Isolation and Characterization of Initiation Fragments from Lens 10S and 14S α-Crystallin Messenger Ribonucleic Acids†

John H. Chen and Abraham Spector*

ABSTRACT: The α-crystallin 10S and 14S messenger ribonucleic acids (mRNAs) for the B and A chains, respectively, were isolated from calf lenses. Initiation complexes were formed with both mRNAs after which the unprotected regions were digested with ribonuclease T_1. A single fragment of approximately 45 nucleotides was obtained from both the 10S and 14S mRNAs. The fragments retained the ability to reform initiation complexes under standard conditions. Two-dimensional fractionation of ribonuclease T_1 digests indicated considerable similarity between the 10S and 14S fragments. However, marked differences in the (U)G region were observed. The addition of the methylating agent *S*-adenosyl-L-methionine to the mRNA initiation system increases complex formation from two to five times, suggesting that methylation may be required for initiation.

Recent investigation of the mechanism for the initiation of protein synthesis suggests that this process is similar in both prokaryotes and eukaryotes (Lengyel and Söll, 1969; Heywood, 1970). The first step involves the formation of an initiation complex containing a ribosome, the initiator $tRNA^{fMet}$ and mRNA. On the basis of present information, the binding of mRNA in this tertiary complex occurs only at its initiation site (Kondo et al., 1968; Lengyel and Söll, 1969). This binding specificity provides the basis for experiments to isolate the initiation region(s) of the mRNA. Such initiation sequences would be protected from the ribonuclease degradation by the ribosome complex (Takanami et al., 1965) but permit the digestion of the remainder of the mRNA.

With bacteriophage RNAs, the specificity of the initiation complex has been established by isolating the mRNA binding regions and elucidating their nucleotide sequence and relating them to the amino acid sequences of the phage proteins (Steitz, 1969; Gupta et al., 1970; Staples and Hindley, 1971; Min Jou et al., 1972; Adams et al., 1972; Pieczenik et al., 1974). These studies indicated that the ribosome-protected fragments clearly contain the initiation sites of the mRNAs as well as unique untranslated sequences at the 5′ end of the initiator AUG codon (Hindley and Staples, 1969; Steitz and Jakes, 1975). Recently work on the ribosome binding site of a plant virus RNA utilizing a wheat germ ribosomal system has also been reported (Dasgupta et al., 1975). However, the initiation binding sites of mammalian mRNAs remain obscure. Recent progress in the isolation of such mRNAs and the development of in vitro translation systems now has made such studies feasible.

α-Crystallin is a major structural protein of the mammalian lens. It consists of two different polypeptide chains, A and B, held together by noncovalent forces in large macromolecules (Spector et al., 1971; Bloemendal et al., 1972). In the bovine lens, the A and B chains have molecular weights of approximately 2×10^4 and a sequence homology of approximately 67% (van der Ouderaa et al., 1974). The mRNAs for the A and B chain have been isolated and characterized (Berns et al., 1971; Chen et al., 1974). A 10S mRNA codes for the B_2 chain and a 14S species for the A_2 chain (Mathews et al., 1972). The 3′-terminal region of both 10S and 14S mRNAs contains poly(rA)-rich segments of approximately 50 residues (Lavers et al., 1974) which are required for reverse transcription but do not effect translation significantly (Chen et al., 1975). The availability of these mRNAs which code for polypeptides of known sequence makes them attractive for investigation of the initiation regions.

The lens also contains an unique system for regulating the synthesis of particular proteins. With the transformation of the epithelial cells of the lens to fibers, the synthesis of α-crystallin is dramatically increased (Delcour and Papaconstantinou, 1972). However, the ratio of 2A to 1B chain (Stauffer et al., 1973) appears to remain constant. Among the possible mechanisms for regulating this amplification and the relative rates of synthesis of the α-crystallin polypeptides may be the chemistry of the initiation site.

The 5′ end of a number of viral (Furuichi and Miura, 1975; Wei and Moss, 1975; Furuichi et al., 1975a; Urushibara et al., 1975) and cellular mRNAs (Rottman et al., 1974; Furuichi et al., 1975b; Lavi and Shatkin, 1975) has been shown to contain methylated nucleosides which are essential in the translation process (Both et al., 1975a). Moreover, methylation of Reovirus mRNA utilizing SAM has been shown to be involved in the formation of initiation complex (Both et al., 1975b).

In this communication, the isolation and characterization of the putative initiation sites of both 10S and 14S α-crystallin mRNAs are reported. Preliminary observations of the effect of SAM on formation of the initiation complex is also included.

Materials and Methods

Oligo(dT)-cellulose was obtained from Collaborative Research Inc. Iodine-125 (low pH, ~17 Ci/mg), [*methyl*-^{3}H]-L-methionine, (100 Ci/mmol), [^{32}S]-L-methionine, (300 Ci/mmol), and [*methyl*-^{3}H]SAM[1] (8.82 Ci/mmol) were pur-

† From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received August 2, 1976.* Supported by grants from the National Eye Institute, National Institutes of Health.

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate; T_1-RNase, ribonuclease T_1; SAM, *S*-adenosyl-L-methionine; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

chased from the New England Nuclear Co. Purified $tRNA^{fMet}$ from rat liver was produced by the Biopolymer Co. T_1-RNase and actinomycin D were obtained from the Sigma Chemical Co. Yeast tRNA and 16S and 23S ribosomal RNAs from *Escherichia coli* were purchased from Miles Laboratory. Sparsomycin and fusidic acid were generously made available by Dr. L. Skogerson of Columbia University. Standard ^{32}P-labeled oligonucleotide markers were obtained from Dr. S. Weissman of Yale University. Ribosomes (80S) and 40S ribosomal subunits were isolated from the Ehrlich ascites S-30 fraction while α-crystallin subunits A_1, A_2, B_1, and B_2 were prepared from calf lenses as previously described (Stauffer et al., 1973).

