Binding of Protein Synthesis Initiation Factor 4E to Oligoribonucleotides: Effects of Cap Accessibility and Secondary Structure†

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ABSTRACT: The binding of rabbit globin mRNA to the 25-kDa cap binding protein eIF-4E from human erythrocytes was found to be 5.3-fold stronger than the binding of the cap analogue m^7GpppG to eIF-4E [Goss et al. (1990) *Biochemistry 29*, 5008–5012]. In order to investigate whether this effect is due to the longer sequence of nucleotides in globin mRNA or to other features such as cap accessibility or secondary structure, oligoribonucleotide analogues of rabbit α-globin mRNA were synthesized by T7 RNA polymerase from a synthetic oligodeoxynucleotide template in the presence of m^7GpppG; these oligoribonucleotide analogues possess varying degrees of cap accessibility and secondary structure. Equilibrium association constants for the interaction of these oligoribonucleotides and purified human erythrocyte eIF-4E were obtained from direct fluorescence titration experiments. The data indicate that while the presence of the m^7G cap is required for efficient recognition by eIF-4E, the cap need not be completely sterically accessible, since other structural features within the mRNA also influence binding.

Discrimination among mRNA by components of the translational machinery is important in the control of translation. Several structural features of mRNA play a role in this discrimination process, including the presence and accessibility of the m^7G(5′)ppp(5′)N cap[1] structure. Several initiation factors have been shown to interact at or near the cap, including eIF-4A, eIF-4B, eIF-4E, and eIF-4F [reviewed by Rhoads (1988) and Sonenberg (1988)]; however, only eIF-4E and eIF-4F have been shown to *directly* interact with the cap (Sonenberg, 1981; Sonenberg et al., 1981; Tahara et al., 1981; Hellmann et al., 1982; Grifo et al., 1983; Webb et al., 1984).

Spectroscopic techniques have been used to characterize the interaction of cap analogues with eIF-4E isolated from either human erythrocytes (Carberry et al., 1989, 1990) or yeast (McCubbin et al., 1988); the interaction of rabbit globin mRNA with human eIF-4E has also been described (Goss et al., 1990). These studies have shown that eIF-4E binds globin mRNA 5.3-fold more tightly than the cap analogue m^7GpppG. However, it is unclear whether this effect is due to the presence of a longer sequence of nucleotides in globin mRNA which facilitates the eIF-4E binding, to differences in the accessibility of the m^7G cap, or to other structural features of the mRNA. A correlation between the accessibility of the cap and the efficiency of translation of mRNA has been illustrated for AMV mRNA (Godefroy-Colburn et al., 1985a,b), reovirus mRNA (Lawson et al., 1988), and HIV-1 mRNA (Parkin et al., 1988). The relative rates of translation among AMV mRNA have also suggested that the amount of secondary structure present in the 5′-terminus of mRNA may also affect the efficiency of translation, since AMV-4 mRNA, which has little secondary structure (Gehrke et al., 1983), is translated 150-fold more efficiently than any of the other AMV mRNAs (Godefroy-Colburn et al., 1985a). Lawson et al. (1988) correlated the relative accessibility of reovirus mRNA caps with eIF-4F cross-linking; a 2.5–3.5-fold difference in cross-linking was observed among the different reovirus mRNAs. However, the efficiency of cross-linking does not provide a quantitative measurement of binding affinity, especially since oxidized caps do not interact efficiently with eIF-4F (Darzynkiewicz et al., 1987).

In order to determine whether cap accessibility or other structural features of the mRNA are important for efficient recognition of globin mRNA by eIF-4E, we have prepared oligoribonucleotide analogues of the 5′-terminus of rabbit α-globin mRNA and measured their affinity for human erythrocyte eIF-4E by direct fluorescence titration. These oligoribonucleotides have different secondary structures, some of which alter cap accessibility. A comparison of the K_{eq} values for the interaction of these analogues with eIF-4E indicates that the presence of the m^7G cap is absolutely required for efficient eIF-4E binding, although the cap need not be completely accessible, since other structural features within the mRNA may also influence eIF-4E binding.

