Translational Repression Dependent on the Interaction of the
Xenopus Y-box Protein FRGY2 with mRNA

ROLE OF THE COLD SHOCK DOMAIN, TAIL DOMAIN, AND SELECTIVE RNA SEQUENCE RECOGNITION*

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We have examined the determinants of the translational repression of mRNA by the Xenopus oocyte-specific Y-box protein FRGY2 using in vitro and in vivo assays. In vitro reconstitution of messenger ribonucleoprotein (mRNP) complexes demonstrates that the sequence-specific RNA-binding cold shock domain is not required for translational repression, whereas the RNA-binding C-terminal tail domain is essential. However, microinjection of reconstituted mRNPs into Xenopus oocytes demonstrates that although translational repression occurs in the absence of consensus RNA binding sequences for FRGY2, the presence of FRGY2 recognition elements within mRNA potentiates translational repression. Analysis of the in vitro assembly of mRNP shows that the cold shock domain alone is not stably incorporated into mRNP, whereas the C-terminal tail domain is sufficient for stable incorporation. We suggest that translational repression of mRNA by FRGY2 is favored by sequence-selective recognition of RNA sequences by the cold shock domain. However, translational repression in vitro and the assembly of mRNP in vivo requires the relatively nonspecific interaction of the C-terminal tail domain with mRNA. Thus two distinct domains of FRGY2 are likely to contribute to translational control.

Translational control of gene expression is particularly important during early metazoan development (Davidson, 1986; Spirin, 1966, 1994). The molecular mechanisms that determine the translational repression (masking) of maternal mRNA are only partially defined. Much attention has focused on the role of regulated polyadenylation of mRNA as a means of controlling translation in the Xenopus oocyte (Jackson and Standart, 1990; Standart, 1992; Wickens, 1992; Wormington, 1994). Cytoplasmic adenylation of mRNA has a major role in the activation (unmasking) of particular maternal mRNAs on oocyte maturation (Wickens, 1992). An additional contribution to translational repression derives from the packaging of maternal mRNA with RNA-binding proteins including the Y-box proteins (Standart and Jackson, 1994; Wolfe, 1994).

In Xenopus laevis, two major proteins, mRNP3 and mRNP4, are associated with maternal RNA in the storage ribonucleoprotein particles (mRNPs) of mature oocytes (Darnbrough and Ford, 1991; Murray et al., 1991). These proteins associate both with mRNAs that are translated in the oocyte and with mRNAs that are masked (Tafuri and Wolfe, 1993). mRNP4 is identical to frog Y-box protein 2 (FRGY2), and mRNP3 shares more than 80% identity with FRGY2 (Murray et al., 1992; Tafuri and Wolfe, 1990). The FRGY2 protein was originally identified as an oocyte-specific transcription factor that associates with the Y-box, a regulatory element found in the promoters of genes that are selectively active in oocytes (Tafuri and Wolfe, 1990, 1992; Wolfe et al., 1992). Thus FRGY2 might be an example of a multifunctional protein capable of associating with DNA and RNA, comparable to the role of TFIIB in the synthesis and storage of 5 S rRNA (Wolffe and Brown, 1988).

An active role for FRGY2 in masking mRNA is suggested from several independent experiments. The reconstitution of FRGY2 with mRNA in vitro represses translation (Richter and Smith, 1984; Kick et al., 1987). Expression of FRGY2 in somatic cells leads to both an increase in mRNA accumulation from promoters containing a Y-box and the translational silencing of that mRNA (Ranjan et al., 1993). Overexpression of FRGY2 in the Xenopus oocyte facilitates the silencing of mRNA synthesized in vivo (Bouvet and Wolfe, 1994). Antibodies that bind the FRGY2 family of proteins relieve the inhibition of translation when introduced into a Xenopus oocyte (Braddock et al., 1994; Gunkel et al., 1995).

The FRGY2 protein has a modular structure containing an amino (N)-terminal nucleic acid binding cold shock domain (CSD) that is conserved between prokaryotic and eukaryotic organisms (Wolffe, 1994). The CSD consists of a five-stranded β-barrel containing a well-characterized RNA binding motif RNP-1 (Schindelin et al., 1993; Schnuchel et al., 1993; Landsman, 1992; Burd and Dreyfuss, 1994a, 1994b). The CSD confers sequence-specific RNA recognition to the FRGY2 protein (Bouvet et al., 1995). The carboxyl (C)-terminal tail domain of FRGY2 also interacts with RNA (Murray, 1994). However, this interaction shows no apparent sequence selectivity (Bouvet et al., 1995). The C-terminal tail domain contains islands of basic/aromatic amino acids and of acidic amino acids containing sites of phosphorylation (Tafuri and Wolfe, 1990; Sommerville, 1992). Phosphorylation of FRGY2 has been implicated in the repression of translation (Kick et al., 1987; Murray et al., 1991).

