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Articles

Fluorescence Study of the Binding of m^7GpppG and Rabbit Globin mRNA to Protein Synthesis Initiation Factors 4A, 4E, and 4F†

Dixie J. Goss,*,‡ Susan E. Carberry,‡ Thomas E. Dever,§ William C. Merrick,§ and Robert E. Rhoads‖

Department of Chemistry, Hunter College of the City University of New York, New York, New York 10021, Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, and Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536

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ABSTRACT: The interactions of protein synthesis initiation factors eIF-4E from human erythrocytes and eIF-4A and eIF-4F from rabbit reticulocytes with the cap analogue m^7GpppG and rabbit globin mRNA were investigated. The equilibrium binding constants for the binary complex formation of eIF-4E–eIF-4A, m^7GpppG–eIF-4E, m^7GpppG–eIF-4F, globin mRNA–eIF-4E, globin mRNA–eIF-4F, and globin mRNA–eIF-4A were measured by direct fluorescence titration experiments. The binding of eIF-4E to globin mRNA was found to be 5.5-fold tighter than its binding to m^7GpppG; the binding of eIF-4F for globin mRNA and m^7GpppG was similar to that of eIF-4E. Association equilibrium constants were determined for the ternary system mRNA–eIF-4E–eIF-4A; four thermodynamically independent equilibria characterize the system. These equilibrium binding constants were used to calculate coupling free energies, which provided an estimate of the cooperativity of the interaction of eIF-4E, eIF-4A, and mRNA. These coupling energies were all found to be small and positive, indicative of anticooperative binding.

The recognition of mRNA by the components of the translational machinery is a crucial step in the initiation of protein synthesis. Several mRNA structural features are important in this recognition process; one such feature is the 5′-terminal $m^7G(5')ppp(5')N$ moiety,[1] termed the cap structure. Several initiation factors have been shown to interact at or near the cap, including eIF-4A, eIF-4B, and eIF-4E [for reviews, see Shatkin (1985), Rhoads (1988), and Sonenberg (1988)]. In addition, the complex of eIF-4E, eIF-4A, and a 220-kDa polypeptide, termed eIF-4F, binds to the cap (Edery et al., 1983; Grifo et al., 1983). eIF-4E and eIF-4F, unlike eIF-4A and eIF-4B, bind directly to the m^7G cap (Tahara et al., 1981; Sonenberg, 1981; Sonenberg et al., 1981; Hellmann et al., 1982; Grifo et al., 1983; Webb et al., 1984; Goss et al., 1987). The eIF-4E component of eIF-4F has been shown to be responsible for the cap binding activity of eIF-4F, while the 46-kDa component, which has been shown to be a mixture of eIF-4AI and eIF-4AII (Nielsen and Trachsel, 1988; Dever and Merrick, unpublished), is responsible for ATP-dependent mRNA unwinding activity (Grifo et al., 1984; Lawson et al., 1989).

The interaction of eIF-4E and eIF-4A with mRNA has been extensively studied. eIF-4E, either separately or as part of eIF-4F, binds to the cap in the absence of ATP (Sonenberg, 1981; Hellmann et al., 1982) and independent of the degree of mRNA secondary structure (Pelletier and Sonenberg, 1985; Lawson et al., 1986). eIF-4A binds to single-stranded regions of mRNA with concomitant hydrolysis of ATP and may unwind mRNA secondary structure (Grifo et al., 1982; Ray et al., 1985; Goss et al., 1987). However, cross-linking studies have shown that eIF-4F binds more tightly to mRNA caps than eIF-4E alone (Abramson, 1989), implying that other polypeptides are involved in the mRNA recognition process. In the presence of ATP, eIF-4A is also capable of cross-linking to the mRNA cap, but only as part of the eIF-4F complex or in the presence of eIF-4E and eIF-4B (Grifo et al., 1983; Edery et al., 1983; Abramson, 1989). The inability of eIF-4A to bind the mRNA cap in the absence of eIF-4F or eIF-4E may indicate that eIF-4A does not interact with the cap per se but rather with the cap-binding component of eIF-4F (Rhoads, 1988). eIF-4A thus may influence cap binding as part of the eIF-4F complex.

