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A Spectroscopic Study of the Binding of N-7-Substituted Cap Analogues to Human Protein Synthesis Initiation Factor 4E†

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ABSTRACT: The binding of N-7-substituted cap analogues to eIF-4E from human erythrocytes is described. Data presented here indicate that there is a correlation between the tightness of binding of these cap analogues to eIF-4E and their potency as inhibitors of protein synthesis. This result indicates that the inhibitory activity of the cap analogues is strictly a function of the affinity of the analogue for eIF-4E under equilibrium conditions. The pH dependence of binding of the cap analogues to eIF-4E indicates that the enolate form of the cap is preferred, as originally postulated by Rhoads et al. [(1983) *Biochemistry 22*, 6084-6088]. Data indicate that there are differences in the mode of binding of alkyl-substituted and aryl-substituted cap analogues to eIF-4E arising from favorable interactions of the phenyl ring with the guanosine moiety. These differences may explain the enhanced recognition of the aryl-substituted cap analogues by eIF-4E.

All eukaryotic cellular mRNA contains a 5′-cap structure which has been shown to facilitate ribosome binding to mRNA during initiation (Rhoads, 1985; Shatkin, 1985; Sonenberg, 1988) and impart stability to mRNA (Furuichi et al., 1977). Important structural features of the mRNA cap include an N-7-substituted, positively charged guanosine (Adams et al., 1978; Furuichi et al., 1979), the C-2 amino and C-6 keto substituents of the capping guanosine (Adams et al., 1978), and a hydrogen-bond acceptor α-phosphate (Canaani et al., 1976; Hickey et al., 1976; Darzynkiewicz et al., 1981, 1987). Increasing the number of phosphates was shown to enhance the inhibitory effects of m^7G[1] cap analogues by maintaining

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[1] Abbreviations: m^7G, 7-methylguanosine; m^7GMP, 7-methylguanosine 5′-monophosphate; m^7GTP, 7-methylguanosine 5′-triphosphate; e^7GMP, 7-ethylguanosine 5′-monophosphate; bn^7GMP, 7-benzylguanosine 5′-monophosphate; (2-phet)^{7}GMP, 7-(2-phenylethyl)-guanosine 5′-monophosphate; $m^{2,7}GMP$, 2,7-dimethylguanosine 5′-monophosphate; $m^{2,2,7}GMP$, 2,2,7-trimethylguanosine 5′-monophosphate; CD, circular dichroism; eIF, eukaryotic initiation factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate disodium salt.

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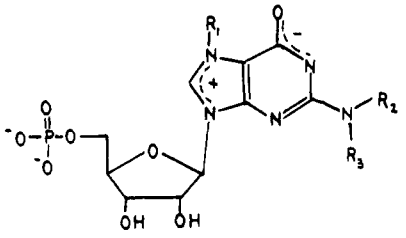

m^7GMP	$R_1 = -CH_3$	$R_2 = R_3 = -H$
e^7GMP	$R_1 = -CH_2CH_3$	$R_2 = R_3 = -H$
bn^7GMP	$R_1 = -CH_2C_6H_5$	$R_2 = R_3 = -H$
$(2\text{-phet})^7GMP$	$R_1 = -CH_2CH_2C_6H_5$	$R_2 = R_3 = -H$
$m^{2,7}GMP$	$R_1 = R_2 = -CH_3$	$R_3 = -H$
$m^{2,2,7}GMP$	$R_1 = R_2 = R_3 = -CH_3$	

FIGURE 1: Structures of N-7-substituted cap analogues employed in this study.

the cap in a more rigid conformation (Kim & Sarma, 1977; Hickey et al., 1977).

Early work by Adams et al. (1978) indicated that the presence of any alkyl or aryl substituent at the N-7 position of the capping guanosine was sufficient for recognition by the initiation machinery, as these authors reported that m^7, e^7, and bn^7GDP all blocked the attachment of wheat germ ribosomes to capped reovirus mRNA to an equal extent. More recent studies by Darzynkiewicz et al. (1989) have shown that alkyl derivatives larger than ethyl at the N-7 position were ineffective inhibitors of protein synthesis, while the aryl derivatives bn^7GMP and $(2\text{-phet})^7GMP$ were found to be better inhibitors of protein synthesis than m^7GMP.