In this work we have explored the determinants of translational repression by FRGY2. We make use of in vitro reconstitution of FRGY2 interactions with mRNA and in vitro translation to demonstrate a major role for the tail domain in this translational repression. We establish that FRGY2 can bind selectively to specific sequences in vivo, but find that although

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§The abbreviations used are: mRNP, messenger ribonucleoprotein; CSD, cold shock domain; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

22706
this selectivity augments translational repression, it is not essential for the translational repression of mRNA following injection of naked mRNA or reconstituted mRNPs into the *Xenopus* oocyte. Finally, we examine the role of the individual domains of FRGY2 in the assembly of mRNP in *vivo*. We find that the tail domain is essential for the stable incorporation of exogenous FRGY2 into mRNP. Our results provide evidence for an essential role for the tail domain in stabilizing the interaction of FRGY2 with mRNA in *vivo* and in directing the translational repression of mRNA in *vivo*.

**MATERIALS AND METHODS**

**Plasmids**—Plasmid DNAs ps2YW, -pM1, -D4, -D6, and -C6 used to synthesize the flag epitope-tagged FRGY2 mRNA for microinjection were constructed as follows. DNA fragments encoding the FRGY2 cDNA were produced by polymerase chain reaction amplification using Vent DNA polymerase (New England Biolabs Inc.) with oligonucleotide primer sets of K28 (5′ AATTTGGAATTCGAGCTTAAAGATGGTAGGAGGCAGAGCCCGAG3′ for WT, PM1, and D4), K29 (5′ AAT TTGAGTTCAACGTAGCTGAGGAAGGAAGGTTGCGACCCATCTTACAA3′ for D6), and K30 (5′ AAT TTGAGTTCAACGTAGCTGAGGAAGGAAGGTTGCGACCCATCTTACAA3′ for CSD) and either K33 (5′ TCCCGGGATTGTCGACTCTGGTAGCTGTCGTGTCCTTGAGGGCGAG TGTTACGTCG3′ for WT, PM1, and D4) or K34 (5′ TCCCGGGATTGTCGACTCTGGTAGCTGTCGTGTCCTTGAGGGCGAG TGTTACGTCG3′ for CSD). After amplification, the products were cloned into the pSP64 poly(A) vector (Promega).

Histone H1 cDNA was cloned by polymerase chain reaction from *X. laevis* genomic DNA using the primers 5′ GAATTTAAGCTTCAAGATGACGAGG3′ and 5′ GGAACCTCTAGATGTACTTTT AGC3′. This fragment was subcloned into the HindIII and XbaI sites of pSP64poly(A) to give psP H1.11 (Bouvet and Wolffe, 1994). Plasmid pH1.10 was described previously (Bouvet and Wolffe, 1994). pH1Np contained the histone H1 cDNA with the flag epitope sequence under control of the SP6 promoter (kindly provided by Dr. L. Freeman in this laboratory). SP6 H1 + YRS was constructed by kinasing and annealing oligonucleotides 1BS1 (5′ AGCTTAACTCGAGCATAATTTGAACGAGG3′, sense strand) and 1BS2(5′ AGCTTAACTCGAGCATAATTTGAACGAGG3′, antisense strand) and ligating the annealed fragment into the HindIII site of pSP H1.11. pSPD4 was constructed by subcloning the coding sequence of B4 (Cho and Wolffe, 1994) into the HindIII/XbaI site of psP64poly(A) vector by polymerase chain reaction using the primers 5′ CTGGAACTCTAGAGTTACTTTT AGC3′ and 5′ ATATCTGAAATTTGAATTCAAGCTTAAAGATGACGAGG3′.

**Proteins**—GST-fusion FRGY2 protein, its derivatives, and GST protein were overexpressed in *E. coli* and purified as described previously (Bouvet et al., 1995). The proteins were dialyzed against a buffer consisting of 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride. The FRGY2 protein with T7 gene 10 leader peptides (T7-FRGY2) was prepared as described by (Cho and Wolffe, 1994).

