Cistron Specificity of 30S Ribosomes Heterologously Reconstituted with Components from *Escherichia coli* and *Bacillus stearothermophilus*†

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ABSTRACT: *Escherichia coli* ribosomes *in vitro* initiate and synthesize all three proteins encoded by bacteriophage R17 RNA, whereas ribosomes from *Bacillus stearothermophilus* recognize almost exclusively the A protein initiator region. Previous mixing experiments traced this difference in cistron selectivity to the 30S ribosomal subunit. To localize the specificity-determining agent in the 16S RNA or protein fraction of the 30S ribosome, we have assayed the initiation specificity of 30S particles heterologously reconstituted with RNA and protein components from the two species. Examination of both ribosome binding to ^{32}P-labeled R17 (and Qβ) RNA and phage RNA directed [^{35}S]fMet dipeptide synthesis indicates that cistron specificity is conferred primarily by the protein fraction of the 30S subunit.

During the initiation of polypeptide chains in bacteria, ribosomes not only select true initiator regions on a messenger RNA but also differentiate among them, such that various cistrons may be translated with quite different efficiencies (for a review, see Steitz (1974)). The discovery of translational systems which exhibit different cistron specificities when programmed with the same mRNA *in vitro* has facilitated identification of the components responsible for this discrimination between initiation sites. In the example best characterized so far, ribosomes from *Escherichia coli* recognize the beginnings of all three genes on bacteriophage R17 or f2 RNA (those for the A or maturation protein, the coat protein, and the replicase or synthetase enzyme), while under comparable conditions ribosomes from *Bacillus stearothermophilus* translate the A cistron almost exclusively (Lodish, 1969; Steitz, 1969). Lodish (1969, 1970a) demonstrated that the specificity of *E. coli* or *B. stearothermophilus* ribosomes could not be altered by the addition of initiation factors, tRNA, or supernatant enzymes from the other species. In mixing experiments, he further traced the cistron selectivity to the 30S subunit of the ribosome.

The possibility of localizing the specificity-determining agent in the RNA or protein component of the 30S ribosome was suggested by the work of Nomura *et al.* (1968), who achieved the heterologous reconstitution of 30S particles using protein and 16S RNA fractions from *E. coli* and *B. stearothermophilus*. Here, we report an examination of the cistron specificity of such hybrid particles. Their ability to recognize the beginnings of the three cistrons on R17 (and Qβ) RNA has been assayed both by ribosome protection of initiator regions on ^{32}P-labeled phage messenger and by [^{35}S]formylmethionyl dipeptide synthesis directed by unlabeled R17 RNA. The results clearly demonstrate that cistron selectivity in this interspecies system is conferred primarily by the protein fraction of the 30S ribosomal subunit.

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Experimental Section

(*a*) *Reconstitution of the 30S Ribosomes. E. coli* ribosomal components were prepared from Q13, a strain lacking RNase I. Cells were grown in 1-l. cultures of R broth (8 g of NaCl, 1 g of glucose, 1 g of Difco yeast extract, and 10 g of Bactotryptone per liter) at 37° with vigorous shaking and harvested in mid-log phase after rapid chilling. The resultant cell paste was frozen in Dry Ice–acetone. *B. stearothermophilus* cells (strain N.C.A. 1503-4R from P. Lengyel) were similarly prepared, except that each liter of R broth was supplemented with 1 ml of 1 M $MgSO_4$ and with 1 ml of a solution containing, per liter: 0.57 g of $CaCl_2 \cdot 2H_2O$, 16.7 g of $FeCl_3 \cdot 6H_2O$), 0.18 g of $ZnSO_4 \cdot 7H_2O$, 0.16 g of $CuSO_4 \cdot 5H_2O$, 0.18 g of $CoCl_2 \cdot 6H_2O$, and 20.1 g of $Na_2EDTA \cdot 2H_2O$. The inoculated culture was incubated without shaking for 30 min at 69° before vigorous shaking at the same temperature.

To prepare ribosomal subunits, frozen cell paste (per gram) was ground with 2 g of levigated alumina; 25 μg (per gram) of DNase I was added, and the mixture was incubated for 15 min at 4°. After extraction with 2.5 ml (per gram) of buffer A (containing 0.1 M NH_4Cl–10 mM $Mg(OAc)_2$–20 mM Tris-HCl (pH 7.5)–0.5 mM EDTA–3 mM 2-mercaptoethanol), the resulting suspension was clarified at 15,000 rpm in the SS34 Sorvall rotor for 20 min and again for 30 min; 5.8 ml of the final supernatant was layered over 5.2 ml of 1.1 M sucrose–0.5 M NH_4Cl–10 mM $Mg(OAc)_2$–20 mM Tris-HCl (pH 7.5)–0.5 mM EDTA, and centrifuged for 16 hr at 48,000 rpm in a Spinco 50Ti rotor. The upper one-half of the supernatant (S-100) was dialyzed extensively against 10 mM $Mg(OAc)_2$–10 mM Tris-HCl (pH 7.5)–80 mM NH_4Cl–6 mM 2-mercaptoethanol, and then frozen over Dry Ice–acetone and stored at −70°. The ribosome pellets were resuspended overnight in buffer B (0.1 M NH_4Cl–1 mM $Mg(OAc)_2$–20 mM Tris-HCl (pH 7.5)) and dialyzed against the same buffer. After dilution to a concentration of 10 mg/ml, 1-ml aliquots were layered on 36-ml, 5–20% sucrose gradients in buffer B and centrifuged for 16 hr at 20,000 rpm in the Spinco SW27 rotor. The fractionated 30S and 50S peaks were pooled separately and the Mg^{2+} concentration raised to 10 mM. The 50S ribosomes were precipitated by the addition of 0.47 g of $(NH_4)_2SO_4$/ml and collected by centrifugation at 15,000 rpm for 20 min. The 30S particles were concentrated by centrifugation at 50,000 rpm for 10 hr in

