Cap proximity is a requirement to enable secondary structures and RNA-binding proteins to repress translational initiation via the 5'-untranslated region (5'-UTR) of mammalian mRNAs. We show that in Saccharomyces cerevisiae, unlike mammalian cells, the in vitro translational repressive effect of the mammalian iron regulatory protein 1 (IRP1) is independent of the site of its target in the 5'-UTR, the iron-responsive element (IRE). In vitro studies demonstrate that the binding affinity of IRP1 is also unaffected by the position of the IRE. Using IRE loop mutants, we observe an almost complete loss of IRP1-dependent repression in yeast concomitant with a 150-fold reduction in binding affinity for the IRE target. This mirrors the natural quantitative range of iron-induced adjustment of IRE/IRP1 affinity in mammalian cells. By enhancing the stability of the IRE stem-loop, we show that its intrinsic folding energy acts together with the binding energy of IRP1 to give an additive capacity to restrict translational initiation. An IRE-IRP1 complex in a cap-distal position in yeast blocks scanning 40 S ribosomes on the 5'-UTR. It follows that the positional effect of mammalian site-specific translational repression is dictated by the competence of the mammalian preinitiation complex to destabilize inhibitory structures at different steps of the initiation process.

RNA-RNA and RNA-protein interactions are dominant features of posttranscriptional gene expression. In eukaryotic translational initiation, ribosomes and a large number of initiation factors follow a complex series of steps leading to recognition of the first codon of a reading frame on the mRNA (1–3). The primary pathway of eukaryotic translational initiation for the majority of cellular mRNAs is currently described by a working model which envisages that 40 S ribosomal subunits progressively scan the 5'-UTR from the 5' end in search of start codons (4). Global regulation of translational initiation can be achieved via modifications of the initiation factors (5). Control of initiation on individual mRNAs, on the other hand, is related to specific properties of the structure of mRNA and in particular the 5'-untranslated region (5'-UTR). The most frequently observed form of control mediated by the 5'-UTR is attributed to intrinsic structural properties that impose restrictions on the movement of the ribosomal preinitiation complex along the mRNA (6, 7). For example, sequence elements within the 5'-UTR that are internally complementary can form stem-loop structures that most likely have to be completely disrupted if an initiation complex is to scan through them. The ability of a given secondary structure in the 5'-UTR to act as a barrier to translational initiation is a function of this structure's free energy of formation; increased stability generally leads to stronger inhibition (7–11). However, the sensitivity of the host translational apparatus to this type of inhibition depends on the organism. Thus, mammalian ribosomes are not detectably impeded by structures that are strongly inhibitory to ribosomes of the yeast Saccharomyces cerevisiae (12). Indeed, a stem-loop structure with an estimated stability of approximately −18 kcal/mol inhibits translation in S. cerevisiae by approximately 90% (8–11), whereas an equivalent degree of inhibition in animal cells can only be achieved by a stem-loop with a stability exceeding −50 kcal/mol provided this is not cap-proximal (6).

The impact of intramolecular folding within the 5'-UTR on translational initiation can be a function of the position of the resulting structures relative to other key elements of the mRNA. In higher eukaryotic cells, a given stem-loop structure is more inhibitory when it is cap-proximal than when it is positioned nearer the start codon (13). A proposed explanation for this is that a structure close to the 5' end of the mRNA interferes with the initial steps of ribosome mRNA binding, whereas a structure further away from the 5' end interferes with the scanning process (13). This implies that either the free energy changes of the early ribosome-mRNA interactions are smaller than the thermodynamic driving force intrinsic to the scanning process, or ribosomes can "skip" structural barriers more readily in a cap-distal position. However, neither of these principles seems to apply to S. cerevisiae, in which the position of a stem-loop structure within the 5'-UTR is of little significance in terms of the degree of translational inhibition observed (10, 11). This discrepancy must reflect inherent differences in the pathways of translational initiation in the respective higher and lower eukaryotic systems.

