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Discriminatory Ribosome Rebinding of Isolated Regions of Protein Synthesis Initiation from the Ribonucleic Acid of Bacteriophage R17

(messenger RNA/specificity in polypeptide chain initiation/RNA structure/ *E. coli* ribosomes/*B. stearothermophilus* ribosomes)

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ABSTRACT To determine whether bacterial ribosomes recognize a distinguishing feature in the immediate vicinity of actual initiator codons or are directed to these sites through involvement of other portion(s) of the mRNA molecule, the interaction between ribosomes and defined ^{32}P-labeled initiator fragments from R17 RNA was studied. When incubated with mixtures of the three sites, ribosomes from *Bacillus stearothermophilus* (which initiate only the A protein on intact phage RNA) are able to select out the A fragment and discriminate against the coat and replicase initiator regions. By contrast, *Escherichia coli* ribosomes do not rebind the coat-protein region of R17 most efficiently, as they do in the native RNA, but likewise prefer the A-protein initiator fragment. In both cases, ribosome binding of the isolated A site is comparable by several criteria to normal polypeptide-chain initiation on an intact R17 messenger RNA *in vitro*. *E. coli* ribosomal preference for the A site is confirmed in experiments with randomly fragmented R17 RNA, by both the initiation dipeptide and ribosome protection assay. Thus the A-protein ribosome-binding site of R17 RNA appears intrinsically to be a good initiator, while efficient recognition of the coat and replicase regions requires the participation of some portion of the remainder of the phage RNA molecule.

During initiation of polypeptide chains in bacteria, ribosomes in the presence of initiation factors, formylmethionyl transfer RNA, and GTP, appear to attach and start protein synthesis only at the beginnings of cistrons on a messenger RNA (1). Nucleotide sequence analysis of the three ribosome-binding sites from R17 (2, 3) and Qβ (4–7) bacteriophage genomes represented a first step towards understanding the specificity exhibited in this reaction. However, no single local base sequence or secondary-structure homology has been identified to explain the ribosome's ability to select true initiator regions while discriminating against the many internal AUG and GUG triplets in an mRNA.

Various proteins can also be initiated with quite different efficiencies. For instance, isolated f2 (or R17) RNA in an *in vitro* system from *Escherichia coli* normally directs the synthesis of 20 mol of coat protein and 5 mol of replicase (synthetase) for each mol of A (maturation) protein (8). Ribosomes from *Bacillus stearothermophilus* bind only to the beginning of the A cistron in f2 (9) or R17 (2) RNA, suggesting that initiation at the three cistrons might be mediated by different cellular components especially designed to recognize different features of initiator regions. In *E. coli*, the existence of heterogeneous subpopulations of ribosomes (10), initiation factors (11–14), and the recently identified interference factors (15, 16) may be related to cistron selectivity.

On the other hand, ribosome recognition of initiator regions is equally likely to be influenced by the overall secondary and tertiary structure of the messenger molecule. Sequence studies of the RNA bacteriophage genome suggest the prevalent formation of hydrogen-bonded loops, which apparently serve as potent negative regulators of ribosome binding to actual initiator regions. Min Jou *et al.* (18) have presented evidence that the initiation site of MS2 replicase is rendered inaccessible through hydrogen-bonding to an early portion of the coat-protein cistron (see Fig. 4*f*), until this configuration is disrupted by ribosomes translating the coat gene. Likewise, inefficient synthesis of both the f2 (19, 20) and Qβ (6) A proteins can be ascribed to structural masking of their initiator codons in intact RNA molecules. However, since simple availability does not seem adequate to explain ribosomal selection of the correct sites, the relative importance of the nucleotide sequence surrounding each initiator and the overall structure of the mRNA molecule remains unclear.

Here I describe a direct experimental approach to the question of where the information required for initiation resides. Defined R17 initiator fragments are used to assess the ribosome's affinity for the three initiator regions in the absence of the remainder of the phage RNA molecule.

