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Binding of Wheat Germ Ribosomes to Fragmented Viral mRNA

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The specificity of binding of wheat germ ribosomes to mRNA was greatly altered by cleavage of the message. Fragmentation of reovirus mRNA allowed wheat germ ribosomes to bind and protect a variety of internal sequences which were not accessible to ribosomes in the intact message. In experiments using the polycistronic mRNA from bacteriophage R17, wheat germ ribosomes bound preferentially at the beginning of the lysis peptide and synthetase cistrons, and at a third site which may be derived from the C-terminal region of the A protein cistron. This result is similar to that reported previously in a mammalian translational system (J. F. Atkins et al., Cell **18**:247-256, 1979) except that, in the present study, limited cleavage of the phage RNA was necessary to activate these sites. More extensive fragmentation of R17 RNA permitted wheat germ ribosomes to bind and protect a great many additional sites. Thus, presence of an (exposed) 5'-terminus on an RNA molecule appears to be necessary and sufficient for attachment of eucaryotic ribosomes.

The degree to which perturbation of mRNA structure can alter the specificity of ribosome binding provides insight into the initiation mechanism. Studies carried out with procaryotic messages in *Escherichia coli* extracts, for example, revealed an important role for secondary structure in modulating initiation. Denaturation of f2 RNA (with or without cleavage of the RNA backbone) dramatically increased binding of *E. coli* ribosomes to the synthetase and A protein cistrons in phage RNA, and also allowed initiation at a few spurious sites (17). The mechanism of translational initiation in eucaryotes appears to be quite different from that in procaryotes, however. Attachment of eucaryotic ribosomes seems to be restricted to the 5'-terminal portion of the message. Indeed, templates lacking a 5'-terminus, such as circular ribopolymers, are completely unable to bind to eucaryotic ribosomes (10). These and other observations led to formulation of a "scanning model" (9) which postulates that a 40S ribosomal subunit binds initially at or near the 5'-terminus of the message and subsequently migrates toward the interior of the mRNA, stopping when it encounters the first AUG codon. According to this mechanism, in a eucaryotic message the initiation site is defined simply by its position: the AUG closest to the 5'-terminus is the one used to initiate protein synthesis. The scanning mechanism makes three predictions regarding the consequences of perturbing the structure of mRNA. First, the model predicts that denaturation of a message should neither impair selection of the authentic 5'-proximal AUG-containing site nor allow eucaryotic ribosomes to attach (directly) to spurious internal sites exposed by unfolding the message. This prediction has been verified (12). Second, fragmentation of a eucaryotic message should lead to promiscuous binding of ribosomes. If the presence of an end is necessary and sufficient for ribosome attachment, upon cleavage of a message a wide variety of internal sequences should become functional initiation sites. There have been two reports in which fragmentation of mRNA was shown to result in synthesis of new polypeptides, implying activation of spurious initiation sites. Both of those studies used uncapped RNAs, in one case from cowpea mosaic virus (21), and in the other from foot-and-mouth disease virus (23). The assay used in those studies might underestimate the multiplicity of spurious initiation events, since translation out of the normal frame would probably lead to termination before the polypeptide became large enough to detect. Thus, a more direct assay of initiation would be informative. The only study in which ribosome-protected regions of the message were analyzed after incubation with fragmented mRNA (derived from satellite tobacco necrosis virus) suggested that ribosomes select and protect the same unique initiation site in fragmented as in intact mRNA (26). The question of whether cleavage of mRNA increases the complexity of ribosome-protected initiation sites is further explored, in the present communication, by using reovirus mRNA. A third prediction of the scanning mech-

anism is that eucaryotic ribosomes should bind to internal sites in a message only if the message has been cleaved. Rosenberg and Paterson (22) have presented evidence in support of this point. A particularly useful system for further testing this prediction involves the polycistronic genomic message of the RNA bacteriophages R17, MS2, and Qβ. It seems clear that authentic phage polypeptides—coat protein (located in the center of the genome), synthetase (located near the 3'-end), and lysis peptide (overlapping the coat and synthetase cistrons)—can be synthesized in eucaryotic cell-free extracts (1, 4, 19, 24). Indeed, the lysis peptide was discovered because of its efficient synthesis by mammalian ribosomes (1). In the study just cited, the sequences in MS2 RNA that were selected and protected by mammalian ribosomes were remarkably specific. Although oligonucleotides from the coat protein initiation site could not be detected in fingerprints of the ribosome-protected fragments (indicating a profound difference between eucaryotic and procaryotic ribosomes, since the latter prefer the coat cistron above all others [27]), oligonucleotides derived from the synthetase and lysis peptide initiation sites were quite prominent (1). There was, in addition, a rather complex background, suggesting low-level binding to a variety of sequences. The present study addresses the question of whether these internal sites are accessible to wheat germ ribosomes in the intact message, or whether cleavage of phage R17 RNA is a prerequisite for binding of eucaryotic ribosomes to the lysis peptide and synthetase initiation regions.