The above considerations are relevant to the function of trans-acting regulators of translation that interact with the 5'-UTR. In particular, RNA-binding proteins are instrumental in the regulation of translational initiation in both prokaryotes (14) and eukaryotes (15, 16). In contrast to the constitutive limitation of translational initiation that can be imposed by intramolecular RNA-RNA interactions, the inducible synthesis or activation of an mRNA-binding repressor protein allows
negative regulation within a defined period. The best characterized eukaryotic example of this type of regulation is based on the binding of the iron regulatory proteins (IRP1 and IRP2) to the iron-responsive element (IRE) in the 5'-UTRs of the mRNAs encoding ferritin and erythroid 5-aminolevulinic acid synthase in vertebrate cells (17). Moreover, both vertebrate and insect IRPs bind to an IRE in the 5'-UTR of the succinate dehydrogenase subunit b mRNA of Drosophila melanogaster (18). Although most studies of the vertebrate system have focused on the role of IRP1, it was shown that both IRPs are expressed in all tissues and bind consensus IREs present in eukaryotic transcripts with equal affinity (19). The affinity of IRP1 for IREs in these mRNAs is high ($K_d = 10^{-10}$–$10^{-11}$ M) at low iron concentrations and sufficient to block translation but is reduced 50- to 100-fold in the presence of iron levels in excess of the requirements of the cell. The expression of the IRP1 gene was also found to be sufficient for strong translational repression of an mRNA bearing an IRE-containing 5'-UTR in S. cerevisiae thus demonstrating that regulation requires no mammalian components other than IRP1 and IRE to function (20).

The influence of the binding of two other RNA-binding proteins to the 5'-UTR has also been investigated in yeast (21). By inserting the appropriate recognition elements in the 5'-UTR of a reporter gene in S. cerevisiae, it was possible to demonstrate translational repression induced by the synthesis of either the human spliceosomal protein U1A or the bacteriophage MS2 coat protein in vivo. This confirmed that a translational regulatory system can in principle be created using the binding energy of any RNA-binding protein for its target.

As with most other RNA-RNA and RNA-protein interactions involved in eukaryotic gene expression, the mechanistic basis of translational control is poorly understood. In this paper, we use the IRP-IRE interaction as a tool in experiments designed to address the principles governing the access of eukaryotic 40 S ribosomal subunits to the initial initiation codon in an mRNA. Analogously to a stem-loop structure, the function of a protein acting as a repressor of initiation depends on its ability to regulate, directly or indirectly, this access. Translational regulation by IRP1 in higher cells is mediated by binding to an IRE that is proximal to the 5' end of the mRNA (reviewed in Ref. 17). Indeed, increasing the distance of the IRE beyond more than 50 nucleotides from the cap greatly reduces the repressive effect of IRP1 binding (22). Moreover, in vitro experiments have indicated that IRP1 binding to an IRE in a suitably capproximal position prevents the formation of stable 43 S complex-mRNA interactions at the 5' end (23). This seems to indicate that IRP1 can only function as a repressor if it acts directly on the initial 43 S-mRNA interaction, which raises the question of whether the impact of the binding of a trans-acting factor such as IRP1 is dictated by specific binding properties of the protein. Alternatively, the ability of 40 S ribosomal subunits to overcome structural resistance (which is a form of "thermodynamic competence") at different steps of initiation has been suggested to play a key role (15). It is therefore evident that resolution of this issue will provide insight into the mechanism of translational regulation via the 5'-UTR. In the current study we have used the specific behavior of the S. cerevisiae translational apparatus to show how the significance of position and repressor binding affinity can only be understood in terms of the thermodynamic principles governing the interactions between the host translational apparatus and the mRNA.

**Position Effects in Eukaryotic Translational Regulation**

**Materials and Methods**

**Strains and Media**—The yeast strain W303 (MATa ade 2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1) was grown in YEP or YNB medium containing either 2% glucose or 2% galactose. Yeast transformation was performed by means of the lithium acetate method (24).