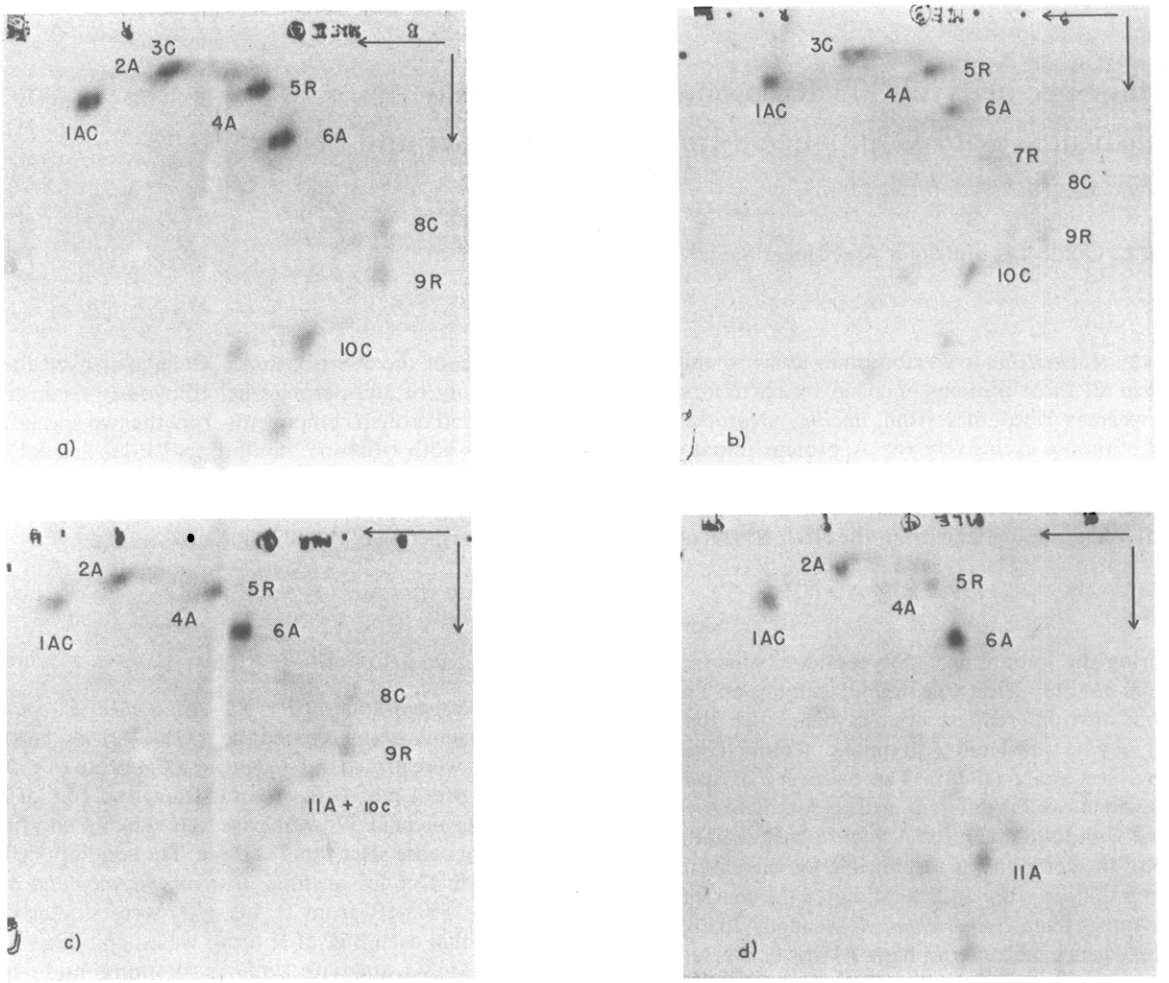

FIGURE 1: T_1 ribonuclease fingerprints of ^{32}P-labeled R17 initiator regions bound and protected in reactions containing 30S ribosomes reassembled from *E. coli* and *B. stearothermophilus* components. The maps are from experiment 3, Table I. The 30S particles contained: (a) *E. coli* proteins and *E. coli* 16S RNA; (b) *E. coli* proteins and *B. stearothermophilus* RNA; (c) *B. stearothermophilus* proteins and *E. coli* RNA; (d) *B. stearothermophilus* proteins and *B. stearothermophilus* RNA. Electrophoresis was from right to left at pH 3.5 on cellulose acetate and from top to bottom in 7% formic acid on DEAE paper. All designated oligonucleotides, if detectable, were routinely counted to assess the relative recognition of the three R17 initiator regions. Sequences of the oligonucleotides (see Steitz, 1969), as confirmed by analysis after counting, are: 1AC = U-U-U-G, 3C = C-U-U-C-U-A-A-C-U-U-U, 8C = C_{2-3}U-C-A-A-C-C-G, and 10C = C-A-U-G from the coat protein initiation site; 1AC = U-U-U-G, 2A = C-U-U-U-U-A-G, 4A = A-U-U-C-C-U-A-G, 6A = A-C-C-U-A-U-G, and 11A = C-U-A-G from the beginning of the A cistron; and 5R = A-U-U-A-C-C-C-A-U-G, 7R = A-A-A-C-A-U-G, and 9R = A-C-A-A-C-A-A-A-G from the replicase initiator region. Initiator A-U-G's are underlined. Especially in the cases of spots which nearly coelectrophorese (2A and 3C, and 10C and 11A), subsequent analysis is essential to determine which oligonucleotide is present.

the Spinco 50Ti rotor. The resultant subunit pellets were resuspended in and extensively dialyzed against buffer A before storage at −70°.

16S RNA was prepared from 70S ribosomes by phenol extraction as described by Traub *et al.* (1971). Total 30S proteins from both *E. coli* and *B. stearothermophilus* were prepared by extraction of 30S particles in urea–LiCl, following the procedure of Nomura *et al.* (1968). All reconstitutions were performed as described (Nomura *et al.*, 1968) except that the amounts of the reactions were halved. Incubation was for 15 min at 40° when only *E. coli* components were present, at 55° for only *B. stearothermophilus* components, and at 50° for mixed components. After concentration by centrifugation, the reconstituted 30S particles were resuspended in a small volume (about 70 μl) of TMA I buffer (Nomura *et al.*, 1968).

