Characterization of Ribosome-protected Fragments from Reovirus Messenger RNA

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The 5'-terminal methylated cap (m'G(5')ppp(5')Gm) in reovirus messenger RNA comprises part of the ribosome binding site, since attachment of 40 S wheat germ ribosomal subunits to reovirus small (s), medium (m), and large (l) RNA classes conferred almost complete protection of the cap against RNase digestion. After joining of the 60 S ribosomal subunits, however, the cap continued to be protected against T1 RNase within the 80 S initiation complexes formed with only some messenger species; namely the three l-messages, one of the m-messages, and one or two of the s-messages. When protected fragments were recovered from 40 S and 80 S complexes and tested for ability to rebind to ribosomes, those fragments which retained the cap were able to rebind most efficiently.

The protected fragments recovered from 40 S initiation complexes with several of the s- and m-RNA species were larger than the messenger fragments recovered from 80 S complexes. The medium size class of reovirus RNA, which consists of three messenger species, gave rise to three discrete 5'-terminal fragments after digestion of 40 S complexes with T1 RNase, and to three somewhat smaller fragments after T1 RNase digestion of 80 S complexes. Fingerprints of the T1 oligonucleotides derived from these fragments are consistent with the interpretation that each messenger species within the m-RNA class gives rise to a protected fragment of a unique size and that, with each message, there is extensive overlap between the regions of the message protected by 40 S and 80 S ribosomes. The ratio of the three protected fragments recovered from 40 S complexes with m-RNA was highly reproducible under a given set of binding conditions, but could be shifted by varying the messenger/ribosome ratio in the binding reaction. Thus, one of the fragments, which was preferentially recovered when the ribosome concentration was limiting, could be tentatively identified as the binding site of the most efficiently translated message within the m-RNA class.

Although considerable information has accumulated concerning the binding of prokaryotic ribosomes to messenger RNA, little is known about the binding of eukaryotic ribosomes. The nearly ubiquitous presence of methylated capped termini (m'G(5')ppp(5')Gm) in eukaryotic messenger RNA (1) suggested the possibility that these distinctive termini might comprise part of the recognition site for ribosome binding. Such a mechanism would necessarily limit ribosome entry to the 5' end of the messenger molecule, consistent with the observed monocistronic nature of most eukaryotic messages. Indeed, in the single instance in which the ribosome binding sequence of a eukaryotic messenger RNA is known, namely, the brome mosaic virus coat protein cistron, that site includes the methylated capped 5' terminus (2).

The observation that only the methylated form of reovirus messenger RNA is translatable in wheat germ extracts under appropriate conditions (3) is consistent with a key role of 5'-terminal methylation in protein synthesis. Furthermore, ribosome binding experiments revealed that discrimination between methylated and unmethylated reovirus messenger RNA occurs at or before binding of 40 S ribosomal subunits (4).

To obtain further information about the involvement of the 5'-terminal methylated cap in ribosome attachment, binding experiments were performed using wheat germ ribosomes and reovirus messenger RNA, labeled specifically at the 5' end. The position of the ribosome in both 40 S and 80 S initiation complexes, relative to the 5' terminus of the messenger RNA, was determined by measuring the nuclease resistance of the [3H]methyl-labeled cap.

EXPERIMENTAL PROCEDURES

Reovirus messenger RNA was synthesized by the virion-associated transcriptase as described previously (5) in the presence of 10 µM S-adenosyl-L-[methyl-3H]methionine (Amerham/Searle, 7.5 Ci/mmol) and four nonradioactive nucleoside triphosphates at a concentration of 2 mM each, or with the nucleoside triphosphate concentration reduced to 0.5 mM and all four α-[3H]labeled nucleoside triphosphates added, at a final specific activity of 0.4 Ci/mmol each. After fractionation of the messenger RNA into large, medium, and small size classes by velocity sedimentation in sucrose gradients (6), an aliquot of each RNA class was heated in 8 M urea and recentrifuged to assess the degree of cross-contamination. The s- and l-RNAs were homogeneous.

1The abbreviations used are: s-, m-, and l-RNA, respectively, the small, medium, and large size classes of reovirus messenger RNA; Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; GMP-P(CH2)3P, guanosine 5'-(β,γ-methylene)triphosphate; AMP-P(CH2)3P, adenosine 5'-(β,γ-methylene)triphosphate; S23, 23,000 x g supernatant fraction from wheat germ extract.
and the m-class contained less than 10% contamination by the s-class.

