ABSTRACT

STUDIES ON THE INFLUENCE OF SECONDARY STRUCTURE AND 2'-O-METHYLATION ON THE TRANSLATION OF RIBOSOMAL RNA

By

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Two factors which might influence the translation of ribosomal RNA have been examined. The first of these is secondary structure and the second 2'-O-methylation. The effect of secondary structure was examined by using B. subtilis ribosomal RNA, which starts to thermally denature at 46° and has a Tm of about 70°, as template RNA in a thermophilic amino acid incorporating system. The incorporating system was prepared from B. stearothermophilus and was found to retain its incorporating activity to 70°. Controls of B. stearothermophilus and B. subtilis whole cell RNA, and poly uridylic acid, were active as templates in the B. stearothermophilus incorporating extract at all temperatures tested (40-65°). A fourth control, the RNA from the virus R17, had a thermal denaturation profile similar to B. subtilis ribosomal RNA, and had template activity which increased as the temperature of incorporation reaction increased. B. subtilis ribosomal RNA, however, had no template activity at any temperature tested (40-65° C) or

any Mg^{++} concentration tested (0-12 mM). If neomycin was included in the incorporation reaction mixture, the ribosomal RNA had template activity, which was found to increase as the temperature of the incorporation reaction increased. These results indicate that secondary structure is not the primary inhibiting factor in preventing ribosomal RNA translation, but may influence translation once the primary inhibiting factor is removed.

The effect of 2'-O-methylation on translation was investigated by preparing synthetic polynucleotides containing 2'-O-methylnucleosides, and testing them for template activity in an E. coli cell free amino acid incorporating system. The heteropolymer, poly(Cm,U), directed the incorporation of significant levels of phenylalanine, serine, leucine, and proline, and small amounts of isoleucine and tyrosine. The total incorporation of amino acids was slightly greater with poly(Cm,U) than with poly(C,U). The heteropolymer poly(Am,C) directed the incorporation of proline, threonine, and histidine, but its template activity was lower than that of poly(A,C). Poly(Cm,U) was active as a template for a longer period of time than poly(C,U) in directing the incorporation of phenylalanine. Both poly(Am,C) and -(Cm,U) were degraded more slowly than their unmethylated analogs when incubated in reaction mixtures used for cell-free protein synthesis. These

results indicate that the level of 2'-O-methylnucleosides (0.1-1.0%) found in ribosomal RNA would not be high enough to eliminate its template activity.

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DEDICATED

to

My Family

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INTRODUCTION

The question of whether or not ribosomal ribonucleic acid (rRNA) can serve as a template for *in vitro* protein biosynthesis has not yet been conclusively answered. When early studies showed that proteins were synthesized on ribosomes, it was hypothesized that ribosomal RNA was the template. The demonstration that rRNA has a very slow turnover rate, and that the two types of rRNA are too homogeneous to code for the wide variety of proteins found *in vivo*, soon caused this hypothesis to be abandoned (Watson, 1963). More recently (Osawa, 1965) it has been suggested that rRNA may serve as a template only for ribosomal proteins, a hypothesis based on the following three findings; (1) during recovery of methionine starved *E. coli* RC^{rel} cells or in *E. coli* cells previously treated with chloramphenicol (CM), ribosomal proteins are preferentially synthesized. During the recovery phase, accumulated precursor rRNA is converted to mature rRNA, even though further RNA synthesis is inhibited with actinomycin D (Nakada, 1965). This was interpreted as indicating that the accumulated precursor rRNA acted as a template for the ribosomal proteins. Midgley and Gray (1971), however, have shown that appreciable messenger RNA (mRNA) also accumulates during inhibition with CM,

and thus the synthesis of proteins which takes place during recovery may just be the expression of this accumulated mRNA; (2) most of the accumulated nascent rRNA appears to exist combined with ribosomes, the cellular site of protein synthesis (Muto, et al., 1966); (3) nascent ribosomal particles accumulate in E. coli B cells during treatment with CM, and the RNA extracted from these particles has template activity in cell-free amino acid incorporating systems, while mature rRNA does not (Otaka et al., 1964). Attempts by others to repeat these experiments have led to conflicting results (Manor and Haselkorn, 1967), and several authors have pointed out that mRNA contamination is possibly the reason for the template activity. This is a pertinent criticism in light of Midgley and Gray's (1971) demonstration that CM treated cells accumulate mRNA, and Levinthal's finding that CM treatment of B. subtilis cells prevents mRNA degradation (Levinthal et al., 1962).

Another argument against ribosomal precursor RNA acting as a template for ribosomal protein stems from the fact that the molecular weight of rRNA is too low to code for all the proteins found in ribosomes (Moore et al., 1968). This assumes that rRNA molecules are homogeneous, an assumption that may not be completely warranted

(Santes et al., 1961; Aronson and Halowyczyk, 1965). It has been shown that there are several cistrons from rRNA in both B. subtilis (Smith et al., 1968; Oishi et al., 1965) and E. coli (Cutler and Evans, 1967) and it is not known if these are identical. Midgley and McIlreavy, (1967) have found that the 3' terminal nucleotide sequence of 16S E. coli rRNA varies, depending on the growth conditions of the cells. Fellner and Sanger (1968) however, who have done extensive sequence analysis of rRNA from E. coli have found no evidence for heterogeneity in this rRNA.

An alternative approach to determining whether rRNA serves as a template in vivo is to ask what prevents mature rRNA from serving as a template in vitro. Once this is known, one could then determine if the rRNA even exists in vivo without this inhibitory factor present. Comparison of the properties of rRNA with those of various template active RNA molecules, indicates that rRNA contains more secondary structure and a number of modified nucleotides. This thesis will be primarily concerned with the effect of secondary structure which will be the subject of Part I, and to a more limited degree with the effect of modified nucleotides, the subject of Part II.

The main types of modified nucleotides which occur in rRNA are pseudouridine, various base methylated nucleotides, and 2'-O-methyl nucleotides (Attardi and Amaldi, 1970).

One experiment approach to determine the effect of these modifications in rRNA is to study their effect on the template activity of known synthetic polynucleotide templates. This has been done for pseudouridine (Pochon et al., 1964), and several base methylated nucleotides (Wahba et al., 1963 Ludlum et al., 1964, McCarthy et al., 1966), but no work has been done to date on the effects on 2'-O-methylation on template activity. Thus Part II of this thesis is an investigation into the effects of 2'-O-methylation on the template activity of synthetic polynucleotides. The discussion following Part I will attempt to synthesize the results of the two parts.

PART I - HISTORICAL

The formulation of the hypothesis that secondary structure may influence the template capacity of RNA molecules was based upon the findings of two separate lines of research. The first involved studies on the species of RNA which could direct the incorporation of amino acids into proteins *in vitro*, and the second was a more detailed analysis of the secondary structure of the various types of natural and/or synthetic molecules of RNA. The first of these lines of research was developed during investigations on the mechanism of protein synthesis. Several workers (Nirenberg and Matthaei, 1961, Tissieres and Hopkins, 1961) reported that *E. coli* whole cell RNA added to *E. coli* cell-free extracts produced a 10 to 20 fold stimulation of protein synthesis. The stimulatory RNA was found to differ in properties from the ribosomal RNA fraction, which by itself was essentially devoid of stimulatory ability. It was subsequently postulated that the stimulatory fraction contained a specific type of RNA called "messenger RNA." In bacterial systems, this mRNA was characterized by rapid turnover and a base composition resembling that of deoxyribonucleic acid (DNA) (Watson, 1963). Difficulty in separating this

messenger RNA from the large amounts of rRNA and transfer RNA (tRNA) in cells has precluded any further studies on purified cellular mRNA in bacteria. Whole cell RNA preparations from a wide variety of eukaryotic sources, for example liver (Barondes et al., 1962) brain (Zomzely et al., 1970) reticulocytes (Arnstein et al., 1964) have been tested and found to have template activity. In the specialized reticulocyte system it has been found that the haemoglobin mRNA is "long lived," (Nathans et al., 1962) and several laboratories are engaged in isolating this mRNA.

In 1961 Nirenberg and Matthaei made the importand discovery that the single stranded synthetic polynucleotide, poly U, was an efficient template for directing the incorporation of phenylalanine to form polyphenylalanine. In addition to its importance in unraveling the genetic code, this result also substantially supported the hypothesis that mRNA was a single stranded polynucleotide intermediate carrying the genetic information from DNA to ribosomes where it could be translated (the mRNA hypothesis). Soon other synthetic homopolynucleotides were found to be active as templates, but there was substantial variation in their relative activity; the order being poly U $>$ poly A $\geq$ poly C, with poly G being inactive (Speyer et al., 1963). Random

copolymers of all four nucleotides were also active. However, polydeoxynucleotide polymers were inactive in extracts free of RNA polymerase activity (McCarthy et al., 1966).

The third type of RNA which has been found to have template activity is viral RNA. Nirenberg and Matthaei (1961) tested tobacco mosaic virus (TMV) RNA in their E. coli B cell free extracts and found it to have template activity. Since then the RNA from several viruses, such as turnip yellow mosaic virus (Ofengand and Haselkorn, 1962), the RNA phages f2 (Nathans, 1962b), MS2 (Nathans, 1965), and R17 (Gussin et al., 1966), have been found to make excellent templates in cell-free systems. Viral RNA has the advantage that it can be obtained from preparations of pure phage, thus eliminating cellular rRNA and tRNA contamination. This has made possible analysis of both primary and secondary structure of these RNA molecules and has led to the interesting findings that these molecules are single stranded, contain extensive secondary structure, and have long stretches of untranslated sequences at 5' and 3' ends (Cory, 1970). It remains to be seen, however, if the findings gleaned from these RNA molecules can be extrapolated to cellular mRNA.

Concurrently with these studies on cell free template activity, work was also being done on the secondary structure

of rRNA, the second line of research mentioned above. The potential importance of secondary structure in RNA was suggested by the dramatic correlation between structure and function of DNA as shown by Watson and Crick (1953).

Much work has been done to elucidate the nature and relative contribution of the forces responsible for the secondary structure of DNA and RNA, but there are still many unanswered questions. Qualitatively there are two main factors responsible for the secondary structure of nucleic acids; hydrogen bonding (H-bonding) and base stacking. H-bonding occurs between the bases guanine and cytosine (G-C pairs) and between adenine and thymine (or uracil) (A-T or A-U pairs). Recent studies on these free bases, performed in organic solvents to minimize base stacking interactions, indicates that the pairings are very specific, i.e., mixtures of the incorrect pairs (A-C or G-U) showed no tendency to interact (Felsenfeld and Miles, 1967). The importance of this specificity for maintaining the correct sequence of bases in DNA and the genetic consequences thereof, was recognized by Watson and Crick (1953) in their original proposal of the structure of DNA. Hydrogen bonding also occurs in the formation of complementary duplexes of synthetic polynucleotides such as poly adenylic acid (poly A) complexed with poly uradylic acid (poly U)

(poly (A:U)) or poly (G:C). H-bonding can also occur between strands of the same homopolymer, such as poly G at neutral pH or poly A at acid pH. In addition to interstrand base pairing, polymers such as poly (A, U) consisting of alternating adenine and uracil bases, can exhibit intra-strand base pairing, where sections of the polymer loop back and form "hairpin" sections of H-bonded paired duplexes (Michelson et al., 1967).

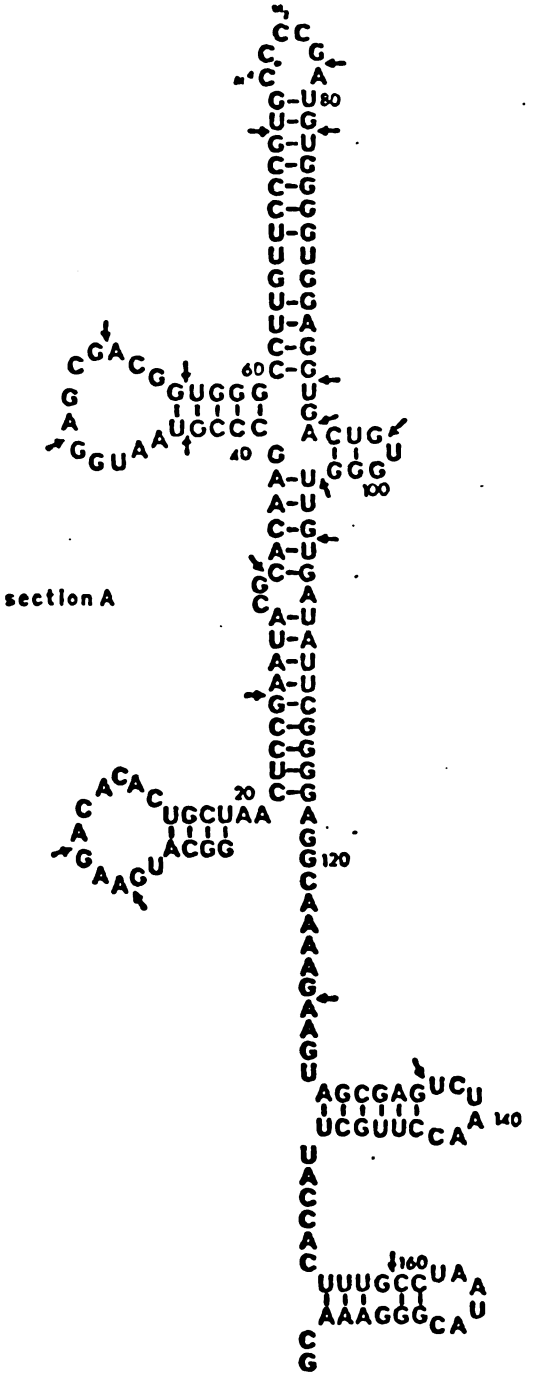
Experiments with single stranded synthetic polymers where H-bonding possibilities have been eliminated (such as poly N^{6} - dimethyladenylic acid), indicate that these polymers still possess considerable secondary structure as evidenced by their hyperchromicity and optical activity (Griffin et al., 1964).

