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A Fluorescence Study of the Binding of Eucaryotic Initiation Factors to Messenger RNA and Messenger RNA Analogues†

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ABSTRACT: The binding of the eucaryotic polypeptide chain initiation factors (eIFs) 4A, 4B, and 4F to poly(1,N^6-ethenoadenylic acid) [poly(εA)] was investigated by fluorescence spectroscopy. Competition experiments allowed us to determine the relative affinity of these proteins for mRNA cap analogues and the triplets AUG, GUG, UUU, UAA, and UGA. The salt dependence of eIF-4A binding to poly(εA) and mRNA suggested that the binding was largely electrostatic and was enhanced in the presence of Mg^{2+} and ATP. The size of the binding site of eIF-4A, eIF-4B, and eIF-4F on poly(εA) was approximately 13, 25, and 35 nucleotides, respectively. Fluorescence studies with the cap analogue 7-methylguanosine triphosphate as well as competition studies with poly(εA) provide further evidence for a direct interaction of eIF-4F with the cap region. There was no evidence that either eIF-4B or eIF-4A bound the mRNA cap directly. In contrast to the other two factors, eIF-4B was found to bind preferentially to AUG, and of all the triplets tested, AUG was the most effective competitor for poly(εA) binding.

The interaction of the polypeptide chain initiation factors with mRNA is an important step in the initiation of protein synthesis. This interaction involves the recognition of various structural features of the message including the 7-methylguanosine cap at the 5′ mRNA terminus, the secondary structure, and the AUG initiation codon. Several proteins have been implicated in this process, including polypeptide chain initiation factor 4A (eIF-4A),[1] eIF-4B, and eIF-4F (Grifo et al., 1983; Benne & Hershey, 1978). Recent work has described the activity of these proteins with respect to recognition of the structural features of the mRNA (Shatkin, 1976; Kozak, 1982; Boss et al., 1981; Lomedico & Andrew, 1982; Butler & Clark, 1984; Goss et al., 1985) and changes in the secondary structure of the message induced by these factors (Ray et al., 1985). Butler and Clark (1984) reported that eIF-4B from wheat germ binds to the AUG initiation codon region of satellite tobacco necrosis viral (STNV) messenger RNA. In addition, Ray et al. (1985) reported that eIF-4A has an ATP-dependent mRNA unwinding activity and this activity was more efficient when eIF-4A was part of the eIF-4F complex. It was also suggested that eIF-4F may be a RNA unwinding enzyme which catalyzes the melting of mRNA secondary structure (Sonenberg et al., 1982; Lee et al., 1983;

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[1] Abbreviations: poly(εA), poly(1,N^6-ethenoadenylic acid); eIF, eucaryotic polypeptide chain initiation factor; CD, circular dichroism; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IF-3, procaryotic polypeptide chain initiation factor 3; STNV, satellite tobacco necrosis virus; Tris, tris(hydroxymethyl)aminomethane; AMPPNP, 5′-adenylyl imidodiphosphate.

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Edery et al., 1984; Ray et al., 1985).

It is still not clear which factors recognize specific features of messenger RNA. The purpose of this study is to characterize further how specific initiation factors interact with specific regions of mRNA by monitoring directly the interaction of eIF-4A, eIF-4B, and eIF-4F with fluorescently labeled nucleotides. Competition experiments allowed us to determine the relative affinity of these proteins for mRNA cap analogues and RNA codon triplets. These methods allow direct measurements of the binding of single proteins to RNA without possible perturbing effects of nitrocellulose membrane filtration or nuclease digestion. These protein–RNA binary interactions provide an indication of which factors are responsible for recognition of particular features of the mRNA. By examining the problem in this manner, together with subsequent studies with the initiation factors added in combination, we may be able to formulate a more precise sequence of events in the process of polypeptide chain initiation.

Our data indicate that eIF-4A does not have a strong specificity for either the cap or the AUG regions of mRNA whereas eIF-4B preferentially binds AUG or GUG. Of the three initiation factors tested, only eIF-4F binds well to cap analogues. These data further suggest that individual proteins have distinct binding specificities.

Materials and Methods

Buffer A contained 20 mM HEPES–KOH, pH 7.8, 1 mM dithiothreitol, and magnesium acetate and potassium chloride as indicated. Poly(1,N^6-ethenoadenylic acid) was purchased from P-L Biochemicals. Adenosine triphosphate, 5′-adenylylimidodiphosphate, guanosine triphosphate, and 7-methylguanosine triphosphate were products of P-L Biochemicals.

