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cis-Acting mRNA Structures in Gene-Specific Translational Control

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I. INTRODUCTION

Translation in eukaryotic cells is regulated both globally and in a gene-specific manner. Global control of the rate of cellular protein synthesis occurs through modulating the level and/or activity of the components of the translational apparatus [Hershey, 1991; Merrick, 1992; Morris, 1995; Donahue, this volume]. Gene-specific control of mRNA translation must be exerted through structural features unique to particular mRNA molecules. This review concentrates on those aspects of mRNA structure that result in gene-specific translational control. Aspects of translational control not discussed here, such as regulation by initiation factor phosphorylation (Donahue, this volume) and mRNA polyadenylation (Baker, this volume), are covered elsewhere in this book.

II. FEATURES OF mRNA STRUCTURE THAT INFLUENCE TRANSLATIONAL EFFICIENCY

In the simplest model for translational initiation in eukaryotic cells [Hershey, 1991; Kozak, 1994; Pantopoulos et al., 1994; Donahue, this volume], the 43S preinitiation complex, consisting of the 40S ribosomal subunit and the ternary complex between eIF-2, guanosine triphosphate (GTP), and met-tRNA, enters at the 5′ cap and migrates ("scans") in a net 3′ direction until it encounters an AUG codon in the appropriate context. Initiation factor eIF-4F is a key participant in this process, both by recognizing the cap structure through its cap-binding component (eIF-4E) and by disrupting RNA secondary structure through the RNA helicase activity of its eIF-4A subunit in conjunction with eIF-4B [Fredrickson and Sonenberg, 1993]. Structural features are known to contribute to the inherent translational efficiency of a particular mRNA molecule. These include the environment around initiator AUG codons and other specific characteristics of 5′ leaders. These aspects of mRNA structure have been reviewed recently [Kozak, 1994; Pantopoulos et al., 1994] and will be summarized briefly below.

Efficiency of translational initiation at an AUG codon seems to depend on several features of mRNA structure that are potentially important for regulation, including the nucleotide sequence and RNA structure adjacent to the initiation codon and the proximity of this codon to the 5′ cap. Based on both a survey of available sequences [Kozak, 1987a] and experimental analysis [Kozak, 1987b], the optimal sequence surrounding an initiator AUG seems to be . . . **Pu**CC*AUG***G** . . . , with the critical residues being the purine at −3 and the G at +4. When these residues are not optimal, purine nucleotides at positions −6 and −9 become important [Kozak,

1987b]. Also, in a nonoptimal context, initiation is stimulated by secondary structure downstream of the initiation codon [Kozak, 1990]. This effect of secondary structure is thought to result from slowing down scanning by the preinitiation complex, thereby facilitating AUG recognition. Secondary structure downstream of an initiation site may be important in encouraging initiation at non-AUG codons, as has been found in some genes [discussed in Kozak, 1991a]. The molecular mechanism by which AUG context promotes recognition is not understood; however, mutational studies in yeast strongly implicate eIF-2 in this process [Donahue et al., 1988; Cigan et al., 1989].

The distance between the initiator AUG and the cap seems to be critical in vertebrate cells. Although it has not been studied in precise detail, it appears that initiation from an AUG in an optimal context drops off strongly as the distance from the cap falls below 20–30 nucleotides [Sedman et al., 1990; Kozak, 1991b]. This correlates with the observation that few natural mRNAs are found with 5′ leaders this short [Kozak, 1987a].

With the special exception of a hairpin structure located downstream of an initiation codon in the weak context (discussed above), stable secondary structure located in the 5′ leader of an mRNA generally inhibits translation. Artificial secondary structure with stability greater than −50 kcal/mol strongly inhibits translation both in intact cells and in cell-free extracts [Kozak, 1986, 1989]. Such structures are thought to block scanning by the 43S preinitiation complex and are perhaps too stable to be unwound by the helicase activity associated with eIF-4F/eIF-4B. Less stable structures inhibit only when placed within 12 nucleotides of the cap [Pelletier and Sonenberg, 1985; Kozak, 1989], a position that may obstruct access to the cap by eIF-4F and the preinitiation complex. It should be noted that secondary structures that are otherwise too unstable to influence translation could be stabilized by specific protein binding and thereby block translation (Section IV).

III. REGULATED TRANSLATIONAL SUPPRESSION BY SECONDARY STRUCTURE

As noted in Section II, secondary structure in the 5′ leader of an mRNA generally inhibits its translation. The RNA helicase activity of the eIF-4A subunit of eIF-4F, in combination with eIF-4B, may be important in preparing the mRNA for entry of the 43S preinitiation complex [discussed in Fredricksen and Sonenberg, 1993]. The level of active eIF-4E in cells seems to limit assembly of the multimeric eIF-4F complex, and the number of eIF-4E molecules is considerably lower than the number of mRNA molecules. These results suggest that eIF-4E may be rate-limiting for translational initiation, an influence that may be most pronounced on those mRNAs with highly structured leaders. Consistent with this picture, overexpression of eIF-4E in cells can overcome the inhibitory influence of artificial secondary structure introduced into 5′ mRNA leaders [Koromilas et al., 1992].

Both the cellular activity and level of eIF-4E are regulated in response to growth stimuli [summarized in Morris, 1995]. In this context, it is of interest that many proto-oncogenes and other growth-related genes have long 5′ leaders with extensive secondary structure [Koromilas et al., 1992], which makes them potential targets for translational control through eIF-4E. There are two known examples in which eIF-4E may be involved in regulating the expression of specific mRNA molecules: cyclin D1 and ornithine decarboxylase (ODC).

