Dissociation of Double-Stranded Polynucleotide Helical Structures by Eukaryotic Initiation Factors, As Revealed by a Novel Assay†

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ABSTRACT: A new technique has been applied to the study of the RNA secondary structure unwinding activity of the eukaryotic initiation factors (eIFs) 4F, 4A, and 4B. Secondary structures were generated at the 5′ ends of reovirus and globin mRNA molecules by hybridization with ^{32}P-labeled cDNA molecules 15 nucleotide residues long. The dissociation of the labeled cDNAs from the mRNAs was assayed by a gel filtration chromatography procedure which separates the free cDNAs from mRNAs and mRNA/cDNA hybrids. When the three factors were tested alone, only eIF-4F stimulated dissociation of hybrids. The combination of eIF-4A plus eIF-4B also exhibited a strong hybrid dissociating activity, which was markedly temperature dependent. Under optimum conditions, up to 90% of the hybrid structures are disrupted in 60 min. These results demonstrate for the first time that stable double-stranded regions can be melted and dissociated by eIFs. They also characterize more precisely the first step in the structure unwinding reaction.

The initiation of protein synthesis in mammalian systems is a complicated process requiring several initiation factors (Pain, 1986; Rhoads, 1985).[1] An early step in the initiation mechanism, which is also a site for the regulation of the overall protein synthesis rate, is the binding of initiation factors to the messenger ribonucleic acid (mRNA)[2] to be translated. Three initiation factors[3] have been implicated in this process: eIF-4A, eIF-4B, and eIF-4F (Grifo et al., 1983). The first step is the interaction of eIF-4F with the mRNA cap, which can be regulated by the secondary and tertiary structure of the mRNA (Gehrke et al., 1983; Sonenberg et al., 1983; Godefroy-Colburn et al., 1985; Lawson et al., 1986, 1988). Subsequently, all three factors are involved in the unwinding of mRNA structure (Edery et al., 1984; Ray et al., 1985). Several of these early steps are message-discriminatory, in the sense that some mRNAs react more rapidly than others, leading to differential rates of translation (Ray et al., 1983; Gehrke et al., 1983; Godefroy-Colburn et al., 1985; Lawson et al., 1986, 1988; Sarkar et al., 1984).

In a previous study of the structure unwinding reaction, it was shown that eIF-4A and eIF-4F in the presence of ATP can increase the sensitivity of native reovirus mRNAs toward cleavage by ribonuclease, a result consistent with a reduction in the overall structure of the mRNAs (Ray et al., 1985). However, the nuclease sensitivity assay does not differentiate between secondary structure and tertiary structure alterations. Thus, it remained to be determined whether stable helical regions in mRNA could be completely disrupted by initiation factors. In order to answer this question, it was necessary to develop a new assay for the dissociation of duplex structure in polynucleotides. The details of such an assay are described in this paper. This dissociation assay relies on the use of model secondary structures formed by hybridizing radioactively labeled complementary oligonucleotides to mRNAs: the active dissociation of these oligonucleotides by initiation factors is then monitored by gel filtration chromatography. The technique is simple and rapid and could be applied to the study of other proteins which affect polynucleotide structure.

Using this assay, we demonstrate for the first time that double-helical regions are melted and separated into single strands by these initiation factors. Moreover, we show that a 15-mer of cDNA base-paired to the first 15 nucleotides of a mRNA can be dissociated by eIF-4F alone, without the assistance of eIF-4A or -4B. This defines the interaction of the p46 subunit of eIF-4F with a 5′ proximal region of the mRNA as the first step in the unwinding reaction.

Materials and Methods

Preparation of Reovirus, Globin mRNAs, and cDNAs. Capped reovirus mRNAs were prepared by in vitro transcription from protease-activated reovirus cores in the presence of [5,6-^{3}H]uridine 5′-triphosphate (ICN) and purified as previously described (Ray et al., 1985; Lawson et al., 1986). Rabbit globin mRNA was purified by using the procedures described earlier (Brendler et al., 1981). Chemically synthesized pentadecamer DNAs were prepared as described (Lawson et al., 1986). Purified DNAs and oligoriboadenylic acid (12–18 residues in length, Pharmacia) were labeled at the 5′ end with [γ-^{32}]ATP and T4 polynucleotide kinase (Amersham) (Gough et al., 1979).

Preparation of Initiation Factors. Eukaryotic initiation factors eIF-4F, eIF-4A, and eIF-4B were purified from rabbit reticulocytes according to the procedures published earlier (Grifo et al., 1982, 1983). The molecular weight of eIF-4A

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[1] See also Mathews (1987).