MATERIALS AND METHODS

Purification of viral mRNA. Capped and methylated reovirus mRNA, labeled with [α-^{32}P]GTP or [α-^{32}P]CTP to a specific activity of 1.2×10^6 cpm/μg, was synthesized in vitro by the virion-associated transcriptase, as described previously (3, 13). Transcription products were purified and fractionated into small-, medium- and large-size classes, as described (3). All experiments in this study involving reovirus mRNA were done with the medium-size class.

^{32}P-labeled RNA from phage R17 was prepared as described by Steitz (29). Virions were purified by CsCl gradient centrifugation, and the RNA was extracted with phenol on ice in the presence of 1% sodium dodecyl sulfate and 0.2% bentonite. All RNA preparations were stored in 75% ethanol at −20°C.

Cleavage of mRNA. Limited fragmentation of R17 RNA occurred during incubation in 3% formaldehyde (Fisher Scientific Co.) at 37°C for 11 min, using the procedure described by Lodish (17). More extensive fragmentation was achieved by incubating the phage RNA for 2 min at 22°C in 0.1 N NaOH, followed by neutralization with Tris-hydrochloride. Reovirus mRNA was cleaved to fragments 150 to 200 nucleotides in length either by controlled alkaline hydrolysis or by limited digestion with T1 RNase. The alkaline hydrolysis was carried out for 3 min at 95°C in 0.1 M NH_4HCO_3 (pH 9.6), followed by rapid chilling and neutralization. Partial digestion with RNase was accomplished by incubating reovirus mRNA with T1 RNase (5 U/ml) for 8 min at 22°C in 20 mM Tris-chloride (pH 8), 10 mM $MgCl_2$, and 100 mM NaCl. The reaction was terminated by phenol extraction.

In vitro protein synthesis. Reaction conditions for formation of 80S initiation complexes with a wheat germ S23 cell-free extract were as described previously (13). Sparsomycin (a gift from the Drug Research and Development Division of the National Cancer Institute) was present at a concentration of 200 μM to inhibit elongation. Yeast RNA was included (5 μg/100 μl) to minimize nonspecific adsorption of labeled mRNA to ribosomes. Conditions for binding R17 RNA to low-salt-washed *E. coli* ribosomes were as described (10). After incubation for 8 min at 20°C (wheat germ) or 35°C (*E. coli*), reaction mixtures were chilled and pancreatic RNase was added (10 μg/ml, 15 min, 4°C). Nuclease-resistant fragments associated with 80S (wheat germ) or 70S (*E. coli*) ribosomes were recovered after centrifugation through 10 to 30% glycerol gradients, as described (14).

Analysis of ribosome-protected sites. Nuclease-resistant ^{32}P-labeled mRNA fragments recovered from 70S or 80S initiation complexes were phenol extracted and then separated from high-molecular-weight rRNA by electrophoresis through 20% polyacrylamide gels (13). The peak of radioactive material migrating slightly slower than the xylene cyanol marker dye was eluted, digested with T1 RNase (2,000 U/ml), and fingerprinted (14). Separation in the first dimension involved electrophoresis at pH 3.5 on cellulose acetate strips, and the second-dimension fractionation was accomplished by homochromatography at 65°C on polyethyleneimine thin-layer plates. Oligonucleotides, located by autoradiography, were eluted from the thin-layer chromatogram and subjected to secondary digestion with pancreatic RNase. The secondary digestion products were fractionated and tentatively identified by electrophoresis on diethylaminoethyl paper at pH 3.5 (14). Identification of AC and AU was confirmed by two-dimensional chromatography (31). The composition of each oligonucleotide in the pancreatic RNase digest was determined by hydrolysis with T2 RNase, followed by chromatographic separation on polyethyleneimine thin-layer plates (30).

