The Two Upstream Open Reading Frames of Oncogene mdm2 Have Different Translational Regulatory Properties

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Few details are known of the mechanisms through which multiple upstream open reading frames (uORFs) interact to regulate translation in higher eukaryotes. The predominant transcript of oncogene mdm2 in normal human cells (L-mdm2) contains two upstream open reading frames in its 5′ leader. Elimination of these two uORFs raises the translational efficiency of the transcript by over 10-fold in HeLa cells. The 5′-most uORF (uORF1) alone suppresses downstream translational activity by over 5-fold, whereas uORF2 contributes <2-fold to the inhibition by the intact leader. The different activities of the two uORFs do not depend on the nucleotide sequence surrounding the uORFs in the 5′ leader, the order of the two uORFs in the 5′ leader, or the occurrence of secondary structure or rare codons within the uORFs. Specific features of the amino acid sequence encoded by uORF1 contribute to its stronger suppressive activity, suggesting that it belongs to the class of “sequence-specific” uORFs. The weaker inhibitory activity inherent in uORF2 is potentiated by a suboptimal nucleotide context surrounding its initiator AUG. The occurrence of two uORFs with differing activities in both the human gene and the mouse orthologue suggests that this pair of elements may play a fundamental role in regulating expression of the mdm2 gene.

Open reading frames in the 5′ leaders of messenger RNAs, which are termed upstream open reading frames (uORFs), are emerging as important mediators of transcript-specific translational control (1, 2). uORFs can occur singly and multiply, depending on the particular transcript, and there are regulatory implications associated with both of these situations. In order for a single uORF to regulate ribosome access to downstream coding sequences, it seems that two conditions must be met. 1) Translation of the uORF must block reinitiation that could lead to translation further downstream on the mRNA; and 2) there must be a regulated mechanism for attenuating or bypassing the block. “Sequence-dependent uORFs” block movement of ribosomes on the 5′ leader as a result of specific interactions of uORF-encoded nascent peptides with targets that are thought to be components of the translation machinery (2). Ribosomes have been found arrested on sequence-dependent uORFs either during the elongation phase of translation or at termination, depending on the sequence of the particular peptide and, therefore, the identity of its target. Another situation leading to inhibition is where a uORF is out of frame with and overlapping the initiator AUG of the major open reading frame. One way of regulating both overlapping uORFs (3) and sequence-specific uORFs is through modulated recognition of the initiation codon of the uORF, which leads to “leaky scanning.” Another mechanism of regulation is mediated through elimination of inhibitory uORFs in alternative forms of transcripts (4, 5). Specific regulation of sequence-dependent uORFs occurs when interaction of the nascent peptide with its target is modulated (2).

In addition to single uORFs, multiple uORFs are found in mammalian genes, particularly those encoding oncoproteins, growth factors, and growth factor receptors (2, 6, 7). A fungal model for regulation through multiple uORFs is the yeast GCN4 gene, the translation of which is controlled through interactions of four uORFs (8). There is no similarly detailed model for regulation through multiple uORFs in higher eukaryotes. One of the human oncogene mdm2 transcripts contains two uORFs, and these two elements cooperate to inhibit translation of the oncoprotein product (5). Inhibition by the two uORFs seems to be of biological significance, because a class of tumors arises from overexpression of a shortened mdm2 mRNA with both uORFs eliminated (5, 9–11). This “short form” of the mdm2 mRNA (S-mdm2) is generally present at low levels in normal cells, and its 5′ leader contains no obvious regulatory elements. mRNAs containing the S-mdm2 leader are very efficiently loaded with ribosomes (5). The “long form” transcript (L-mdm2 mRNA) is constitutively expressed and has a relatively long 5′ leader of 297 nucleotides. mRNAs containing the L-mdm2 5′ leader are >10 times less efficiently translated than S-mdm2 mRNAs in the cell types tested. This inhibitory activity of the L-mdm2 leader is due to the presence of the two uORFs, which seem to cooperate in inhibiting translation (5).