Materials and Methods

Transcription of Oligoribonucleotides. The T7 RNA polymerase primer was purchased from Pharmacia Molecular Biologicals. Deoxyoligonucleotides incorporating both the desired template and the complement of the T7 RNA polym-

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[1] Abbreviations: m^7G, 7-methylguanosine; AMV, alfalfa mosaic virus; HIV, human immunodeficiency virus; eIF, eukaryotic initiation factor; kDa, kilodalton(s); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; Tris, tris(hydroxymethyl)aminomethane; TAP, tobacco acid pyrophosphatase.

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erase primer sequence were synthesized by the Hunter College Center for Gene Structure and Function on an Applied Biosystem DNA synthesizer, Model 380, and purified using oligonucleotide purification cartridges (Applied Biosystems) according to the manufacturer's protocol. The purified DNA strands were dried on a Speed-Vac concentrator (Savant Instruments) and redissolved in 10 mM Tris-HCl buffer, pH 7.0; the solutions were stored at −150 °C. The T7 primer sequence strand and the complementary template strand (each prepared to be 800 nm) were annealed by heating together to 65 °C for 3 min and cooling the sample on ice.

Synthesis of RNA oligonucleotides from the T7 transcription templates was carried out according to the protocol of Milligan et al. (1987) with some modifications. All glassware was treated by heating in a solution of 0.1% DEPC in sterile distilled water at 37 °C overnight, followed by baking at 150 °C overnight to dryness in order to remove all the DEPC. All plasticware was purchased as sterile; all chemicals and reagents were RNase- and DNase-free. All solutions were prepared using sterile distilled water from a MilliQ water system. A typical 0.5-mL transcription reaction mixture contained 40 mM Tris-HCl buffer (pH 8.1 at 37 °C), 1 mM spermidine, 5 mM DTT, 50 μg/mL BSA, 0.01% Triton X-100, 80 mg/mL poly(ethylene glycol) (MW 8000), 1.5 mM each of ATP, CTP, UTP, and m^7GpppG, 0.3 mM GTP, 1000 units/mL RNA Guard (Pharmacia), 800 units/mL T7 RNA polymerase, 9 mM $MgCl_2$, and 100 nM transcription template. The transcription incubation was carried out at 37 °C for 2–3 h. The 1.5 mM ATP, CTP, UTP, and m^7GpppG and the 0.3 mM GTP were prepared from 25 mM stock solutions which were adjusted to pH 8.1. Under these conditions, approximately 90% of the transcripts were determined to be capped. The transcribed oligoribonucleotide was then purified by gel filtration using a 1.6 × 50 cm Superose 12 column (Pharmacia) and 0.1 M NaCl/20 mM Tris-HCl, pH 7.6, buffer pumped at a rate of 0.6 mL/min (Draper et al., 1988). The purity of the transcripts (and extent of capping) was checked on 15% acrylamide gels containing 50% urea, according to the protocol of Sambrook et al. (1989) and as described by Draper et al. (1988); the transcripts were estimated to be ≥90% homogeneous. Typical yields of the transcript were 1–2 mg.

5′-Labeling of Oligoribonucleotides. In order to determine the steric accessibility of the m^7G cap in the oligoribonucleotides prepared, the hairpin-containing oligonucleotides and a "control" linear oligonucleotide (I, Figure 1) were labeled at the 5′-terminus with acriflavine according to the procedure of Millar and Steiner (1965). The only modification of this protocol was that it was scaled down for an oligoribonucleotide concentration of 0.2 mg/mL and the final precipitation step was replaced by exhaustive dialysis.