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* To whom correspondence should be addressed.
‡ Hunter College of the City University of New York.
§ Case Western Reserve University.
‖ University of Kentucky.

[1] Abbreviations: m^7G, 7-methylguanosine; kDa, kilodalton; eIF, eukaryotic initiation factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol.

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From these results, it is clear that the activities of the various polypeptides involved in mRNA binding are profoundly influenced by each other. In order to better understand these interactions, we have begun to investigate some of the simpler binary interactions. One of the simplest of these is the binding of eIF-4E or eIF-4F with cap analogues (McCubbin et al., 1988; Carberry et al., 1989) and mRNA (Goss et al., 1987). In the present study, we compare by direct fluorometric titrations the interaction of eIF-4E and eIF-4F with the cap analogue m^7GpppG and rabbit globin mRNA. The effect of eIF-4A on eIF-4E cap binding has also been analyzed. These studies were performed in the absence of ATP to assess the effects of eIF-4A on the affinity of eIF-4E for caps, since eIF-4A in the presence of ATP may alter the mRNA structure. We have found that (1) both eIF-4F and eIF-4E bind more tightly to mRNA than to cap analogue, (2) there is no significant difference between eIF-4E and eIF-4F in binding affinity to either cap analogues or mRNA (in the absence of ATP), and (3) eIF-4E and eIF-4A form a complex in the absence of ATP. From these data, information was obtained on the relative stability and cooperativity of these complexes.

Materials and Methods

Rabbit globin mRNA (lot no. 58F3928) was purchased from Sigma Chemical Co. (St. Louis, MO); m^7GpppG was purchased from Pharmacia Molecular Biologicals (Milwaukee, WI). eIF-4E was isolated from human erythrocytes as described by Webb et al. (1984) and Rychlik et al. (1986); eIF-4A and eIF-4F were isolated from rabbit reticulocytes as described by Grifo et al. (1982, 1983). All solutions were prepared in buffer A, consisting of 20 mM HEPES and 1 mM DTT, pH 7.6. Fluorescence measurements were carried out at 23 °C, and the data were collected as described previously (Carberry et al., 1989). Equilibrium constants obtained under a variety of protein concentrations (data not shown) were the same, within experimental error, showing the reversibility of the reaction. All fluorescence emission spectra of the eIF-4E–eIF-4A complexes were corrected for the small eIF-4A contribution at 330 nm by subtracting the contribution of the free eIF-4A to the fluorescence; the contribution of m^7GpppG or globin mRNA to the eIF-4E fluorescence intensity at 330 nm was negligible. Furthermore, the inner filter effect was found to be less than 3% at the highest cap analogue (20 μM) or mRNA (2 μM) concentration; therefore, correction of the observed fluorescence intensity due to this effect was not necessary (Lakowicz, 1983). The equilibrium association constants, K, were obtained from the construction of binding isotherms and Eadie–Hofstee plots (Eadie, 1942).

Results

Fluorescence titrations were utilized to directly determine the affinity of eIF-4E and eIF-4F for m^7GpppG and rabbit globin mRNA, as well as the affinity of eIF-4E for eIF-4A. The fluorescence emission spectra of eIF-4E and eIF-4A are shown in Figure 1. eIF-4E (solid line) has a fluorescence emission maximum at 330 nm and a shoulder at 360 nm; the intensity of the spectrum has been attributed mainly to tryptophan residues (McCubbin et al., 1988; Carberry et al., 1989); a similar fluorescence spectrum was obtained for eIF-4F (data not shown). In contrast to mammalian eIF-4E, which contains eight tryptophan residues (Rychlik et al., 1987; McCubbin et al., 1988) corresponding to 3% of the total number of amino acid residues, mammalian eIF-4A contains only three tryptophan residues (Nielsen et al., 1985) corresponding to 0.5%. The lower tryptophan content of eIF-4A is consistent with its lower fluorescence (dashed line) at 330 and 360 nm relative