**In Vitro Translation**—The capped histone H1 mRNA was synthesized by SP6 RNA polymerase transcription from EcoRI-linearized psP H1.11 (Bouvet and Wolffe, 1994). RNA was heated at 65°C for 10 min and quickly chilled on ice before use. In *vitro* translation was performed with the nuclease-treated rabbit reticulocyte lysate system (Promega). Histone H1 mRNA (0.25 μg) was incubated at 30°C for 60 min in a 25-μl reaction mixture consisting of 15 μl of rabbit reticulocyte lysate, 50 μl each of amino acids including 87.5 μCi of [3H]lysine and 17.5 μCi of [3H]arginine, 20 units of RNasin (Promega), and GST-GRF2 protein as indicated. The mixture was then digested with 5 μl of RNase A at 30°C for 5 min. The aliquots were subjected to electrophoresis in a 4%–20% polyacrylamide gel containing SDS. The gel was fixed, treated with Amplify (Amersham Corp.), and dried. The translation products were detected by fluorography. To detect the phosphorylation of FRGY2 in reticulocyte lysate, GST-GRF2 WT protein was incubated in the same reaction mixture as above except that 40 μl of [γ-32P]ATP (3000 Ci/mmol) was included instead of [3H]-labeled amino acids.

**Oocyte Microinjection**—*In vitro* transcription reactions to obtain capped mRNA with poly(A) tail for microinjection were performed with EcoRI-linearized plasmids by SP6 RNA polymerase as described previously (Krieg and Melton, 1984; Bouvet and Wolffe, 1994). 32P-Labeled primer HI FM4 5′ TCTCTAGATTGGTTGTCGACCCGGGGAGG3′ was used to detect histone H1 mRNA, and primer 5′ GTTGAGGTAGTGTGGTGTCGACAG3′ was used to detect B4 mRNA.
Translational Repression by FRGY2

**RESULTS AND DISCUSSION**

Reconstitution of mRNP Complexes In Vitro and Translational Repression of mRNA in a Rabbit Reticulocyte Lysate—

Earlier work has clearly shown that proteins now known to be of the Y-box family (Spirin, 1994) have the capacity to direct the translational repression of mRNA in a rabbit reticulocyte lysate (Richter and Smith, 1984; Minich and Ovchinnikov, 1992; Evdokimova et al., 1995). Translational repression depends on the mass of protein per mRNA. mRNPs assembled in vivo or reconstituted in vitro have a protein/RNA mass ratio of 5:1, or approximately one protein molecule per 50 nucleotides (Darnborough and Ford, 1981; Richter and Smith, 1984; Marello et al., 1992). We wished to determine the molar ratio of protein to mRNA necessary to repress the translation of histone H1 mRNA in vitro. Reconstitution of in vitro-synthesized H1 mRNA (735 nucleotides in length) with the wild type FRGY2 protein (Fig. 1) led to the progressive repression of H1 protein synthesis (Fig. 2A). Translational repression is almost complete once 2 μg (30 pmol) of FRGY2 have associated with 0.25 μg (1 pmol) of H1 mRNA (Fig. 2C), indicative of one

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**Fig. 2.** Translational repression by FRGY2 protein in rabbit reticulocyte lysate. A, translation reactions in rabbit reticulocyte lysate were directed by 0.25 μg (1 pmol) of histone H1 mRNA in the presence of 3H-labeled lysine and arginine. Lane 1 shows the reaction containing no mRNA. 0.2 μg (3 pmol, lane 3), 0.5 μg (7.5 pmol, lane 4), 1 μg (15 pmol, lane 5), or 2 μg (30 pmol, lane 6) of GST-FRGY2 WT protein was added to the translation reaction. Translation products were analyzed by SDS-PAGE. The gel was dried and subjected to fluorography. The position of histone H1 protein is indicated. Lane M contains 14C-methylated protein molecular weight markers (Amersham Corp.). B, phosphorylation of FRGY2 protein in rabbit reticulocyte lysate. Reactions were performed as in A in the presence of [γ-32P]ATP instead of 3H-labeled amino acids. Two μg of GST-FRGY2 WT protein was added to the reaction shown in lane 2. Proteins were separated by SDS-PAGE and visualized by autoradiography. The position of GST-FRGY2 WT is indicated. Proteins around 70–50 kDa as seen in lane 2 are degradation products of GST-FRGY2 (Fig. 1B). C, titration of FRGY2 in rabbit reticulocyte lysate translation system. Translation reactions directed by 0.25 μg of histone H1 mRNA were performed in the presence of various amounts of GST-FRGY2 WT protein. Translation product was quantified by densitometer. Relative translation activity normalized by the value of the reaction with no GST-FRGY2 WT as 100% is shown.

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**Fig. 3.** Translational repression by GST-FRGY2 and its derivatives. Translation reactions were performed as in Fig. 2A in the absence (lanes 1 and 8) or presence of 15 pmol (lanes 2, 4, 6, 9, 11, and 13) or 45 pmol (lanes 3, 5, 7, 10, 12, and 14) of GST-fusion FRGY2 proteins (Fig. 1) or GST protein as indicated.