The structures of the human and mouse L-mdm2 5′ leaders are diagrammed in Fig. 1. Both uORFs in human mdm2 are 15 codons in length (including the termination codons) and share four codons in common (excluding the initiator methionine). Both uORFs are required for maximal inhibition of translation by the L-mdm2 leader, but uORF1 seems to contribute more to inhibition than uORF2 (5). Two uORFs are also found in the mouse L-mdm2 leader (Fig. 1), suggesting that this conserved arrangement could have functional significance. The hORF2 and mORF2 sequences (hORF2 and mORF2 refer to the human and mouse versions, respectively) have diverged widely between the two species, whereas hORF1 and mORF1 are conserved in both overall length and coding at four of thirteen positions. Three of the four amino acids conserved in the pep-

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Fig. 1. Structure of the 5′ leaders of the human (huMDM2) and murine mdm2 (muMDM2) mRNAs. The relative lengths of the leader sequences are indicated below. The amino acids conserved in the peptides encoded by human uORF1 and uORF2 are underlined, while amino acids conserved between human and murine uORF1 are shown in boldface type.

EXPERIMENTAL PROCEDURES

Reporter Constructs—Chimeric expression constructs between the 5′ leader of the mdm2 gene and human growth hormone (hGH) as a reporter gene were detailed previously (5). Building on our previously described construct, pGH-E1, by means of “back-to-back” PCR (12), uORFs 1 and 2 were deleted from the 5′ leader and replaced with restriction sites MluI and StyI, respectively. The primer sets used (restriction sites in boldface) were, for the MluI site, 5′-GACACAGGCGCCACAGGCG-3′ and 5′-ACGGCGTCCTGCTGTTCTCCGACGGCGACGGG-3′, and for the StyI site, 5′-CTCGGCGCCAGGGCAGCTGGCGC-3′ and 5′-CAAGGGCTGGGATCTCTGAGGGACGGG-3′. The resulting construct enabled the insertion of specifically designed, double stranded oligonucleotides in place of the wild-type uORFs. For example, wild-type uORF1 was inserted into the MluI site, and uORF2 was inserted into the StyI site, restoring the wild-type 5′ mdm2 leader sequence, with the exception of the restriction sites, through use of the following oligonucleotides (restriction sites in boldface, initiation site underlined, sense strand shown): uORF1, 5′-GACACAGGCGCCACAGGCG-3′ and 5′-ACGGCGTCCTGCTGTTCTCCGACGGCGACGGG-3′, and uORF2, 5′-CAAGGGCTGGGATCTCTGAGGGACGGG-3′ and 5′-CAAGGGCTGGGATCTCTGAGGGACGGG-3′. The resulting construct enabled double-stranded oligonucleotides encoding the desired uORF to be inserted into the 5′ leader by standard cloning procedures. All constructs were sequenced prior to transfection to verify orientation and sequence of the uORF.

Cell Culture—HCo. cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, penicillin (100 units/ml) and streptomycin (50 μg/ml). The general procedures for generation of stable transfecants and sample collection for hGH expression analysis were detailed previously (13), with a few modifications as follows. For expression analysis, 7.5 × 10⁵ cells were plated in replicates (10-cm² plates) in the absence of G-418 selection. On the following day, culture medium was changed using conditioned medium (13), and samples of the medium were removed immediately (T₀) and after incubation for 4 h (T₄). At T₄, the cells were lysed using TRIzol reagent (Molecular Research Center Inc.) for extraction of RNA. Measurements of growth hormone accumulation (T₄ - T₀) and ribonuclease protection assays for hGH mRNA level were performed as outlined below.

Human Growth Hormone Assay—The level of hGH protein in the culture medium was analyzed with an enzyme-linked immunosorbent assay (ELISA) kit provided by Roche Applied Science. Duplicate aliquots were measured after dilution to within the linear range of the assay.

Ribonuclease Protection Assay—The template for synthesizing the hGH riboprobe was derived by PCR amplification of a hGH cDNA kindly provided by R. Braun. Primers used were 5′-TTGAGAGCATGCTCACCTAGCTG-3′ (upper strand) and 5′-CCGGTGATCACCCAGCTGGTG-3′ (lower strand). The resulting PCR product was analyzed by gel electrophoresis, and the riboprobe was synthesized utilizing T7 RNA polymerase (Stratagene). As an internal control, actin riboprobe was produced from the pTRI-ß-actin-human plasmid (Ambion) and annealed to RNA simultaneously with the hGH riboprobe. Digestion with RNase A was carried out as described (14). A 6% urea-polyacrylamide gel was used for electrophoresis, and phosphorimage analysis was used for quantitation. For each sample, the assay was carried out in triplicate, and the hGH mRNA levels were normalized to the levels of actin mRNA.