The rate of decapping of mRNA by tobacco acid pyrophosphatase (TAP) and subsequent dephosphorylation and [γ-^{32}P]ATP labeling of the 5′-terminus were shown to be proportional to the cap accessibility of the mRNA (Godefroy-Colburn et al., 1985b). The oligoribonucleotides were decapped according to the protocol of Godefroy-Colburn et al. (1985b), with the following modification: 50 pmol of oligoribonucleotide was reacted with 4 units of TAP (Sigma) in the presence of 50 mM sodium acetate, pH 6.0, 10 mM 2-mercaptoethanol, and 1 mM EDTA; the hydrolysis reaction was carried out for 5, 15, and 60 min. The oligoribonucleotides were then dephosphorylated with calf intestinal alkaline phosphatase (Bohringer-Manheim) according to the manufacturer's protocol. The dephosphorylated oligoribonucleotide was labeled with approximately 60–80 μCi of [γ-^{32}P]ATP

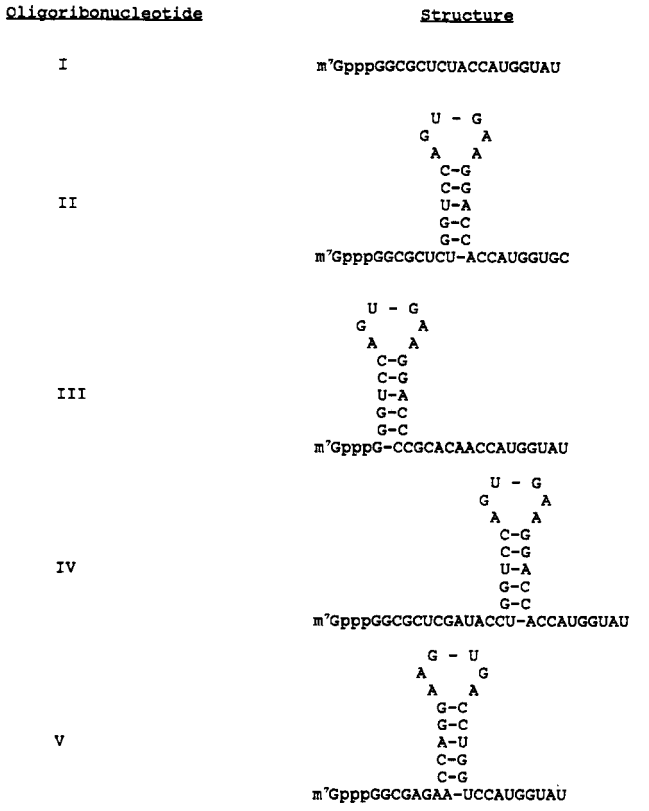

FIGURE 1: Oligoribonucleotides employed in this study.

(ICN Biomedicals) in the presence of T4 polynucleotide kinase (Promega) as specified by Boseley et al. (1978). The 5′-labeled oligoribonucleotides were then precipitated on Whatman GC/F glass filters, washed, combined with a scintillation cocktail (ChemFluor I, ICN Biomedicals), and scintillation-counted on a Beckman Model 600 scintillation counter using the ^{32}P channel (Muench, 1971; Sambrook et al., 1989).

Fluorescence Studies. The steric accessibility of the 5′ cap of the acriflavine-labeled oligoribonucleotides was determined fluorometrically by monitoring the quenching of the acriflavine fluorescence by iodide; the excitation and emission wavelengths were 460 and 510 nm, respectively, and a 1.4-mm slit was employed. Stern–Volmer plots were constructed, and the dynamic quenching constant (K_D) was determined.

The binding of eIF-4E [isolated from human erythrocytes as described by Webb et al. (1984) and Rychlik et al. (1986)] to the oligoribonucleotides shown in Figure 1 was carried out as described previously in detail (Carberry et al., 1989, 1990). The solutions for these fluorescence studies were prepared in buffer A, consisting of 20 mM HEPES–KOH and 1 mM DTT, pH 7.6, and measurements were carried out at 23 °C. The data were collected and analyzed according to the Eadie–Hofstee method (Eadie, 1942).

