MOLECULAR AND CELLULAR BIOLOGY, Jan. 1995, p. 235–245 Vol. 15, No. 1
0270-7306/95/$04.00+0

Long-Range RNA Interaction of Two Sequence Elements Required for Endonucleolytic Cleavage of Human Insulin-Like Growth Factor II mRNAs

WIEP SCHEPER,[1]* DURK MEINSMA,[2] P. ELLY HOLTHUIZEN,[1] AND JOHN S. SUSSENBACH[1]

Laboratory for Physiological Chemistry[1] and Wilhelmina Childrens Hospital,[2] Utrecht University, Utrecht, The Netherlands

Received 25 July 1994/Returned for modification 13 September 1994/Accepted 5 October 1994

Human insulin-like growth factor II (IGF-II) mRNAs are subject to site-specific endonucleolytic cleavage in the 3′ untranslated region, leading to an unstable 5′ cleavage product containing the IGF-II coding region and a very stable 3′ cleavage product of 1.8 kb. This endonucleolytic cleavage is most probably the first and rate-limiting step in degradation of IGF-II mRNAs. Two sequence elements within the 3′ untranslated region are required for cleavage: element I, located approximately 2 kb upstream of the cleavage site, and element II, encompassing the cleavage site itself. We have identified a stable double-stranded RNA stem structure (ΔG = −100 kcal/mol [−418.4 kJ/mol]) that can be formed between element I and a region downstream of the cleavage site in element II. This structure is conserved among human, rat, and mouse mRNAs. Detailed analysis of the requirements for cleavage shows that the relative position of the elements is not essential for cleavage. Furthermore, the distance between the coding region and the cleavage site does not affect the cleavage reaction. Mutational analysis of the long-range RNA-RNA interaction shows that not only the double-stranded character but also the sequence of the stable RNA stem is important for cleavage.

Regulation of mRNA stability is a common mechanism of controlling gene expression in eukaryotic cells. In general, degradation of mRNAs proceeds through the action of exo- and endonucleases in cooperation with regulatory factors. *cis*-acting sequences involved in stability regulation are often found in the 3′ untranslated region (3′-UTR), although there are also reports of stability determinants present in the coding region or 5′-UTR (29). Because degradation intermediates are usually very unstable, it is difficult in most cases to determine whether the degradational pathways involve exonucleases, endonucleases, or a combination of the two (6). Often, degradation is initiated by a rate-limiting endonucleolytic cleavage step in which protective structures such as caps, poly(A) tails, or stem-loops are removed, thereby targeting the mRNA to rapid exonucleolytic degradation (4, 8, 39, 40). Only a few systems in which mRNA degradation is regulated are well characterized. This is mainly due to the instability and resulting low levels of the mRNA involved and its degradation intermediates. The analysis of the mechanisms of mRNA degradation is also hampered by the fact that secondary and tertiary structures, rather than the primary sequence, play a very important role in recognition of RNA by *trans*-acting factors. For example, transferrin receptor mRNA is protected from nuclease attack under low-iron conditions because an iron-responsive factor is bound to stem-loop structures in the 3′-UTR (23). Several lymphokine and proto-oncogene mRNAs contain an A+U-rich region in the 3′-UTR that is able to destabilize heterologous transcripts (36). Although it seemed initially that the presence of AUUUA pentamers in the 3′-UTR was sufficient to regulate the stability of these mRNAs, the mechanism was later shown to be more complex. Various proteins have been found to bind the A+U-rich sequences (5, 7, 20, 43), but sometimes different proteins show different specificities for A+U-rich-element-containing mRNAs (5). For several of these mRNAs, additional sequences in the 3′-UTR and also in the coding region have been identified that are involved in the control of stability (37, 45, 47).

Human insulin-like growth factor II (IGF-II) is a 67-amino-acid polypeptide with strong structural and functional homology to insulin that can be detected throughout the entire life span in a wide variety of tissues (11). It has been implicated in both proliferative and differentiating effects (reviewed in reference 14). The human IGF-II gene consists of nine exons, of which exons 7 and 8 and the first 237 nucleotides (nt) of exon 9 encode pre-pro-IGF-II (41). Furthermore, it contains 4 kb of 3′-UTR that is all present within exon 9. IGF-II expression is regulated in a tissue- and development-specific manner by differential activation of four promoters, resulting in a family of IGF-II mRNAs with different leader sequences derived from untranslated exons 1 to 6 (13, 44). The human and mouse IGF-II genes are also subject to genomic imprinting (12, 26, 31). In addition, regulation takes place at the levels of translation (25) and mRNA stability (21, 27, 48).