RESULTS

Influence of Organization of the L-mdm2 5′ Leader on Translational Repression—Site-directed mutagenesis of the L-mdm2 construct previously demonstrated that both hORF1 and hORF2 were necessary to achieve maximum suppression of translation of an associated reporter gene (5). To facilitate further analysis, unique restriction sites were engineered into the 5′ leader in positions flanking the uORFs (see “Experimental Procedures”), which allowed defined oligonucleotides to be cloned into the constructs in place of the wild-type uORFs. hGH was used as the reporter gene (15) in these constructs, and the data are presented as translational efficiency, which is defined as the rate of hGH production normalized to hGH mRNA (13). In each experiment, the results were compared with a control construct in which the initiator AUG codons of both human uORFs had been altered to AAG (Fig. 2, construct X-X). As shown in Fig. 2, the L-mdm2 leader with both human uORFs intact (Fig. 2, construct 1-2) reduced the translational efficiency of the construct to 7% of the control. The activities of the constructs with just hORF1 (Fig. 2, construct 1-X) or just hORF2 (Fig. 2, construct X-2) were 19 and 66% of the control, respectively. These activities compared well with the corresponding constructs generated by site-directed mutagenesis; in three independent experiments, constructs from mutagenesis, which corresponded to 1-2, 1-X, and X-2, displayed translational efficiencies relative to the corresponding control-lacking uORFs of 8 ± 2, 24 ± 3, and 65 ± 15%, respectively (data not shown). Therefore, introduction of restriction sites flanking the uORFs did not alter their regulatory properties.

The above results demonstrate that construct 1-X, which contains only hORF1, has about one-third the activity of the construct with only hORF2 (X-2). To test whether these large differences in inhibitory activity was due to position within the
5' leader, the locations of the two human uORFs were reversed, yielding constructs 2-1, X-1, and 2-X, as seen in Fig. 2). The general conclusion remained the same, i.e. hORF1 is much more strongly inhibitory than hORF2 irrespective of position in the leader and, therefore, the surrounding sequence (Fig. 2, compare X-1 with X-2 and 1-X with 2-X). When compared in the same position in the 5' leader, hORF1 is >4-fold more inhibitory than hORF2. Substitution of sequence from the human β-actin 5' leader between the two human uORFs in the L-mdm2 leader had no significant effect on its inhibitory activity (data not shown), reinforcing the conclusion that the surrounding sequence does not influence the inhibitory activities of the two human uORFs. There is a small contribution of position within the leader; proximity to the 5' cap seems to decrease the inhibitory activity of both uORFs (Fig. 2, compare X-1 with 1-X and X-2 with 2-X). This may arise from the known decrease in recognition of an initiator AUG with increased proximity to the cap (16).

As discussed in the Introduction, the mouse L-mdm2 mRNA also contains two uORFs. The human and mouse uORF1 sequences have some characteristics in common, whereas the two uORF2s are quite divergent between the two species (Fig. 1). When the two mouse uORFs were inserted into the human L-mdm2 leader (Fig. 2, construct M-M), suppression of downstream translation was quite similar to that observed with the human uORFs. The results of swapping the mouse and human uORFs to create hybrid constructs (Fig. 2, constructs H-M and M-H) suggested that the mORF1 is somewhat less inhibitory than hORF1, whereas human and mouse uORF2s have similar activities.

Origins of the Enhanced Suppressive Properties of hORF1 Relative to uORF2—To examine the functional properties of mdm2 hORF1 in isolation, it was cloned into the AdoMetDC 5' leader in place of the endogenous uORF. The regulatory properties of the AdoMetDC leader and its uORF have been well characterized (13, 17–20). The activities of the mdm2 uORFs were compared in all cases with an AdoMetDC uORF sequence in which the initiator AUG codon had been inactivated by alteration to a GUG (Fig. 3, construct [ADC]). Also for comparison, we include a construct in which the regulatory activities of the AdoMetDC uORF had been abolished through changing a critical aspartic acid codon to arginine (Fig. 3, construct ADC[D/R]), thus illustrating, for comparison, the activity of a uORF with no unusual suppressive properties placed in the AdoMetDC 5' leader (13). There was 60% residual translation present in the ADC[D/R] construct, which probably resulted from translation of the uORF, followed by reinitiation at the downstream reporter gene (2).

The human mdm2 uORF1, when introduced into the AdoMetDC 5' leader (Fig. 3, construct hORF1), inhibited downstream translation by 82% (18% residual activity), whereas the mouse uORF1 (Fig. 3, construct mORF1) was somewhat less inhibitory, consistent with the conclusion drawn from the human-mouse hybrid constructs (Fig. 2). Translational suppression by human uORF2 (Fig. 3, construct hORF2) was over three times less than suppression by hORF1. The activities of the uORFs assayed individually in this context were very similar to those found in the context of the native 5' leader (Fig. 2), reinforcing the conclusion that the surrounding sequence does not contribute to the suppressive activities of these uORFs.