Isolation of α-Crystallin mRNAs. The initial isolation of α-crystallin mRNAs was carried out as described previously (Chen et al., 1974). The isolated mRNAs were further fractionated on a linear 15–30% sucrose gradient in 0.05 M Tris-HCl buffer, pH 7.4 (buffer A). Sedimentation was performed at 38 000 rpm for 20 h at 4 °C in a SW 41 rotor after which the 10S and 14S regions were isolated and concentrated. Standard RNA markers, *E. coli* 16S and 23S rRNAs, and yeast tRNA were run in parallel gradients. The peak 10S and 14S positions were individually pooled, ethanol precipitated, and dissolved in 0.1 M Tris-HCl, pH 7.5, containing 0.5 M NaCl (buffer B). This sedimentation and isolation process was then repeated two to three times until a clear resolution of the individual sedimentation peaks was obtained. The mRNAs were applied to a 2.5 × 2.0 cm oligo(dT)-cellulose column which had been previously equilibrated with buffer B. After all the unbound RNAs were removed with buffer B, the bound mRNAs were eluted with H_2O. The mRNA fractions were pooled, precipitated with 0.3 M LiCl in 75% ethanol, and then redissolved in buffer B and recycled through the oligo(dT)-cellulose column two more times. Then, the oligo(dT)-cellulose-purified mRNAs were refractionated through another 15 to 30% sucrose gradient as described above. The resultant 10S and 14S lens mRNAs peaks from the gradient were used for the subsequent studies.

In Vitro Protein Synthesis. In vitro protein synthesis directed by the lens 10S and 14S mRNAs was conducted in both Ehrlich ascites and wheat germ cell-free translation systems. In the Ehrlich ascites system, the growth of the cell culture and the preparation of the S-30 extract were performed as previously described (Gupta et al., 1973). An Ehrlich ascites S-30 extract provided by Dr. S. Gupta of the Sloan-Kettering Institute for Cancer Research was also used. The Ehrlich ascites polysomal extracts were passed twice through a column of Sephadex G-25 (medium) equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 80 mM KCl, 4 mM magnesium acetate, and 2 mM dithiothreitol. The reaction mixture for amino acid incorporation in a total volume of 50 μl contained 25 mM Tris-HCl, pH 7.5, 90 mM KCl, 4 mM magnesium acetate, 1 mM ATP, 0.5 mM CTP, 0.1 mM GTP, 2 mM dithiothreitol, 5 mM phosphenolypyruvate, 0.4 mg/ml pyruvate kinase, 19 unlabeled common amino acids, 40 μmol each; 5 μl of [^{35}S]-L-methionine (300 Ci/mmol), and 15 μl of polysomal extract (~12 mg/ml proteins). In most cases mRNAs were added to the incubation mixture at a concentration of 30 μg/ml. The reactions were incubated at 37 °C for 75 min in glass test tubes.

The wheat germ cell-free translation system was prepared according to the method of Marcu and Dudock (1974) except for the following modifications. The 16 000-rpm supernatant was layered onto a 10-ml Sephadex G-25 (fine) syringe column. The G-25 column was prepared by equilibration with 20 mM Hepes buffer, pH 7.6, containing 120 mM KCl, 5 mM magnesium acetate, and 1 mM dithiothreitol and centrifuging the column two times at 2000 rpm at 4 °C. Centrifugation of the loaded column was then carried out for 5 min at 2000 rpm in the cold. The resultant extract was routinely used for protein synthesis assays according to the method of Roberts and Patterson (1973). Reactions were incubated at 22 °C for 90 min. For measuring amino acid incorporation into protein, 0.5 ml of 5% CCl_3COOH containing 0.1% methionine was added to each sample and the resulting suspensions were heated at 100 °C for 10 min. The precipitates were collected on Millipore filters (HA 0.45 μm), washed with 5% CCl_3COOH, twice in 95% ethanol, and finally with acetone. The radioactivity was measured in Brays solution. For large scale ^{35}S-labeled preparations, volumes of 200 μl were usually utilized.

The processing of the samples and urea-polyacrylamide gel electrophoresis were performed as described previously (Lavers et al., 1974).

Preparation of ^{125}I-Labeled α-Crystallin mRNAs. Iodination of mRNAs was performed by previously described methods (Chen et al., 1975). After iodination and immediately before the ribosome binding assay, the iodinated mRNA were centrifuged through a 15–30% sucrose gradient to remove any small RNA fragments.

Preparation of [^{3}H]-Met-$tRNA^{fMet}$. The reaction mixture for the preparation of [^{3}H]-Met-$tRNA^{fMet}$ contained the following components: 100 mM sodium cacodylate buffer, pH 7.2, 10 mM magnesium acetate, 10 mM KCl, 1 mM ATP, pH 7.0, 0.1 ml of $tRNA^{fMet}$ (4.2 mg/ml), 5 mM [^{3}H]-L-methionine (100 Ci/mmol), 0.05 ml of methionine (100 μg/ml), and 50 μg of crude supernatant protein from *E. coli* Q13. Incubation was carried out at 37 °C for 15 min. The reaction was stopped by addition of 0.1 ml of 2 M sodium acetate buffer, pH 4.5, containing 20% sodium dodecyl sulfate and 1 ml of water-saturated phenol. The phenol phase was extracted with 0.05 M sodium acetate (pH 5.0) and 5 mM magnesium acetate. The combined aqueous phase was then dialyzed against 0.02 M sodium acetate (pH 5.0) for 12 h.