[2] Abbreviations: mRNA, messenger ribonucleic acid; eIF, eukaryotic initiation factor; ATPase, ATP hydrolysis activity; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; m^{7}GTP, 7-methylguanosine 5′-triphosphate; AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate); 5′ UTR, 5′-untranslated region of a mRNA.

[3] eIF-4F is a complex composed of a 25-kDa cap binding protein (CBPI or eIF-4E), a 46-kDa protein, and a 220-kDa protein. SDS–PAGE analyses showing the purity of these and similar initiation factor preparations have been published earlier (Abramson et al., 1987; Grifo et al., 1984).

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is approximately 46K, whereas that of eIF-4B is approximately 160K (i.e., a dimer of two 80-kDa polypeptides). Using these values, it may be calculated that 1 μg of eIF-4A contains 22 pmol, and 1 μg of eIF-4B contains 6.3 pmol, of protein. The p26 content of 1 μg of eIF-4F was determined (by SDS–PAGE, staining with Coomassie blue, and densitometric scanning) to be 2.4 pmol.

Preparation of Hybrids. The reovirus and globin mRNAs were hybridized to the cDNA 15-mers according to the previously employed procedures (Lawson et al., 1986). Typically, 10–40 μg (about 50 pmol) of mRNA was combined with a 20-fold molar excess of the ^{32}P-labeled cDNA(s) in 20 mM HEPES, pH 7.5, and 1 mM DTE. This mixture was heated at 70 °C for 1 min followed by incubation at 30 °C for 5 min. The solutions were then made 100 mM in potassium chloride and 3 mM in magnesium acetate and brought to a final volume of 50 μL. The samples were incubated at 30 °C for 90 min. RNA/RNA hybrids were prepared by hybridizing polyuridylic acid (Pharmacia; an average sedimentation coefficient of 4.1 S) to ^{32}P-labeled oligo(A) under similar conditions. The hybrids were purified from the free cDNAs or oligo(A) by gel filtration chromatography. The solutions were loaded onto 18 × 0.5 cm columns of 5 mL of Sephadex G-100 jacketed with ice water and equilibrated with 20 mM HEPES, pH 7.5, 100 mM potassium chloride, and 4 mM magnesium acetate. The columns were eluted with the same buffer. Fractions of 250 μL were collected and assayed by liquid scintillation counting in 3a70 cocktail (Research Products International). Fractions containing hybrids (^{32}P-labeled material collecting with the mRNA or polyuridylic acid) were pooled and stored in aliquots at −70 °C.

Assays for the Dissociation of Hybrids by Initiation Factors. For the assay of dissociation of the mRNA/cDNA hybrids, typical reaction mixtures contained 0.06 μg (0.25 pmol) of globin mRNA hybridized to ^{32}P-labeled cDNAs (20 000–30 000 cpm/pmol), and the indicated quantity of initiation factor preparations (from 0.1 to 3 μg) in a volume of 50 μL, which included 20 mM HEPES, pH 7.5, 100 mM potassium chloride, 4 mM magnesium acetate, and 10 mg/mL bovine serum albumin. Similar amounts of a mixture of reovirus mRNAs were used. Either ATP or AMP-PNP (each at 1 mM) was also included, and these were each chelated with 2 mM additional magnesium acetate. The reactions were incubated at the indicated temperatures (25–35 °C) for 20 min to 1 h. The reactions were terminated by adding 150 μL of ice-cold 20 mM HEPES, pH 7.5, containing 100 mM potassium chloride, 4 mM magnesium acetate, and 1% blue dextran, and the samples were then immediately loaded onto columns of 5-mL Sephadex G-100 identical with those described above. The columns were eluted with the same buffer minus blue dextran. Three fractions were collected from each column: fraction 1 (1.5–2-mL total volume) contained material eluting prior to the blue dextran; fraction 2 (2-mL total volume) consisted of eluent containing visible blue dextran, mRNA, and mRNA/cDNA hybrids (excluded material); and fraction 3 (5–6-mL total volume) contained free cDNA (included material). Ninety-five percent or more of the total radioactivity loaded onto the columns was consistently recovered, both in the absence and in the presence of factors. The cDNA eluting in fraction 3 remained intact. These results indicates the absence of phosphatase, DNase, or phosphodiesterase in the factor preparations. The amount of cDNA in each fraction was determined by quantitating the ^{32}P present by liquid scintillation counting of 500 μL in 10 mL of 3a70 cocktail. In order to determine the percentage of cDNA dissociated by initiation factors, the amount of cDNA eluting with the mRNA at zero time and in the absence of added factors under the conditions studied was determined. The percentage of cDNA which was separated from the mRNA (i.e., included in the column) in the absence of factors was then subtracted from the percentage of cDNA separated from the mRNA in the presence of factors to give te amount of factor-induced dissociation. Similar procedures were employed to assay for the dissociation of the RNA/RNA hybrids containing poly(U) and ^{32}P-labeled oligo(A).