To obtain ribosome-protected fragments, radioactive reovirus RNA was bound to ribosomes in reaction mixtures containing, per ml, 35 to 45 A260 units of preincubated wheat germ S32 extract, 120 µg of creatine phosphokinase, 30 mM Hepes buffer (pH 7.4), 72 mM KCl, 2.8 mM magnesium acetate, 8 mM creatine phosphate, 2 mM dithiothreitol, 220 µM sparsomycin, 1 mM ATP and either 0.24 mM GTP or 0.48 mM GMP-P(CH)2P. After incubation of the reaction mixtures for 10 min at 23°C, pancreatic RNase (3 µg/ml) or T1 RNase (250 to 320 units/ml) was added, and incubation continued for 15 min at 23°C. The initiation complexes were then isolated by centrifugation at 39,000 rpm for 4 hours at 4°C in the SW 41 rotor, through 10 to 30% glycerol gradients containing 20 mM Tris-HCl (pH 7.4)/75 mM KCl/2.5 mM magnesium acetate. RNA from appropriate gradient fractions was precipitated overnight at -20°C with 2.5 volumes of ethanol, extracted twice with phenol, reprecipitated with ethanol, and dissolved in water.

Polyacrylamide gel electrophoresis of nuclelease-resistant, ribosome-protected fragments was carried out in 10-cm cylindrical gels containing 20% acrylamide/0.15% bisacrylamide/9 M urea/90 mM Tris/90 mM boric acid/2.5 mM EDTA/10% glycerol. Prior to electrophoresis, all samples were denatured by heating for 3 min at 55°C in 6 M urea. 3H-Labeled 5 S RNA, tRNA, and reovirus oligo(A), analyzed in a parallel gel, were used as markers for estimating the apparent size of the ribosome-protected fragments. After electrophoresis at 130 V for 18 to 20 hours at 23°C, gels were sliced into 1.3-mm sections, dissolved in 15% H2O2, and counted in Aquasol (New England Nuclear). Alternatively, RNA was eluted from gel sections by incubation for 24 hours at 37°C in 10 mM Tris-HCl (pH 7.5) and 0.2% sodium dodecyl sulfate with 10 µg of tRNA added as carrier. The eluted fragments were then dissolved in water and used for binding experiments or for fingerprint analysis.

The RNA fingerprinting technique was essentially as described by Kedl and Stein (6). RNA samples were digested with 10 µl of T1 RNase (1500 units/ml, enzyme/substrate ratio 1/10) at 37°C for 30 min, then applied onto cellulose acetate strips (3 x 55 cm). After electrophoresis for 18 to 20 hours at 23°C, gels were sliced into 1.3-mm sections, dissolved in 6 M urea, and counted in Aquasol (New England Nuclear). As a control, 10 µl of RNase digestion was carried out in the presence of the antisense oligo(A). In this case, the RNA was not digested and could be isolated from the gel. RNase digestion of the RNA from the ribosome-protected fragments resulted in the production of RNA fragments of different sizes, indicating that the RNA was protected by the ribosome.

RESULTS

Protection of 5'-Terminal Caps against Nuclease Digestion—As reported for other eukaryotic systems (7), addition of GMP-P(CH)2P to wheat germ extracts directed by reovirus messenger RNA partially inhibited the joining of 40 S ribosomal subunits to 40 S-mRNA initiation complexes, causing about one-third to one-half of the complexes to remain at the 40 S level, as shown by centrifugation through glycerol gradients (Fig. 1, A and C). Thus, from reaction mixtures containing GMP-P(CH)2P as well as sparsomycin, an inhibitor of polypeptide elongation, both 40 and 80 S ribosomal complexes were able to accumulate during the initial 10 min of incubation.

The degree of protection, by 40 S or 80 S ribosomes, of the [3H]methyl-labeled 5' terminus of each class of reovirus messenger RNA was determined by centrifuging equal aliquots of an initiation mixture, before and after digestion with pancreatic RNase or T1 RNase. Comparison of the amount of radioactivity recovered at 40 S complexes before and after nuclease treatment shows that, with the small and medium size classes of reovirus RNA, the 40 S ribosome conferred almost complete protection of the cap against both pancreatic (Fig. 1D) and T1 RNase (Fig. 2). In the case of I-RNA, omission of sparsomycin from the incubation mixture resulted in incomplete protection of the cap by 80 S ribosomes against T1 RNase when GTP was used instead of GMP-P(CH)2P. (It is noteworthy that, in the presence of GTP, which allowed 80 S complex formation, the 5' terminus of 80 S complexes was not protected.) In contrast with 80 S complexes with s- or m-RNA, binding of the 40 S ribosome conferred nearly complete protection of the 5'-terminal cap against nuclease digestion, indicating that with all three classes of reovirus message, the cap is an integral part of the 40 S initiation complex.