The C-2 amino group has also been shown to be important for cap function. Removal of the amino group decreases the inhibitory activity of the cap, and replacement by a keto substituent abolishes the activity (Adams et al., 1978). Work by Darzynkiewicz et al. (1988) has shown that $m^{2,7}GMP$ was a much more effective inhibitor of protein synthesis than m^7GMP, while $m^{2,2,7}GMP$ was ineffective. These findings have led several groups to postulate that the C-2 amino group may be important for cap binding (Ishida et al., 1983; Ueda et al., 1988).

Although mRNA caps can be cross-linked to several initiation factors, only eIF-4E has been shown to bind specifically to the mRNA cap (Tahara et al., 1981; Sonenberg, 1981; Sonenberg et al., 1981; Hellmann et al., 1982; Webb et al., 1984). A model for the interaction of eIF-4E with the mRNA cap has been recently proposed which involves a tryptophan residue stacking with the capping guanosine (Carberry et al., 1989). In this study, the cap–eIF-4E interaction is further characterized by utilizing various alkyl and aryl N-7-substituted cap analogues.

Materials and Methods

The cap analogues e^7GMP, e^7GpppG, bn^7GMP, bn^7GpppG, $(2\text{-phet})^7GMP$, $m^{2,7}GMP$, $m^{2,7}GpppG$, and $m^{2,2,7}GpppG$ were synthesized as described elsewhere (Darzynkiewicz et al., 1988, 1989); m^7GTP (Lot OI614364) and m^7GpppG (Lot QK82463501) were purchased from Pharmacia Molecular Biologicals (Milwaukee, WI), and m^7GMP (Lot 59C-7470) was purchased from Sigma Chemical Co. (St. Louis, MO). The structures of the cap analogues are shown in Figure 1. eIF-4E was isolated from human erythrocytes as described by Webb et al. (1984) and Rychlik et al. (1986). Buffer A was 20 mM HEPES and 1 mM DTT; the pH of buffer A was varied between 6.6 and 8.5. Buffer B was 10 mM sodium phosphate and 0.2 mM EDTA, pH 7.0.

Table I: Equilibrium Binding Constants (K_{eq}) for eIF-4E–Cap Analogue Complexes at pH 7.6[a]

cap analogue	$K_{eq} \times 10^{-5}$ (M^{-1})	cap analogue	$K_{eq} \times 10^{-5}$ (M^{-1})
m^7GMP	1.64 ± 0.30	bn^7GMP	5.26 ± 0.28
m^7GTP	3.86 ± 0.15	bn^7GpppG	3.17 ± 0.26
m^7GpppG	3.77 ± 0.11	$m^{2,7}GMP$	3.27 ± 0.20
e^7GMP	0.452 ± 0.023	$m^{2,7}GpppG$	2.25 ± 0.24
e^7GpppG	1.27 ± 0.10	$m^{2,2,7}GpppG$	0.54 ± 0.05
$(2\text{-phet})^7GMP$	2.06 ± 0.31		

[a] All solutions were prepared in buffer A. Measurements were made at 23 ± 0.2 °C.

Fluorescence measurements were performed on a custom-designed spectrophotometer, as previously described (Carberry et al., 1989). For all measurements, an excitation wavelength of 258 nm, a 1.4-mm slit, and a 1-cm cell path were employed. Typical titration experiments were performed by adding 1–5-μL aliquots of cap analogue to a 100-μL solution containing 4–5 μM eIF-4E in buffer A, which had been previously adjusted to the appropriate pH; the concentrations of cap analogues utilized were in the range 1–20 μM. Since the inner filter effect was less than 3% at the highest cap concentration, correction of the observed fluorescence was not necessary (Lakowicz, 1983); however, corrections were made for the dilution of the sample in the course of the titration. The fluorescence of eIF-4E was typically monitored at 330 nm; this maximum has been attributed mainly to the fluorescence of the tryptophan residues of eIF-4E. Upon addition of cap analogue, a quenching of this maximum is observed; thus, binding isotherms were constructed by monitoring the decrease in the fluorescence intensity at 330 nm as a function of cap analogue concentration. The equilibrium binding constants (K_{eq}) were estimated from the midpoint of the binding isotherm and calculated from the slopes of Eadie–Hofstee plots (Eadie, 1942).

Circular dichroism (CD) measurements were performed on a Jobin-Yvon Mark V circular dichrograph interfaced with an Apple IIe microcomputer with fast arithmetic processors. All samples for CD measurement were prepared in buffer B, pH 7.0.