Results

cDNAs were selected to construct the model secondary structures because they are easily prepared and purified. Although the mRNA/cDNA duplex structures are not identical with natural RNA/RNA structures, they do exist as A-form helices. In addition, it has previously been demonstrated that the eukaryotic initiation factors studied here behave similarly toward mRNA/cDNA duplexes structures as they do toward mRNA/RNA hybrids in (1) assays measuring the reduction of higher order structure as determined by nuclease sensitivity (Ray et al., 1985) and (2) their effect on cross-linking of oxidized mRNA cap structures to initiation factors (Lawson et al., 1986). These mRNA/cDNA hybrids therefore would seem to be satisfactory model substrates for the study of isolated secondary structure dissociation reactions that are stimulated by purified factors.

The mRNA/cDNA hybrids employed in these studies were prepared by hybridizing ^{32}P-labeled 15-mers of cDNA to the first 15 bases of reovirus s_3, s_4, and m_3 mRNAs or to the first 15 bases of rabbit α- and β-globin mRNAs. The hybrids were purified from the hybridization reaction mixtures by passage through columns of Sephadex G-100. The same columns were used subsequently to study the dissociation of these purified hybrids by initiation factors. The ability of these columns to separate mRNAs from free 15-mer cDNAs is demonstrated in Figure 1A. This shows the elution profiles of ^{3}H-labeled reovirus mRNAs and ^{32}P-labeled cDNAs when passed individually through a column of 5-mL Sphadex G-100. The elution volumes of the mRNA and cDNA peaks were sufficiently different to allow virtually complete resolution of these components from a mixture of the two. In addition, it was determined that blue dextran coeluted with the mRNA in the void volume, which provided a convenient marker for the mRNA. The mRNA/cDNA hybrids also eluted in the void volume, and therefore could be completely separated from unhybridized cDNA. This is demonstrated in Figure 1B, which shows the analysis of hybridization reaction mixtures by gel filtration chromatography. That this coelution of the cDNA with the mRNA represents the specific hybridization of the cDNAs to complementary sequences in the mRNA was demonstrated previously by analysis of the hybridization mixtures with ribonuclease H, and by primer extension (Lawson et al., 1986).

The conditions for the formation of the hybrid structures were optimized to achieve the maximum possible involvement of each mRNA. Typically, coincubation of a 15-fold molar excess of cDNA with its mRNA complement resulted in conversion of more than 90% of the latter to hybrid structures in 1 h at 35 °C. Similar results could be achieved with a 5-fold molar excess of cDNA after 4 h of incubation. It thus appears that secondary structures within the native mRNAs examined (reovirus s_3, s_4, and m_3, and α- and β-globin mRNAs) do not substantially interfere with the formation of the mRNA/cDNA hybrids. This is consistent with computer modeling studies of potential secondary structures of the s_3, s_4, and m_3