In contrast with the nearly complete protection of caps with all classes of reovirus message by 40 S ribosomes, upon conversion to 80 S initiation complexes the caps remained protected with only some messenger species. While complexes between 80 S ribosomes and I-RNA, formed in the presence of sparsomycin, were almost completely resistant to pancreatic RNase digestion (Fig. 3, C and D), 80 S complexes with s- or m-RNA conferred only 25 to 30% protection of the cap against T1 RNase (Fig. 2), and even less (6 to 12%) protection against pancreatic RNase (Fig. 1, A to D). Failure of 80 S ribosomes to protect the 5' termini of s- and m-RNA cannot be attributed to incomplete inhibition by sparsomycin, since l-message was completely protected under the same conditions. Furthermore, the presence of diphtheria toxin in addition to sparsomycin, each at a concentration sufficient to inhibit amino acid incorporation by greater than 99%, did not increase the pancreatic RNase-resistance of the 5' terminus of s-RNA in 80 S complexes. Incomplete protection of the cap in 80 S complexes with s- and m-RNA also cannot be attributed to the presence of GMP-P(CH)2P in the incubation mixtures, since identical values (±3%) were obtained for percent protection of the cap by 80 S ribosomes against T1 RNase when GTP was used instead of GMP-P(CH)2P. (It is noteworthy that, in the presence of GTP, which allowed 80 S complex formation but prevented accumulation of complexes between 40 S ribosomes and intact messenger RNA, after addition of nuclease there were no messenger fragments sedimenting in the 40 S region of the gradient. Thus, recovery of nuclease-resistant fragments from the 40 S region occurred only when messenger RNA-40 S ribosomal complexes were able to accumulate during the initial binding reaction, eliminating a possible artifactual origin for the fragments protected within 40 S complexes.)

In the case of I-RNA, omission of sparsomycin from the 10-min binding reaction completely abolished retention of [3H]methyl-labeled fragments in 80 S complexes, indicating that protection of caps by ribosomes is transient and does not persist when elongation is permitted.

Characterization of Ribosome-protected Fragments—The size of the ribosome-protected fragments derived from each class of reovirus RNA was determined by polyacrylamide gel electrophoresis. In cases where the ribosome conferred complete protection of the cap, [3H]methyl-labeled fragments were used. In the case of 80 S complexes with s- and m-RNA, however, in which there was minimal protection of the [3H]methyl-labeled cap, fragments labeled internally with [32P]P were used for size determinations. After addition of RNase to these

*Data not shown.
Fig. 1. Sedimentation of initiation complexes before and after pancreatic RNAse digestion. [3H]Methyl-labeled reovirus RNA, at concentrations of 39 μg/ml for s-RNA (A and B), 43 μg/ml for m-RNA (C and D), and 100 μg/ml for l-RNA (E and F), was incubated under standard binding conditions, in the presence of GMP-P(CH)2P. After 10 min at 20°C, an aliquot from each reaction mixture was chilled and centrifuged without further treatment (A, C, and E). A second aliquot, equal to the first, was incubated with pancreatic RNAse prior to centrifugation (B, D, and F). In the absence of nuclease, a considerable amount of the input RNA adhered to particulate material in the wheat germ extract, and pelleted upon centrifugation. This pelleted RNA is not shown in A, C, and E because quenching prevented accurate counting. The pelleted RNA was sensitive to nuclease, contributing to the counts at the top of gradients B, D, and F.

Fig. 2. Sedimentation of initiation complexes before and after T1 RNase digestion. Reaction conditions were as described in Fig. 1. Binding of s-RNA is shown before (A) and after (B) digestion with T1 RNase. On the right, binding of m-RNA is shown before (C) and after (D) T1 digestion.

Binding reactions, only about 1% of the input 32P-labeled material was recovered in ribosome-protected fragments, indicating absence of extensive random binding of ribosomes to many internal regions of the messenger molecules. Fig. 4 shows that the purified fragments recovered from 80 S complexes (open circles) were relatively small (28 to 37 nucleotides). In contrast, some of the fragments protected by 40 S initiation complexes (closed circles) with s- and m-RNA were considerably larger than the corresponding fragments from 80 S complexes. Fig. 4A, for example, shows that the pancreatic RNAse-resistant fragments derived from 40 S complexes with s-RNA were 11 to 31 nucleotides longer than the fragments protected by 80 S ribosomes. Similarly, after pancreatic RNAse treatment of complexes with m-RNA, fragments approximately 29 to 37 nucleotides long were recovered from 80 S complexes, versus 37-, 44-, and 63-nucleotide fragments from 40 S complexes (Fig. 4C). A similar size difference between 40 S and 80 S-
protected fragments was observed with the T₁-resistant fragments derived from 40 S complexes. The pancreatic RNase-resistant fragments obtained from 40 S complexes were only slightly larger than the corresponding fragments from 80 S initiation complexes (Fig. 4E), and the fragments recovered from 40 S and 80 S complexes after T₁ RNase digestion were almost identical in size (Fig. 4F).