Michelson was the first to show that these properties result from the "stacking" of the bases of a single stranded polynucleotide with their planes parallel to one another, because of favorable free energy contribution from electro-static interactions or solvent exclusions (Michelson, 1963). Support for this theory comes from the analysis of the Optical Rotatory Dispersion (ORD) spectra of dinucleotides, which indicates that even with this short oligonucleotide, base stacking occurs (DeLuca and McElroy, 1965). The hydrodynamic properties of non-hydrogen-bonded polymers

show that they are not rigid rods, however, like H-bonded duplexes, but more closely resemble flexible chains. Studies with poly A indicate that about two-thirds of the bases are in the stacked conformation at any given instant at 20° (Leng and Felsenfeld, 1966), thus giving considerable flexibility to the molecule. The exact origin of the energy of stabilization of single stranded structures is still under debate. Some of the factors which have been considered are dipole-dipole interactions of the bases, solvent exclusion and solvent interactions with the sugar-phosphate moiety (Felsenfeld and Miles, 1967).

Hydrogynamic studies of the natural RNA's, rRNA, tRNA and several viral RNA's show that they are not rigid double-stranded duplexes like DNA but are flexible single-stranded chains. Analysis of ORD and circular dichroism (CD) spectra, along with hyperchromic measurements, showed the presence of considerable secondary structure, with both H-bonding and base stacking contributions present (Spirin, 1964). Cox and Kangalingan (1967) have estimated that about 75% of the secondary structure (as measured by hyperchromicity) of both rRNA and tRNA is due to H-bonding, the rest due to base stacking. The model which has emerged from these studies is shown in Figure 1. Short sections of the polynucleotide chains are seen to loop back over complementary

Figure 1. Proposed secondary structure of a fragment of *E. coli* ribosomal RNA (Ehresmann *et al.*, 1970). Note the "folding back' of complementary sections and "looping out" of the non complementary hairpin turns. A structure similar to this has been proposed for MS2 RNA.


section A
20
40
60
80
100
120
140
160

sections and form hydrogen-bonded stretches. In these, and in the non-hydrogen-bonded sections, base stacking is present. Upon heating, the H-bonds are broken and base stacking is disrupted. Thus the increased ultraviolet absorbance (hyperchromism) represents the loss of both types of ordering forces. Because the "hairpin" loops are of variable length, and the loss of base stacking is non-cooperative, the melting profile is rather broad.

The finding of considerable secondary structure in rRNA and its lack of template ability prompted Willson and Gros (1964) to hypothesize that the secondary structure of rRNA was preventing its translation. This was consistent with Nirenberg and Matthaei's (1961) finding that whereas poly U was an excellent template by itself, when complexed with poly A to form a double stranded duplex (poly A:U), it was completely inactive. Nirenberg et al., (1963) had also observed that there was a correlation between synthetic homopolymers which were most active as templates and those which had the least secondary structure.

Singer et al., (1963) were the first to systematically test the hypothesis that secondary structure influences template activity. They prepared synthetic polymers containing varying amounts of guanosine (G) and used these as templates in a cell-free system. They found that polymers

with large amounts of G were almost inactive as templates, had the highest thermal denaturation temperature, and were the most resistant to phosphorolysis by polynucleotide phosphorylase. They concluded that the presence of extensive secondary structure inhibits template activity.

Szer and Ochoa (1964) arrived at similar conclusions by measuring the template activity of poly ribothymidylic acid (rT) both below and above its Tm of 36°. Poly rT is similar to poly U with the addition of a methyl group at position 5 of the uracil residues, and thus both have the same H-bonding capabilities. Poly U has a Tm (the temperature at which the polymer is 50% denatured) of 8.5° while poly rT has a Tm of 36°, at pH 7.0 and 0.01 M $MgCl_2$. At 37° or higher, poly rT was as effective as poly U in promoting the incorporation of phenylalanine, while at 20° the activity of poly rT was almost negligible compared to poly U. These authors also found that poly A becomes more effective for polylysine synthesis as the temperature is raised from 20 to 45°. They interpret these results as indicating that extensive secondary structure of a polymer inhibits its attachments to ribosomes and hence its effectiveness as a template.

A more recent experiment of this type using natural RNA molecules was done by Lodish (1970). He used the RNA

from bacteriophage f2 (a virus which normally infects E. coli) which is a very efficient template for protein synthesis in E. coli cell-free extracts (Nathans, 1962b). Analysis of peptides labelled in E. coli B extracts with ^{14}C-labelled N-formylmethionine under the direction of f2 RNA had shown that initiation of only two of the three genes takes place independently (coat and maturation); the initiation of the third (polymerase) gene can take place only after translation of the maturation gene. After mild formaldyhyde treatment, which partially disrupts H-bonding in the RNA, he was able to show that initiation could take place independently at all three initiation sites, thus implying that secondary structure prevented initiation at the polymerase gene. He obtained further evidence for the involvement of secondary structure in the translation of f2 RNA by measuring the incorporation of amino acids directed by f2 RNA at various temperatures, using an in vitro system from B. stearothermophilus (Lodish, 1971). At temperatures where the f2 RNA was partially unfolded, as evidenced by increasing A_{280}, the incorporation increased with the temperature. Thus at 65°, where the f2 RNA is approximately 5% denatured, the incorporation of amino acids is 20 times greater than at 50°, where it is still in its fully-folded, compact form.

The above results indicate that secondary structure

affects the ability of both synthetic and natural RNA polymers to function as templates in the cell-free synthesis of proteins. However, there has been no direct test of this hypothesis as it applies to rRNA. McCarthy et al., (1966), attempted to test this hypothesis by including neomycin in a cell-free amino acid incorporating system from E. coli. The neomycin stimulation of the translation of rRNA is greatly stimulated if the rRNA is first boiled. They interpret this promotion of template activity by heating to a loss of rRNA secondary structure or exposure of new ends, since they calculated that heating of the rRNA would have caused an average of one break per chain. However, they also point out that the inhibition in translation of rRNA may be due to the methylated bases it contains, and the neomycin may be overriding this inhibition.

Other indirect evidence for the involvement of secondary structure in translation of rRNA comes from the work of Willson and Gros (1964) and Osawa (1965) on the template activity of precursor rRNA. These workers found that precursor ribosomal particles accumulate in E. coli cells treated with chloramphenicol, or in methionine-requiring relaxed mutants which were starved for methionine. When the RNA was extracted from these particles, it was found to be less methylated than mature rRNA and had template activity in cell-free protein synthesizing systems. It also is reported to have less

secondary structure, on the basis of its slightly lower ($\sim 4^{o}$) Tm and an aggregation in Mg^{++} containing buffers. Osawa (1965) has thus advanced the hypothesis that the precursor rRNA can be translated because it is not yet methylated and has a different (or less) secondary structure than mature rRNA. After it is translated, it "matures," i.e., it becomes methylated, assumes its complete or final secondary structure, and can no longer be translated.

Since Lodish (1971) has shown that unfolding or reducing the secondary structure of f2 RNA affects its template activity, it seems reasonable to ask whether this holds true for rRNA as well. This hypothesis can be tested by using rRNA as a template for amino acid incorporation at a temperature sufficiently high to cause disruption of secondary structure. Although the Tm of bacterial rRNA is about 50^{o} in NaCl-phosphate buffers (pH 7), (Spirin, 1964), the addition of Mg^{++} to these buffers shifts the Tm upward almost 15^{o}. Mg^{++} must be included in the buffers used to measure the Tm since it is a necessary factor in cell-free incorporation systems. The resulting Tm of the rRNA ($60 - 70^{o}$) is above the temperature at which mesophilic bacterial cell-free amino acid incorporation can occur. Thus experiments using a homologous system of rRNA and an <u>in vitro</u> amino acid incorporating system in <u>E. coli</u> is

not possible. However, Friedman and Weinstein (1966), and Algranati and Lengyel (1966), have prepared amino acid incorporating systems from B. stearothermophilus which are active to almost 70°. Thus it should be possible to use mesophilic rRNA which has a Tm of 60 - 70°, and test it for template activity in B. stearothermophilus extracts at temperatures near its Tm. This will provide a direct test of the influence of secondary structure on the template activity of rRNA. If no activity is found at any temperature, under conditions in which control RNA molecules are active, it is probable that the lack of rRNA template activity cannot be ascribed solely to its secondary structure. If incorporation takes place at temperatures where rRNA is thermally denatured, but not below these temperatures, then the hypothesis that secondary structure is preventing template activity remains valid.

MATERIALS AND METHODS

RNA Extractions

(a) Whole Cell RNA. *B. subtilis* (strain 168)[1] cells were grown in one liter of media in a 3.8 liter Fernbach flask on a rotary shaker at 37°. The media consisted (per liter) of 10 gms Difco Bacto-tryptone, one gm Difco yeast extract, three gms Tris Cl pH 7.5. *B. stearothermophilus* (strain NCA2184)[2] cells were grown in the same media with agitation in a shaking water bath at 60°. When cells reached mid log phase ($A_{660} \sim 0.6$), they were poured over 500 gms of ice containing 15 ml 1.0 M NaN_3, and then centrifuged at 7,000 x g in a Sorvall refrigerated centrifuge for four minutes. The pellet was washed with 40 ml 0.1 M sodium acetate pH 5.0 and centrifuged at 7,000 x g for four minutes. The pellet was frozen, thawed and resuspended in six ml of 10 mM sodium acetate, pH 5. The cells were sonicated for 45 seconds at position 8 of a Bronwill Biosonik II ultrasonic tissue disruptor sonicator using a needle probe, and 60µl of 25% sodium lauryl sulfate was added with mixing. Six ml of redistilled, water saturated, phenol was added, and the sonicate mixed for

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[1] Courtesy of Dr. Alan Price, Univ. of Michigan, Ann Arbor, Mich.
[2] Courtesy of Dr. Koffler, Purdue Univ., Lafayette, Indiana

ten minutes on a vortex mixer, keeping it cool ($\sim 4^{o}$) by periodic chilling in an ice bath. The extract was centrifuged at 30,000 x g for ten minutes, the aqueous layer withdrawn and extracted twice more with an equal volume of water saturated phenol. After the third extraction, 0.10 volume of 1.0 M sodium acetate pH 5 and 2 volumes of cold 95% ethanol were added. The RNA was allowed to precipitate for several hours at -20^{o}, and then centrifuged at 5,000 x g for five minutes. The pellet was taken up in two ml of 0.01 sodium acetate (pH 5), 0.01 M $MgCl_2$) and approximately 20 μg of electrophoretically purified DNA ase I (Worthington Biochemical Company) added, and allowed to incubate at 4^{o} for five minutes. Four ml of cold 95% ethanol was added, the RNA allowed to precipitate for one hour at -20^{o}, and then centrifuged for five minutes at 5,000 x g. The pellet was washed three times with 2.0 ml of 3.0 M sodium acetate (pH 5.0) by vigorous resuspension and recentrifugation, then rinsed twice with 95% ethanol, once with ethyl ether, dried, and dissolved in 1.0 ml of water. The yield of total RNA was approximately 150 - 200 A_{260} units per gm of cells.

(b) Ribosomal RNA. *B. subtilis* cells were grown as described in section (a). Upon reaching mid log ($A_{660} \sim 0.6$) rifampicin (Calbiochem Company) was added to a final concentration of 30 μg/ml. Rifampicin has been shown (Di Mauro *et al.*, 1969) to specifically inhibit RNA synthesis, and hence was added to

reduce the level of endogenous mRNA. The cells were incubated in the presence of rifampicin for 15 minutes, after which they were cooled, and harvested by centrifugation. All the following operations were carried out at 0 - 4°. The packed cells were washed once with TKM buffer (0.05 M Tris Cl (pH 7.5), 0.05 M KCl, 0.01 M $MgCl_2$, 0.001 M dithiothreotol), centrifuged, and the pellet suspended in 10 ml TKM buffer per gm (wet weight) of packed cells. The cell suspension was passed twice through a French press (Aminco Company, Silver Springs, Maryland) at 8,000 psi. After the first pass DNase I was added to a concentration of 10 μg per ml. The broken cell extract was centrifuged at 30,000 x g for 20 minutes, the supernatant removed and again centrifuged at 30,000 x g for 20 minutes. The supernatant contained in the upper three-fourths of the tube was centrifuged at 105,000 x g for two hours to pellet the ribosomes. The supernatant (S-100) was carefully removed and the tube rinsed with TKM buffer in which the KCl concentration had been increased from 0.05M to 2.0 M (HS-TKM) taking care not to disturb the surface of the pellet. The ribosomal pellet was gently suspended in 5 ml of the HS-TKM, allowed to stand overnight at 0°, and then centrifuged at 105,000 x g to pellet the ribosomes. The supernatant was removed, the tube rinsed carefully with water, and the ribosomal pellet suspended in 3 ml of 0.05 sodium acetate,

pH 5. The ribosomal suspension was transferred to a glass stoppered test tube, the ribosomes disrupted by adding sodium lauryl sulfate to a final concentration of 0.5% and the ribosomal proteins removed by extracting the solution three times with an equal volume of water saturated phenol. The RNA was then precipitated by addition of two volume of 95% ethanol and allowing to stand at -20° for two hours. The precipitate was washed three times with 3.0 M NaAc pH 5.0, rinsed twice with 95% EtOH, once with ether, dried and then dissolved in 1.0 ml of water.

