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Intrinsic RNA Binding by the Eukaryotic Initiation Factor 4F Depends on a Minimal RNA Length but Not on the m^{7}G Cap*

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The eukaryotic initiation factor 4F (eIF4F) is thought to be the first factor to bind mRNA during 7-methylguanosine (m^{7}G) cap-dependent translation initiation. The multipartite eIF4F contains the cap-binding protein eIF4E, and it is assumed that eIF4F binds mRNAs primarily at the 5′ m^{7}G cap structure. We have analyzed equilibrium binding of rabbit eIF4F to a series of diverse RNAs and found no impact of the 5′-cap on the stability of eIF4F-RNA complexes. However, eIF4F preferentially and cooperatively binds to RNAs with a minimum length of ~60 nucleotides *in vitro*. Furthermore, translation activity in rabbit reticulocyte lysate is strongly inhibited by RNAs exceeding this length, but not by shorter ones, consistent with the notion that eIF4F in its physiological environment preferentially binds longer RNAs, too. Collectively, our results indicate that intrinsic RNA binding by eIF4F depends on a minimal RNA length, rather than on cap recognition. The nonetheless essential m^{7}G cap may either function at steps subsequent to eIF4F-RNA binding, or other factors facilitate preferential binding of eIF4F to the m^{7}G cap.

In eukaryotes, the synthesis of more than 95% of all proteins is initiated via pathways involving a 7-methylguanosine (m^{7}G)[2] cap at the 5′ end of the mRNA. During this cap-dependent translation initiation, multiple initiation factors assemble in a translation initiation complex at the 5′-untranslated region of the mRNA (1). Subsequently, the initiation complex locates the start codon on the mRNA and further orchestrates the start of protein synthesis (1).

The first translation initiation factor to bind the mRNA during cap-dependent eukaryotic translation initiation is thought to be the eukaryotic initiation factor 4F (eIF4F) (1–4). eIF4F is a stable tripartite protein complex composed of the scaffolding and RNA-binding protein eIF4G, the DEAD-box protein eIF4A, and the m^{7}G cap-binding protein eIF4E (1). eIF4E is the only known initiation factor to directly contact the m^{7}G cap (1). Structural information has illuminated how eIF4E binds to the m^{7}G cap (5–8), and interaction with eIF4G has been shown to enhance m^{7}G cap binding by eIF4E (9, 10). The ability of eIF4E to bind m^{7}G cap structures is thought to enable eIF4F to associate directly with the m^{7}G cap of mRNAs. Yet the presence of RNA binding regions on eIF4G and eIF4A suggests that contacts to RNA may also play a role in mRNA recognition by eIF4F. It is not known which contributions to the stability of eIF4F-mRNA complexes come from binding to the m^{7}G cap and which contributions arise from binding to RNA, and it is thus unclear to what extent eIF4F can discriminate between capped and uncapped RNAs.

To delineate contributions from RNA *versus* those from the m^{7}G cap to the binding by eIF4F, we analyzed binding of eIF4F to RNA in isolation from other processes of translation initiation. By measuring equilibrium binding of rabbit eIF4F to a diverse series of capped and uncapped RNAs, we found no significant effect of the cap on the stability of eIF4F-RNA complexes, even though the m^{7}G cap was verified to be essential for efficient translation initiation. We discovered, however, that eIF4F preferentially and cooperatively bound RNAs with more than ~60 nucleotides (nt) *in vitro*. Consistent with this observation, only RNAs exceeding this length, but not shorter ones, strongly inhibited translation activity in rabbit reticulocyte lysate (RRL). This inhibition was also cap-independent. Collectively, these observations show that intrinsic RNA binding by eIF4F depends on RNA length, not on m^{7}G cap recognition, and these binding characteristics are recapitulated in RRL. The essential m^{7}G cap may thus either function at steps subsequent to eIF4F-RNA binding, or other factors facilitate a preferential binding of eIF4F to the m^{7}G cap.

EXPERIMENTAL PROCEDURES

Protein Purification—eIF4F and all other proteins used in this study were purified from RRL and quantified as described previously (11–14). For the purification of eIF4F, the low salt gel filtration step was omitted. RRL was purchased from Promega. The integrity of the eIF4F preparation was assessed on SDS-PAGE by Coomassie Blue staining as described (11–14).

RNA Preparation—RNAs were either generated by *in vitro* transcription or purchased from Dharmacon. The latter were deprotected, purified, and quantified as described (15, 16). The other RNAs were transcribed from linearized plasmids (β-globin RNA, pBG DNA digested with EcoRI; 158 RNA, pPET22B$^+$ DNA digested with XhoI; and TRAP RNA, pB222 DNA digested with KpnI) by T7 MegaScript transcription kits from Ambion; m^{7}G capped, unlabeled RNAs were produced by including either m^{7}GpppG or anti-reverse cap analog in the transcription mix as directed by the manufacturer (Ambion, T7

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[2] The abbreviations used are: m^{7}G, 7-methylguanosine; eIF4F, eukaryotic initiation factor 4F; nt, nucleotide; RRL, rabbit reticulocyte lysate; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; GMP-PNP, guanyl imidodiphosphate; MOPS, 4-morpholinepropanesulfonic acid.

mMessage mMachine kit). Capping efficiency approached completion, as verified by gel electrophoresis and UV shadowing. 5′-^{32}P-Labeled uncapped RNAs were generated by treatment of transcripts with calf intestinal alkaline phosphatase (Roche Applied Science) and subsequent labeling with [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs). Capped labeled RNAs were generated using guanylyltransferase (Ambion) and [α-^{32}P]GTP according to the manufacturer's protocol. All RNAs were purified and recovered from 7 M urea, 6–8% polyacrylamide gels prior to use. RNA concentrations were determined by UV absorbance.