The nucleotide-resistant, ribosome-protected fragments derived from each messenger size class were tested for ability to rebind to wheat germ ribosomes. Fragments derived from 40 S complexes, with either s-, m-, or l-RNA, were able to rebind, in each case with 50% or more of the input counts appearing in 80 S complexes, as summarized in Table I. When the protected fragments were first fractionated by polyacrylamide gel electrophoresis, fragments of every size were able to rebind, as shown in Fig. 5 (closed circles) for the three T₁-resistant fragments from m-RNA, and in Table I for the two T₁-resistant fragments from 40 S complexes with s-RNA. In contrast with the ability of all fragments derived from 40 S complexes (all of which retained the [³H]methyl-labeled cap) to rebind with high efficiency, Table I shows that the fragments recovered from 80 S complexes with e- and m-RNA showed considerably less ability to rebind. In an experiment using fragments from RNA that was labeled at the 5' terminus with [³H]methyl radioactivity and labeled internally with [³²P], the ability of 80 S-derived fragments to rebind to ribosomes appeared to be correlated with retention of the methylated 5' terminus. The pancreatic RNase-resistant, [³²P]-labeled fragments recovered from 80 S complexes with s- or m-RNA retained very little [³H]methyl radioactivity, and only about 10% of these fragments were able to rebind to ribosomes (Fig. 6A). On the other hand, when 80 S complexes formed with the same double-labeled s- or m-RNA were treated with T₁ RNase, about 25% of the recovered fragments were able to rebind to ribosomes (Fig. 6B), consistent with the more extensive protection of the cap against T₁ RNase (Fig. 2) than against pancreatic RNase (Fig. 1). In fact, although the amount of [³H] radioactivity shown in Fig. 6 is low, the increased ratio of [³H] to [³²P] in the bound versus the free region of the gradients suggests that the [³H]methyl-containing fragments were selected for rebinding. Thus, ability of the protected fragments to rebind efficiently to ribosomes was correlated with retention of the 5'-terminal cap. Presence of a cap was not an absolute requirement for fragment re-binding, however. The T₁-resistant fragments derived from 80 S complexes with m-RNA could be fractionated by polyacrylamide gel electrophoresis into three species (Fig. 4D), two of which lacked caps, as indicated by absence of [³H]methyl radioactivity, while the third, intermediate-sized (32-nucleotide) species retained the cap. Although the 32-nucleotide capped fragment rebound with the highest efficiency, as shown in Table I, the two uncapped fragments derived from 80 S complexes with m-RNA could rebind to a limited extent.

Correlation between Ribosome-protected Fragments and Individual Messenger Species—In the case of 40 S initiation complexes, the presence of a limited set of discrete fragments (Fig. 4), in highly reproducible ratios, is consistent with the possibility that individual messenger species within a size class give rise to particular fragments. Since the various messenger species are often not synthesized in equimolar amounts during in vitro transcription by reovirus cores (9), the exact ratio of fragments expected from such a situation cannot be predicted. In the case of s-RNA, however, it is possible that two of the messenger species within this class yielded a 56-nucleotide fragment from 40 S initiation complexes after T₁ digestion, while the other two s-messages yielded 46-nucleotide fragments (Fig. 4B). Fingerprints of the oligonucleotides derived from complete T₁ digestion of the 56- and 46-nucleotide fragments are consistent with this possibility, since the two fingerprints are distinctly different from each other, and the complexity of each is consistent with the presence of two fragments (from two different messages) contributing to the 56 nucleotide material, and two fragments contributing to the 46-nucleotide material.