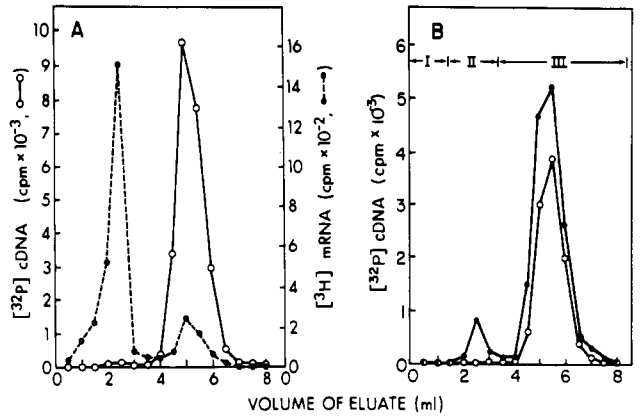

FIGURE 1: Separation of mRNA and cDNA by gel filtration chromatography. (A) Separation of mRNA and cDNA molecules. Reovirus mRNAs synthesized by in vitro transcription in the presence of [5,6-^{3}H]UTP and a cDNA 15-mer labeled at the 5′ end with ^{32}P were individually passed through a column containing 5 mL (18 × 0.5 cm) of Sephadex G-100 as described under Materials and Methods. Fractions were collected and assayed by liquid scintillation counting. (●) mRNA; (○) cDNA. (B) Separation of mRNA/cDNA hybrids from free cDNAs. A mixture of reovirus mRNAs was hybridized to the ^{32}P-labeled cDNA complementary to the first 15 bases of reovirus s_3 mRNA as described under Materials and Methods. The reaction mixture was then passed through a column containing 5 mL of G-100. Fractions were collected and assayed by liquid scintillation counting. (●) ^{32}P in cDNA from hybridization; (○) a slightly smaller quantity of ^{32}P in cDNA in the absence of mRNA. I, II, and III indicate the fractions collected in the hybrid dissociation assays (see text).

reovirus mRNAs which have shown that while several regions of secondary structure can exist, the free energies of the potential structures involving the bases which hybridize to the cDNAs are less than 0.4 kcal/nucleotide.[4] In contrast the free energies of the mRNA/cDNA hybrids range from about 1.8 to 2.1 kcal/nucleotide.

On the basis of these observations, we devised a protocol for the large-scale preparation, purification by gel chromatography, and storage of the mRNA/cDNA hybrids (see Materials and Methods). It was important to determine if these purified hybrid preparations were kinetically stable in the absence of excess unhybridized cDNA, under the conditions employed for the assays of the dissociation activity of initiation factors. To this end, an aliquot of reovirus mRNAs hybridized to cDNAs complementary to the 5′ ends of s_3, s_4, and m_3 mRNAs was incubated in the presence of 100 mM potassium chloride and 4 mM magnesium acetate at 35 °C. (This temperature is the highest that was employed in these studies, and these conditions would therefore be the least conducive to the maintenance of secondary structures.) The amount of total cDNA which remained associated with the mRNA was measured as a function of time. The results are presented in Figure 2, where it is evident that approximately 10% of the hybrids dissociated in 1 h of incubation. Hybrids were slightly less stable at 3 mM magnesium acetate, so the higher concentration was preferred for subsequent studies. However, significant results were usually repeated using the lower concentration, which is more physiological. No qualitative differences attributable to magnesium concentration within this range were observed.

In contrast to the demonstrated stability of the hybrids when incubated alone, they became destabilized in the presence of initiation factors. As shown in Figure 2, when eIF-4F, -4A, and -4B (1 μg of each) were incubated with the mRNA/

[4] R. E. Thach, unpublished observation.

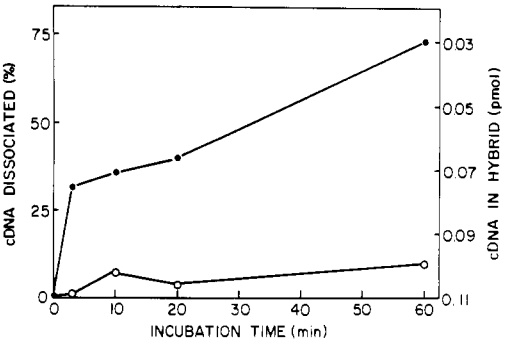

FIGURE 2: Stabilty of the mRNA/cDNA hybrids in the absence and presence of initiation factors. For each measurement, 0.25 μg of a mixture of reovirus mRNAs hybridized to the ^{32}P-labeled cDNAs complementary to the first 15 bases of s_3, s_4, and m_3 mRNAs was incubated alone or in the presence of 1 μg each of eIF-4F, -4A, and -4B in 100 mM potassium chloride, 4 mM magnesium acetate, and 1 mM ATP at 35 °C. At the indicated times, the incubations were terminated and analyzed by gel filtration chromatography as described under Materials and Methods, and the amount of cDNA eluting as included material was determined. This is shown both as picomoles of cDNA remaining in hybrid and as the percent of original hybrid that is dissociated. The molar amounts of each reactant were the following: 22 pmol of eIF-4A, 6.3 pmol of eIF-4B, 2.4 pmol of eIF-4F (based upon the amount of p26 subunit present), and 0.6 pmol of total reovirus mRNA (containing 0.24 pmol of m_3 plus s_3 plus s_4 mRNAs, hybridized to 0.11 pmol of cDNAs). (○) mRNA/cDNA alone; (●) mRNA/cDNA plus 1 μg each of eIF-4F, eIF-4A, and eIF-4B.