To generate capped and uncapped 37-nt fragments of β-globin RNA, a DNAzyme was used to cleave a longer β-globin transcript (17). A 6.75-fold excess of DNAzyme over RNA was incubated in 75 mM NaCl, 20 mM Tris-HCl, pH 8, for 3 min at 95 °C, quickly cooled on ice for 5 min, briefly centrifuged, and incubated at 25 °C for 10 min. Cleavage reactions were started by adjusting the reaction buffer to 150 mM NaCl, 40 mM Tris-HCl, pH 8, and 60 mM $MgCl_2$. After incubation at 42 °C for 3 h, the DNAzyme was degraded with RNase-free DNase I (Roche Applied Science), and the RNA was recovered and quantified as described above.

eIF4F-RNA Binding Reactions—eIF4F-RNA binding was measured at the eIF4F concentrations indicated at 37 °C in a reaction volume of 10 μl with buffer containing 2.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 8% (v/v) glycerol, 40 mM HEPES, pH 7.2, 0.3 mM dithiothreitol, and 0.01% Nonidet P-40 (v/v). RNA concentrations were 1 nM. Different RNA concentrations did not change the results. Reactions were incubated for 6 min. Incubation times between 2 and 10 min did not alter results, indicating that equilibrium was reached during the reaction time. Samples were applied to polyacrylamide gels consisting of 3.6% polyacrylamide, 50:1 bis/acrylamide, 0.5× TBE, 2.5 mM $MgCl_2$, 5% glycerol (1-cm wells, 1.5 mm thick). A running buffer containing 0.5× TBE, 2.5 mM $MgCl_2$, and 5% glycerol was used, and gels were electrophoresed at 5 watts and 4 °C.

The extent of eIF4F-RNA binding was quantified with a Storm PhosphorImager and the ImageQuant software (GE Healthcare). The fraction of RNA bound to eIF4F ($Frac^{[bound]}$) was calculated from the intensity of the bound ($I^{[bound]}$) and of the free ($I^{[free]}$) RNA species according to Equation 1,

$$Frac^{[bound]} = I^{[bound]}/(I^{[bound]} + I^{[free]}) \quad \text{(Eq. 1)}$$

Apparent binding constants $K_{½,app}$ were determined by plotting $Frac^{[bound]}$ *versus* concentrations of eIF4F (Fig. 1) using KaleidaGraph (Synergy Software). The resulting curves were fit to the nonlinear form of the Hill equation, Equation 2,

$$Frac^{[bound]} = [eIF4F]^n \cdot Frac^{[bound]}_{max}/([eIF4F]^n + K^n_{1/2,app}) \quad \text{(Eq. 2)}$$

where [eIF4F] is the concentration of eIF4F; n is the Hill constant; $Frac^{[bound]}_{max}$ is the fraction bound at eIF4F saturation; and $K_{½,app}$ is the average apparent dissociation constant for all bound eIF4F molecules (18). The values for $K_{½,app}$ and n shown in Table 2 are the average of at least three independent experiments, and the error represents the standard deviation of these averages.

Toeprint Analysis—48 S and 80 S translation initiation complexes were assembled as described (19–21) using GMP-PNP or cycloheximide, respectively. RNAs include the 5′-terminal 419 nt of β-globin mRNA. Capped RNAs were generated cotranscriptionally as described above. A 5′-^{32}P-labeled DNA primer (5′-TCACCACCAACTTCTTCCAC-3′) complementary to nt 114–133 was hybridized to the RNA. Sequence ladders were generated using the same primer and RNA with the Thermosequenase cycle sequencing kit (United States Biochemical Corp.).

Native Western Blot Analysis—Binding reactions (20 μl) were performed with 350 nM eIF4F and 1.05 μM RNA as described. Samples were then applied to nondenaturing PAGE, also as described above. In addition, Precision Plus Protein Standards (Bio-Rad) were loaded as markers for size and transfer from the native gel. Protein was transferred from the native gel to PVDF paper with a Bio-Rad transfer apparatus in 0.5% SDS, 20% methanol (v/v), 25 mM Tris-HCl, and 192 mM glycine at 4 °C and 30 V for 16 h. Subsequently, the PVDF was quickly blotted with Whatman paper to remove excess acrylamide and washed twice in PBS (136 mM NaCl, 27 mM KCl, 43 mM Na_2HPO_4, 15 mM KH_2PO_4) for 5 min. Nonspecific antibody adsorption was suppressed with 10 ml of 10% (w/v) nonfat milk dissolved in PBST (PBS plus 0.1% Tween) for 2 h at 25 °C with constant agitation. The PVDF was then washed twice in PBST (5 min, 25 °C) and once in PBS (5 min, 25 °C) and incubated for 2 h at 25 °C with 10 ml of primary antibody eIF4E-FL (Santa Cruz Biotechnology) diluted 1:100 in PBS and 5% nonfat milk. The PVDF was then washed twice in PBST (5 min at 25 °C) and once in PBS (5 min, 25 °C), incubated for 2 h at 25 °C with 10 ml of the secondary antibody anti-rabbit horseradish peroxidase (Sigma) diluted 1:1000 in PBS and 5% nonfat milk, and then washed three times with PBST (5 min, 25 °C). Last, 1.5 ml of West Pico chemiluminescent substrate (Pierce) was mixed according to the manufacturer's instructions and evenly applied to the PVDF paper. After a 1 min incubation at 25 °C, the PVDF was blotted dry, wrapped in Saran™ wrap, and immediately exposed to film.

Measurement of the RNA Length Requirement for eIF4F Binding—Uncapped RNAs were 5′ end-labeled with [γ-^{32}P]ATP and T4 PNK, as described above. 5′-Labeled capped RNAs were generated with [α-^{32}P]GTP and guanylyltransferase (Ambion) according to the manufacturer's protocol. The capping reaction with guanylyltransferase transfers the m^7G cap to only about 5% of the RNAs (Ambion product literature). To obtain sufficient signal (cpm/μl), it was therefore necessary to use 20-fold more RNA in the labeling reaction than for the virtually quantitative labeling of the uncapped RNAs. Following the respective labeling reactions, the RNAs (in 0.01% Nonidet P-40) were partially hydrolyzed at 75 °C in 27 μl of freshly prepared buffer containing 2 M urea, 17 mM NaOH. Several aliquots (6 μl) were removed over 15 min and quenched on ice with 100 μl of a buffer containing 1 mM HCl, 10 mM MOPS, pH 6.5. The extent of hydrolysis in each aliquot was assessed by denaturing PAGE. Aliquots were occasionally combined to create a uniform distribution of the different RNA species. The RNAs were then ethanol-precipitated and resuspended in 0.1% (v/v) Nonidet P-40. These RNA preparations were used as a substrate in bind-

TABLE 1
Sequences of RNA and DNA oligonucleotides used in the translation inhibition experiments