* Data not shown.
Table I

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<th>RNA class</th>
<th>Source of fragment</th>
<th>Ribosomal complex</th>
<th>Fragment properties</th>
<th>Retention of cap</th>
<th>Percent of fragments able to rebind</th>
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<td>60</td>
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<td></td>
<td>T1</td>
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<td>Complete</td>
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<td>29</td>
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<tr>
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<td>≤5%</td>
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<td>11</td>
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<td>78%</td>
<td>42-55°</td>
<td>49-58°</td>
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<td>≤5%</td>
<td>17</td>
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<td>Complete</td>
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<tr>
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*Ability of fragments to rebind to ribosomes was determined by incubating labeled fragments for 10 min in standard reaction mixtures containing GTP and sparsomycin, followed by centrifugation in glycerol gradients. For those fragment preparations in which the cap was almost completely (80 to 100%) protected against nuclease digestion, the fragments were terminally labeled with \[^32\text{P}\]labeled RNA. The lower value is based on \[^32\text{P}\] and the higher value on \[^3\text{H}\]methyl radioactivity. The difference is due to contamination of the 52-nucleotide fragment by the less efficiently binding \[^32\text{P}\]-labeled 38-nucleotide fragment.

Fig. 5. Rebinding of T1-resistant fragments recovered from 40 S initiation complexes with m-RNA. \[^3\text{H}\]Methyl-labeled fragments were fractionated by polyacrylamide gel electrophoresis, eluted, then tested for ability to rebind to ribosomes in standard reaction mixtures containing GTP and sparsomycin. Equal aliquots were applied to glycerol gradients before (\(\text{---}\)) or after incubation with T1 RNase (\(\text{O---O}\)). A, rebinding of 36-nucleotide fragment; B, 43-nucleotide fragment; C, 52-nucleotide fragment.

Fig. 6. Rebinding of nuclease-resistant fragments to ribosomes. Protected fragments, derived from \[^3\text{H}\]methyl- \[^32\text{P}\]-labeled m-RNA, were tested for ability to rebind to ribosomes by incubating for 10 min in standard reaction mixtures containing GTP, followed by centrifugation through glycerol gradients. \[^3\text{H}\] (1200 cpm) was used for each binding reaction. A, fragments recovered from 80 S complexes after pancreatic RNase digestion; B, fragments from 80 S complexes after T1 RNase digestion; C, fragments from 40 S complexes after T1 digestion. Fragments recovered from complexes with \(\text{s-RNA}\) gave results almost identical with those shown here for m-RNA. Fragments remained essentially unchanged when the T1 concentration was varied from 120 to 460 units/ml (although, at the highest nucleotide concentration, a small amount of a new ~32-nucleotide fragment appeared), it is unlikely that the 43- and 36-nucleotide fragments are derived by more extensive digestion of the 52-nucleotide fragment. The alternative explanation, that each of these fragments arises from a unique messenger species, is supported by several further observations. When the fragments were eluted from gels and tested separately for ability to rebind to ribosomes, each of the three fragments was capable of rebind only the 36-nucleotide fragment was resistant to T1 RNase in the 80 S initiation complex (Fig. 5). Since the 36-nucleotide fragment is thus qualitatively different from the larger fragments, it cannot be derived by more extensive digestion of either of the two larger fragments. The 25 to 30% protection against T1 RNase noted above (Fig. 2) for the m-RNA class bound to 80 S ribosomes thus appears to represent complete protection of the 5' terminus of one of the messenger species within this class, while the cap on the other two messenger species is completely sensitive to T1 RNase hydrolysis within 80 S initiation complexes. (Parenthetically, the 25 to 30% protection against T1 RNase observed with s-RNA in 80 S complexes probably also represents complete protection of one or two species, and complete sensitivity of the remaining species within this class, since analysis of the 36- and 46-nucleotide fragments recovered from 40 S complexes after T1 digestion showed that, while each could rebind to form an 80 S complex, resistance to T1 RNase within the 80 S complex was associated only with the 46-nucleotide fragment.) While the T1 RNase sensitivity of the rebound fragments distinguishes the 36-nucleotide fragment from the 52- and 43-nucleotide fragments recovered from 40 S complexes with m-RNA, the possibility remained that the 52- and 43-nucleotide fragments were related, rather than originating from different messenger species. We, therefore, obtained T1 fingerprints from these two fragments. As shown in Fig. 7, A and B, each fragment yielded a distinctive fingerprint, and the oligonucleotides derived from the 43-nucleotide fragment are not merely a subset of the oligonucleotides obtained from the 52-nucleotide fragment. Although spots 2 to 4 appear rather diffuse in the fingerprint shown in Fig. 7B, these spots were well resolved in other fingerprints of the 52-nucleotide frag-