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**Fig. 4.** Sequence-specific RNA binding in Xenopus oocytes. 32P-Labeled in vitro-transcribed RNA containing YRS (S) or its one point mutant (NS) was injected into the cytoplasm of Xenopus oocytes. The oocytes were harvested at 90 min after injection. Lysates from total oocytes were homogenized in ice (lanes 1 and 2) or irradiated with UV light (lanes 3–6) and digested with RNase A. Aliquots of UV-irradiated samples were immunoprecipitated with anti-FRGY2 antibody (lane 5 and 6). Samples of 2 oocyte equivalent (lanes 1–4) or 4 oocyte equivalent (lanes 5 and 6) were analyzed by 4–20% SDS-PAGE.

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UV Cross-linking—Oocytes were injected with 32P-labeled RNA, and at 90 min after injection the oocytes were homogenized in 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 50 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride (Bouvet and Wolffe, 1994). The homogenate was centrifuged at 4°C for 10 min, after which the supernatant was irradiated with UV light for 10 min in a Stratalinker (Stratagene). The samples were digested with a final concentration of 0.5 mg/ml RNase A at 37°C for 1 h and analyzed by SDS-PAGE. Ethanol precipitation. RNA from each fraction was resolved in a 1% agarose gel containing formaldehyde. The RNA was transferred to Zeta-probe blotting membrane (Bio-Rad), and histone H1 cDNA was used as the probe to detect its mRNA.

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Endogenous FRGY2 was detected by Western blotting with anti-FRGY2 antibody (Tafuri and Wolffe, 1992). FRGY2 protein and its derivatives expressed from the injected mRNA had flag epitopes at their C termini, thus they were detected by Western blotting with anti-flag M2 antibody (Eastman Kodak Co.).

RNA was isolated from each fraction by phenol-chloroform extraction and ethanol precipitation. RNA from each fraction was resolved in a 1% agarose gel containing formaldehyde. The RNA was transferred to Zeta-probe blotting membrane (Bio-Rad), and histone H1 cDNA was used as the probe to detect its mRNA.

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FRGY2 protein bound for every 25 nucleotides. Consistent with earlier suggestions (Darnborough and Ford, 1981; Marelo et al., 1992; Evdokimova et al., 1995), we suggest that FRGY2 has to interact with mRNA throughout most of the nucleotide sequence in order to efficiently repress translation.

It has been suggested that FRGY2 must be phosphorylated in order to efficiently repress translation in vitro (Kick et al., 1987) or in vivo (Murray et al., 1991; Braddock et al., 1994). We did not explore this hypothesis other than to demonstrate that FRGY2 was phosphorylated in the rabbit reticulocyte extract (Fig. 2B).

In earlier work we defined the RNA binding characteristics of the CSD and tail domains (Bouvet et al., 1995). Having established conditions under which the full length FRGY2 protein directs the repression of histone H1 mRNA in vitro (Fig. 2), we next wished to explore the role of the sequence-specific RNA binding CSD relative to non-sequence-specific tail domain in repressing translation. In the current study we compared the capacity of a limited set of mutants shown in Fig. 1 to repress translation. These include the full length FRGY2 protein (WT); a mutant in which the RNPI motif of the CSD has been altered to significantly reduce sequence-specific RNA binding (PM1); and the CSD domain alone (CSD), the entire N terminus of FRGY2 including the CSD (D4) and the tail domain (D6). The CSD, D4, and D6 all bind to RNA approximately 10-fold less stably than wild type FRGY2. The CSD and D4 proteins retain the capacity to bind to RNA with sequence selectivity (Bouvet et al., 1995).

We find that the WT, PM1, and D6 proteins repress translation of histone H1 mRNA at an excess of 45 pmol of protein to 1 pmol of mRNA (Fig. 3, lanes 1–7). In contrast, the same excess of the D4 or CSD proteins do not significantly influence the translation of H1 mRNA (Fig. 3, lanes 9–14). We suggest that in this assay the sequence-selective CSD domain does not have an essential role in inhibiting translation. In contrast, proteins that retain the tail domain (WT and PM1) or that consist of only the tail domain (D6) can repress translation. We suggest that translational repression reconstituted by FRGY2
in vitro will be sequence-independent, and we next explored whether this might also be true in vivo.

The Role of Sequence-specific RNA Recognition in Translational Repression in Vivo—In earlier work we defined a specific RNA sequence recognized by FRGY2 protein (Bouvet et al., 1995). This FRGY recognition sequence (YRS) is the hexanucleotide 5’ AACAU C3’. We also defined mutants in the YRS that failed to be selectively recognized by FRGY2 (Bouvet et al., 1995). We wished to determine whether the presence of a YRS element immediately adjacent to a translation initiation codon in the H1 mRNA might confer the selective repression of translation in vivo. This synthetic mRNA might provide an enhanced opportunity for sequence-selective repression of translation by the Y-box proteins to be manifest. It should be noted that the sequence of the H1C mRNA used in the experiment lacks a YRS element. We first had to demonstrate that the sequence of the H1C mRNA used in the experiment enhanced opportunity for sequence-selective repression of translation in vivo. Our earlier analyses of sequence-specific interactions of FRGY2 with RNA had all occurred in vitro under conditions that might not reflect those within the oocyte (Bouvet et al., 1995).