RESULTS

Ribosome binding to fragmented reovirus mRNA. Previous studies showed that when wheat germ ribosomes were incubated with native, intact reovirus mRNA in the presence of sparsomycin, a single 80S ribosome bound to the 5' region of each message. The ~30-nucleotide-long mRNA fragments (one from each of the three messages comprising the medium-size class) that were protected against nuclease digestion by 80S ribosomes have been purified and sequenced (8, 14). The present study addresses the question of whether limited cleavage

of reovirus mRNA, before incubation in a wheat germ extract, alters the specificity of ribosome-mRNA interactions. Figure 1 shows glycerol gradient profiles of 80S initiation complexes formed with intact (Fig. 1A) and with fragmented reovirus mRNA (Fig. 1B). Since the RNA was labeled internally with [α-^{32}P]GTP, cleavage of the message necessarily reduced the amount of radioactivity associated with 80S ribosomes. However, it is clear from Fig. 1B that fragmented RNA could still interact with ribosomes. Whereas the binding of native, intact reovirus mRNA was completely inhibited by the cap analog pm^{7}G (Fig. 1A), the same concentration of pm^{7}G caused only a slight reduction in binding of fragmented RNA (Fig. 1B). Insensitivity to the cap analog was not due to use of fragmented RNA per se, since binding of capped 5'-terminal reovirus mRNA fragments was shown to be inhibited by pm^{7}G in earlier studies (15). Thus, failure of pm^{7}G to inhibit binding in Fig. 1B suggests that ribosomes might be attaching to uncapped fragments derived from the interior of the mRNA. This was confirmed by two-dimensional fingerprint analysis of the ribosome-protected regions of the RNA.

The reaction conditions described in Fig. 1 were used to form 80S initiation complexes with [α-^{32}P]GTP-labeled reovirus mRNA. Parallel incubations were carried out with intact and with alkali-fragmented RNA. The initiation complexes were trimmed with pancreatic RNase, and the nuclease-resistant mRNA fragments protected by 80S ribosomes were purified and subjected to two-dimensional fingerprint analysis. Figure 2A shows a T1 RNase fingerprint of the protected material recovered from 80S ini-

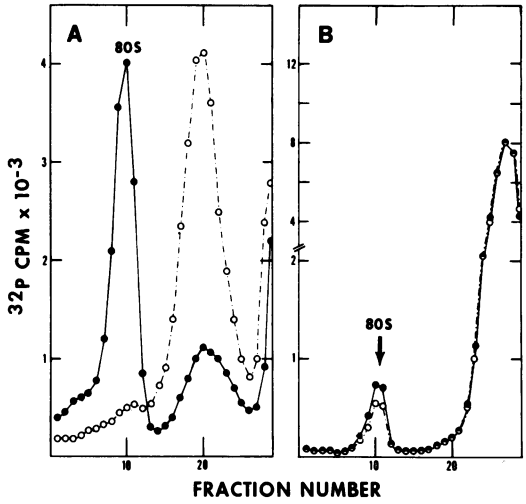

FIG. 1. *Binding of reovirus mRNA to wheat germ ribosomes. Initiation complexes were formed by incubating ^{32}P-labeled reovirus mRNA in a wheat germ S23 extract, in the presence of sparsomycin. Intact mRNA was used in (A), and alkali-cleaved RNA was used in (B). Binding was carried out in the presence (○) and absence (●) of 0.8 mM pm^{7}G. Samples, each containing 40,000 cpm, were layered onto glycerol gradients which were centrifuged (right to left) for 3.5 h at 39,000 rpm in the SW41 rotor.*