(b) *Poly(U) Directed [^{14}C]Phenylalanine Incorporation System.* The activity of reconstituted 30S particles relative to native *E. coli* or *B. stearothermophilus* 30S subunits was assayed by poly(U) directed incorporation of [^{14}C]phenylalanine into hot Cl_3CCOOH precipitable material. Reaction mixtures (60 μl) contained: 0.033 M Tris-HCl (pH 7.5), 0.014 M $Mg(OAc)_2$, 0.056 M NH_4Cl, 4 mM phosphoenolpyruvate, 0.3 mM GTP, 3.5×10^{-5} M unlabeled phenylalanine, 4 mM ATP, 0.1 A_{260nm} unit of uncharged *E. coli* tRNA, 0.8 μg of pyruvate kinase (Worthington), 12 μl of S-100, 9 μg of poly(U) (Miles), 0.5 A_{260nm} unit of *E. coli* 50S ribosomes, 0–0.5 A_{260nm} unit of 30S particles, crude initiation factors from *E. coli* (see below), and 0.1 μCi of [^{14}C]phenylalanine (New England Nuclear, 400 Ci/mol). Incubation was for 1 hr at 49°. The system responded linearly to increasing amounts of all 30S particles, leveling off at approximately 0.3 A_{260nm} unit of 30S particles per reaction.

(c) *Analysis of Ribosome-Protected R17 Initiator Regions.* ^{32}P-labeled R17 or Qβ RNA (1 to 8 Ci/g) and charged, formylated mixed *E. coli* tRNA (from CA244) were prepared as before (Steitz, 1969, 1972). Crude initiation factors, isolated from *E. coli* strains Q13 or MRE600 as described by Anderson *et al.* (1967), were titrated against *E. coli* 70S ribosomes washed in 2 M NH_4Cl to determine amounts needed for maximum stimulation of ^{35}S-labeled fMet-tRNA binding.

^{32}P-labeled RNA was bound to ribosomes in 55-μl reaction mixtures containing: 0.05 M ammonium cacodylate, 0.13 M NH_4Cl, 8 mM $Mg(OAc)_2$, 2 mM 2-mercaptoethanol, 2.2 A_{260nm} units of *B. stearothermophilus* 50S ribosomes, 0.15–0.5 A_{260nm} unit of native or reconstituted 30S ribosomes, *E. coli* initiation factors, 2.5 A_{260nm} units of charged formylated tRNA, and approximately 1 A_{260nm} unit of [^{32}P]R17 RNA. Incubation was at 49° for 10 min. Pancreatic ribonuclease digestion of the resulting initiation complexes, fractionation on sucrose gradients, and isolation and analysis of the protected initiator regions were performed as described previously (Steitz *et al.*, 1970). After quantitation, all T_1 oligonucleotides labeled in Figure 1 were analyzed by pancreatic ribonuclease digestion to ascertain both their identification and purity.