*Data not shown.*
Ribosome-protected Reovirus Messenger RNA Fragments

**Fig. 7.** T1 RNase fingerprints of 32P-labeled protected fragments from reovirus m-RNA. After m-RNA was bound under standard conditions and digested with T1 RNase, the protected fragments were recovered from glycerol gradients, fractionated by polyacrylamide gel electrophoresis, then subjected to complete hydrolysis by T1 RNase followed by fingerprinting. The first dimension was from left to right and the second dimension was upward. The dotted circle indicates the position of the blue marker. The RNA preparations contained traces of acrylamide fragments, which may have trapped some RNA, accounting for the streak labeled ac observed near the origin of the second dimension. A, 43-nucleotide fragment from 40 S complexes; B, 52-nucleotide fragment from 40 S complexes; C, 28-nucleotide fragment from 80 S complexes; D, 38-nucleotide fragment from 80 S complexes. The inset in Panel B shows another T1 fingerprint of the 52-nucleotide fragment, in which the spots are somewhat sharper. The oligonucleotide labeled in represents an incompletely digested product, which disappeared when the T1 RNase concentration was raised.

**Fig. 8.** Effect of varying input RNA concentration on recovery of T1-resistant fragments. [3H]Methyl-labeled m-RNA at a concentration of 20 or 300 µg/ml was incubated under standard binding conditions, with GMP-P(CH2)4P present. After digestion with T1 RNase, the protected fragments from 40 S initiation complexes were purified and analyzed by polyacrylamide gel electrophoresis. ○—○, fragments recovered from the reaction mixture containing 20 µg of RNA/ml; O—O, fragments recovered from the reaction mixture containing 300 µg of RNA/ml.

ment, such as the one shown in the inset. Both the 52- and 43-nucleotide fragments gave very simple fingerprints, indicating the absence of random binding of 40 S ribosomes to a large number of internal sites in the messenger RNA molecules. Thus, each messenger species within the m-RNA class, when bound to 40 S ribosomes, appears to give rise to a nuclease-resistant 5'-terminal fragment of a unique size.

Finally, an experiment was done to determine the effect of varying the messenger RNA concentration, during the incubation in wheat germ extracts. The set of fragments shown in Fig. 4D was obtained from a reaction containing a low concentration of m-RNA, such that nearly all of the RNA was bound to ribosomes. It seemed reasonable to expect that if the m-RNA concentration were increased, above the saturating level, the most efficient messenger species would then bind preferentially, with a corresponding increase in the particular fragment derived from that message. Fig. 8 shows that, indeed, the ratio of T1-resistant fragments derived from 40 S complexes was significantly altered by increasing the messenger RNA concentration above saturation. The 43-nucleotide fragment appears to come from the most efficient message, since its proportion among the recovered fragments increased from 48% under conditions of limiting messenger concentration, to 76% under conditions of saturation by message.

**Relationship between 40 S and 80 S Ribosomal Binding Sites**—With the m-class of reovirus RNA, there was extensive overlap between the regions of each message protected by 40 S and 80 S ribosomes. Comparison of Fig. 7A and C shows that, of the five major T1 oligonucleotides derived from the 40 S-protected 43-nucleotide fragment, four corresponded to oligonucleotides present in the 80 S-protected, 28-nucleotide fragment, and those oligonucleotides have been numbered accordingly. Overlap was also evident between the 52- and 38-nucleotide fragments. Of the seven major T1 oligonucleotides derived from the 40 S-protected 52-nucleotide fragment (Fig. 7B), four had counterparts of almost identical mobility in the 80 S-protected 38-nucleotide fragment (Fig. 7D). The
38-nucleotide fragment yielded one additional prominent spot, labeled X, of uncertain origin. We are also uncertain about the nature of the material that formed a streak (labeled ac) near the origin of the second dimension. This material sometimes had a segmented appearance, as in Fig. 7C, suggesting that it might consist of RNA trapped by acrylamide fragments. Since the position of the streak in fingerprints of other fragments was quite reproducible however, it is possible that some fragments contain an oligonucleotide of unusual base composition, which causes trailing. This possibility does not significantly alter our interpretation of the data.

In the case of the third messenger species within the m-class, which at the 40 S level gave rise to a 36-nucleotide T 4-resistant fragment, overlap between 40 S and 80 S binding sites can be inferred from the fact that the 5'-terminal cap was protected by 80 S as well as by 40 S ribosomes (Fig. 5A). Thus, we assume that the 36-nucleotide 40 S-protected fragment would yield a fingerprint almost identical with that of the 32-nucleotide 80 S-protected fragment which, as mentioned in the legend to Fig. 4, is the only 80 S-protected fragment that retained substantial amounts of [3H]methyl radioactivity. Neither the 36- nor the 32-nucleotide fragment has yet been obtained in sufficient quantities and in a pure enough form to allow fingerprint analysis.