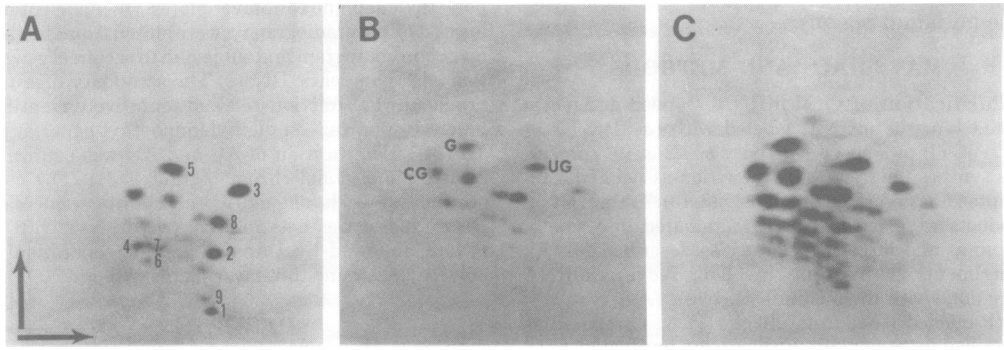

FIG. 2. *Comparison of sequences protected by wheat germ 80S ribosomes, using intact and fragmented reovirus mRNA. Initiation complexes were formed by using intact (A) or alkali-fragmented (B, C) [α-^{32}P]GTP-labeled reovirus mRNA from the medium-size class. After the complexes were trimmed with pancreatic RNase, the ribosome-protected fragments were recovered, digested with T1 RNase, and subjected to two-dimensional fingerprinting. The first-dimension electrophoresis was from left to right, and the second-dimension homochromatography was upwards. Sequences protected by 80S ribosomes, using intact reovirus mRNA, have been analyzed previously in detail. The earlier studies (8, 14) revealed that three fragments (designated m35, m26, and m30; one from the 5' region of each message in the medium-size class) are protected when 80S ribosomes bind to intact mRNA. By comparison with the earlier studies, oligonucleotides 1 to 5 in (A) can be assigned to initiation fragment m26; 6 to 8 are from fragment m35; and 9 is from fragment m30. These assignments were confirmed by analysis of secondary digestion products derived from each oligonucleotide. The amount of radioactivity used for each fingerprint was 3,000 cpm. Autoradiograms were exposed at −70°C with intensifying screens for 40 h (A, B) or for 11 days (C).*

tiation complexes formed with intact reovirus mRNA. The fingerprint revealed only those oligonucleotides which were previously (8, 14) shown to originate from the 5'-proximal regions of the three medium-sized messages. Fingerprints of the 80S ribosome-protected material obtained with fragmented reovirus mRNA are shown in Fig. 2B and 2C. The same amount of radioactivity was used for the fingerprints in Fig. 2A and 2B, and the autoradiograms were exposed for the same period of time. Thus, the abundance of small (di- and tri-) oligonucleotides and the absence of large oligonucleotides in Fig. 2B, in contrast with 2A, suggests that the protected fragments analyzed in Fig. 2B were a relatively complex mixture. Prolonged exposure of the autoradiogram (Fig. 2C) confirmed that the protected sequences recovered from 80S ribosomes after incubation with alkali-fragmented reovirus mRNA were far more complex than the normal initiation sequences.

When reovirus mRNA was cleaved to a limited extent by incubation with T1 RNase, the resulting fragments displayed only a low level of binding to wheat germ ribosomes. Thus, although there was a marked increase in the background in Fig. 3B, oligonucleotides representing the authentic initiation sites predominated. The efficiency of ribosome binding to adventitious sites was markedly stimulated, however, when the nuclease-cleaved RNA was briefly exposed to formaldehyde (Fig. 3C). Formaldehyde treatment of intact reovirus mRNA, on the other hand, did not increase the complexity of the sequences selected and protected by ribosomes (Fig. 3A). Thus, activation of internal ribosome binding sites appears to require both cleavage and partial unfolding of the message.

Binding of wheat-germ ribosomes to phage R17 RNA. Phage R17 RNA, uniformly labeled with ^{32}P, was extracted from virions and sedimented through a sucrose gradient to ascertain that the RNA was intact. Figure 4 shows a sharp peak of ^{32}P radioactivity, with only small amounts of trailing material. When this RNA was incubated in a wheat germ extract (under conditions that routinely allow 50 to 95% of reovirus mRNA to associate with 80S ribosomes), the level of binding of R17 RNA was only 0.2% of the input radioactivity (Fig. 5A). The efficiency of binding was markedly stimu-

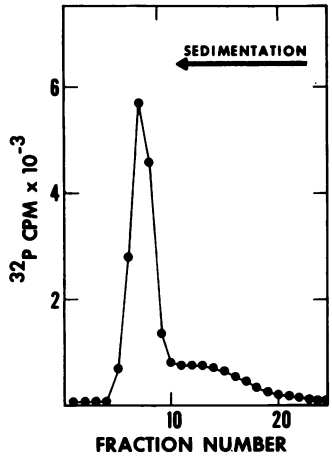

FIG. 4. *Sedimentation of ^{32}P-labeled R17 RNA through a 5 to 20% sucrose gradient. Centrifugation was for 18 h at 26,000 rpm in the SW41 rotor.*