Polypeptide chain initiation factors were purified from the 0.5 M KCl wash of rabbit reticulocyte polysomes. The buffers used in the preparation of the initiation factors were similar to buffer A, except that 20 mM Tris-HCl, pH 7.5, was used instead of the HEPES–KOH (Mehta et al., 1983; Dratewka-Kos et al., 1984). The ribosomal salt wash was applied to a phosphocellulose column equilibrated with buffer containing 150 mM KCl (Grifo et al., 1983). The fraction not adsorbed was used for the preparation of eIF-4A, and eIF-4B and eIF-4F were purified from the fraction that eluted with buffer containing 450 mM KCl. The fraction containing eIF-4A was precipitated by 70% saturated ammonium sulfate and dialyzed against buffer containing 100 mM KCl. eIF-4A (M_r 46 000) was purified to apparent homogeneity by two successive gradient elutions from Mono Q (Pharmacia) at room temperature. The eIF-4A eluted as a sharp peak at 240 mM KCl. eIF-4B and eIF-4F were further purified by centrifugation through a sucrose gradient containing 0.5 M KCl (Grifo et al., 1983). These factors were separated by chromatography on a Bio-Gel A-0.5m column (1.5 × 65 cm) equilibrated with buffer containing 0.5 M KCl. The fraction containing eIF-4B was purified by gradient elution from Mono Q (eluted at 205 mM KCl). eIF-4F was applied to DEAE-cellulose in buffer containing 100 mM KCl and eluted stepwise by 300 mM KCl. The eIF-4B (M_r 78 000) and eIF-4F (M_r 200 000, 46 000, and 26 000) preparations did not contain any detectable amounts of the other factor as determined by Coomassie Blue R-250 staining of a dodecyl sulfate/polyacrylamide gel (10%, 5–8 g of protein/gel) (Laemmli, 1970), as well as their activity in stimulating in vitro protein synthesis in extracts of VSV-infected L cells (Dratewka-Kos et al., 1984). On the basis of these analyses, we estimated the preparations of eIF-4B and eIF-4F were 65–85% pure. These preparations are similar in purity to those reported elsewhere (Dratewka-Kos et al., 1984; Ray et al., 1986). Preparations with differing levels of impurity, but the same activity, show identical binding results. The molarity of the solutions was calculated from the total protein concentration after subtracting the amount of protein impurity determined from dodecyl sulfate/polyacrylamide electrophoresis.

Fluorescence measurements were performed in a specially designed spectrophotometer in which the excitation light source was a Liconix 4100 He–Cd laser with an output of 2 mW at 325 nm. Temperature was controlled by circulating thermostated water through a brass cuvette holder. Data were collected by a Zenith Z-100 computer using software and interfacing by On-Line Instrument Systems.

Fluorescence titrations were performed in buffer A by adding 2–5-μL aliquots of the factors to an initial volume of 200 μL. All fluorescence titrations were corrected for dilution of the sample in the course of the titration. The contribution to the observed emission intensity of each individual component in a multicomponent sample was determined by parallel control experiments performed on the same day as the titration. For KCl titrations, such as Figure 2, in some cases small amounts of solid KCl were added to raise the salt concentration at 100–250 mM to verity the dilution correction.

The binding of initiation factors to poly(ϵA) was assumed to be the binding of a ligand to a homogeneous polymer. The measured fluorescence intensity (F_M) is the average fluorescence intensity of free poly(ϵA) (F_0) and poly(ϵA)·eIF (F_B). The observed fluorescence is analogous to the ribosome–triplet binding described by Goss et al. (1984). Where X is the experimental fraction of poly(ϵA) bound, then $F_M = XF_B + (1 - X)F_0$ and

$$X = [\text{poly}(\epsilon\text{A})\cdot\text{eIF}] / [\text{poly}(\epsilon\text{A})]_T$$

The binding of proteins to poly(ϵA) was analyzed according to the equations derived by McGhee and von Hippel (1974).