Binding of [^{125}I]mRNA and [^{3}H]-Met-$tRNA^{fMet}$ to Ribosome. The ribosome binding assay was conducted in a total volume of 50 μl containing: 40 mM Tris-acetate buffer, pH 7.5, 80 mM potassium acetate, 3 mM magnesium acetate, 1 mM GTP, 100 pM Met-$tRNA^{fMet}$, 4 mM dithiothreitol, 3.4 A_{260} units of crude ribosomes, and 1.5 μg of α-crystallin 10S or 14S mRNA. In some binding assays, 200 μM sparsomycin, 1.2 mM puromycin, or 1.6 mM fusidic acid was added. Depending on the binding system, [^{3}H]-Met-$tRNA^{fMet}$ or [^{125}I]mRNAs were employed. Unless stated otherwise, incubations were carried out at 37 °C for 4 min.

For Millipore binding assays, the reactions were stopped by addition of 1 ml of cold 40 mM Tris-acetate buffer, pH 7.5, containing 100 mM NH_4Cl and 10 mM $MgCl_2$. The resultant suspensions were chilled and filtered through a 0.45-μm nitrocellulose filter. The filters were washed three times with 5 ml of the above buffer and then dissolved in Bray's solution for the measurement of radioactivity. In some experiments, at the termination of the incubation the reaction mixture was immediately layered on top of a 6 to 21% linear sucrose gradient in 0.05 M Tris-HCl (pH 7.5) buffer containing 8 mM magnesium acetate and 100 mM NH_4Cl. Centrifugation was performed in a SW 41 rotor at 38 000 rpm for 3.5 h. The gradients were collected in 20–22 fractions of about 0.55 ml. The absorbancy at 260 nm and radioactivity were measured as before.

Isolation of Initiation Fragments and Characterization by

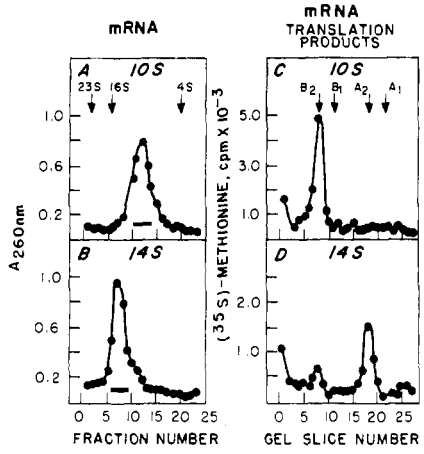

FIGURE 1: Purification of α-crystallin mRNAs and characterization of translation products. Sucrose gradient centrifugation profiles of 10S and 14S mRNA (A and B). Gel electrophoretograms of translation products from 10S and 14S mRNA (C and D). Arrows in A, which apply to B as well, indicate standard RNA markers of yeast tRNA and 16S and 23S ribosomal RNAs from *E. coli*. Arrows in C represent authentic A_1, A_2, B_1, and B_2 chains of α-crystallin and also apply to D. In vitro protein synthesis programmed by α-crystallin mRNAs was conducted in Ehrlich ascites cell-free system as described in Materials and Methods. Similar results were also obtained with a cell-free system from wheat germ.

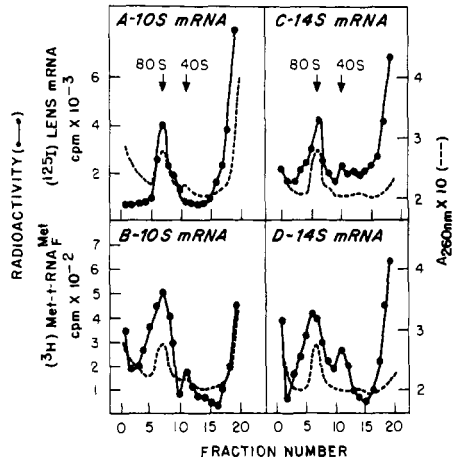

FIGURE 2: Sucrose gradient centrifugation profiles of initiation complexes. Details of initiation complex formation and the centrifugation procedure are described in Materials and Methods. Incubations were carried out at 37 °C for 4 min in the presence of 200 μM of sparsomycin. In A and C, 10S and 14S [^{125}I]mRNAs were respectively used to detect complex formation. In B and D, [^{3}H]-Met-tRNA^{fMet} was used as the label. Arrows in A and C indicate the sedimentation peak positions of 80S ribosomes and the 40S ribosomal subunit. The markers have a similar position in B and D.

Polyacrylamide Gel Electrophoresis. The binding conditions and centrifugation procedure are as described in Materials and Methods except that, before layering on the sucrose gradient for centrifugation, 200 units of T_1-RNase was added to the reaction mixture and incubated in the ice bath for 10 min. The radioactive fractions from the 80S region, containing the ribosome-protected mRNA initiation segments, were pooled and the RNAs were extracted with phenol. The extracted RNAs were analyzed by electrophoresis through a 10% polyacrylamide gel in 3.3 mM Tris buffer containing 30 mM diethylbarbituric acid, pH 8.5, in the presence of 7 M urea (Lavers et al., 1974).