Length/composition	ΔG[a]	Sequence
	kcal/mol	
RNA 27A	−1.00	5′-GCUUUACGGUGCUUAAAACAAAACAAA
RNA 27B	+0.80	5′-AAAACAAAACAAAAUAGCACCGUAAGC
RNA 28	−3.60	5′-GACAGCAUUGUACCCAGAGUCUGUACGG
DNA 28	−1.55	5′-ATGACCATGATTACGAATTCGAGCTTTT
RNA 29	−7.40	5′-CCCAGACAGCUUGUACCAGGUCUGUACGG
RNA 36		5′-UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU
RNA 37	−7.80	5′-(m7G)GGACACUUGCUUUUGACACAACUGUGUUUACUUGCAA
RNA 38	−1.00	5′-GCUUUACGGUGCUUAAAACAAAACAAAACAAAACAAAA
DNA 38	−4.23	5′-TTTTTGGGGATCCTCTAGAGTCGACCTGCAGGCATGCA
RNA 51	−2.80	5′-GCGUCUUUACGGUGCUUAAAACAAAACAAAACAAAACAAAACAAAACAAAA
DNA 51	−4.23	5′-AGGTGTAAGACATAGAGCAGCAAACAACGTCTTCGGTGTCCATGGGGTGGG
RNA 56	−14.10	5′-CCGUACAGACAUUGCACCUGGCGCUGUCUGGGACGUACUAACAGCAUCAAUGACAU
RNA 61	−5.60	5′-CUGACAGACCCUGCAUGAUUGCGUCUUUACGGUGCUUAAAACAAAACAAAACAAAACAAAA
DNA 63	−4.51	5′-GCCCTTGGATCCTAATACGACTCACTATAGGGAGAACCCCACCCCACCCCATGGACACCGAAG
RNA 65	−14.10	5′-CCGUACAGACAUUGCACCUGGCGCUGUCUGGGACGUACUAACAGCAUCAAUGACAUCAGCAUCAA
RNA 66	−10.80	5′-AUGACCAUGAUUACGAAUUCGAGCUUUUUUUUUGGGGAUCCUCUAGAGUCGACCUGCAGGCAUGCA
DNA 66	−4.04	5′-ATGACCATGATTACGAATTCGAGCTTTTTTTTTGGGGATCCTCTAGAGTCGACCTGCAGGCATGCA
RNA 70	−1.72	5′-(m7G)GGGAATTGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGATGAGGTACCCGGGGATCCT
DNA 71A	−4.41	5′-CCCAGACAGCATTGTACCCAGAGTCTGTACGCGTCTTTACGGTGCTTAAAACAAAACAAAACAAAACAAAA
DNA 71B	−4.00	5′-AAAACAAAACAAAACAAAACAAAATAGCACCGTAAAGACGCCCCAGACAGCATTGTACCCAGAGTCTGTAC
RNA 71	−5.60	5′-CUGACAGACCCUGCAUGAUUGCGUCUUUACGGUGCUUAAAACAAAACAAAACAAAACAAAACAAAACAAAA
RNA 73	−12.70	5′-CCGUACAGGCUCUGGGUACAAUGCUUGUUUUUUUUCUGUCUGGGACGUACUGCAUCAAUGACAUCAGCAUCAA
DNA 73	−1.77	5′-GCGTCTTTACGGTGCTTCCTCGTCTCCCTCGTTAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAAC

[a] ΔG indicates the free energy of the most stable structure predicted from Mfold software (by M. Zuker) for either RNA or DNA folding. For capped RNAs, the m^7G cap was not included in the folded sequence.

ing reactions that were conducted as described above. Bound and free RNAs were separated by nondenaturing PAGE, also as described above, except that glycerol was omitted from the gel. Next, RNAs were visualized in the gel with Kodak Biomax MS maximum sensitivity film. Bound and free RNAs were excised and eluted in 3 ml of elution buffer (300 mM NaOAc, 0.5% SDS, 10 mM Tris-HCl, pH 8, 1 mM EDTA) overnight and recovered by phenol/chloroform extraction and ethanol precipitation. Subsequently, the samples were electrophoresed on 8% denaturing gels. These gels were dried, and the individual RNA species were visualized with the Storm PhosphorImager (GE Healthcare).

Translation Inhibition Assays in RRL—Translation was measured in a nuclease-treated RRL system (Promega). Reactions were performed in a volume of 7.5 μl, with 60% (v/v) RRL, 20 ng/μl Promega luciferase control RNA (RNA concentrations were saturating; data not shown), and inhibitor RNAs, as indicated, for 30 min at 30 °C (linear phase of translation). To quantify light output, the entire reaction was diluted 10-fold with water, and 1 μl of the dilution was added to 100 μl of Firefly luciferase assay reagent (Promega). The mixture was briefly vortexed, and light units were read with a Turner Biosystems TD-20/20 single tube luminometer set to pause for 2 s and read for 10 s. The degree of inhibition is given as the ratio of light units measured with and without inhibitor. Length, composition, sequence, and stability of the RNAs used are given in Table 1.

RESULTS

m^7G Cap Does Not Affect the Affinity of eIF4F for RNA—To analyze RNA binding by eIF4F, we first measured equilibrium binding of eIF4F to an uncapped, 158-nt RNA using a PAGE-based gel shift assay (Fig. 1*A*). eIF4F, purified from RRL (12), bound the RNA with an apparent dissociation constant of $K_{½,app} = 31 \pm 13$ nM (Fig. 1*B* and Table 2). A sigmoidal binding curve was recorded, consistent with cooperative binding of multiple eIF4F complexes to the RNA (Fig. 1*B*). We then measured eIF4F binding to the same 158-nt RNA with a m^7G cap. We observed a sigmoidal binding curve with an apparent dissociation constant highly similar to the one measured for the uncapped RNA (Fig. 1*B*). Filter binding assays also yielded highly similar dissociation constants for capped and uncapped

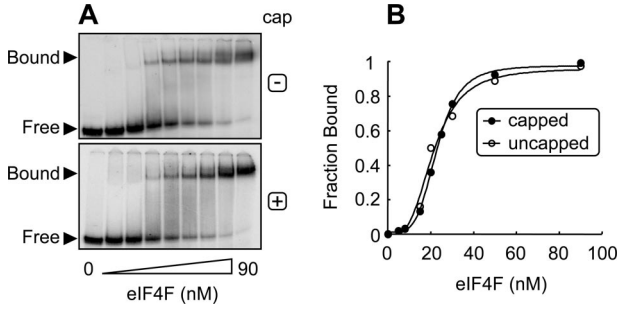

FIGURE 1. **eIF4F binding to capped and uncapped RNA.** *A,* representative nondenaturing PAGE of eIF4F (0, 5, 8, 15, 20, 25, 30, 50, and 90 nM) binding to radiolabeled uncapped (*top*) and capped (*bottom*) 158-nt RNA (1 nM). Mobilities of free and bound RNA are indicated on the *left*. *B,* representative plot of the dependence of the fraction of bound RNA on eIF4F concentration. *Lines* represent the best fit to the nonlinear form of the Hill equation ("Experimental Procedures").