(d) *Dipeptide Synthesis. E. coli* tRNA (from CA244) was charged and formylated in the presence of [^{35}S]methionine (isolated from *E. coli* grown on $^{35}SO_4$ (New England Nuclear) and the kind gift of R. Condit) and 19 unlabeled amino acids according to the method of Bretscher (1969). Dipeptide synthesis was performed in reaction mixtures (50 μl) comparable to those used in ribosome binding except that the ^{32}P-labeled R17 RNA and unlabeled *E. coli* tRNA were replaced with 50 μg of unlabeled R17 RNA and 15 μg of ^{35}S-labeled charged, formylated tRNA, respectively. After incubation at 49° for 15 min, dipeptides were released from the tRNA, oxidized, and analyzed in a two-dimensional electrophoresis–chromatography system as described by Bretscher (1969) (Figure 2).

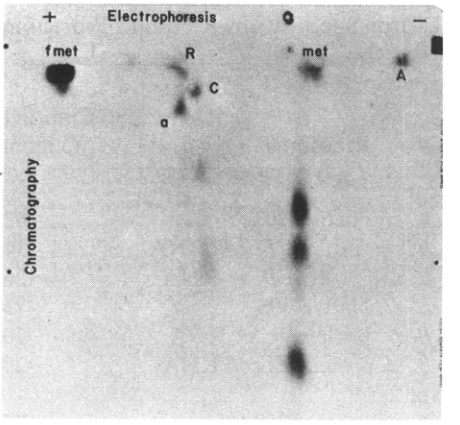

FIGURE 2: Dipeptide map, illustrating the type of pattern obtained, was prepared as described in the Experimental Section: A, fMet–Arg; C, fMet–Ala; R, fMet–Ser; Met, methionine; fMet, formylmethionine. a is an (unknown) oxidized derivative of [^{35}S]fMet-tRNA (see Bretscher, 1969). The identity of the three phage-specific dipeptides was confirmed by electrophoresis at pH 6.5 against synthetic markers. Fainter dipeptide spots visible in the map were not stimulated by the addition of R17 RNA (see Condit *et al.*, 1973).

Results

Total 30S ribosomal proteins and 16S RNAs were isolated from *E. coli* and *B. stearothermophilus* and reassembled in all four possible combinations as described by Nomura *et al.* (1968). Before assaying for cistron specificity, we first checked each set of reconstituted particles for their ability to incorporate [^{14}C]phenylalanine into hot Cl_3CCOOH precipitable material in a poly(U) directed *in vitro* protein synthesis system. Tables I and II show that in most experiments homologously reconstituted particles were greater than 40% as active as the corresponding native 30S ribosomes. The activity of particles containing both *E. coli* and *B. stearothermophilus* components was more variable, but no correlation between the efficiency of reassembly and the cistron specificity of the particles is observed. 30S ribosome activity recovered from the reconstitution reactions was not due to the presence of intact subunits in either the protein or 16S RNA fractions: pellets obtained from control reconstitution mixtures from which protein had been omitted did not stimulate phenylalanine incorporation, and no detectable pellets were obtained from complementary reactions lacking 16S RNA.

We used two different assays to assess the cistron specificity of the reconstituted 30S ribosomes. (1) T_1 fingerprint analysis of the regions on ^{32}P-labeled R17 RNA which are protected from nuclease digestion in an initiation complex provided a measure of the relative ribosome binding at the beginnings of the three phage cistrons. (2) Examination of R17 RNA directed [^{35}S]fMet dipeptides tested, in addition, the ability of the ribosomes to synthesize the first peptide bond of each nascent protein. It has been previously demonstrated that these two assays give similar results (Yoshida and Rudland, 1972; Steitz, 1973b). Also note that both assays measure relative initiation at the three sites directly; hence, the absolute efficiency with which any reconstituted 30S particle functions in initiation is unimportant.

Because it is known that alterations in the secondary and tertiary structure of the R17 RNA molecule drastically affect ribosome recognition of the beginnings of the three phage genes (Lodish, 1970b, 1971; Steitz, 1973b), all assays were performed at 49°. At this temperature in the presence of *E. coli* initiation factors, *E. coli* ribosomes initiate coat, replicase, and A protein synthesis, whereas the addition of *B. stearothermophilus* factors to native ribosomes from either species produces a temperature-dependent anomalous recognition of additional sites on R17 RNA (Steitz, 1973a). For this reason, *E. coli* initiation factors were utilized in all assays of reconstituted particles. 50S ribosomal subunits came from *B. stearothermophilus* to ensure that recognition of the coat or replicase initiator region could not arise from contamination by 30S particles.