Two-Dimensional Fractionation of the mRNA Initiation Fragments by T_1-RNase Digestion. The ^{125}I-initiation fragments were digested with T_1-RNase with an enzyme-to-substrate ratio of 1:20. The reactions were carried out in a sealed micropipet at 37 °C for 30 min. The digests were then fractionated on a two-dimensional high-voltage electrophoretic system, using cellulose acetate, pH 3.5, in 7 M urea, for the first dimension and DEAE-7% formic acid for the second. The electrophoretograms were autoradiographed by using Kodak Cronex 4 film. Other details of the fractionation procedure have been described previously (Gupta et al., 1970). The radioactivity in the electrophoretograms was determined in a Packard Auto γ spectrometer.

Results

Purification of α-Crystallin in mRNAs and Characterization of Translation Products. Lens polysomes were isolated from young calf lens homogenates as described previously (Chen et al., 1974). After repeated fractionation through a sucrose gradient, the mRNA species were chromatographed on an oligo(dT)-cellulose column as described in the Materials and Methods section. Purified poly(rA)-rich 10S and 14S mRNAs were further fractionated by centrifugation through a linear 15–30% sucrose gradient as shown in Figures 1A and 1B. It is evident that essentially all of the oligo(dT)-cellulose-purified mRNAs sedimented as discrete 10S and 14S species with no apparent contamination of small- or large-sized components. Both the 10S and 14S α-crystallin mRNAs have also been analyzed by 7 M urea polyacrylamide gel electrophoresis and on sucrose gradients containing 70% formamide. Such experiments confirm that both 10S and 14S mRNA preparations contain one discrete species.

Translation of the 10S and 14S mRNAs was carried out in an Ehrlich ascites S-30 system as previously described (Graziadei and Lengyel, 1972). The translation products were analyzed by urea polyacrylamide gel electrophoresis. The 10S mRNA preparation synthesized a polypeptide chain having the same electrophoretic mobility as that of the α-crystallin B_2 chain (Figure 1C), while the major translation product produced by the 14S mRNA preparation migrated to the α-crystallin A_2 region (Figure 1D). A minor peak with the same mobility as the B_2 chain was also observed with the 14S mRNA template. Similar results were also obtained with a cell-free system from wheat germ.

Demonstration of the Initiation Complex Formation. The initiation complex was formed by incubating Met-tRNA^{fMet}, GTP, α-crystallin 10S or 14S mRNA, and the Ehrlich ascites ribosomal system at 37 °C for 4 min. In these experiments from 50- to 100-fold excess of ribosomes and tRNA^{fMet} were used. Demonstration of [^{3}H]-Met-tRNA^{fMet} and [^{125}I]-mRNA binding to the ribosomes was determined by centrifugation of the incubation mixture through sucrose gradients. Figure 2 shows the radioactivity and optical density distribution patterns utilizing the different labeled components. In all cases, a binding complex in the 80S region was observed. Figures 2A and 2C represent the sedimentation profiles obtained with [^{125}I]-α-crystallin 10S and 14S mRNA. Approximately 10–15% of the total radioactivity sedimented at the 80S region in such experiments. Figures 2B and 2D utilize [^{3}H]-Met-tRNA^{fMet} as the label. In these experiments the binding ratio of [^{3}H]-Met-tRNA^{fMet} to mRNAs is approximately 1 with both 10S and 14S mRNAs. The A_{260nm} and radioactivity profiles are similar in the 80S region. In some cases, a minor radioactive peak in the 40S region was also observed. Chain elongation was inhibited by introducing sparsomycin, puromycin, or fusidic acid in some experiments. No change in the results was observed under such conditions.

TABLE I: Binding of [^{125}I]mRNAs to Ribosomes.[a]

	[^{125}I]mRNA Ribosomal Binding			
	10S mRNA		14S mRNA	
	cpm	%	cpm	%
1. Complete system	7450	100	13326	100
2. RNase preincubation of mRNAs[b]	987	13	1158	9
3. −Met-tRNA^{fMet}	1022	14	1453	11
4. −GTP and −Met-tRNA^{fMet}	674	9	489	4
5. −GTP and +GDPCP[c]	1960	26	4044	30

[a] Conditions of the binding assay are given in Materials and Methods. At the termination of incubation, the reaction mixtures were layered on a linear 6–21% sucrose gradient. After centrifugation at 38 000 rpm for 3.5 h, the 80S regions were pooled and the radioactivity was measured. mRNAs were used at a concentration of 30 μg/ml and contained 100 000 cpm. Puromycin (1.2 mM) was added to the reaction mixtures to prevent elongation. [b] Preincubation of mRNAs was carried out with 15 μg of RNase A at 37 °C for 10 min. The incubations were then phenol extracted at pH 9.0 and the remaining mRNAs were isolated for assay. Controls were treated in a similar manner. [c] GDPCP concentration was 1 mM.

TABLE II: Binding of [^{3}H]-Met-tRNA^{fMet} to Ribosomes.[a]

	[^{3}H]-Met-tRNA^{fMet} Binding	
	10S mRNA (pM)	14S mRNA (pM)
Complete system	4.5	6.2
−mRNA	0.3	0.2
−GTP	0.8	0.9

[a] Details of Millipore binding assay are described in Materials and Methods. Incubations were performed at 37 °C for 10 min. At the end of the incubation 1 ml of cold 25 mM Tris-acetate buffer, pH 7.5, containing 100 mM NH_4Cl and 10 mM $MgCl_2$ was added to the reaction mixtures. [^{3}H]-Met-tRNA^{fMet} (100 pM) was employed (specific activity, 1600 cpm/pmol).