Measurement of Melting Profile of RNA

RNA samples which were to be measured were diluted to a final concentration of approximately one A_{260} units/ml. The buffer used to dilute the RNA was the same as that which was used in the amino acid incorporating assays, 0.05 TrisCl pH 7.5, 0.10 M NH_4Cl, 3 mMCl_2, 3 mM Spermidine and 1mM dithiothreotol. The samples were degassed by placing *in vacuo* for two minutes, placed in cuvettes, and the cuvettes sealed with plastic caps. One cuvette contained only buffer as a control blank. The temperature of the cuvettes was measured directly by placing a thermistor probe into one of the cuvettes filled with water. The temperature of the samples was raised in 5 degree increments every 15 minutes using a Haake FJ thermostatically controlled water bath which circulated water through special heating spacers

fitted on a Beckman DU spectrophotometer. After allowing the cuvettes to equilibrate for two to five minutes at each temperature, the A_{260} of the sample was measured. No corrections for volume changes have been made in plotting the resultant melting profile.

Preparation of Amino Acid Incorporating Extracts

Since it was not possible to anticipate the temperature required to demonstrate rRNA template activity, it was desirable to prepare a cell-free amino acid incorporating system which would be active at the highest temperatures possible. Two species of bacteria, B. stearothermophilus and Thermus aquaticus were used in attempts to achieve these goals.

The bacteria Bacillus stearothermophilus grows in the temperature range 45 - 68°. Cell-free amino acid incorporating extracts have been prepared from this organism, which had endogenous activity up to 70° (Friedman and Weinstein, 1966), and were stimulated by added synthetic RNA (Algranati and Lengyel, 1966). In order to prepare extracts of high activity towards added natural mRNA an attempt was made to deplete endogenous mRNA in vivo using the method of Forchammer and Kjeldgaard (1967). When an E. coli uracil auxotroph was starved for uracil (causing a preferential depletion of endogenous mRNA) the resulting

amino acid incorporating extract had very low endogenous amino acid incorporation and was more highly stimulated by added template RNA than extracts prepared in the conventional way (i.e., Nirenberg's technique (1961)). Initially, attempts were made to obtain a B. stearothermophilus uracil auxotroph using nitrosoguanidine as a mutagen; enriching for the mutant using the penicillin enrichment technique, and finally identifying the mutant by replica plating. However, after checking over 500 replica plated colonies, which should have theoretically yielded 50 uracil auxotrophs, and finding none, this procedure was abandoned.

We reasoned that another theoretically attractive technique for depleting mRNA in vivo might employ the use of a specific inhibitor of RNA synthesis, since Levinthal et al., (1962), and Forchhammer and Kjeldgaard (1967), found that the inhibition of RNA synthesis does not prevent its degradation. This would be an easier and more general approach than the use of uracil mutants, since the difficult and time consuming preparation of such mutants would be eliminated. Two likely inhibitors were actinomycin D and rifamycin (or its derivatives). Actinomycin D is prohibitively expensive for large scale preparations, it is not effective with E. coli, and its specificity has been questioned. Rifampicin (a derivative of rifamycin) however, has been

shown (Di Mauro et al., 1969) to specifically inhibit initiation of RNA synthesis, it is effective against a wide spectrum of bacteria, and is inexpensive.

Initially, amino acid incorporating extracts were prepared using B. stearothermophilus cells which had been incubated with rifampicin for 15 minutes after reaching mid log phase of growth. Since the half life of bacterial mRNA is about two to five minutes, this should allow time for most of the mRNA to decay (Levinthal et al., 1962). After incubation with the rifampicin, a crude S30 was prepared by lysing the harvested, washed cells in a French pressure cell, centrifuging the lysate at 30,000 x g for 30 minutes, and using the supernatant (S30) as the cell extract in the amino acid incorporating system. The activity of such extracts, while high with synthetic polymers, was not satisfactory with whole cell RNA (only about three to five times background). Therefore, the S30 system was fractionated into ribosomes and 105,000 x g supernatant (S100), the ribosomes washed with high salt, and then recombined with the S100 supernatants for use in in vitro amino acid incorporating studies. The disadvantage of this approach is that one might wash off the initiation factors, since this has been shown to happen with E. coli (Stanley et al., 1966), and would result in washed ribosomes which would not

work with native mRNA. When this procedure was tried with B. stearothermophilus, the resulting reconstituted extract was found to have very high activity, both with synthetic polymers and whole cell RNA. In addition, the Mg^{++} optimum for the template activity of whole cell RNA was low (3 mM) which indicates that the washing procedure used here does not remove the initiating factors, since high Mg^{++} concentrations are needed for initiation when the initiation factors are removed. Therefore, the following procedure was used to prepare the cell free extracts for the studies in this thesis.

B. stearothermophilus cells were grown, harvested, lysed, separated into S100 and washed ribosomes as described in the previous section. After the washed ribosomes had been resedimented from the high salt wash, the supernatant was removed and the tube rinsed carefully with TKM buffer. The ribosomal pellet was dissolved in 3 ml of TKM buffer. Both ribosomes and the S100 were frozen in small aliquots in a dry ice-acetone bath and stored frozen in liquid nitrogen. Equal volumes of the thawed ribosomes and S100 were combined to form the reconstituted extract, which was used in the template studies. The protein content of the extracts was determined by the method of Lowry et al., (1951).

A second species of bacteria from which cell-free extracts were prepared was Thermus aquaticus. This extremely thermophilic bacteria which grows from 40 - 79° has been isolated and characterized by Brock and Freeze (1969). Presumably, amino acid incorporating extracts from this organism would permit testing the template activity of rRNA over a wider range of the melting profile since the extracts would be expected to be active to approximately 75 - 78°, as compared to 65 - 68° for B. stearothermophilus. Cultures of this organism which were obtained from Dr. Brock (University of Indiana, Bloomington, Indiana), were grown and cell free extracts prepared. However, they were completely inactive when tested for amino acid incorporation even with the usually active template poly U. Variation of several parameters of the assay, such as Mg concentration, etc., did not stimulate activity. These bacteria produce copius slime layers which heavily contaminated the cell free extracts and were probably responsible for its inactivity. Because of this, further experiments with this bacterial species were discontinued.

Assay for Template Activity of RNA

The conditions for amino acid incorporation were similar to those of Nirenberg and Matthaei (1961) and the assay included a modification of Bollum's paper filter disc method

(1966). Each reaction contained the following components in a total volume of 70 µl; 0.05 M Tricine (adjusted to pH 7.8 with NH_4OH), 0.10 M ammonium chloride, 5.0 mM phosphoenolpyruvate, 1.0 mM ATP, 0.2 mM GTP, 2.0 mM each of nineteen (^{12}C) amino acids, 2.0 mM of the ^{14}C-labelled amino acid being studies (5-40 m Ci/mM), 2.0 mM dithiothreitol, 1.0 A_{260} unit of B. stearothermophilus tRNA, 5 µg of phosphoenolpyruvate kinase, 3 mM magnesium acetate, and 3 mM spermidine HCl. The polymer concentration is given for each experiment. Ten µl of reconstituted extract was used, composed of approximately 70 µg of ribosomal protein and 20 µg of S100 protein. When poly U was the template, the ammonium chloride concentration was reduced to 0.01 M. After addition of the reconstituted extract, the reaction mixtures were incubated at the temperatures indicated for each experiment for 20 minutes, chilled rapidly in an ice bath, and a 60 µl sample from each reaction mixture was spotted on Whatman No. 3MM disks (2.3 cm diameter). The disks were dried under a heat lamp, placed in a beaker of 5% trichloroacetic acid (approximately 10 ml/disk), and heated at 90 - 95° for 20 minutes. The disks were placed on a wet filter paper in a Buchner funnel and washed with 5% trichloroacetic acid, 95% ethanol, and finally dietyl ether. The dried disks were placed in 10 ml of

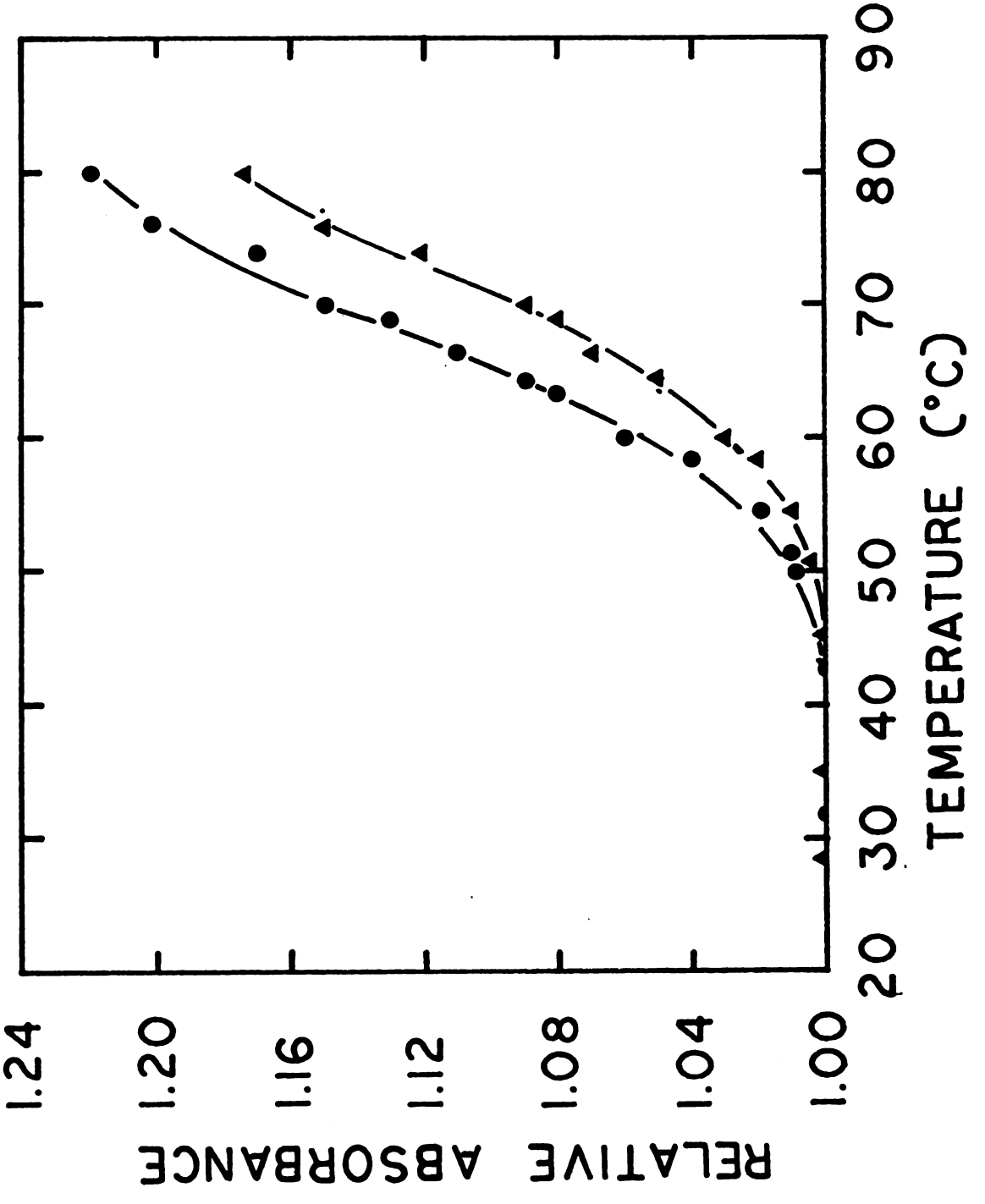
toluene containing 0.4% 2,5-bis (2(5-tert-butylbenzoxazolyl)) thiophene and counted in a Beckman LS100 scintillation counter. Background incorporation of amino acids in the absence of added polynucleotide has been substracted from the values reported.

RESULTS

Before testing an rRNA molecule for its ability to direct the synthesis of protein at elevated temperatures in a B. stearothermophilus cell-free incorporating system, three basic criteria must be fulfilled. First, under conditions of Mg^{++}, salt, etc., used in the cell-free system, the rRNA must be shown to undergo thermal denaturation at a temperature within the termperature range of the amino acid incorporating system. Second, mRNA isolated from the same bacterial source as the rRNA to be used in the experiments must be able to act as a template in the B. stearothermophilus amino acid incorporating system. The necessity for this requirement will be demonstrated later. Third, the incorporating system should be capable of detecting changes in template activity which are due to loss of RNA secondary structure.

The melting profile of purified B. subtilis rRNA is shown in Figure 2. This rRNA was extracted from the ribosomes of rifampicin treated cells, as described in Materials and Methods. Gel electrophoresis of this RNA showed that only the 16S and 23S ribosomal species were present. The maximum thermal hyperchromicity of B. subtilis rRNA is about 30% (Stenesh and Holazo, 1967). On this basis the Tm or temperature at 50% maximum hyperchromicity of B. subtilis rRNA (Figure 2) is about 70°. Since the rRNA

Figure 2. Thermal denaturation profile of B. subtilis ribosomal RNA and R17 RNA. The profiles were obtained as described in Materials and Methods. The relative absorbance (the absorbance of the heated sample divided by the absorbance of the sample at 28°) is plotted against the temperature of the sample after it has reached temperature equilibrium. (—●—), rRNA; (—▲—), R17 RNA.