Radiolabeled in vitro-transcribed RNA that contained a YRS or a mutant YRS (M3/4–1, see Bouvet et al., 1995) incapable of binding FRGY2 in vitro were injected into the cytoplasm of Xenopus oocytes. The oocytes were incubated for 90 min prior to lysis and irradiation with UV light. After RNase A treatment the proteins were immunoprecipitated with antibodies specific for FRGY2. The RNA containing the native YRS element demonstrated a clear preference for association with FRGY2 compared to that containing the mutant YRS (Fig. 4, compare lanes 5 and 6). We conclude that FRGY2 selectively binds to RNAs that contain the YRS element in vivo. Although it would be of great interest to examine the selective association of mutant or deletion forms of FRGY2 with the YRS element using the in vitro cross-linking assay, this did not prove possible due to the low levels of mutant protein expression compared to the large stores of endogenous FRGY2 protein (see Figs. 6–9).

Our next experiments explored the role of sequence-selective recognition of mRNA in translational repression. H1 mRNA that contained or did not contain a YRS immediately 5’ to the translation start site (see under “Materials and Methods”) was injected into oocyte cytoplasm either naked or reconstituted with FRGY2. The FRGY2 to mRNA molar ratio was either high, 30:1 (Fig. 5A), or low, 1:1 (Fig. 5B). As an internal control we made use of B4 mRNA that was mixed into each sample as naked RNA. In each case we monitored the recovery of mRNA by primer extension (mRNA) and the synthesis of protein by radiolabeling with [3H]arginine and [3H]lysine (Protein). The translational efficiency of H1 mRNA was assessed by normalizing the synthesis of protein to the mass of mRNA recovered (right panel). We find that the introduction of a YRS element does not lead to the selective inhibition of translation of H1 mRNA when injected in naked form (right panel, solid bars). In the presence of a 30-fold molar excess of FRGY2 translation of in vitro-reconstituted mRNPs is inhibited after injection into oocytes. Inhibition of translation attributed to FRGY2 association from the mRNA containing the YRS is approximately 20-fold compared to a 3-fold repression in the absence of the YRS (Fig. 5A, right panel). It should be noted that in presenting this data normalizing protein synthesis to the mRNA level remaining at the end of the incubation period may be misleading. A low level of mRNA at the end of the experiment does not necessarily mean a low level throughout the incubation, thus normalization may exaggerate the translation response. With this reservation, we suggest that significant translational repression occurs in the absence of a YRS element in the H1 mRNA (Fig. 5A, Protein, compare lanes 2 and 3), but that the presence of a YRS element augments translational repression when the FRGY2 protein is present at a 30-fold molar excess over mRNA. We obtained a very different result at equimolar ratios of FRGY2 to H1 mRNA (Fig. 5B). Under these conditions the prior association of FRGY2 with mRNA enhances histone H1 mRNA translation (Fig. 5B, right panel). Again the influ-

![Fig. 6. Expression of FRGY2 protein and its derivatives in Xenopus oocytes. Ten ng of mRNA encoding flag epitope-tagged FRGY2 protein and its derivatives was injected into the cytoplasm of stage VI oocytes together with [3H]arginine and [3H]lysine alone. Lanes M contains [14C]-methylated protein markers.](http://www.jbc.org/)

![Fig. 7. Cytoplasmic localization of FRGY2 and its derivatives expressed from the injected mRNA. Stage VI oocytes were injected with 10 ng of mRNA encoding FRGY2 derivatives, histone H1 or H4 as indicated. [3H]-Labeled lysine and arginine were injected alone (lanes 1 and 2) or together with mRNA (lanes 3–20). The oocytes were incubated at 18°C for 18 h (lanes 1–16) or 6 h (lanes 17–20). Acid-soluble proteins from cytoplasmic (C) or nuclear (N) fractions were analyzed by SDS-PAGE and detected by fluorography. Lane M shows the proteins from oocytes injected with labeled amino acids alone. Lane M contains [14C]-methylated protein markers.](http://www.jbc.org/)
Translational Repression by FRGY2