Requirements for Initiation Complex Formation. The binding of α-crystallin mRNAs to 80S ribosomes in initiation complexes was determined by incubating the labeled mRNA under a number of conditions and then isolating the 80S ribosomal binding complexes by sucrose gradients as previously shown in Figure 2. When all components required for initiation complex formation were present, considerable radioactivity was found in the 80S region for both 10S and 14S mRNAs (Table I). Preincubation of the mRNAs with ribonuclease reduced the binding to 13 and 9% of the controls for the 10S and 14S mRNAs, respectively. If Met-tRNA^{fMet} was omitted from the incubation mixture, the radioactivity in the 80S complex region was decreased to about 11–14% of the control level. Omission of GTP as well as Met-tRNA^{fMet} caused an even greater reduction in mRNA ribosomal binding.

Supportive evidence for the formation of the initiation complex was obtained utilizing 80S ribosomes washed with 1 M KCl. Complex formation was only observed when the ribosomal wash was added to the incubation mixture. Under such conditions the preparation gave similar sucrose gradient sedimentation profiles to that obtained with unwashed ribosome preparations.

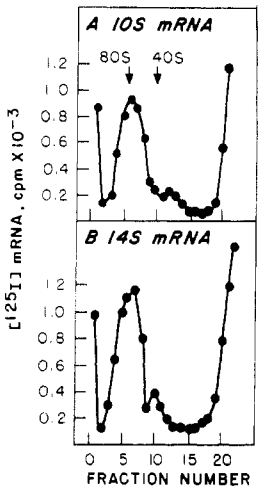

FIGURE 3: Sucrose gradient profiles of 10S and 14S mRNA binding complexes following T_1-RNase digestion. The same binding conditions as described in the legend of Figure 2 were used, except that 200 units of T_1-RNase was added to the reaction mixture. After 10 min at 0 °C the preparations were layered on sucrose gradients. See Materials and Methods for further information.

In order to study the GTP requirement for the formation of the initiation complex, GDPCP, an analogue of GTP, was tested for its ability to substitute for GTP in the binding of mRNA to the ribosome. As shown in Table I only 26 to 30% of the binding activity remained, suggesting GTP is specifically required for optimal formation of the initiation complex.

Studies with [^{3}H]-Met-tRNA^{fMet} were also carried out to specifically follow the binding of this constituent in the initiation complex. Complex formation was determined by measuring the retention of [^{3}H]-Met-tRNA^{fMet} on Millipore filters. The binding to ribosomes is dependent on mRNA as shown in Table II. Omission of GTP resulted in a five- to six-fold decrease in the binding. Similar results were obtained with both 10S and 14S α-crystallin mRNAs.

Isolation of the Initiation Fragment. The mRNA initiation fragment was isolated by introducing T_1-RNase after the usual 4-min, 37 °C incubation for forming the initiation complex. Elongation was prevented by the addition of an inhibitor such as puromycin or sparsomycin. The reaction mixture was then centrifuged through a sucrose gradient. As shown in Figure 3, with both 10S and 14S [^{125}I]mRNAs, the radioactivity profile gave a prominent peak in the 80S region. Such results indicate that the labeled polynucleotide fragments are still associated with the ribosomes. The amount of radioactivity in the 80S region represents approximately 1% of the total initial count. The radioactive fractions, containing the ribosome-protected polynucleotide fragments, were pooled and then extracted with phenol, releasing the initiation fragments for further analyses.

Characterization of the Isolated Initiation Fragment. The released fragments were dissolved and characterized by determining their size on urea-polyacrylamide gel electrophoresis and testing their ability to bind ribosomes under the usual conditions. Figure 4 shows the electrophoretic pattern obtained with 10S and 14S binding fragments. A single binding fragment was observed with each mRNA preparation. Furthermore, the fragments arising from both the 10S and 14S mRNA have similar sizes of about 45 nucleotide residues. Thus it appears that the ribosomes are able to protect oligonucleotide

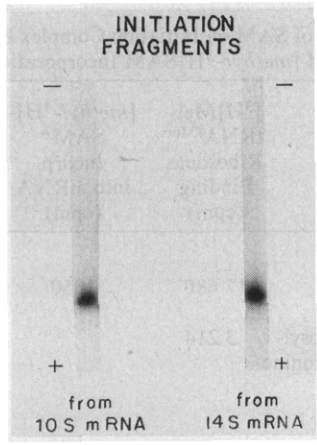

FIGURE 4: Electrophoretic pattern of 10S and 14S initiation fragments isolated from initiation complexes pooled from sucrose gradient 80S regions (See Figure 3). The labeled fractions were ethanol precipitated and redissolved in the electrophoretic running buffer. Electrophoreses were run in 10% polyacrylamide gels in the presence of 7 M urea.

TABLE III: Binding of the Initiation Fragments to Ribosomes.[a]

	^{125}I Initiation Fragment Ribosomal Binding			
	10S		14S	
	cpm	%	cpm	%
Millipore assay[a]				
Complete system	1415	75	806	90
−Met-tRNA^{fMet}	247	13	110	12
Sucrose gradient[b]				
Complete system	876	46	544	60

[a] Binding conditions were essentially the same as described for the mRNA Millipore retention assay, except that the magnesium acetate concentration was 4 mM. Incubations were carried out for 10 min at 37 °C. [b] Binding conditions are as described in Materials and Methods.

sequences of such length from T_1-RNase digestion. Such results are in general agreement with previous observations obtained with ribosome-protected initiation fragments of bacteriophage RNAs (Steitz, 1969; Gupta et al., 1970) and of a plant virus RNA (Dasgupta et al., 1975).