RESULTS

Structure of Oligoribonucleotides. Capped oligoribonucleotides were prepared by cell-free transcription in the presence of the cap analogue in order to investigate the effects of oligonucleotide length and secondary structure on the eIF-4E–mRNA interaction. These oligoribonucleotide sequences were chosen so that the desired secondary structure was significantly more stable than any other possible secondary structures or dimers. Small changes in the sequence of the linear portion of the oligoribonucleotide were required in some cases in order to maintain the desired secondary structural features. These structures are shown in Figure 1. In order

Table I: Summary of the Dynamic Quenching Constants (K_D) and Bimolecular Quenching Constants (k_q) for Acriflavine-Labeled Oligoribonucleotides

oligoribonucleotide	% labeled	K_D (M^{-1})	$k_q \times 10^{-10}$ (M^{-1} s^{-1})[a]
I	48	96.1 ± 6.0	2.18 ± 0.14
II	31	82.7 ± 7.0	1.87 ± 0.16
III	16	33.2 ± 3.0	0.76 ± 0.07
IV	48	89.1 ± 9.0	2.03 ± 0.20

[a] $k_q = K_D/\tau_0$ where τ_0 is 4.4 ns (Churchich, 1963).

to predict the most stable structures for the oligoribonucleotides constructed, all probable secondary structures and dimers for oligoribonucleotides I–V were determined using the Genbank Prophet and OLIGO (Rychlik & Rhoads, 1989) programs, and the relative stabilities of these structures have been calculated using Turner's modifications of Tinoco's rules (Tinoco, 1973; Freier et al., 1986) and the OLIGO program. In addition, the melting temperatures (T_m) of the constructed oligoribonucleotides were determined in buffer A, pH 7.6, containing 1 mM NaCl, utilizing a Cary-3 UV/VIS spectrophotometer outfitted with a Cary temperature controller accessory (data not shown); the experimentally determined T_m values were found to be within 2 °C of the values calculated by the OLIGO program for the structures shown in Figure 1. These data are consistent with the most stable structures of the oligoribonucleotides presented in Figure 1.

Oligoribonucleotide II contains a hairpin structure with a ΔG(25 °C) value of −12.6 kcal/mol according to Tinoco's rules; this oligoribonucleotide is an analogue of the 5′-terminal region of rabbit α-globin mRNA, which contains a hairpin with a calculated ΔG(25 °C) value of −7.6 kcal/mol (Baralle, 1977). The difference between the ΔG values for oligoribonucleotide II and α-globin mRNA may be accounted for by two differences in structure: α-globin mRNA has a single base bulge in the hairpin stem and has eight more nucleotides in the hairpin loop than oligoribonucleotide II (Baralle, 1977). Both of these changes increase the stability of the stem in oligoribonucleotide II.

Characterization of Cap Accessibilities of Oligoribonucleotides. The percent labeling of the oligoribonucleotides with acriflavine was found to correlate with the relative cap accessibilities one would infer from comparing the structures shown in Figure 1. Oligoribonucleotide III would be expected to have a relatively sterically inaccessible cap, and was 16% labeled; oligoribonucleotide II (the globin mRNA analogue) with intermediary cap accessibility was 31% labeled, and the presumed most cap-accessible oligoribonucleotide IV and linear oligoribonucleotide I were 48% labeled. In order to quantify the cap accessibilities, iodide quenching of these oligoribonucleotides was performed, and Stern–Volmer plots were constructed. The dynamic quenching constants (K_D) were obtained from the slopes of these plots, and the bimolecular quenching constants ($K_q = K_D/\tau_0$) were calculated using a τ_0 value of 4.4 ns for acriflavine (Churchich, 1963); these values are summarized in Table I. The magnitude of the K_D values reflects the relative accessibility of the fluorophore to the quencher; a larger K_D value indicates greater accessibility. The values of K_D (and K_q) decrease in the order oligoribonucleotide I > IV > II ≫ III.