Representative results obtained using ribosome protection to assay cistron specificity are shown in Figure 1 and Table I. The data, within the approximate twofold reproducibility of this technique (Steitz, 1973b), allow two conclusions to be drawn concerning the reassembled 30S particles. First, the reconstitution process *per se* does not greatly alter the cistron specificity of 30S ribosomes. Second, the ability of heterologously reconstituted particles to recognize the three R17 initiator regions reflects primarily the source of the protein fraction. Ribosomes containing *E. coli* proteins and either RNA efficiently initiate coat protein and replicase as well as A protein synthesis. By contrast, particles reconstituted with *B. stearothermophilus* proteins in most instances discriminate 10- to 20-fold against the R17 coat site and three- to fivefold against the replicase initiator region.

The relatively high level of recognition of the R17 replicase initiation site by 30S ribosomes containing *B. stearothermophilus* proteins was surprising in light of previous results (Steitz, 1973a). Such binding is observed not only with reconstituted particles but also with native *B. stearothermophilus* subunits. It is not likely to be due to the presence of *E. coli* 30S ribosomes in the initiation factor preparations: control binding reactions omitting 30S particles yielded less than 10% the amount of protected [^{32}P]RNA as in the 70S region of fractionated reactions containing the least active reconstituted ribosomes. Alternatively, severe autoradiolytic degradation of

TABLE I: Cistron Specificity by Ribosome Protection Assay.[a]

Ribosomes	Cpm/PO_4 in A Site Oligonucleotide	Ratio of R17 Initiation Sites Bound A : Coat : Replicase			Activity in Poly(U) Assay[b] (%)
Expt 1[c]					
E. coli native 30S	681	1	2.3	1.4	100
E. coli protein/*E. coli* RNA	345	1	1.7	1.0	97
E. coli protein/*B. st.* RNA	261	1	1.6	1.1	43
B. st. Native 30S	732	1	0.06	0.4	100
B. st. protein/*B. st.* RNA	200	1	0.08	0.2	47
B. st. protein/*E. coli* RNA	208	1	0.08	0.4	111
Expt 2					
E. coli native 30S	874	1	1.3	0.6	100
E. coli protein/*E. coli* RNA	198	1	0.5	0.5	61
E. coli protein/*B. st.* RNA	275	1	1.2	1.0	7
B. st. native 30S	213	1	0.04	0.3	100
B. st. protein/*B. st.* RNA	335	1	0.09	0.2	24
B. st. protein/*E. coli* RNA	236	1	0.1	0.06	102
Expt 3					
E. coli native 30S	56	1	4.1	2.0	100
E. coli protein/*E. coli*	32	1	1.0	0.5	35
E. coli protein/*B. st.* RNA	50	1	2.0	1.5	19
B. st. native 30S	6.6	1	<0.2[d]	0.5	100
B. st. protein/*B. st.* RNA	24	1	<0.1[d]	0.1	44
B. st. protein/*E. coli* RNA	35	1	0.3	0.3	17

[a] 30S ribosomes were reassembled and assayed for poly(U) synthesis and cistron specificity as described in the Experimental Section. Ratios of the three R17 initiator regions recovered from 70S ribosomes (Steitz *et al.*, 1970) were calculated from the cpm/PO_4 values for the three initiator AUG-containing oligonucleotides (see Figure 1). The actual cpm/PO_4 observed in the A site initiator oligonucleotide (ACCUAUG) of each fingerprint are presented. Specific activities of oligonucleotides arising from more distal portions of each initiator region were consistent with those of the corresponding AUG-containing oligonucleotide. [b] Activity of the reconstituted particles, assayed as described in the Experimental Section, is compared with that of the native ribosomes (100%) from which the 30S protein fraction was derived. [c] The same set of reconstituted ribosomes was assayed by ribosome protection in experiment 1, Table I, and dipeptide synthesis in experiment 1, Table II. [d] Note that less than 20 total cpm (above machine background = 12 cpm) were detected in these AUG-containing oligonucleotides.

several of the [^{32}P]R17 RNA preparations used here may have produced structural alterations in the message which allowed *B. stearothermophilus* ribosomes to recognize the replicase initiator region. Lodish (1971) previously reported that unfolding of f2 RNA by treatment with formaldehyde significantly stimulated the synthesis of replicase by thermophilic ribosomes at temperatures between 37 and 65°. We, however, observe detectable recognition of the R17 replicase initiation site only at 49°; *B. stearothermophilus* ribosomes bind the A site exclusively at 65°.

Dipeptide synthesis in the presence of heterologously reconstituted ribosomes and R17 RNA is reported in Table II. Because no-template values generally are quite high and the results therefore more variable, we consider this assay to be less reliable than ribosome protection. Also, note that reconstituted *B. stearothermophilus* particles show significantly less reading of the coat and replicase sites than do native ribosomes. Nonetheless, the data do confirm the conclusion that specificity in cistron recognition is a function of the 30S ribosomal protein fraction.