The concentration of free protein (L) can be calculated by subtracting the amount of bound protein from the total protein added. The concentration of base residues for solutions of poly(ϵA) (ϵ^{mM} = 16 A_{260} units) or mRNA [ϵ^{mM} = 20 A_{260} units] was determined from absorbance measurements. The value ν is the average number of moles of bound protein per mole of total nucleotides. Plots of ν/L vs. ν were used to calculate K_{obsd} and the cooperativity constant, ω, using the site size n, which was determined from saturation plots (see Figure 6). The cooperativity constant, ω, is the (unitless) equilibrium constant for the process of moving a bound ligand from an isolated site to a singly contiguous site or from a singly to a doubly contiguous site. The value of ω can be >1 (ligands attract), <1 (ligand repel), or zero (no interaction). Data were fit with a Fletcher and Powell (1963) sum of squares minimization for the best values of K and ω.

The data for competitive binding of mRNA and poly(ϵA) with initiation factors can be treated as an extension of eq 15 of McGhee and von Hippel (1974). However, we cannot independently determine the site size. Therefore, there is an insufficient number of separately measurable parameters to determine K and ω for factor binding to globin message. If one assumes that n and ω are the same for poly(ϵA) and mRNA, then using K_1 determined independently, the total concentrations of mRNA and poly(ϵA), and the initiation factor, one can determine K_2, the equilibrium constant for mRNA binding. The implications of these assumptions are discussed later.

Circular dichroism spectra were recorded by using a Jobin–Yvon Mark V circular dichrograph interfaced to an Apple

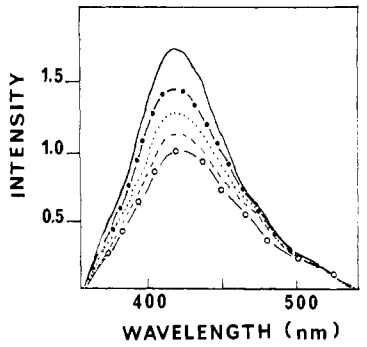

FIGURE 1: Emission spectra (excitation at 325 nm) for poly(εA) with varying amounts of eIF-4A. The concentration of poly(εA) was 3.7 μM in base residues, and the concentrations of eIF-4A were 0.0 (O–O), 0.03 (---), 0.1 (●–●), and 0.25 μM (—). The samples were in buffer A.

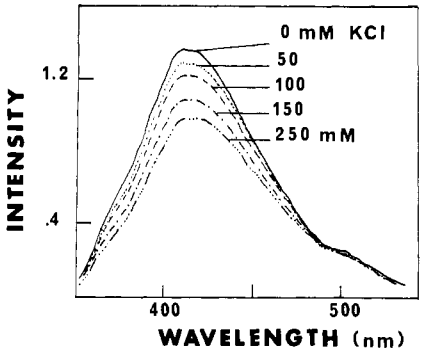

FIGURE 2: Emission spectra (excitation at 325 nm) for poly(εA) in the presence of eIF-4A. The concentration of poly(εA) was 3.9 μM in base residues, and the concentration of eIF-4A was 0.5 μM. The spectra show the decrease in fluorescence intensity after additions of KCl to give the final concentrations indicated on the figure. The y axis is the fluorescence intensity (arbitrary units).

IIe microcomputer with a fast arithmetic processor.

RESULTS

The fluorescence emission and absorption spectra of poly(εA) were found to be essentially identical with those reported previously (Janik et al., 1973; Schmidt et al., 1985). In the absence of KCl, the emission spectrum resulting from excitation of poly(εA) at 325 nm was a single band with a maximum at 406 nm. With increasing levels of eIF-4A, there was a gradual enhancement of the poly(εA) fluorescence (Figure 1 and Figure 6, left panel). Upon saturation of poly(εA) with eIF-4A, the net fluorescence enhancement was 1.7-fold, and in the presence of 4 mM Mg^{2+}, this fluorescence enhancement was approximately 1.2-fold.

The titration of poly(εA) with eIF-4A produced a definite end point, thereby allowing a determination of the number of bases bound by each protein molecule (n), or more precisely the lattice occupancy of the factor. The site size was found to be 13 ± 2 nucleotide residues per eIF-4A.