TABLE 2
Binding constants for eIF4F association with different RNAs
$K_{½,app}$ and n were measured as shown in Fig. 1 and described under the "Experimental Procedures." Error ranges are the standard deviations of at least three independent experiments.

RNA name/ length	−ATP		+ATP	
	Capped	Uncapped	Capped	Uncapped
Trap (70 nt)				
$K_{½,app}$ (nM)	9 ± 1	16 ± 4	12 ± 6	16 ± 5
n	3.4 ± 1.5	3.9 ± 1.8	3.7 ± 0.5	3.5 ± 0.4
pET22B$^+$ (158 nt)				
$K_{½,app}$ (nM)	40 ± 7	31 ± 13	70 ± 25	30 ± 10
n	3.6 ± 0.7	3.5 ± 1.2	2.8 ± 0.8	2.5 ± 0.5
β-Globin (419 nt)				
$K_{½,app}$ (nM)	17 ± 3	25 ± 4	18 ± 7	23 ± 7
n	4.6 ± 1.2	3.3 ± 0.9	4.6 ± 1.9	3.6 ± 0.8

RNA, demonstrating that this observation did not depend on the nature of the binding assay (data not shown). Addition of ATP and/or eIF4B did not change the sigmoidal shape of the binding curves or the apparent dissociation constant for capped or uncapped RNA (Table 2 and data not shown). Collectively, these data indicate that the m^7G cap does not affect equilibrium binding of eIF4F, and multiple units of eIF4F associate cooperatively with this RNA.

To examine whether these observations were specific for the 158-nt RNA used, we measured eIF4F binding to two additional RNAs, both capped and uncapped (Table 2). One of the RNAs, a 70-nt unstructured RNA derived from the binding site of the *Escherichia coli* TRAP protein (22), is not a natural substrate for eIF4F. The other RNA, a 419-nt 5′-terminal fragment of the β-globin mRNA that included the entire 55-nt 5′-untranslated region, is a natural substrate for eIF4F. For all three RNAs, $K_{½,app}$ for capped and uncapped RNAs differed by less than a factor of 2 (Table 2), which is miniscule in energetic terms ($\Delta\Delta G^0 < 0.4$ kcal·mol^{-1}). eIF4F bound cooperatively to all RNAs ($n > 2$), indicating association of multiple eIF4F units to all substrates (Table 2). Addition of ATP and/or eIF4B did not have significant effects on the measured parameters (Table 2 and data not shown). Inclusion of the cap analog at concentrations 3.5-fold higher than eIF4F saturation did not inhibit capped or uncapped RNA binding or alter the measured affinities (data not shown). Taken together, the equilibrium binding data for the three different RNAs provide two main insights. First, the m^7G cap does not provide a significant energetic contribution to RNA binding by eIF4F, irrespective of whether or not a given RNA represents a physiological substrate for eIF4F. Second, multiple eIF4Fs bind RNA in a cooperative fashion.

Because these observations were not consistent with preferential binding of eIF4F to the m^7G cap, it was important to confirm that the RNAs contained a functional m^7G cap and that the cap-binding protein eIF4E remained part of the eIF4F complex during the experimental procedure. To verify the presence of a functional m^7G cap, we employed toeprint analysis of the RNA in RRL (Fig. 2). This assay monitors assembly of the initiation complex and the subsequent scanning step on the RNA through inhibition of primer extension (21, 23). In the presence of cycloheximide, which stabilizes 80 S complex formation, on capped RNA, we readily detected the hallmark toeprint of the edge of the 80 S complex at (+)14 to (+)18 nt from the start codon (Fig. 2, *lane 4*). With uncapped RNA, the toeprint was much less pronounced (Fig. 2, *lane 3*). These data indicate that the initiation complex reaches the initiation codon more efficiently on the capped RNA. Similar results were obtained in the presence of GMP-PNP, which stabilizes the 48 S complex on the start codon. The corresponding toeprint at (+)14 to (+)16 also appeared more readily with capped than with uncapped RNA (Fig. 2, *lanes 5* and *6*). Incubation of the samples on ice, which greatly slows the formation of the initiation complexes (20), verified that the toeprints marked initiation complexes (Fig. 2, *lanes 1* and *2*). The results of the toeprint analysis thus clearly indicated a functional m^7G cap, which enhanced assembly of the 80 S and 48 S complex on the start codon, compared with uncapped RNA.

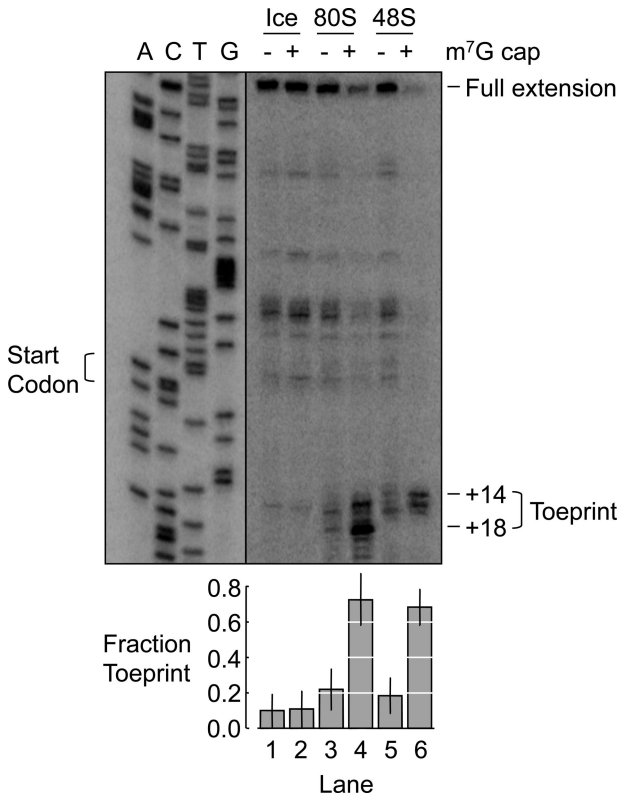

FIGURE 2. **Toeprint analysis of capped and uncapped RNA.** The sequence ladder indicates the AUG start codon. Primer extension of the β-globin RNA (full extension) and toeprint are indicated on the *right*. Presence (+) or absence (−) of an m^7G cap on the RNA is indicated at the *top* of the gel. *Lanes 3* and *4* (*80S*) denote reactions in the presence of cycloheximide; *lanes 5* and *6* (*48S*) denote reactions in the presence of GMP-PNP. The fraction toeprint, indicated in the *bar graph*, represents the fraction of cpm at +14 to +18 compared with cpm for full extension. The values shown are the average, and the *error bars* indicate the standard deviation of multiple independent experiments.