Regulation of a key enzyme of polyamine biosynthesis, ODC, shows a significant element of translational control in some cell types [Blackshear et al., 1987; White et al., 1987]. The long and potentially structured 5′ leader of ODC inhibits translation in both cell-free extracts and intact cells [Grens and Scheffler, 1990; Manzella and Blackshear, 1990]. Up-regulation by insulin of a reporter construct containing the 5′ leader of ODC correlated with phosphorylation of both

eIF-4E and eIF-4B [Manzella et al., 1991]. Furthermore, expression of endogenous ODC, as well as that of a construct containing the 5′ leader of ODC mRNA, is specifically activated by overexpression of eIF-4E in cells [Shantz and Pegg, 1994]. These results argue strongly for a role for eIF-4E and its phosphorylation in the translational control of ODC.

The cellular content of cyclin D1 protein does not correlate with the level of its mRNA, which suggests post-transcriptional regulation [Matsushime et al., 1991; Rosenwald et al., 1993]. The level of this protein is elevated in response to overexpression of eIF-4E, with no effect on the expression of several other proteins [Rosenwald et al., 1993]. Surprisingly, it seems that in this instance, eIF-4E overexpression acts to enhance cyclin D1 export from the nucleus [Rouseau et al., 1996].

Therefore, there is good reason to think that secondary structure in the 5′ leaders of certain mRNA molecules, besides providing binding sites for regulatory proteins (Section IV) and sites for internal ribosome entry (Section VI), can provide a mechanism for gene-specific modulation of the translation rate, which is mediated through the activity level (phosphorylation state) of eIF-4E.

IV. REGULATION THROUGH REPRESSOR-BINDING SITES

Translation of some mRNA molecules can be regulated through specific binding of translational repressor proteins. Depending on the particular mRNA molecule, binding sites for these regulatory proteins can occur in either the 5′ leader or the 3′ untranslated region (UTR). The mechanism of inhibition by binding to the 5′ leader can easily be interpreted according to the scanning model for ribosome entry, but inhibition by those proteins that bind to the 3′ end of the mRNA must be more complex.

IV.A. Iron Response Element

Regulation of the synthesis of the iron storage protein ferritin is the classic example of regulation in vertebrates through a translational repressor [for recent reviews, see Klausner et al., 1993; Pantopoulos et al., 1994; Mascotti et al., 1995; Rouault et al., 1996]. In an iron-deficient environment, synthesis of ferritin is inhibited and ferritin mRNA is found in messenger ribonucleoprotein (mRNP) particles. When iron is restored to deficient cells, ferritin mRNA moves from mRNP particles into actively translating polysomes. Translational control of ferritin expression mRNA is mediated by a 27-nucleotide element in the 5′ leader referred to as the *iron response element* (*IRE*).The IRE (Fig. 1) consists of a variable base-paired stem, containing an invariant unpaired C, and a conserved six-base loop [Mascotti et al., 1995]. An IRE is also found in another iron-regulated mRNA, the one encoding the erythroid 5-aminolevulinate synthase (eALAS). The IRE seems to be a negative regulatory element, since mutational ablation of protein binding results in a constitutively translated mRNA.

A 98-kDa protein, iron regulatory protein (IRP), binds specifically to the IREs from ferritin and eALAS mRNAs, suppressing translation. The binding activity of this protein is regulated by the availability of iron. IRP has been identified as a cytosolic form of the Krebs cycle enzyme aconitase. Cytosolic aconitase–IRP contains a cubane [4Fe-4S] cluster, and this form of the protein does not bind the IRE but is enzymatically active. The loss of iron from the cluster results in the apo form of IRP, which is enzymatically inactive but binds with high affinity to the IRE. The IRE has been shown to bind in the vicinity of the aconitase-active site of IRP. Based on the crystal structure of the homologous mitochondrial aconitase, a detailed molecular model for the role of this iron-sensing molecule has been proposed [Basilion et al., 1994].

The mechanism by which IRP inhibits translation is becoming clear (Fig. 1). The IRE must be located within approximately 40 nucleotides of the 5′ cap in order to function