When eIF-4A was added to poly(εA) in buffer containing 150 mM KCl, the fluorescence amplitude immediately after adding eIF-4A was comparable to that expected in buffer without KCl. However, within 2–3 min, the intensity decreased to a lower value. No further decrease was observed for periods up to 3 h. For all titrations performed in the presence of KCl, the fluorescence spectrum was not recorded until at least 5 min after the factor was added. The increase in poly(εA) fluorescence upon eIF-4A binding was less in the presence of high salt (150 mM KCl). Similarly, when poly(εA) was titrated to saturation with eIF-4A in the absence of salt, the subsequent addition of KCl caused a reduction in the fluorescence intensity (Figure 2). At 250 mM KCl, the fluorescence intensity was reduced to the same level as in the absence of eIF-4A. Identical results were obtained when NaCl was used as the monovalent cation. Blank titrations of poly(εA) with KCl in the absence of eIF-4A indicated a 10% change in fluorescence intensity, in agreement with other reports (Toulme & Helene, 1980).

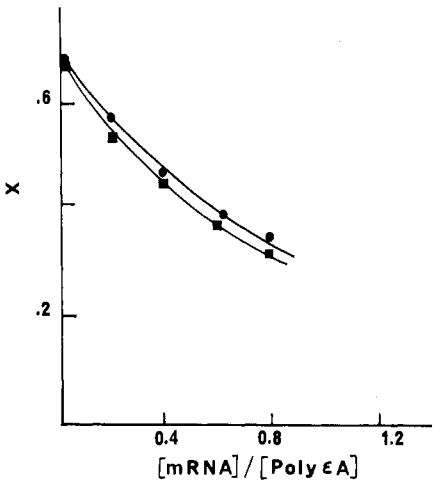

FIGURE 3: Effect of [KCl] on the competitive binding of mRNA and poly(εA) with eIF-4A. The vertical axis is the fraction (X) of poly(εA) with eIF-4A bound as defined in the text. The abscissa is the ratio of moles of mRNA nucleotide/moles of poly(εA) nucleotide. The circles and squares represent the data for competition in buffer A with 0 and 150 mM KCl, respectively. The solid lines represent the calculated curves (see text for details).

Table I: Equilibrium of eIF-4A with Poly(εA) and Globin mRNA

	$K \times 10^6$ M	n^a	ω
(A) eIF-4A + poly(εA)			
no KCl	0.2	13 ± 2	22 ± 5
150 mM KCl	9	13 ± 2	21 ± 5
150 mM KCl, 4 mM Mg^{2+}	8	12 ± 3	27 ± 6
150 mM KCl, 4 mM Mg^{2+}, 2 mM ATP	3	14 ± 3	23 ± 5
(B) eIF-4A + globin mRNA			
no KCl	0.6		
150 mM KCl	2.4		

[a] n for poly(εA) was determined from saturation titrations (see text); for mRNA, n and ω were assumed to be the same as for poly(εA), and K was determined from least-squares analysis. Errors in K were ±10% of the value given (1 standard deviation). Values for the equilibrium constant are for $K_{dissociation}$.

To determine if eIF-4A had the same affinity for binding to poly(εA) and natural mRNA, competition experiments were performed with rabbit globin mRNA (Collaborative Research). Figure 3 shows the competition of natural mRNA with poly(εA) at 0 and 150 mM KCl. Samples of poly(εA) were titrated to the same level of saturation at 0 and 150 mM KCl. The experiment at 150 mM KCl required increased concentration of initiation factor. If mRNA binding were not also inhibited by KCl, it would compete much better for eIF-4A relative to poly(εA) at high KCl concentration. These results demonstrate that eIF-4A binding to both poly(εA) and natural mRNA is inhibited by increased KCl concentrations.

Equilibrium constants (K), site size (n), and the cooperativity constant (ω) for eIF-4A binding to poly(εA) and mRNA are summarized in Table I. In the presence of 150 mM KCl and 4 mM Mg^{2+}, the addition of 1–2 mM ATP increased the binding affinity of eIF-4A to poly(εA) 2.5-fold. The affinity of eIF-4A for either globin RNA or poly(εA) was reduced by the addition of 150 mM KCl. Both ATP and the nonhydrolyzable ATP analogue AMPPNP further increased the

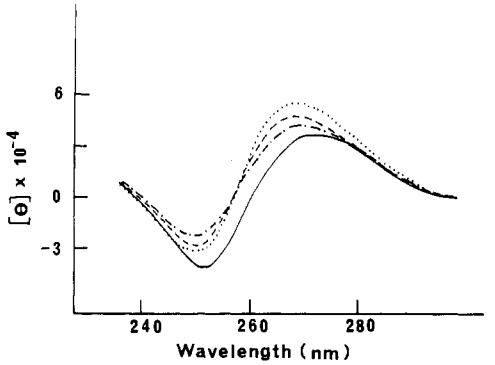

FIGURE 4: Effect of eIF-4A and ATP on the CD spectrum of poly(A) in buffer A with 100 mM KCl. The ratios of eIF-4A to nucleotide are 0 (···), 0.02 (---), 0.09 (-·-), and 0.09 with 0.04 mM ATP present (—). The total absorbance for all samples was less than 1.8. The temperature was 23 °C.