We have reported previously the existence of an unusually stable RNA intermediate in degradation of human IGF-II mRNAs (13, 21, 22). This RNA is an 1.8-kb uncapped 3′-cleavage product of a specific endonucleolytic cleavage in the 3′-UTR of IGF-II mRNAs and still contains a poly(A) tail. The capped 5′-cleavage product, which contains the entire IGF-II coding region, can be detected, but it is usually very unstable, probably because it lacks a poly(A) tail. Although the precise function of this cleavage reaction is not known, there are indications that it is the initial step in degradation of IGF-II mRNAs that can be influenced by the growth conditions of the cells (21).

In a search for *cis*-acting stability determinants, we showed that in vivo cleavage of IGF-II mRNAs requires two widely separated sequence elements (elements I and II) of about 300 nt each in the 3′-UTR (22) (see Fig. 3B). Element II encom-

* Corresponding author. Mailing address: Laboratory for Physiological Chemistry, Utrecht University, Stratenum, P.O. Box 80042, 3508 TA Utrecht, The Netherlands. Phone: 31(30)538204. Fax: 31(30) 539035.

passes the cleavage site and consists of two contiguous domains. The first domain upstream of the cleavage site exhibits two stable stem-loop structures that are conserved among human, rat, and mouse RNAs. Disruption of this structure abolishes cleavage, whereas the presence of an additional 40 nt upstream of these stem-loops markedly enhances cleavage. The second domain that can be distinguished in element II is an extremely well-conserved G-rich region (46 of 74 nt) containing the cleavage site itself. Surprisingly, a region at the beginning of exon 9, element I (located at −2286 to −1960 upstream of the cleavage site), is also essential for cleavage. In this element, no obvious structural features could be identified. Introduction of elements I and II into the 3′-UTR of the β-globin gene was sufficient for mRNAs derived from these hybrid β-globin/IGF-II genes to become a substrate for the cleavage reaction (22). From these experiments it is clear that both elements I and II are necessary and sufficient to confer the cleavage reaction to a heterologous transcript.

In this report we further characterize elements I and II and show that a very stable long-range RNA-RNA interaction, which is well conserved among human, rat, and mouse mRNAs, can occur between them. Mutational analysis reveals that not only the double-stranded character but also the sequence of this stem-structure is important for cleavage of IGF-II mRNAs.

MATERIALS AND METHODS

Materials. Plasmid pBluescript II (KS^+) was obtained from Stratagene, La Jolla, Calif. Restriction enzymes, T4 DNA ligase, and T7 RNA polymerase were purchased from Boehringer, Mannheim, Germany; Pfu DNA polymerase was purchased from Stratagene; RNase-free DNase was purchased from Promega, Madison, Wis.; and RNase T_1 was purchased from CalBiochem, La Jolla, Calif. Enzymes were used as specified by the manufacturers. Nucleoside triphosphates (NTPs) and dNTPs were obtained from Kabi-Pharmacia, Uppsala, Sweden. BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] was purchased from Sigma, St. Louis, Mo. A random-priming DNA-labeling kit was purchased from Boehringer, and a DNA sequencing kit was purchased from Kabi-Pharmacia. Guanidinium thiocyanate was obtained from Fluka, Buchs, Switzerland, and GeneScreen membranes were purchased from Du Pont de Nemours, Dreieich, Germany. [α-^{32}P]dCTP (3,000 Ci/mmol) and [α-^{32}P]CTP (760 Ci/mmol) were purchased from Amersham, Amersham, United Kingdom.

Construction of plasmids. Molecular cloning was performed by established methods (34). Where necessary, 3′ recessed ends were filled in with Klenow enzyme and 3′ protruding ends were removed with T4 DNA polymerase. All positions within exon 9 of the IGF-II gene are indicated relative to the cleavage site (+1). When exon 9 sequences are deleted or additional restriction sites are introduced, the numbers indicated still refer to their original position in exon 9 relative to the cleavage site.