Effect of Formaldehyde Treatment on Recovery of Nuclease-resistant Fragments from Ribosomes—The relatively large size of some of the fragments protected within 40 S initiation complexes might be explained, in part, by the presence of hairpin loops within, or immediately adjacent to, the nucleotide sequences that directly interact with the ribosome. To test this possibility, m-RNA was first treated with formaldehyde under conditions that should relieve some conformational constraints (10), and the treated RNA was then used to obtain pancreatic RNase-resistant fragments from 40 S initiation complexes. Glycerol gradient analysis of binding reactions, before and after nuclease digestion, showed that, while there was no change in the overall extent of messenger binding after exposure to formaldehyde, protection of the cap within 40 S complexes decreased from 90 to 50% as a result of the formaldehyde treatment. Analysis of the protected fragments by polyacrylamide gel electrophoresis (Fig. 9) revealed that the 44-nucleotide fragment, which was the most prominent fragment recovered from initiation complexes with untreated m-RNA, was greatly reduced after formaldehyde treatment. Thus, the structure of at least one of the 5'-terminal fragments can be perturbed by formaldehyde, suggesting the presence of hydrogen-bonded loops within the fragment.

DISCUSSION

Since these studies were carried out using a set of messenger RNAs, rather than a single species, it is possible to generalize as to which features of messenger-ribosome interaction might be common to many messages, and which features vary from one message to another. The most striking general characteristic, with reovirus messenger RNA in wheat germ extracts, is the nearly complete protection of the 5'-terminal cap against nuclease digestion in the 40 S initiation complex. The fact that the 40 S ribosomal subunit conferred almost complete protection of the cap, in turn, suggests that the protected fragments included the initiation codon. The limited ability of such fragments to rebind to ribosomes, once the cap had been removed, suggests that the initiation codon and the sequences directly adjacent to it are inadequate to promote efficient ribosome attachment. In the case of reovirus messenger RNA or fragments thereof, the apparent dependence on a methylated 5' terminus for efficient binding of the cap in all three classes of reovirus message (and thus, presumably, in all 10 reovirus messages), even though the size of the protected fragments ranged from 31 to 65 nucleotide residues, strongly suggests that protection of the cap is not merely a coincidence. On the contrary, the data suggest that a portion of the 40 S initiation complex directly interacts with, and thus protects, the 5' terminus of the message. This is consistent with earlier studies which showed that binding of reovirus message to 40 S wheat germ ribosomes was almost completely dependent on the presence of a methylated 5' terminus (4). While the requirement for a methylated cap, observed with native reovirus messenger RNA, could be obviated by fragmenting or unfolding the RNA, presence of a methylated 5' terminus always caused a significant increase in the efficiency of ribosome binding. For example, unmethylated reovirus m-RNA, which in its native form showed virtually no binding to either 40 S or 80 S wheat germ ribosomes, was able to bind to the extent of 14% after treatment with formaldehyde;* but this was still far less than the 90% level of binding obtained under the same conditions with methylated reovirus m-RNA, before or after formaldehyde treatment (Figs. 1 and 9). Similarly, the ability of purified protected fragments to rebind to ribosomes, although not absolutely dependent on the presence of a methylated 5' terminus, was far more efficient for capped than for uncapped fragments (Table 1 and Fig. 6). Since the 80 S complexes from which the uncapped fragments were derived were blocked with sparsomycin, an inhibitor of polypeptide elongation which allows normal initiation complex formation, it is likely that the protected fragments included the initiation codon. The limited ability of such fragments to rebind to ribosomes, once the cap had been removed, suggests that the initiation codon and the sequences directly adjacent to it are inadequate to promote efficient ribosome attachment. In the case of reovirus messenger RNA or fragments thereof, the apparent dependence on a methylated 5' terminus for efficient binding of the cap in all three classes of reovirus message (and thus, presumably, in all 10 reovirus messages), even though the size of the protected fragments ranged from 31 to 65 nucleotide residues, strongly suggests that protection of the cap is not merely a coincidence. On the contrary, the data suggest that a portion of the 40 S initiation complex directly interacts with, and thus protects, the 5' terminus of the message. This is consistent with earlier studies which showed that binding of reovirus message to 40 S wheat germ ribosomes was almost completely dependent on the presence of a methylated 5' terminus (4). While the requirement for a methylated cap, observed with native reovirus messenger RNA, could be obviated by fragmenting or unfolding the RNA, presence of a methylated 5' terminus always caused a significant increase in the efficiency of ribosome binding. For example, unmethylated reovirus m-RNA, which in its native form showed virtually no binding to either 40 S or 80 S wheat germ ribosomes, was able to bind to the extent of 14% after treatment with formaldehyde;* but this was still far less than the 90% level of binding obtained under the same conditions with methylated reovirus m-RNA, before or after formaldehyde treatment (Figs. 1 and 9).