The rate of decapping of the oligoribonucleotides with TAP and subsequent labeling of the 5′-terminus with [γ-^{32}P]ATP have been shown to be a measure of cap accessibility (Godefroy-Colburn et al., 1985b). The results of this assay for our oligoribonucleotide analogues are shown in Table II. The lag observed for some of the oligoribonucleotides may be attributed to the high concentration of oligoribonucleotide (relative to TAP concentration) employed, which could lead to longer reaction times required for an observable effect and completion of the reaction. Therefore, a comparison of the percent incorporation of the radioactive label after 60 min is a more reliable indicator of the extent of decapping and subsequent labeling. The extent of decapping of oligoribonucleotide III does not vary significantly even after the completion of the reaction at 60 min and has only 0.3% of the radioactive label incorporated. In contrast, the extent of decapping of oligoribonucleotides II and IV increases 8.5- and 17-fold, respectively, over the course of the reaction, with 2.5% and 24% of the label incorporated, respectively, after 60 min. Furthermore, after only 5 min, oligoribonucleotides II and IV have 2- and 9-fold greater extents of decapping than oligoribonucleotide III, respectively. The linear oligoribonucleotide I shows the greatest (36%) incorporation of the radioactive label after 60 min. These data therefore suggest that the m^{7}G cap of oligoribonucleotide IV is the most sterically accessible of the oligoribonucleotides and that of oligoribonucleotide II is moderately accessible and oligoribonucleotide III has a relatively inaccessible cap. These data are also consistent with the data obtained from the iodide quenching of the acriflavine-labeled oligoribonucleotides (Table I) and with the structure of the oligoribonucleotides shown in Figure 1.

Table II: Decapping and [γ-^{32}P]ATP Labeling of Oligoribonucleotides as a Function of Time[a]

oligoribonucleotide	time (min)	cpm[b]	% incorporation[c]
I	5	2000 ± 40	0.2
	15	2700 ± 55	0.3
	60	476000 ± 690	36.0
II	5	3700 ± 60	0.3
	15	6300 ± 79	0.5
	60	30000 ± 175	2.5
III	5	2000 ± 45	0.2
	15	2800 ± 52	0.2
	60	3200 ± 57	0.3
IV	5	18000 ± 133	1.5
	15	25000 ± 158	3.0
	60	304000 ± 552	24.0

[a] Oligoribonucleotides were decapped in the presence of TAP for the times indicated and subsequently 5′-labeled with [γ-^{32}P]ATP as described under Materials and Methods. [b] cpm reported were corrected for a background value of 50. [c] Percent incorporation was calculated from [(cpm$_{washed}$/cpm$_{unwashed}$) × 100, where cpm$_{unwashed}$ represents the cpm for the total amount of [γ-^{32}P]ATP added per sample (1.2×10^6) and cpm$_{washed}$ are the values listed in the above table.

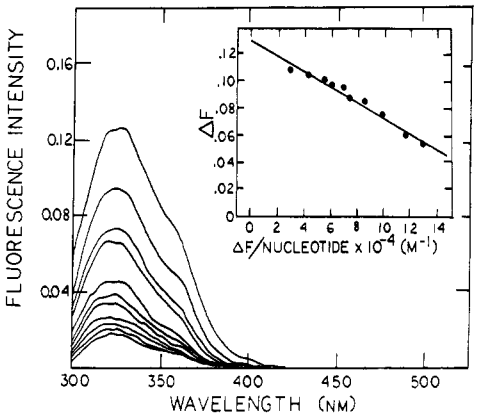

FIGURE 2: Fluorescence emission spectra of eIF-4E (5 μM) complexed with oligoribonucleotide II in buffer A, pH 7.6. The concentration of oligoribonucleotide was (top to bottom) 0. 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.8, 2.5, and 4.0 μM. The excitation wavelength was 258 nm. Inset: Eadie–Hofstee plot of the fluorescence data.

Table III: Equilibrium Association Constants (K_{eq}) for the Interaction of Oligoribonucleotides with eIF-4E in the Absence and Presence of 100 mM KCl and 2 mM $MgCl_2$[a]

oligoribonucleotides	$K_{eq} \times 10^{-5}$ (M^{-1})	
	0 mM KCl/ 0 mM $MgCl_2$	100 mM KCl/ 2 mM $MgCl_2$
I	10.6 ± 1.05	7.14 ± 0.29
II	17.4 ± 0.80	13.1 ± 0.10
III	4.32 ± 0.30	6.73 ± 0.53
IV	5.00 ± 0.11	9.88 ± 0.61
V	6.41 ± 0.41	5.97 ± 0.60
m^7GpppG[b]	3.77 ± 0.11	0.91 ± 0.03
rabbit globin mRNA	20.9 ● 1.0[c]	24.6 ± 1.2
AIDS	5.74 ± 0.60	6.88 ± 0.20

[a] All solutions in buffer A, pH 7.6, measured at 23 ± 0.2 °C. [b] Data from Carberry et al. (1989). [c] Data from Goss et al. (1990).