RELATIVE ABSORBANCE
1.24
1.20
1.16
1.12
1.08
1.04
1.00
20
30
40
50
60
70
80
90
TEMPERATURE (°C)

starts to denature at 46° and the B. stearothermophilus amino acid incorporating system is active to 70°, the first criteria mentioned previously is satisfied. Since Lodish (1971) has found that a complete unfolding of f2 RNA is not necessary for a large increase in template activity in B. stearothermophilus extracts, the amount of unfolding of the rRNA in the range 46 - 65° should be adequate to unmask template activity.

To show that mRNA from B. subtilis could be translated in the B. stearothermophilus incorporation system, whole cell B. subtilis RNA was used as a template. Whole cell RNA is frequently used as a source of template active RNA (Barondes et al., 1962, Arnstein et al., 1964) since no general procedure is available for purifying mRNA from bacterial cells. Messenger RNA only comprises a small percent of the total extracted cell RNA (8% for B. subtilis Levinthal et al., 1962, and 3% for E. coli) the bulk of the RNA being rRNA, and thus the assumption is made that the template activity of this total RNA represents its mRNA component and in addition, there is no inhibition due to the presence of the rRNA. Initially, it was assumed any bacterial mRNA would be translated by the B. stearothermophilus amino acid incorporating extracts. However, it was found (see Table 1) that whole cell RNA from E. coli (which was

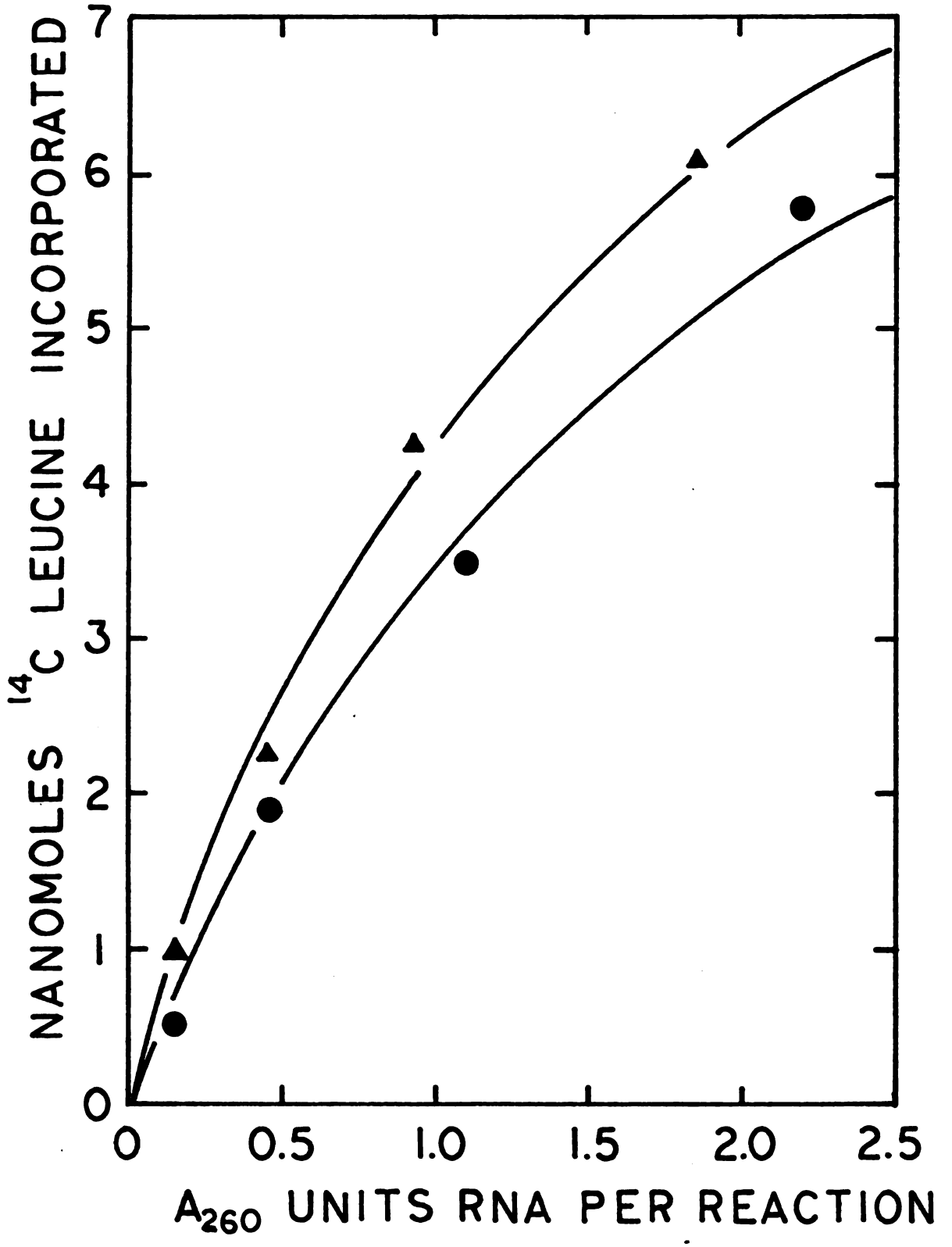
Table 1. Incorporation of amino acids directed by whole cell RNA from *E. coli* and *B. stearothermophilus*.

Source of amino acid incorporating extract	Source of whole cell RNA: *E. coli* — p moles of ^{14}C leucine incorporation	Source of whole cell RNA: *B. stearothermophilus* — p moles of ^{14}C leucine incorporation
B. stearothermophilus S-30	30	162
E. coli S-30	150	21

Conditions for the *E. coli* S30 assay are described in Part II, Materials and Methods, and conditions for the *B. stearothermophilus* S30 assay are described in Part I, Materials and Methods. The incubation temperature was 37° for the *E. coli* assay and 60° for the *B. stearothermophilus* assay. Approximately 1 A_{260} unit of RNA was used per reaction. Incorporation is expressed as picomoles of ^{14}C leucine incorporated per milligram of S30 protein.

active in E. coli extracts) had low template activity in B. stearothermophilus extracts. Thus we were prompted to try B. subtilis RNA, reasoning that related organisms would be more likely to translate each others RNA. This reasoning was borne out, since B. subtilis RNA turned out to have template activity in the B. stearothermophilus extracts. The RNA was extracted from both B. subtilis and B. stearothermophilus log phase cells, as described in Materials and Methods, and then tested for its template activity in the B. stearothermophilus amino acid incorporating system. The results are shown in Figure 3. As can be seen, the template activity increased as the RNA concentration increased, and on an A_{260} basis, the B. stearothermophilus RNA was slightly more active. This could be due to a higher percent of mRNA in the B. stearothermophilus RNA, more efficient recognition of B. stearothermophilus mRNA or degradation of some of the B. subtilis mRNA. The incorporation stimulated by whole cell RNA is comparable to the active E. coli in vitro system using comparable RNA fractions (Forchhammer and Kjeldgaard, 1967), and to the incorporation stimulated by synthetic polynucleotides in a similar B. stearothermophilus reconstituted in vitro system (Algranati and Lengyel, 1966). Hence, it appears that mRNA from B. subtilis is recognized and translated

Figure 3. Cell free incorporation of amino acids directed by B. stearothermophilus or B. subtilis whole cell RNA. Conditions for the assay are described in Materials and Methods. Incubation was at 60° for 20 minutes. Incorporation is expressed as nanomoles ^{14}C leucine incorporation per milligram of ribosomal protein. (—▲—) B. stearothermophilus RNA; (—●—) B. subtilis RNA.


NANOMOLES ^{14}C LEUCINE INCORPORATED
0
1
3
4
5
6
7
0
0.5
1.0
1.5
2.0
2.5
A_{260} UNITS RNA PER REACTION

by B. stearothermophilus extracts, thus fulfilling the second criterion mentioned above.

To insure that the in vitro incorporating system would be able to detect changes in template activity in the temperature range employed, four different RNA samples were used as controls and are shown in Figures 4 and 5. The first two controls shown in Figure 4 demonstrate the effect of temperature on the template activity of whole cell RNA from either B. subtilis or B. stearothermophilus. As is shown, both template activities are optimal at 60°, falling off at both higher and lower temperatures. The third control tests the response of the incorporating system to poly U, a polymer known to be free of secondary structure under these conditions (Richards et al., 1963). The results in Figure 4 show that activity is found at all the temperatures tested, with a maximum at 60°.

A further, and perhaps more meaningful control tests the response of the incorporation system to an RNA molecule in which the observed template activity is proportional to the degree of secondary structure. In order to mimic the rRNA as closely as possible, a natural RNA was used, i.e., from the bacteriophage R17. A melting profile of this RNA is shown in Figure 2. As can be seen, the R17 melts at a slightly higher temperature than the rRNA

Figure 4. Temperature dependence of cell free incorporation of amino acids directed by polyuridylic acid or the whole cell RNA from B. subtilis or B. stearothermophilus. Conditions for the assay are given in Materials and Methods. Incubation was at temperature indicated for 20 minutes. Incorporation is expressed as nanomoles (^{14}C) amino acid incorporated per milligram of ribosomal protein. (^{14}C) phenylalanine was used to assay poly U, and (^{14}C) leucine was used to assay B. subtilis and B. stearothermophilus whole cell RNA. Each reaction mixture included 2 A_{260} units of B. subtilis or B. stearothermophilus RNA, or 0.5 A_{260} units of poly U. Mg^{++} concentrations were optimum for each temperature. For B. stearothermophilus and B. subtilis at 40°, 50°, 55°, and 60°, the Mg^{++} concentration was 3 mM, at 65°, it was 6 mM. For poly U at 40°, the Mg^{++} concentration was 1 mM, 50°, 55°, and 60°, 3 mM, 65°, 6 mM. (—■—) B. subtilis RNA; (—●—) B. stearothermophilus RNA; (- -▲- -) poly U.

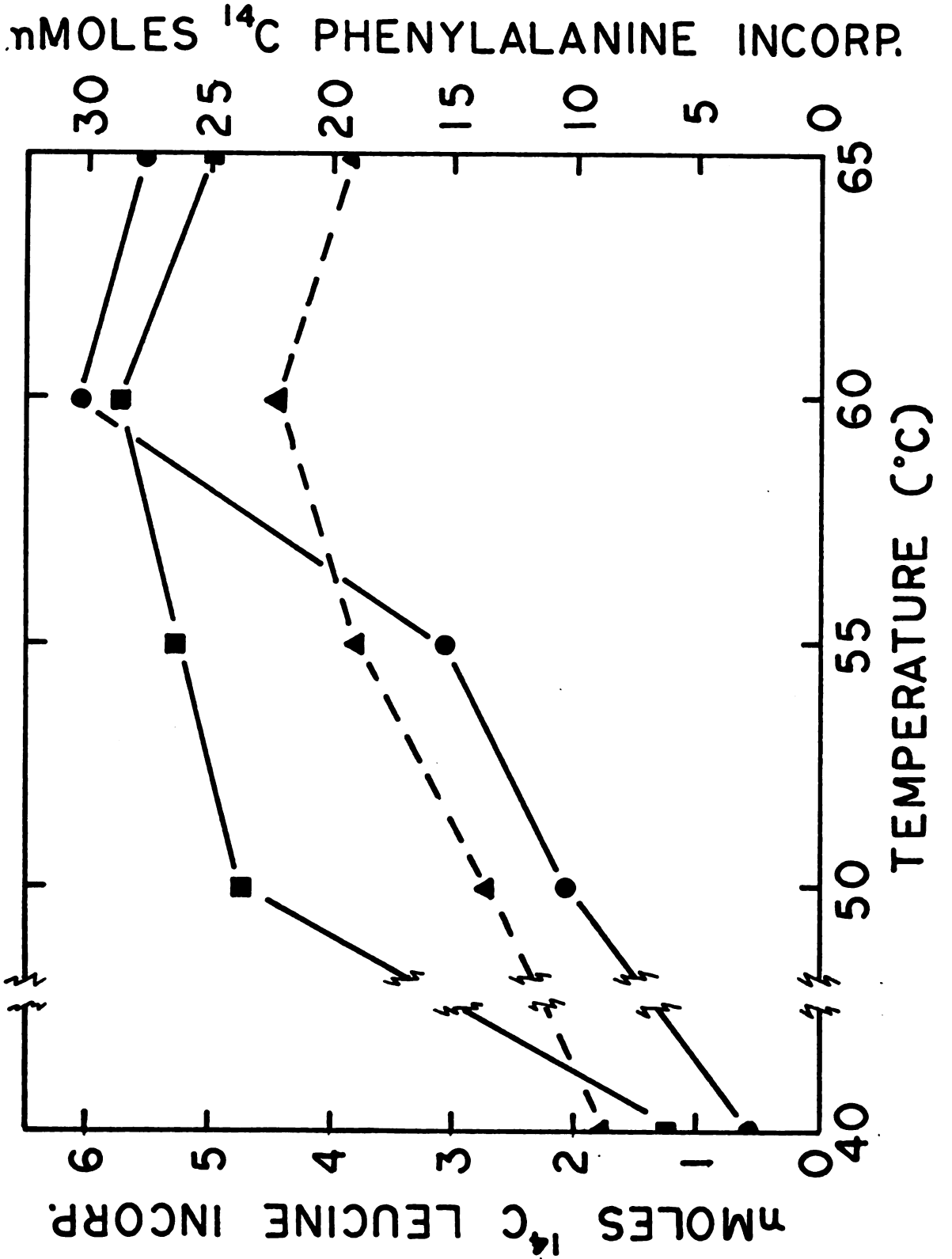
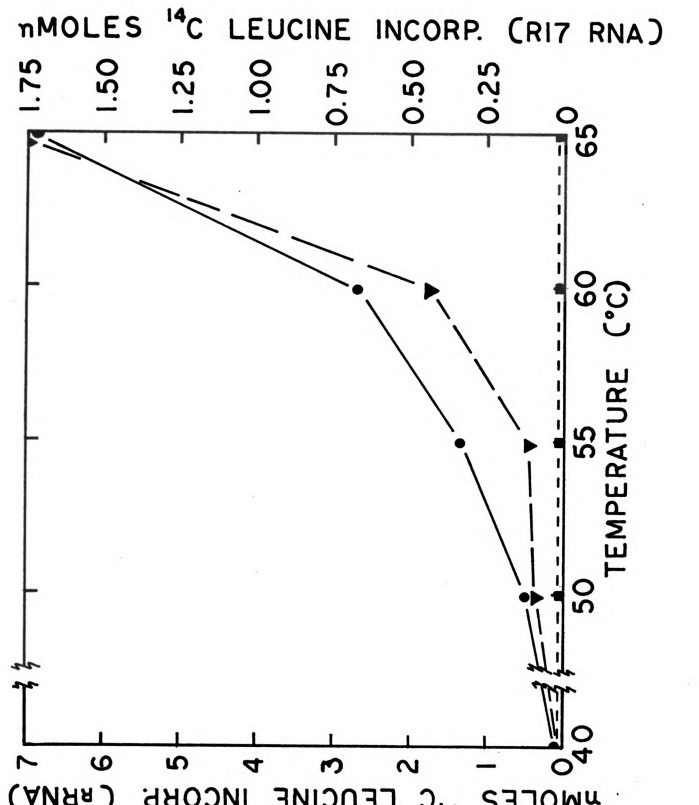

nMOLES ^{14}C PHENYLALANINE INCORP.
30
25
20
15
10
5
0
65
60
55
50
40
TEMPERATURE (°C)
nMOLES ^{14}C LEUCINE INCORP.
6
5
4
3
2
1
0