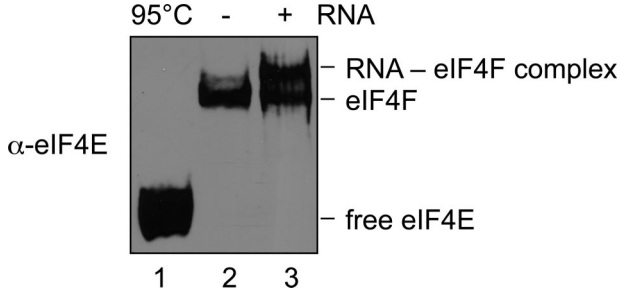

FIGURE 3. **Native Western blot for eIF4E before and after eIF4F-RNA binding.** Western blot of eIF4F in the presence and absence of RNA on nondenaturing PAGE probed with anti-eIF4E. To generate free eIF4E (*95 °C*), 350 nM eIF4F was incubated with SDS at 95 °C for 2 min.

To test whether the cap-binding protein eIF4E remained part of the eIF4F complex during the binding reaction, we employed native Western blot analysis (Fig. 3). We incubated eIF4F with RNA and applied the samples to nondenaturing PAGE to separate bound from free eIF4E. The protein was then transferred to a membrane and probed with anti-eIF4E antibody. eIF4F without RNA was used to determine the mobility of eIF4E/eIF4F not bound to the RNA, and denatured eIF4F was used to determine the mobility of eIF4E dissociated from the eIF4F complex (Fig. 3). eIF4F without RNA showed reduced mobility compared with the heat-denatured complex, indicating com-

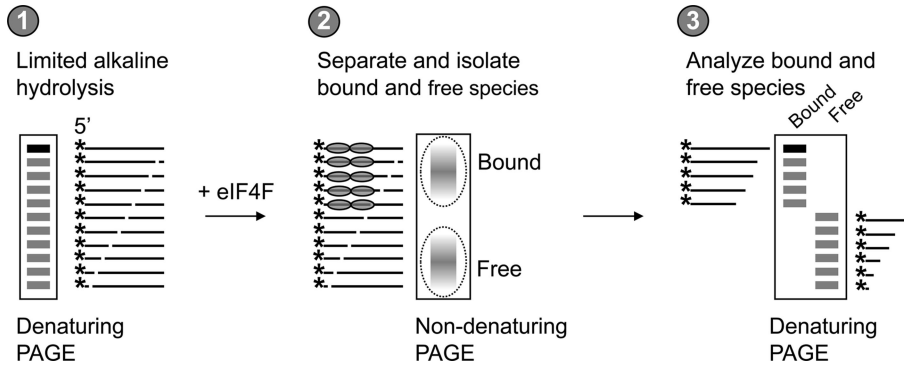

FIGURE 4. **Experimental design probing the minimal RNA-binding site of eIF4F.** *Step 1,* labeled (*asterisk*) capped or uncapped RNA is subjected to limited alkaline hydrolysis. The extent of hydrolysis is monitored by denaturing PAGE. *Step 2,* pool of hydrolyzed RNA is used as a substrate for eIF4F binding. The free and eIF4F-bound RNAs are excised and eluted from nondenaturing PAGE. *Step 3,* eIF4F-bound and free RNAs are purified and separated by denaturing PAGE.

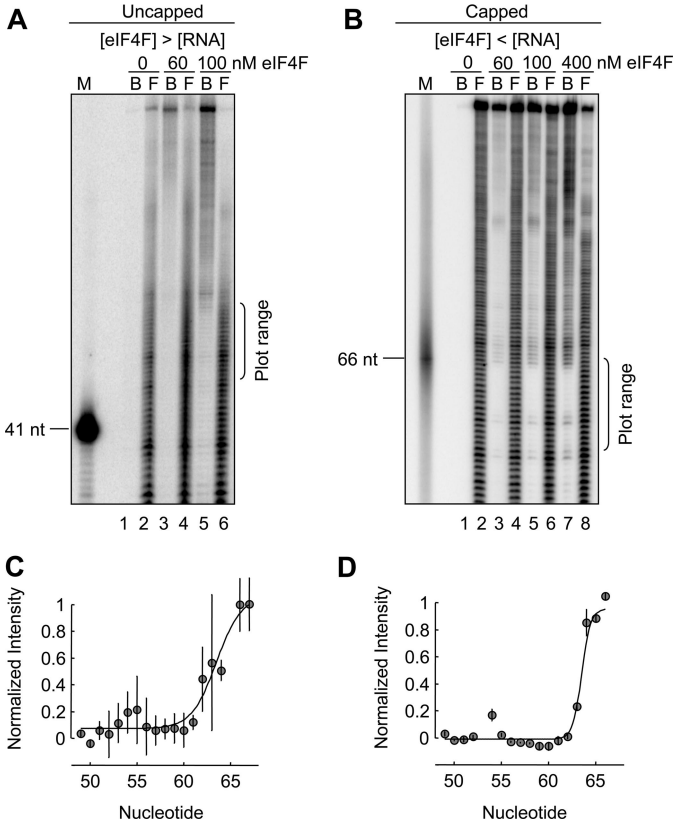

FIGURE 5. **Determination of the minimal binding site size of eIF4F.** *A* and *B,* representative PAGE of eIF4F bound (*B*) and free (*F*) uncapped (*A*) and capped (*B*) RNAs (*i.e. step 3* in Fig. 4) at increasing eIF4F levels. Concentrations of eIF4F are marked at top of panels. Size markers (*M*) are shown at the *left* of panels. Plot range indicates the individual RNA lengths shown in the quantitative analysis below. *C* and *D,* plots of normalized band intensities for bound fractions of uncapped (*C*) and capped (*D*) RNAs around the inflection point between bound and free RNA. Values represent averages of three independent measurements, and the *error bars* indicate the standard deviation. Band intensities for the independent experiments were normalized to bound and free RNA species, to facilitate comparison between the measurements. The smaller errors for the reaction with capped RNA reflect the higher signal of the radiolabeled RNAs.