Table II: Binding of eIF-4B and eIF-4F with Poly(ϵA) and Globin mRNA

	$K \times 10^7$ M	n	ω
(A) eIF-4B + poly(ϵA)			
no KCl	6	25 ± 5	16 ± 4
150 mM KCl	24	24 ± 4	18 ± 3
150 mM KCl, 4 mM Mg^{2+}, 2 mM ATP	10	23 ± 2	17 ± 2
(B) eIF-4B + globin mRNA			
no KCl	8		
150 mM KCl	6		
(C) eIF-4F + poly(ϵA)			
no KCl	2	35 ± 5	32 ± 7
150 mM KCl	12	25 ± 5	30 ± 6
150 mM KCl, 4 mM Mg^{2+}, 2 mM ATP	6	30 ± 5	34 ± 5
(D) eIF-4F + globin mRNA			
no KCl	3		
150 mM KCl	4		

[a] Values and errors in n, K, and ω were determined as described in Table I and the text.

fluorescence intensity of poly(ϵA) resulting from eIF-4A binding. The binding of eIF-4A to poly(ϵA) was increased 2.5-fold in the presence of ATP independent of the effect of $[Mg^{2+}]$ (Table I).

Circular dichroism (CD) spectra were recorded for eIF-4A binding to poly(A). Poly(A) was used because the CD bands occur in a spectral region free of contributions from the protein. The CD spectra (Figure 4) showed changes consistent with eIF-4A causing disruption of the poly(A) base stacking at a physiologically relevant salt concentration. The presence of ATP enhanced this effect. The concentration of ATP was 40 μM, so that high absorbance of UV light was avoided. A background spectrum of ATP was subtracted from the poly A/eIF-4A/ATP spectra; ATP did not cause a change in the poly(A) spectrum in the absence of eIF-4A.

Experiments similar to those described in Table I were performed for the binding of eIF-4B or eIF-4F to poly(ϵA) and globin mRNA. Saturation of poly(ϵA) with eIF-4B in the absence of KCl resulted in a 1.8-fold enhancement of fluorescence (Table II). For eIF-4F, this enhancement was 2.1-fold. The presence of 4 mM Mg^{2+} and 150 mM KCl did not significantly change the maximum fluorescence enhancement attainable. In the absence of KCl, both eIF-4B and eIF-4F had similar affinities for poly(ϵA) and globin message. However, in the presence of 150 mM KCl, the affinity of each factor for the synthetic nucleotide, but not for mRNA, was reduced 4–6-fold. ATP enhanced the binding about 2-fold at 150 mM KCl. This suggests that these factors recognize base-specific residues by nonionic bonding. The site

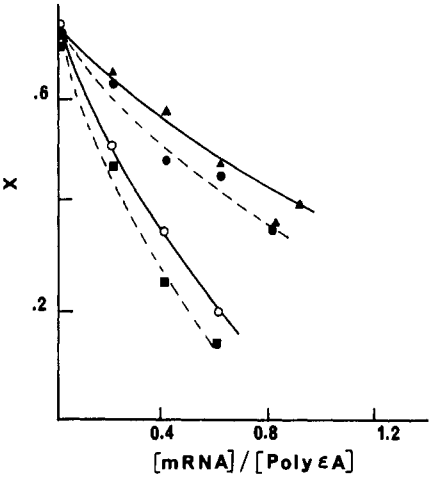

FIGURE 5: Effects of [KCl] on the competition of globin mRNA and poly(ϵA) with eIF-4B or eIF-4F. The triangles and squares show the data for eIF-4F in buffer A with 0 and 150 mM KCl, respectively. The closed circles and open circles depict the data for eIF-4B in buffer A with 0 and 150 mM KCl, respectively. The solid lines are the calculated curves.