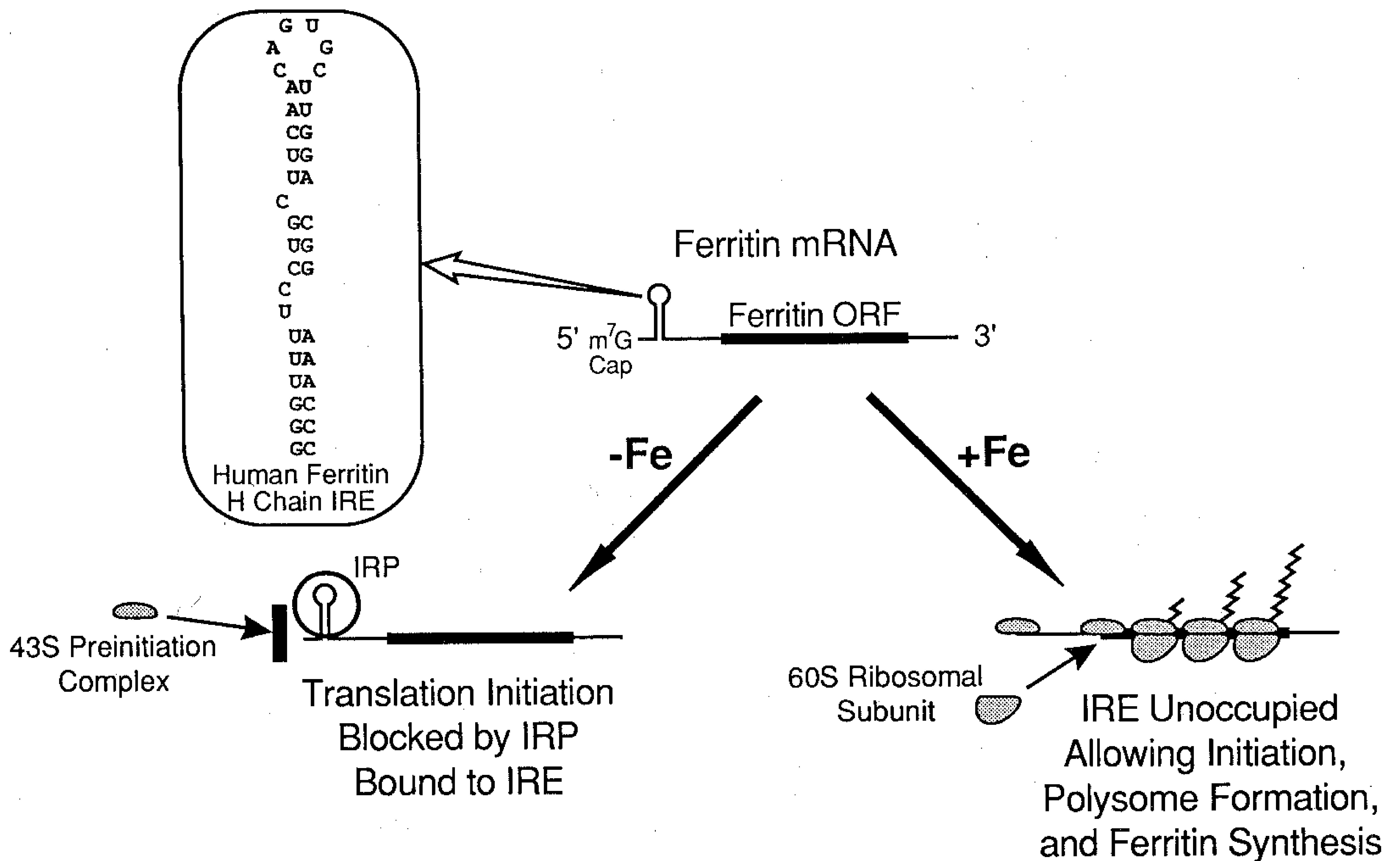

Fig. 1. *Expression of ferritin is controlled translationally through interaction between the mRNA and the RNA-binding protein IRP.*

as a regulatory element. This suggests a model in which the IRP–IRE complex may interfere sterically with the function of the cap in translational initiation. Consistent with this picture, binding of IRP inhibits association of the 43S preinitiation complex with ferritin mRNA [Gray and Hentze, 1994]. Also compatible with this model is the observation that other pairs of proteins and their specific binding sites can replace the IRE–IRP pair, as long as the location is close to the cap. For example, phage MS2 coat protein or snRNP U1A protein can replace IRP if the appropriate binding site is incorporated close to the 5′ end of the mRNA [Stripecke and Hentze, 1992]. These results are inconsistent with a requirement for any specific interactions of the binding protein other than binding to the mRNA and support the model of translational inhibition by steric hindrance of the cap.

IV.B. Polypyrimidine Tracts

Polypyrimidine (pPy) tracts are located at the immediate 5′ end of a class of translationally controlled mRNA molecules that chiefly encode components of the translational machinery, including the structural proteins of the vertebrate ribosome [Morris et al., 1993]. Synthesis of the ribosomal proteins is under translational control in response to mitogenic and developmental signals in a variety of cell types [Kaspar et al., 1993]. All of the vertebrate ribosomal protein mRNAs that have been characterized contain a pPy tract of 5 to 14 nucleotides in length, located immediately adjacent to the 5′ cap. These pPy tracts have no obvious consensus sequence and seem to require only consecutive pyrimidines. Mutation of the pPy tract results in constitutive translation [Levy et al., 1991; Kaspar et al., 1992], suggesting that it acts as a component of a negative regulatory system.

A 56-kDa protein ($p56^{L32}$) that binds to the wild-type 5′ leader of ribosomal protein L32 has been identified in cytosolic extracts of mouse cells. This protein does not bind to mutant leaders that have lost their regulatory properties [Kaspar et al., 1992]. A protein

with similar properties has been identified in *Xenopus* [Cardinali et al., 1993]. Messenger RNAs other than those for ribosomal proteins contain the distinctive 5′ pPy tract, including those encoding eEF-1α [Jefferies and Thomas, 1994] and a protein of unknown function [Yenofsky et al., 1983; Chitpatima et al., 1988], and probably interact with the same binding protein [RL Kaspar and MW White, personal communication]. The $p56^{L32}$ protein binds strongly to poly(U) but differs in several other respects from previously described pPy tract-binding proteins [Morris et al., 1993; Severson et al., 1995]. Interaction of $p56^{L32}$ with 5′ leaders is complex in that high-affinity binding requires not only the pPy tract but also a G-rich region immediately downstream of the tract [Severson et al., 1995]. This downstream region is also required for regulation *in vivo* [Avni et al., 1994].

Repressed ribosomal protein mRNAs reside in mRNP particles that sediment at 35S to 45S. Therefore, they must contain more components than mRNA and $p56^{L32}$ alone. Although it has not been proven, it is suspected that $p56^{L32}$ remains with the mRNA in the translationally repressed particles and may serve as a nucleation site for assembly of these particles [discussed in Morris et al., 1993]. In contrast to regulation of ferritin translation, where interaction of IRP with the mRNA is regulated, binding of $p56^{L32}$ to the 5′ leader of L32 mRNA is unchanged in extracts from resting or activated lymphocytes [Kaspar et al., 1992]. Therefore, in this case, binding of $p56^{L32}$ seems to be constitutive, and assembly of the mRNP particles may be the regulated step in translational suppression [Morris et al., 1993]. Translation of the pPy tract family of mRNAs seems to be regulated through a protein kinase pathway that is sensitive to the drug rapamycin [Jefferies et al., 1994].