Finally, 30S particles heterologously reconstituted from *E. coli* and *B. stearothermophilus* components were tested for cistron specificity by binding to ^{32}P-labeled Qβ RNA (not shown). *B. stearothermophilus* ribosomes at 65° had previously been shown to bind only to a site on Qβ RNA which does not correspond to any of the three initiator regions recognized by *E. coli* ribosomes at 37 to 49° (Steitz, 1973a). At 49°, on the other hand, *B. stearothermophilus* ribosomes exhibited much less efficient anomalous binding and marginal but detectable recognition of the actual Qβ initiation sites. When the reconstituted particles were examined, the anomalous recognition was observed to transfer only with the thermophilic protein fraction, providing further evidence that selectivity in initiation is conferred by some protein component(s) of the 30S ribosome.

A trivial explanation for the observed difference in cistron specificity between *E. coli* and *B. stearothermophilus* ribosomes is that the thermophilic 30S proteins are contaminated by a nuclease activity which destroys features of the phage mRNA required for recognition of the coat and replicase initiator regions. Even partial fragmentation of R17 RNA may significantly reduce ribosome binding to the beginnings of these two cistrons relative to the A protein initiation site (Steitz, 1973b). The possibility of nuclease contamination was tested by preincubating R17 RNA with *B. stearothermophilus* 30S ribosomes before adding *E. coli* particles. Results obtained in both types of assays (Table III) demonstrate that, with the reaction components and conditions used here, *E. coli* ribosomes can subsequently initiate at the coat and replicase sites. Thus, interaction with *B. stearothermophilus* ribosomes does not cause an irreversible alteration in the R17 RNA template (see also Lodish (1969) and Held *et al.* (1974)).

TABLE II:[a] Cistron Specificity by Dipeptide Synthesis.

Ribosomes	R17 RNA	Cpm Obsd in fMet-Arg	Cpm Obsd in fMet-Ala	Cpm Obsd in fMet-Ser	Total ^{35}S (cpm)	Ratio of A	Ratio of Coat	Ratio of Replicase	Activity in Poly(U) Assay[b] (%)
Experiment 1[c]									
E. coli native 30S	+	6139	21991	4131	318,200	1	5.4	0.6	100
	−	2788	3230	2078	343,500				
E. coli protein/*E. coli* RNA	+	4437	11051	1953	358,500	1	3.8	0.5	97
	−	1900	1568	753	342,800				
E. coli protein/*B. st.* RNA	+	3984	8506	1617	356,100	1	3.2	0.08	43
	−	2085	2223	1520	367,700				
B. st. native 30S	+	7504	7297	3317	316,800	1	0.6	0.2	100
	−	4287	4851	2526	304,300				
B. st. protein/*B. st.* RNA	+	3781	2426	1229	358,500	1	0.02	<0[d]	47
	−	1693	2275	1447	338,300				
B. st. protein/*E. coli* RNA	+	4022	3027	1553	338,500	1	0.5	<0[d]	111
	−	1765	1816	1664	322,600				
Experiment 2									
E. coli Native 30S	+	22665	45908	16271	1,547,200	1	2.6	0.6	100
	−	8271	9615	6815	1,430,000				
E. coli protein/*E. coli* RNA	+	7174	10010	4057	905,400	1	1.4	0.3	70
	−	3658	5175	3640	1,225,900				
E. coli protein/*B. st.* RNA	+	6214	6963	3187	1,307,000	1	0.7	0.6	14
	−	4580	5969	2185	1,381,900				
B. st. Native 30S	+	26385	17155	8809	1,168,400	1	0.3	0.1	100
	−	9060	12770	7665	1,250,000				
B. st. protein/*B. st.* RNA	+	9843	5998	3680	1,208,000	1	<0[d]	0.02	24
	−	4271	7191	3813	1,311,400				
B. st. protein/*E. coli* RNA	+	11206	6866	2189	1,308,600	1	0.1	<0[d]	71
	−	5004	6375	3721	1,347,500				

[a] Reconstituted 30S ribosomes were assayed by dipeptide synthesis in the presence of unlabeled R17 RNA as described in the Experimental Section. For each type of 30S ribosome, dipeptide maps (such as that shown in Figure 2) were prepared from two reactions, one with and one without R17 RNA. All ^{35}S radioactivity appearing on each map was counted (column 6) and the fraction of the total radioactivity in each dipeptide spot calculated (columns 3, 4, and 5). The % radioactivity incorporated into fMet-Arg (for the A protein), fMet-Ala (for the coat protein) or fMet-Ser (for replicase) in the reaction containing R17 RNA was then corrected by subtracting the comparable values from the reaction lacking template. Ratios of the three dipeptides (column 7) were calculated from these corrected numbers. [b] See Table I, footnote *b*. [c] See Table I, footnote *c*. [d] Note that no stimulation of dipeptide synthesis over the no-template control was observed in these cases.

Discussion

Our results indicate that the difference in the ability of *E. coli* and *B. stearothermophilus* ribosomes to initiate polypeptide synthesis at RNA phage cistrons is determined primarily by the protein fraction of the 30S subunit. The responsible component is apparently not a nuclease or a contaminating initiation factor, and therefore is most likely to be one or several of the 21 characterized 30S proteins.