Figure 5. Temperature dependence of cell free incorporation of amino acids directed by B. subtilis rRNA in the presence or absence of neomycin, and R17 RNA. Conditions for the assay are given in Materials and Methods. Each reaction mixture included 1 A_{260} unit B. subtilis rRNA or 0.3 A_{260} unit R17 RNA. Incubation was for 20 minutes at the temperature indicated. Mg^{++} concentrations were optimum for each temperature. For R17 at 40° and 50°, the Mg^{++} concentration was 3 mM; at 55° and 60°, 6 mM; 65°, 9 mM. For B. subtilis rRNA at all temperatures, the Mg^{++} concentration was 3 mM. For B. subtilis rRNA in the presence of neomycin, the Mg^{++} concentration was 3 mM at 40°, 9 mM at 50°, 15 mM at 55° and 60°, and 18 mM at 65°. The neomycin concentration in the reaction mixture was 25μg/ml. Incorporation is expressed as nanomoles of amino acid incorporated per milligram of ribosomal protein. (— ▼ —) R17 RNA; (- - ■ - -) B. subtilis rRNA; (— ● —) B. subtilis rRNA plus neomycin. The right hand scale applies to R17 RNA, the left hand scale to B. subtilis rRNA.


nMOLES ^{14}C LEUCINE INCORP. (RI7 RNA)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
65
60
55
50
40
TEMPERATURE (°C)
μMOLES ^{14}C LEUCINE INCORP. (RNA)
7
6
5
4
3
2
1
0

from B. subtilis. The R17 RNA was tested for template activity from 40 - 65° in the B. stearothermophilus amino acid incorporating system. The results (Figure 5) show no incorporation at 40° while there is some incorporation at 50° and slightly more at 55°. At 60°, however, there is a large increase in template activity and at 65° a still further increase. There is an overall 20 fold increase in activity between 55 and 65°. This is in contrast to the small change in template activity of polu U and B. subtilis RNA at these three temperatures, and it especially contrasts with the decrease in template activity between 55° and 60° which is exhibited by all three of the RNA's in Figure 3. Thus it appears that the amino acid incorporation system used here responds to changes in the secondary structure of the mRNA, i.e., the increase in template activity is coincident with melting out of R17 RNA secondary structure.

Purified B. subtilis rRNA, from the same preparation that had been used for determination of the melting profile, was then tested for template activity in the B. stearothermophilus amino acid incorporating system over a temperature range of 40° to 65°. Two levels of rRNA were used which were chosen on the basis of the activity of whole cell RNA. Figure 3 shows that the template activity of whole

cell RNA is detectable from 0.15 to 2.5 A_{260} units/reaction mixture. If one assumes that whole cell RNA contains about 8% mRNA (Levinthal et al., 1962) and amino acid incorporation is due solely to its presence, the levels of incorporation found (Figure 3) correspond to .012 to 0.20A_{260} units of mRNA/reaction. Assuming that under non-restrictive conditions rRNA would possess template activity comparable to messenger RNA, one should easily detect the activity of B. subtilis rRNA at levels of 0.05 and 1.0 A_{260} units/reaction. At the 1.0 A_{260} unit/reaction level, even if only 5% of the rRNA was effective as a template, significant incorporation would be detected. The results are shown in Table 2 and plotted in Figure 5. No increase in incorporation over background was detected at any temperature, the range of which included a significant portion of the melting profile of the added rRNA. At the lower temperature the rRNA has not lost any of its secondary structure while at the higher temperature it has lost about one-third of its secondary structure as reflected by hyperchromicity measurements. The possible necessity for a different Mg^{++} concentration was assessed by varying the Mg^{++} concentration from 0 to 12 mM at the temperatures of 50°, 60°, and 65°. The results in Table 3 indicate no stimulation of activity at any Mg^{++} concentration.

Table 3. Incorporation of ^{14}C leucine directed by B. subtilis ribosomal RNA at different Mg concentration.

Temperature	Mg^{++} concentration (mM)	Incorporation of ^{14}C leucine for RNA level shown: No RNA	1.0 A_{260} unit/reaction
50	0	80	78
	3	108	106
	6	105	102
	12	96	110
60	0	97	89
	3	120	115
	6	125	125
	12	117	101
65	0	112	90
	3	92	82
	6	110	116
	12	115	118

Conditions for the incorporation assay are described in Materials and Methods. Incorporation is expressed as picomoles ^{14}C leucine incorporated per milligram ribosomal protein. In these experiments the background incorporation was not subtracted since this is represented by the column headed "no RNA."

One possible reason for the inactivity of the rRNA as a template is that it is non-specifically absorbing proteins or some necessary factor for amino acid incorporation or that it contains some inhibitory substance and hence may mask any inherent activity present in the rRNA. To test this, an excess of B. subtilis rRNA was mixed with B. subtilis whole cell RNA and this combined sample of RNA was used as a template. As shown in Table 4, no inhibition of template activity of the B. subtilis whole cell RNA was found, thus indicating that the rRNA does not inhibit template activity.

Finally, the possible influence of secondary structure on translation was probed by testing the template activity of B. subtilis rRNA in the presence of neomycin. McCarthy et al., (1966), have shown that in the presence of neomycin, E. coli rRNA will act as a template in a cell-free amino acid incorporating extract. These authors found that if the rRNA is first heated to 100° and then quickly cooled, its template activity is greatly increased. They have hypothesized that neomycin interacts with a protein on the ribosome and allows the ribosome to recognize rRNA as a template by overriding the rRNA secondary structure or methylation that normally prevents its translation. I have found that neomycin enables B. subtilis rRNA to act as a template in the B. stearothermophilus amino acid

Table 4. Effect of B. subtilis ribosomal RNA on incorporation of ^{14}C leucine directed by B. subtilis whole cell RNA.

Incorporation (picomoles ^{14}C leucine incorporated per milligram ribosomal protein)	RNA in reaction mixture	
	Whole cell RNA A_{260} units/reaction	rRNA A_{260} units/reaction
2200	0.6	0
2175	0.6	1.8
1000	0.2	0
1015	0.2	2.0

Conditions for the incorporation assay are described in Materials and Methods. Incubation was at 60° for 20 minutes.

incorporating system. However, in contrast to McCarthy's results with E. coli rRNA, prior heating of the rRNA produced only a slight increase in its template activity. If the secondary structure of the rRNA did affect its template ability, one would expect that at higher temperatures, where unfolding takes place, the addition of neomycin would stimulate rRNA template activity. This experiment was done by using B. subtilis rRNA as a template with neomycin in the B. stearothermophilus incorporating system from 40 - 65°. The results are shown in Figure 5. As can be seen, the profile of template activity is similar to that of R17 RNA, i.e., there is an increase in activity with temperature which parallels the melting curve of rRNA shown in Figure 2. Thus it seems there is template activity when secondary structure is lost, but that it is not evident because of some other type of inhibition which is not temperature dependent. Neomycin is evidently overcoming the primary inhibition, thus allowing the template activity to become noticeable at temperatures where the secondary structure is reduced.

DISCUSSION

The data in Figure 3 indicate that whole cell RNA, and presumably mRNA, from B. subtilis is recognized and translated by B. stearothermophilus extracts. In contrast, the data in Table 2 shows that B. subtilis rRNA has no detectable template activity at any temperature under conditions which would have permitted the possible detection of 5% of the activity of the B. subtilis mRNA. The melting profile shown in Figure 2, in combination with hyperchromicity data on complete thermal denaturation, indicates that the rRNA is approximately one-third melted by 65°. Thus it appears evident that the secondary structure of rRNA is not the deciding factor in preventing its translation. However, several other possible reasons for the failure of the rRNA to act as a template must be examined before this conclusion is justified.

First of all it is possible that the rRNA lacks an initiating codon. However, Nirenberg et al., (1963) and others, have shown that artificial polynucleotides which have no initiating codons nevertheless have high template activity. The Mg^{++} optimum is usually higher for these polymers (Revel and Hiatt, 1965) but as the results in Table 3 show, varying the Mg^{++} concentration did not enable the rRNA to act as a template.

A second possibility is that the rRNA binds some

protein or factor necessary for translation or some inhibitory substance is present in the rRNA preparations. The experiments using an excess of rRNA in the presence of whole cell RNA would seem to rule this out since no inhibition by the rRNA preparation was found. These experiments do not rule out the possibility that there is a tightly bound (perhaps covalently linked) substance which would prevent rRNA template activity but would not affect other template molecules.

A more subtle consideration is whether complete unfolding is necessary before any template activity could be seen or alternatively what level of template activity could be expected from only partially unfolded rRNA. Lodish's (1969) work with the f2 phage has shown that in B. stearothermophilus extracts the intact f2 RNA codes for only one of the three genes which it contains. This is the maturation protein, which is also the protein made in smallest amounts in the homologous E. coli system. He further showed (Lodish, 1971) that only a small increase in the A_{280} of the f2 RNA (and hence only a small amount of unfolding) is necessary for a very large increase in the ability of the f2 RNA to code for the maturation protein. Both formaldehyde treated f2 RNA in E. coli extracts and untreated f2 RNA in B. stearothermophilus

extracts were used in these studies. The net template ability is still low however. This means that R17 RNA (which is almost identical to f2 RNA (Gussin et al., (1966)) provides a good test to see if a B. stearothermophilus cell free extract can respond to changes in the template ability of an RNA molecule due to thermal unfolding, even when the net activity is low. As shown in Figure 5, the B. stearothermophilus system used here can detect these changes in R17 RNA. The data in Figure 2 demonstrate that B. subtilis rRNA unfolds at a slightly lower temperature than R17 RNA, and is unfolded at least as much at any given temperature. Since the unfolding of the R17 RNA is accompanied by a change in incorporation, it seems reasonable that even if only a small amount of the total template ability of the rRNA were exhibited when it was partially unfolded, this would have been detected.

The stimulation of incorporation by the whole cell RNA also supports this argument. Levinthal et al., (1962) have reported that mRNA comprises about 8% of the total whole cell RNA in B. subtilis. Figure 3 shows that at 60^{o} 5,100 μμ moles of ^{14}C leucine are incorporated for 2 A_{260} units of added RNA. Assuming this incorporation is due to the 8% mRNA present in

total cellular RNA, the stimulation results from only $0.16A_{260}$ units of mRNA. Therefore, 1.0 A_{260} unit of rRNA per reaction was tested at 65°, where the rRNA is about one-third melted. By analogy with the R17 data, we could make a reasonable estimate that the template active portion of the rRNA would be 10% of the rRNA A_{260}. Thus we would expect 0.1 A_{260} units of mRNA activity or about 3,500 μμ moles of ^{14}C leucine incorporated. The fact that we did not detect even 10% of this incorporation indicates that the template activity of rRNA must be extremely low in the partially unfolded form, and hence it is doubtful if the secondary structure is inhibiting its template ability.

The results from the experiments using neomycin to induce template activity are of twofold importance. First, they show that the rRNA can, under special conditions, act as a template. Hence the rRNA is not degraded or modified by the extraction technique thus rendering it inherently incapable of ever acting as a template. Secondly, since the site of action of neomycin is most likely to be a protein on the ribosome and not the rRNA, (McCarthy et al., 1966), we would expect the incorporation at higher temperatures to increase if the neomycin were overcoming some inhibition other than secondary structure of the rRNA.

The results in the presence of neomycin show that there was a constant increase in incorporation from 50° to 65° which indicates that there may be some effect of secondary structure on template activity. This would imply that there is some block to template activity other than secondary structure which is removed by neomycin, but once this block is overcome the secondary structure has some influence on the resulting template activity. It is possible that this primary block to template activity resides in the modified nucleotides present in rRNA, or in the absence of a specific primary sequence of nucleotides needed for ribosome recognition. If the latter were true, then since neomycin is a potent inducer of ambiguity in the translation of polynucleotides (Davies et al., 1965), the ambiguity induced by neomycin may cause the ribosome to recognize a sequence which normally would not be recognized.