**Plasmid Constructs**—The IRE-containing sequences (Table I) were inserted into the A/I site of the YCp22FL1 plasmid (Fig. 1). The YCp22FL1 plasmid contains the constitutive TEF1 promoter carrying the firefly luciferase reporter gene (LUC) preceded by an IRE containing 5'-UTR (2). A range of mutations was introduced into the loop of the minimal IRE (D). The wild-type IRE was also extended and stabilized by adding G:C base pairs (E and F).

**DNA/RNA Preparation and Luciferase Assay**—DNA cloning and sequencing were performed using standard methods (26). Oligodeoxynucleobonucleotides were synthesized using an Applied Biosystems DNA synthesizer. Total yeast RNA was isolated using the hot phenol method (27) from 20 ml of yeast culture. The Northern blots and luciferase assays were performed as described previously (20).

**Polysonal Analysis in Vivo**—Yeast cell extracts were prepared from cultures in 50 ml of YEP-Gal medium (4°C–20°C) and loaded on 12-mi 15–38% linear sucrose gradients (28). The gradients were centrifuged for 60,000 × g for 2.5 h at 4°C using a Beckman SW40 rotor. RNA was extracted from 600-μl fractions and analyzed by Northern blot analysis as described previously (28).

**In Vitro Transcription, Translation, and Sucrose Gradient Analysis**—DNA templates linearized with Nael (located 33 nucleotides downstream of the LUC start codon) were transcribed in vitro using T7 RNA polymerase (Promega) according to the manufacturer's specifications in the presence of the cap analogue m7GpppG and [α-32P]CTP (Amersham Corp.). The RNA transcripts were separated from free nucleotides by electrophoresis on a 6% acrylamide (acrylamide:bisacrylamide 38:2),...
8 x urea gel. The full-length bands were cut out after visualization by autoradiography and eluted overnight in 400 μl of 300 mM NaOAc, pH 5.2, 100 μM of phenol, shaking at 37 °C. Eluates were extracted with phenol-chloroform and precipitated by adding 3 volumes of 100% ethanol at −70 °C. Extracts from yeast strains W303 and W303 bearing the YCpSUP-IRP1 plasmid grown on YEP medium with 2% galactose were prepared as described (29). 5 ng of labeled RNA was incubated with 12 μl of each extract at 20 °C under translation conditions (29) in the presence of 700 μM methionine, 0.25 mM spermidine, and 400 μg ml⁻¹ yeast tRNA (30) for 5 min and immediately loaded onto the 5–30% linear sucrose gradients. Gradient analysis was performed as described previously (31).

Competition Experiments, Gel Retardation Assays, and Quantification—The relative binding affinities of mutated IRE RNAs were determined by their abilities to compete with IRE-wt for IRP1 binding. The sizes of the in vitro synthesized RNAs were equivalent to the lengths of the leaders indicated in Table I. In vitro transcription vectors linearized with NdeI (the NdeI site contains the ATG start codon of the luciferase gene) were used as templates for the synthesis of the full-length leader RNAs bearing the IREs. The IRE-wt-bearing RNA was labeled with [α-32P]CTP (Amersham Corp.) to a specific activity of 10,000 cpm/ng. The competitor RNAs were labeled with [5,6-3H]UTP (DuPont) to a specific activity of approximately 500 cpm/ng. Synthesized RNAs were visualized by autoradiography in the case of 32P-labeled RNAs and by ethidium bromide staining in the case of 3H-labeled RNAs and purified as described above. After purification, RNAs were reconstituted in diethyl pyrocarbonate-treated water, and the RNA concentrations were measured by scintillation counting. Gel-retardation assay mixtures were prepared in 20-μl reaction volumes. 1 ng of 32P-labeled RNA and serially diluted 3H-labeled competitor RNAs were mixed in a final volume of 10 μl, heated to 65 °C for 5 min, and chilled in ice. Yeast extracts were prepared from cells bearing the YCpSUP-IRP1 plasmid that had been grown on YEP-Gal medium for 8 h. 0.5–1.75 μg of extract in 10 μl of binding buffer (100 mM potassium phosphate, pH 7, 10 mM MgCl₂, 50 mM KCl, 2 mM dithiothreitol) were pretreated for 10 min with 2% 2-mercaptoethanol and 20 units of RNasin ribonuclease inhibitor (Promega) (32) and then incubated with RNA probe at 25 °C for 30 min. 2-Mercaptoethanol improved the sharpness of the shifted bands but did not influence the measured binding affinities (20). After adding heparin to a final concentration of 5 mg/ml, the samples were incubated for an additional 10 min, mixed with 3 μl of loading buffer (100% glycerol, 0.05% bromphenol blue), and loaded on 6% nondenaturing polyacrylamide gels. Electrophoresis was performed on a 1 × TBE at 60 V. The gels were dried, and the amount of RNA-protein complex was determined on a Molecular Dynamics PhosphorImager using the ImageQuant software, version 3.3. The data were analyzed as described previously (33, 34).