IV.C. Sites Located in the 3′ UTR

The 3′ UTR of mRNA molecules plays a major role in regulation of polyadenylation, localization, and stability, topics that are covered elsewhere in this book [Baker, Singer, Parker, Green, four chaters in this volume]. In addition, several examples exist of binding sites for translational regulatory proteins in the 3′ UTRs of mRNAs. All of the limited examples available to date occur in developmental systems.

Given our current models for translation initiation, it is more difficult to envision mechanisms for inhibition of translation through protein binding to 3′ sites than to sites in the 5′ leader. Binding of a protein to the 3′ UTR could interfere with a required interaction between the 5′ and 3′ ends of the mRNA during initiation, mediated, for example, by the poly(A)-binding protein [Tarun and Sachs, 1995]. Alternatively, binding of a regulatory protein to the 3′ UTR could trigger sequestration of an mRNA into an untranslatable mRNP particle [Morris et al., 1993]. In the following examples, it is not yet possible to distinguish between these classes of mechanisms.

Expression of the vertebrate protamine genes (*Prm-1* and *Prm-2*) is developmentally regulated during sperm maturation. The *Prm* mRNAs are synthesized early in development but are translated only at late times when protamines are required for DNA packaging in the sperm head [reviewed in Braun et al., 1989]. This temporal delay in protamine translation is required for normal sperm development [Lee et al., 1995]. Temporal translational control was transferred with the 3′ UTR of *Prm-1* to a chimeric mRNA in transgenic mice, and a region sufficient to mediate this regulation was localized to a 62-nucleotide fragment [Braun et al., 1989]. As expected for involvement of a trans-acting suppressor of translation, stage-specific differences were lost when deproteinized mRNA was translated *in vitro* [Fajardo et al., 1994]. Complementary DNA clones have been obtained that encode a protein, Prbp, capable of binding to the cis-acting 62-nucleotide sequence of the *Prm-1* 3′ UTR [Lee et al., 1996]. Antibody to Prbp coprecipitated *Prm-1* mRNA from testis extracts, and Prbp was not detected in elongated spermatids in

which *Prm-1* mRNA is actively translated [Lee et al., 1996]. These results are consistent with the presence of Prbp in mRNP particles containing repressed *Prm-1* mRNA. The situation is more complex, however, since there are regions outside the 62-nucleotide sequence that are conserved between the *Prm-1* and *Prm-2* genes and contain protein-binding sites [Kwon and Hecht, 1991; Kwon and Hecht, 1993; Fajardo et al., 1994]. One of these other binding sites has now also been shown to confer developmental control on a reporter gene [M Fajardo and R Braun, unpublished results], suggesting the existence of multiple translational control elements that could act either redundantly or synergistically. In addition, the 3′ UTR of the *Prm-1* mRNA contains a binding site for a 71-kDa protein, Spnr, that is localized in a spermatid-specific microtubular array and may be involved in other aspects of mRNA metabolism [Schumacher et al., 1995].

The heterochronic gene, *lin-14*, of *Caenorhabditis elegans* seems to be translationally regulated through a sequence in its 3′ UTR. Its product, Lin-14 protein is located in the nucleus and functions during early larval development [Ambros and Moss, 1994]. The synthesis of Lin-14 protein is down-regulated at later developmental stages, but its mRNA remains [Wightman et al., 1993]. Sequences controlling suppression of *lin-14* mRNA expression lie within the 3′ UTR, as demonstrated by mutation as well as by transfer to a reporter gene [Wightman et al., 1993]. Down-regulation of Lin-14 synthesis is controlled by the *lin-4* gene, which is located in an intron of a gene of unknown function [Lee et al., 1993]. The products of *lin-4* are two RNA molecules that seem not to be translated; the most abundant species is only 22 nucleotides in length, and the minor form is about twice as long. The abundant *lin-4* product shows partial complementarity to seven sequences within the *lin-14* 3′ UTR that are evolutionarily conserved between related species [Lee et al., 1993]. Although the data suggest strongly that the *lin-4* RNA acts through duplex formation with multiple sites in the 3′ UTR of *lin-14* mRNA, the mechanism by which this interaction inhibits translation is not clear. Possible mechanisms include directing the *lin-14* mRNA into mRNP particles, interfering with possible interactions between the 3′ and 5′ ends of the mRNA, mislocating it within the cell, or causing a modification in the mRNA structure, such as altering the degree of polyadenylation. Furthermore, it has not yet been determined whether the *lin-4* RNA is acting alone or as part of a nucleoprotein complex.

Erythroid 15-lipoxygenase (LOX) participates in degradating the phospholipids of the mitochondrial membrane during maturation of reticulocytes into erythocytes. LOX mRNA is present during the early stages of erythropoiesis but is translated only when reticulocytes move to the circulation. A 48-kDa protein was identified in extracts of bone marrow cells and reticulocytes, which would bind to the 3′ UTR of LOX mRNA [Ostareck-Lederer et al., 1994]. Purified 48-kDa protein inhibited translation in reticulocyte lysates of both LOX mRNA and a reporter mRNA containing the LOX 3′ UTR. Inhibition required the region of the 3′ UTR that bound the protein. The mechanism of this inhibition is of interest: does the 48-kDa protein promote formation of mRNP particles in the reticulocyte lysate or does it interfere with an interaction between the 5′ and 3′ ends of the mRNA that is necessary for translational initiation? One other critical issue is whether the protein–RNA interaction in this instance—inhibits translation in intact cells as well as *in vitro*.

