Ribosome Binding Site Analysis of Ovalbumin Messenger Ribonucleic Acid†

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ABSTRACT: The region of the ovalbumin messenger ribonucleic acid ($mRNA_{ov}$) molecule bound to the 40S ribosomal subunit and its associated initiation factors in the wheat germ cell-free translation system were isolated and characterized. Two $mRNA_{ov}$ fragments, 87 and 92 nucleotides in length, were protected from T1 ribonuclease digestion by binding in a wheat germ preinitiation complex formed in the presence of guanosine 5′,β,γ-methylenetriphosphate and were shown by hydridization and fingerprint mapping to be derived from the 5′ end of $mRNA_{ov}$. Both these $mRNA_{ov}$ fragments were of sufficient length to contain both the cap structure and the AUG initiation codon. Four T1-resistant oligonucleotides, prepared by direct digestion of $mRNA_{ov}$ with T1 ribonuclease, were also found to bind to the wheat germ 40S ribosomal subunit. Nucleotide sequence analysis of these oligonucleotides revealed (1) that they were not a subset of the ribosome binding fragments described above, (2) that they were derived from within the $mRNA_{ov}$ molecule (one from within the coding region and three from the noncoding region at the 3′ end of the $mRNA_{ov}$ molecule), and (3) that three of the four Ti-resistant oligonucleotides contained 3′-terminal AUG trinucleotides. These data suggested that features of the $mRNA_{ov}$ molecule in addition to the nucleotide sequence might be important in specifying the correct ribosome binding site for the initiation of protein synthesis. The amount of $mRNA_{ov}$ bound to the wheat germ 40S ribosomal subunit in a preinitiation complex was found to vary inversely with the potassium ion concentration. Lowering the potassium concentration to levels suboptimal for translation also resulted in the protection of larger fragments of the $mRNA_{ov}$ molecule derived from the same 5′-end region as the ribosome binding fragments described above. The ability of the cap analogue 7-methylguanosine 5′-phosphate ($m^7G^{5'}p$) to reduce the amount of $mRNA_{ov}$ bound to the wheat germ 40S ribosomal subunit was found to depend directly on the potassium concentration. Interestingly, the effects of potassium on the amount of $mRNA_{ov}$ bound in a preinitiation complex and the inhibition of this binding by $m^7G^{5'}p$ could be observed by changing the potassium concentration after binding had occurred. These data suggested that the interaction between the wheat germ 40S ribosomal subunit and $mRNA_{ov}$ was very sensitive to the ionic environment.

Recently, the nucleotide sequence of the 5′ end of ovalbumin messenger ribonucleic acid (Kuebbing & Liarakos, 1978) and of the entire ovalbumin messenger ribonucleic acid molecule (McReynolds et al., 1978) has been reported. These data demonstrated the presence of a 64-nucleotide, noncoding leader sequence preceding the first AUG codon at the 5′ end of the $mRNA_{ov}$[1] molecule. Furthermore, this first AUG codon immediately preceded the codon for the amino-terminal glycine residue of ovalbumin, thereby identifying it as the putative initiation codon for protein synthesis and demonstrating the absence of a nucleotide sequence coding for an amino terminal transitory signal peptide. On the basis of this sequence information, potential hairpin structures formed by intramolecular base pairing and consistent with the $mRNA_{ov}$ secondary structure studies of Van et al. (1976, 1977) were postulated for both the 5′ end of the $mRNA_{ov}$ molecule and the region containing the AUG initiation codon. We have previously reported two possible regions of base pairing between the 5′ end of $mRNA_{ov}$ and the 3′ end of 18S rRNA (Kuebbing & Liarakos, 1978).

For a better understanding of the functional significance of the nucleotide sequence and possible secondary structure at the 5′ end of the $mRNA_{ov}$ molecule, studies were undertaken to isolate and identify the "ribosome binding site" of this mRNA. As with previous studies of this nature (Legon, 1976; Kozak & Shatkin, 1976), the ribosome binding site was generally defined as that region of a mRNA molecule which is protected from ribonuclease digestion either by the ribosome itself or by the smaller (40 S) ribosomal subunit and its associated initiation factors. In this case, we chose to determine what elements of the $mRNA_{ov}$ sequence were protected from T1 ribonuclease digestion by the wheat germ 40S ribosomal subunit in the preinitiation complex which is formed as the first step in protein synthesis. In the experiments described below, the 5′-end region of $mRNA_{ov}$ is identified as the area protected from T1 ribonuclease digestion by the wheat germ 40S ribosomal subunit. In addition, four potential ribosome binding sites in the intact $mRNA_{ov}$ molecule, but not recognized by the 40S ribosomal subunit, are described, and their possible significance in terms of the relationship between mRNA structure and function is discussed.

Materials and Methods

Dithiothreitol and ATP were obtained from Sigma. Creatine phosphokinase and creatine phosphate were obtained from Calbiochem and were freshly dissolved just prior to use. Ribonucleases T1 and U2 were Sankyo products obtained from Calbiochem. Bovine pancreatic ribonuclease A was obtained from Worthington. Physarum 1 ribonuclease was the generous

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[1] Abbreviations used: $mRNA_{ov}$, ovalbumin messenger ribonucleic acid; GMP-PCP, guanosine 5′,β,γ-methylenetriphosphate; $m^7G^{5'}p$, 7-methylguanosine 5′-phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; ATP, adenosine triphosphate.

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gift of Professor J. P. Bargetzi. pOV_{230} DNA, which is plasmid pMB9 DNA containing a cloned ovalbumin cDNA molecule with all of the $mRNA_{ov}$ sequence except the first 12 nucleotides, was generously supplied by Dr. Achilles Dugaiczyk. 7-Methylguanosine 5′-phosphate and guanosine 5′,β,γ-methylenetriphosphate were obtained from PL Biochemicals. Ultrapure sucrose for gradient analysis was from Schwarz/Mann. Restriction endonucleases *Hin*fI, *Sst*I, and *Taq*I were obtained from Bethesda Research Laboratories. Technical grade acrylamide and *N,N′*-methylenebis(acrylamide) were obtained from Eastman and were recrystallized once from chloroform (50 °C) and acetone (45 °C), respectively.

Ovalbumin mRNA was prepared with White Leghorn hen oviducts to greater than 95% purity and radiolabeled with $Na^{125}I$ (New England Nuclear) to a specific activity of (1–2) $\times 10^6$ dpm/μg as described by Woo et al. (1975). All ^{125}I radioactivity determinations were made by direct measurement in a Packard γ counter. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964), as described by Maxam & Gilbert (1977). A 30000*g* supernatant, cell-free translation fraction (S30) was prepared from wheat germ (generously donated by General Mills, Inc.) by the procedure of Roberts & Paterson (1973), as modified by Means et al. (1975), and stored as frozen pellets at −70 °C for over a year without loss of translation activity. These wheat germ S30 preparations had ~14% of their ribosomes present in the dissociated form (i.e., as 40S and 60S subunits), as determined by sucrose gradient analysis, and also contained the necessary tRNAs and initiation, elongation, and termination factors for protein synthesis.

Formation of the 48S Preinitiation Complex. Standard reaction conditions for the formation of a preinitiation complex for protein synthesis were a variation of the method of Kozak & Shatkin (1976) and involved adding [^{125}I]$mRNA_{ov}$ to a binding reaction mixture (100 μL final volume) containing 24 mM Hepes (pH 7.6), 2.0 mM DTT, 1.0 mM ATP, 0.48 mM GMP-PCP, 2.5 mM $Mg(OAc)_2$, 8.0 mM creatine phosphate, 0.8 unit of creatine phosphokinase, and 20 μL of wheat germ S30. It should be noted that the wheat germ S30 contributed 1 mM $Mg(OAc)_2$ to the final concentration listed above as well as 24 mM KCl. Since the pH of the Hepes buffer was adjusted with KOH, the potassium ion concentration (i.e., from wheat germ S30 and Hepes) in the binding reaction mixture described above was 52 mM. Unless otherwise noted, this reaction mixture was also supplemented with KOAc to a final potassium ion concentration of 112 mM, which has been shown to be the optimal concentration for translation of $mRNA_{ov}$ in the wheat germ system (Means et al., 1975). Potassium was added as KOAc since the chloride ion at high concentrations (>80 mM) has been demonstrated to inhibit protein synthesis (Weber et al., 1977a). A final [^{125}I]$mRNA_{ov}$ concentration (10–15 nM at 112 mM potassium) sufficient to produce saturable binding was used unless otherwise indicated. After incubation at room temperature for 10 min, T1 ribonuclease was added (enzyme/$mRNA_{ov}$ mass ratio = 1:20) and the incubation continued for 20 min at room temperature. The reaction mixture was then fractionated by centrifugation at 4 °C through a 10–30% sucrose gradient containing 20 mM Tris-HCl (pH 7.5), 60 mM KCl, and 2.5 mM $Mg(OAc)_2$. Analytical reaction mixtures were centrifuged at 35 000 rpm for 10 h in a Spinco SW40 rotor, and 0.33-mL fractions were collected. Preparative reaction mixtures were centrifuged at 27 000 rpm for 12 h in a Spinco SW27 rotor, and 1-mL fractions were collected. The A_{254} was continuously recorded on an ISCO UA-5 absorbance monitor as the sucrose gradient fractions were collected, and each fraction was counted for ^{125}I as described above.