The inhibitory activity of human uORF2 is very similar to the AdoMetDC uORF that was mutated to abolish its unique regulatory properties (compare constructs hORF2 and ADC[D/R] in Fig. 3). Conversely, it seems that the human and mouse uORF1s must have special properties that produce the observed potent inhibition of downstream translation. It seems that uORF1 must be translated in order to display these properties, because mutation of the human initiator AUG yielded a construct that is indistinguishable in activity from the uORF2 and AdoMetDC uORF constructs with mutant initiation codons (Fig. 3, compare the activities of constructs [ADC], [hORF1], and [hORF2]). Therefore, even though energy minimization analysis predicts a secondary structure for human uORF1 with a free energy of ~15.8 Kcal (not shown), it appears that this putative structure does not contribute to the inhibitory activity of the uORF. Also consistent with this conclusion, simultaneous introduction of 11 synonymous alterations in the codons of hORF1, abolishing the putative secondary structure (ΔG = −0.7 kcal) and retaining identical protein coding capacity, had no effect on the inhibitory activity of this uORF (data not shown).

There are two relatively rare codons within hORF1, i.e. those encoding proline 11 (CCG) and arginine 12 (CGA), which have frequencies in humans of 0.7% and 0.63%, respectively. To test whether these two codons were contributing to the suppressive activity of the uORF, they were changed to CCA and AGA, which encode the same amino acids and have frequencies of 1.67% and 1.15%, respectively. In three independent experiments (data not shown), this construct showed no significant difference in expression compared with the hORF1 construct, which contains the wild-type human uORF1. Therefore, the potent suppressive activity of hORF1 cannot be due to the presence of these two relatively rare codons.

One difference between human uORF1 and uORF2 is the context surrounding the initiation codon. Whereas uORF1 in both human and mouse has a strong context (21) with guanine at position 4, human uORF2 has adenine at that position. Substituting the dipeptide Ile2-Pro3 of hORF2 with Glu2-Gln3 (Fig. 4, construct ORF2/IP/EQ), the same sequence as in hORF1, significantly strengthens inhibition of downstream translation (Fig. 4). Two other substitutions, alanine and valine at position 2 of hORF2, also strengthen the suppressive activity of the uORF by about the same amount (Fig. 4, constructs ORF2[IA] and ORF2[IV]). The only thing that these three codon substitutions have in common is strengthening the initiation context by introducing a guanine at position 4. Although hORF2 with the strong initiation context is still less
suppressive than hORF1 (Fig. 4), these results strongly suggest that at least one factor reducing the suppressive activity of uORF2 is non-optimal initiation context. It should be noted that the hORF2 constructs with the optimal initiation contexts are more suppressive that the “generic” uORF, ADCD/I/R (compare Fig. 4 with Fig. 3), suggesting that this uORF2 may have some unique inhibitory properties of its own.

Independent of the context surrounding the initiation codon, there is still an inherent 2-fold difference in inhibitory activity between hORF1 and hORF2 (Fig. 4). To locate the region responsible for this difference in activity, the human uORFs were divided into three regions (A, B, and C). Constructs were assembled in which these regions were swapped between the uORFs, and the resulting chimeras were tested in the AdoMetDC leader (Fig. 5). Thus, constructs A1B1C1, A2B2C2, A1B2C2, and A2B1C2, respectively, and these show the expected activities (compare Fig. 5 with Fig. 3). The effect of initiation context is important in interpreting these data; for example, the differences between A1B1C1, A2B2C2, and A1B2C2, and the corresponding wild-type constructs (hORF2 and hORF1, respectively) can be accounted for entirely by differences in initiation context (contrast the activities in Fig. 5 with those in Fig. 4). Therefore, constructs with the same regions A must be compared in order to identify other differences between the two uORFs. In this way, region B was identified as a dominant determinant of the differences in hORF1 and hORF2 activities. Comparison of constructs A1B1C1 with A1B1C2, and A2B2C2 with A2B2C1, clearly demonstrates that region B from hORF1 has greater suppressive activity (Fig. 5). The same conclusion can be reached with respect to region B by comparing A1B1C2 with A1B1C2, and A2B2C1 with A2B2C1. A similar analysis of region C leads to the conclusion that this part of hORF1 is slightly more inhibitory than the corresponding region from hORF2 (compare constructs A1B1C2 with A1B1C1, A1B1C2 with A1B1C2, A2B2C2, with A2B2C1, and A2B2C2 with A2B2C1). Although the difference in activity between the regions C is smaller than that found between the regions B and, in some cases, is barely significant, it is found consistently among the four comparisons.