Interaction of eIF-4E with Oligoribonucleotides. The interaction of oligoribonucleotides with eIF-4E was monitored fluorometrically; the titration of eIF-4E with mRNA oligoribonucleotide II is shown in Figure 2. The maximum in the emission spectrum of eIF-4E at 330 nm has been primarily attributed to tryptophan residue(s) (McCubbin et al., 1988; Carberry et al., 1989; Goss et al., 1990); as the concentration of the oligoribonucleotide is increased, the eIF-4E fluorescence is quenched as a result of stacking interactions between tryptophan residue(s) within the binding site of eIF-4E and the nucleotide bases of the mRNA cap (McCubbin et al., 1988; Carberry et al., 1989). The change in fluorescence intensity at 330 nm as a function of nucleic acid concentration was used to construct Eadie–Hofstee plots, as shown in the inset of Figure 2. The equilibrium association constants (K_{eq}) for the oligoribonucleotide–eIF-4E interactions were calculated from the negative of the reciprocal of the slope of these plots. The K_{eq} values for the interaction of oligoribonucleotides I–V with eIF-4E in the presence and absence of 100 mM KCl and 2 mM $MgCl_2$, pH 7.6, are given in Table III.

Rabbit globin mRNA has 5.3-fold greater affinity for eIF-4E than the cap analogue m^7GpppG [Table II; see also Goss et al. (1990)], while the oligoribonucleotide analogue of the 5′-terminus of rabbit α-globin mRNA (oligoribonucleotide II) has 4.6-fold greater affinity for eIF-4E than m^7GpppG. The similarity in affinity of globin mRNA and oligoribonucleotide II for eIF-4E suggests that the mRNA structural elements required for efficient eIF-4E binding are contained in the 5′-terminal fragment represented by oligoribonucleotide II. In the presence of 100 mM KCl and 2 mM $MgCl_2$, globin mRNA and oligoribonucleotide II have 27- and 15-fold greater affinity for eIF-4E, respectively, than m^7GpppG (Table III). The enhanced effect observed in the presence of salt suggests that secondary structure (and possibly tertiary structure in the case of globin mRNA) may play a role in the interaction, since these structures are stabilized in the presence of salt (Holder & Lingrel, 1975).

We next wanted to determine whether the cap accessibility of oligoribonucleotides I–V could be correlated with their relative affinities for eIF-4E. Oligoribonucleotide III has a relatively sterically inaccessible cap as determined by acriflavine labeling (Table I) and TAP assay (Table II). This oligoribonucleotide has 4-fold less affinity for eIF-4E than the globin mRNA analogue oligoribonucleotide II. A similar effect is observed for the interaction of the 5′-terminus of HIV-1 mRNA with eIF-4E. This oligoribonucleotide, denoted "AIDS" (Table III), has a sterically inaccessible cap and a hairpin which is 3-fold more stable than that of oligoribonucleotide III [structure not shown; see Edery et al. (1989) and Parkin et al. (1988)]. In the presence of 100 mM KCl and 2 mM $MgCl_2$, both oligoribonucleotide III and AIDS have only 2-fold lower affinity for eIF-4E than oligoribonucleotide II, as the salt stabilization of the hairpin structure (and the 3′-linear segment in the case of oligoribonucleotide III) enhances the oligoribonucleotide–eIF-4E interaction.