Other recently discovered mechanisms of translational control involving the 3′ UTR are implicated in the production of gradients of regulatory molecules that control pattern formation in the developing *Drosophila* embryo [reviewed in Curtis et al., 1995]. Translation of the mRNA encoded by the *hunchback* gene is repressed by an opposing gradient of the protein product of *nanos*. *Nanos response elements* in the 3′ UTR of *hunchback* mRNA bind the product of the *pumilio* gene; this pro-

tein–RNA complex is thought not to be inhibitory by itself, but to form the binding site for Nanos protein, thereby assembling the final inhibitory complex [Murata and Wharton, 1995]. The existence of a second 55-kDa protein that binds to the nanos response elements suggests that the mechanism may have additional complexities. Regulation of *caudal* mRNA translation by an opposing gradient of the *bicoid* gene product is similar to the relationship between *hunchback* and *nanos* in that the Bicoid protein binds to the 3′ UTR of *caudal,* inhibiting translation. Interestingly, Bicoid is also a transcription factor, and this interaction with *caudal* mRNA seems to be mediated through the DNA-binding homeodomain of Bicoid [Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996]. One wonders how far this bifunctional role of homeodomain proteins extends beyond the present example. A glimpse into the possible mechanism of translational regulation of *caudal* mRNA is provided by the observation that cap-dependent translation is inhibited through binding of Bicoid but not through initiation driven by an internal ribosome entry site [Dubnau and Struhl, 1996]. This result is clearly inconsistent with a model in which the primary result of Bicoid binding is to mask the mRNA by triggering assembly of an mRNP particle.

IV.D. Autoregulatory Feedback Systems

Autogenous regulation of gene expression occurs commonly in phage and bacteria [Goldberger, 1974; Nomura et al., 1984; Maloy and Stewart, 1993], but few examples, particularly at the translational level, have been reported in vertebrate organisms. Recently, however, notable examples in mammalian cells have appeared in which translation of an mRNA seems to be regulated by its protein product.

The best defined of these autoregulatory systems involves expression of thymidylate synthase (TS), a key enzyme in the synthesis of DNA precursors. The TS mRNA contains two high-affinity binding sites for TS protein, one at the translational initiation site and one in the coding region [Chu et. al., 1993a]. Recombinant human TS specifically inhibited translation of TS mRNA in rabbit reticulocyte lysates [Chu et al., 1991]. Inhibition of translation was completely relieved by the TS substrates, dUMP or 5,10-methylene-tetrahydrofolate, or by the enzyme inhibitor 5-fluoro-dUMP, suggesting interaction between the active site of the enzyme and the RNA-binding site. Physiological credence for these *in vitro* observations was provided by the fact that TS was found in association with its mRNA in extracts of human colon cancer cells [Chu et al., 1994]. Interestingly, TS also seems to interact with c-*myc* mRNA both *in vitro* and *in vivo* [Chu et al., 1995], suggesting that the role of TS protein in the translational control of growth-related genes may be broader than simply autogenous regulation of its own synthesis.

Another example of a key enzyme in nucleotide biosynthesis that possibly regulates its own synthesis is dihydrofolate reductase (DHFR). Treatment of human cancer cell lines with the DHFR inhibitor methotrexate led to an elevation in the level of DHFR protein that seemed to be regulated post-transcriptionally [Domin et al., 1982]. More recently, it has been found that DHFR protein binds specifically to its mRNA and prevents its translation in an *in vitro* system [Chu et al., 1993b]. Both mRNA binding and translational inhibition were prevented by the presence of the DHFR substrate dihydrofolate. The parallel between translational regulation of DHFR and TS is inescapable. However, TS protein did not inhibit DHFR translation, and DHFR protein did not inhibit the translation of c-*myc* mRNA [Chu et al., 1993b]. It is noteworthy and intriguing that three proteins with primary metabolic roles—IRP–aconitase, TS, and DHFR—have apparently provided a source of evolutionary raw material for generation of specific mRNA-binding regulatory proteins.

When resting vertebrate cells are stimulated to reenter the cell cycle, the level of mRNA encoding tumor suppressor protein

p53 is elevated rapidly, whereas the level of p53 protein increases only after entry of the cells into the S phase [Mosner et al., 1995]. When G_1-phase cells, containing the translationally repressed p53 mRNA, are exposed to γ-irradiation, expression of p53 protein is activated in a manner that is resistant to actinomycin D. At least one mechanism of translational control seems to be through autogenous regulation, since p53 protein binds specifically to the 5′ leader of its mRNA and suppresses its own translation when added to a cell-free system, possibly by stabilizing an extensive stem-loop structure in the mRNA [Mosner et al., 1995]. The putative role of p53 in translational control may not be restricted to autogenous regulation, since a dominant negative mutant of p53 relieves translational repression mediated by the 5′ leader of cyclin-dependent kinase-4 (CDK4), while wild-type p53 enhances repression [Ewen et al., 1995]. In the latter instance, it has not been established whether translational suppression is due to direct interaction of p53 with the CDK4 mRNA or to an indirect effect.

V. UPSTREAM INITIATION CODONS AND OPEN READING FRAMES

According to the simple scanning model for translational initiation, the presence of initiation codons within the 5′ leader of an mRNA should influence initiation at the major open reading frame by capturing scanning ribosomes [Kozak, 1991a]. As expected, most mRNA molecules do not contain upstream initiation codons in a strong context, but a significant fraction do [Kozak, 1987a]. Upstream initiation codons, when in frame with the major cistron, can alter the structure and physiological function of the protein product. Upstream AUGs, when they occur as part of either an overlapping, out-of-frame open reading frame (ORF) or a free-standing upstream open reading frames (uORF) of particular coding sequence (Fig. 2), can dramatically inhibit translation, sometimes in a regulated fashion.