Results

Recent studies have shown that the structure of the N-7 substituent of the cap analogue affects the potency of the analogue as an inhibitor of protein synthesis (Darzynkiewicz et al., 1989). Since initiation of protein synthesis is a complex process, the step(s) that are directly affected by the analogue cannot always be clearly identified. Therefore, to further characterize the interaction between the cap analogues and eIF-4E, direct binding measurements were performed, making use of the fluorescence change in eIF-4E upon interaction with a cap analogue. A summary of the equilibrium binding constants (K_{eq}) obtained for the interaction of the N-7-substituted cap analogues with eIF-4E at pH 7.6 is given in Table I. The relative levels of binding of these analogues to eIF-4E were then correlated with their reported efficacy as inhibitors of globin synthesis by an in vitro reticulocyte lysate translation assay as reported by Darzynkiewicz et al. (1988, 1989); in this assay, the efficacy of the cap analogue as an inhibitor was defined to be the concentration of cap analogue required for half-maximal inhibition. The result of this correlation is shown in Figure 2; all values are taken relative to m^7GMP (relative inhibition = relative binding = 1). The data are linearly

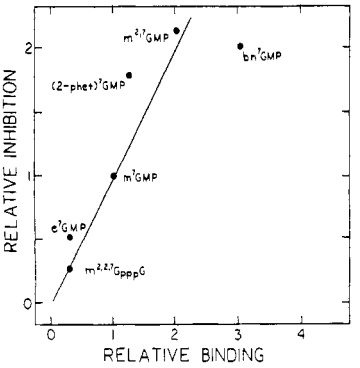

FIGURE 2: Correlation of relative levels of inhibition of protein synthesis and eIF-4E binding of N-7-substituted cap analogues. Inhibition and binding levels are taken relative to m^{7}GMP (relative binding = relative inhibition = 1). Inhibition assay data were taken from Darzynkiewicz et al. (1988, 1989). Binding studies were carried out in buffer A, pH 7.6, at 23 °C. The solid line denotes the least-squares linear correlation fit for the data.

correlated, as would be expected for a competitive binding model. However, the two aryl derivatives bn^{7}GMP and (2-phet)^{7}GMP do not show as high a correlation as the alkyl derivatives.

It has been suggested that the increase in cap recognition that occurs with increasing pH, whether observed by cell-free protein synthesis (Rhoads et al., 1983) or by direct binding of cap analogues to eIF-4E (Carberry et al., 1989), is due to the ionization of the N-1 proton and preferential recognition of the resulting enolate form by eIF-4E. The availability of N-7-substituted cap analogues offered the possibility to determine their pK values and, if significantly different, correlate these with the ability to bind eIF-4E. Each analogue was titrated and the ionization of the N-1 proton determined fluorometrically. Surprisingly, the pK values varied substantially: the pK values for bn^{7}GMP, (2-phet)^{7}GMP, e^{7}GMP, m^{7}GMP, m^{7}GTP, and m^{2,7}GMP were found to be 6.8, 6.9, 7.0, 7.2, 7.4, and 7.5, respectively. These values can be compared to those obtained by Darzynkiewicz and Lonnenberg (unpublished experiments) in an independent determination utilizing absorbance spectroscopy under different experimental conditions; the pK values of bn^{7}GMP, (2-phet)^{7}GMP, m^{7}GMP, and e^{7}GMP were found to be 7.18, 7.3, 7.2, and 7.26, respectively.

The pH dependence of binding of these analogues to eIF-4E are shown in Figure 3. The profiles of m^{7}GTP, m^{2,7}GMP, and, to a lesser extent, e^{7}GMP all demonstrate optimum binding at pH 7.6. Bn^{7}GMP and (2-phet)^{7}GMP show little variation in the level of binding to eIF-4E in the pH range 6.5–7.6, but the level of binding decreases above pH 7.6 in a manner similar to that of m^{7}GTP and m^{2,7}GMP. The pH dependence of the binding profile of e^{7}GMP is somewhat ambiguous due to the large error (up to 30%) incurred in these measurements as a result of the low levels of binding observed. The differences in the pH dependence of binding for the cap analogues can be more clearly seen in the inset of Figure 3, where the binding constants have been normalized to a value of 1 at the pH 7.6 optimum. In the low pH range, the data fall into two sets: the aryl N-7-substituted cap analogues [bn^{7}GMP and (2-phet)^{7}GMP], which have normalized K_{eq} values in the range 0.9–1.0; and the alkyl N-7-substituted cap analogues (m^{7}GTP, e^{7}GMP, and m^{2,7}GMP), which have normalized K_{eq} values that vary over a 3-fold range. The differences in the profiles of the two sets of cap analogues may be attributed to two factors. First, the pK values of the aryl N-7-substituted cap analogues were found to be slightly lower