RESULTS AND DISCUSSION

Defining the Limits of Binding Affinity Required for Translational Repression by an RNA-binding Protein in Yeast—The 5′-UTR has been shown to act as the site of translational control mediated by secondary structures or protein binding sites. To investigate the relationship between RNA protein binding and the regulation of translation, we used the IRE/IRP1 system, which has previously been found to function in both mammalian cells and yeast (17, 20). Since other work has also shown that the binding of either the human U1A spliceosomal protein or the bacteriophage MS2 coat protein to their respective RNA target elements provides an analogous mechanism of translational regulation in yeast (21), the IRE/IRP1 system can be expected to provide representative data on the principles of action of a range of RNA-binding proteins in eukaryotic translation. At the outset of this work, specific changes in the loop sequence were known to affect the affinity of IRP1 for in vitro synthesized IRE stem-loop structures (33). In the first phase of the present work, we wanted to determine how translational regulation via the IRE-IRP1 interaction in yeast responded to changes in the binding affinity of the repressor/target pair. We therefore made use of a number of the IRE loop mutants already described in vitro by Jaffrey et al. (33). Since our objective was to examine the role of IRE/IRP1 binding affinity in translational regulation in vivo, we sought to obtain binding affinity data that would take into account the potential influence of the RNA environment of the IRE in the 5′-UTR used in...
our expression studies. We therefore synthesized the complete leaders containing the respective mutant versions of IRE as ³H-labeled transcripts in vitro and examined their individual abilities to compete with the equivalent ³²P-labeled leader containing the wild-type IRE in a band-shift assay (Figs. 1 and 2, Tables I and II). Competition curves were constructed using the relative band-shift intensities obtained at different ratios of mutant IRE leader RNA to wild-type IRE leader RNA (see Fig. 2 for example). The concentration of each competitor RNA required in the standard assay to attenuate wild-type IRE binding by 50% (IC₅₀) was assessed graphically. By using an excess of IRE ligands (labeled and unlabeled) over IRP in the extracts, we achieved conditions in which the relative IC₅₀ values corresponded directly to relative dissociation constants (e.g. Ref. 33).

The ³H-labeled IRE-wt RNA as self-competitor yielded an IC₅₀ value of 1, while the substitution or deletion of single nucleotides in the IRE loop (Fig. 1D) decreased the relative affinity of IRP1 by a factor ranging from 3 to 158. This fully covers the range of decrease in IRP binding affinity observed in mammalian cells under conditions of nonlimiting iron. We conclude that the context for the IRE provided by the leader sequence was of secondary importance with regard to the determination of IRP1 binding affinity since the relative values we obtained are similar to those of Jaffrey et al. (33) who used only the IRE sequences. This would be less likely to apply to the interaction between IRP1 and the various IRE-containing leader sequences that interfere with IRP folding or the interaction with IRP1 (see also Ref. 35).