In one series of experiments, [^{125}I]$mRNA_{ov}$ was digested for 20 min at room temperature with T1 ribonuclease in 10 mM Hepes, pH 7.6 (enzyme/RNA mass ratio = 1:20), prior to addition of the ribosome binding reaction mixture. The complete binding reaction mixture was then incubated for 10 min at room temperature and analyzed by sucrose gradient centrifugation as described above.

Experiments to measure inhibition of $mRNA_{ov}$ binding using the cap analogue m^7G^5p (PL Biochemicals) were performed at both 52 and 112 mM potassium by adding m^7G^5p to the binding reaction mixture (final concentrations 0, 0.5, and 1.0 mM) prior to the addition of [^{125}I]$mRNA_{ov}$ and analyzed as above.

Extraction of RNA from the 48S Preinitiation Complex. Sucrose gradient fractions containing the 48S preinitiation complex (see Results) were diluted 1:1 with water, and the RNA–protein complex was precipitated at −20 °C with 2 volumes of 2% KOAc in 95% ethanol. The precipitate was dissolved in 10 mM NaOAc (pH 5.2) and 10 mM EDTA, and the RNA was extracted with an equal volume of phenol–*m*-cresol (8:1 v/v) containing 8-hydroxyquinoline (1 mg/mL) and saturated with 10 mM NaOAc (pH 5.2) and 10 mM EDTA by vigorously shaking for 5 min at room temperature, followed by gentle mixing for 5 min at 65 °C. The aqueous phase plus the protein interphase was collected by centrifugation, adjusted to 0.5 M NaCl, and reextracted with 0.5 volume of saturated phenol–*m*-cresol–8-hydroxyquinoline as before. The aqueous phase was again collected and the RNA precipitated with 2% KOAc in 95% EtOH.

Polyacrylamide Gel Electrophoresis. RNA extracted from the 48S preinitiation complex was fractionated by electrophoresis on 12% polyacrylamide [acrylamide/bis(acrylamide) = 29:1] slab gels (40 × 20 × 0.3 cm) containing 7 M urea, 90 mM Tris–borate (pH 8.3), and 2.5 mM EDTA as described by Maniatis et al. (1975). Gels were prerun for 6 h at 200 V before application of the RNA. The RNA was applied in a solution containing 0.05 mM EDTA, 30% sucrose, 0.02% bromophenol blue, and 0.02% xylene cyanol FF, heated for 30 s at 70 °C, and quick-chilled on ice before electrophoresis. Gels were run at room temperature at 400 V until the bromophenol blue dye had migrated approximately two-thirds of the way through the gel. ^{125}I-Labeled $mRNA_{ov}$ fragments were visualized by autoradiography at −20 °C and extracted from the gel by using the "crush and soak" technique of Maxam & Gilbert (1977). Oligonucleotide sizes (N = nucleotides) were estimated by comparison with the marker dyes xylene cyanol FF (57 N) and bromphenol blue (13 N) as described by Maniatis et al. (1975).

Oligonucleotide Fingerprint Mapping. The 40S ribosomal subunit bound fragments of [^{125}I]$mRNA_{ov}$, extracted from the 48S preinitiation complex and isolated by polyacrylamide gel electrophoresis (see above), were each digested for 1 h at 37 °C with T1 RNase as described above. The resulting oligonucleotides were fractionated in two dimensions as described by Busch et al. (1976). The first dimension consisted of electrophoresis on 3 × 95 cm Cellogel strips (Kalex) in 7 M urea–5% HOAc–3 mM EDTA (pH 3.5), followed by DEAE-cellulose thin-layer homochromatography using 90% homomixture C-15–10% homomixture B in the second dimension. Autoradiography was for 60 days.

Hybridizations. (a) The 87- and 92-nucleotide fragments of [^{125}I]$mRNA_{ov}$ (extracted from the 48S preinitiation complex and isolated by polyacrylamide gel electrophoresis as described

above) were hydridized to denatured pOV_{230} DNA immobilized on Millipore filters essentially as described by Roop et al. (1978). The effective amount of hybridizable (i.e., with $mRNA_{ov}$) ovalbumin DNA bound per 25-mm filter was ~4 μg. Hybridization was accomplished by adding 0.6 mL of [^{125}I]RNA (10000–20000 cpm) in hybridization buffer [1 M NaCl–60 mM sodium citrate–70 mM Tes-HCl (pH 7.2)–7 mM EDTA in 50% formamide] to a DNA filter prewet with 0.6 mL of the same hybridization buffer and incubating for 16 h at 37 °C. The reaction buffer was then removed and the filter washed at 37 °C with 0.6 mL of fresh hybridization buffer (minus RNA). Filters containing DNA–RNA hybrids were equilibrated for 15 min at 0 °C in 0.6 mL of denaturation buffer [10 mM Tes-HCl (pH 7.4)–3 mM EDTA]; hybridized RNA was eluted from the filters by heat denaturation (2 min at 90 °C) in 0.6 mL of fresh denaturation buffer and precipitated with 2% KOAc–95% ethanol. Nonspecific binding of [^{125}I]RNA to filters carried through the hybridization procedure, but containing no DNA, was undectable (<0.1%).

(b) Hybridization reactions at a 1:1 molar ratio of DNA/RNA were performed in solution by using the 92-nucleotide fragment of [^{125}I]$mRNA_{ov}$ (first purified by hybridization to filter-bound pOV_{230} DNA as described above) and restriction endonuclease fragments of pOV_{230} DNA. Restriction endonuclease digestion of pOV_{230} DNA with *Hin*fI (primary) and *Sst*I or *Taq*I (secondary), polyacrylamide gel electrophoresis, and subsequent extraction of the separated restriction fragments from the gel were as described by Kuebbing & Liarakos (1978). DNA and [^{125}I]RNA were dissolved together in 10 μL of 0.14 M phosphate buffer (pH 6.8), heat denatured (5 min at 100 °C), incubated for 1 h at 62 °C (DNA C_0t = 0.13), and diluted into 1 mL of ice-cold 0.14 M phosphate buffer to stop the reaction. Pancreatic ribonuclease A (50 μg) and T1 ribonuclease (50 units) were added, and after incubation for 10 min at 37 °C to destroy any unhybridized [^{125}I]RNA, single- and double-stranded nucleic acids were separated by chromatography at 60 °C on a 2-mL hydroxylapatite (Clarkson) column (Rosen et al., 1974). [^{125}I]RNA alone (i.e., in the absence of DNA) was also subjected to the hybridization reaction and subsequent hydroxylapatite chromatography with and without ribonuclease treatment.

Preparation and ^{32}P Labeling of T1 RNase Resistant Oligonucleotides. Forty micrograms of $mRNA_{ov}$ was digested at 37 °C for 30 min with 10 units of T1 RNase in 12 μL of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA, diluted to 1 mL with water, precipitated at −20 °C with 2.5 volumes of 2% KOAc–95% ethanol, and collected by centrifugation. The resulting T1-resistant oligonucleotides were 5′-end radiolabeled with ^{32}P by incubation at 37 °C for 30 min in 25 μL of 40 mM Tris-HCl (pH 7.4)–8 mM $MgCl_2$–8 mM DTT containing an approximate 10-fold molar excess of [γ-^{32}P]ATP (1000–2000 Ci/mmol) and 10–20 units of T4 polynucleotide kinase (Boehringer-Mannheim). The reaction mixture was diluted with an equal volume of 60% sucrose–0.05% bromophenol blue–0.05% xylene cyanol FF, heated for 30 s at 70 °C, quick-chilled on ice, and immediately applied to a 12% polyacrylamide slab gel containing 90 mM Tris–borate (pH 8.3) and 2.5 mM EDTA. After electrophoresis as described above, the ^{32}P-labeled oligonucleotides were visualized by autoradiography and extracted from the gel as described above.

RNA Sequence Analysis. RNA sequence analysis was performed by a slight modification of published enzymatic procedures (Donis-Keller et al., 1977; Simoncsits et al., 1977; Gupta & Randerath, 1977). One microgram of 5′-end, ^{32}P-labeled RNA was used in each reaction. Unlabeled tRNA was used to provide additional mass where required. The partial degradation products of the various sequencing reactions were separated by simultaneous electrophoresis in adjacent lanes of a polyacrylamide slab gel [20% acrylamide–0.67% bis(acrylamide)] containing 7 M urea, 50 mM Tris–borate (pH 8.3), and 1 mM EDTA. The electrophoresis buffer was the same but contained no urea. (Gels were allowed to polymerize overnight and were preelectrophoresed for 12 h at 400 V before use.) Electrophoresis was performed at 1000 V until the bromophenol blue dye had migrated two-thirds of the length of the gel. The gel was allowed to warm to 40–50 °C during electrophoresis to ensure complete denaturation of the RNA. The sequencing reactions were as follows.

(*a*) *Undegraded RNA Control.* One microgram of [^{32}P]-RNA in 10 μL of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA was combined with 15 μL (99%) of formamide.