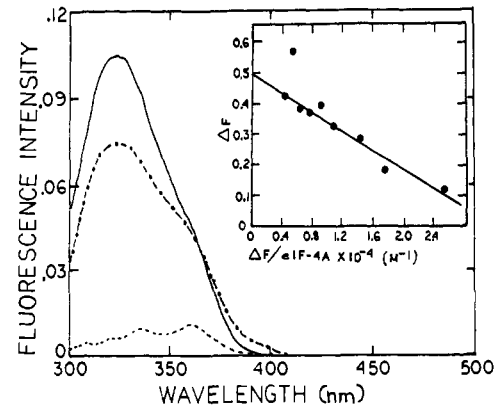

FIGURE 1: Fluorescence emission spectra of 5 μM eIF-4E (—), 4 μM eIF-4A (---), and an eIF-4E–eIF-4A complex (-·-) of equimolar (5 μM) concentrations of eIF-4E and eIF-4A. An excitation wavelength of 258 nm and a 1.4-mm slit were used; measurements were made at 23 °C. Inset: Eadie–Hofstee plot for the titration of eIF-4E with eIF-4A. The equilibrium association constant, K (M^{-1}), is obtained from the negative reciprocal of the slope.

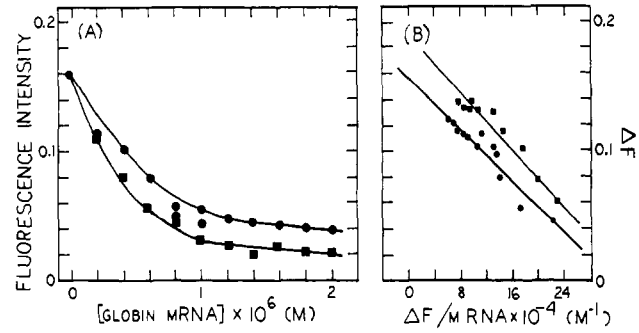

FIGURE 2: (A) Binding isotherm for the interaction of rabbit globin mRNA with 5 μM eIF-4E (circles) and 3 μM eIF-4F (squares) at 23 °C. The data have been normalized so that the initial fluorescence of the eIF-4E and eIF-4F solutions (no mRNA added) were identical. (B) Eadie–Hofstee plot of the data in (A). The equilibrium constant was determined as in Figure 1.

to eIF-4E; thus the eIF-4A contribution to the eIF-4A–eIF-4E complex is small. The fluorescence emission spectrum for the eIF-4A–eIF-4E complex is shown in Figure 1 (solid circles). At equimolar (5 μM) concentrations of eIF-4E and eIF-4A, there is a 36% fluorescence quenching. The Eadie–Hofstee plot for the complete titration of eIF-4E with eIF-4A is shown in the inset of Figure 1 and indicates that these two factors bind to each other with an equilibrium constant of $(7.1 \pm 0.6) \times 10^5\ M^{-1}$. The linearity of the plots shows no indication of multiple binding sites or cooperativity of binding.

The interaction of eIF-4E derived from yeast and human erythrocytes with the cap analogue m^7GpppG has been previously described in detail (McCubbin et al., 1988; Carberry et al., 1989); an equilibrium binding constant of $(3.8 \pm 0.1) \times 10^5\ M^{-1}$ was reported for the human erythrocyte eIF-4E–m^7GpppG interaction (Carberry et al., 1989) and under similar conditions with 0.1 M KCl a value of $(2.1 \pm 0.2) \times 10^5\ M^{-1}$ was reported for the yeast eIF-4E–m^7GpppG interaction (McCubbin et al., 1988). It was of interest to compare this binding with the binding of eIF-4E to its physiological ligand, mRNA itself. The binding isotherm and Eadie–Hofstee plot for the binding of rabbit globin mRNA to eIF-4E are shown in parts A and B of Figure 2, respectively (circles); an average equilibrium binding constant of $(21 \pm 1.0) \times 10^5\ M^{-1}$ was obtained. Similar experiments were performed for the binding of eIF-4F to globin mRNA (Figure 2A,B, squares) and to m^7GpppG (data not shown). The equilibrium binding constant for the formation of the eIF-4F–m^7GpppG complex was found

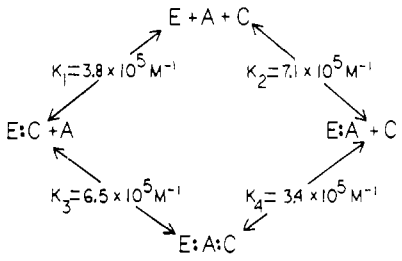

FIGURE 3: Scheme for the interaction of the cap analogue m^7GpppG with eIF-4E and eIF-4A (denoted as C, E, and A, respectively). K_1 to K_4 refer to equilibrium binding constants for the interactions shown and were obtained as described in the text. The K_{eq} value for the m^7GpppG–eIF-4F interaction was found to be $(2.04 \pm 0.1) \times 10^5$ M^{-1}.