V.A. In-Frame, Overlapping uORFs

When upstream initiation codons in frame with the major cistron (Fig. 2A), alternative protein products with different amino termini can be produced [reviewed in Kozak, 1991a]. One well-studied example is that of the C/EBP family of leucine-zipper transcription factors, in which activators and inhibitors are encoded by the same mRNA molecules. LAP (C/EBPβ) is a 36-kDa protein that is highly enriched in liver nuclei and a potent transactivator of several liver-specific genes, including serum albumin. The initiator AUG of LAP has a suboptimal context (CCC**AUGG**), which contributes to inefficient translational initiation and formation of a second protein, the 20-kDa LIP, through leaky ribosome scanning [Descombes and Schibler, 1991]. LIP contains dimerization and DNA-binding domains but lacks the amino-terminal transactivation domain of LAP. Therefore, LIP acts as a naturally occurring, dominant-negative inhibitor of LAP-activated transcription, probably through formation of inactive heterodimers and competition with LAP for DNA binding. The LAP:LIP ratio increases approximately fivefold during postnatal rat development, suggesting that the regulation of AUG codon use is an important determinant of the transcriptional activity of this factor [Descombes and Schibler, 1991]. A similar situation is found in the related gene encoding C/EBPα, with the synthesis of a full-length, active transcription factor and an inactive, amino-truncated product [Ossipow et al., 1993]. In the case of C/EBPα, an evolutionarily conserved uORF located seven nucleotides upstream of the first AUG of the major cistron, together with a nonoptimal context, contributes to the leakiness and to the formation of the truncated product [Calkhoven et al., 1994]. Other, less well characterized examples of in-frame, overlapping ORFs have been catalogued by Kozak [1991a].

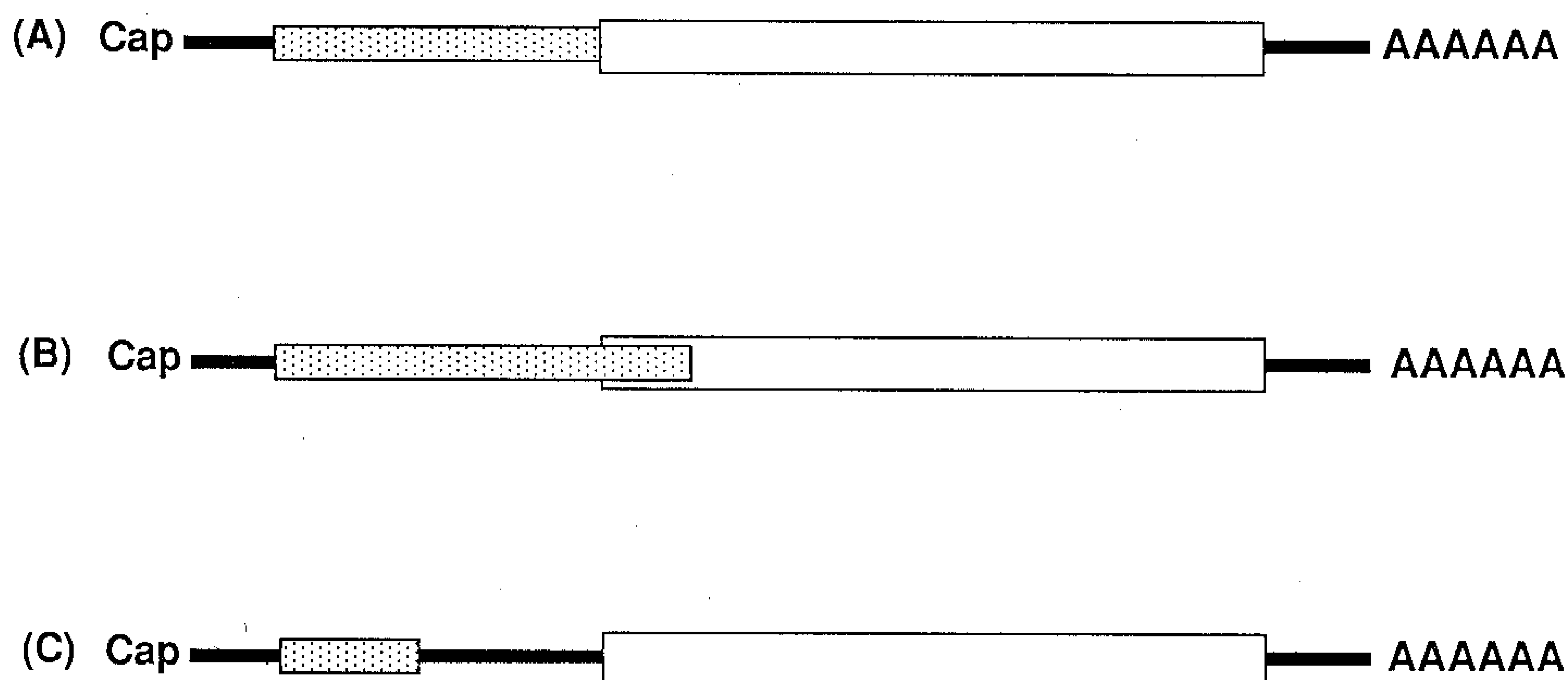

Fig. 2. *Naturally occurring configurations in eukaryotic mRNAs of OREs upstream of the major protein-coding cistron. (**A**) In-frame, overlapping. (**B**) Out-of frame, overlapping. (**C**) Free-standing.*