Isolated R17 initiator fragments

The relative affinity of *E. coli* ribosomes for the three R17 initiator regions is markedly affected by fragmentation of the RNA molecule. Various mixtures of the sites can therefore be prepared by ribosome protection (2) of ^{32}P-labeled phage RNA in progress states of degradation.

Before proceeding, each preparation is characterized with respect to the length and relative amounts of the three R17 initiator fragments present. Direct analysis by homochromatography (2) provides estimates of the proportion of the several size variants (30–40 nucleotides long) corresponding to each initiator region (Fig. 1). The exact molar ratio of the three initiator AUGs is determined by counting the radioactivity of the appropriate oligonucleotide spots from a T1

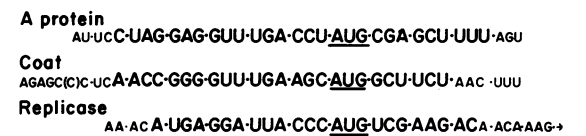

FIG. 1. Oligonucleotide sequences of the isolated R17 initiator fragments. *Bold type* indicates that portion that is present in the major size variant from each site as judged by homochromatography and the relative yields of component T1 oligonucleotides (Fig. 2).

Abbreviation: IF, initiation factor.

TABLE 1. *Rebinding of R17 initiator fragments*

Exp.	Ratio of sites A:coat:replicase (a) In fragment preparation	(b) After incubation with ribosomes	(c) Bound to 70S ribosome peak	(d) Percent of input A sites bound
	To B. stearothermophilus ribosomes			
1	1:7.6:1.9	1:9.5:1.8	1:<0.5:<0.2	70
2	1:2.9:1.6	1:2.0:1.2	1:<0.07:<0.07	25
3	1:17:3.3	1:13:2.0	1:<0.25:<0.25	60
	To E. coli ribosomes			
4	1:2.0:1.1	1:1.8:0.95	1:<0.05:<0.1	30
5	1:1.7:0.36	1:1.9:0.19	1:<0.1:<0.05	30

Mixtures of ^{32}P-labeled R17 initiator fragments were prepared (2). Rebinding to *Bacillus* ribosomes was performed in 50–75 μl containing per ml: 180–300 A_{260nm} units of low-salt-washed *B. stearothermophilus* ribosomes (21); 50 A_{260nm} units of charged, formylated *E. coli* mixed tRNA; 8 mM Mg acetate; 0.1 M NH_4 cacodylate (pH 7.1); 0.06 M NH_4Cl; 2 mM 2-mercaptoethanol; 0.2 mM GTP; and ^{32}P-labeled R17 initiator fragments originally isolated from half to the same number of *E. coli* ribosomes as there are *B. stearothermophilus* ribosomes present in the rebinding reaction. Rebinding of fragments to *E. coli* ribosomes was in 20–60 μl containing per ml: 125–280 A_{260nm} units of high-salt-washed ribosomes from MRE600 (21); purified initiation factors from A. Wahba, per A_{260nm} unit of ribosomes, 0.1 μg of IF-1, 0.08 μg of IF-2, and 0.48 μg of IF-3; 110–220 A_{260nm} units of charged, formylated *E. coli* mixed tRNA; buffers and salts as described in ref. 2 with addition of 2 mM 2-mercaptoethanol; and ^{32}P-labeled R17 initiator fragments originally extracted from 2.5- to 3.5-fold as many *E. coli* ribosomes as are included in the rebinding mixture. The fragment preparations added from 5–50 A_{260nm} units/ml of RNA to the reactions. Incubation was at 61–65° for 8 min with ribosomes from *B. stearothermophilus* or at 38–39° for 10 min with *E. coli* ribosomes. The reaction mixtures, without ribonuclease treatment, were then fractionated directly on sucrose gradients (as in Fig. 3). Before sedimentation, an aliquot was removed, extracted with phenol, precipitated with ethanol, and reanalyzed by direct homochromatography and by counting the oligonucleotides from a T1 ribonuclease fingerprint (column *b* and Fig. 2, *left*). To determine the ratio of sites bound to 70S ribosomes (column *c*), the appropriate gradient fractions were pooled; the RNA was extracted and analyzed by T1 fingerprints (ref. 39; see Fig. 2, *right*). Oligonucleotide spots that were too faint to be detected in the fingerprints contained less than 20 cpm; they are assigned this value in calculation of ratios. The fraction of input A sites bound (column *d*) was determined by comparison of the fraction of total counts appearing in the 70S region of the gradient with the fraction of A sites in the fragment mixture after incubation with ribosomes (column *c*); since up to 20% of the radioactivity in some fragment preparations is not assignable to any of the three known R17 initiator regions (Fig. 2), the numbers in column *d* represent minimum values. *E. coli* rebinding reactions performed in parallel with lower concentrations of initiator fragments (equivalent to amounts extracted from half to the same number of ribosomes, rather than 2.5- to 3-fold) yielded comparable ratios but lower rebinding efficiencies. The fraction of A sites rebound did not appear to be systematically correlated with the amount of unlabeled RNA fragments in any particular preparation.