IGF-II expression plasmids are derived from EP7-9, EP7-9/*Not*I, or I-II (Δ−1955/−174) (21, 22) (see Fig. 3A). EP7-9 contains a human genomic IGF-II fragment that starts 373 bp upstream of exon 7 and extends to 4.7 kb downstream of the gene. Expression of EP7-9 in cultured cells was driven by the immediate-early cytomegalovirus enhancer/promoter. EP7-9/*Not*I contains a unique *Not*I site introduced at position +84. Clone I-II was obtained from EP7-9 deleting the region from −1955 to −174 from exon 9. Construct Δ+151/+644 was obtained by insertion of a double-stranded oligomer representing the region from +84 to +150 with *Not*I-compatible (5′) and *Nhe*I-compatible (3′) ends into the *Not*I(+84) and *Nhe*I (+644) sites of EP7-9/*Not*I. Construct II (Δ−2165/−1962) was derived from EP7-9. First the *Xho*I-*Cla*I fragment of exon 9 was subcloned in the *Eco*RV and *Sma*I sites of pBluescript II KS^+ (pBS), such that the *Xho*I (−2291) and *Cla*I (−1961) sites are restored. Subsequently, a *Pst*I (−2166)-*Hin*dIII (pBS polylinker) fragment was deleted and the plasmid was religated. From the resulting clone, an *Xho*I (IGF-II)-*Cla*I (pBS polylinker) fragment was isolated and cloned into the *Xho*I-*Cla*I sites of EP7-9, resulting in clone Δ−2165/−1962. Constructs II/I-II and I-II/I-II were obtained by cloning a fragment containing elements I and II into the *Nhe*I site (+644) of clones II and I-II, respectively. The I-II fragment was obtained by *Xba*I (polylinker)-*Nhe*I (exon 9) digestion of a pBS subclone containing the *Xho*I-*Xba*I fragment (−2290/+1051) from I-II in the *Eco*RV-*Sma*I sites and was cloned in the *Nhe*I sites of II (generating construct II/I-II) and I-II (yielding I-II/I-II). Constructs Sense/Sense (S/S) and Antisense/Sense (AS/S) were derived from I-II by replacing the *Xho*I-*Cla*I fragment by a PCR product synthesized with primers corresponding to positions 5′ −2116/−2097 3′ (*Cla*I-*Xho*I linker attached at the 5′ end) and 5′ −2013/−2032 3′ (*Cla*I-*Sal*I linker attached at the 5′ end). The PCR product was either digested with *Xho*I and *Cla*I (S/S) or digested with *Cla*I and *Sal*I (AS/S), so that it could be cloned bidirectionally into the *Xho*I-*Cla*I sites of I-II. For the cloning of construct Antisense/Antisense (AS/AS), an *Eco*RV-*Xba*I fragment (−557 to +1052) of Δ+12/+82 (22) was subcloned in the *Sma*I-*Xba*I sites of pUC19. A double-stranded DNA (dsDNA) fragment was prepared by annealing the two oligomers 5′-GGCCGG**TT**CCAC**G**TCCTACCC**TGGGGC**TT**C**T**CC**T** GCCCAG**T**CCCCC**GT**TGCCCCCC**G**CC**T**ACCCCTCCAAC-3′ and 5′-GGCC GTTGGAGGGGT**A**GG**C**GGGGGGCA**AC**GGGGGA**C**TGGGC**A**GG **A**G**A**AG**CCCCA**GGGTAGGA**C**GTGG**AA**CC-3′. This dsDNA fragment is an inversion of nt +12 to +82, with the exception of the bases printed in boldface type. Because of the presence of G·U base pairs at these positions in the authentic mRNA, adjustments were made to retain the secondary structure after inversion. The underlined bases represent positions of additional adjustments that were made to restore the secondary structure. Because the fragment contains a 5′-GGCC protruding end, it could be cloned into the *Not*I site at the +11/+83 border in the pUC19 subclone. From the resulting clone, a *Bgl*II (−110)-*Nhe*I (+644) fragment was isolated to replace the *Bgl*II-*Nhe*I fragment of AS/S, resulting in the construct AS/AS. For the preparation of in vitro synthesized RNAs, oligonucleotides 5′ *Bam*HI −2340/−2324 3′ and 5′ *Eco*RI +170/+152 3′ were used as primers in PCRs on S/S, AS/S, and AS/AS. The PCR products were digested with *Bam*HI and *Eco*RI and subcloned into the *Bam*HI and *Eco*RI sites of pBluescript, resulting in plasmids BS-S/S, BS-AS/S, and BS-AS/AS, respectively. After linearization with *Eco*RI, the RNAs could be synthesized with T7 RNA polymerase, resulting in RNAs of 571, 571, and 578 nt, respectively. The predicted stem structure between elements I and II for the S/S and AS/AS RNAs is as follows (unpaired bases are underlined):