V.B. Out-of-Frame, Overlapping uORFs

When two ORFs overlap, but in an out-of-frame configuration, a very different situation occurs (Fig. 2B). Two unrelated peptides are produced, and translation of the downstream ORF is inhibited to an extent determined by the strength of upstream initiation. This condition has been observed primarily in viral systems [Kozak, 1991b], perhaps reflecting pressure for compact viral genomes. One notable example of a cellular gene with this arrangement is that encoding tissue inhibitor of metalloproteases (TIMP). The mRNA encoding this glycoprotein, which regulates the breakdown of extracellular matrix, contains in its 5′ leader an ORF that overlaps the TIMP cistron by 65 nucleotides in the -1 reading frame [Edwards et al., 1986; Coulombe et al., 1988]. This uORF inhibits translation of TIMP by a factor of 3 in cell-free systems [Waterhouse et al., 1990]. Translation is regulated by eliminating the uORF from the mRNA through the use of an alternative promoter (Section VII).

V.C. Free-Standing uORFs

Free-standing uORFs, those that do not overlap the major cistron (Fig. 2C), occur commonly in vertebrate genes encoding oncoproteins and other products related to growth control. Some, but not all, of these uORFs suppress downstream translation [Geballe and Morris, 1994]. The characteristics of a uORF that lead to translational suppression are discussed in this section.

In some genes, translational suppression depends on the amino acid coding information of the uORF. Well-studied examples with this characteristic are the vertebrate *AdoMetDC* gene and the human cytomegalovirus *gpUL4* (*gp48*) gene [Geballe and Morris, 1994]. The 5′ leaders of both genes contain uORFs that share many characteristics. Both uORFs must be translated to inhibit translation of the downstream cistron. Furthermore, missense mutations in the uORF, particularly close to the carboxy terminus of the peptide, destroy the inhibitory activity of both. Synonymous mutations in these same codons, which do not alter the structure of the peptide, uniformly preserve the inhibitory effect. Although the amino acid sequence of the peptide product at its carboxy terminus is critical for inhibition of expression of the downstream cistron, the nucleotide sequence downstream of the termination codon of the uORF is unimportant. Both the *AdoMetDC* and the *gp48* uORFs seem to act only in cis, at least in intact cells.

These experimental results with cis-acting, sequence-dependent uORFs suggest a class of models for the mechanism by which they inhibit downstream translation [Geballe and Morris, 1994]. Interference with the process of termination by the peptide product of the uORF would result in the ribosome with the nascent peptide stalling at the termination codon. This stalled ribosome would create a blockade to scanning by other ribosomes loading at the 5′ cap, thus inhibiting translation of the downstream cistron. Both *AdoMetDC* and *gp48* mRNAs sediment in sucrose gradients at a position coincident with the monosome fraction, a result that is consistent with this model. Furthermore, recent results using the ribosome "toeprint" technique have localized the arrested ribosome to the 3′ end of the *gp48* uORF [Cao and Geballe, 1996]. The targets of the uORF-encoded peptides have not yet been identified, but likely candidates are structures in the ribosome channel, the peptidyl transferase, or the release factors involved in termination. In an analogous prokaryotic regulatory system, the peptide product of a uORF in the *cat-86* mRNA interacts specifically with the bacterial peptidyl transferase to inhibit translation [Gu et al., 1994].

Another example of inhibition by uORFs is provided by the yeast gene encoding transcription factor GCN4. The leader of the GCN4 mRNA contains four short uORFs that are involved in its translational control [Hinnebusch, 1990; Hinnebusch et al., 1993]. Translation of either uORF 2, 3, or 4 leads to strong repression of expression of the downstream GCN4 cistron. This strong block has been studied extensively with uORF 4 [Grant and Hinnebusch, 1994]. Although the detailed mechanism is not understood, the high (G+C) content of the final codon and the 10 nucleotides immediately downstream of the termination site seem to be responsible for the strongly suppressive properties of uORF 4. It is these properties that preclude further scanning and reinitiation at the GCN4 cistron.

In order for these free-standing uORFs to participate in a regulatory scheme, there must exist a regulated mechanism for circumventing or bypassing their suppressive influence on the downstream cistron [discussed in Morris, 1995]. One straightforward mechanism to achieve this is to simply eliminate the uORF from the mRNA through the use of alternative promoters or processing (Section VII). In the case of the *AdoMetDC* gene, the positioning of the uORF 14 nucleotides from the cap seems to be critical for determining its cell-specific recognition [Ruan et al., 1994]. The regulation of GCN4 translation is complex in that the termination region of uORF 1, which is (A+U) rich (in contrast to uORFs 2, 3, and 4), allows reinitiation farther downstream. Subsequent reinitiation at one of the three downstream uORFs blocks further translation (see above), while reinitiation at the GCN4 cistron produces GCN4 protein. The site of reinitiation is controlled by the state of phosphorylation of the α subunit of eIF-2, which thereby regulates production of GCN4 protein [Dever et al., 1992]. Although examples of regulation through the activity of uORFs are currently limited, given the regulatory importance of the genes with which they are associated, one suspects that this list will be expanding rapidly.

One property of regulation through uORFs should be emphasized. The system contains an inherent property of amplification. If a system is poised appropriately [discussed in Morris, 1995], a small change in recognition of a uORF can result in profound alteration in the rate of translation of the downstream cistron. For example, if a uORF blocked scanning 98%, only 2% of the scanning ribosomes would reach the downstream cistron. If recognition of the uORF is then decreased to 80%, a minor change, 20% of the ribosomes now reach the downstream cistron, resulting in a 10-fold increase in the rate of production of the protein product. Therefore, with genes such as *AdoMetDC* or *GCN4,* small changes in the activity of the factor re-

sponsible for recognition of the uORF could produce large effects on the synthesis of the gene product.