(*b*) *Alkaline Hydrolysis.* One microgram of [^{32}P]RNA plus 4 μg of tRNA was incubated for 30 min at 90 °C in 10 μL of 50 mM $NaHCO_3/Na_2CO_3$ (pH 9.0)–1 mM EDTA, followed by the immediate addition of 20 μL of 10 M urea and rapid cooling to 0 °C.

(*c*) *Partial Degradation with Pancreatic Ribonuclease A.* A reaction mixture containing 1 μg of [^{32}P]RNA, 4 μg of tRNA, and 1 ng of pancreatic ribonuclease A in 10 μL of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA was incubated for 2 min at 0 °C; 5 μL of the reaction mixture was combined with 8 μL of 99% formamide. After 15 min, the remaining 5 μL of reaction mixture was treated in the same way and the two time points were pooled for gel electrophoresis.

(*d*) *Partial Degradation with U2 Ribonuclease.* Two separate reactions were used. In the first, 1 μg of [^{32}P]RNA was incubated for 40 min at 0 °C with 1 μg of U2 ribonuclease in 10 μL of 40 mM sodium citrate (pH 5.0)–1.6 mM EDTA, followed by the addition of 15 μL of 99% formamide. In the second reaction, 1 μg of [^{32}P]RNA plus 4 μg of tRNA was incubated for 5 min at 50 °C in 20 μL of 7 M urea–20 mM sodium citrate (pH 5.0)–1 mM EDTA. One microgram of U2 ribonuclease was added and the incubation continued for 10 min at 50 °C, followed by rapid cooling to 0 °C.

(*e*) *Partial Degradation with Physarum 1 Ribonuclease.* One microgram of [^{32}P]RNA plus 4 μg of tRNA in 20 μL of 20 mM NaOAc (pH 4.5)–2 mM EDTA was digested at room temperature with 0.014 unit of Phy 1 ribonuclease. After 2 and 12 min of digestion, respectively, one-half of the reaction mixture was combined with 15 mg of urea (added as a solid). The two time points were pooled for gel electrophoresis.

After each of the sequencing reactions, the marker dyes xylene cyanol FF and bromophenol blue were added and the reaction mixture was heated for 1 min at 100 °C and rapidly cooled to 0 °C. Reaction mixtures not immediately applied to a polyacrylamide gel were stored at −70 °C.

Results

Isolation of the Ribosome Binding Fragments of Ovalbumin mRNA. The formation of a stable preinitiation complex between ^{125}I-labeled ovalbumin mRNA and the wheat germ 40S ribosomal subunit was accomplished in the presence of GMP-PCP. This nonhydrolyzable analogue of GTP inhibits the formation of the 80S initiation complex by preventing the GTP hydrolysis-dependent binding of the 60S ribosomal subunit to the mRNA–40S ribosomal subunit preinitiation complex (Levin et al., 1973; Kozak & Shatkin, 1976). Radiolabeling of globin mRNA with ^{125}I, under the conditions used, has been shown to have no effect on its ability to code for "initiation" peptides or to bind ribosomes (Legon et al., 1976, 1977). The binding reactions were performed at a

potassium ion concentration (112 mM) optimal for the faithful translation of $mRNA_{ov}$ in the wheat germ cell-free system (Means et al., 1975). Although the molar ratio 40S ribosomal subunits/$mRNA_{ov}$ in the binding reaction was ~10:1, the amount of $mRNA_{ov}$ bound was found to be a saturable function of $mRNA_{ov}$ concentration. This suggested that either mRNA stability or some essential factor in the wheat germ S30 preparation was limiting the amount of $mRNA_{ov}$ binding. The degradation of $mRNA_{ov}$ did not appear to significantly limit the extent of its binding since doubling the amount (see Materials and Methods) of wheat germ S30 in the reaction mixture after saturable binding had been achieved resulted in a 50–70% increase in the amount of $mRNA_{ov}$ bound. The actual amount of $mRNA_{ov}$ which bound on a molar basis was 10–20% of the added $mRNA_{ov}$ and varied somewhat between wheat germ preparations.

Figure 1a shows a typical sucrose gradient profile in which a fragment of the [^{125}I]$mRNA_{ov}$ molecule was protected from T1 RNase digestion by the 40S ribosomal subunit and migrated as a 48S "shoulder" on the peak (A_{254}) of 40S ribosomal subunits. Extraction of the [^{125}I]RNA from this 48S preinitiation complex and analysis by polyacrylamide gel electrophoresis revealed two oligonucleotides, 92 and 87 nucleotides in length, respectively, present in approximately equal amounts (Figure 1a′). The same binding reaction was also performed with [^{125}I]$mRNA_{ov}$ which had been predigested with T1 RNase so that the only RNA species available for binding were T1-resistant oligonucleotides. Sucrose gradient analysis again gave a 48S peak of protected [^{125}I]RNA (Figure 1b); however, extraction and subsequent gel analysis of this [^{125}I]RNA revealed four T1 RNase resistant oligonucleotides, 42, 30, 25, and 18 nucleotides in length, respectively (Figure 1b′).

Identification of the Ribosome Binding Fragments of Ovalbumin mRNA. The location of the various (40 S) ribosome binding fragments within the $mRNA_{ov}$ molecule was established by using RNA sequence analysis and hybridization techniques. For performance of the sequence analysis on the 87- and 92-nucleotide RNA fragments, they had to first be purified away from the large amount of unlabeled RNA with which they comigrate on a polyacrylamide gel. This was necessary since the RNA sequencing procedure (see Materials and Methods) required a relatively pure RNA species labeled with ^{32}P at its 5′ end. To accomplish this, the 87- and 92-nucleotide RNA fragments were hybridized to Millipore filter-bound, single-stranded pOV_{230} DNA as described under Materials and Methods. The RNA hybridized with an efficiency of 60–70% and was recovered by thermal elution. The 30–40% of the RNA which remained unhybridized also failed to hybridize to any measurable extent upon incubation with a second DNA filter under the same conditions. This was apparently the result of the degradation of this RNA, during the first reaction, to a size too small to form stable hybrids.

Several unsuccessful attempts were made to ^{32}P label the 5′ ends of the 87- and 92-nucleotide RNA fragments purified by filter hybridization both with and without prior enzymatic decapping with tobacco acid phosphatase. The decapping reaction was used since both the 87- and 92-nucleotide ribosome binding fragments were observed (data not shown) to bind to DBAE-cellulose and elute in the presence of sorbitol. This is indicative of a *cis*-diol group on these RNA molecules which is consistent with but not diagnostic for the presence of a cap structure (McCutchan et al., 1975; Duncan & Gilham, 1975). The labeling reaction involved 5′-end dephosphorylation with bacterial alkaline phosphatase followed by T4 polynucleotide kinase catalyzed addition of ^{32}P to the 5′

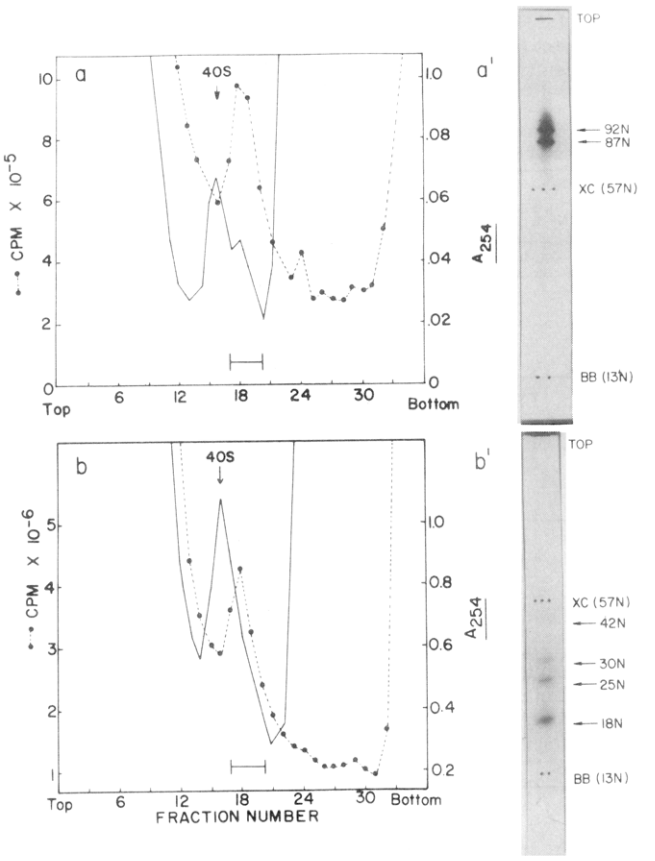
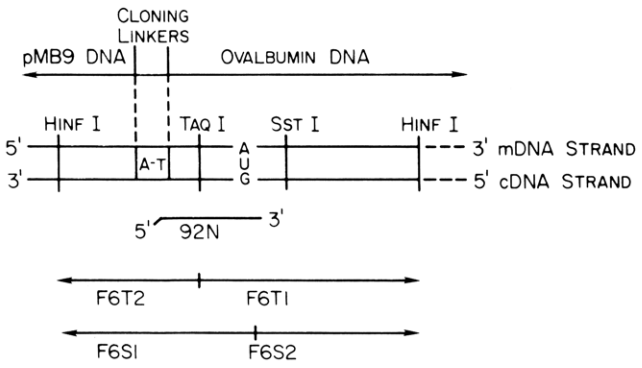