S/S I 5′ UCCCGAAAAUCUCUCGGUUCCACGUCC CCCUGGGGCUUCUCCUGACCCAGUCCCCG UGCCCCGCCUCCCCG 3′

S/S II 3′ AGGU UUUAGAGGGCCCUGGUGAAGGAUGGGGUCUUGAGGGGGGC GGGUCGGGGGUGACGGGG GGU GGGU 5′

AS/AS I 5′ GUUUC GGGG AGGCGGGGC ACGGGG ACUGGGUCAGGAGAAGCCCCAGGG GGACGUGGAACCG 3′

AS/AS II 3′ CAACCUCCCCAUCCGCCCCCGUUGCCCCCUGACCC GUCCUCUUCGGGGUCCCAUCCUGCACCUUGGC 5′

All constructs were checked by restriction analysis or sequencing when necessary.

Cell culture and transfection. Human 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 300 μg of glutamine per ml. Cells were transfected in 75-cm^2 flasks at a confluency of about 40%. Precipitates were prepared with BES-buffered saline by calcium phosphate coprecipitation (9) and contained 10 μg of plasmid DNA. After 4 h the medium was aspirated, and the cells were treated for 1 min with medium containing 10% dimethylsulfoxide and subsequently refed with complete medium. After 40 to 48 h the cells were harvested in 0.025% trypsin–0.02% EDTA for RNA isolation.

RNA isolation and analysis. Total RNA was isolated by the single-step guanidinium thiocyanate method (10). RNA (10 μg) was glyoxalated, size separated on a 1% agarose–10 mM sodium phosphate gel, and transferred to a GeneScreen membrane. The RNA was fixed on the membrane by irradiation with long-wavelength UV light for 2.5 min and baking at 80°C for 2 h. Northern (RNA) blots were hybridized in the presence of 50% formamide in glass cylinders with continuous rotation at 42°C by following GeneScreen protocols. Two human IGF-II exon 9 probes were used: a 531-bp *Eco*RV-*Ava*I fragment encompassing the region between positions −557 and −27 (5′-specific probe) and a 1.0-kb *Sma*I fragment (positions +84 to +1096; 3′-specific probe). The DNA fragments were labeled by random priming with [α-^{32}P]dCTP by following Boehringer protocols and added at a final concentration of 10^6 cpm/ml after a 3-h prehybridization. After overnight hybridization, blots were washed to a final stringency of 0.5× or 0.1× SSC–1% SDS at 65°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed on Fuji RX X-ray film. The cleavage efficiencies were determined by densitometric scanning of the autoradiographs.

RNA structure analysis. The pBluescript constructs BS-S/S, BS-AS/S, and BS-AS/AS were each linearized with *Eco*RI, and 1 μg was used as a template to

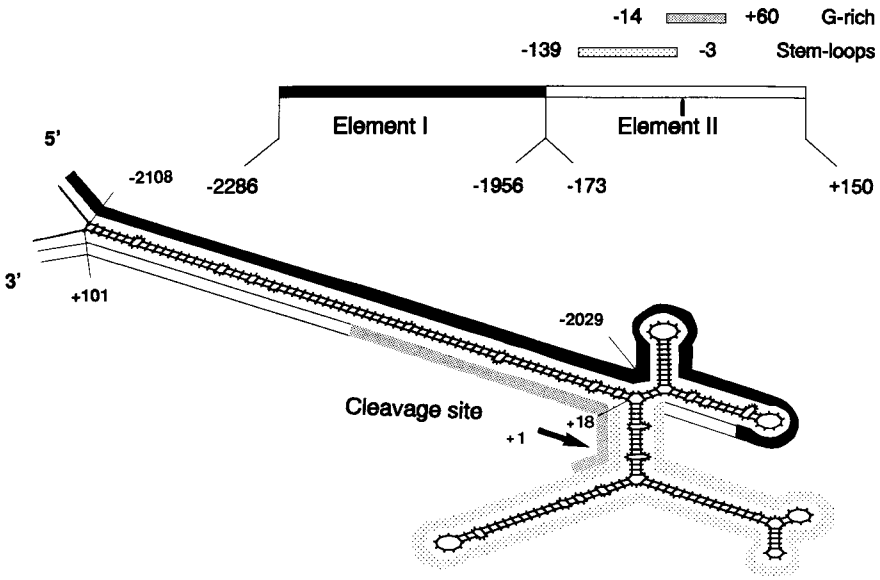


FIG. 1. A putative RNA stem-structure between elements I and II in human IGF-II mRNAs. Computer prediction of the secondary structure of elements I and II was performed by the algorithm of Zuker and Stiegler (49). Shown is a folding where the spacing between the elements is identical to that in construct I-II (see Fig. 3B). At the top of the figure, the relative positions of the complete elements, the stem-loop structures, and G-rich region are depicted in a linear format. The interaction shown in this figure is predicted regardless of flanking sequences or sequences spacing the elements. The cleavage site (position +1) is indicated by an arrow.