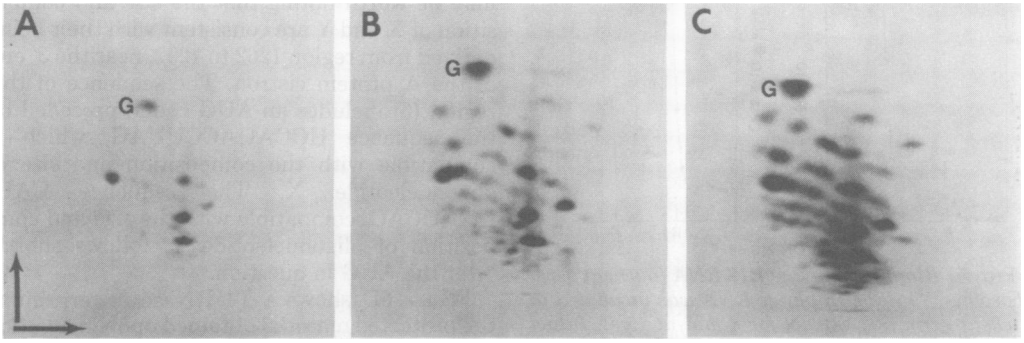

FIG. 3. *Effect of mRNA fragmentation and denaturation on the specificity of ribosome binding. Reovirus mRNA labeled with [α-^{32}P]CTP was incubated with wheat germ ribosomes in the presence of sparsomycin. The 80S ribosome-protected material was cleaved with T1 RNase, and the resulting oligonucleotides were fingerprinted as in Fig. 2. The protected fragments analyzed in (A) were obtained by binding ribosomes to intact, formaldehyde-treated mRNA. The pattern of oligonucleotides corresponds to that of the authentic 5'-proximal initiation sites from the medium-sized messages. The mRNA used in (B) and (C) was cleaved with T1 RNase to produce fragments 150 to 200 nucleotides in length. In (C), the fragmented RNA was briefly heated in formaldehyde and then repurified, as prescribed by Lodish (17), before ribosome binding. The same regimen was followed with sample (B), except that formaldehyde was omitted. The letter "G" indicates the position of GMP in each fingerprint.*

lated, however, by limited cleavage of the RNA (Fig. 5B). This could be accomplished by controlled exposure to alkali or by brief incubation in 3% formaldehyde at 37°C, as described in Materials and Methods. In the above studies with reovirus mRNA as well as in previously published work (17), incubation in 3% formaldehyde was shown not to hydrolyze mRNA. Thus, cleavage of R17 RNA in the present study was probably effected by exposure of virions to trace amounts of nuclease during purification, with formaldehyde serving only to expose the breaks.

To ascertain which regions of the phage RNA were selected and protected by wheat germ ribosomes, initiation complexes were trimmed with pancreatic RNase and the nuclease-resistant material recovered from 80S ribosomes was fingerprinted. As a control, the protected mRNA fragments obtained upon incubating intact or broken R17 RNA with *E. coli* ribosomes were also analyzed. Figure 6A shows a T1 RNase fingerprint of the protected material resulting from incubation of intact R17 RNA with *E. coli* ribosomes. The most prominent oligonucleotides were derived from the initiation region of the coat protein cistron. The oligonucleotides in Fig. 6A and 6B were identified by analysis of the products obtained upon secondary digestion with pancreatic RNase, and also by comparison with the fingerprints published by Atkins et al. (1). (To facilitate comparison with previously published work, I have used the same numbering system in Fig. 6 of this manuscript as was used in Fig. 3 of reference 1.) The minor oligonucleotides numbered 10 through 14 in Fig. 6A were derived from the initiation regions of the synthetase and A protein cistrons, as indicated by their secondary digestion products (not shown) and by the fact that they were the predominant oligonucleotides protected by *E. coli* ribosomes when partially degraded R17 RNA was used as template (Fig. 6B). Enhanced initiation of the synthetase and A protein cistrons after cleavage of phage RNA has been amply documented (16, 27). Thus, the low yield of oligonucleotides 10 through 14 (relative to oligonucleotides derived from the coat protein ribosome binding site) in Fig. 6A is further evidence for the integrity of the starting preparation of R17 RNA.

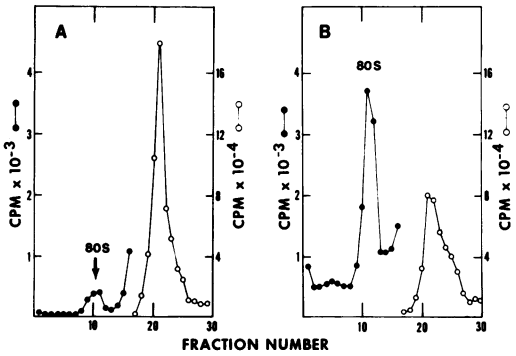

FIG. 5. *Binding of phage R17 RNA to wheat germ ribosomes. ^{32}P-labeled phage RNA was incubated in a wheat germ S23 extract for 8 min at 19°C. Intact R17 RNA was used in (A), and RNA cleaved during exposure to formaldehyde was used in (B). Samples were layered onto glycerol gradients and centrifuged (right to left) in the SW41 rotor at 39,000 rpm for 3.25 h. The amount of [^{32}P]RNA analyzed was 580,000 cpm in (A) and 456,000 cpm in (B). The scale shown on the left ordinate was used to graph fractions 1 to 16, and the scale on the right was used for the unbound RNA in fractions 17 to 30.*