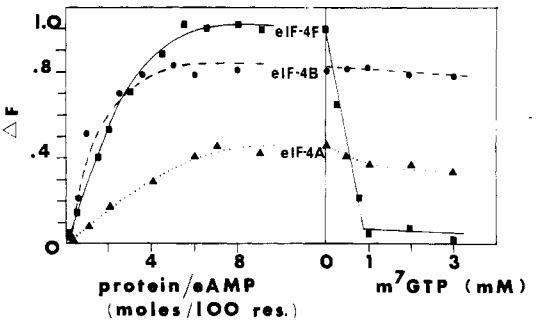

FIGURE 6: Binding of initiation factors to poly(ϵA). Titrations were carried out in 20 mM HEPES buffer, pH 7.6, 100 mM KCl, and 4 mM Mg^{2+} with 2 mM ATP added. Poly(ϵA) concentration was 4 μM in base residues. The ordinate represents the fluorescence intensity change observed for protein binding (normalized to the change obtained with eIF-4F). The left panel shows the increase in fluorescence with additions of initiation factors. The right panel depicts the effects of increasing m^7GTP concentrations. The protein concentration was 0.4 μM.

size for eIF-4B and eIF-4F was 25 ± 5 and 35 ± 5 bases, respectively.

Competition experiments with globin mRNA similar to those performed with eIF-4A and poly(ϵA) were performed. Globin mRNA competes effectively with poly(ϵA) for binding either eIF-4B or eIF-4F. At 150 mM KCl, the globin message competes more effectively than poly(ϵA) for binding to either factor (Figure 5), as also suggested by the data in Table II.

To assess further the specificity of binding of the initiation factors, experiments were carried out with the cap analogue m^7GTP and several nucleotide triplets (AUG, UUU, GUG, UAA, and AGU). Figure 6 shows the effect of adding m^7GTP to the initiation factor–poly(ϵA) complex. m^7GTP competes effectively with poly(ϵA) only for eIF-4F binding as demonstrated by the fluorescence intensity decrease. There is a very slight decrease in eIF-4A binding at high levels of m^7GTP. The addition of similar concentrations of GTP did not alter poly(ϵA) fluorescence for any of the proteins. For all of these experiments, the proteins were titrated separately with m^7GTP, and the fluorescence intensity at 406 nm was subtracted.

The cap analogue m^7GTP is fluorescent when excited at 325 nm and has an emission peak at 390 nm. Addition of eIF-4F caused an increase in m^7GTP fluorescence. Because of the relatively weak fluorescence intensity of m^7GTP, equilibrium

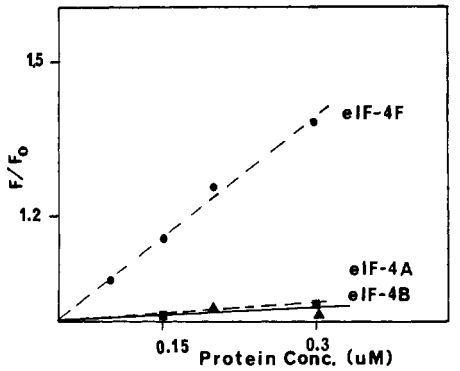

FIGURE 7: Change in intrinsic fluorescence intensity of m^{7}GTP (1 mM) upon addition of initiation factors. F_0 is the fluorescence intensity in the absence of added protein. Titrations were carried out in buffer A (100 mM KCl). The circles, squares, and triangles depict the data for additions of eIF-4F, eIF-4A, or eIF-4B, respectively.

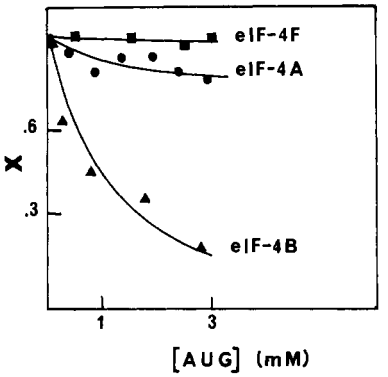

FIGURE 8: Effect of nucleotide triplet, AUG, on the binding of eIF-4A, eIF-4B, and eIF-4F to poly(ϵA). The y axis is the fraction of poly(ϵA) with initiation factor bound. The protein concentrations were 0.72–0.80 μM, and the poly(ϵA) concentration was 12 μM in base residues. The titrations were performed in buffer A (100 mM KCl) at 23 °C.

constants for m^{7}GTP–eIF-4F association could not be obtained. Figure 7 shows qualitatively the fluorescence intensity change for eIF-4F, eIF-4A, and eIF-4B additions to solutions of m^{7}GTP. Only eIF-4F caused a significant increase in fluorescence intensity consistent with the competition experiments.