ribonuclease fingerprint of the mixed fragments (Fig. 2, *left*).

Rebinding to *B. stearothermophilus* ribosomes

Table 1, experiments *1–3*, show the results of incubation of three different fragment mixtures with *B. stearothermophilus* ribosomes at 65° under conditions identical to those normally used for initiation with intact R17 RNA. Fingerprint analysis of the radioactive RNA sedimenting with the 70S ribosomes (Fig. 3*a*) reveals that the A-protein initiator region is specifically rebound (Table 1, column *c*). This is the case even when the R17 coat site is present in greater than 10-fold molar excess over the A site (Table 1, Exp *3*), giving discrimination ratios of more than 40:1 and 8:1 for the A site relative to the coat and replicase initiators, respectively.

Binding of the isolated A-protein initiator region to *B. stearothermophilus* ribosomes is, moreover, as efficient as initiation at this site in intact R17 RNA (17). Even though a vast (30- to 100-fold) excess of similarly-sized unlabeled rRNA and tRNA pieces contaminate the fragment mixture, up to 70% of the A site can be rebound (Table 1, column *d*). Specific degradation of the other two sites during incubation does not account for the data; reexamination of an aliquot at the end of each reaction by both homochromatography and fingerprint analysis (Table 1, column *b*) reveals no alteration in fragment length and less than 2-fold changes in the ratios of the three initiator regions. (I find this extent of variation to be within the limits of experimental error when using ribosome protection to assay polypeptide-chain initiation.)

Not only the high efficiency, but other characteristics of the rebinding reaction indicate that the interaction between *B. stearothermophilus* ribosomes and the A-initiator fragment is comparable to that which occurs during initiation on intact R17 RNA. In Table 1, low-salt-washed (70S) ribosomes were used. However, Fig. 3*b* shows that after incubation with *B. stearothermophilus* ribosomes that have been exposed to high salt and are therefore primarily subunits, initiator fragments sediment only in the 70S and 30S regions of the gradient, not at 50 S where a sizable fraction of the counts might be ex-

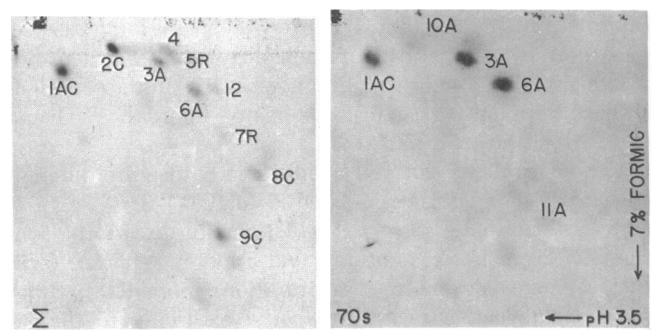

FIG. 2. T1 ribonuclease fingerprints of initiator fragments after incubation with *E. coli* ribosomes (*left*) and rebound to the 70S peak (*right*). Fingerprints (from Exp. 7, Table 1) illustrate the oligonucleotide spots that are routinely counted to assess the ratio of R17 initiator regions present in a fragment preparation or after binding to ribosomes. The A site contains: *1AC* = UUUG, *10A* = CUUUUAG, *6A* = ACCU*AUG*, and *11A* = CUAG. The coat site contains: *1AC* = UUUG, *2C* = CUUCUAACUUU, *8C* = C_{1-2} UCAACCG, and *9C* = C*AUG*. The replicase site contains: *5R* = AUUACCC*AUG* and *7R* = AAACAUG. Spots *4* = (AC,AAC, U_x,C_x)G and *12* = C(AC,C)UUAG are of unknown origin and are not rebound.