FIGURE 1: Ribosome binding site analysis of ^{125}I-labeled $mRNA_{ov}$. (a) A complex containing [^{125}I]$mRNA_{ov}$ bound to the wheat germ 40S ribosomal subunit was subjected to T1 RNase digestion and isolated as a 48S preinitiation complex by centrifugation on a 10–30% sucrose gradient. Fractions 17–20, containing a 48S peak of [^{125}I]RNA, were pooled, and the RNA was phenol extracted and analyzed by electrophoresis on a 12% polyacrylamide gel (a′). The size of each gel band was estimated by comparison of relative electrophoretic mobility with the dye markers xylene cyanol FF (XC) and bromophenol blue (BB), whose relative sizes in nucleotides (N) are also shown. For purposes of discussion, the nucleotide sizes shown were selected to be consistent with the actual guanosine positions in the $mRNA_{ov}$ molecule (see Figure 9) and are within 2 nucleotides of the estimated size for each band. (b) ^{125}I-Labeled $mRNA_{ov}$ was digested with T1 RNase and carried through the binding reaction with the wheat germ S30 system. A 48S complex containing T1-resistant oligonucleotides from [^{125}I]$mRNA_{ov}$ was isolated as above, and the RNA was extracted and analyzed (b′) also as above. The oligonucleotide sizes shown correspond to actual T1-resistant oligonucleotides identified by inspection of the $mRNA_{ov}$ sequence and are within 2–6 nucleotides of the size estimates obtained by comparison of relative electrophoretic mobilities.

end of the RNA molecule. Similar attempts to label the 5′ end of intact ovalbumin mRNA with ^{32}P have also been unsuccessful (Chu et al., 1978; C. D. Liarakos, unpublished experiments). The resistance of $mRNA_{ov}$ to enzymatic ^{32}P labeling of its 5′ end may reflect either inefficient decapping of the 5′ end or steric hindrance of the dephosphorylation and labeling reactions. Chu et al. (1978) have demonstrated a change in the salt optimum for translation of $mRNA_{ov}$ after decapping, similar to that observed with decapped globin mRNA. This suggests that the decapping reaction itself is not the problem. If the dephosphorylation and labeling reactions were sterically hindered by the structure of the $mRNA_{ov}$ molecule, the isolated ribosome binding site of $mRNA_{ov}$ might be free of such steric hindrance and therefore more readily decapped and ^{32}P labeled than intact $mRNA_{ov}$. This was not found to be the case, however, and hybridization, rather than sequence analysis, had to be used to locate the

Table I: Hybridization of the 92-Nucleotide Ribosome Binding Fragment from [^{125}I]mRNA$_{ov}$ with Restriction Fragments from pOV$_{230}$ DNA[a]



pOV$_{230}$ DNA restriction fragment	% hybridization (bound to HAP)
none[b] (−RNase)	78.6
none (+RNase)	0.1
F6S1	30.6
F6T1	23.5
F6S2, F2, F4, F7, F9	6.1

[a] The extent of hybridization was measured as the percentage of the initial [^{125}I]RNA counts per minute which became ribonuclease resistant and bound to hydroxylapatite. The *Hin*f I sites define fragment F6. [b] Measures the ability of the ^{125}I-labeled, 92-N RNA fragment to bind to hydroxylapatite.

mRNA$_{ov}$ ribosome binding fragments within the mRNA$_{ov}$ molecule. Differential hybridization was performed with restriction fragments from pOV$_{230}$ DNA which corresponded to different parts of the mRNA$_{ov}$ molecule. Table I shows both the derivation and designation of the restriction fragments from the "left" end of the ovalbumin cDNA in pOV$_{230}$ (i.e., corresponding to the 5′ end of mRNA$_{ov}$) and the extent of hybridization of the ^{125}I-labeled 92-nucleotide fragment of mRNA$_{ov}$ (first purified by filter hybridization with pOV$_{230}$ DNA as described above) with these various restriction fragments. Since the 92-nucleotide RNA fragment itself readily bound to hydroxylapatite (78.2%), a posthybridization ribonuclease treatment was necessary to reduce the binding of unhybridized RNA to relatively negligible levels (0.1%) so that only true DNA–RNA hybrids were detected by hydroxylapatite binding. Restriction fragment F6S1 was prepared by secondary digestion of *Hin*fI fragment F6 and contains plasmid (pMB9) DNA, the dA–dT linkers used in cloning, and DNA corresponding to the 5′ end of mRNA$_{ov}$ from nucleotides 13–118. If the 92-nucleotide ribosome binding fragment corresponds to the 5′ end of mRNA$_{ov}$, complete hybridization with F6S1 DNA should protect all but the first 12 ribonucleotides from ribonuclease digestion, resulting in a maximum theoretical [^{125}I]RNA hybridization of 87%. (This of course assumes a uniform distribution of the cytosine residues containing the ^{125}I, which inspection of the mRNA sequence reveals is essentially the case.) The observed hybridization with F6S1 DNA was 32% (37% of the theoretical maximum). Secondary digestion of DNA fragment F6 with restriction endonuclease *Taq*I gave a DNA fragment (F6T1) containing no plasmid or linker DNA and corresponding to mRNA$_{ov}$ nucleotides 41–258. Again, if the 92-nucleotide RNA fragment corresponds to the 5′ end of mRNA$_{ov}$, hybridization with F6T1 DNA should protect 51 nucleotides from ribonuclease digestion, resulting in a maximum theoretical [^{125}I]RNA hybridization of 55%. The observed hybridization was 23.5% (43% of the theoretical maximum). Thus, both hybridization reactions occurred with approximately the same efficiency in the presence and absence of plasmid and linker DNA. This is consistent with the observed hybridization being specific for ovalbumin DNA. The similar hybridization efficiencies (i.e., percentage of the theoretical maximum) also indicated the complete hybridization of each RNA molecule which reacted. Reaction of the 92-nucleotide [^{125}I]RNA fragment with a mixture of *Hin*fI restriction fragments (F2, F4, F7, and F6S2), each present at a molar ratio of 1:1 with the RNA and containing the remainder of the mRNA$_{ov}$ sequence information (i.e., minus the 5′ end), gave a hybridization value of 6%. Although the relative contribution of each of these DNA fragments to the 6% total hybridization was not determined, separation of these *Hin*fI fragments of pOV$_{230}$ DNA by gel electrophoresis generally results in some cross-contamination of neighboring gel bands. Thus, F7 DNA usually contains some F6 DNA, which may largely account for the 6% hybridization value observed above. In any case, the 92-nucleotide ribosome-protected fragment of mRNA$_{ov}$ exhibited a strong preference for hybridization with DNA restriction fragments corresponding to the 5′ end of mRNA$_{ov}$.

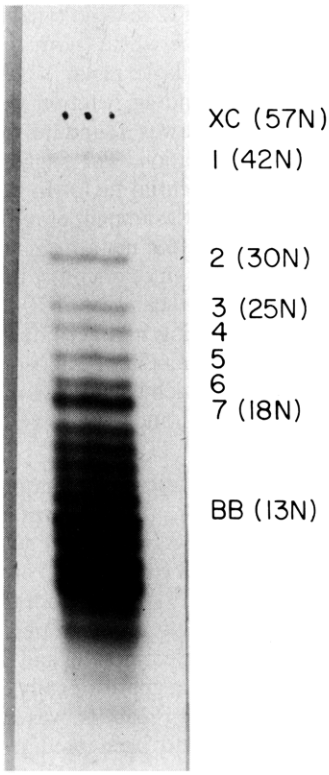


FIGURE 2: 12% polyacrylamide gel electrophoresis pattern of T1 RNase digested mRNA$_{ov}$. mRNA$_{ov}$ was digested to completion with T1 RNase, and the resulting T1-resistant oligonucleotides were 5′-end radiolabeled with ^{32}P, using T4 polynucleotide kinase. The largest gel bands are numbered (1–7) in order of decreasing size. The sizes in nucleotides (N) of those which correspond in relative mobility (±1 nucleotide) to those T1-resistant oligonucleotides which bound to the wheat germ 40S ribosomal subunit (Figure 1b′) are shown in parentheses. Marker dyes were as in Figure 1.