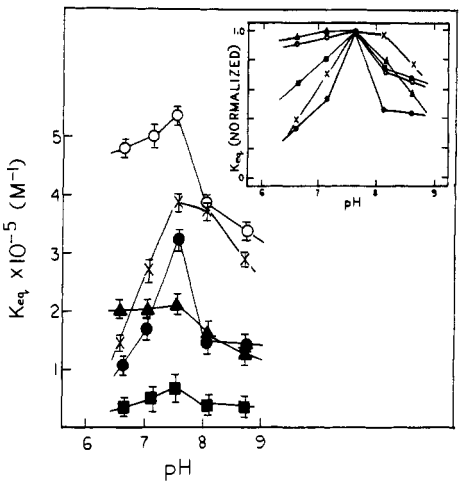

FIGURE 3: pH dependence of binding of bn^{7}GMP (○), m^{7}GTP (×), m^{2,7}GMP (●) (2-phet)^{7}GMP (▲), and e^{7}GMP (■) to eIF-4E. All solutions were prepared in buffer A, adjusted to the appropriate pH at 23 °C. The variance in the eIF-4E fluorescence intensity over the pH range was within experimental error (±10%). (Inset) pH dependence of binding profiles of the cap analogues to eIF-4E normalized to 1.0 at pH 7.6.

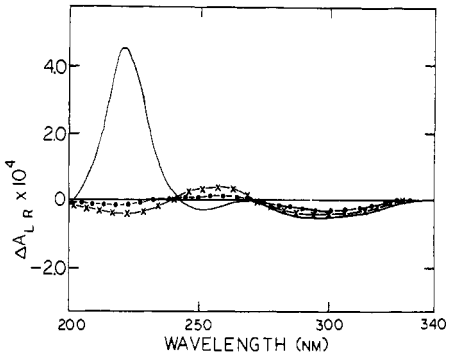

FIGURE 4: CD of bn^{7}GMP (—), m^{7}GMP (●), and m^{2,7}GMP (×). Solutions containing 24 μM cap analogue were prepared in buffer B, pH 7.0; the spectra were obtained at 23 °C.

than those of the alkyl N-7-substituted cap analogues; thus, the aryl cap derivatives are nearly fully ionized in the low pH range, and so the level of binding of these analogues to eIF-4E does not change. Second, the aryl substituents may provide the cap with a different electronic and/or steric configuration which is not possible for the alkyl-substituted cap analogues. Thus, the observed pH dependence of binding is determined not only by the pK of the cap analogue but also by the nature of the N-7 substituent. In the high pH range, there is no distinct pattern of falloff in the level of binding for the alkyl- or aryl-substituted cap analogues, and this decrease was found to be similar for all the analogues tested. The change in the pH dependence of binding at high pH values is attributed to the deprotonation of an interacting amino acid residue in eIF-4E, possibly a histidine, which is common for all analogue–eIF-4E interactions.

To further investigate the differences between the binding of aryl- and alkyl-substituted cap analogues to eIF-4E, the CD of bn^{7}GMP, m^{7}GMP, and m^{2,7}GMP were obtained (Figure 4). Both m^{7}GMP and m^{2,7}GMP were found to have negligible CD spectra; the spectrum of e^{7}GMP was found to be essentially the same as that of m^{7}GMP (data not shown). In contrast, bn^{7}GMP has a strong spectrum, with a maximum at 220 nm; (2-phet)^{7}GMP was found to have a small negative peak in the 210–220-nm range (data not shown). These findings suggest that the interaction between the phenyl ring of the N-7 aryl substituent and the guanosine residue is very

different from the interaction of the alkyl N-7 substituent with the guanosine.