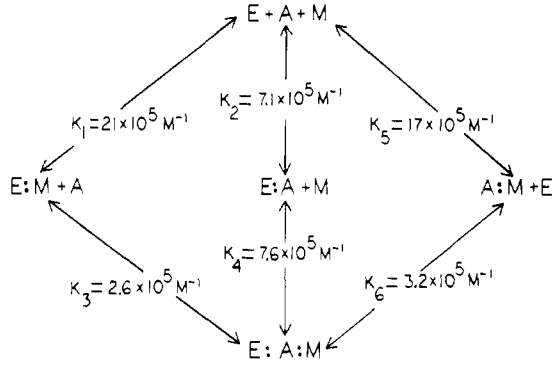

FIGURE 4: Scheme for the interaction of rabbit globin mRNA with eIF-4E and eIF-4A (denoted as M, E, and A, respectively). K_1 to K_6 refer to equilibrium binding constants for the interactions shown and were obtained as described in the text. The K_{eq} value for the globin mRNA–eIF-4F interaction was found to be $(18.6 \pm 1.1) \times 10^5$ M^{-1}.

to be $(2.04 \pm 0.1) \times 10^5$ M^{-1} and that of the eIF-4F–globin mRNA complex to be $(18.6 \pm 1.1) \times 10^5$ M^{-1}. These values are similar to the values obtained for the binding of m^7GpppG and globin mRNA to eIF-4E.

Having demonstrated binary interactions of eIF-4E with eIF-4A and of eIF-4E with m^7G-containing moieties, we were next interested in examining the simultaneous interaction of all three. A scheme can be developed to describe the interaction of m^7GpppG with both eIF-4E and eIF-4A, as shown in Figure 3. The values of K_1, K_2, and K_4 were determined in independent fluorescence titration experiments similar to those described above. K_1 and K_2 were obtained from the titration of eIF-4E with m^7GpppG and eIF-4A, respectively; K_4 was obtained from the titration of an eIF-4E–eIF-4A complex (in which 50% of the eIF-4A and 96% of the eIF-4E was determined to be bound) with m^7GpppG. Such a scheme contains three thermodynamically independent equilibrium constants. K_3 was chosen to be the thermodynamically dependent equilibrium constant and was obtained from the relationship

$$K_3 = (K_2K_4)/K_1 \quad (1)$$

The scheme presented in Figure 3 indicates that the formation of an eIF-4E–eIF-4A complex does not alter the affinity of the cap analogue m^7GpppG for eIF-4E (comparing K_1 and K_4). Similarly, the formation of the eIF-4E–m^7GpppG complex does not alter the affinity of eIF-4E for eIF-4A (comparing K_2 and K_3).

A similar scheme can be written for the interaction of rabbit globin mRNA with eIF-4E and eIF-4A, as shown in Figure 4. Since eIF-4A can interact directly with globin mRNA, in contrast with cap analogues, additional terms are included in the scheme. K_1, K_2, K_3, and K_4 were obtained as described above for m^7GpppG; the value of K_5 was taken from Goss et al. (1987), and K_6 is given by