Table I: Dissociation of Reovirus mRNA/cDNA Hybrids in the Presence of Various Combinations of Initiation Factors[a]

factor(s) present	dissociation (%)
none	13
4A + 4B	78
4F + 4A + 4B	89

[a] The same reovirus mRNAs (0.25 μg) described in the legend to Figure 2 were incubated at 35 °C in the presence of 1 μg of the indicated initiation factor(s) in 100 mM potassium chloride, 4 mM magnesium acetate, and 1 mM ATP. After 1 h, the reactions were terminated and analyzed as described under Materials and Methods, and the percentage of cDNA dissociated was determined.

cDNA hybrids in the presence of ATP, there was a marked time-dependent dissociation of the hybrids. This factor-dependent disruption of the duplex was not due to the presence of degradative enzyme contaminants: no contaminating phosphatase, ribonuclease, or deoxyribonuclease activity was found in the factor preparations, and the dissociated products remained intact as demonstrated by polyacrylamide gel electrophoresis. This result is consistent with that of Ray et al. (1985), who showed that an increase in the susceptibility of reovirus mRNAs toward cleavage by added ribonuclease A or T1 occurred in the presence of these factors under similar conditions. In addition, it is now apparent that these initiation factors actually melt the hybrid structures, resulting in the dissociation of the two complementary strands.

The time course of the dissociation reactions shown in Figure 2 is similar to that seen when the cross-linking of purified factors to oxidized mRNA caps is studied (Lawson et al., 1988). This suggests that the binding of factors to mRNA is the rate-determining step in the dissociation reaction. Thus, the dissociation steps per se may be very much faster than the overall reaction rate suggested by Figure 2.

We next sought to determine the specific requirements for the dissociation of 5′ proximal secondary structures by these initiation factors. Our expectation was that eIF-4F would be absolutely required for this reaction. However, under the conditions used in Figure 2, this was not the case. Indeed, eIF-4A plus eIF-4B was almost as effective in the absence of

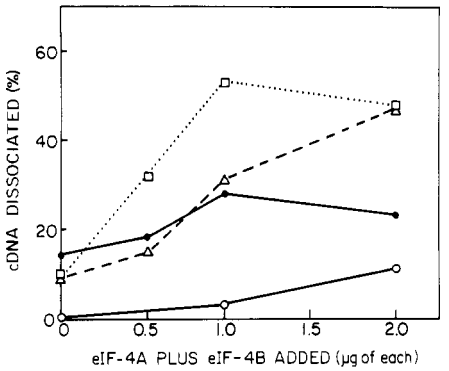

FIGURE 3: Effects of temperature and eIF-4F on the dissociation of hybrids by eIF-4A plus eIF-4B. For each measurement, 0.25 μg of the same reovirus mRNAs described in the legend to Figure 2 was incubated alone or in the presence of initiation factors in 100 mM potassium chloride, 4 mM magnesium acetate, and 1 mM ATP at the indicated temperature. After 20 min, the incubations were terminated and analyzed by gel filtration chromatography as described under Materials and Methods, and the percentage of cDNA eluting as included material was determined. (○) eIF-4A plus eIF-4B at 25 °C; (●) eIF-4A plus eIF-4B plus 2 μg of eIF-4F, at 25 °C: (△) eIF-4A plus eIF-4B at 30 °C; (□) eIF-4A plus eIF-4B at 35 °C.

eIF-4F as in its presence (Table I). A subsequent investigation of reaction conditions revealed that the predicted dissociation activity of eIF-4F, in the absence of other factors, could be optimized by shortening the reaction time, increasing the eIF-4F concentration, and lowering the reaction temperature to 25 °C. This last change in particular dramatically reduced the dissociation activity of eIF-4A plus -4B, as shown in Figure 3. Under these conditions, dissociation is now strongly dependent upon eIF-4F. Moreover, under these conditions, it is evident that eIF-4F is significantly more efficient on a molar basis than eIF-4A plus eIF-4B. For example, at 25 °C, 1 μg each of eIF-4A and -4B (22 and 6.3 pmol, respectively) dissociates only 6% of the hybrids formed; in contrast, 2 μg of eIF-4F (4.8 pmol) dissociates 20% of the hybrids. This result is again consistent with earlier results (Ray et al., 1985). It is not clear why the dissociation activity displayed by eIF-4A plus eIF-4B should be so markedly sensitive to temperature. This may be an effect, at least in part, of the increased stability of helices at low temperature. In any event, this result indicates that when eIF-4A is part of the eIF-4F complex (as the p46 subunit), its dissociation activity is significantly altered.