Discussion

Correlation between the Binding of N-7-Substituted Cap Analogues to eIF-4E and Their Efficacy as Inhibitors of Protein Synthesis. The correlation between the levels of binding of N-7-substituted cap analogues to eIF-4E and the efficacy as inhibitors of protein synthesis (Figure 2) demonstrates that differences in inhibition can be explained by binding affinity under equilibrium conditions. This direct correlation eliminates other possible explanations for the inhibitory activity of the analogues such as kinetic effects, interaction of the analogues with the active sites of other factors, or changes in eIF-4E induced upon binding of the analogue which render it nonfunctional in the subsequent step of initiation (e.g., ribosome binding).

The direct binding measurements reported here and the inhibition assays of Darzynkiewicz et al. (1989) also indicate that the aryl derivatives bn^{7}GMP and (2-phet)^{7}GMP have a greater affinity for eIF-4E than the alkyl derivatives. Steric considerations alone would predict that these aryl derivatives would be less effective inhibitors, as it has been shown that alkyl derivatives with N-7 substituents larger than an ethyl moiety were ineffective inhibitors of protein synthesis. Thus, there must be an interaction of the aryl group that compensates for the steric bulk of the phenyl ring and enhances the affinity of these analogues for eIF-4E. One such model is discussed below.

pH Dependence of Binding of N-7-Substituted Cap Analogues to eIF-4E. The pH dependence of binding profiles of N-7-substituted cap analogues to eIF-4E shown in Figure 3 can be divided into two categories: (1) those that have a binding optimum (m^{7}GTP, m^{2,7}GMP, and, to a lesser extent, e^{7}GMP) and (2) those that have maximum binding in the pH 6.5–7.6 range and a substantial decrease in binding above pH 7.6 [bn^{7}GMP and (2-phet)^{7}GMP]. The profile of category 1 has been previously described for m^{7}GTP and m^{7}GpppG (Carberry et al., 1989) as being attributed to the enolate (deprotonated) form of the cap interacting with a protonated amino acid residue, presumably histidine; the increase in binding in the pH 6.5–7.6 range is due to the increased affinity of the protonated histidine residue for the enolate form of the cap, while the decrease in the pH 7.6–8.5 range is due to the decreased affinity of the enolate form of the cap for the deprotonated histidine residue.

The analogues in category 2, bn^{7}GMP and (2-phet)^{7}GMP, have pK values of 6.8 and 6.9, respectively. These analogues are therefore in the enolate form over the pH 6.5–7.6 range, and so maximum binding is observed in this range. The decrease in binding over the pH 7.6–8.5 range is attributed to the same factors as described for category 1. This finding provides further evidence in support of the model postulated in which the enolate form of the cap is preferred for interaction with a protonated amino acid residue.

Model for the Interaction of eIF-4E with Aryl N-7-Substituted Cap Analogues. The result that the aryl-substituted cap analogues bn^{7}GMP and, to a lesser extent, (2-phet)^{7}GMP possess CD spectra, while the alkyl-substituted analogues m^{7}GMP, e^{7}GMP, and m^{2,7}GMP do not, indicates that the interaction of the aryl substituent with the guanosine residue must be different from the interaction of the guanosine with the alkyl substituent. One such possible interaction is an edge-to-face arrangement, in which the plane of the phenyl ring interacts with the edge of the guanosine. This alignment has been reported to be favorable in energy calculations with benzene (Karlstrom et al., 1983) and from model building. In such a conformation, the edge of the phenyl ring can also interact with the oxygens of the phosphate backbone and O-6 keto substituent on the guanosine; this orientation was found to be favorable in a benzene–water model system (Karlstrom et al., 1983) and to impart additional stability to the conformation (Thomas et al., 1982). Therefore, the effectiveness of bn^{7}GMP and, to a lesser extent, (2-phet)^{7}GMP as inhibitors of protein synthesis may lie in the ability of the N-7 aryl substituent to interact in the edge-to-face manner described, thereby compensating for the bulkiness of the substituent.

This study has shown that there is a correlation between the tightness of binding of N-7-substituted cap analogues to eIF-4E and their potency as inhibitors of protein synthesis. The relative binding efficiencies of these cap analogues to eIF-4E reflect independent contributions of the pK of the N-1 proton, steric effects, π–π electronic contributions, and possibly three-dimensional configurations of the cap analogue. It appears that alkyl-substituted cap analogues differ qualitatively in their binding to eIF-4E; these differences presumably result from distinct interactions of the aryl substituent with the tryptophan and guanosine moieties.