* Recent fingerprints of preparations from which the acrylamide was removed by filtration continued to show the presence of a streak when cellulose acetate electrophoresis was performed in the absence of urea. This material migrated as a more discrete spot in the presence of 7 M urea and upon digestion with pancreatic RNase yielded a unique set of oligonucleotides.

* M. Kozak, unpublished results.
attachment to ribosomes is consistent with the observed complete, or nearly complete, protection of the cap in 40 S initiation complexes formed with all three size classes of viral message.

The size of the fragments protected within 40 S initiation complexes varied from one message to another. With several messenger species, the fragments recovered from 40 S complexes were larger than the corresponding fragments protected by 80 S initiation complexes, which were closer to the expected size, based on the diameter of the ribosome and the size of protected fragments observed in prokaryotic systems (11, 12). Although the size difference between protected fragments from 40 S and 80 S complexes might be explained in part by changes in conformation of the ribosome upon addition of the 60 S subunit, as occur with prokaryotic ribosomes (13, 14), or by loss of initiation factors after subunit joining (15–17), such mechanisms fail to account for the variation observed from one reovirus messenger species to another. Therefore, it seems reasonable to postulate that for each message the minimal length of the fragment protected within the 40 S initiation complex might be determined by the distance between the cap and the AUG initiation codon; and that continued protection of the cap upon formation of 80 S complexes occurs only with those messages in which this distance is relatively short. In other words, the 40 S complex protects the entire stretch from the cap to the initiation site, while the 80 S complex continues to protect only those ~30 residues which are closest to the initiation codon. Several observations are consistent with this mechanism. The first is that there is extensive overlap between the internal sequences protected by 40 S and 80 S ribosomes on a given message, as shown in the fingerprints in Fig. 7 and as observed previously for globin messenger RNA (18, 19). The second is that, among reovirus messenger RNAs, the species in which the cap continued to be protected within the 80 S initiation complex were precisely those messages which gave rise to the smallest protected fragments at the level of the 40 S initiation complex; namely, the three l-messages and one message from the m-size class which at the 40 S level gave rise to a 36-nucleotide T1-resistant fragment. As shown in Fig. 5, among the fragments recovered from 40 S complexes with m-RNA, only this 36-nucleotide fragment rebound to form 80 S complexes in which the cap was highly resistant to T1 RNase. A further observation, consistent with the idea that the relatively large size of some fragments recovered from 40 S initiation complexes reflects a relatively long distance between the cap and the initiation codon, concerns the effect of formaldehyde treatment on the fragments recovered from 40 S complexes with the m-class of reovirus RNA. Fig. 9 shows that treatment of m-RNA with formaldehyde prior to binding and digestion with pancreatic RNase caused loss of the 44-residue [3H]methyl-labeled fragment. This is consistent with the idea that, normally, the 44-nucleotide fragment might contain a hairpin loop between the cap and the initiation codon, and that this loop takes up the slack, allowing both cap and initiation codon to be protected by the 40 S ribosome. After the loop has been opened by formaldehyde, the 5′ terminus of the message must still interact with the ribosome to promote binding, but this interaction is apparently inadequate to protect the cap on the conformationally extended RNA molecule. Failure to observe new 5′-terminal fragments after formaldehyde treatment, smaller than the fragments obtained with native RNA, contradicts an alternative hypothesis in which the occurrence of a hairpin loop on the 3′ side of the initiation codon could account for the larger-than-expected size of the fragments protected within 40 S initiation complexes. (While the latter mechanism seems to be ruled out as an explanation for the 44-nucleotide fragment derived from m-RNA, it obviously may still be a contributing factor in other situations.)

Finally, the experiment in which the ratio of ribosome-protected fragments was shifted (Fig. 8) by increasing the m-RNA concentration above saturating levels for the wheat germ system seems to be a useful beginning toward identifying which ribosome binding sites derive from efficiently versus inefficiently translated messages. Since the species of message that gives rise to the 43-nucleotide protected fragment seems to bind more efficiently than the species giving rise to the 52- and 36-nucleotide fragments, there seems to be no simple correlation between translational efficiency and the distance from the cap to the initiation codon, as reflected in the size of the fragment protected within the 40 S initiation complex.

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