Fig. 8. Incorporation of FRGY2 into mRNP. Ten ng of mRNA encoding FRGY2 protein or its derivatives was injected into oocyte cytoplasm. At 18 h after injection oocytes were harvested. Oocyte homogenates were subjected to glycerol density gradient centrifugation as described under "Materials and Methods." A, aliquots of oocyte homogenate (input) and each fraction of glycerol density gradient were electrophoresed in a polyacrylamide gel containing SDS and transferred to a polyvinylidene difluoride membrane. Endogenous FRGY2 protein from oocytes without mRNA injection was detected by Western blotting with anti-FRGY2 antibody (top panel). FRGY2-WT, -PM1, -D4, and -D6 proteins expressed from injected mRNA were detected with anti-flag antibody (lower panels). B and C, RNA from each fraction was electrophoresed in an agarose gel under denaturing conditions. RNA was visualized by ethidium bromide staining (B). Endogenous histone H1 mRNA was detected by Northern blotting (C).

ence of FRGY2 on the translation process is more pronounced when the mRNA contains a YRS element (Fig. 5B, right panel). These results suggest that the repression of translation manifested once high molar excesses of FRGY2 interact with mRNA require the association of FRGY2 throughout a large segment of the RNA sequence. The influence of the YRS in facilitating translational repression under these conditions might be to nucleate the association of FRGY2 with a particular mRNA. Since the Y-box proteins assemble large homomultimeric complexes (Tafuri and Wolffe, 1992; Evdokimova et al., 1995) perhaps such nucleation could facilitate ribonucleoprotein complex assembly. The capacity of equimolar amounts of FRGY2 to enhance translation might be related to the capacity of a targeted RNA binding protein to destabilize any inhibitory secondary structure around the initiation codon of the H1 mRNA (Evdokimova et al., 1995). Since the translational repression due to FRGY2 binding to mRNA previously described (Richter and Smith, 1984; Kick et al., 1987) and recapitulated in our experiments (Figs. 2, 3 and 5) occurs at large molar ratios of FRGY2 to mRNA, we focus here on the fact that translational repression can occur on H1 mRNA (Figs. 2, 3, and 5) or globin mRNA (Richter and Smith, 1984; Kick et al., 1987) apparently irrespective of sequence. This is consistent with our earlier observation that prokaryotic mRNAs could also be translationally silenced through interaction with the Y-box proteins (Bouvet and Wolffe, 1994; Ranjan et al., 1995).

Determinants of Translational Repression of mRNA Transcribed in Vivo—Expression of FRGY2 in somatic cells or oocytes facilitates the translational repression of mRNA synthesized in vivo (Ranjan et al., 1993; Bouvet and Wolffe, 1994). Antibodies against FRGY2 microinjected into oocytes relieve the repression of translation of mRNA (Braddock et al., 1994; Gunkel et al., 1995). Thus there is significant experimental evidence suggesting that the interaction of FRGY2 with mRNA contributes to translational masking of maternal mRNA in vivo. Nevertheless the determinants of translational repression in vivo might differ markedly from those operating in vitro (Figs. 2, 3, and 5). Our working hypothesis to account for translational repression by FRGY2 is that the protein interacts with mRNA packaging the majority of the nucleotide sequence, thereby precluding the access of key elements of the translational regulatory machinery (Bouvet and Wolffe, 1994). A comparable role for nucleic acid packaging proteins exists for the histones in regulating the access of the transcriptional machinery to DNA in chromatin (Wolffe, 1995). It has also been proposed that the Y-box proteins including FRGY2 have active roles within the nucleus (Ranjan et al., 1993; Braddock et al., 1994; Gunkel et al., 1995). We next wished to explore any roles of the CSD and tail domain of FRGY2 in establishing a translationally repressed state in vivo.

We expressed wild type FRGY2 and the various mutants in oocytes (Fig. 6) and determined their nuclear or cytoplasmic location in the oocyte. Without exception, the wild type FRGY2 and the mutant proteins accumulated in the cytoplasm (Fig. 7, lanes 1–12). Control experiments using histone H4 and H1 demonstrated the preferential localization of these proteins in the nucleus (Fig. 7, lanes 13–20). Note that the Xenopus oocyte nucleus has 1/10 of the volume of the cytoplasm (Dingwall and Allan, 1984), thus the accumulation of equal amounts of protein in nuclear and cytoplasmic compartments represents a 10-fold higher concentration of protein in the nucleus. The failure of the tail domain to accumulate in oocyte nuclei is in contrast to our earlier demonstration of the accumulation of the tail domain in the nucleoli of Xenopus somatic cells (Ranjan et al., 1993). The proteins used are identical in sequence, and thus we suggest that determinants of nuclear versus cytoplasmic localization differ from the oocyte to somatic cells. We do not find any known nuclear export signals within the FRGY2 sequence (Fischer et al., 1995). However, all of the mutant proteins will retain the capacity for nonspecific interactions with mRNA (Bouvet et al., 1995), which might facilitate their cytoplasmic retention.