T1 RNase fingerprints of the protected material obtained with wheat germ ribosomes are shown in Fig. 6C and 6D. In Fig. 6C, the phage RNA had been cleaved to only a limited extent, as in Fig. 5B. Oligonucleotides L1 and L2 derived from the lysis peptide initiation region as well as oligonucleotides 10, 11′, and 12 from the synthetase initiation region are prominent in Fig. 6C. Secondary digestion with pancreatic RNase was carried out on these oligonucleotides, and the products are listed in Table 1. The result shown in Fig. 6C with wheat germ ribosomes is quite similar to that described for the mammalian translational system (1) except that, in the present study, it was necessary to cleave the R17 RNA in order to obtain this pattern of binding. Three additional large oligonucleotides (X, Y, and Z) are also abundant in Fig. 6C. Although their secondary digestion products (listed in Table 1) do not permit a unique identification, it may be worth noting that the size and composition of X and Y are consistent with their being derived from region 1262 to 1292, near the 3′ end of the A protein cistron. The sequence of this region (6) includes an AUG codon preceded by the sequence UCCAUACCUUAG, which is compatible with the composition and size of oligonucleotide X. The sequence CAUUAAUCAG, compatible with the size and composition of oligonucleotide Y, follows shortly after the AUG in question.

Figure 6D shows a T1 RNase fingerprint of the protected material obtained upon incubation of alkali-fragmented R17 RNA with wheat germ ribosomes. The increased complexity of the fingerprint in Fig. 6D relative to 6C indicates that as the phage RNA was cleaved more extensively, additional sequences became available for interaction with wheat germ ribosomes.

The binding experiment in Fig. 5A shows that only a trace amount of intact R17 RNA attached

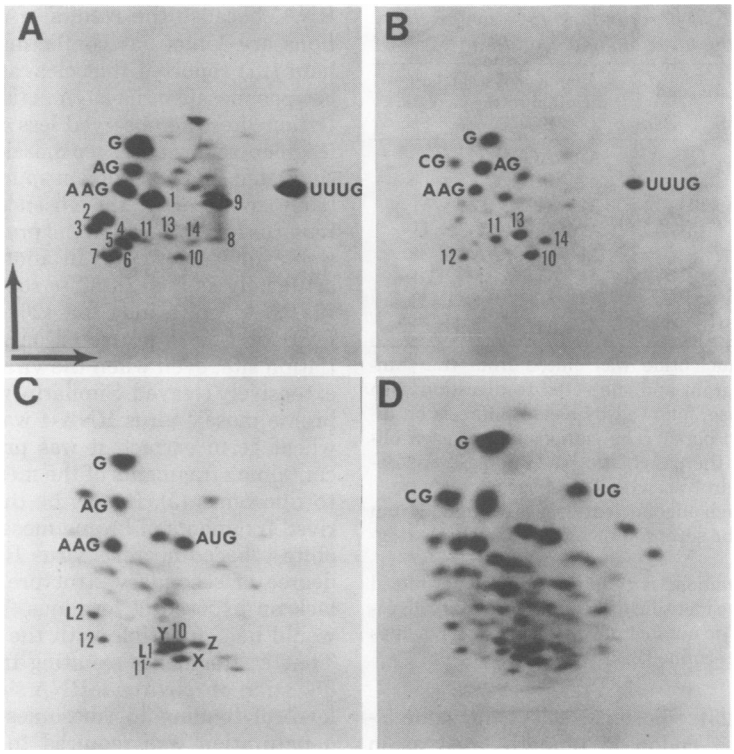

FIG. 6. *T1 RNase fingerprints of ribosome-protected fragments obtained with phage R17 RNA. ^{32}P-labeled R17 RNA was incubated with E. coli ribosomes (A, B) or wheat germ ribosomes (C, D), under initiation conditions. Intact phage RNA was used in (A); alkali-cleaved RNA was used in (B) and (D); RNA cleaved during exposure to formaldehyde was used in (C). In each case, the fragments protected by 70S or 80S ribosomes were purified, digested with T1 RNase, and fingerprinted. Secondary digestion with pancreatic RNase was carried out on each numbered oligonucleotide in panels (A), (B), and (C). The results of these analyses, and comparison with earlier published work (1), permit oligonucleotides 1 to 9 to be assigned to the R17 coat protein initiation site; 10 to 12 are from the synthetase initiation region; and 13 and 14 are from the A protein ribosome binding site. (The sequences of these oligonucleotides are given in reference 1.) Oligonucleotide 12 comigrates with and is hidden by oligonucleotide 7 in (A). In (C), oligonucleotide 11' appears to be a longer version (CCAUUCAAACAUG) of oligonucleotide 11 (AAACAUG). Table 1 lists the results of secondary pancreatic RNase digestion on oligonucleotides from (C).*