While it is possible to explain the results of this thesis on the basis of inhibition by secondary structure which becomes observable only after the temperature independent inhibition is removed by neomycin, there are some data which imply that the strong correlation between the degree of secondary structure and template activity may not be as straightforward as it first

appears. Thus Singer's work with synthetic polynucleotides containing guanosine (Singer et al., 1963) and that of Szer and Ochoa (1964) using poly rT support the conclusion that secondary structure does inhibit template activity. However, both TMV RNA and f2 RNA have as much secondary structure at 37° as rRNA, yet they are both translated very efficiently in their normal host at this temperature. Since the secondary structure in these cases is obviously not preventing translation, it may be that the effect of secondary structure in natural messenger is not the same as its effect in artificial polymers.

Further evidence for this latter point comes from the work of Lodish (1971) who has shown that even when the secondary structure of f2 viral RNA is disrupted enough to allow translation of the maturating gene (in B. stearothermophilus extracts) the other two genes are not translated. Also, other studies by Steiz (1969) have shown that in f2 RNA there are several internal AUG codons not normally used as initiation codons. Following formaldyhyde treatment of f2 RNA (Lodish, 1970) some of these normally inaccessable initiating sites begin to function as initiating codons. The above data has led Lodish (1970, 1971) to suggest that there may be a specific but subtle interaction which occurs between native mRNA

and ribosomes before translation can begin. This interaction would only be allowed to take place at the correct starting codons on the mRNA because of a specific configuration of the mRNA, or a specific sequence of nucleotides just before the AUG codon, or a combination of both. The possibility that a pre-initiation sequence of nucleotides may be of importance is suggested by several lines of research. Sequencing of R17 viral RNA indicates that there are up to 150 nucleotides at the 5' end of the virus before the first AUG initiating codon is reached (Cory et al., 1970). Further evidence comes from studies on E. coli ribosomal initiation factors. Template competition experiments (Revel et al., 1970) showed that ribosomes containing the initiating factor f2 (C) have equal affimity for synthetic polynucleotides and natural mRNA. Addition of initiation factor f3 (B) leads to preferential binding of the ribosomes to natural mRNA. Iwasaki et al., (1968) have shown that MS2 binding to ribosomes occurs only in the presence of f3 (B). Berissi et al., (1971), have found that while ribosomes will only bind to one site on intact MS2 RNA, mild formaldehyde treatment of the MS2 RNA allowed ribosomes to bind to all three initiation codons of the RNA, in the absence of f3 (B). Addition of factor f3 (B) however, selectively stimulates the attachment of

ribosomes to the coat protein cistron initiation site. Experiments on species specificity of translation in this laboratory also are consistent with these findings. As shown in Table 1, E. coli whole cell RNA, which has high template activity in E. coli cell free amino acid incorporating extracts has almost no template activity in B. stearothermophilus extracts. This is in contrast to B. subtilis or B. stearothermophilus whole cell RNA's which are very active as templates in B. stearothermophilus amino acid incorporating extracts. It appears that B. stearothermophilus ribosomes (possibly the factor f3 (B)) can discriminate between the types of mRNA.

The lack of rRNA template activity at various temperatures is consistent with the above hypothesis of Lodish (1970, 1971). If rRNA does not have a correct primary and/or secondary configuration of a pre-initiation sequence, it cannot be recognized by a ribosome. Heating above its Tm would not help since this only reduces the secondary structure but does not introduce any change in primary sequence and may not produce the necessary conformational change. Thus template activity would not be expected to be found at any temperature, not because it does or does not have any secondary structure, but because it does not have the correct secondary structure.

An alternative hypothesis for the lack of template activity of the rRNA is that the presence of 2'-O-methyl groups on the ribose or the methylated bases in the rRNA may prevent its translation. This, of course, would not be affected by temperature, since the methylated nucleotides and their position in the rRNA are not affected by temperature. There is some circumstantial evidence which makes this hypothesis rather unlikely. Several of the modified bases such as thymine, pseudouracil, N^7 - methyl guanosine and N^6 - methyl adenosine do not seriously affect template activity of synthetic polynucleotides, especially at the levels found in rRNA. N^6 - methyl poly adenylic acid retains much of its template activity (McCarthy et al., 1966), as do copolymers of thymidylic and uridylic acid (Grunberg-Manago and Michelson, 1964) or pseudouridylic and uridylic acids (Pochon et al., 1969). The bases present in rRNA which are known to have severely altered H-bonding capabilities include N^6 - dimethyl adenine and N-1 methylguanine, but these amount to only four nucleotides per 16S RNA and 2 nucleotides per 23S RNA molecule respectively. Thus we must hypothesize that only a few methyl groups in a polymer 3,100 nucleotides long (for the 23S rRNA) are responsible for completely eliminating its template activity. Experiments with

artificial polynucleotides methylated at the H-bonding position (i.e., N-3-methyl poly U and N-1 methyl poly A) indicate that reasonably high levels of methylation are necessary to severely reduce template activity (Ludlum et al., 1964). For example, even when poly U is 10% substituted with N-3-methyl U, it still retains 10% of its template activity (Wahba et al., 1963).

One group of nucleotides which occur in rRNA which have not been tested for their effect on template activity, however, are the 2'-O-methyl nucleotides. Although they are present in low amounts, they conceivably could have a large effect on template activity since workers have shown previously that alterations at the 2' position renders polymers inactive as templates (Nirenberg and Leder, 1964). Thus experiments were designed to test the template activity of synthetic polynucleotides which contained 2'-O-methyl groups. These experiments constitute Part II of this thesis and are contained in an article published in the journal, Biochemistry.

The results of Part II show that polymers containing up to 15% 2'-O-methylnucleotides still function effectively as templates, and in some cases are better than their non-methylated analogues. Hence on the basis of levels of these modified nucleotides found in rRNA (0.1 - 1.7%),

we would expect a large percent of the template activity to be retained, but it is not.

So we are still forced to ask the question; "What property of rRNA prevents it from acting as a template?" This is even more of an enigma, since it does not seem to be the obvious factors of secondary structure or methylation. One likely possibility is that one of the initiation factors on the ribosome (possible the f3 (B)) is strongly inhibiting recognition of rRNA and hence preventing binding. This is consistent with experiments by Takonami (1963) who showed that while E. coli rRNA normally does not bind to E. coli ribosomes, formylated rRNA does. These results are reminiscent of the findings of Berissi et al., (1971), with formylated MS2 RNA, mentioned earlier. Thus it would be interesting to combine these two approaches of formylation and temperature variation and use formylated rRNA in a thermophilic system which was known to be free of f3 (B) initiation factor. If the rRNA was active under these conditions, the f3 (B) factor could then be restored to see if it specifically inhibited the template activity of the rRNA.

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PART II

The article "2'-O-Methyl Polynucleotides as Templates for Cell-Free Amino Acid Incorporation" by Brian E. Dunlap, Karen H. Friderici, and Fritz Rottman, which appeared in Biochemistry, 10, 2581 (1971). Reprinted by permission of the copyright owner, the American Chemical Society.

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2′-*O*-Methyl Polynucleotides as Templates for Cell-Free Amino Acid Incorporation*

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ABSTRACT: The 2′-*O*-methyl-containing heteropolymers, poly(Cm,U) and poly(Am,C), and the three homopolymers, poly(Am), poly(Cm), and poly(Um), were tested for template activity in a cell-free amino acid incorporation system from *Escherichia coli* B. The heteropolymer, poly(Cm,U), directed the incorporation of significant levels of phenylalanine, serine, leucine, and proline, and small amounts of isoleucine and tyrosine. The total incorporation of amino acids was slightly greater with poly(Cm,U) than with poly(C,U). The heteropolymer poly(Am,C) directed the incorporation of proline, threonine, and histidine, but its template activity was lower than that of poly(A,C). Poly(Cm,U) was active as a template for a longer period of time than poly(C,U) in directing the incorporation of phenylalanine. Both poly(Am,C) and -(Cm,U) were degraded more slowly than their unmethylated analogs when incubated in reaction mixtures used for cell-free protein synthesis. Poly(Am), poly(Cm), and poly(Um) had no template activity when tested under conditions that were optimum for the template activity of the corresponding nonmethylated polymers. However, neomycin induced the template activity of the homopolymer, poly(Um), and stimulated the amino acid incorporation directed by the heteropolymers poly(Cm,U) and poly(Am,C). Thus RNA polymers only partially methylated in the 2′ position can still direct the incorporation of amino acids into protein while complete 2′-*O*-methylation renders an RNA molecule inactive as a template.

Since Nirenberg and Matthaei's discovery in 1961 of the template activity of poly(U) in a cell-free extract, RNA polymers of known nucleotide composition have been extensively used as artificial messengers to study the properties of the RNA code and the mechanism of protein synthesis. Many of these studies have examined the changes in template properties following modifications of the RNA polymer. Most of the modifications have been in the ring moiety, *e.g.*, poly(m^2A), poly(m^6A), and poly(m^5U) (McCarthy *et al.*, 1966). However, several investigations have employed polymers containing modifications in the ribose phosphate backbone. Single-stranded DNA has been reported by McCarthy *et al.* (1966), and Morgan *et al.* (1967), to lack template activity, except in the presence of certain amino glycoside antibiotics, such as streptomycin or neomycin. In another study, Knorre *et al.* (1967), examined the effect of acetylation of the 2′-hydroxyl group in RNA. They found that neither poly(U) that was 88% acetylated in the 2′-hydroxyl position, nor poly(A) that was 98% acetylated, directed the incorporation of amino acids in a cell-free system from *Escherichia coli* B.

In contrast to 2′-*O*-acetyl ribonucleotides and deoxyribonucleotides, 2′-*O*-methyl ribonucleotides are found in RNA isolated from natural sources (Smith and Dunn, 1959; Hall, 1964; Wagner *et al.*, 1967). Nucleotides containing 2′-*O*-methylribose have been used to synthesize both 2′-*O*-methyl homopolymers (Rottman and Heinlein, 1968; Janion *et al.*, 1970), and heteropolymers containing both normal and methylated nucleotides (Rottman and Johnson, 1969). Since natural RNA species contain methyl groups on both the base and sugar moieties, the synthesis of these polymers has made it possible to ex-

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amine the effects of sugar methylation on the physical and biological properties of RNA, unobscured by the effects of base methylation. Thus 2′-*O*-methyladenosine oligonucleotides were shown to stimulate the binding of lysyl-tRNA to ribosomes (Price and Rottman, 1970), indicating that 2′-*O*-methylation does not destroy template activity. However the binding assay only monitors recognition of aminoacyl-tRNA by codons in the presence of ribosomes and not the ability of adjacent codons to direct the subsequent polymerization of amino acids. Therefore the present investigation was undertaken to determine the effect of 2′-*O*-methyl nucleotides on the ability of RNA polymers to direct the incorporation of amino acids into peptides.

Materials and Methods

Preparation of S-30 Extracts. Cultures of *E. coli* B were grown in a 12-l. Microfermenter (New Brunswick Scientific Co.) with forced aeration at 37° in a medium containing 1.0% tryptone, 0.5% yeast extract, 0.35% K_2HPO_4, and 0.15% KH_2PO_4 (pH 7.0). At mid-log phase (A_{660} *ca.* 0.5) the antibiotic rifampicin (Calbiochem) was added to a final concentration of 30 μg/ml. Rifampicin, which specifically inhibits initiation of RNA synthesis (Di Mauro *et al.*, 1969), was added to reduce endogenous mRNA levels. Fifteen minutes after adding rifampicin, the culture was chilled rapidly to 4° and the cells harvested by centrifugation. The yield was approximately 1 g of cells (wet weight) per l.

The harvested cells were washed once in a buffer containing 20% sucrose, 0.05 M Tricine, [*N*-tris(hydroxymethyl)methylglycine, adjusted to pH 7.8 with concentrated NH_4OH], and 0.01 M magnesium acetate, centrifuged, and resuspended in 10 ml of the above buffer per g (wet weight) of packed cells. Lysozyme was added to a final concentration of 0.1 mg/ml followed by EDTA to a final concentration of 1.0 mM. After 3-min incubation at 0°, the cells were centrifuged at 3000*g* and resuspended (2 ml/g wet weight of cells) in a buffer consisting of 0.05 M Tricine (pH 7.8), 0.04 M KCl, 0.04 M NH_4Cl, 0.01 M magnesium acetate, 2.0 mM dithiothreitol, and approximately 10 μg/ml of DNase (type I, Worthington Biochemical Co.). The cell suspension was passed through a French pressure cell (Aminco), at 8000–10,000 psi after which it was centrifuged at 30,000*g* for 30 min and the supernatant (S-30) withdrawn. The S-30 extract was incubated for 6 hr at 0° prior to freezing. This procedure increased the incorporating activity threefold when poly(A,C) or poly(Am,C) were used as templates. After this incubation the S-30 was frozen in 0.3-ml fractions in a Dry Ice–acetone bath, and stored in liquid nitrogen. The protein content of the S-30 extract was determined by the method of Lowry *et al.* (1951).