We next examined whether the wild type FRGY2 protein or epitope-tagged mutant proteins would be incorporated into mRNP in vivo. We microinjected mRNA encoding the various proteins into oocytes and examined their association with various RNP complexes fractionated on glycerol gradients (Fig. 8). Only the wild type FRGY2 protein and the tail domain (D6) are found in the mRNP fraction, presumably in direct association with RNA. The N terminus of FRGY2 including the CSD (D4) is not found in the mRNP fraction and surprisingly neither is PM1 containing a mutant CSD and an intact tail domain. Both the intact CSD and the tail domain must be required for the stable association of FRGY2 with mRNA in vivo. Our results also demonstrate that a determinant of cytoplasmic localization for FRGY2 and mutants is not the capacity of the proteins to be incorporated into mRNP. An unknown mechanism tethers these highly basic proteins in the cytoplasm.

Our final experiments examined whether expression of the mutant FRGY2 proteins would enhance or interfere with the translational repression of histone H1 mRNA synthesized in vivo. In agreement with earlier work (Bouvet and Wolffe, 1994) in vitro-synthesized H1 mRNA is translationally repressed compared to in vitro-synthesized H1 mRNA injected into the oocyte (Fig. 9A, compare lanes 2 and 3). This translational repression
FRGY2 mRNA
H1 RNA or DNA
\(^{3}H\) amino acids
protein RNA

Fig. 9. FRGY2 mutant proteins expressed from injected mRNA have no effect on translation. Three ng (lane 4) or 10 ng (lanes 5–9) of mRNA encoding FRGY2 or its derivatives was injected into stage VI oocyte cytoplasm. At 6 h after the first injection, 3 ng of histone H1 mRNA (lane 2) or 1 ng of plasmid pH1.10 (lanes 3–9) was injected into the oocyte nucleus. The oocytes were incubated at 18°C for 18 h and then injected with \(^{3}H\)-labeled lysine and arginine. Six hours later oocytes were harvested. Acid-soluble proteins were analyzed by SDS-PAGE, and histone H1 mRNA was detected by primer extension (B).

is maintained in the presence of exogenous FRGY2 (Fig. 9A, lanes 3–5). Expression of the mutant FRGY2 proteins is without significant effect on translational repression (Fig. 9A, lanes 6–9). Thus expression of these proteins in this experiment does not enhance or relieve the translational repression of histone H1 mRNA coupled to transcription operative in oocytes. This could be due to relatively low level of expression of the FRGY2 proteins from injected mRNA compared to the abundance of endogenous FRGY2 (data not shown). Alternatively, this failure could be due to failure of the mutant proteins to stably associate with RNA synthesized in vivo (Fig. 8) compared to their stable association in vitro (Figs. 2, 3, and 5; see also Bouvet et al., 1995). This failure to interact stably with RNA in vivo might be due to competition for RNA binding with endogenous RNA binding proteins including FRGY2. This is not the case for the tail domain (Fig. 8; D6); nevertheless, association with the tail domain rather than intact FRGY2 does not alleviate translational silencing of H1 mRNA (Fig. 9A). It is also likely that protein–protein interaction among RNA-bound FRGY2 and other mRNPs proteins plays an essential role in the formation of mRNP particles.

We find that the determinants of translational repression depend on the assay system used to reconstitute complexes of FRGY2 and mRNA. In vitro reconstitution (Richter and Smith, 1984; Kick et al., 1987; Evdokimova et al., 1995) indicates that the tail domain has a key role in inhibiting translation (Fig. 3). The tail domain binds RNA with no apparent sequence selectivity consistent with the high molar excesses (30–50) over mRNA necessary to repress translation (Figs. 2, 3, and 5). The tail domain is also the site of FRGY2 phosphorylation, a known regulator of translation (Kick et al., 1987; Murray et al., 1991).

In vivo we find that overexpressed mutant FRGY2 proteins with the exception of the tail domain are not stably incorporated into mRNP (Fig. 8). Wild-type FRGY2 requires both the CSD and tail domain for stable association with RNA. A mutant CSD appears to destabilize interaction of FRGY2 with RNA (Fig. 8). The lack of effect of the mutant FRGY2 proteins on translational repression in vivo appears likely to reflect their failure to be incorporated into mRNP. Although the tail domain can bind RNA, translational repression established in vivo is not relieved (Fig. 8) consistent with the capacity of this domain to independently repress translation (Fig. 3). The lack of incorporation into mRNP in vivo may well reflect the failure of FRGY2 derivatives to compete for RNA binding with either endogenous FRGY2 in the cytoplasm or the nuclear RNA binding proteins that will be associated with newly synthesized RNA in the nucleus (Fig. 7). Future experiments will further explore the nature of these nuclear proteins and their potential role in the coupling of translational silencing to the transcription process (Fig. 9) (Bouvet and Wolffe, 1994; Wolffe and Meric, 1996).

