydrogen bonding) with increasing G content for, at pH 12, where the secondary structure of poly G collapses,[25, 28] all the polymers have similar sedimentation characteristics. (2) The sharp drop in phosphorolysis rate observed on going from a poly UG with a U/G ratio of 1.6/1 to one with a U/G ratio of 0.58/1 can also be explained by an increase in secondary structure. Ochoa[31] and Grunberg-Manago[32] have shown that polyribonucleotides having ordered secondary structures are phosphorolyzed much less rapidly than polymers existing as random coils. Poly G, for example, is completely resistant to phosphorolysis by polynucleotide phosphorylase.[19, 32] (3) Only polymers containing relatively large amounts of G (U/G, 1.6/1, and 0.58/1) showed any increase in ultraviolet absorption at raised temperatures, and the increase is greater the higher the G content. (4) The relatively high optical rotation of poly UG (U/G, 0.58/1) also indicates ordered secondary structure.[4]

Thus, the first three polynucleotides listed in Table 1 contain only moderate amounts of secondary structure and direct amino acid into protein with high efficiency. The last polymer, which contains almost 70 per cent G, exhibits a great deal of secondary structure and a markedly decreased ability to serve as a template for protein synthesis. This correlation between template efficiency and secondary structure is consistent with our earlier observation that the ability of poly U to direct polyphenylalanine synthesis is lost when poly A-poly U helices are formed.[9] Furthermore, we have found[10] that the extent of inhibition of polyphenylalanine synthesis by oligoadenylic acid preparations increases with the length of the oligonucleotide chain and can be correlated with the stability of oligoadenylic-poly U helices.

Thus, single strandedness and lack of extensive intramolecular hydrogen bonding appear to be requisite for messenger RNA activity in this *in vitro* system. Results obtained with natural RNA preparations are in accord with this conclusion. For

example, TMV-RNA directs protein synthesis with high efficiency[9, 34] in this system, compared to the efficiency of other RNA preparations.[9] Melting curves for TMV-RNA[4, 5] indicate that a larger per cent of its secondary structure is destroyed at 37° than is destroyed with ribosomal or transfer RNA preparations.

From an experimental point of view, caution should be used in handling template RNA to be used in *in vitro* systems. In the course of experiments on the melting of polymers we noted that the change in absorption with temperature is not always readily reversible. Thus the storage (for example, freezing and thawing) of polymer solutions may cause significant changes in secondary structure and therefore in template efficiency.

At present three factors influencing template activity are known—the size of the polymer chain,[14, 15, 33, 35] the nucleotide sequence, and the secondary structure. Previously we showed that poly U fractions of relatively high molecular weight are more active as templates than smaller molecules[14] and more recent data indicate that high template activity corresponds to average chain lengths of 100 or greater.[33, 35] The effect of nucleotide sequence on template efficiency is difficult to assess. Recent data show that the code is very degenerate[15] but it is not known whether all nucleotide sequences will code. Although nonsense sequences may exist, thus far none have been definitively demonstrated. As indicated in the present report, the secondary structure of a polynucleotide must also be considered in any evaluation of its ability to serve as template RNA in the *in vitro* system.

The molecular basis for the genetic information specifying the beginning or end of a protein is unknown. Previously we suggested that nonsense nucleotide sequences might function in this manner.[14] The results of the present study suggest an alternative explanation. Thus, a limited region of messenger RNA containing a high degree of secondary structure as a result of intramolecular hydrogen bonding (for example, G-G interaction) might also specify the beginning or end of a protein. Areas of marked secondary structure, which may be likened to knots in a rope, might separate the messenger molecules containing information for the synthesis of more than one protein into functional units. Mechanisms enabling certain proteins to be synthesized in close geographic proximity may be advantageous as, for example, in the synthesis of proteins containing different subunits[36, 37] or for the synthesis of coordinately repressed enzymes. In a more general sense, we expect that future studies of the three distinct RNA fractions, transfer RNA, ribosomal RNA, and messenger RNA, will indicate functional significance for the specific conformational aspects of their structures. With regard to messenger RNA, we would suggest that both the nucleotide sequence and secondary structure are relevant to the decipherment of the genetic code.

Summary.—A series of copolymers containing varying amounts of uracil and guanine have been studied as templates in a cell-free system for protein synthesis. Polymers containing relatively large proportions of guanine have low template activity. Evidence indicating that the guanine-rich polymers contain a high degree of ordered secondary structure is presented. It is suggested that the existence of such secondary structure accounts for the poor template activity of these polymers. The possible functional significance of RNA secondary structure in the coding mechanism is discussed.

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* The following abbreviations are used: Poly U, polyuridylic acid; poly A, polyadenylic acid; poly C, polycytidylic acid; poly G, polyguanylic acid; poly UC, copolymer of uridylic and cytidylic acids; poly UG, copolymer of uridylic and guanylic acids; G, guanylic acid; U, uridylic acid; A, adenylic acid; C, cytidylic acid; TMV, tobacco mosaic virus. All other abbreviations conform to those acceptable to the *Journal of Biological Chemistry*.

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