Therefore, the stronger translational suppression activity of hORF1 relative to hORF2 seems to be the cumulative result of several structural features. The first is the stronger initiation context present in hORF1. The second is the amino acid sequence of the peptide product of hORF1, primarily in region B, which contributes strongly to its enhanced activity. Thirdly, there seems to be a minor contribution of the amino acid sequence of region C. Thus, mdm2 hORF1 seems to be a member of the class of sequence-dependent uORFs, the activities of which are mediated by the encoded peptides (2).

Influence of Single-Codon Changes on the Activity of Mdm2 uORF1—The results of swapping large regions of the two human mdm2 uORFs suggested that the exceptional inhibitory activity of uORF1 results, in part, from the amino acid sequence of the peptide product. To test this hypothesis, we introduced selected single-codon changes into hORF1 and examined the activities of the altered uORFs using the reporter gene construct containing the AdoMetDC 5’ leader, as above. The activity of construct A1B2C1 (Figs. 5 and 6) suggested that the central region of the peptide product was likely to play a role in the inhibitory properties of hORF1. Within this region, glycine 9 is conserved between the human and mouse uORFs, but valine occurs in this position in human uORF2 (Fig. 1). Converting the conserved glycine to valine resulted in considerable reduction of the inhibitory activity of human uORF1 (Fig. 6, construct 1XG/V). Curiously, the moderating influence of the valine 9 substitution on inhibition by hORF1 was abolished by introduction of a second valine at position 5 (Fig. 6, construct 1XAG/VV). These relative activities of constructs 1XG/V and 1XAG/VV were confirmed with three independently transfected cell lines, and the average values are those presented in Fig. 6. The glutamate 4 and alanine 5 positions are conserved between human uORF1 and uORF2, as is alanine 5 in mouse uORF1 (Fig. 1). A double substitution of valine at positions 4 and 5 significantly reduced the inhibitory activity of human uORF1 (Fig. 6, construct 1XEA/VV), suggesting a role for this region of the peptide in inhibition. This was not identified in
the experiment of Fig. 5 because of the conservation in this region between human uORFs 1 and 2. The activities of constructs A2B2C1 and A1B1C2 (Fig. 5) suggested that the carboxyl-terminal region of the hORF1-encoded peptide could play a role in the inhibition. Conversion of proline 11 and arginine 12 both to alanine (Fig. 6, construct 1XPR/AA) had no significant effect on inhibition by hORF1. This result suggests that these two highly conserved residues (Fig. 1) do not play a detectable role in the inhibitory activity of the peptide. Shortening the peptide by deleting the carboxyl terminal leucine had no appreciable influence on the activity of hORF1, nor did extending the peptide by one alanine residue (Fig. 6, constructs 1X-L and IX+A, respectively).

**DISCUSSION**

Oncogene *mdm2* is highly regulated, probably because of the important role that the MDM2 protein plays in controlling p53 and other proteins involved in cell growth (22-24). The ramifications of deregulation of *mdm2* expression are profound; oncogenesis as a result of overexpression, and lethality in the case of underexpression. Besides transcriptional control of *mdm2* expression (25), translation provides a second target for regulation of this key gene. At least one class of tumors results from deregulation of translation of *mdm2* (5, 9-11), which underscores the physiological importance of regulation at this second level.

Two uORFs occur in the long form of *mdm2* mRNA in both human and mouse, the two species for which we have information. Together, these uORFs have a major impact on translation of this form of the mRNA in HeLa cells (5); however, uORFs 1 and 2 contribute quite differently to the net influence on translation. Several features of the 3' most uORF, uORF2, cause it to have the weakest effect on translation in both species. Both human and mouse uORF2s have weak contexts for translation initiation, with adenine at position 4 in human and cytosine at the same position in mouse. In addition, as shown in this paper, features of the coding sequence of human uORF2 diminish its influence on downstream translation relative to uORF1. Also, the location of uORF2 in mouse, just 11 nucleotides downstream of uORF1 (26), would contribute to diminished recognition by a reinitiation mechanism (2), in contrast to human, where the distance between the two uORFs is 88 nucleotides (9). Thus, several features of the uORF2e of both mouse and human combine to weaken their influence on downstream translation.