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REFERENCES

Almouzni, G., and Wolffe, A. P. (1993) Genes Dev. 7, 2033–2047
Bouvet, P., and Wolffe, A. P. (1994) Cell 77, 831–841
Bouvet, P., Matsumoto, K., and Wolffe, A. P. (1995) J. Biol. Chem. 270, 28297–28303
Braddock, M., Muckenthaler, M., White, M. R. H., Thorburn, A. M., Sommerville, J., Kingsman, A. J., and Kingsman, S. M. (1994) Nucleic Acids Res. 22, 5255–5264
Burd, C. G., and Dreyfuss, G. (1994a) Science 265, 615–621
Burk, C. G., and Dreyfuss, G. (1994b) EMBO J. 13, 1197–1204
Che, H., and Wolffe, A. P. (1994) Gene 143, 233–238
Darnbrough, D. H., and Ford, P. J. (1981) Eur. J. Biochem. 113, 415–424
Davidson, E. H. (1986) Gene Activity in Early Development, Academic Press, Orlando, FL
Dingwall, C., and Allan, J. (1984) EMBO J. 3, 1933–1943
Evdokimova, V. M., Wei, C.-L., Stitikov, A. S., Simonenko, P. N., Lazarev, O. A., Vasilienko, K. S., Ustinov, V. A., Hershey, J. W. B., and Ovchinnikov, L. P. (1995) J. Biol. Chem. 270, 3186–3192
Fischer, U., Haber, J., Boelles, W. C., Mattaj, I. W., and Ludhrman, R. (1995) Cell 82, 475–483
Gunkel, N., Braddock, M., Thorburn, A. M., Muckenthaler, M., Kingsman, A. J., and Kingsman, S. M. (1995) Nucleic Acids Res. 23, 405–412
Jackson, J. R., and Standart, N. C. (1990) Cell 62, 18–24
Kick, D., Barrett, P., Cummings, A., and Sommerville, J. (1987) Nucleic Acids Res. 15, 4099–4109
Krieg, P. A., and Melton, D. A. (1984) Nucleic Acids Res. 12, 7057–7070
Landsdell, N., and Wolffe, A. P. (1995) Mol. Cell. Biol. 15, 6013–6024
Landisman, D. (1992) Nucleic Acids Res. 20, 2861–2864
Marella, K., La Rovere, J., and Sommerville, J. (1992) Nucleic Acids Res. 20, 5590–5600
Minich, W. B., and Ovchinnikov, L. P. (1992) Biochimie (Paris) 74, 477–483
Murray, M. T. (1994) Biochemistry 33, 13910–13917
Murray, M. T., Krohne, G., and Franke, W. W. (1991) J. Cell Biol. 112, 1–11
Murray, M. T., Schiller, D. L., and Franke, W. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11–15
Rajani, R. M., Tufari, S. R., and Wolffe, A. P. (1995) Genes Dev. 7, 1725–1736
Richter, J. D., and Smith, L. D. (1984) Nature 309, 378–380
Schindelin, H., Marahiel, M. A., and Heinemann, U. (1990) Nature 364, 164–168
Schumacher, A., Wiltscheck, R., Czisz, M., Herrier, M., Willinsky, G., Graumann, P., Marahiel, M. A., and Holak, T. A. (1993) Nature 364, 169–171
Sommerville, J. (1992) Bioessays 14, 337–339
Spinar, A. S. (1966) Curr. Top. Dev. Biol. 1, 1–63
Spinar, A. S. (1994) Mol. Rep. Dev. 30, 107–117
Standert, N., and Jackson, R. (1994) Curr. Biol. 4, 939–941
Tafuri, S. R., and Wolffe, A. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9029–9032
Tafuri, S. R., and Wolffe, A. P. (1992) New Biol. 4, 349–359
Tafuri, S. R., and Wolffe, A. P. (1993) J. Biol. Chem. 268, 24255–24261
Toyoda, T., and Wolffe, A. P. (1992) Dev. Biol. 153, 150–157
U.S. A. 87, 9208–9213
Wickens, M. (1992) Semin. Dev. Biol. 3, 399–412
Wolffe, A. P. (1994) Bioessays 16, 245–251
Wolffe, A. P. (1995) Chromatin: Structure and Function, Academic Press, London
Wolffe, A. P., and Brown, D. D. (1988) Science 241, 1626–1632
Wolffe, A. P., and Mercie, F. (1996) Int. J. Biochem. Cell Biol. 28, 247–257
Wolffe, A. P., Tafuri, S., Rajani, M., and Familiari, M. (1992) New Biol. 4, 290–298
Wormington, M. (1994) Bioessays 16, 533–535
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