Since they were too small to form stable hybrids, but were readily 5′-end labeled with ^{32}P, the four T1-resistant oligonucleotides which bound to the 40S ribosomal subunit (Figure 1b′) were localized by indirectly determining their individual nucleotide sequences and comparing them with the complete sequence of mRNA$_{ov}$ as reported by McReynolds et al. (1978). Ovalbumin mRNA was digested to completion with T1 RNase, and the resulting oligonucleotides were 5′-end labeled

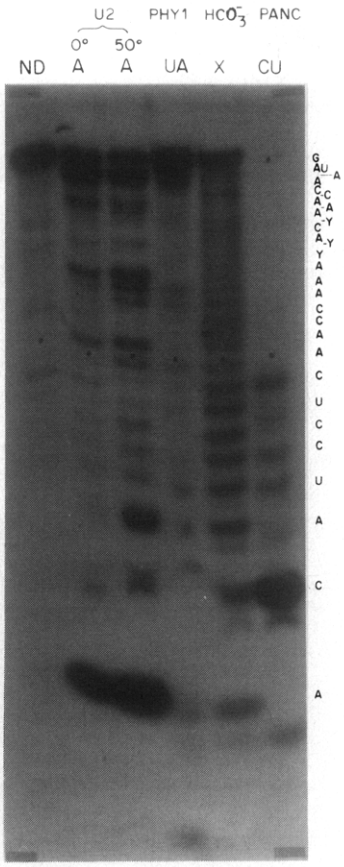


FIGURE 3: RNA sequencing gel for T1-resistant oligonucleotide no. 2. Each vertical pattern of bands corresponds to a different partial digestion of the 5′-end, ^{32}P-labeled RNA molecule. ND = no digestion (used to monitor nonspecific degradation). Ribonucleases U2, Phy 1, and Panc are shown with their base specificities, A, U and A, and pyrimidines (C and U), respectively. Bicarbonate (HCO_3^-) digestion yields a nested set of fragments corresponding in length to the distance from the 5′-^{32}P label to each of the nucleotides in the fragment.

with ^{32}P (see Materials and Methods) and separated by gel electrophoresis as shown in Figure 2. The population of T1-resistant oligonucleotides derived from $mRNA_{ov}$, whether internally labeled with ^{125}I or 5′-end labeled with ^{32}P, gave coincident separation patterns upon simultaneous gel electrophoresis (data not shown). Therefore, the relative electrophoretic mobilities of ^{125}I-labeled and ^{32}P-labeled oligonucleotides could be effectively compared. Furthermore, any significant degradation produced by the wheat germ system should alter the relative electrophoretic mobilities of the T1-resistant oligonucleotides from those observed in Figure 2. This should be especially apparent for the largest oligonucleotides (no. 1–3, Figure 2), which are characteristic of the $mRNA_{ov}$ molecule because of their significant difference in size from the rest of the T1-resistant oligonucleotides. In fact, the three largest gel bands (no. 1–3) in Figure 2 as well as band no. 7 had sizes (as determined from their relative electrophoretic mobilities) essentially identical (±1 nucleotide) with those observed in Figure 1b′. Therefore, the [^{32}P]RNA was extracted from these bands and subjected to sequence analysis as described under Materials and Methods.

Figure 3 shows a typical pattern of partial digestion products from which a nucleotide sequence (that of oligonucleotide no. 2) can be read. T1-resistant oligonucleotide no. 7 gave a mixed sequence analysis (data not shown), indicating the presence of more than one oligonucleotide of this size in $mRNA_{ov}$. Two possible T1-resistant oligonucleotides of this size (18 N) were found by searching the nucleotide sequence of $mRNA_{ov}$ (no. 7 and 7′, Figure 4). The one containing a 3′-AUG trinucleotide was assumed to correspond to the smallest T1-resistant oligonucleotide which bound to the 40S ribosomal subunit (Figure 1b′) since this was consistent with the sequence information obtained for oligonucleotides 2 and 3. A summary of this sequence information is presented in Figure 4 and discussed further below. Figure 5 shows the relative positions of the functional ribosome binding site at the 5′ end of $mRNA_{ov}$, as determined by hybridization, and of the four T1-resistant oligonucleotides which bind to the 40S ribosomal subunit, as determined by comparison of their sequence with that of $mRNA_{ov}$. One of these oligonucleotides (no. 2) is located within the coding region of $mRNA_{ov}$ while the other three are located in the noncoding region at the 3′ end of the $mRNA_{ov}$ molecule.

Characterization of the Ribosome Binding Reaction. It is well-known that the optimal potassium ion concentration for translation of eucaryotic mRNAs in the wheat germ system varies for different mRNAs (Roberts & Paterson, 1973). More recent evidence (Weber et al., 1977b, 1978; Chu & Rhoads, 1978; Bergmann & Lodish, 1979) has indicated that decapped mRNAs have lower optimal potassium concentrations for translation in the wheat germ system than their capped counterparts and that the ability of cap analogues to

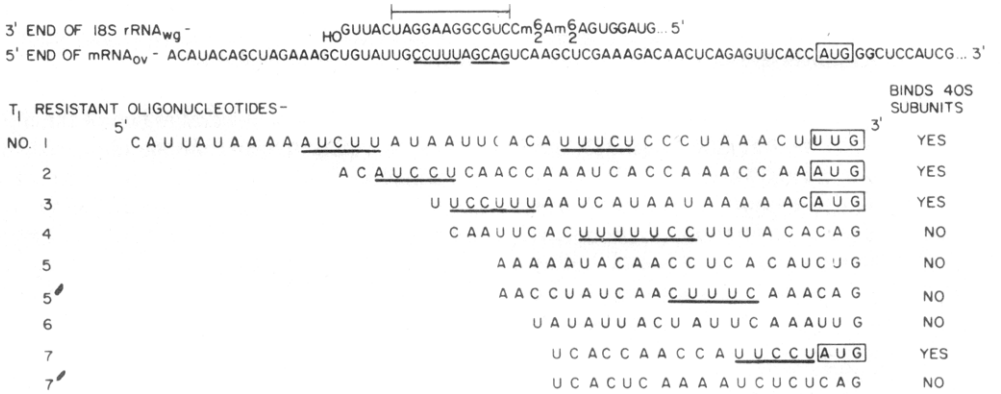


FIGURE 4: Nucleotide sequences shown are those at the 3′ end of 18S rRNA (Alberty et al., 1978; Hagenbuchle et al., 1978), at the 5′ end of ovalbumin mRNA (McReynolds et al., 1978; Kuebbing & Liarakos, 1978), and of the large T1-resistant oligonucleotides from ovalbumin mRNA numbered as in Figure 2. Only the sequences of the four oligonucleotides with relative electrophoretic mobilities corresponding to those which bound to the 40S ribosomal subunit were determined experimentally. The sequences of nonbinding oligonucleotides 4, 5, 5′, and 6 were deduced by examination of the $mRNA_{ov}$ sequence for T1-resistant oligonucleotides of the correct size. The AUG initiation codon of ovalbumin mRNA is boxed as well as the 3′-terminal trinucleotides of the T1-resistant oligonucleotides which bound to the 40S ribosomal subunit. Underlined sequences show base complementarity within the region of 18S rRNA shown under the bracketed line (⊢⊣).

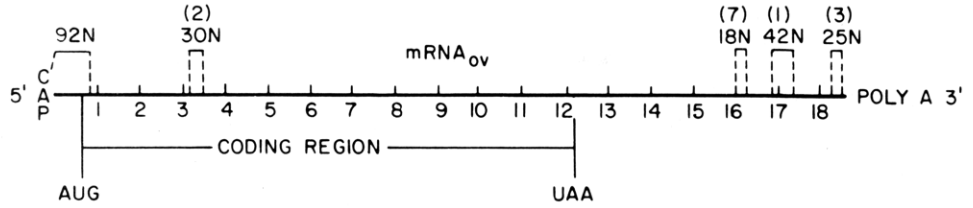


FIGURE 5: Schematic representation of ovalbumin mRNA showing its size in hundreds of nucleotides and the location of the coding region bounded by a 5′-AUG initiation codon and a 3′-UAA termination codon. Also shown are the relative locations of the 5′-terminal, 92-nucleotide ribosome binding site and the T1-resistant oligonucleotides 1, 2, 3, and 7 (numbered as in Figures 2 and 4). The size of each RNA fragment is expressed in nucleotides (N).

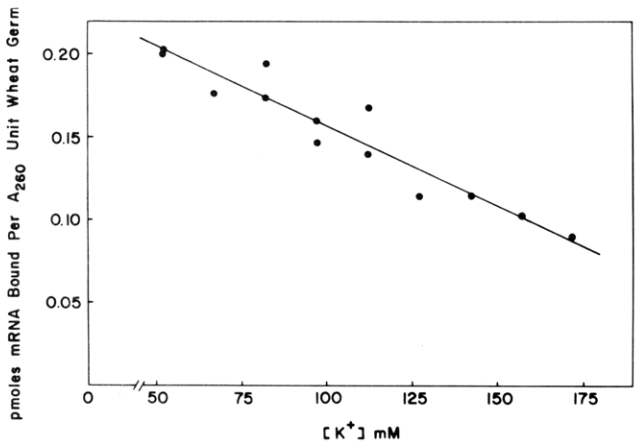


FIGURE 6: The amount of [^{125}I]ovalbumin mRNA protected in a 48S preinitiation complex was measured as a function of the potassium ion concentration in the binding reaction by 10–30% sucrose gradient analysis (see Materials and Methods). The number of picomoles of ovalbumin mRNA bound was estimated by extrapolation from the total ^{125}I counts per minute (minus background) bound in the 48S complex by assuming an average protected [^{125}I]RNA fragment size of 90 nucleotides having the same specific activity as the ovalbumin mRNA molecule. The potassium ion concentration was varied from 52 (no added potassium) to 170 mM by the addition of KOAc (see Materials and Methods).