The results shown in Figure 3 do not rule out the possibility that either eIF-4A or eIF-4B alone might stimulate the activity intrinsic to eIF-4F, at least under certain conditions. In particular, it was of interest to know whether eIF-4A could interact with eIF-4F to supplement the activity of the p46 subunit. To test this possibility, experiments were conducted under the conditions shown in Figure 3, except that the temperature was raised in order to amplify any synergistic effects between eIF-4F and eIF-4A. As shown in Table II, even at 35 °C eIF-4A does not stimulate eIF-4F activity. Under these conditions, the activity of eIF-4F alone is still substantial, being almost half that obtainable when all three factors are present.

It is not clear in Table II whether the added eIF-4B acts on eIF-4F directly or on the eIF-4A present (see below). However, an approximately 2-fold effect of eIF-4B on eIF-4F activity in the absence of eIF-4A was routinely observed. This modest effect could be due to the recycling of eIF-4F by eIF-4B (Ray et al., 1986). Further experiments must be done in order to define the effect of eIF-4B on eIF-4F more precisely.

Little dissociation activity was seen when eIF-4A or eIF-4B was tested alone, under a variety of conditions. For example,

Table II: Dissociation of Reovirus mRNA/cDNA Hybrids in the Presence of Higher Concentrations of Initiation Factors[a]

factor(s) present	dissociation (%)
none	3
4F	28
4F + 4A	22
4F + 4A[b]	23
4F + 4A + 4B	62

[a] Reovirus mRNAs (0.3 μg) hybridized with a ^{32}P-labeled cDNA complementary to the first 15 bases of s_3 mRNA were incubated at 35 °C in the presence of 2 μg of the indicated initiation factors in 100 mM potassium chloride, 4 mM magnesium acetate, and 1 mM ATP. After 20 min, the reactions were terminated as described under Materials and Methods, and the percentage of cDNA dissociated was determined. [b] 4 μg of eIF-4A was used in this experiment.

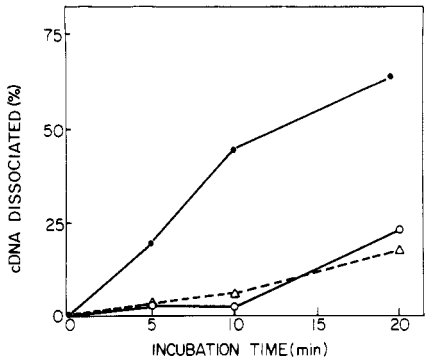

FIGURE 4: Dissociation of globin mRNA/cDNA hybrids by eIF-4A plus eIF-4B in the presence of ATP or AMP-PNP. Globin mRNAs (0.06 μg, 0.25 pmol) hybridized to ^{32}P-labeled cDNAs complementary to the first 15 bases of both α- and β-globin mRNAs (0.18 pmol of cDNA hybridized) were incubated in the presence of 1 μg each of eIF-4A and eIF-4B in 100 mM potassium chloride and 4 mM magnesium acetate at 25 °C. 1 mM ATP, AMP-PNP, or no nucleotide was also present. At the indicated times, the reactions were terminated and analyzed by gel filtration chromatography as described under Materials and Methods, and the percentage of cDNA dissociated was determined. The molar equivalents of 1 μg of each factor are given in the legend to Figure 2. (●) ATP; (○) AMP-PNP; (△) no added nucleotide.

even after 60 min of incubation, 1 μg of eIF-4B stimulated dissociation of only 11% of hybrids present (using 0.5 μg of the mRNA/cDNA hybrids described in Figure 2). Under similar conditions, 1 μg of eIF-4A caused only 10% dissociation. This result contrasts with that reported previously, where eIF-4A alone sensitized single-stranded reovirus mRNA to ribonucleases (Ray et al., 1985). The reason for this difference between the two types of assays is unclear. In any case, eIF-4A and eIF-4B together actively promote hybrid dissociation, especially at higher temperatures (Table I and Figure 3). As previously observed in the RNase sensitivity assay, this dissociation activity also shows a strong requirement for ATP (Figure 4). A similar ATP requirement was observed for the hybrid dissociation catalyzed by eIF-4F.