plete retention of eIF4E within eIF4F (Fig. 3, *lanes 1* and *2*). Incubation with RNA produced a supershift, indicating eIF4E bound to the RNA through eIF4F (Fig. 3, *lane 3*). No eIF4E corresponding to dissociated eIF4E was observed, demonstrating that eIF4E remained part of the eIF4F complex during the RNA binding procedure.

eIF4F Preferentially Binds RNAs with More than 60 Nucleotides—The integrity of the eIF4F complex and the presence of functional m^7G cap on the RNA ruled out trivial explanations for the lack of a significant contribution of the m^7G cap to eIF4F-RNA binding. However, the RNAs used in the binding experiments above were all longer than 70 nt, and it remained possible that the m^7G cap contributed more significantly to eIF4F binding to shorter RNAs. To test this possibility, we investigated binding of eIF4F to several capped and uncapped RNAs with 37 and fewer nucleotides. The affinity of eIF4F for all of these RNAs was too low to measure with our PAGE-based binding assay (data not shown). Supplementation of other translation factors (eIF4B, eIF4H, eIF3, additional eIF4A, all with or without 1 mM ATP/$MgCl_2$) did not facilitate measurable binding of eIF4F to the RNAs (data not shown). These results suggested that eIF4F was unable to bind to RNAs with less than 37 nt with high affinity, irrespective of the presence of an m^7G cap.

To more systematically determine the minimal RNA length required by eIF4F for high affinity binding, we measured eIF4F binding to a pool of RNAs with different lengths (Fig. 4). The RNA pool was generated by subjecting a 5′-radiolabeled 158-nt RNA to limited alkaline hydrolysis (Fig. 4, *step 1*). Uncapped RNAs were labeled at the 5′-triphosphate and capped RNAs at the m^7G cap ("Experimental Procedures"). The limited alkaline hydrolysis yielded a population of 5′ end-labeled RNAs with all lengths represented (Fig. 4, *step 1*). Increasing concentrations of eIF4F were incubated with this RNA pool, and these reactions were then applied to nondenaturing PAGE to separate bound and free RNA species (Fig. 4, *step 2*). Bound and free RNA species were isolated from the gel, and the size of the RNA was identified on denaturing PAGE (Fig. 4, *step 3*).

For uncapped RNA, long RNAs were preferentially bound by eIF4F, whereas shorter RNAs remained free (Fig. 5*A*). A sharp cutoff, more pronounced with increasing eIF4F concentrations, was observed for RNAs longer than ~60 nt (Fig. 5*A*, *lane 5* and *C*). Nucleic acid-binding proteins are known to generally prefer longer nucleic acids, because of the increased number of registers available for association (24). However, these statistical factors lead to a gradual increase in the fraction of bound nucleic acid, not to a sharp cutoff. Our data thus indicate that the preferential binding of longer RNAs by eIF4F is not because of higher binding probability from the higher number of binding sites.

The experiments with capped RNA could only be performed with subsaturating concentrations of eIF4F relative to the overall concentration of RNA. This is because, under labeling conditions, guanylyltransferase forms an m^7G cap on only a fraction (~5%) of the RNA (see "Experimental Procedures"). To nonetheless obtain a strong signal, we needed to increase the

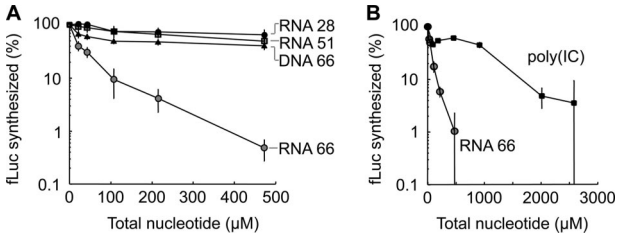

FIGURE 6. **Translation inhibition by different RNAs in rabbit reticulocyte lysate.** *A,* inhibition by RNAs and DNAs of different lengths. *fLuc* synthesized indicates measured light units with inhibitor normalized to the light units without inhibitor, given in percent. Total nucleotide represents the number of nucleotides added with the RNAs of different length, as explained in the text. *Error bars* represent the standard deviation of at least three independent measurements. Sequences of the inhibitors are given in Table 1. *B,* inhibition by poly(IC). Total nucleotide represents the number of nucleotides added, as determined from absorption measurements at 260 nm. Inhibition was measured with an RRL lot different from the one used in the measurements shown in *A*. For normalization, an inhibition curve with RNA 66 is shown for this lot.

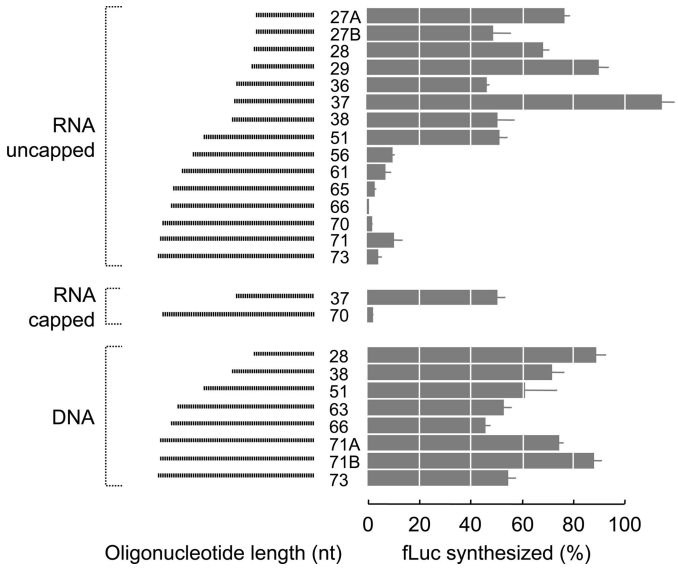

FIGURE 7. **RNA length dependence of translation inhibition.** Inhibition is given at 500 μM total nucleotide. The degree of inhibition is measured as in Fig. 6. Values represent averages of at least three independent measurements, and the *error bars* indicate the standard deviation. Inhibitor length is represented visually and by actual nucleotide number. Capped RNAs as well as DNA oligonucleotides are labeled accordingly. RNA and DNA sequences are given in Table 1.

overall RNA level in the binding reaction, and significant concentrations of unlabeled uncapped RNA remained present. Although unlabeled RNAs were invisible, they could compete with the binding of eIF4F to the labeled, capped RNA. Consequently and expectedly, not all labeled RNA was quantitatively bound, even at high eIF4F concentrations (Fig. 5*B*, *lanes 6* and *8*). Notwithstanding, a sharp cutoff for eIF4F binding at RNA length slightly above 60 nt was seen (Fig. 5*B*), similar to the observations made with uncapped RNA (Fig. 5*B*). The cutoff again increased in strength at higher eIF4F concentrations but did not change nucleotide position (Fig. 5*B*, *lanes 5* and *7*, and *D*). The presence of unlabeled RNAs emphasizes the strength of the cutoff, compared with reactions without unlabeled RNAs (Fig. 5*C* and *D*).