pected to appear if binding were nonspecific. The radioactivity at 70 S is more than 50% resistant to ribonuclease; RNA associated with the 30S peak also arises exclusively from the beginning of the R17 A cistron. The nearly total lack of dependence upon added *E. coli* fMet-tRNA (Fig. 3*b*) has been observed in binding of complete R17 RNA to *B. stearothermophilus* ribosomes (21).

Rebinding to *E. coli* ribosomes

In R17 RNA, the beginning of the coat-protein gene has significantly higher affinity for ribosomes than the other two sites. If this advantage results solely from the overall conformation of the RNA molecule, all three isolated R17 initiator fragments should bind equally to *E. coli* ribosomes. On the other hand, if differential rates of initiation can be ascribed to some special feature in the binding-site region, preferential recognition should be retained.

Table 1, experiments *4–7*, show that the defined R17 initiator fragments are not rebound to *E. coli* ribosomes (column *c*) in ratios reflecting their relative concentrations in the initiation reaction (columns *a* and *b*). However, it is not the coat site (as might have been expected) that is preferred. Instead, the A site attaches with highest efficiency. In experiment *7*, for example, *E. coli* ribosomes discriminate in favor of the A-initiator fragment some 40- and 11-fold over the coat and replicase sites, respectively.

Purified *E. coli* initiation factors IF-1, IF-2, and IF-3 were used to eliminate any selective RNA degradation (Table 1, column *b*) that might account for this surprising result. These homogeneous factors (22) can recognize all three R17 cistrons, and Table 2 accordingly shows that they initiate protein synthesis very efficiently at the coat and replicase sites in half-molecules of R17 RNA. The possible specific interference of the many unlabeled RNA fragments present in the preparations of R17 initiation sites was tested in several ways. Although such pieces can stimulate binding of fMet-tRNA to ribosomes, their addition to a reaction mixture containing whole ^{32}P-labeled R17 RNA does not significantly alter the relative ribosome recognition of the three sites. Likewise, each of the radioactive R17 initiator regions is totally susceptible to degradation by T1 or pancreatic ribonuclease, indicating that fortuitous hybridization to the unlabeled fragments has not effectively removed the coat or replicase sites from the reaction. Finally, elimination of more than 90% of the unlabeled fragments by preparative polyacrylamide-gel electrophoresis did not alter the ratios of radioactive fragment rebinding.

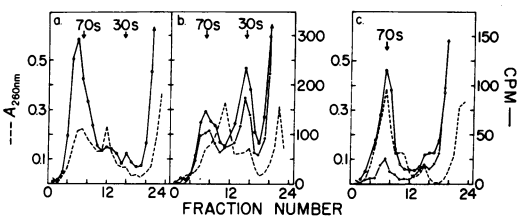

FIG. 3. Binding of R17 initiator fragments to (*a*) low-salt-washed ribosomes (retaining their initiation factors) from *B. stearothermophilus* (21); (*b*) high-salt-washed ribosomes supplemented with crude initiation factors (21) from *B. stearothermophilus;* or (*c*) high-salt-washed ribosomes from *E. coli* MRE600 supplemented with purified IF-1, IF-2, and IF-3 from A. Wahba. Reactions were performed in 25 μl containing 5.2 A_{260nm} units of ribosomes and all other components as described in Table 1. In (*b*) and (*c*), data from two gradients with equivalent *A* profiles have been superimposed. O——O, reactions containing formylated charged *E. coli* tRNA; ●——●, reactions containing no tRNA in (*b*) and an equivalent amount of uncharged tRNA in (*c*).