Which 30S ribosomal protein(s) is involved? Our preliminary heterologous reconstitution experiments using split proteins and core particles (Hosokawa *et al.*, 1966) from the two species suggest that the specificity-determining agent is among those proteins retained in the core. Indeed, Held *et al.* (1974) present evidence in the accompanying paper that a core protein (S12, the streptomycin protein (Ozaki *et al.*, 1969)) and its *B. stearothermophilus* counterpart play a critical role in the efficient translation of R17 RNA. Higo *et al.* (1973) had previously performed heterologous reconstitution experiments to demonstrate functional correspondence between the fractionated 30S ribosomal proteins of *E. coli* and *B. stearothermophilus*. Clearly, use of the above assays to examine the initiation specificity of 30S ribosomes which have been reassembled using fractionated proteins from the two species will establish whether a single protein replacement is sufficient to alter cistron selectivity.

The data presented here, however, do not completely rule out some participation of the 16S RNA in determining ribosome specificity with respect to initiation on the phage mRNAs. Variations between trials indicate that our assays are not sufficiently sensitive to detect a slight enhancement of cistron selectivity in homologously compared to heterologously reconstituted particles. Several observations suggest that 16S RNA could be involved in RNA phage cistron selection. Although resistance to most ribosome-directed antibiotics has been assigned to one or another ribosomal protein (Nomura, 1970), sensitivity to kasugamycin is related to a change in the methylation pattern of the RNA (Helser *et al.*, 1972); Kozak and Nathans (1972) have detected differential inhibition of the initiation of the three MS2 proteins by *E. coli* ribosomes in the presence of the drug. Likewise, Held *et al.* (1974) in the preceding paper show that the origin of the RNA in 30S particles heterologously reconstituted from fractionated *E.*

TABLE III:[a] Recovery of Coat and Replicase Sites After Incubation with *B. stearothermophilus* Ribosomes.

	Cpm/PO_4 in A Site Oligonucleotide	Ratio of R17 Initiation Sites Bound		
		A	: Coat	: Replicase
Experiment 1				
B. st. 30S ribosomes at 0 min	181	1	<0.1[b]	0.2
B. st. 30S ribosomes at 0 min, then *E. coli* 30S ribosomes at 3 min	175	1	1.3	1.1
E. coli 30S ribosomes at 3 min only	47	1	1.0	0.9

		Cpm Observed in				Ratio of		
	R17 RNA	fMet–Arg	fMet–Ala	fMet–Ser	Total ^{35}S (cpm)	fMet–Arg	: fMet–Ala	: fMet–Ser
Experiment 2								
B. st. 30S ribosomes at 0 min	+	3119	1000	1950	169,900	1	0.06	<0[c]
	−	989	828	2442	163,500			
B. st. 30S ribosomes at 0 min, then *E. coli* 30S ribosomes at 5 min	+	3908	2530	3476	170,300	1	0.5	<0[c]
	−	1908	1539	3985	172,200			
E. coli 30S ribosomes at 5 min only	+	1538	1092	1353	184,800	1	0.7	0.2
	−	761	544	1174	179,000			

[a] Data were obtained and calculated as detailed in Tables I and II. All reactions included 3.8 $A_{260\ nm}$ units of *B. stearothermophilus* 50S subunits, and where indicated, 1.6 $A_{260\ nm}$ units of *E. coli* native 30S ribosomes and 1.5 $A_{260\ nm}$ units of *B. stearothermophilus* native 30S ribosomes. Total incubation time was 10 min at 49°. [b] SeeTable I, footnote *d*. [c] See Table II, footnote *d*.

coli and *B. stearothermophilus* components does significantly alter the R17 RNA/poly(U) translation capability of the subunit. Thus, ribosomal recognition of initiator regions may involve not only direct interaction between the messenger and ribosomal protein(s) but may, in fact, be mediated by a ribosomal site created by both the protein and the 16S RNA.

Finally, do initiation factors also participate in the selection of mRNA initiator regions by ribosomes from these two species? They are not the primary specificity determining agent, as shown previously (Lodish, 1970a; Steitz, 1973a) and confirmed here. However, we have noted (Steitz, 1973a) that (1) addition of crude *E. coli* initiation factors to washed *E. coli* ribosomes stimulates relatively more recognition of the R17 coat and replicase initiator regions than of the A site, and (2) recognition of the A cistron by *B. stearothermophilus* ribosomes is largely independent of initiation factors. Thus, the specificity-determining protein(s) in the *E. coli–B. stearothermophilus* system may function by subtly altering the interaction between the 30S ribosome and initiation factors, which has a more pronounced effect on recognition of the R17 coat and replicase sites. This idea is supported by the observation of Held *et al.* (1974) that ribosomes containing *B. stearothermophilus* S12 are relatively inactive in general initiation assays. Likewise, Ozaki *et al.* (1969) have shown that 30S ribosomes totally lacking S12 are defective in AUG and f2 RNA directed initiation; Lelong *et al.* (1971) observe that streptomycin causes release of fMet-tRNA from a preformed initiation complex; J. C. Lelong, D. Gros, F. Gros, A. Bollen, R. Maschler, and G. Stöffler (manuscript submitted for publication), using antibodies to individual ribosomal proteins, deduce a critical role for S12 in initiation-dependent fMet-tRNA binding; and A. Bollen, L. Kahan, J. W. B. Hershey, A. Cozzone, and R. Traut (personal communication) observe that S12 can be cross-linked to IF_3 initiation factor on the 30S ribosome.