In contrast to uORF2, the structure of uORF1 in both species seems to be optimized for recognition and inhibition of downstream translation. The initiation context in both human and mouse is quite favorable, with guanine in position 4 and a purine located at -3. Additionally, the coding sequence of uORF1 contributes significantly to its inhibitory influence. This effect of the coding sequence is not due to secondary structure or the occurrence of rare codons. Instead, the strong influence of uORF1 on translation of downstream cistrons seems to be mediated by the structure of the encoded peptide. The central portion of the encoded peptide and, in particular, the glycine residue at position 9, seems especially important in the inhibitory activity of the uORF1 peptide.

Human *mdm2* uORF1 seems to fall into the category of sequence-dependent uORFs (2) in that missense alterations within the coding region perturb the inhibitory activity, while synonymous changes in the codons have no effect. However, the behavior of *mdm2* uORF1 seems more complex than that of other sequence-dependent uORFs that have been studied. With two of the best characterized uORFs, those from the mammalian AdoMetDC gene and the cytomegalovirus UL4 gene, their activities can be totally abolished by single-codon alterations (13, 27), which are thought to destroy direct interactions between the uORF-encoded peptides and their targets. In contrast, the only alteration in hORF1 that has been observed to completely eliminate its unique inhibitory activity is the replacement of the central block of four amino acids in generating the construct A1B1C2. Complexity in either the structure of the hORF1 peptide or its contact with its target is also suggested by the apparent functional interaction between residues 5 and 9. These residues are conserved as alanine and glycine, respectively, in human and mouse. Changing residue 9 to valine, as found at this position in human uORF2, reduced the inhibitory activity of hORF1, but the effect of this change was moderated by simultaneously introducing an alteration at position 5.

The known sequence-dependent uORFs seem to act by inhibiting movement of ribosomes along the mRNA, trapping them on the 5' leader. This probably occurs as a result of the interaction between the nascent peptides and their targets (2). With the UL4 and AdoMetDC mRNAs, the ribosomes seem to arrest at the termination step after completing translation of the uORF (18, 28). Therefore, with these two uORFs, the structural relationship between the critical residues in the interior of the peptide and the carboxyl terminus (termination codon) is vital in lining up the peptide with its target to achieve optimal inhibition; the addition or removal of even a single codon abolishes activity (discussed in Ref. 2). In contrast, another sequence-dependent uORF, that from the *Neurospora* arg-2 gene, arrests ribosomes at the elongation step (29, 30) and seems to be far less demanding in the position of the inhibitory peptide sequence relative to the termination codon. In this regard, human *mdm2* uORF1 seems to be similar to arg-2 in that adding or removing a codon from the 3' end of the uORF had no discernable effect on its inhibitory activity.

It is noteworthy that the organization of the 5' leaders of the human and mouse *mdm2* mRNAs both feature two uORFs with similar properties. In both instances, uORF1 is quite inhibitory to downstream translation and, in fact, shows sequence similarities between the two species, whereas uORF2 shows no unusual inhibitory properties and is evolutionarily diverse. There are examples in both yeast (8) and mammals (3) of cooperation between multiple uORFs that result in regulation of translation initiation. It is tempting to suggest that the evolutionary conservation of these two functionally similar uORFs may have resulted from use of this arrangement for translational control of the long form of the *mdm2* mRNA under some set of physiological conditions yet to be defined.

Although the two human *mdm2* uORFs do not require specific surrounding nucleotide sequences to exhibit their individual inhibitory properties, additional translational control elements may exist in the L-mdm2 5' leader. Recently, a binding site for La antigen has been identified in the intercistronic region that lies to the 3' side of mouse uORF2 (31). This binding site is evolutionarily conserved between mouse and human and is necessary for activation of *mdm2* mRNA translation by the BCR/ABL oncogenes. If, as suggested by the authors (31), the La binding element constitutes part of a site of internal ribosome entry (IRES), the active IRES would likely circumvent the inhibitory activities of the two uORFs. Initiation codons are sometimes found upstream of IRES elements, for example in poliovirus (32, 33) and the *attenuapedia* gene of *Drosophila* (34), perhaps serving to block ribosome scanning upstream of the entry site. The presence of an IRES at the 3' end of the L-mdm2 leader could provide a regulated mechanism for bypassing the inhibitory influence of the two uORFs. A regulated IRES might also account for the enhancement of translation initiation at an internal AUG of the L-mdm2 transcript, which generates a protein with altered biological properties (35).
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