$$K_6 = (K_2K_4)/K_5 \quad (2)$$

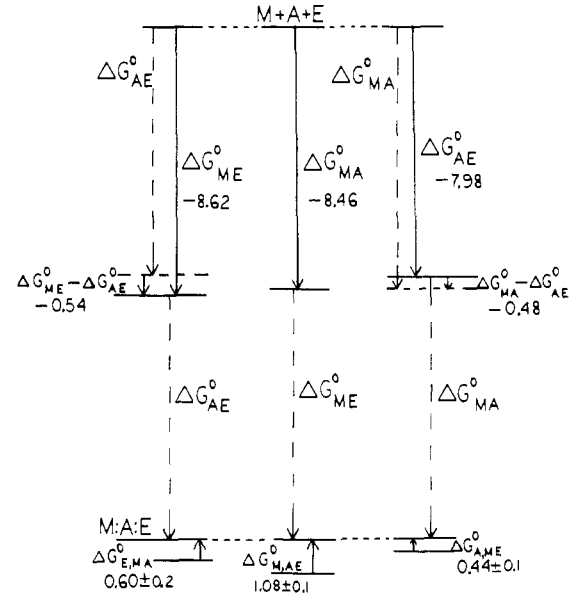

FIGURE 5: Representation of the binding and coupling free energies for the interaction of rabbit globin mRNA (M), eIF-4E (E), and eIF-4A (A). The interactions are drawn to scale. All ΔG° values are reported in kilocalories per mole.

In order to quantitate these interactions, the coupling energies were calculated according to the method of Weber (1975). The coupling energies provide an indication of the overestimation or underestimation of the free energy of binding of eIF-4A and eIF-4E to globin mRNA (ΔG°_{MAE}) from the component binding energies for the interaction of mRNA with eIF-4E (ΔG°_{ME}), mRNA with eIF-4A (ΔG°_{MA}), and eIF-4E with eIF-4A (ΔG°_{AE}). These interactions can be expressed in several ways, some of which appear to be paradoxical; however, each value represents a different binding perspective. The coupling energies are defined as (Goss et al., 1984)

$$\Delta G^\circ_{M,AE} = \Delta G^\circ_{MAE} - \Delta G^\circ_{MA} - \Delta G^\circ_{ME} \quad (3a)$$

$$\Delta G^\circ_{E,MA} = \Delta G^\circ_{M,AE} + \Delta G^\circ_{MA} - \Delta G^\circ_{AE} \quad (3b)$$

$$\Delta G^\circ_{A,ME} = \Delta G^\circ_{M,AE} + \Delta G^\circ_{ME} - \Delta G^\circ_{AE} \quad (3c)$$

where E, A, and M denote eIF-4E, eIF-4A, and rabbit globin mRNA, respectively. ΔG°_{MA}, ΔG°_{ME}, and ΔG°_{AE} were obtained from K_5, K_1, and K_2 of Figure 4, respectively; ΔG°_{MAE} was obtained from the sum of the ΔG° values of K_1 and K_3. The interactions are shown in Figure 5, where we have defined $\Delta G^\circ_{M,AE}$ as the free energy for the binding of eIF-4E and eIF-4A to mRNA. Looked at from the perspective of mRNA, this gives an indication of the effect of the binding of one protein on the affinity of the mRNA site for the other protein. For example, the binding of eIF-4E could potentially affect the affinity of mRNA for eIF-4A. Effectively, each component is treated as having two binding sites, and the coupling energy expresses the interaction of those sites. These coupling energy values may be negative, positive, or zero, indicating that the binding of the first "ligand" to the component has a cooperative effect, an anticooperative effect, or no effect on the binding of the second ligand, respectively.

The coupling energies for the interaction of eIF-4E and eIF-4A with mRNA, $\Delta G^\circ_{M,AE}$, $\Delta G^\circ_{E,MA}$, and $\Delta G^\circ_{A,ME}$, are all found to be small and positive, indicative of anticooperative binding. The value obtained for $\Delta G^\circ_{A,ME}$ (0.44 ± 0.10 kcal/mol) is quantitatively similar to that of $\Delta G^\circ_{E,MA}$ (0.60

± 0.20 kcal/mol); thus, the binding of eIF-4E to eIF-4A only slightly reduces the affinity of mRNA for its respective binding sites on either eIF-4A or eIF-4E in the eIF-4E–eIF-4A complex. However, a slightly greater degree of anticooperactivity is obtained for $\Delta G°_{M,AE}$ (1.08 ± 0.10 kcal/mol), which represents the binding of eIF-4E and eIF-4A to their respective sites on mRNA. In this latter case, the binding of the first factor (e.g., eIF-4A to the mRNA) may partially overlap or block the mRNA binding site of the second factor (eIF-4E).