We also investigated the influence of changes in two additional properties of the IRE on the binding to IRP1: its distance to the 5′ end of the mRNA and the G/C content and stability of its stem. Both properties are relevant to the mode of action of the IRE/IRP1 system. The introduction of a spacer between the cap and the IRE (IRE-wt + 50sp; Fig. 1D) did not change the binding affinity of IRP1 (Table II). On the other hand, increasing the stability of the stem by introducing G:C base pairs (IRE-s1 and IRE-s2; Fig. 1, E and F) did influence IRP1 binding. These additional G:C pairs are likely to change the overall structure of the IRE stem significantly (see Ref. 36) also possibly reducing the ability of the IRE to accommodate itself to an “induced-fit” type of binding to IRP. These findings are also consistent with previous work emphasizing the importance of the overall structure of the IRE in determining its affinity for IRPs (37, 38).

**Table I**

| Constructs | IC₅₀ ± S.D. |
|------------|------------|
| IRE-wt     | 1.0 ± 0.2  |
| IRE-mut1   | 3.4 ± 0.4  |
| IRE-mut2   | 14.3 ± 1.6 |
| IRE-mut3   | 109.6 ± 8.2|
| IRE-mut4   | 138.0 ± 12.8|
| IRE-mut5   | 158.5 ± 16.0|
| IRE-s1     | 10.2 ± 1.3 |
| IRE-s2     | 13.2 ± 1.7 |

**Table II**

IC₅₀ values of the wild-type and altered IRE sequences

These are the concentrations of competitor required for 50% inhibition of IRE-wt ligand binding to IRP1 in in vitro band-shift assays. Since these data were obtained under conditions of excess IRE ligand, the values can be directly converted to relative dissociation constants (see e.g. Ref. 33).

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**Fig. 1.** A, B, C, and D: Western blotting analysis of IRP binding to IRE-wt leader RNA (A) and to IRE-wt leader RNA carrying a spacer (B), a G:C pair (C) or both (D). E and F: Effect of changes in the distance to the 5′ end of the leader on IRP binding to IRE-wt leader RNA (E) and to IRE-wt leader RNA carrying a spacer (F).
folding to a predicted $-15 \text{ kcal/mol}$ (IREs1) inhibited translation by 95%, whereas the binding of IRP for this version of IRE gave a greater combined total level of inhibition of 98%. The even more stable IREs2 imposed a constitutive level of inhibition of 99.3% so that the additional influence of IRP1 gene induction was barely detectable (Fig. 3B). These data show that the binding of IRP1 to these extended IRE structures does indeed provide an additive inhibitory effect. Interestingly, however, the affinity of IRP1 was reduced by a factor of 10 and 13, respectively, relative to the wild-type IRE.

In any analysis of the effect of 5′-UTR function on the translation of mRNA, it is essential to determine whether there are concomitant changes in mRNA stability. This may be of significance to any regulatory mechanism involved and is certainly relevant to the accurate assessment of the translational effects under study. Previous work has shown that translational inhibition can modulate mRNA stability; where this occurs the type of response depends on the nature of the gene. LUC mRNA is stabilized upon inhibition of its translation (20, 39). Therefore we analyzed the steady-state abundance of each of the constructs. Whereas the mRNA levels of the respective constructs in glucose medium were all the same, the induction of IRP1 synthesis (in galactose) resulted in degrees of stabilization that were a positive function of the binding affinities for the respective IRE target (panel A) and with enhanced stabilization of the IRE element (IRE-s1 and IRE-s2; panel B). The samples loaded were from cells in which IRP1 synthesis had been induced by growth in galactose medium. The relative abundance data were used to correct the luciferase activities presented in Fig. 3. Northern blot hybridization was performed using radioactive probes for LUC and PGK1 mRNAs, the latter taken as an internal control.

The original leader sequence FL lacking the IRE was taken here as a reference point (normalized to 100%). This leader contains no IRE sequence and therefore supports a higher absolute level of initiation than those leaders bearing an IRE. All values presented are the results of averaging the data from at least three independent measurements and are corrected for mRNA abundance (B and C, compare Fig. 4).
FIG. 5. \textit{In vivo} polysomal gradient analysis interactions with mRNAs containing IRE sequences. Sucrose gradient analysis was performed on extracts from cells containing \textit{LUC} reporter constructs after induction of IRP1 synthesis for 12 h in galactose medium. The diagrams...
tive IREs (Fig. 4). These values were used to correct the raw luciferase activity data to obtain reliable estimates of relative translation rates (Fig. 3).