It was important to determine whether the apparent activities of these initiation factors were artifacts resulting from the use of mRNA/cDNA hybrids. To this end, assays were carried out to test the ability of the initiation factors to dissociate RNA/RNA hybrids. These hybrids were prepared by annealing ^{32}P-labeled oligo$(A)_{12-18}$ to poly(U). In the standard assay, neither eIF-4A, eIF-4F, nor eIF-4B alone dissociated the poly(U)/oligo(A) duplexes, nor did eIF-4F plus eIF-4B. However, the presence of eIF-4A and eIF-4B together resulted in a substantial portion of the hybrid structures being dissociated during 60 min of incubation at 35 °C. This is indicated by the data shown in Table III. No dissociation occurred when AMP-PNP was substituted for ATP. The level

Table III: Dissociation of RNA/RNA Hybrids in the Presence of eIF-4A and eIF-4B[a]

factor(s) present	dissociation (%)
none	5
4A (4 μg)	8
4A (4 μg) + 4B (1 μg)	20
4A (8 μg) + 4B (2 μg)	31

[a] Polyuridylic acid (4.1 S, 0.3–0.6 pmol) hybridized with ^{32}P-labeled oligo(A)$_{12-18}$ [molar ratio of oligo(A)/poly(U) = 1.2] was incubated at 35 °C in the presence of the indicated quantities of eIF-4A and eIF-4B in 100 mM potassium chloride, 4 mM magnesium acetate, and 1 mM ATP. After 1 h, the reactions were terminated and analyzed as described under Materials and Metods, and the percentage of oligo(A) dissociated was determined.

of duplex dissociation obtained with these RNA/RNA structures is considerably lower than dissociation observed in the presence of the same initiation factors for the mRNA/cDNA hybrids (Table I), even with larger quantities of factors. This is likely due to the presence of multiple sequential hybridization sites on the poly(U) molecules that are available for oligo(A) binding. This allows the oligo(A) molecules to "stack" on one another, eliminating end effects. Indeed, the theoretical melting temperature for such a structure is about 65 °C (Freier et al., 1985), which is approximately 20 °C higher than that of the mRNA/cDNA hybrids (Lawson et al., 1986). Such a large difference in melting temperature could easily account for the quantitative difference seen.

Discussion

The results presented here demonstrate the effective application of a technique for monitoring the dissociation of duplex structures in polynucleotides by structure unwinding enzymes. The data obtained in this study provide the first direct evidence that eIF-4F, -4A, and -4B can melt and dissociate stable helical regions. Under carefully defined conditions, dissociating activity was observed with eIF-4F alone. Neither eIF-4A nor eIF-4B alone displayed significant dissociating activity, although in combination they promoted extensive hybrid dissociation.

These results provide insights into the first step in the mechanism involved in the unwinding of mRNA secondary structure. Most importantly, they indicate that the p46 component of eIF-4F, which possesses nuclease sensitization activity (Ray et al., 1985), can cause the melting of secondary structure within the first 15 nucleotides adjacent to the cap. This probably occurs as a result of the binding of the p46 subunit to a single-stranded region of the mRNA, perhaps by a direct displacement mechanism. This would be consistent with the observation that when 5′ proximal secondary structure is extremely stable, the interaction with either p46 or eIF-4A is strongly inhibited (Lawson et al., 1986; Abramson et al., 1987). Supplementation of eIF-4F with eIF-4A did not increase the extent of unwinding. Supplementation with eIF-4B produced a modest (2-fold) enhancement. Whether the latter effect is due simply to the recycling of eIF-4F (Ray et al., 1986) or a direct involvement of eIF-4B in the initial unwinding event remains to be determined.

These results can be combined with information from earlier studies (Ray et al., 1985, 1986; Grifo et al., 1982, 1984; Abramson et al., 1987; Lawson et al., 1986, 1988) to provide a more detailed picture of how eIF-4F, -4A, and -4B interact with mRNA molecules during the early steps of translation initiation. This is summarized by Figure 5, which depicts a number of reactions that have been shown to occur independently of one another. However, we believe that they represent a coordinated sequence of events that leads to the