Discussion

The data presented in this direct binding study provide insight into the mechanism of interaction of eIF-4A, eIF-4E, and eIF-4F with rabbit globin mRNA and the cap analogue m^7GpppG. One notable difference between m^7GpppG and rabbit globin mRNA is the affinity with which they bind eIF-4E. Chu and Rhoads (1980) previously reported that globin mRNA was a better inhibitor of ovalbumin synthesis then the cap analogue m^7GTP. However, in that study, the effects were measured in a complete translation system; therefore, it was not possible to distinguish between inhibition caused by globin mRNA binding to eIF-4E and depletion of other essential components for ovalbumin synthesis due to globin synthesis. The present results, however, clearly demonstrate that globin mRNA has a greater affinity for eIF-4E than does the cap analogue (21.0×10^5 M^{-1} versus 3.8×10^5 M^{-1}). We have also shown here that the affinity of globin mRNA for eIF-4F is greater than that of m^7GpppG (18.6×10^5 M^{-1} versus 2.0×10^5 m^{-1}). This implies that eIF-4E and eIF-4E component of eIF-4F may interact with structural features of mRNA in addition to the cap. A quantitatively similar result for the value of the equilibrium binding constant for the eIF-4F–mRNA complex was previously reported (Goss et al., 1987). The value of the equilibrium binding constant for the eIF-4F–mRNA complex is also in agreement with that calculated from nitrocellulose filter binding assays [Abramson et al. (1987), viz., Table IV]; the value we calculate from this assay was found to be 14.8×10^5 M^{-1}, which is similar to that reported in the present study (within experimental error).

Although the affinity of either factor for globin mRNA is greater than its affinity for cap analogue, eIF-4E and eIF-4F have similar affinities for cap analogue (3.8×10^5 M^{-1} and 2.0×10^5 M^{-1}, respectively). Also, the two factors bind globin mRNA with nearly the same affinity (21.0×10^5 M^{-1} and 18.6×10^5 M^{-1}, respectively). This is surprising since it has been reported that eIF-4F cross-links to mRNA caps more strongly than eIF-4E (Abramson, 1989).

The equilibrium binding constants for the interaction of mRNA with eIF-4E and eIF-4A are also very similar. However, unlike eIF-4E, eIF-4A is known to bind in a structure specific, sequence nonspecific way to mRNA (Abramson et al., 1987), and the binding of the first eIF-4A to mRNA cooperatively enhances the binding to subsequent eIF-4A molecules (Goss et al., 1987). This situation may appear to complicate the interpretation of the fluorescence data obtained in this study, since one could potentially be monitoring several binding events. However, the design of the fluorescence titration experiment allowed us to focus on the binding of the eIF-4A–eIF-4E complex to mRNA. By monitoring the eIF-4E fluorescence at 330 nm upon titration of the eIF-4E solution (whether or not eIF-4A had been previously complexed with the eIF-4E), we were able to primarily measure the cap binding. Binding of additional eIF-4A would not contribute to the fluorescence change at this wavelength. Furthermore, ATP was not employed in these studies, since we were specifically monitoring the binding of the factors to the cap region of the mRNA. Cross-linking data have shown (Abramson et al., 1987) that the binding of eIF-4F to the mRNA cap is an ATP-independent step; however, ATP is required for the secondary structure unwinding and single-stranded mRNA binding activity of eIF-4A and eIF-4F (Grifo et al., 1982; Ray et al., 1985).