Having determined the steady-state levels of translation and mRNA abundance of the constructs, we proceeded to examine the interactions between ribosomes and mRNA. We initiated this part of the work with sucrose gradient analysis of in vivo polysome distributions. The first set of experiments revealed that the LUC mRNA was shifted into the monosomal region upon binding of IRP1 to the wild-type IRE (Fig. 5A) as would be expected where translation is inhibited at the initiation step. This accumulation of monosomes was not observed using an mRNA lacking an IRE (Fig. 5B) or under conditions in which IRP1 synthesis is not induced (data not shown). The extent of the shift to the monosomal fractions was strongly reduced with IRE loop mutants that had attenuated IRP1 binding affinity (Fig. 5C). Therefore we conclude that the degree of exclusion of 40 S ribosomal subunits from polysomal fractions is related to the affinity of IRP1 for its binding site in the 5' UTR.

The Properties of the Host Translational Apparatus Dictate the Position Dependence of Translational Repression via an RNA-binding Protein—It is known that for efficient regulation of translation by the IRE-IRP1 complex in higher eukaryotes, the IRE must be located within 40 nucleotides of the 5' end (22). If it is located at a position more distal from the cap, the regulation both in vivo and in vitro is very much weakened. At least in vitro a similar position effect was observed in the case of translational repression by the U1A protein and bacteriophage MS2 coat protein (40). In contrast to these data, our results indicate that there is no position requirement for IRE function in yeast. Increasing the length of the spacer between the 5' end of the mRNA and the IRE-wt (IRE-wt 1 50sp) did not reduce the degree of translational inhibition in vivo in comparison with that of the control IRE-wt construct (Fig. 3). Polysomal gradient analysis revealed that the LUC mRNA with the extended leader was primarily found associated with monosomes and show the fractional distribution of optical absorbance in a sucrose gradient experiment above a Northern blot prepared using the numbered fractions. These data indicate how the binding of ribosomal subunits to the respective mRNAs is affected by the IRP1-IRE interaction. In these blots, cohybridization with a PGK1-specific probe reveals the polysomal distribution of chromosomally encoded PGK1 mRNA. The data shown are those obtained with the following LUC constructs: IRE-wt (A), the control construct FL, which lacks an IRE (B), IRE-mut5 (C), and IRE-wt+50sp (D). To confirm that the observed luciferase mRNA is the full-length nondegraded species containing the spacer region, samples were hybridized with a spacer probe (E and F). The lanes in panel F marked IREwt+50sp and IREwt are controls demonstrating that the spacer probe (E) hybridized only with mRNA including the 5' spacer region.
Thermodynamic Principles Dictate the Lack of a Position Effect in the Yeast 5'-UTR—In conclusion, our data indicate how the molecular basis of translational repression by an RNA-binding protein in the eukaryotic cell is governed by thermodynamic principles (Fig. 7). The position effect of the IRE/IRP1 system is not an intrinsic property of these components but rather derives from the nature of the host translational machinery. The restriction of translation in cap-proximal and cap-distal positions in S. cerevisiae corresponds to what are apparently thermodynamically equivalent processes with there being no effective distinction between initial cap-related mRNA binding of the 40 S ribosomal subunit and scanning in terms of the free energy required to achieve a given level of inhibition. In this respect, the mode of action of the IRE-IRP1 interaction is mechanistically equivalent to that of a stem-loop structure placed either proximally or distally to the 5' end.