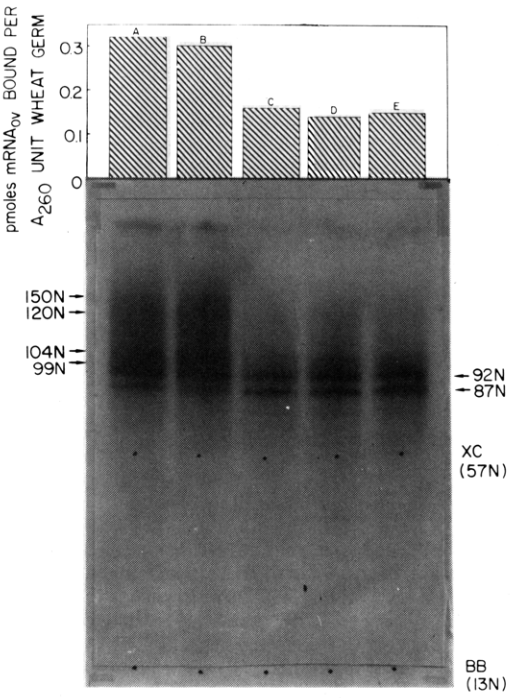


FIGURE 7: Analysis of [^{125}I]ovalbumin mRNA fragments extracted from 48S preinitiation complexes formed at two different potassium concentrations. The bar graph at the top shows the relative amounts of [^{125}I]ovalbumin mRNA protected in a 48S preinitiation complex as estimated from 10–30% sucrose gradient analysis (see Figure 6). [^{125}I]RNA extracted from each 48S complex was analyzed by electrophoresis on a 12% polyacrylamide gel (see Materials and Methods), and the results are shown below each respective bar graph. (A) 10-min binding reaction at 52 mM potassium; (B) 20-min binding reaction at 52 mM potassium; (C) 10-min binding reaction at 52 mM potassium followed by addition of KOAc to 112 mM final concentration and reaction for an additional 10 min; (D) 10-min binding reaction at 112 mM potassium; (E) 20-min binding reaction at 112 mM potassium. The size of each gel band is given in nucleotides (N), and the dye markers were as in Figure 1.

inhibit translation in the wheat germ system is directly proportional to the potassium ion concentration. We therefore decided to examine the effect of the potassium ion concentration on the formation of the 48S preinitiation complex described above and on the inhibition of 48S preinitiation complex formation by the cap analogue 7-methylguanosine 5′-phosphate. The amount of [^{125}I]mRNA$_{ov}$ bound in the 48S preinitiation complex (see Figure 1a) was measured as a function of the potassium ion concentration in the binding reaction by sucrose gradient analysis. Figure 6 shows that the amount of mRNA$_{ov}$ bound was inversely proportional to the potassium concentration used. Comparative polyacrylamide gel electrophoresis was performed on [^{125}I]RNA extracted from preinitiation complexes formed at low (52 mM) and optimal (112 mM) potassium concentrations. [^{125}I]RNA extracted from the 48S preinitiation complex formed at 52 mM potassium revealed an extensive population of protected fragments (Figure 7A). These included the 87- and 92-nucleotide fragments observed at 112 mM potassium (Figure 7E) as well as several larger fragments up to 150 nucleotides in length. Four of these fragments (87, 92, 99, and 104 N) were isolated from a polyacrylamide gel (see Materials and Methods) and digested with T1 ribonuclease. Fractionation of the T1 RNase digest of each fragment in two dimensions (see Materials and Methods) gave fingerprint patterns in which similarities were especially notable for the larger, more characteristic T1-resistant oligonucleotides (Figure 8, areas "a" and "b"). This indicated that these fragments were derived from the same general region of the mRNA$_{ov}$ molecule. Thus, it would appear that the increased mRNA$_{ov}$ binding obtained at 52 mM potassium is the result, at least in part, of more of each mRNA$_{ov}$ molecule being protected from T1 RNase digestion by the 40S ribosomal subunit. It is also apparent from this experiment that the four large, T1-resistant oligonucleotides which bind to the 40S ribosomal subunit (Figure 1b′) are not a subset of the 92-nucleotide RNA fragment (Figure 1a′).

For a test of the sensitivity of the 40S ribosomal subunit–mRNA$_{ov}$ interaction to a changing ionic environment, an experiment was also performed to determine the effect of changing the potassium concentration after binding had occurred (parts B–D of Figure 7). Increasing the potassium concentration from 52 to 112 mM after the mRNA$_{ov}$ had bound to the 40S ribosomal subunit resulted in a decrease in

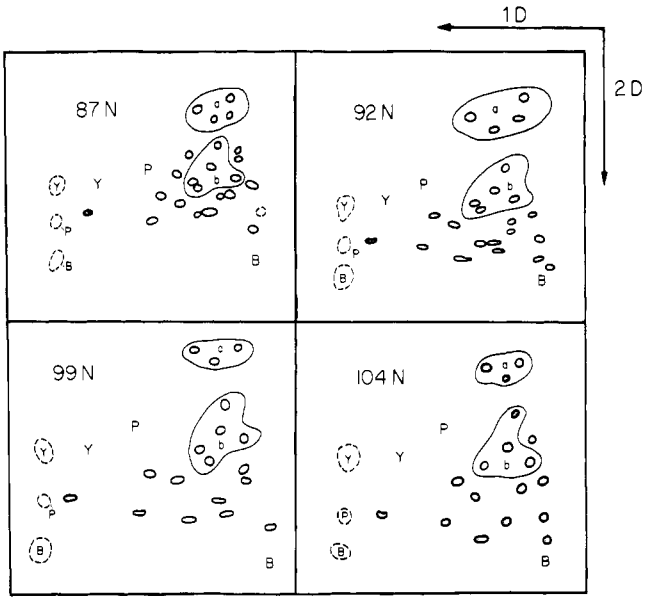


FIGURE 8: Two-dimensional fingerprint analysis of T1 RNase digested [^{125}I]RNA fragments extracted from 48S preinitiation complexes formed at 112 mM potassium (87 and 92 N) or at 52 mM potassium (99 and 104 N) and separated by polyacrylamide gel electrophoresis (see Materials and Methods). The first dimension (1D) was electrophoresis at pH 3.5 in 7 M urea; the second dimension (2D) was DEAE-cellulose thin-layer homochromatography at 60 °C. Each panel shows a schematic representation of the faint but discernible pattern of T1-resistant oligonucleotide obtained after autoradiography for 60 days. Each RNA fragment is designated by its length in nucleotides (N).

the amount of $mRNA_{ov}$ protected from T1 RNase digestion to the level normally observed at 112 mM potassium. Polyacrylamide gel electrophoresis of the [^{125}I]RNA extracted from the resulting preinitiation complex revealed a change in the pattern of 40S subunit protected fragments from the extensive population observed at 52 mM potassium to the two predominant fragments (87 and 92 N) observed at 112 mM potassium. The increase in the binding reaction time from 10 to 20 min had no effect on the observed amount or pattern of protected RNA fragments at either potassium concentration (parts B and D of Figure 7).

The ability of the cap analogue 7-methylguanosine 5′-phosphate to inhibit the binding of [^{125}I]$mRNA_{ov}$ to the wheat germ 40S ribosomal subunit is summarized in Table II. The molar ratio $m^7G^{5'}p$ (1 mM)/[^{125}I]$mRNA_{ov}$ in the binding reaction was 10^5. The minimum ratio of the cap analogue 7-methylguanosine 5′-phosphate to $mRNA_{ov}$ required to produce the partial inhibition of the ribosome binding reaction reported in Table II was not determined; however, Hickey et al. (1977) have reported that the 5′-phosphate derivative of 7-methylguanosine is a relatively less efficient inhibitor of translation when compared with its di- and triphosphate derivatives. Thus, a relatively high ratio of $m^7G^{5'}p$/mRNA might be expected to be necessary to achieve inhibition of the ribosome binding reaction. Even at this excessive ratio, however, no inhibition was observed at 52 mM potassium. Some inhibition of binding (33%) was apparent at 112 mM potassium. Interestingly, this partial inhibition could be induced by raising the potassium concentration from 52 to 112 mM after 40S subunit binding to $mRNA_{ov}$ had occurred in the presence of $m^7G^{5'}p$ and was essentially the same as that of the inhibition experiments performed directly in 112 mM potassium (33%). Gel analysis of the [^{125}I]RNA extracted from the 48S preinitiation complex formed in the presence or absence of $m^7G^{5'}p$ gave potassium-dependent patterns of protected RNA fragments identical with those described in Figure 7.

Table II: Inhibition of [^{125}I]$mRNA_{ov}$ Binding to the 40S Ribosomal Subunit by 7-Methylguanosine 5′-Phosphate

[K] (mM)	$[m^7G^{5'}p]$ (mM)	% $mRNA_{ov}$ bound	% inhibn
52	0	28	0
	0.5	31	0
	1.0	28	0
112	0	18	0
	0.5	12	33
	1.0	12	33
52 → 112[a]	1.0	11.5	35

[a] In this experiment [^{125}I]$mRNA_{ov}$ was bound to the 40S ribosomal subunit at 52 mM potassium in the presence of $m^7G^{5'}p$. KOAc was then added to a final concentration of 112 mM, and the amount of [^{125}I]RNA bound in the 48S preinitiation complex was measured by sucrose gradient analysis (see Materials and Methods).