Binding of the isolated R17 A protein initiator region to *E. coli* ribosomes is remarkably efficient (Table 1, column *d*), comparable to the level of ribosome attachment to the coat site in intact R17 RNA (2). It is moreover 5-fold dependent upon the presence of *E. coli* fMet-tRNA (Fig. 3*c*) and 2- to 3-fold dependent upon inclusion of initiation factors in the reaction mixture. Once rebound, the site is about 80% resistant to digestion with pancreatic ribonuclease. Thus, *E. coli* ribosomes select out the isolated initiator region of the R17 A-protein gene in what appears to be the same type of binding as normally occurs during polypeptide-chain initiation on an intact mRNA molecule (2).

Initiation on severely fragmented R17 RNA

To confirm the preference of *E. coli* ribosomes for the R17 A-site fragment, I examined the effect of progressive alkali degradation of the entire R17 RNA molecule on the initiation capacity of its three cistrons (Table 2). This approach was taken because direct use of an alternative initiation assay, such as fMet-dipeptide synthesis (36, 37), is precluded by the excess unlabeled RNA present in preparations of isolated radioactive initiator regions. When assayed by either ribosome binding site protection (Table 2, columns *a* and *b*) or by fMet-dipeptide synthesis (columns *c* and *d*), a striking decrease in relative initiations at the coat and replicase sites is observed as the length of the degraded genome diminishes

TABLE 2. *Initiation on fragmented R17 RNA*

	Ribosome protection assay		fMet dipeptide synthesis	
Fragment size	(*a*) cpm $^{32}PO_4$ in oligonucleotide containing initiator **AUG**	(*b*) Ratio of sites A:coat:replicase	(*c*) % total [^{35}S]fMet in dipeptide	(*d*) Ratio of sites A:coat:replicase
13–20S	19:499:102	1:26:5.3	0.07:0.82:0.11	1:12:1.6
8–13S	34:254:76	1:7.5:2.2	0.80:0.82:0.51	1:1.0:0.64
4–8S	117:119:129	1:1.0:1.1	1.2:0.21:0.33	1:0.13:0.20
1–4S	545:172:419	1:0.32:0.77	0.76:0.09:0.19	1:0.12:0.25

For preparation of nonspecific fragments of R17 RNA, 15 $A_{260\ nm}$ units of unlabeled RNA or 0.8 $A_{260\ nm}$ units of ^{32}P-labeled RNA at 1.2 × 10^6 cpm/μg (already somewhat autoradiolyzed) were incubated in 400 μl of 25 mM Na_2CO_3 for 60 min at 50° (23). After sedimentation in parallel on sucrose gradients, fractions of various S values (determined relative to intact R17 RNA at 28 S) were pooled and concentrated by ethanol precipitation. Ribosomes were bound to the ^{32}P-labeled fragments in 30 μl containing: 4.2 $A_{260\ nm}$ units of high-salt-washed MRE600 ribosomes (21); 0.6 μg of IF-1, 0.3 μg of IF-2, and 0.5 μg of IF-3 [all purified initiation factors (IF) from A. Wahba]; 1.6 $A_{260\ nm}$ units of formylated charged *E. coli* fMet-tRNA; 3.3 × 10^7 cpm of RNA; and buffers and salts as described (2) with addition of 2 mM 2-mercaptoethanol. After 10 min at 39°, reaction mixtures were digested with 7 μg/ml of pancreatic ribonuclease and fractionated on gradients. The 70S region was extracted and analyzed by T1 fingerprint analysis (39). The initiator-containing oligonucleotides were ACCU**AUG** for the A site, C**AUG** for the coat site and AUUACCC**AUG** for the replicase ribosome binding site. Their absolute amounts (*a*) can only be taken as an approximate measure of the relative binding in the several reactions since the procedure contains many steps in which differential losses may occur. Dipeptide synthesis was performed in 40 μl containing the same amounts of ribosomes, purified initiation factors, buffers and salts as above, but 0.9 $A_{260\ nm}$ units of unlabeled fragments of R17 RNA, 3.5 $A_{260\ nm}$ units of *E. coli* [^{35}S]fMet-tRNA (about 1000 Ci/mol), and 1 mM fusidic acid. After 15 min at 37°, dipeptides were released, oxidized, and fractionated in two dimensions (41). After they were counted, the identity of dipeptides was checked by electrophoresis at pH 6.5 against synthetic markers. Dipeptides were fMet-Ala for coat protein, fMet-Ser for replicase, and fMet-Arg for A protein.