The ^{125}I-labeled fragments were further tested for their ability to reform binding complexes with ribosomes utilizing the Millipore filter method. From Table III, it is evident that the fragments do bind to the ribosomes and are highly dependent upon the addition of Met-tRNA^{fMet}. Under such conditions most of the radioactivity appears to bind to the ribosomes. Thus these fragments not only possess ribosome binding activity but also retain their dependence upon Met-tRNA^{fMet}. These results suggest that the fragments continue to retain sufficient structural information for binding to specific ribosome sites and appear to require the usual initiation cofactors. Examination of the reformed complex by sucrose gradients indicates, however, that some of the Millipore binding may be involved with degraded ribosomal components.

Sequence Analysis of the Isolated Fragment. Preliminary sequence studies of the isolated ^{125}I-initiation fragments suggest considerable similarity. Two-dimensional fractionation of T_1-RNase digests of the labeled fragments (Figure 5) shows closely related sequence complexity. Seventeen oligonucleotide

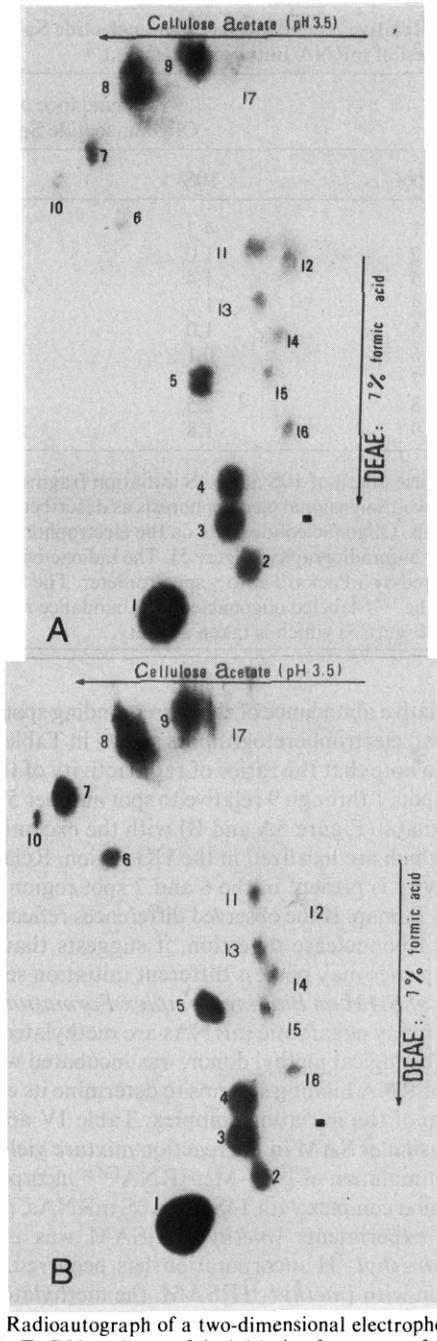

FIGURE 5: Radioautograph of a two-dimensional electrophoretic fractionation of a T_1-RNase digest of the initiation fragments of α-crystallin mRNAs. (A) The 10S initiation fragment; (B) the 14S initiation fragment; (■) electrophoretic position of xylene cyanol. The ribonuclease-protected initiation fragments preparations were obtained from the 80S region of a sucrose gradient as described in the legend in Figure 3. Without further purification, the preparations were digested with T_1-RNase and fractionated according to Sanger's procedure (Sanger et al., 1965). Electrophoreses were performed first on cellulose acetate membrane strips (Schleicher and Schuell, Inc.) at pH 3.5 in 7 M urea and in the second dimension on DEAE-cellulose paper at pH 1.7. It should be noted that the photographs only give a qualitative picture of the radioactivity in the various zones. The spots were cut out and counted for quantitative evaluation. See Table IV and the text for further information.

regions can be detected on the autoradiographs with similar relative positions for the 10S and 14S initiation fragments. The delineated areas were cut out, and their radioactivities were determined. Regions 10 through 17 are probably caused by contaminants and frayed ends. Such a conclusion is based on the relative abundance of cytosine and/or little radioactivity in these areas relative to zones 1 through 9.

TABLE IV: Relative Abundance of Oligonucleotide Spots from T_1-RNase Digest of mRNA Initiation Fragment.[a]

Spot	Rel Abundance of Oligonucleotide Spot	
	10S	14S
1	4.3	4.5
2	1.0	0.9
3	1.4	1.2
4	1.2	1.1
5	1.0	1.0
6	0.1	0.5
7	0.2	1.0
8	2.5	2.4
9	1.8	2.1

[a] T_1-RNase digest of 10S and 14S initiation fragments were separated by two-dimensional electrophoresis as described in Materials and Methods. Oligonucleotide spots on the electrophoretogram were localized by autoradiography (Figure 5). The radioactivity of the spots was measured by a Packard auto γ spectrometer. The results are expressed as the ^{125}I-labeled oligonucleotide abundance relative to spot number 5 (Figure 5) which is taken as unity.

TABLE V: Effect of SAM on Initiation Complex Formation and Demonstration of [*methyl*-^{3}H]SAM Incorporation into mRNAs.[a]

	[^{3}H]Met-tRNA^{fMet} Ribosome Binding (cpm)	[*methyl*-^{3}H]-SAM[b] Incorp into mRNA (cpm)	Molar Ratio[c] [^{3}H]Methyl/ mRNA
A. 10S mRNA			
Complete system	17 680	1850	0.11
−*S*-Adenosyl-L-methionine-	3 214		
B. 14S mRNA			
Complete system	11 290	1143	0.15
−*S*-Adenosyl-L-methionine-	5 836		

[a] For measuring the [^{3}H]Met-tRNA^{fMet} binding the Millipore retention method was employed as detailed in Materials and Methods. SAM (50 μM) was neutralized with 1 M Tris buffer before use in the standard system. [b] For the incorporation assay, [*methyl*-^{3}H]SAM (5 μM) was used with the complete system. At the end of the incubation, the mRNAs were reisolated by sequential phenol extraction at pH 7.0 and pH 9.0. The aqueous phase of the pH 9.0 extraction was passed through an oligo(dT)-cellulose column (2.0 × 2.5 cm) and the mRNA-associated radioactivity was measured. [c] Molar ratios were calculated on the basis of the following molecular parameters: specific activity of [*methyl*-^{3}H]SAM, 8.82 Ci/mmol; molecular weight, 10S mRNA 2.5×10^5 and 14S mRNA 5.1×10^5 (Berns et al., 1974).