The nucleotide triplet AUG and also other nucleotide triplets were tested for competitive binding with poly(ϵA). Figure 8 shows the decrease in fluorescence on addition of nucleotide triplets for poly(ϵA) saturated with eIF-4A, eIF-4B, or eIF-4F. eIF-4B clearly shows a greater affinity for AUG than do the other two factors. This is consistent with the observations of Butler and Clark (1984), who suggested that eIF-4B binds to the initiator AUG codon region of STNV RNA. eIF-4A and eIF-4F showed a decrease in poly(ϵA) binding only at very high triplet concentrations (Figure 8).

Figure 9 shows a comparison of the competition of various trinucleotides with poly(ϵA) for binding eIF-4B. AUG competes most efficiently for eIF-4B binding. GUG, an alternate initiation codon, also appears to have some binding affinity. The trinucleotides UGA, UUU, and UAA had only very small affinities for eIF-4B as compared with poly(ϵA). It therefore appears that eIF-4B is specific for the trinucleotide AUG.

Discussion

At low salt concentrations, the binding of initiation factors eIF-4A, eIF-4B, and eIF-4F to poly(ϵA) produced a steady increase in the fluorescence emission presumably due to unstacking of the nucleotide base residues. In the case of eIF-4A, the fluorescence enhancement was reduced at high salt. One

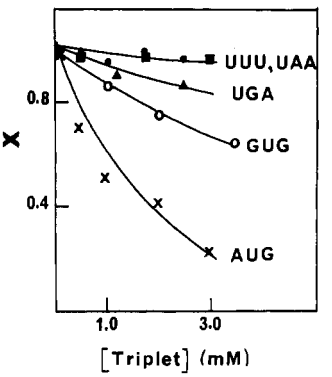

FIGURE 9: Effects of nucleotide triplets on the binding of eIF-4B to poly(ϵA). The y axis is the normalized fluorescence change for eIF-4B binding to poly(ϵA). The data show the decrease in fluorescence that results from additions of UUU (closed circles), UAA (squares), UGA (triangles), GUG (open circles), or AUG (×). The titrations were performed in buffer A (100 mM KCl) at 23 °C.

would expect that the same fluorescence enhancement would occur only at much higher protein concentrations since K_{obsd} decreased with increasing KCl concentrations. Gene 32 protein binding to poly(ϵA) exhibited this behavior (Kowalczykowski et al., 1981). The eIF-4A binding behavior was very similar to that reported by Schmidt et al. (1985) for procaryotic IF-3 binding to poly(ϵA). They reported a decreased fluorescence enhancement at high salt concentrations and also an immediate increase in fluorescence intensity which relaxed to a reduced stable value when IF-3 was added to poly(ϵA) solutions. We observed a similar phenomena for eIF-4A binding. Mayer et al. (1979) proposed a general binding scheme for the binding of tyrosine-containing peptides to polynucleotides:

$$P + N \overset{K_1}{\rightleftharpoons} PN' \overset{K_2}{\rightleftharpoons} PN$$

where N and P are the unbound poly(ϵA) residues and eIF-4A, respectively, PN′ is a protein–nucleic acid complex with unstacked nucleotide bases, and PN is an eIF–4A-poly(ϵA) complex with tyrosine or phenylalanine stacked between the bases. The first step would involve an enhancement of fluorescence due to nucleic acid base unstacking, and the second step would result in a quenching of fluorescence due to stacking of the aromatic amino acid side chains between the bases. Schmidt et al. (1985) discussed an interpretation of the IF-3 binding data with regard to this model. They suggested that K_1 should decrease in magnitude with increasing salt concentrations since it would involve electrostatic interactions. K_2 should be less dependent on salt since it involves the stacking of aromatic amino acid side groups with the nucleotide base.

Our data showed a decrease in affinity (K_{obsd}) and a decrease in fluorescence enhancement for eIF-4A binding to poly(ϵA), consistent with this model. The competition data demonstrated that the binding of both natural mRNA and poly(ϵA) is salt dependent. This would suggest that the binding is largely electrostatic, and unlike IF-3, there is not a specific mRNA sequence recognized by eIF-4A.