Discussion

The formation of preinitiation complex containing ovalbumin mRNA and the 40S ribosomal subunit of the wheat germ cell-free translation system resulted in the protection, from T1 ribonuclease digestion, of a $mRNA_{ov}$ fragment containing ~92 nucleotides at the 5′ end of the molecule. This conclusion is supported by the observations of a strong preferential hybridization of this 92-nucleotide ribosome binding fragment of $mRNA_{ov}$ to DNA restriction fragments corresponding to the 5′ end of the $mRNA_{ov}$ molecule relative to those from the remainder of the molecule. A slightly smaller (~87 nucleotides) ribosome binding fragment was also observed, which appears to be similar to the 92-nucleotide fragment since it gave a similar T1 RNase fingerprint. Complete T1 RNase digestion of $mRNA_{ov}$ produced a population of T1-resistant oligonucleotides, only four of which could still bind to the 40S ribosomal subunit. Furthermore, this binding was not simply a function of size since several T1-resistant oligonucleotides of comparable size failed to bind (Figure 4). Nucleotide sequence analysis was used to identify the four T1-resistant oligonucleotides which bound to the 40S subunit and to demonstrate that they were not a subset of the 87- and 92-nucleotide RNA fragments described above but were derived from other parts of the $mRNA_{ov}$ molecule.

The amount of $mRNA_{ov}$ bound in a preinitiation complex was found to vary inversely with the potassium concentration used in the binding reaction. A more extensive population of $mRNA_{ov}$ fragments was protected from T1 RNase digestion at the lowest potassium concentration used (52 mM) than at that determined to be optimal for translation (112 mM). Fingerprint mapping of some of these RNA fragments indicated that they came from the same region of the $mRNA_{ov}$ molecule. As reported by Weber et al. (1977b, 1978) for other mRNAs, the ability of the cap analogue $m^7G^{5'}p$ to inhibit $mRNA_{ov}$ binding in a preinitiation complex was found to depend directly on the potassium concentration used.

The upper model in Figure 9 illustrates the hypothetical structure at the 5′ end of $mRNA_{ov}$. The numbered guanosine residues are the proposed sites of T1 RNase digestion which delimit the experimentally observed $mRNA_{ov}$ ribosome binding fragments relative to the 5′ end of the molecule and are within 2 nucleotides of the actual size estimates made from the gel (Figure 1a′). The 87- and 92-nucleotide RNA fragments were observed at both optimal and suboptimal potassium concentrations whereas the 99- and 104-nucleotide RNA fragments were observed only under the latter condition. All four appear,

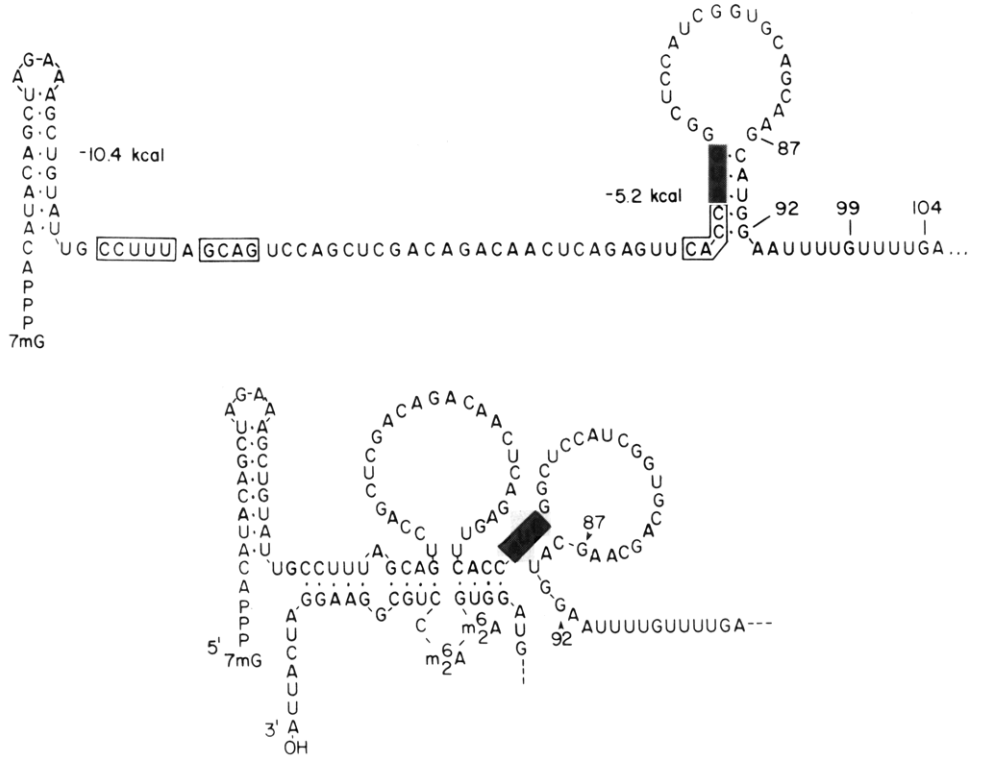


FIGURE 9: (Upper) Postulated structure at the 5′ end of ovalbumin mRNA showing hairpin structures at the 5′ end and in the region of the AUG initiation codon (shaded) with stabilization energies as estimated from Tinoco's rules (Tinoco et al., 1973). Numbers show the experimentally suggested limits of the ribosome binding fragments of $mRNA_{ov}$ and represent the distance in nucleotides from the 5′ end of $mRNA_{ov}$. (Lower) Postulated 40S ribosomal subunit–initiation factor facilitated interaction between the 3′ end of 18S rRNA and the 5′ end of ovalbumin mRNA resulting in the illustrated base pairing.

by fingerprint mapping, to be derived from the same part of the $mRNA_{ov}$ molecule. Since we have some preliminary evidence that suggests that a cap structure is protected in the preinitiation complex, the ribosome binding fragments defined in Figure 9 seem to offer the best (but not the only) representation of the experimental data. This evidence includes the following: (1) the ability of both the 87- and 92-nucleotide ribosome binding fragments of $mRNA_{ov}$ to bind to DBAE-cellulose, indicating the presence of a *cis*-diol structure; (2) the decrease in binding of these $mRNA_{ov}$ fragments in a preinitiation complex in the presence of $m^7G^5'p$ under optimal translation conditions, which may reflect competition with a cap structure; (3) the resistance of both intact $mRNA_{ov}$ and the 87- and 92-nucleotide $mRNA_{ov}$ fragments to enzymatic 5′-end labeling with ^{32}P either before or after treatment with a decapping enzyme, suggesting a common "blocking" structure and therefore an identity between the 5′ end of the $mRNA_{ov}$ molecule and the 87- and 92-nucleotide RNA fragments. It should be noted, in contrast, that the populations of T1-resistant oligonucleotides derived from $mRNA_{ov}$ were readily 5′-end labeled with ^{32}P by T4 polynucleotide kinase.

The exposure of the preinitiation complex containing $mRNA_{ov}$ and the wheat germ 40S ribosomal subunit of T1 RNase apparently does not significantly alter the nature of their interaction. Hybridization of the ribosome binding fragment of $mRNA_{ov}$ with overlapping $cDNA_{ov}$ (restriction) fragments indicates that the region of $mRNA_{ov}$ which is protected from T1 RNase digestion by the preinitiation complex contains the AUG initiation codon (Table I). Fingerprint analysis of both the 87- and 92-nucleotide $mRNA_{ov}$ fragments also shows that this protected region is highly labile to T1 RNase when not bound in a preinitiation complex. Furthermore, destruction of this region of the $mRNA_{ov}$ molecule with T1 RNase prior to binding in a preinitiation complex results in the binding of a set of T1-resistant oligonucleotides not observed when intact $mRNA_{ov}$ is used. These oligonucleotides are derived from different parts of the $mRNA_{ov}$ molecule than the 92-nucleotide ribosome binding region. Thus, the interaction of intact $mRNA_{ov}$ with the 40S subunit appears to involve a relatively high degree of specificity. This specificity may reflect a combination of (a) the presence of an AUG codon near the 5′ end of the $mRNA_{ov}$ molecule, (b) the ability of $mRNA_{ov}$ to base pair with the 3′ end of 18S rRNA, and (c) the topological conformation of the $mRNA_{ov}$ molecule.

The upper model in Figure 9 indicates that the AUG initiation codon is the first AUG codon in both the 87- and 92-nucleotide RNA fragments and is located relatively close to the 3′ end of both fragments. This was also observed for T1-resistant oligonucleotides 2, 3, and 7 (Figure 4). Since oligonucleotides 1 and 3 have similar cytosine contents, their ^{125}I specific activities should also be similar. Therefore, the apparently weak binding of oligonucleotide 1 relative to oligonucleotides 2, 3, and 7 may not reflect a difference in specific activity but rather the relatively limited ability of the 3′-terminal UUG trinucleotide to initiate protein synthesis (Stegge, 1977).