If the relative accessibility of the cap is increased, either by omission of the hairpin (resulting in the linear oligoribonucleotide I) or by insertion of an additional six-base linear sequence to the 5′ side of the hairpin (oligoribonucleotide IV), the affinity for eIF-4E does not increase. The linear oligoribonucleotide I has slightly (1.7-fold) lower affinity for eIF-4E than oligoribonucleotide II regardless of the presence of KCl and $MgCl_2$ (Table III). The interaction of eIF-4E with the linear oligoribonucleotide which has had the m^7G cap removed yields a K_{eq} value of $(0.37 \pm 0.15) \times 10^5$ M^{-1}; this result is representative of an average 50-fold decrease in binding affinity upon removal of the cap. Oligoribonucleotide IV, with the longer linear insert, has 3.5-fold lower affinity for eIF-4E than oligoribonucleotide II in the absence of 100 mM KCl and 2 mM $MgCl_2$. In the presence of these salts, however, this difference is only 1.3-fold, and may be due to the additional stability of the 3′-linear segment (and possible folding) in the presence of salt.

If cap accessibility, as determined in this study, was solely responsible for the effects observed, then one would predict that oligoribonucleotides II and V would have approximately equal affinity for eIF-4E, since these structures have equally accessible caps. The only structural differences between oligoribonucleotides II and V are that oligoribonucleotide V has a slightly different sequence to the 5′ side of the hairpin and the sequence of the hairpin has been reversed in oligoribonucleotide V (Figure 1). Oligoribonucleotide V has approximately 2.5-fold lower affinity for eIF-4E than oligoribonucleotide II, suggesting that the primary sequence near the hairpin, or of the hairpin itself, may be important for eIF-4E binding. These results suggest the presence of the m^7G cap is required for eIF-4E binding; however, factors other than cap accessibility alone determine the efficiency of eIF-4E binding.

Discussion

The effects of cap accessibility and secondary structure in the 5′-terminal region of rabbit α-globin mRNA analogues on the efficiency of eIF-4E binding were investigated. These effects were found to change the K_{eq} values for the eIF-4E–oligoribonucleotide interactions from 1.3- to 4.5-fold. In contrast, the presence of the 5′ cap itself increases the affinity by approximately 50-fold. The magnitude of the effects of cap accessibility and secondary structure is similar to that reported for the RNA–coat protein interactions of R17 (Romaniuk et al., 1987) and Qβ (Witherell & Uhlenbeck, 1989), in which relatively small (2–4-fold) changes in the association constants were reported for the introduction of sequence variants at nonessential protein binding sites on the RNA, in contrast to changes of several orders of magnitude for essential protein binding sites. Similar small changes in affinity have been observed in an in vitro rabbit reticuloyte system which was deficient in certain protein synthesis factors (Abramson et al., 1988; viz., Tables II and III): the removal of both eIF-4F and eIF-4B results in a 4.8-fold decrease in translation, and removal of either factor results in a 2–3-fold decrease in globin translation; similarly, removal of eIF-4A results in a 4.4-fold decrease in translational efficiency. Lawson et al. (1988) have also found that the cross-linking of the most efficiently translated reovirus RNAs 1_{1+2+3} to rabbit reticulocyte eIF-4F is 2.5–3.5-fold greater than to the most poorly

translated reovirus RNA m_3 [viz., Figure 3D,E in Lawson et al. (1988)]. Therefore, the relative differences in affinity we report for binding are consistent with the magnitude of changes for the manipulation of components necessary for efficient translation.

Cap accessibility has been argued to play an important role in the efficiency of mRNA translation. If the steric accessibility of the m^7G cap alone were responsible for optimum eIF-4E binding, one would predict the affinity of eIF-4E for the hairpin-containing oligoribonucleotides to correlate with the K_D values of the acriflavine-labeled oligoribonucleotides (Table I) and the extent of decapping (Table II) and therefore the binding affinity would decrease in the order IV > II, V > III (Figure 1). However, this is not the case.

A comparison of the K_{eq} values for the interaction of our α-globin oligoribonucleotide analogues with eIF-4E can provide information as to which structural features, other than the m^7G cap and cap accessibility alone, can account for the different binding affinities observed. The effects of these other features (linear inserts, hairpins, and their position relative to the cap) additively contribute to the stability of the eIF-4E–mRNA interaction; therefore, the globin mRNA would bind eIF-4E more strongly than the cap analogues alone.