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Replacement of Residues 8-22 of Angiogenin with 7-21 of RNase A Selectively Affects Protein Synthesis Inhibition and Angiogenesis†

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ABSTRACT: The region of human angiogenin containing residues 8-21 is highly conserved in angiogenins from four mammalian species but differs substantially from the corresponding region of the homologous protein ribonuclease A (RNase A). Regional mutagenesis has been employed to replace this segment of angiogenin with the corresponding RNase A sequence, and the activities of the resulting covalent angiogenin/RNase hybrid, designated ARH-III, have been examined. The ribonucleolytic activity of ARH-III is unchanged toward most substrates, including tRNA, naked 18S and 28S rRNA, CpA, CpG, UpA, and UpG. In contrast, the capacity of ARH-III to inhibit cell-free protein synthesis is decreased 20-30-fold compared to that of angiogenin. The angiogenic activity of ARH-III is also different; it is actually more potent. It induces a maximal response in the chick chorioallantoic membrane assay at 0.1 ng per egg, a 10-fold lower dose than required for angiogenin. In addition, binding of ARH-III to the placental ribonuclease inhibitor is increased by at least 1 order of magnitude ($K_i \leq 7 \times 10^{-17}$ M) compared to angiogenin. Thus, mutation of a highly conserved region of angiogenin markedly affects those properties likely involved in its biological function(s); it does not, however, alter ribonucleolytic activity toward most substrates.

Angiogenin is a potent inducer of blood vessel growth in the chick chorioallantoic membrane (CAM)[1] and rabbit cornea assays (Fett et al., 1985). In vitro, the protein induces multiple responses in endothelial cells including activation of phospholipase C and secretion of prostacyclins (Bicknell & Vallee, 1988, 1989); it also inhibits cell-free protein translation by specific cleavage of 18S RNA within the 40S ribosomal subunit (St. Clair et al., 1987, 1988). Angiogenin is homologous to the ribonuclease family of enzymes. It has 33% sequence identity to the most extensively studied member of this group, bovine pancreatic RNase A (Strydom et al., 1985; Kurachi et al., 1985); its tertiary structure is similar as well, based on conservation of three of four disulfide bonds, extremely tight binding to the common ligand PRI (Shapiro & Vallee, 1987; Lee et al., 1988b; Blackburn et al., 1977), and a computer-generated three-dimensional structure (Palmer et al., 1986). The three essential catalytic residues of RNase A are conserved in angiogenin (His-13, Lys-40, and His-114) as are many other key active-site and structural residues. Indeed, angiogenin possesses ribonucleolytic activity, but of a type far different from other RNases; its activities toward most substrates are 5-6 orders of magnitude below those of RNase A (Shapiro et al., 1987, 1988; Harper & Vallee, 1989).

The vast difference in in vivo and in vitro activities of angiogenin compared to the RNases (which have not been shown to induce angiogenesis or second-messenger activities) is clearly indicative of a distinct physiological role for this protein. Two lines of evidence implicate the amino-terminal region of angiogenin in this function. The first comes from experiments with noncovalent hybrids formed between synthetic angiogenin peptides and fragments of RNase A (Harper et al., 1988). Maximal activities of both the Ang(1-21)/RNase(21-124)[2] and the RNase(1-118)/Ang(108-123) hybrids toward small substrates are decreased compared to those of RNase A. Of the two hybrids, however, only the first is able to generate the pattern of cleavage products from 18S and 28S rRNA that is characteristic of angiogenin. Therefore, the amino-terminal region of angiogenin may be involved in determining specificity.

The second line of evidence comes from a comparison of the sequences of angiogenins from four different species. There

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[1] Abbreviations: ARH-III, angiogenin/RNase hybrid in which residues 8-22 of angiogenin are replaced by 7-21 of RNase A; RNase, ribonuclease; RNase A, bovine pancreatic ribonuclease A; PRI, placental ribonuclease inhibitor; C18, octadecylsilane; CAM, chorioallantoic membrane; TFA, trifluoroacetic acid; CpA, cytidylyl (3′-5′)adenosine; CpG, cytidylyl(3′-5′)guanosine; UpA, uridylyl(3′-5′)adenosine; UpG, uridylyl(3′-5′)guanosine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol.

[2] Ang(n-n') refers to an angiogenin peptide whose N- and C-terminal residues are denoted by n and n', respectively, indicating their positions in the primary structure of angiogenin. The same terminology is used for RNase A fragments.

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