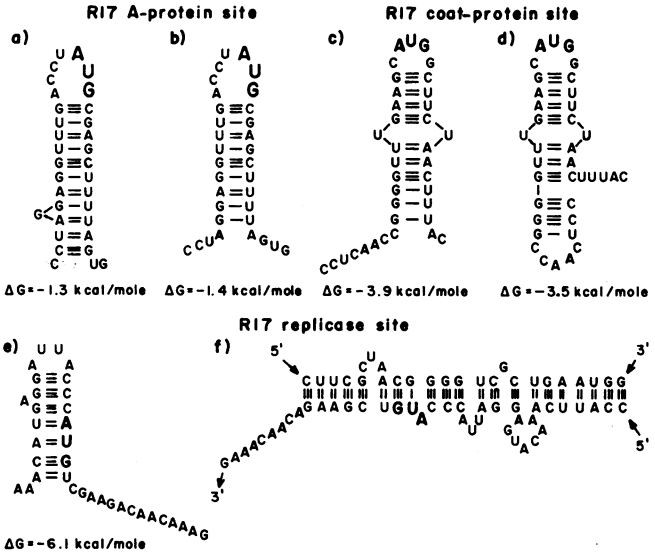

FIG. 4. Possible secondary structures for R17 initiator regions. The free energies of formation of hairpin loops in 1 M NaCl at 25° are calculated according to Gralla and Crothers (31). ΔG values for structures containing many internal GU base pairs are considered less reliable than those for structures that do not. Structure *f* (from ref 18) shows the replicase initiator and preceding intercistronic region hydrogen-bonded to a segment of the coat cistron encoding aminoacids 24–32. Initiator AUGs are in **bold type.**

from approximate half-molecules. This decrease is coupled with a steady increase in absolute activity of the A-protein initiator region, which is the most efficient site in the smallest RNA pieces (1–4 S). Only with this fragment size did the binding of oligonucleotides other than those corresponding to the three known initiator regions and the appearance of spurious fMet-dipeptides become significant enough that analysis of the oligonucleotide or dipeptide fingerprints may no longer provide a highly accurate absolute measure of initiation at the three sites.

Data obtained by use of ribosome protection compared to dipeptide synthesis for measurement of initiation are quantitatively different, as observed by Yoshida and Rudland (14) using these same two procedures. Neither the reason for the discrepancy nor which method constitutes the more reliable assay is known. Nonetheless, it is apparent from Table 2 that initiation at the coat and replicase sites is overestimated by the ribosome-binding assay relative to dipeptide synthesis. Were the latter assay possible with the defined radioactive initiator fragments the discrimination ratios would be expected to be even more dramatically in favor of the A site than are observed in Table 1.

Messenger RNA structure and initiation

The specificity of phage-directed polypeptide-chain initiation with *E. coli* ribosomes is drastically altered upon fragmentation of the R17 genome. Results obtained with either the isolated ribosome-binding sites or the randomly cleaved phage RNA show that the coat site diminishes in absolute activity, whereas the A site is transformed into a very potent initiator (equal to the level of the coat site in native RNA) upon release from the remainder of the messenger molecule. Behavior of the replicase initiator appears more complex: initially its activity rises as constraints causing polarity are relieved, but more severe RNA fragmentation brings about a decline. Slight decreases in coat-protein initiation coupled with increases in replicase and A-protein synthesis have also been observed after the overall structure of the phage messenger has been altered in other ways—by limited nuclease cleavage (8, 24–27), by unfolding with formaldehyde (19), by heat treatment under various ionic conditions (32), or by use of a partially double-stranded replicative ensemble as template (38). Lack of ribosome binding of an R17 RNA fragment containing 56 nucleotides from the coat-protein initiator region was previously reported by Adams *et al.* (42).