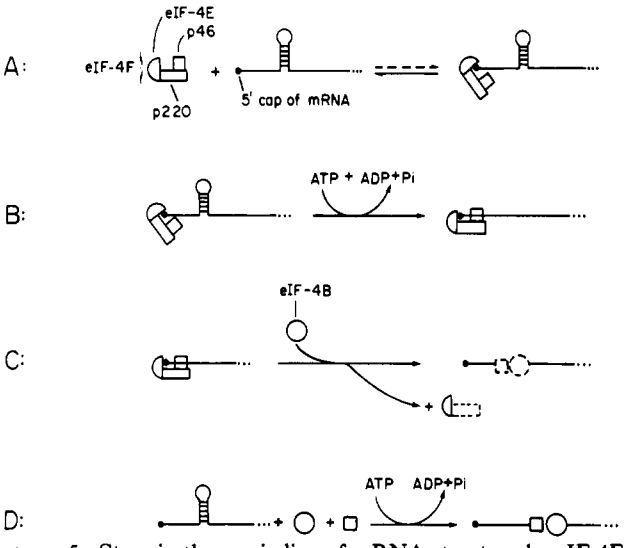

FIGURE 5: Steps in the unwinding of mRNA structure by eIF-4F, -4A, and -4B. Each of the reactions shown has been studied independently of the others. They are arranged here in a logical sequence which could lead to the melting of all secondary and tertiary structures present in the 5′ UTR of a mRNA. Details of each step are discussed in the text.

complete unwinding of structures in the 5′ untranslated region (5′ UTR) of a mRNA, and therefore present them in a logical order. As demonstrated previously, the eIF-4F complex first interacts with mRNAs through the formation of a weak complex between the eIF-4E cap binding component and the cap structure (Figure 5A). This reaction, which is one of the rate-limiting steps for mRNA translation, is facilitated by a sterically exposed cap (Lawson et al., 1988). Subsequently (Figure 5B), a stable complex forms between the p46 subunit of eIF-4F and the mRNA, in a reaction which requires the hydrolysis of ATP (Lawson et al., 1986; Abramson et al., 1987). This step can also be rate limiting for translation if stable 5′ proximal secondary structure is present (Gehrke et al., 1983; Lawson et al., 1986). As demonstrated in this paper, this binding of p46 actually melts 5′ proximal secondary structure, and thus may be viewed as the first step in the unwinding of the mRNA molecule. In a subsequent reaction (Figure 5C), eIF-4B can act as a recycling factor, causing the release of the eIF-4E (and probably the p220) component from the mRNA (Ray et al., 1986). This released eIF-4E can then participate in a second mRNA binding event. As shown in Figure 5D, eIF-4B can also stimulate the ATP-dependent melting of secondary structure by free eIF-4A. This reaction is also demonstrated for the first time by the data in this paper. This unwinding by eIF-4A plus eIF-4B may represent a continuation of the mRNA structure melting process which begins with the stable binding of eIF-4F, or it may represent a cap-independent unwinding mechanism (Abramson et al., 1988; Pelletier & Sonenberg, 1988; Jang et al., 1988), or both.

A number of questions remain to be answered concerning the mechanism of action of these factors. For example, the apparent multiple functions of eIF-4B and its interactions with the other factors will be thoroughly understood only after further investigation. In particular, the potential role this factor may play during the binding of eIF-4F with mRNA remains to be elucidated. In addition, the fate of the p46 component of eIF-4F associated with the mRNA during the initial unwinding reaction is uncertain. It may remain bound to the mRNA (as suggested in Figure 5C), or it may be released along with eIF-4E and p220 as a result of eIF-4B activity. Similarly, whether additional eIF-4A (or eIF-4B) molecules actually bind to the mRNA to facilitate the un-

winding steps initiated by eIF-4F, or whether the original p46 and eIF-4B molecules can unwind an entire 5′ UTR region, must be determined. It is also not yet known precisely at what point the 43S ribosomal complex enters the sequence of events and binds to the mRNA. In addition, questions exist concerning the effects of covalent modification on the activities of these factors. It has been observed that both eIF-4E and eIF-4B can be phosphorylated (Duncan & Hershey, 1984; Rychlik et al., 1986; Duncan et al., 1987; McMullin et al., 1988). However, there is as yet no evidence as to whether changes in the phosphorylation state of these components may affect their interaction with mRNAs.

The assay for structure unwinding activity described here may be useful for the study of other proteins which act to disrupt double-stranded polynucleotides (Pinkham & Platt, 1983; Finch & Emmerson, 1984; Nakayama et al., 1984; Finch et al., 1986; Stahl et al., 1986; Ford et al., 1988). Of special interest are the recent observations that a mammalian nuclear protein of 68 kDa shares considerable amino acid sequence homology with eIF-4A (Ford et al., 1988) and that an eIF-4A-like protein may be involved in bacterial ribosome assembly (Nishi et al., 1988).

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