Procaryotic mRNAs generally contain a sequence of 3–9 nucleotides which are complementary to a nucleotide sequence at the 3′ end of 16S rRNA and which are located in a relatively fixed position ~10 nucleotides 5′ to the AUG initiation codon of the mRNA (Shine & Dalgarno, 1975). The importance of base pairing between this "initiation region" of procaryotic mRNAs and the 3′ end of 16S rRNA was indicated by the isolation, from an initiation complex, of an RNA–RNA hybrid between the 3′ end of 16S rRNA and the initiation region of an R17 bacteriophage mRNA (Steitz & Jakes, 1975; Steitz & Steege, 1977) and by the greatly reduced

ability of a T7 bacteriophage mRNA to direct protein synthesis after the introduction of a single base-change mutation in its initiation region (Dunn et al., 1978). The initiation of protein synthesis in eucaryotes may also involve base pairing between the 5′ end of a mRNA and the 3′ end of 18S rRNA. Although the nucleotide sequences at the 3′ ends of 16S and 18S rRNAs are very similar, the sequence at the 3′ end of 16S mRNA which base pairs with procaryotic mRNAs is absent from 18S rRNA (Alberty et al., 1978; Hagenbuchle et al., 1978). Nevertheless, there is a pyrimidine-rich sequence in the 5′ end of $mRNA_{ov}$ with the potential to base pair with the 3′ end of 18S rRNA (Figure 4). In addition, all four of the binding, as well as some of the nonbinding, T1-resistant oligonucleotides from $mRNA_{ov}$ contain similar regions of sequence complementarity to the 3′ end of 18S rRNA. This indicates that the ability to base pair with the 3′ end of 18S rRNA does not, in itself, result in binding to the 40S ribosomal subunit. Furthermore, unlike procaryotic mRNAs, the presence of these regions of potential base pairing and their position relative to the AUG codon are quite variable both in the T1-resistant oligonucleotides described in Figure 4 and in eucaryotic mRNAs in general (Baralle & Brownlee, 1978). Thus, base pairing between the 3′ end of 18S rRNA and the pyrimidine-rich sequence in the 5′ end of $mRNA_{ov}$ described in Figure 4 does not appear likely to be an obligatory feature of either binding of $mRNA_{ov}$ to the 40S ribosomal subunit or protein synthesis.

In addition to the pyrimidine-rich sequence discussed above, the upper model in Figure 9 identifies a second pyrimidine-rich region of potential base pairing with the 3′ end of 18S rRNA. This is the tetranucleotide $^{5'}CACC^{3'}$, which immediately precedes the AUG initiation codon of $mRNA_{ov}$. The lower model in Figure 9 shows base pairing between the 3′ end of 18S rRNA and both this CACC sequence and the pyrimidine-rich sequence in the 5′ end of $mRNA_{ov}$ described in Figure 4. This would result in a contraction of the 5′ end of the mRNA, drawing the capped 5′ end of the molecule closer to the AUG initiation codon by looping out the nucleotides between the base-paired regions. This combined interaction may explain the ability of the 40S ribosomal subunit to protect both the cap structure and the AUG initiation codon and may have a facilitative role in "highly read" mRNAs such as ovalbumin and globin, which contain both potential base-pairing regions. Base pairing of the 3′ end of 18S rRNA with the CACC sequence preceding the AUG initiation codon of $mRNA_{ov}$ would also result in destabilization of the hairpin stem containing the AUG initiation codon and the juxtaposition of this codon with a $^{3'}AUG^{5'}$ trinucleotide in 18S rRNA. Although this four-base pairing may not be necessary for $mRNA_{ov}$ binding to the 40S ribosomal subunit (especially since the four T1-resistant oligonucleotides which bind to the 40S ribosomal subunit do not have it), it may be necessary to free the AUG initiation codon from structural constraints and to properly position it in the 40S ribosomal subunit for the efficient initiation of protein synthesis. This possibility is further suggested by the observation that the potential for base pairing between the tetranucleotide preceding the AUG initiation codon and the $^{3'}GUGG^{5'}$ sequence at the 3′ end of 18S rRNA appears to be a feature of many of the eucaryotic cellular and viral mRNAs [see, for example, Baralle & Brownlee (1978)].

The upper model in Figure 9 illustrates two possible hairpin structures, one at the 5′ end of $mRNA_{ov}$ (McReynolds et al., 1978) and the other in the region of the AUG initiation codon and involving the base pairing of this codon in the hairpin stem. We have also suggested an alternative hairpin structure of similar stabilization energy for this region of the $mRNA_{ov}$ molecule (Kuebbing & Liarakos, 1978). The existence of these hairpins in the $mRNA_{ov}$ molecule seems likely given their estimated stabilization energies and the ionic environment of the cell. Like the $mRNA_{ov}$ itself (Van et al., 1976, 1977), the ribosome binding fragments of $mRNA_{ov}$ may contain some helical structure in the form of hairpins since they bind to hydroxylapatite under conditions in which single-stranded DNA fails to bind (Table I). It is also interesting that the guanosine residues which are 87 and 92 nucleotides from the 5′-terminal adenosine are at either end of a hairpin stem. The resulting symmetrical placement of T1-labile bonds on either side of this hairpin stem may explain the apparently equal probability of obtaining an 87- or 92-nucleotide ribosome binding fragment from $mRNA_{ov}$. On the other hand, this hairpin stem may be destabilized by base pairing with the 3′ end of 18S rRNA as suggested above. This would free these guanosine residues from the constraints of the hairpin structure and result in their (equal) lability to T1 RNase.

As with other eucaryotic mRNAs (Kozak, 1978), intact $mRNA_{ov}$ has only one site to which the 40S ribosomal subunit will bind. Disruption of the $mRNA_{ov}$ molecule by T1 RNase digestion destroys this functional ribosome binding site but generates four T1-resistant oligonucleotides which can also bind to the 40S ribosomal subunit. Thus, all four T1-resistant oligonucleotides represent potential ribosome binding sites at different locations (Figure 5) within the $mRNA_{ov}$ molecule. Even though T1 RNase digestion of a preinitiation complex containing intact $mRNA_{ov}$ results in the generation of these T1-resistant oligonucleotides, they were never observed bound to the 40S ribosomal subunit when intact $mRNA_{ov}$ was used. This suggests that the binding of these T1-resistant oligonucleotides to the 40S ribosomal subunit is similar in nature to that of the functional ribosome binding site. Furthermore, the failure of the wheat germ 40S ribosomal subunit to recognize potential binding sites other than the functional one at the 5′ end of intact $mRNA_{ov}$ may reflect the nature of the recognition process. The potential ribosome binding sites presented by the four T1-resistant oligonucleotides could be "hidden" by the $mRNA_{ov}$ structure itself (as, e.g., is the case with polycistonic $Q\beta$ RNA; Weissman, 1974). Alternatively, the 40S ribosomal subunit may slide the 5′ end of a RNA molecule and stop at the first AUG codon in order to initiate protein synthesis (Kozak, 1978). This mechanism would also explain the binding of the T1-resistant oligonucleotides described above to the 40S ribosomal subunit. In either case, the topology of the $mRNA_{ov}$ molecule could be very important in the recognition, by the 40S ribosomal subunit, of the functional ribosome binding site at the 5′ end of a mRNA molecule. For example, Laycock (1978) has reported that enzymatic recapping of decapped β-globin mRNA is not sufficient to restore its original translational efficiency. Rather, the recapped β-globin has to be heat denatured and renatured by slow cooling before translational activity is restored to the level of native β-globin mRNA. This may represent the restoration of the proper mRNA conformation for either direct recognition by or "sliding on" of the 40S ribosomal subunit. Likewise, the increased size of the ribosome binding fragments of $mRNA_{ov}$ obtained by lowering the potassium concentration may reflect a change in $mRNA_{ov}$ conformation, resulting from secondary structure destabilization as well as an increased affinity for basic proteins such as eucaryotic initiation factors. Interestingly, this effect could be reversed by increasing the potassium concentration to optimal translation levels after binding $mRNA_{ov}$ to the 40S ribosomal subunit. The ability

of $m^7G^5'p$ to partially inhibit this binding could also be induced by increasing the potassium concentration after binding had occurred. This apparently reflects a high degree of sensitivity of the protein–RNA interaction in the preinitiation complex to the ionic environment.

Finally, it is interesting that the noncoding sequence at the 5′ end of $mRNA_{ov}$ is not represented by a continuous sequence in the genomic DNA but is interrupted by an intervening sequence between mRNA nucleotides 45 and 46 (Catterall et al., 1978). The ribosome binding site, as defined by the wheat germ 40S ribosomal subunit, must therefore be "constructed" by the processing of the 5′-noncoding sequence into the final $mRNA_{ov}$ product. Thus, the inclusion and retention of the noncoding sequence at the 5′ end of $mRNA_{ov}$ may reflect either a direct functional role in the initiation of protein synthesis or a more indirect role, perhaps as the site of $mRNA_{ov}$ capping. In any case, further studies will be required to precisely define the interaction of the ribosome with $mRNA_{ov}$ and the role of $mRNA_{ov}$ conformation in this interaction.

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