Assay for Template Activity of Polymers. The conditions for amino acid incorporation were similar to those of Nirenberg and Matthaei (1961), and the assay included a modification of Bollum's paper filter disk method (1966). Each reaction mixture contained the following components in a total volume of 70 μl: 0.05 M Tricine (adjusted to pH 7.8 with NH_4OH), 0.04 M KCl, 0.04 M ammonium acetate, 5.0 mM phosphoenolpyruvate, 1.0 mM ATP, 0.2 mM GTP, 2.0 mM each of nineteen [^{12}C]amino acids, 2.0 mM of the ^{14}C-labeled amino acid being studied (5–40 mCi/mmole), 2.0 mM dithiothreitol, 1.0 A_{260} unit of *E. coli* B tRNA, 5 μg of phosphoenolpyruvate kinase (Sigma Chemical Co.), 10 mM magnesium acetate with poly(C,U), poly(Cm,U), poly(U), or poly(Um) as templates, 11.5 mM magnesium acetate with poly(A,C), and 13.5 mM with poly(Am,C). The polymer concentration varied, and is given for each experiment. S-30 (10–15 μl) was used, which represents about 0.3 mg of protein. After addition of the S-30, the reaction mixtures were incubated at 37° for 20 min, chilled rapidly in an ice bath, and a 60-μl sample from each reaction mixture was spotted on Whatman No. 3MM disks (2.3 cm diameter). The disks were dried and placed in a bath of 5% trichloroacetic acid (approximately 10 ml/disk), and heated at 90–95° for 20 min. The disks were placed on a wet filter paper in a Büchner funnel and washed with 5% trichloroacetic acid, ethanol, and finally diethyl ether. When poly(C) or poly(Am) was the template, 5% trichloroacetic acid–2% sodium tungstate (pH 2.0) was used in place of 5% trichloroacetic acid. The dried disks were placed in 10 ml of toluene containing 0.4% 2,5-bis[2(5-*tert*-butylbenzoxazolyl)]thiophene and counted in a Beckman LS-100 scintillation counter. Background incorporation of amino acids in the absence of added polynucleotide has been substracted from the values reported.

Preparation of Polymers. To determine if the introduction of relatively small amounts of 2′-*O*-methyl nucleotides had an effect on the template activity of an RNA polymer, series of 2′-*O*-methyl and nonmethylated copolymers were synthesized and their template activities compared. Poly(Am,C), poly(A,C), poly(Cm,U), and poly(C,U) were synthesized as described previously (Rottman and Heinlein, 1968; Rottman and Johnson, 1969). Poly(Um) was prepared by the polymerization of UmDP with polynucleotide phosphorylase under conditions similar to those used for poly(Am). UmDP was made by deamination of CmDP (Basilio *et al.*, 1962). Poly(Cm) was prepared as described by Janion *et al.* (1970), in which Mn^{2+} was used in place of Mg^{2+}. Heteropolymers formed from a mixture of 2′-*O*-methyl and nonmethylated nucleotides reflect a tendency for paired incorporation of the 2′-*O*-methyl species (Rottman and Johnson, 1969). This was suppressed in the poly(Am,C) by using dimethyl sulfoxide in the polymerization reaction, but no way has been found to eliminate clustering of the (Cm) bases in the poly(Cm,U), and hence there was a higher per cent of Cm–Cm sequences in the poly(Cm,U) polymer than C–C sequences in the poly(C,U) polymer.

Analysis of Polymers. The heteropolymers poly(A,C), poly(Am,C), poly(C,U), and poly(Cm,U) were analyzed for their nucleotide composition after completely digesting each polymer to its component nucleosides using a combination of snake venom phosphodiesterase and *E. coli* alkaline phosphatase. The nucleosides were separated by paper chromatography on acid-washed Whatman No. 1 paper in the following systems: 1-butanol water ammonia (86:14:5, v/v) for the separation of A, Am, and C; ethyl acetate 1-propanol H_2O (4:1:2, v/v, upper phase) for the separation of C and U; isopropyl alcohol ammonia 0.1 M boric acid (7:1:2, v/v) for the separation of Cm and U. Ultraviolet-absorbing material on the chromatogram, corresponding to known standards, was eluted and quantitated as described previously (Rottman and Johnson, 1969). Prior to the digestion all polymers were chromatographed on Whatman No. 1 paper in 1-propanol concentrated NH_4OH H_2O (55:10:35, v/v) to determine that they were free of monomer.

The sedimentation coefficients (*s* value) of the polymers were determined as follows. Approximately 3 A_{260} units of polymer was layered over a 4.8-ml linear sucrose gradient of 5–20% sucrose in 0.1 M potassium acetate and 0.02 M Tris-acetate (pH 9.0) and centrifuged at 38,000 rpm for 18 hr in a Spinco SW39 rotor. *E. coli* tRNA was included as a standard and the *s* values of the polymers determined from their pos-

TABLE I: Incorporation of Amino Acids Directed by Poly(Cm,U) and Poly(C,U).[a]

[^{14}C]Amino Acid	Coding Triplet	Poly(C,U) pmoles of Amino Acid Incorpd[b]	Poly(C,U) % of Total Incorpn Exptl	Poly(C,U) % of Total Incorpn Theor[c]	Poly(Cm,U) pmoles of Amino Acid Incorpd[b]	Poly(Cm,U) % of Total Incorpn Exptl	Poly(Cm,U) % of Total Incorpn Theor
Phenylalanine	UU$^{C}_{U}$	2090	69.0	70.5	2530	75.6	73.0
Serine	UC$^{U}_{C}$	400	13.2	13.4	244	7.3	12.5
Leucine	CU$^{U}_{C}$	430	14.3	13.4	414	12.4	12.5
Proline	CC$^{U}_{C}$	110	3.5	2.7	160	4.7	2.1
Total		3030			3348		

[a] Conditions for the S-30 assay are described in Materials and Methods. Approximately 0.2 A_{260} unit of polymer was used per reaction. [b] Incorporation is expressed as picomoles of amino acid incorporated per milligram of S-30 protein. [c] Theoretical per cent incorporation is calculated from the polymer base ratios, which were 1:5.3 and 1:5.8 (C or Cm to U) for poly(C,U) and poly(Cm,U), respectively. Background incorporation of amino acids in the absence of added polynucleotides has been subtracted from the values reported. Polynucleotide stimulated incorporation ranged from twice background for proline to fifteen-times background for phenylalanine.

ions in the gradient relative to that of tRNA. Since Jones *et al.* (1964) have shown that the template activity of a polymer is influenced by its chain length, pairs of heteropolymers were used which were of similar size as indicated by similar s values. The s values of the polymers used in this study were as follows: poly(Cm,U), 4.0; poly(C,U), 4.0; poly(Am,C), 5.2; poly(A,C), 5.3; poly(Am), 5.0; poly(Cm), 4.0; and poly(Um), 9.2. The sedimentation profiles indicated that the polymers were heterodisperse.

Results

Poly(Cm,U)- and Poly(C,U)-Directed Incorporation of Amino Acids. The template activity of both the poly(Cm,U) and poly(C,U) polymers was determined by measuring the incorporation of phenylalanine, leucine, serine, and proline. These four are the only amino acids that can be coded for by a polymer containing C and U. The results, in Table I, are compared to the values calculated from the base ratio of the polymers. As shown in Table I, the total amino acid incorporation directed by the (Cm,U) polymer was slightly greater than the incorporation directed by the (C,U) polymer. Therefore there seems to be no overall inhibition in the template activity of poly(Cm,U) by the Cm nucleoside. The poly(Cm,U) directed incorporation of serine, leucine, and proline into peptides shows that the nucleoside Cm can replace C in a triplet, since these amino acids are coded for by triplets which contain C.

Poly(Cm,U) and poly(C,U) were used as templates to determine whether polymers containing Cm were misread more than polymers containing C. The incorporation of several amino acids was measured whose code words corresponded to these listed in Table I with one of the 5′ or internal C residues replaced by A or G. These amino acids were valine (GUN,N = A, U, C or G), tyrosine $\left(UA^{U}_{C}\right)$, arginine (CGN, N = A, U, C or G), and isoleucine $\left(AU^{U}_{C}\right)$. No incorporation of valine or arginine was detected when either poly(C,U) or poly(Cm,U) was used as a template. Tyrosine and isoleucine were incorporated to a slight extent when either poly(C,U) or poly(Cm,U) was used as a template. Therefore, it is evident that the nucleoside Cm is not extensively misread in the 5′ or internal position as A or G, nor does it cause misreading of adjacent U bases in the polymer.

Since a fixed time assay was used to obtain the results in Table I, experiments were done to determine if the difference in template activity was time dependent. The time course of incorporation of phenylalanine was measured using both poly(Cm,U) and poly(C,U) as templates. The results shown in Figure 1 indicate that although the initial rate of incorporation of phenylalanine is similar for both polymers, incorporation continues for a longer period of time when poly(Cm,U) is present.

The longer duration of the template activity of poly(Cm,U) as compared to poly(C,U) may be related to increased protection of the (Cm,U) polymer from degradation by nucleases in the S-30 extract. To test this, [^{14}C]uracil-labeled copolymers of (C,U) and (Cm,U) were synthesized and their degradation to trichloroacetic acid soluble products measured, using the same conditions employed in the amino acid incorporation assay. The results in Figure 2A indicate that the (Cm,U) polymer is degraded at a much slower rate than the (C,U) polymer, suggesting that the 2′-*O*-methyl group increased the nuclease resistance of the (Cm,U) polymer. Thus it seems likely that poly(Cm,U) can direct the incorporation of phenylalanine for a longer period of time than poly(C,U) because of its slower rate of degradation.

Incorporation of Amino Acids Directed by Poly(Am,C) and Poly(A,C). In contrast to the template activity of poly(Cm,U) relative to poly(C,U), the (Am,C) polymer was found to be much less active as a template than the corresponding (A,C) polymer. Table II contains a summary of the results of experiments which measured the template activity of the (A,C) and (Am,C) polymers. The poly(Am,C)-directed incorporation of histidine and threonine into peptides in the absence of neomy-

TABLE II: Incorporation of Amino Acids Directed by Poly(Am,C) and Poly(A,C) in the Presence and Absence of Neomycin.[a]

[^{14}C]Amino Acid	Coding Triplet	Poly(A,C)[b]		Poly(Am,C)	
		−Neomycin	+Neomycin	−Neomycin	+Neomycin
Proline	CCN	600	314	156	314
Threonine	ACN	53	82	18	82
Histidine	CA$\begin{matrix}C\\U\end{matrix}$	43	143	8	121
Serine	UCN, AG$\begin{matrix}U\\C\end{matrix}$	ND[c]	206	ND	164
Arginine	CGN, AG$\begin{matrix}A\\G\end{matrix}$	ND	54	ND	48
	(N = A, C, U, or G)				

[a] Conditions for the S-30 assay are described in Materials and Methods. Poly(Am,C) (0.12 A_{265} unit) and poly(A,C) (0.2 A_{265} unit) were used per reaction. Neomycin concentration was 5 μg/ml. Incorporation is expressed as picomoles of amino acid incorporated per milligram of S-30 protein. [b] Both poly(A,C) and poly(Am,C) had base ratios of 1:13 (A or Am to C). [c] ND = none detected. Background incorporation of amino acids in the absence of added polynucleotide has been subtracted from the values reported. Polynucleotide-stimulated incorporation ranged from one-times background for histidine to ten-times background for proline.

cin indicates Am can be read as A in a triplet, since both amino acids require A in their code words. The lower stimulation of amino acid incorporation by poly(Am,C) compared to poly(A,C) indicates that the 2′-*O*-methyl groups have an inhibitory effect on the template activity of the (Am,C) polymer.

To determine if the nucleoside Am was misread in the 5′ or internal position, or caused misreading of the cytosine residues in the polymer, the incorporation of the amino acids serine, arginine, alanine, and leucine, was measured. The codons for these amino acids are, respectively, UCN, CGN, GCN, CUN, where N = A, U, C, or G. Neither poly(Am,C) nor poly(A,C) stimulated significant incorporation of any of these four amino acids. Thus the nucleoside Am does not appear to be read as G or U, and apparently does not increase the misreading of the polymer to any detectable extent.

To determine if the inhibition noted in Table II was time dependent, the incorporation of proline was measured as a function of time when directed by either poly(Am,C) or poly(A,C). The results, shown in Figure 3, indicate that the rate of incorporation of proline is significantly slower when directed by poly(Am,C) than when directed by poly(A,C). Since poly(Cm,U) was more resistant than poly(C,U) to degradation by the S-30 extract, it was of interest to see if the same held true for poly(Am,C) when compared to poly(A,C). The results of degradation studies performed with [^{14}C]cytosine-labeled

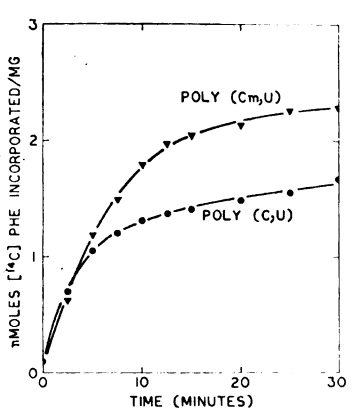

FIGURE 1: Time dependence of incorporation of [^{14}C]phenylalanine directed by poly(C,U) or poly(Cm,U). Conditions for the S-30 assay are given in Materials and Methods. The reaction volume was 700 μl, which contained 2 A_{260} units of poly(C,U) or poly(Cm,U), and 2.9 mg of S-30 protein. After addition of the S-30, 60-μl samples were withdrawn at the times indicated and spotted on Whatman No. 3MM disks, which were then treated as described in Materials and Methods. Incorporation is expressed as nanomoles of amino acid incorporated per milligram of S-30 protein.