VI. INTERNAL RIBOSOME ENTRY SITES

The simple scanning model for initiation, whereby preinitiation complexes enter at the 5′ cap, does not seem to apply to all mRNA species. Some mRNAs possess in their 5′ leader a complex structure known as an *internal ribosome entry site* (*IRES*), which allows initiation in a manner independent of the 5′ cap. Cap-independent initiation has been best characterized for the picornavirus RNAs, which contain IRESs several hundred nucleotides in length and are thought to be folded into complex secondary structures [Jang et al., 1990; Sonenberg, 1990].

Initiation using IRES structures seems to require factors present in the host cell. Translation of poliovirus RNA in reticulocyte lysates is poor and lacks fidelity; these defects can be abrogated by addition of extracts from other mammalian cells [Brown and Ehrenfeld, 1979; Dorner et al., 1984]. Two IRES-binding proteins of 52 kDa and 57 kDa were identified as La autoantigen [Meerovitch et al., 1993] and nuclear polypyrimidine tract–binding protein (PTB) [Borman et al., 1993; Hellen et al., 1993], respectively. Addition of La to reticulocyte lysates seems to specifically restore the ability to translate poliovirus RNA accurately [Svitkin et al., 1994]. Immunodepletion of PTB from HeLa cell extracts inhibited translation of picornavirus RNAs, with no effect of β-globin mRNA translation [Hellen et al., 1993]. Although these results are consistent with a role for La and PTB in IRES-dependent initiation, the molecular mechanisms by which they act have yet to be defined.

Since recognition of IRES structures seems to require factors from the host cell, it is perhaps not unexpected that cellular mRNAs have been identified that contain IRES activity within their 5′ leaders. This has been found for both the mammalian BiP mRNA [Macejak and Sarnow, 1991] and the *Antennapedia* gene of *Drosophila* [Oh et al., 1992]. One does not yet know whether the mechanism of initiation at the IRESs of cellular genes is the same as with the picornaviruses. In this respect, it is interesting to note that there is no apparent structural similarity between the cellular IRESs and those of the picornaviruses.

At this point, we are just beginning to appreciate the importance of IRES elements in cellular mRNAs. Why should a cellular mRNA use this mode of translational initiation in contrast to the widely used cap-dependent mechanism? One suspects that the answer may be related to gene-specific translational control, although at present there are no reports of regulated IRES activity.

VII. REGULATION THROUGH ALTERATION OF mRNA STRUCTURE

As should be clear from the theme of this review, various aspects of mRNA structure—secondary structure, protein-binding sites, uORFs—can influence the translatability of a particular message. One way to regulate translation of an mRNA is simply to include or exclude one or more of these structural features. This could be accomplished through the use of alternative promoters to yield different 5′ leaders or through alternative processing, which could alter any region of a particular mRNA species. These aspects of mRNA structure, as they relate to translation, have been reviewed extensively [Kozak, 1991a], and only selected examples will be presented here.

The translation of tissue inhibitor of metalloprotease (TIMP) is controlled by the growth status of the cell through modification of the 5′ leader of its mRNA by the use of an alternative promoter. In resting fibroblasts, a long form of the mRNA is produced that contains a uORF, which is out of frame with the main cistron and overlaps it (see Section V). Mito-

genic activation of quiescent fibroblasts induces the use of an alternative transcriptional start site, which in turn generates a short form of the TIMP mRNA [Edwards et al., 1986]. The growth-induced short form of the mRNA lacks the initiation codon of the uORF, thereby enhancing the translatability of the message threefold [Waterhouse et al., 1990].

Promoter switches also eliminate uORFs from the 5′ leaders of the mRNAs encoding TGF-β3 [Arrick et al., 1994] and proenkephalin [Rao and Howells, 1993]. Other examples are discussed in Kozak [1991a]. The extent of the possible complexity is illustrated by the c-*fgr* gene, in which the 5′ leader is encoded by seven distinct exons. Two tissue-specific promoters, together with alternative splicing, generate at least six c-*fgr* mRNA species with different 5′ leaders [Link et al., 1992]. Although the translational efficiencies of these mRNA species have not yet been reported, the fact that several of these leader exons contain uORFs suggests that there may be differences.

VIII. CONCLUSIONS AND PERSPECTIVES

In this review, I have summarized those features of mRNA structure that are known to contribute to gene-specific translational control. At this point, we can formulate a series of conclusions that should have some predictive value when considering new systems that are under specific translational control:

- Complex structures in the 5′ leader are generally inhibitory, unless they form an IRES, and can provide selectivity in response to regulated eIF-4F activity.
- Specific, regulated translational repressor molecules exist and can interact with binding sites in either the 5′ leader or the 3′ UTR.
- Some, but not all, uORFs inhibit translation of associated cistrons; various mechanisms seem to exist for regulating this inhibition.

A number of obvious questions remain to be addressed. Through what mechanisms do particular translational repressor molecules down-regulate translation of their mRNAs—through direct inhibition of translational initiation or by triggering assembly of an mRNP particle? What are the targets with which the nascent peptide products of the sequence-specific uORFs interact? What specific mechanisms are available to circumvent, in a regulated manner, the suppressive influence of uORFs? How prevalent are autoregulatory feedback loops such as those represented by TS and DHFR? What is the significance of IRESs in cellular mRNAs, and is their activity regulated in unique ways? How often are sequence-specific DNA-binding proteins involved in regulating expression of mRNA molecules, as has been found in regulation of translation of *caudal* mRNA by the transcription factor Bicoid? These questions, and others too numerous to list, represent a fertile future for research directed to understanding the biology and the molecular details of the roles that cis elements located in mRNA molecules play in translational control.

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