The effect of ATP on eIF-4A binding to poly(ϵA) is quite interesting. It has been suggested (Grifo et al., 1984; Ray et al., 1985) that ATP is required for eIF-4A binding to RNA. Although ATP increased the equilibrium binding of eIF-4A to poly(ϵA) approximately 3-fold, there was not an absolute requirement of ATP for this binding. At high salt concentrations, there was an ATP-dependent increase in fluorescence enhancement. In terms of the model of Schmidt et al. (1985), this would mean that the electrostatic interaction of eIF-4A–mRNA and base unstacking of the nucleic acid are increased

or the stacking between aromatic amino acids of the protein and the nucleic acid bases is reduced. Our studies with the nonhydrolyzable analogue of ATP, AMPPNP, indicated that hydrolysis of ATP is not necessary to obtain this effect. It may be that binding of ATP to the protein induces conformational changes that affect mRNA binding.

In contrast to the fluorescence data with eIF-4A, the binding of eIF-4B and eIF-4F to poly(εA) shows the same fluorescence enhancement at high and low KCl concentrations. The K_{obsd} values (dissociation) for poly(εA) binding to eIF-4B and eIF-4F are increased with increasing salt. ATP enhances the binding of both proteins about 2-fold. The competition with natural globin mRNA suggests that binding to this nucleic acid is much less salt dependent than the binding to poly(εA). We suggest that for these two proteins the interaction with poly(εA) is mainly electrostatic and that for globin message other types of interactions, probably sequence specific, dominate. This same type of salt dependence has been observed for binding of procaryotic IF-3 to RNA. At low salt, nonspecific binding occurs, while at higher salt concentrations sequence-specific interactions are observed (Wickstrom et al., 1986). ATP may affect the binding through protein conformational changes.

The site size determined for eIF-4A binding to poly(εA) was 13 ± 2 nucleotides. This is comparable to the site size obtained for IF-3 binding by Schmidt et al. (1985). Larger site sizes were obtained for eIF-4B and eIF-4F, 25 ± 5 and 35 ± 5 bases, respectively. It should be pointed out that although each preparation is free of detectable levels of known initiation factors, our preparations of eIF-4F, in contrast to eIF-4A, are not homogeneous. The presence of contaminants which do not bind poly(εA) would cause the calculated site size to be somewhat larger. The large site size could also be a reflection of anticooperative binding. If binding of one protein diminishes the affinity for binding a second identical protein in the adjacent base sequence, then the site size will appear quite large even though a small number of bases may actually be involved in binding of the protein.

The studies on the competition of m^7GTP and poly(εA) for binding to the initiation factors demonstrate that the binding of only eIF-4F to poly(εA) is blocked by the addition of the cap analogue. This conclusion is supported by the fluorescence studies using the natural fluorescence of m^7GTP which show that only eIF-4F produces a change in the m^7GTP fluorescence. There is no evidence that either eIF-4A or eIF-4B binds directly to m^7GTP. Our results suggest that although eIF-4F will bind to RNA at other than the 5′ cap it has the greatest affinity for the cap region.

Eucaryotic initiation factor 4B appears to have a preference for AUG binding sites. The AUG triplet showed the greatest affinity and was the only trinucleotide which effectively competed with eIF-4B binding to poly(εA). Butler and Clark (1984) concluded from studies using STNV fragments that eIF-4B from wheat germ binds the translation initiation region and requires up to 20 nucleotides on the 3′ side of the AUG codon. We are currently investigating binding of eIF-4B to oligonucleotides with more base residues on the 3′ side of AUG to see if this increases binding efficiency.

It appears that eIF-4A alone binds nonspecifically, mainly through electrostatic interactions. This protein may function only as part of the larger eIF-4F complex, which shows preferential binding to the m^7-guanine cap region of message. eIF-4A may also interact with eIF-4B, whose binding to the AUG initiation codon could provide specificity for the binding of eIF-4A to the message. Another possibility is that eIF-4A is a general unwinding protein that can act at any number of sites independent of eIF-4F and eIF-4B. It is still a question whether these proteins act independently or in concert. We are currently investigating the mRNA affinities and site specificities of these proteins in multiprotein–RNA interactions.

Registry No. ATP, 56-65-5; AUG, 3494-35-7; GUG, 5746-20-3; UUU, 3152-53-2; UAA, 2889-33-0; UGA, 3438-26-4; 7-Me-GTP, 26554-26-7; poly(εA), 41911-88-0; Mg, 7439-95-4.

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