It is unclear at this time whether the functional factor for cap binding is eIF-4E or eIF-4F. We have presented the first evidence that there is a *direct* interaction between eIF-4E and eIF-4A in the absence of ATP, and one can envisage such an interaction occurring within the eIF-4F complex. If eIF-4F *is* the cap-recognizing factor, however, it is important to note that the complexation of eIF-4A with eIF-4E does not reduce or enhance the affinity of eIF-4E for the mRNA cap. This is in line with the observation, noted above, that eIF-4E and eIF-4F have similar affinities for the cap. Evidence that is consistent with a functional eIF-4E–eIF-4A interaction prior to mRNA binding is that formation of the complex is favored statistically, since eIF-4A is 10 times more abundant than eIF-4E in the cell (Duncan et al., 1987). We have calculated the amounts of eIF-4E and eIF-4A that occur as free factors in the cell. By use of the values determined for the relative abundance of these factors in HeLa cells by two-dimensional separation of proteins (Duncan et al., 1987) and the approximation that there are 5×10^7 cells/mL of packed volume, the solution volume of the cell is 2×10^{-11} L, and the total concentrations of eIF-4E and eIF-4A in the cell are calculated to be 66.4 nM and 830 nM, respectively. By use of the K_{eq} value of 7.1×10^5 M^{-1} reported here, values of 42 nM eIF-4E and 810 nM eIF-4A are obtained for the concentration of free eIF-4E and eIF-4A in the cell. Therefore, 37% of eIF-4E and 7% of eIF-4A are theoretically bound in an eIF-4E–eIF-4A complex (possibly as components of eIF-4F) in the cell. Cellular conditions may, however, alter the amount of complex found. In an earlier study, Hiremath et al. (1985) found somewhat lower levels of eIF-4E (about 8 nM) in reticulocyte lysate, which corresponds to 16 nM in intact reticulocytes; this value is 4-fold lower than that calculated here but assumes that all of the eIF-4E is retained by m^7GTP–Sepharose. A similar calculation for the amount of globin mRNA bound to eIF-4E in the cell has also been performed. When an mRNA concentration of 6 pmol of mRNA/100 μL (lysate) or 12 pmol of mRNA/100 μL (cell) reported by Jagus and Safer (1979) and the K_{eq} value of 21×10^5 M^{-1} (Figure 4) are utilized, values of 53 nM and 110 nM are obtained for the concentration of free eIF-4E and globin mRNA in the cell, respectively; these values correspond to 20% eIF-4E and 12% mRNA bound.

The alternative model is that eIF-4E and eIF-4A bind sequentially to their respective sites on mRNA (represented by $\Delta G°_{M,AE}$). This scheme is energetically less favored, although not by a large margin and is consistent with a mechanism in which the sequential binding of the first initiation factor to mRNA partially blocks the mRNA binding site of the second initiation factor. The data presented here indicate that, using binding constants and cellular concentrations of factors, one cannot rule out either model.

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O^6-Methylguanine and A·C and G·T Mismatches Cause Asymmetric Structural Defects in DNA That Are Affected by DNA Sequence†

Jeffrey M. Voigt and Michael D. Topal*

Lineberger Cancer Research Center and Departments of Pathology and Biochemistry, University of North Carolina Medical School, Chapel Hill, North Carolina 27599-7295

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ABSTRACT: Mismatched and modified base pairs are central to questions of DNA mutation and repair. NMR and X-ray crystallography of mispairs indicate little to no local helical distortion, but these techniques are not sensitive to more global distortions of the DNA molecule. We used polyacrylamide gel electrophoresis and thermal denaturation to examine A·C, G·T, and O^6-methylG·T and O^6-methylG·C mismatches synthesized in place of either of two adjacent G·C base pairs in synthetic DNA duplexes. Substitution for G·C at either position decreased the stability of the duplex; O^6-methylguanine was more destabilizing in place of the 5′G than in place of the 3′G. Comparisons between polymers synthesized so that lesions occurred regularly spaced on the same side of the helix and polymers synthesized so that the lesions alternated from side to side on the helix showed that these lesions introduced helical distortion composed of (i) a symmetric frictional component, probably caused by localized bubble formation, and (ii) an asymmetric component indicative of a more global effect on the DNA molecule. Comparisons between these effects at the two adjacent positions show that the extent of structural perturbation depends on sequence context.

Two types of mutagenic lesions, namely, mismatched and modified base pairs, occur in DNA. Mismatched base pairs occur mainly as the products of natural processes: mismatched base pairs arise normally during genetic recombination (Bianchi & Radding, 1983), as incorporation errors during DNA replication (Loeb & Kunkel, 1982), and during the folding of single-strand DNAs. Mismatched base pairs also occur as the product of potentially detrimental processes such as the deamination of 5-methylcytosine in DNA to give G·T (Zell & Fritz, 1987) and repair of O^6-methylguanine–thymidine

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* Correspondence should be addressed to this author at the Lineberger Cancer Research Center, CB 7295, UNC—Chapel Hill, Chapel Hill, NC 27599-7295.

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