As we have seen, the rate or frequency with which the preinitiation complex moves through the site of an IRE-IRP1 complex in the 5' leader depends on the binding energy of the repressor interaction (Figs. 2 and 3). In yeast, the binding affinity of IRP1 for the wild-type IRE is sufficient to block progression toward the initiation codon irrespective of whether the IRE is cap-proximal or cap-distal (Fig. 7, A and B). In the present work we have shown that this applies to a cap-to-IRE distance of 59 nucleotides, a distance that would nullify the repression effect in vertebrate cells (22). While demonstrating that yeast and vertebrate cells differ markedly in this respect, this does not rule out the possibility that extending the distance further might reduce repression in the yeast system. It should be noted that the IRE used in this work is a minimal
form of the wild-type structure and may not be as tightly bound by IRP1 as the full-size natural IRE (compare with Refs. 20 and 22). The wild-type IRE used here, however, restricts translational initiation by at least 92%. Mutations in the IRE element that reduce the binding affinity of IRP1 allow increased rates (frequencies) of ribosomal scanning through the site of the IRE (Fig. 7C). Figs. 2 and 3 therefore provide us with an indirect measure of the relationship between the thermodynamic stability of the blocking element (IRE/IRP1) and the rate at which ribosomes can proceed through a regulatory site on the 5' -UTR in yeast cells (Fig. 3C). This relationship is shifted considerably toward higher free energy values in higher eukaryotic cells. In the latter, the translational apparatus is capable of destabilizing a much more stable inhibitory structure/complex, albeit only when this is placed in a distal position relative to the cap structure. The spectrum of IRP1 binding affinities provided by the mutant IREs studied in this work reflects a variation in relative binding affinity similar to that induced in IRP1 by changes in iron concentration in mammalian cells. On the other hand, only a 10-fold reduction in IRP1/IRE binding affinity sufficed to abolish iron-dependent regulation in rat fibroblasts (38).

The experiments with the extended and stabilized IRE mutants (IRE-s1 and IRE-s2) have demonstrated that the effect of intramolecular RNA-RNA interactions and of intermolecular RNA-protein interactions (at the same site) are effectively additive. The introduction of the additional G:C base pairs into the IRE stem stabilizes a structure that, in itself, is otherwise relatively inconspicuous in terms of the scanning ribosome. G:C base pairs are particularly effective at blocking yeast translational initiation (10). At the same time they alter the overall structure of the IRE, reducing its affinity to IRP1 (Table II). Despite this, the binding of IRP1 gives an additional repressive effect on translational initiation (Fig. 3B). The additional IRP1-induced repression is approximately equivalent to the inhibitory ratio expected from an IRE-IRP1 interaction whose affinity has been reduced 10-fold relative to that of the wild-type IRE (compare with Table II, IRE-s1 in Fig. 3B, and IRE-mut2 in Fig. 3A). These data are also consistent with the model presented in Fig. 7. They indicate that the intramolecular RNA-RNA interactions and intermolecular mRNA-protein interactions act in thermodynamically and mechanistically equivalent ways with respect to the scanning ribosome. Progress through the inhibitory site requires unwinding of the RNA-RNA structure and/or disruption of the RNAprotein complex. In both cases, the ability of the scanning ribosome to achieve this is dictated by the free energy required to disrupt the element or complex at the given site.

The large discrepancy between higher and lower eukaryotic cells in terms of the translatability of mRNAs with structured leaders must reflect mechanistic differences in the mode of action of the respective translational apparatuses. For example, since scanning is apparently an ATP-driven process, mammalian ribosomes may be coupled to the turnover of a larger number of ATP molecules per given length of mRNA scanned than their lower eukaryotic counterparts. On the other hand, in higher eukaryotic cells, the thermodynamic driving force coupled to the initial interaction of the preinitiation complex with the 5' region of the mRNA is effectively weaker than that coupled to scanning. In contrast, in yeast, no distinction is apparent. An alternative way of looking at this is to assume that the association of the preinitiation complex with the 5' end of the mRNA is thermodynamically equivalent in higher and lower eukaryotic cells, and that the difference is in the scanning process. The greater thermodynamic driving force that is associated with higher eukaryotic scanning could be derived from the closer association of eIF4A/eIF4B with eIF4F and/or the preinitiation complex (2), which might allow more free energy from ATP hydrolysis to be brought to bear on the scanning process. The experiments described in this paper have now provided a framework for examining the mechanistic basis for these thermodynamic effects in more detail.

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