to 80S wheat germ ribosomes, and the amount of ribosome-protected material recovered after nuclease treatment was correspondingly low. Nevertheless, the small amount of ribosome-associated material obtained after nuclease digestion of reaction mixtures containing intact R17 RNA was subjected to fingerprint analysis. The T1 RNase fingerprint, which was too faint to reproduce, was extremely complex. The only abundant oligonucleotides were in the di- and trinucleotide range; the predominant, large oligonucleotides (L1, L2, etc.) seen in Fig. 6C were not evident in the fingerprint of protected fragments obtained with intact R17 RNA. Thus, either wheat germ ribosomes bind with very, very low efficiency to a wide variety of sites in intact R17 RNA or, more likely, the trace amount of "protected" material consists of heterogeneous fragments adsorbed to the surface of 80S ribosomes.

DISCUSSION

Various mechanisms have been proposed to explain how eucaryotic ribosomes select the unique site in a message for initiation of protein synthesis. By analogy with procaryotic systems, it would seem reasonable to expect eucaryotic ribosomes to recognize some primary or secondary structural feature in the vicinity of the AUG initiator codon. Comparison of the nucleotide sequences flanking the ribosome binding sites in several dozen eucaryotic messages, however, has not revealed a common feature which might direct the ribosome to those sites. The m^{7}G cap apparently does not fulfill this function because, although the cap greatly increases the efficiency

TABLE 1. *Analysis of secondary digestion products obtained from the large oligonucleotides in Fig. 6C*[a]

T1 oligonucleotide	Estimated size[b]	Products of secondary digestion with pancreatic RNase[c]
L1	11	AAAAG, C, U
L2	7	AAAC, G, C
10	10	AC, AU, G, C, U
11′	13	AAAC, AU, G, C, U
12	9	AC, AAC, AAAG
X	12	AC, AU, AG, C, U
Y	10	AU, AAU, AG, C, U
Z	9 or 10	AC, AU, G, C, U

[a] Each oligonucleotide was eluted from the thin-layer chromatogram and subjected to digestion with pancreatic RNase, followed by electrophoresis on diethylaminoethyl paper. The composition of each oligonucleotide in the pancreatic RNase digest was determined by hydrolysis with T2 RNase.

[b] Length of each oligonucleotide was estimated from its mobility in the second-dimension homochromatography.

[c] Amount of radioactivity in the samples permitted only a qualitative identification of the secondary digestion products. The number of CMP and UMP residues could not be determined.

of initiation (25), ribosomes select the same 5′-proximal AUG codon in unmethylated as in methylated reovirus mRNA (15). An alternative view is that the unique initiation site in each eucaryotic message is defined merely by its position: a ribosome binds at the 5′-terminus of an RNA molecule (irrespective of sequence) and then moves down the RNA chain, stopping and initiating at the first AUG codon it encounters. This "scanning mechanism" predicts that cleavage of a message should activate many new initiation sites. The contrary finding (i.e., selection by eucaryotic ribosomes of the same unique site in fragmented as in intact RNA) would indicate that binding of eucaryotic ribosomes requires something more than a 5′ terminus on an RNA molecule.