The insertion of a longer linear sequence into a hairpin-containing oligoribonucleotide, which increases the apparent steric accessibility of the m^7G cap (oligoribonucleotide IV), results in a *reduction* of eIF-4E binding (Table III). In the absence of salt, oligoribonucleotide IV would be expected to possess a more accessible cap than at high salt conditions; yet, under high salt conditions, the affinity of eIF-4E for the oligoribonucleotide increases, suggesting that eIF-4E may be interacting with the longer linear sequence which is stabilized in the presence of salt. The longer insert of oligoribonucleotide IV would provide a larger linear target for the binding of eIF-4E, relative to oligoribonucleotide II. A similar length effect has been observed for analogues of oligoribonucleotide I, which vary in length from 12 to 22 nucleotides (data not shown). The shorter oligoribonucleotides bind approximately 2-fold less to eIF-4E than oligoribonucleotide I, yet increasing the length of the oligonucleotide beyond that of oligoribonucleotide I does not significantly increase the binding affinity over that observed for oligoribonucleotide I. This suggests that there is a length effect for optimum eIF-4E binding to mRNA; a similar result has been described for the wheat germ cap binding proteins eIF-4F and eIF-(iso)4F (Carberry & Goss, 1991).

The effect of mRNA primary structure on the interaction can be determined by comparing the affinities of oligoribonucleotides II and V for eIF-4E. These oligoribonucleotides have equally accessible m^7G caps and hairpin structures of identical stability which differ only in the 5′ > 3′ sequence of the hairpin. The differences in the relative K_{eq} values for these oligoribonucleotides (Table II) reflect the ability of eIF-4E to differentially recognize primary sequences near the 5′-terminus. Oligoribonucleotide III, with a relatively inaccessible cap, binds eIF-4E with lower affinity than oligoribonucleotide II; yet this oligoribonucleotide has approximately equal affinity for eIF-4E as the AIDS oligoribonucleotide despite the latter possessing a more stable hairpin. The differences observed for oligoribonucleotide III and AIDS may be attributed to the length of the linear segment to the 3′ side of the hairpin structure. The longer linear segment found in oligoribonucleotide III may fold in a manner similar to that described for oligoribonucleotide IV, bringing this segment into proximity with the m^7G cap, and so provide addition stability for the interaction with eIF-4E. The AIDS oligoribonucleotide, devoid of this linear length, lacks the additional stability. In the presence of salt, however, the more stable AIDS hairpin, like the linear segment of oligoribonucleotide III, provides the additional stability for the eIF-4E interaction.

Therefore, for our globin analogues, the steric accessibility of the m^7G cap alone cannot account for differences in eIF-4E binding. The results presented here suggest that the presence of the m^7G cap is required for efficient eIF-4E binding; however, the steric accessibility of the cap is *not* the only determining factor for eIF-4E binding, since the presence and stability of mRNA secondary structural features, such as the hairpin, appear to influence the affinity of eIF-4E for the m^7G cap. Although the m^7G cap is the primary feature recognized by eIF-4E, the additional primary and secondary structural features of mRNA can additively contribute to the binding of eIF-4E, and thereby enhance the affinity of globin mRNA for this factor.

If cap accessibility is an important determinant in overall translation rates, then it must influence other factors such as eIF-4B. One plausible mechanism is that eIF-4E, either alone or as part of the eIF-4F complex, binds to the cap region. This interaction is particularly stable in the presence of an optimumly placed hairpin, such as in oligoribonucleotide II. eIF-4B may then interact and affect the secondary structure of the mRNA. The fact that inaccessible caps bind eIF-4E, yet are poorly translated, would suggest that eIF-4B may need a linear segment of mRNA near the cap for interaction. The optimum configuration might well be one in which eIF-4B has contact with eIF-4E (or eIF-4F) and both a single- and double-stranded region of mRNA. This is consistent with the fact that the initiation factors require a single-stranded region to initiate helicase activity (Rozen et al., 1990). further studies with eIF-4B are underway to investigate these effects.

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