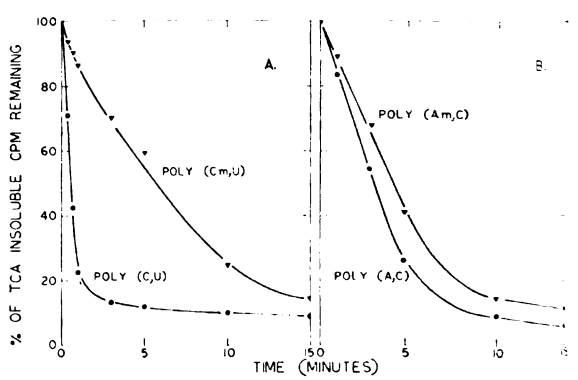

FIGURE 2: Degradation of polymers by S-30 extracts. Labeled polymers, in 700 μl of S-30 incorporation buffer (substituting [^{14}C]- for [^{14}C]amino acid), were incubated at 37°. At the times shown, 60-μl samples were withdrawn, added to 1.0 ml of cold 5% trichloroacetic acid (TCA), and filtered through a Millipore filter (Type HAWP, 0.45 μ pore size). The filter was washed three times with cold 5% trichloroacetic acid, dried, and counted. In part A 2.7 mg of S-30 and 5.0 A_{260} units of ^{14}C-labeled (C,U) or (Cm,U) were used. The specific radioactivity of both the [^{14}C]poly(C,U) and poly(Cm,U) was 1.0×10^4 cpm/A_{260} unit. In part B 5.0 mg of S-30 and 5.0 A_{260} units of ^{14}C-labeled poly(A,C) or poly(Am,C) were used. The specific radioactivity of poly(A,C) was 3×10^4 cpm/A_{260} unit and of poly(Am,C), 1.7×10^4 cpm/A_{260} unit.

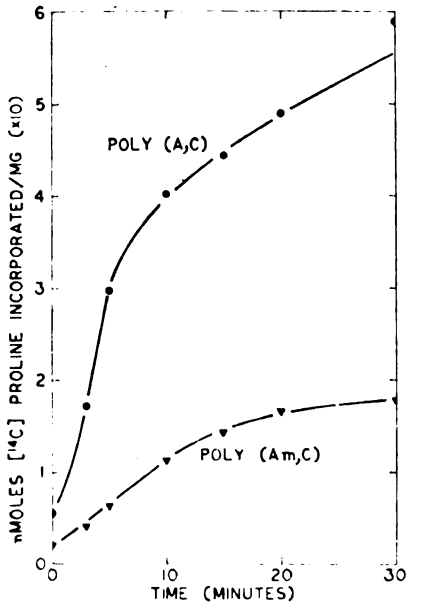

FIGURE 3: Time dependence of incorporation of [^{14}C]proline directed by poly(A,C) or poly(Am,C). Conditions of the S-30 assay are given in Materials and Methods. The reaction volume was 700 μl, which contained 2.6 A_{260} units of poly(A,C) or 1.3 A_{260} units of poly(Am,C) and 5.8 mg of S-30 protein. After addition of the S-30, 60-μl samples were withdrawn at the times indicated and spotted on Whatman No. 3MM disks, which were then treated as described in Materials and Methods. Incorporation is expressed as nanomoles of amino acid incorporated per milligram of S-30 protein.

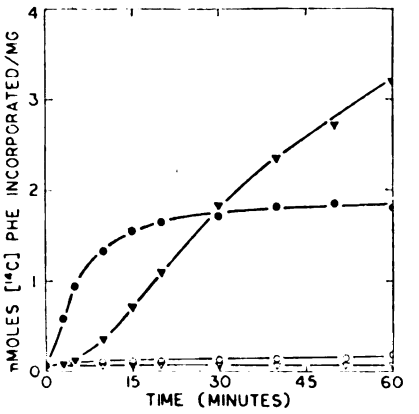

FIGURE 4: Time dependence of incorporation of [^{14}C]phenylalanine directed by poly(U) or poly(Um) with and without neomycin. Conditions of the S-30 assay are given in Materials and Methods. The reaction volume was 350 μl, which contained 2.0 A_{260} units of poly(U) or poly(Um) and 2.0 mg of S-30 protein. Neomycin concentration was 5 μg/ml. After addition of the S-30, 30-μl samples were withdrawn at the times indicated and spotted on Whatman No. 3MM disks, which were then treated as described in Materials and Methods. ● Poly(U), (▽) poly(Um), (○) poly(U) + neomycin, and (▼) poly(Um) + neomycin. Incorporation is expressed as nanomoles of amino acid incorporated per milligram of protein.

poly(Am,C) and poly(A,C) are shown in Figure 2B. Although poly(Am,C) was degraded at a slower rate than poly(A,C), the differences in the rates of degradation are not as pronounced as the differences between poly(Cm,U) and poly(C,U).

In determining the optimum conditions for the amino acid incorporation assays, differences were found between poly(A,C) and poly(Am,C) for both the polymer saturation level and the Mg^{2+} concentration. Poly(A,C) saturated the reaction at 0.25 A_{260} unit/70-μl reaction, and had an optimum Mg^{2+} concentration of 11.5 mM, while poly(Am,C) saturated the reaction at 0.12 A_{260} unit/70 μl, and had an optimum Mg^{2+} concentration of 13.5 mM. A lower saturation level was also found for poly(Cm,U) (0.12 A_{260} unit/70 μl) compared to poly(C,U) (0.25 A_{260} unit/70 μl). However, the optimum Mg^{2+} concentration of 10 mM was the same for both poly(Cm,U) and poly(C,U).

Use of 2′-O-Methyl Homopolymers as Templates. The three 2′-*O*-methylribose homopolymers, poly(Am), poly(Cm), and poly(Um), were tested for template activity under conditions which were optimum for the corresponding nonmethylated polymer. None of the three methylated homopolymers exhibited any template activity under these conditions, which would have permitted detection of 1% of the poly(U)-directed incorporation, and 5% of the poly(C)- or poly(A)-directed incorporation. Since template activity is highly dependent on the Mg^{2+} concentration in the assay, the 2′-*O*-methyl homopolymers were also tested for template activity using a range of Mg^{2+} concentrations from 4 to 20 mM. No activity was found at any Mg^{2+} concentration.

Effects of Neomycin on Template Activity of Homopolymers. McCarthy *et al.* (1966) and Morgan *et al.* (1967) have shown that neomycin enables DNA to act as a template for *in vitro* protein synthesis. Hence it was of interest to see if neomycin would influence the template activity of 2′-*O*-methyl-containing polymers. At neomycin concentrations of 5 μg/ml, neither poly(Cm) nor poly(Am) exhibited any template activity, but poly(Um) now served as an efficient template for the incorporation of phenylalanine. A time study of the template activity of poly(Um) plus neomycin as compared to poly(U) is shown in Figure 4. The results indicate that the rate of phenylalanine incorporation was slower when directed by poly(Um) plus neomycin, but incorporation continued for a longer period of time, perhaps due to the stability of the template. Thus the incorporation of phenylalanine directed by poly(U) is essentially over after 20 min, while the incorporation of phenylalanine directed by poly(Um) plus neomycin is still continuing after 60 min. To determine the relative ambiguity of the neomycin-facilitated translation of poly(Um), the incorporation of the amino acids, leucine, isoleucine, serine, and tyrosine, was measured using both poly(Um) and poly(U) as templates. The results, in Table III, show that the total incorporation of the amino acids listed is about the same when the template was either poly(Um) with neomycin or poly(U) without neomycin. This implies that the total ambiguity in translation of poly(Um) in the presence of neomycin is about the same as that of poly(U) in the absence of neomycin. The pattern of miscoding has changed, however, and the predominant miscoded amino acid with poly(Um) is serine as compared to leucine with poly(U).

Effect of Neomycin on the Template Activity of Heteropolymers. Table IV lists the results of experiments to determine the effect of neomycin on the incorporation of amino acids directed by poly(Cm,U) and poly(C,U). With poly(Cm,U) the effect of neomycin depended on its concentration and the amino acid being tested. Low (0.5–5.0 μg/ml) concentrations of neomycin stimulated the incorporation of all amino acids, whereas higher concentrations of neomycin were inhibitory. The neomycin concentration for maximum stimulation of incorporation depended on the amino acid, and is given in Table IV. With poly(C,U) as a template however, the incorporation of the amino acids listed in Table IV was inhibited at all concentrations of neomycin. The results of similar experiments using poly(Am,C) and poly(A,C) are listed in

TABLE III: Effect of Neomycin on the Misreading of Poly(Um) and Poly(U).[a]

[14C]Amino Acid Incorpd	Poly(U)		Poly(Um)	
	−Neomycin	+Neomycin	−Neomycin	+Neomycin
Leucine	510	330	ND[b]	260
Isoleucine	100	82	ND	51
Serine	61	150	ND	570
Tyrosine	50	ND	ND	ND
Total	721	562		881

[a] Conditions for the S-30 assay are described in Materials and Methods. Approximately 0.50 A_{260} unit of polymer was used per reaction. Neomycin concentration was 5 μg/ml. Results are expressed as picomoles of amino acid incorporated per milligram of S-30 protein. [b] ND = none detected. Background incorporation of amino acids in the absence of added polynucleotide has been subtracted from the values reported. Polynucleotide-stimulated incorporation ranged from two-times background for isoleucine to five-times background for leucine.

TABLE IV: Effect of Neomycin on the Incorporation of Amino Acids Directed by Poly(Cm,U) and Poly(C,U).[a]

[14C]Amino Acid	Poly(C,U)		Poly(Cm,U)	
	−Neomycin	+Neomycin	−Neomycin	+Neomycin
Phenylalanine	1684	897	1868	2182
Serine	355	129	310	414
Leucine	548	330	494	1330
Proline	132	77	126	209

[a] Conditions for the S-30 assay are described in Material and Methods. Approximately 0.50 A_{260} unit of polymer was used per reaction. Neomycin concentration was 0.5 μg/ml for phenylalanine, 2.5 μg/ml for leucine and proline, and 5 μg/ml for serine. Incorporation is expressed as picomoles of amino acid incorporated per milligram of S-30 protein. Background incorporation of amino acids in the absence of added polynucleotide has been subtracted from the values reported. Polynucleotide stimuated incorporation ranged from two-times background for proline to ten-times background for phenylalanine.

Table II. At a concentration of 5 μg/ml, neomycin stimulated the poly(Am,C)-directed incorporation of all the amino acids tested. This concentration of neomycin stimulated the poly-(A,C)-directed incorporation of four of the five amino acids tested, but inhibited the incorporation of the fifth, proline.

Discussion

Both poly(Cm,U) and poly(Am,C) served as templates for amino acid incorporation, as shown in Tables I and II. Compared to their nonmethylated analogs, poly(Am,C) was less active as a template than poly(Cm,U). Other experiments employing poly(Um,U), which was prepared by deamination of poly(Cm,U), indicate that poly(Um,U) also served as a template (data not shown). The effect of 2′-*O*-methyl groups on the template activity of a polymer may depend on which of the four nucleotides is 2′-*O*-methylated, the nature of adjacent nucleotides, and the base composition of the polymer. The important conclusion to be drawn from these studies is that low levels of 2′-*O*-methyl nucleotides in an RNA polymer do not eliminate its template activity and under certain conditions can be stimulatory.

If methylation of the 2′-sugar position caused a significant change in the conformation of the bases in the polymer, it might alter the hydrogen-bonding capabilities of the component nucleotides and thus increase misreading. The results of Tables I and II, however, indicate that both Am and Cm can replace their respective nonmethylated analogs without causing an increase in misreading of the polymer.

Nakada (1965) has postulated that in *E. coli*, methylation of nascent rRNA destroys its template activity. 2′-*O*-Methyl nucleotides comprise from 0.1 to 1.9% of the component nucleotides of rRNA (Starr and Sells, 1969). Our results, which show that even higher levels of 2′-*O*-methyl nucleotides (7–15%) do not completely inhibit template activity, suggest that the amount of 2′-*O*-methyl nucleotides found in rRNA would not be sufficient to prevent translation.

Neomycin had no effect on poly(Am) or poly(Cm) template activity, but promoted that of poly(Um) and stimulated the template activity of both methylated heteropolymers. The promotion of poly(Um) template activity was not accompanied by any appreciable increase in miscoding. In a similar finding, Morgan *et al.* (1967) reported that poly(dI) is inactive as a template, but in the presence of neomycin it efficiently directed the incorporation of phenylalanine. They also noted that neomycin did not cause misreading of this polymer. Davis (1966) has reported that neomycin can cause inhibition of template activity with only a slight increase in miscoding. Our results are in essential agreement with the conclusion that neomycin can affect template efficiency without significantly increasing miscoding.

Bobst *et al.* (1969a–c) have shown that poly(Am) has more secondary structure than poly(A), and Zmudzka *et al.* (1969) obtained similar results for poly(Cm). Thus the failure of the 2′-*O*-methyl homopolymers to serve as templates may be due to their increased secondary structure, since Szer and Ochoa (1964) have reported that increased secondary structure in RNA decreases its template ability.

The degradation studies using poly(Cm,U) and poly(Am,C) indicate that even in a crude cell-free protein-synthesizing system, known to contain a variety of nucleases (Barondes and Nirenberg, 1962) methylated nucleotides confer nuclease resistance to a polymer. We have also noted that the methylated homopolymers are very resistant to mixtures of alkaline phosphatase, snake venom phosphodiesterase, and micrococcal nuclease. Thus 2′-*O*-methylation may provide a mechanism for stabilizing a template without affecting its fidelity of translation. Further studies are in progress to investigate the effect of 2′-*O*-methyl nucleotides on the stability of RNA toward various nucleases.

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