Fragmentation of reovirus mRNA allowed wheat germ ribosomes to bind and protect a wide variety of internal sequences which are not accessible to ribosomes in the intact message (Fig. 2 and 3). This supports the scanning mechanism of initiation. In earlier studies, ribosomes failed to attach (directly) to internal sites in intact reovirus mRNA which had been extensively denatured (12). Thus, cleavage of the RNA, but not unfolding of intact molecules, permits initiation at multiple sites derived from the interior of the message. The degree to which cleavage of reovirus mRNA would activate spurious ribosome binding sites could not be predicted from previous studies with fragmented RNA, because the results of earlier investigations are somewhat conflicting. Although Pelham (21) reported that cleavage of (uncapped) cowpea mosaic virus RNA activates internal initiation sites, he observed less of an effect upon fragmentation of tobacco mosaic virus RNA, and suggested that initiation at spurious sites is more likely to occur in the absence of a cap. The reovirus mRNA used in the present experiments was capped, however. In another study, using artificially capped satellite tobacco necrosis virus RNA, Smith and Clark (26) found that wheat germ ribosomes protected only the correct initiation site, even when the viral RNA had been extensively cleaved. Similarly, when fragmented brome mosaic virus RNA-4 was incubated in a wheat germ extract, it was primarily the cap-containing fragments of the message that bound to ribosomes (5). It may be that internally derived fragments of brome mosaic virus and satellite tobacco necrosis virus RNA have a high degree of secondary structure, such that they lack an exposed 5′ terminus. This explanation would be compatible with the reovirus results. That is, fragments resulting from T1 nuclease digestion of reovirus mRNA showed only a low level of binding to ribosomes (Fig. 3B); mild denaturation was required to obtain efficient binding of the fragmented RNA (Fig. 3C). Complementing the results obtained in the present study with reovirus, there have been several other reports describing the ability of large fragments, derived from the 3′ portion of a viral message, to direct synthesis of polypeptides which are not obtained when the corresponding intact mRNA is used as template (2, 20, 23). These studies support the interpretation that the minimal requirement for initiation by eucaryotic ribosomes is simply that the sequence to be translated be brought near the 5′ terminus of the RNA chain. Addition of a cap would, in most cases, dramatically enhance the efficiency, but presence of an (uncapped) exposed 5′ terminus is sufficient for a low level of ribosome binding to almost any sequence.

The fact that extensive fragmentation of phage R17 RNA permits wheat germ ribosomes to attach (inefficiently) at a wide variety of sequences (Fig. 6D) fits nicely with the above interpretation. More limited cleavage of R17 RNA permitted binding at just a few sites (Fig. 6C): the synthetase and lysis peptide initiation sequences, and an additional site which might be derived from the C-terminal portion of the A protein cistron. Binding of mammalian ribosomes to the synthetase and lysis peptide initiation sites was the subject of a recent detailed investigation by Atkins et al. (1). That elegant

study, which led to the discovery of the lysis peptide, left unanswered the question of whether internal regions of MS2 RNA are directly accessible to eucaryotic ribosomes, or whether cleavage of the phage RNA is a prerequisite. The current study supports that latter interpretation and suggests that the same rules operate with procaryotic messages (in a eucaryotic translational system) as with eucaryotic messages. The same conclusion was reached by Rosenberg and Paterson (22), who used a polycistronic message from phage λ. It seems reasonable to suggest that preferential binding of eucaryotic ribosomes to the initiation region of the R17 synthetase and lysis peptide cistrons is due to those sites being rather exposed (7, 18), and therefore more susceptible to cleavage than the rest of the genome.

The practical implications of this work merit emphasis. Studies concerning eucaryotic "ribosome binding sites" require extreme precautions regarding use of intact mRNA, as well as selection of reaction conditions that minimize migration of 40S subunits (11). It is, unfortunately, very easy to induce eucaryotic ribosomes to initiate at spurious sites. Moreover, the authenticity of purified initiation fragments cannot be established by showing that they are able to rebind to ribosomes. This criterion may be acceptable for procaryotes (28) but not for eucaryotes, since almost any linear single-stranded RNA fragment can bind to eucaryotic ribosomes. The efficiency of rebinding, however, can distinguish authentic, capped initiation fragments from random noninitiator fragments (15).

One might conclude from the experiments described herein that presence of a 5′ terminus on an RNA molecule is necessary and, in view of the great number of spurious sites that are activated by mRNA cleavage, sufficient for binding of eucaryotic ribosomes. The results obtained with R17 RNA, however, suggest that this is an oversimplification. Although cleavage of R17 RNA permitted (and was a precondition for) wheat germ ribosomes to associate with sequences derived from the interior of the phage genome, ribosomes failed to bind to the 5′ terminus of intact R17 RNA. The 5′-terminal portion of the phage genome has a high degree of secondary structure, however (6), which may preclude ribosome attachment. This is consistent with the experiment carried out with reovirus mRNA in which cleavage of the RNA with T1 RNase permitted only a low level of binding to internal sequences (Fig. 3B); ribosome binding was markedly stimulated, however, after brief exposure of the fragmented RNA to formaldehyde (Fig. 3C). Thus, the more correct and circumscribed conclusion may be that presence of a 5′ terminus on an RNA molecule is necessary and sufficient for attachment of eucaryotic ribosomes, provided that the 5′ terminus is exposed.

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