The inability of the isolated R17 coat and replicase regions to be recognized efficiently in fragment form demonstrates that parts of the phage genome beyond these binding sites participate either directly or indirectly in ribosome recognition. Thus, it appears that the remainder of the mRNA molecule can function to enhance, as well as decrease (6, 18, 20), the activity of a particular initiator region. This enhancement could occur if a site resides in so prominent a portion of the messenger that ribosomes invariably bind it (despite a lack of distinguishing features otherwise required of an initiator region) or if its position relative to some other molecular feature (perhaps an adjacent region) is crucial. Such an explanation may well apply to the R17 coat site; the specificity of nuclease cleavage of phage RNA (24, 28–30) indicates that the beginning of the coat gene is particularly exposed, and the presence of only one AUG triplet in the region (Fig. 1) insures that protein synthesis will not be initiated at the wrong codon.

Alternatively, the secondary structure of a binding site may be different and hence unrecognizable in the isolated fragment. For instance, the isolated R17 replicase region appears to have a high probability of forming loop *e* of Fig. 4 (which *sequesters* the initiator AUG), while in the intact MS2 (or R17) genome, the existence of structure *f* is supported experimentally (18). Thus, the beginning of the replicase cistron may be available for initiation only when the entire region is held in an extended form [and the opposite strand is released by translating ribosomes (18) or by conformational rearrangement of the RNA molecule (32)].

How does formation of local hydrogen-bonded loops relate to recognition of initiator regions by ribosomes? So far, the ability to be drawn as a hairpin has been a common feature of the sequences of highly active ribosome-binding sites from phage RNAs [i.e., the coat sites from both R17 (2) and Qβ (4)] and from the single-stranded DNA of φX174 (40). The best current evidence indicates that the beginning of the R17 coat-protein gene does exist as loop *c* (or *d*) of Fig. 4 (either of which exposes the initiator AUG) in both the fragment (31) and the native RNA molecule (18, 33). However, since this site is not efficiently bound as an isolated oligonucleotide, loop formation *cannot* be sufficient for highly efficient recognition of an initiator region by ribosomes. On the other hand, potential hairpin structures for the A site are theoretically of marginal stability (31), and in the intact phage genome this region very likely forms alternative hydrogen-bonded structures (34, 35). In Qβ RNA, Weber *et al.* (23) have demonstrated remarkably efficient (35%) ribosome rebinding of a 100-nucleotide-long fragment that includes the coat protein initiator codon directly at its 3′ terminus and that, therefore, cannot fold into the hairpin originally proposed (4). Hence, the remainder of the R17 RNA molecule may actually be required to facilitate the *opening* of loop structures during ribosome attachment.

Different classes of initiator regions?

By contrast to the R17 coat and replicase fragments, the binding site of the R17 A-protein gene is an excellent initiator when removed from its natural environment. It appears to possess within the immediate vicinity of its AUG triplet some feature that confers high affinity for ribosomes. The intrinsic initiation potential of this region is therefore regulated, as far as we know, only in a negative way by the remainder of the phage RNA molecule (2, 19, 20). In this light, it is perhaps not so surprising that ribosomes from another species, *B. stearothermophilus*, specifically recognize the A-protein initiator region of R17 RNA.

We do not know why the binding site of the R17 A-protein cistron is a good initiator or how nonlocal elements contribute to recognition of the beginnings of the R17 coat and replicase genes by *E. coli* ribosomes. Nonetheless, the contrasting behavior of these regions tempts one to speculate that they might be representative of different types of initiation sites in *E. coli*. A high content of initiator regions of one sort or the other in a particular set of messenger molecules could, in fact, be the basis of the previously reported ability of isolated IF-3 initiation factors (11–13) or interference factors (15) to discriminate amongst *classes* of RNAs (e.g., T4 late mRNA compared to *E. coli*, RNA phage, and early T4 messengers). The capacity of an established initiator region to be recognized in fragment form is an additional parameter to be considered in compiling a complete description of what constitutes a signal for polypeptide-chain initiation.

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