The similar cutoffs with capped and uncapped RNA showed that eIF4F bound with high affinity only to RNAs with more than 60 nt. No profound effects of the m^7G cap on this length preference of eIF4F were seen. Highly similar results regarding length dependence and cap independence were obtained in experiments with a different RNA, indicating that the sequence of the RNA did not play a role in the observed cutoff (data not shown). These observations mirrored the results above with individual RNA oligonucleotides; only long RNAs were stably bound, and the m^7G cap had no discernible effect on RNA binding by eIF4F (Fig. 1 and Table 2). In addition, the requirement of RNA >60 nt for tight eIF4F binding provides a possible rationale for the cooperative binding of eIF4F; only RNAs exceeding a certain length presumably accommodate multiple eIF4F units.

RNA Length Requirements for eIF4F Binding Are Paralleled in Rabbit Reticulocyte Lysate—Having shown a strong preference of purified eIF4F for binding RNAs with more than 60 nt, it was of interest to examine whether this length requirement was also apparent in more complex physiological environments where eIF4F could interact with other components of the translation initiation machinery. We reasoned that preferred binding of eIF4F to long RNAs should lead to a potent inhibition of protein synthesis by long but not by short RNAs. To test this hypothesis, we added RNAs of different length to an RRL translation system where protein synthesis was monitored through the production of firefly luciferase (Fig. 6, *fLuc*). To facilitate a direct comparison of inhibition by short *versus* long RNAs, the RNA concentrations were normalized according to nt concentration in the reaction (*e.g.* 10 μM of a 100-mer RNA equals 1,000 μM "total nucleotide" and 100 μM of a 10-mer RNA also equals 1,000 μM total nucleotide).

Addition of 28-mer and 51-mer RNAs at a concentration of 480 μM total nt (17.1 μM 28-mer RNA; 9.4 μM 51-mer RNA) reduced the level of fLuc to 50%, whereas addition of a 66-mer RNA at 480 μM total nt (7.3 μM 66-mer RNA) reduced the level of fLuc to less than 1% (Fig. 6*A*). We also measured the effect of a 66-mer DNA (sequence identical to the 66-mer RNA) on the translation reaction. We observed inhibition comparable with that seen with the 28- or 51-mer RNAs (Fig. 6*A*), suggesting a 2-fold inhibition could also be caused by DNA. However, the more than 100-fold inhibition seen with the 66-mer RNA was specific for RNA.

Next, we tested whether the inhibition by the long (66-mer) RNA was caused through activation of RNA-regulated protein kinase (PKR) that occurs upon recognition of regions of extensive double-stranded RNA. PKR activation triggers phosphorylation of eIF2α, which inhibits translation (25, 26). As a control for translation inhibition through this pathway, we used poly(IC), a common standard for double-stranded RNA that potently activates PKR (27). As expected, we observed inhibition of translation upon addition of poly(IC) (Fig. 6*B*). However, this type of inhibition was only seen at a concentration of poly(IC) much higher than the concentration of the 66-mer RNA. This observation strongly argued against PKR activation by the 66-mer RNA as the cause of inhibition, thus supporting the notion that inhibition was caused by sequestration of eIF4F.

To determine the length cutoff for this RNA inhibition, we tested the inhibition potency of a larger set of uncapped RNAs and DNAs ranging from 27- to 73-mer (Fig. 7). RNAs shorter

than 56 nt and all DNAs inhibited protein synthesis 2-fold or less (Fig. 7). RNAs with 56 or more nt inhibited translation by factors between 10 and 100. This cutoff (≥56 nt) strikingly resembled the binding cutoff seen with isolated eIF4F (>60 nt), suggesting that the observed inhibition of translation in RRL was because of sequestration of eIF4F.

Finally, we tested translation inhibition by two capped RNAs, one below and one above the inhibition cutoff (37- and 70-mer; Fig. 7). Clearly, strong translation inhibition was observed with the capped RNA above the cutoff (70-mer), but not with the RNA below the cutoff (37-mer, Fig. 7). These data indicate that the length dependence of translation inhibition by RNA oligonucleotides is largely unaffected by the m^7G cap. The RNA length dependence of the inhibition potency strikingly resembles the length dependence seen for RNA binding by purified eIF4F, further supporting the notion that RNA length, not cap recognition, determines binding of eIF4F to RNAs in RRL as well.

DISCUSSION

In this study, we have shown *in vitro* and in RRL that eIF4F binds preferentially to RNAs with more than 60 nt. The m^7G cap structure does not significantly contribute to the binding of eIF4F to these RNAs. Our observations indicate that intrinsic RNA binding by eIF4F depends on a minimal RNA length and not on recognition of the 5′-cap.

It is most important to note that these findings do not preclude contacts of eIF4F to the cap via eIF4E, which have been clearly demonstrated in numerous previous studies (5–8). We specifically verified the presence of a functional m^7G cap on the RNAs used and the presence of the cap-binding protein eIF4E in eIF4F (Figs. 2 and 3). Therefore, our data do not question the significance of the cap for the overall translation initiation process. However, our study reveals that contacts of eIF4F to the cap do not provide energetic contributions comparable with those derived from contacts of eIF4F with RNA. Thus, RNA binding, not cap recognition, determines the stability of the eIF4F-RNA complex. Accordingly, eIF4F alone does not have an inherent ability to bind RNAs preferentially at the m^7G cap.