The relative abundance of the corresponding spots 1 through 9 in the two electrophoretograms is shown in Table IV. It is of interest to note that the ratios of radioactivity of the oligonucleotide spots 1 through 9 relative to spot number 5 are similar for both maps (Figure 5A and B) with the exception of spots 6 and 7 which are localized in the UG region. Relatively little radioactivity is present in the 6 and 7 spot region of the 10S fragment T_1 map. If the observed differences reflect no artifact from the ribonuclease digestion, it suggests that these two mRNA species may possess different initiation sequences.

Effect of SAM on Binding Complex Formation. Since the 5′ end of many eukaryotic mRNAs are methylated, SAM, an effective biological methyl donor, was incubated with the 10S and 14S mRNA binding systems to determine its effect on the formation of the initiation complex. Table IV demonstrates that inclusion of SAM in the reaction mixture yields a two- to fivefold stimulation of [^{3}H]-Met-tRNA^{fMet} incorporation into the initiation complex with 14S and 10S mRNAs, respectively. In some experiments [*methyl*-^{3}H]SAM was used to test whether *methyl*-^{3}H incorporation has occurred. Following incubation with [*methyl*-^{3}H]SAM, the methylated mRNAs were reisolated by phenol extraction and oligo(dT)-cellulose chromatography. The radioactivity associated with the mRNA was measured. As shown in Table V a significant incorporation of the label into the mRNA macromolecules was found, with a *methyl*-^{3}H/mRNA ratio of 0.11 and 0.15 for 10S and 14S mRNA, respectively. These results suggest that the Ehrlich ascites S-30 system contains the necessary components to support the methylation of mRNAs and that methylation of mRNA may be of importance in the formation of the initiation complex.

Discussion

From previous work, it is known that α-crystallin mRNAs isolated from sucrose gradient or oligo(dT)-cellulose are still contaminated with other RNA components (Chen et al., 1974). Therefore in this work extensive purification was employed to eliminate extraneous RNA. On the basis of the data, both the 10S and 14S mRNAs appear physically homogeneous; however, the 14S mRNA continues to direct the synthesis of a minor component in addition to the A_2 polypeptide. This minor component has an electrophoretic mobility similar to that of the B_2 polypeptide. While further work is required to identify this fraction, it is possible that the 14S mRNA may also be coding for a B_2 polypeptide. Other observations from our laboratory also suggest that the 14S mRNA may be a bicistronic messenger.[2] Preliminary sequence studies of the 10S and 14S mRNAs indicate marked similarity between the two species and hybridization studies utilizing the 14S mRNA and cDNA synthesized from the 10S mRNA suggest that 2 mol of cDNA binds to 1 mol of the mRNA. Such observations further support the possibility that the 14S mRNA contains two cistrons. Because of the very considerable homology between the A_2 and B_2 chains (van der Ouderaa et al., 1974), it cannot be ruled out that the 14S mRNA is homocistronic containing two copies of the A_2 message and that the putative B_2 polypeptide produced by the 14S mRNA preparation arises as a result of a minor contaminating mRNA.

The ribosome protected binding fragments appear to be uniform in size containing approximately 45 residues and appear to have similar sequence complexity. However, the low level of radioactivity in the spots 6 and 7 region of the two-dimensional 10S initiation fragment T_1-RNase digest suggests a structural difference in the initiation sequences. Further work will be required in order to understand this observation.

These experiments also indicate the similarity in the initiation complex formation programmed by phage and mammalian mRNAs. Both systems form complexes requiring ribosomes, mRNA, initiator tRNA, and GTP. Recent observations also suggest that in the mammalian system Met-tRNA^{fMet} forms a complex with a 40S ribosome subunit as a precursor to the 80S initiation complex (Schreier and Stae-

[2] Chen, J. H., and Spector, A. (1976), manuscript in preparation.

helin, 1973). As shown in Figure 1, in some experiments a minor 40S Met-tRNA^{fMet} complex was observed. However, the significance of this observation in the present system is unclear.

GDPCP is a poor substitute for GTP in these experiments, suggesting that GTP hydrolysis may be required for formation of the initiation complex. This observation is somewhat similar to that reported with myosin mRNA. In that system an initiation complex could not be formed when GTP was replaced with GDPCP (Heywood, 1970), although in the lens system from 26 to 30% of the binding could still be obtained under such conditions.

Within the past 2 years methylated guanosine has been found at the 5′ terminal end of a number of eukaryotic mRNAs (Urushibara et al., 1975; Rottman et al., 1974; Furuichi et al., 1975b; Lavi and Shatkin, 1975). It has also been reported that the chemical removal of this modified nucleoside from rabbit globin mRNA resulted in a reduction of protein synthesis (Muthukrishnan et al., 1975). Methylation is necessary for the Reovirus mRNA to form initiation complexes (Both et al., 1975b). On the basis of such observations, the effect of SAM was tested in the lens α-crystallin mRNA system and found to markedly stimulate initiation complex formation. The data indicate that methylation of mRNA may have occurred. However, the relatively low level of presumptive methylation suggests that either the mRNAs are already methylated or that, for some unknown reason, there are only a few specific sites available for reaction. The very considerable stimulation of initiation complex formation caused by the presumptive incorporation of only 0.1 to 0.15 mol of methyl groups per mol of mRNA suggests that methylation of mRNA in the 5′-terminal region may be of importance in regulating protein synthesis.