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Comparative Optical Property Studies on Polycistronic R17 Phage Ribonucleic Acid and Rabbit Globin Messenger Ribonucleic Acid†

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ABSTRACT: Optical density and circular dichroism (CD) properties of the polycistronic R17 phage RNA and rabbit globin mRNA have been compared in the presence and absence of Mg^{2+}. If Mg^{2+} is present, the CD melting profile is more complex for R17 RNA than for the eukaryotic messenger, whereas the main positive CD band at about 266 nm is similar for both RNAs at 28°. In the absence of Mg^{2+} and in a buffer of low ionic strength the melting profiles are similar for R17 RNA and rabbit globin mRNA. However, the mRNAs of the two different sources show now a pronounced difference in their CD spectra at 28°. Calculations of CD curves have been performed which show that the observed CD differences are due to stacked adenylate segments of an average length of 40–60 nucleotides, present in the rabbit globin mRNA and absent in the R17 RNA.

Most physical studies on the conformational properties of ribonucleic acids in solution have so far been carried out on synthetic polyribonucleotides, transfer, ribosomal, and viral RNAs. It seemed worthy to us to compare conformational properties of rabbit globin mRNA with those of the R17 polycistronic mRNA. In the first case one is dealing with a messenger which is covalently bound to polyadenylic sequences, whereas no such sequences seem to occur in the R17 RNA.

Much progress has been made in determining nucleotide sequences in the R17 phage RNA (Adams *et al.*, 1972a,b; Cory *et al.*, 1972). Sequencing results at the 5′- and 3′-ends of R17 RNA suggest the presence of tightly hydrogen-bonded hairpin loops at both ends (Adams *et al.*, 1972a; Cory *et al.*, 1972). Besides the existence of hairpin loops there is reason to believe that R17 RNA has a defined tertiary structure (Fukami and Imahori, 1971; Jeppesen *et al.*, 1970; Phillips and Bobst, 1972). The sequencing work of rabbit mRNA is still in its early beginnings. Lim and Canellakis (1970) isolated from rabbit mRNA chain lengths of 50–70 nucleotides, containing 70% AMP. Burr and Lingrel (1971) and Hunt (1973) identified polyadenylate sequences at the 3′-termini of globin mRNA. The sequences appear to be homogeneous and of approximately the same length for both α- and β-globin mRNA (Hunt, 1973). A polyadenylate region of about 50 nucleotides was found in mouse globin mRNA (Morrison *et al.*, 1973). The influence of Mg^{2+} on both mRNAs was examined in view of its importance to the conformation and function of RNAs. It has been shown for certain tRNAs by circular dichroism (CD) that a conformational change takes place upon the addition of Mg^{2+} (Willick and Kay, 1971). With respect to the biological role of Mg^{2+} in the case of polycistronic mRNA f_2, it was found that Mg^{2+} strongly influences the kind of final products formed under the direction of phage f_2 RNA (Zagorski *et al.*, 1972). Significant changes in the incorporation of some amino acids into protein were also seen with R17 RNA, when it was first incubated in the presence of Mg^{2+} before being used as messenger (Fukami and Imahori, 1971).

We report here the results of a comparative optical property study on viral and eukaryotic mRNAs with and without the addition of Mg^{2+}. Although one is far from understanding the exact nature of secondary and tertiary structures for systems as complex as those studied here, the two types of messengers show significant differences with respect to their spectroscopic and thermodynamic properties.

Materials and Methods

R17 RNA. Escherichia coli K12 Hfr RNase $^{-}D_{10}$ was grown in defined medium (Gesteland and Boedtker, 1964) to a cell density of 4×10^8/ml and then infected with R17 at a multiplicity of 10. The phage was purified and its RNA isolated according to the procedure of Gesteland and Spahr (1970). The absence of hidden breaks was verified by sedimenting fractions through a sucrose density gradient containing formaldehyde (Boedtker, 1968).

The rabbit globin mRNA (a mixture, coding for the α- and β-globin chains) was a gift from J. B. Lingrel. It had been prepared according to procedures described previously (Evans and Lingrel, 1969; Lingrel *et al.*, 1971). The degree of contamination of the rabbit globin mRNA with RNA containing no or small poly(A) stretches was determined through oligo(dT) cellulose affinity chromatography; 20% or less of the material used for this study did not bind to the column

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