Although this result may require a re-assessment of the current model for mRNA recognition by the translation initiation machinery, which assumes preferential binding of eIF4F to the m^7G cap (3), our data are consistent with reported affinities of eIF4E to m^7G cap structures (28–33). m^7G cap binding by eIF4E and eIF4F and inhibition of translation reactions by m^7G cap structures are characterized by micromolar to millimolar affinities (28–33), whereas affinities of eIF4F for RNAs exceeding the critical length of 60 nt are in the low nanomolar range (Table 2). The affinities for m^7G cap structures have been previously noted to be too weak to mediate the physiological functions attributed to the interaction between eIF4E/eIF4F and capped mRNAs, and it had been hypothesized that the inherent cap-binding ability of eIF4E required augmentation (10). Yet binding of eIF4G fragments to eIF4E had been shown to only moderately enhance binding of eIF4E to m^7G cap structures and short, capped RNAs (10). Based on qualitative cross-linking assays, it had been hypothesized that contacts of eIF4F with the RNA might stabilize eIF4F-mRNA complexes (34). Our quantitative binding data now demonstrate that contacts of eIF4F with the RNA not only enhance but essentially dictate the stability of complexes formed between eIF4F and RNAs. Thus, contacts of eIF4F with RNA may play important physiological roles.

Consistent with this notion, eIF4F features several RNA-binding sites in eIF4G and an RNA-binding motif in eIF4A (11, 35, 36). Although our experiments were not designed to delineate individual contributions of eIF4A and eIF4G to RNA binding, the absence of notable ATP effects on the eIF4F-RNA dissociation constants in our experiments suggests that eIF4A, a DEAD-box protein whose affinity for RNA is affected by ATP (37, 38), may not contribute significant energy to eIF4F-RNA binding. We therefore favor a scenario where contacts of eIF4G to the RNA provide the main energy for eIF4F-RNA binding. In this context, it is interesting to note that a purified *Saccharomyces cerevisiae* eIF4G-eIF4E complex binds several of the RNAs tested under the same reaction conditions with dissociation constants virtually identical to those measured with rabbit eIF4F in this work.[3]

Our analysis of eIF4F-RNA binding also revealed cooperative eIF4F interactions with RNA and a requirement for a minimal RNA-binding site size of ~60 nt. The cooperative binding indicates that multiple eIF4F units are necessary to bind RNA with high affinity. Because multiple units of eIF4F are likely to occupy larger binding sites, cooperativity and large binding site size are consistent.

The requirement of a large RNA-binding site by a protein interacting with the m^7G cap is not unprecedented. The yeast decapping enzyme Dcp1p/Dcp2p also requires a long RNA-binding site for activity, with efficient decapping occurring for a 99-nt but not for a 29-nt RNA (39, 40). eIF4F is thus a further example of a cap-interacting protein binding to capped RNA primarily through contacts distant from the m^7G cap.

Although cap-independent and cooperative RNA binding as well as the large RNA-binding site of eIF4F were delineated with purified components, these findings were strongly supported by results obtained in RRL, where other translation initiation factors are present. In RRL, only RNAs with 56 or more nt strongly inhibited translation, and this inhibition pattern was cap-independent (Figs. 6 and 7). Although one cannot rule out formally that the inhibition seen in RRL is because of effects of the RNAs on other critical translation factors, the striking correlation between results *in vitro* and in RRL renders this possibility unlikely.

Our findings raise intriguing questions about mRNA recognition during cap-dependent translation initiation and about the role of the m^7G cap in this process. The current model for mRNA recognition rests on the assumption that the m^7G cap recruits eIF4F to the mRNA (3), which requires preferential binding of eIF4F to the m^7G cap. Our data indicate that eIF4F does not favor m^7G cap binding over RNA binding. Yet the m^7G cap is critical for efficient translation initiation (Fig. 2), and eIF4E is the only known cap-binding initiation factor (1). Thus, although RNAs are not preferentially bound at the m^7G cap, eIF4F obviously contacts the m^7G cap.

[3] H. Bowers and E. Jankowsky, unpublished results.

These observations can be reconciled if eIF4F senses the m^7G cap subsequent to RNA binding, or if other factors enable eIF4F to preferentially bind to the m^7G cap. The latter scenario is unlikely in RRL, where we found no notable differences between capped and uncapped RNAs in translation inhibition studies (Figs. 6 and 7). Although it remains possible that in other systems certain factors enable eIF4F to associate preferentially with the m^7G cap, in RRL the data favor a scenario where cap-bound eIF4F is sensed after RNA recognition.

Notwithstanding, contacts of eIF4F to the m^7G cap by eIF4F/eIF4E are likely to serve as a signal for further assembly of the initiation complex (8, 41–44). Other factors will have to detect cap-bound eIF4F/eIF4E, and association of such factors could stabilize the eIF4F complex on the cap. *In vitro*, we have tested several factors that might detect cap-bound eIF4F, including eIF4B and eIF4H, but we found no notable effects of these proteins on the eIF4F RNA binding characteristics (data not shown). Further experiments are thus necessary to identify factors that sense cap-bound eIF4F/eIF4E. Promising additional candidates are the poly(A)-binding protein or the 40 S ribosomal subunit, both of which are known to bind to eIF4G (1, 45). It is important to note that such factors may only function to detect cap-bound eIF4F in a certain context, *e.g.* the poly(A)-binding protein might require binding to poly(A) RNA, presence of the 40 S subunit, or both.

Another intriguing question arising from our study concerns the length of the RNA needed for high affinity eIF4F binding. The translation inhibition studies in RRL suggest that eIF4F strongly prefers binding to RNAs with 56 or more nt. However, mRNAs with that many accessible nucleotides at their 5′ end prior to eIF4F binding seem unlikely to exist in cells, although this is not formally known. Cooperative binding of eIF4F might facilitate access to a larger binding site by nucleating association from a smaller footprint, but more data are required to elucidate by which means the large binding site becomes available to eIF4F. In this context, it is interesting to note recent data suggesting the requirement of multiple poly(A)-binding protein units for efficient translation initiation (46). Contacts of multiple poly(A) proteins to eIF4F might necessitate binding of a corresponding number of eIF4Fs to the 5′-untranslated region.

The cap independence of eIF4F-RNA binding also raises the question of how eIF4F finds RNA regions in the proximity of the 5′ end. Most likely, indiscriminate binding to all available RNA sites in a cell and random searches for capped 5′ ends have to be avoided, and therefore, specific mechanisms may exist, which aid "nucleation" of the cooperative eIF4F binding to RNAs in the proximity of the 5′ end. Thus, our finding that intrinsic RNA binding by eIF4F depends on RNA length, not cap recognition, suggests that mRNA recognition by the translation initiation machinery is more complex than spontaneous association of eIF4F to the RNA at the m^7G cap. Other factors quite clearly function in the mRNA recognition step. It is now important to identify these factors, which may play critical roles in the regulation of eukaryotic translation initiation.

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