Acknowledgments

We express our appreciation to Dr. Mon-Li Chu and Ms. Ta-Wen Chen for their helpful technical assistance with part of this work. We are also grateful to Dr. S. Gupta for his assistance with preparations of the Ehrlich ascites system.

References

Adams, J. M., Spahr, P. F., and Cory, S. (1972), *Biochemistry 11,* 976.

Berns, A. J. M., De Abreu, R. A., van Kraaikamp, M., Benedetti, E. L., and Bloemendal, H. (1971), *FEBS Lett. 18,* 159.

Berns, A. J. M., Janssen, P., and Bloemendal, H. (1974), *Biochem. Biophys. Res. Commun. 59,* 1157.

Bloemendal, H., Berns, A. J. M., Zweers, A., Hoenders, H., and Benedetti, E. L. (1972), *Eur. J. Biochem. 24,* 401.

Both, G. W., Banerjee, A. K., and Shatkin, A. J. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1189.

Both, G. W., Furuichi, Y., Muthukrishnan, S., and Shatkin, A. J. (1975b), *Cell 6,* 185.

Chen, J. H., Lavers, G. C., and Spector, A. (1973), *Biochem. Biophys. Res. Commun. 52,* 767.

Chen, J. H., Lavers, G. C., and Spector, A. (1975), *Biochim. Biophys. Acta 418,* 39.

Chen, J. H., Lavers, G. C., Spector, A., Schutz, G., and Feigelson, P. (1974), *Exp. Eye Res. 18,* 189.

Dasgupta, R., Shih, D. S., Saris, C., and Kaesberg, P. (1975), *Nature (London) 256,* 624.

Delcour, J., and Papaconstantinou, J. (1972), *J. Biol. Chem. 247,* 3289.

Furuichi, Y., and Miura, K.-I. (1975), *Nature (London) 253,* 374.

Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 362.

Furuichi, Y., Morgan, M., Shatkin, A. J., Jelinek, W., Salditt-Georgieff, M., and Darnell, J. E. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1904.

Graziadei, III, W. D., and Lengyel, P. (1972), *Biochem. Biophys. Res. Commun. 46,* 1816.

Gupta, S. L., Chen, J. H., Schaefer, P., Lengyel, P., and Weissman, S. M. (1970), *Biochem. Biophys. Res. Commun. 39,* 383.

Gupta, S. L., Sopori, M. L., and Lengyel, P. (1973), *Biochem. Biophys. Res. Commun. 54,* 777.

Heywood, S. M. (1970), *Nature (London) 225,* 696.

Heywood, S. M., and Thompson, W. C. (1971), *Biochem. Biophys. Res. Commun. 43,* 470.

Hindley, J., and Staples, D. H. (1969), *Nature (London) 224,* 964.

Kondo, M., Eggerston, G., Eisenstadt, J., and Lengyel, P. (1968), *Nature (London) 220,* 368.

Lavers, G. C., Chen, J. H., and Spector, A. (1974), *J. Mol. Biol. 82,* 15.

Lavi, S., and Shatkin, A. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2012.

Lengyel, P., and Söll, D. (1969), *Bacteriol. Rev. 33,* 264.

Marcu, K., and Dudock, B. (1974), *Nucleic Acids Res. 1,* 1385.

Mathews, M. B., Osborn, M., Berns, A. J. M., and Bloemendal, H. (1972), *Nature (London), New Biol. 236,* 5.

Min Jou, W., Haegeman, G., Ysebaert, M., and Fiers, W. (1972), *Nature (London), New Biol. 237,* 82.

Muthukrishnan, S., Both, G. W., Furuichi, Y., and Shatkin, A. J. (1975), *Nature (London) 255,* 33.

Pieczenik, G., Model, P., and Robertson, H. D. (1974), *J. Mol. Biol. 90,* 191.

Roberts, N. E., and Paterson, B. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2330.

Rottman, F., Shatkin, A. J., and Perry, R. P. (1974), *Cell 3,* 197.

Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965), *J. Mol. Biol. 13,* 373.

Schreier, M. H., and Staehelin, T. (1973), *Nature (London), New Biol.* 242, 35.

Spector, A., Li, Lu-Ku, Augusteyn, R. C., Schneider, A., and Freund, T. (1971), *Biochem. J. 124,* 337.

Staples, D. H., and Hindley, J. (1971), *Nature (London), New Biol. 234,* 211.

Stauffer, J., Li, Lu-Ku, Rothschild, C., and Spector, A. (1973), *Exp. Eye Res. 17,* 329.

Steitz, J. A. (1969), *Nature (London) 224,* 957.

Steitz, J. A., and Jakes, K. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4734.

Takanami, M., Yan, Y., and Jukes, T. H. (1965), *J. Mol. Biol. 12,* 761.

Urushibara, T., Furuichi, Y., Nishimura, C., and Miura, K.-I. (1975), *FEBS Lett. 49,* 385.

van der Ouderaa, F. J., deJong, W. W., Hilderink, A., and Bloemendal, H. (1974), *Eur. J. Biochem. 49,* 157.

Wei, C.-M., and Moss, B. (1975), *Proc. Nat. Acad. Sci. U.S.A. 72,* 318.