synthesize RNA with T7 RNA polymerase, as specified by the manufacturer, in the presence of 1 mM ATP, GTP and UTP; 20 to 40 μCi of [α-^{32}P]CTP; and 0.1 mM CTP. After synthesis (1 h), the template was removed by DNase I treatment (1 U for 15 min at 37°C) and the RNA was separated from unincorporated nucleotides by Sephadex G-25 spin-dialysis. Subsequently the samples were phenol-chloroform extracted, ethanol precipitated, washed in 70% ethanol, and dissolved in water or renaturation buffer (20 mM Tris · HCl [pH 7.5], 100 mM KCl, 2 mM $MgCl_2$). The integrity of the RNAs was checked by gel electrophoresis.

The in vitro synthesized RNAs were renatured in renaturation buffer for 5 min at 90°C and for 30 min on ice (similar results are obtained if renaturation is performed for 5 min at 90°C followed by slow cooling to 30°C). RNase T_1 digestions were carried out in renaturation buffer for 15 min at room temperature. The digested samples were phenol-chloroform extracted, ethanol precipitated, washed with 70% ethanol, and analyzed on a 6% polyacrylamide sequencing gel. Radiolabeled RNase T_1 fragments were isolated from gel slices by overnight incubation at 37°C in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate). The eluted fragments were used as a probe in Southern blots containing PCR fragments 1 (−2116 to −2013, element I specific), 2 (−173 to −23, specific for the stem-loop structures of element II), and 3 (+2 to +120, specific for the interacting region of element II). As a negative control, *Alu*I-digested pBluescript KS$^+$ was included. The fragments (100 ng) were separated on a 2% agarose gel, and Southern blotting was performed on GeneScreen membranes by standard methods (34). Blots were hybridized and washed as described for Northern blots.

RESULTS

Putative interaction between elements I and II of IGF-II mRNAs. We have identified two widely separated sequence elements that are required for endonucleolytic cleavage of IGF-II mRNAs (22) (see Fig. 3B). Element II exhibits two conserved features: (i) two stable stem-loop structures upstream of the cleavage site and (ii) a G-rich domain encompassing the cleavage site. Element I is located 1,786 nt upstream of element II and does not display any obvious structural features by itself. Although element I is absolutely required for specific endonucleolytic cleavage of IGF-II mRNAs, it was not clear how this element could exert its effect on cleavage. An attractive possibility would be that elements I and II together form a dsRNA stem structure.

To establish putative direct interactions, the secondary structure of the elements was determined by using the algorithm of Zuker and Stiegler (49). In the wild-type situation, the distance between elements I and II is 1,786 nt, which is too great for the program to handle. However, we have shown previously that the region between the elements (−1955 to −174) can be deleted without causing a decrease of the cleavage efficiency (21). Therefore, we analyzed the folding of human IGF-II mRNAs in which the region from −1955 to −174 was deleted. In addition to the program of Zuker and Stiegler (49), we used the algorithm described by Abrahams et al. (1). This program favors short-range over long-range interactions, because it assigns penalties for the free energies of the loops, which are high in long-range interactions.

Interestingly, both programs predict a very stable interaction between 80 nt in the last part of element I (−2108 to −2029) and a stretch of 84 nt in element II starting at position 18 downstream of the cleavage site (ΔG is approximately −100 kcal/mol [1 kcal is 4.184 kJ]) (Fig. 1). This strongly indicates that two widely separated sequence elements identified by in vivo endonucleolytic cleavage studies are indeed able to form a stable dsRNA structure. That this interaction between elements I and II is of physiological relevance is further suggested by the fact that a similar interaction can be found for rat and mouse IGF-II mRNAs (Fig. 2A). In these species, IGF-II mRNAs undergo endonucleolytic cleavage like their human analogs (16). Although sequence requirements for cleavage in these species have not been studied, we have shown previously that marked sequence conservation exists in the G-rich domain encompassing the cleavage site (72 of 74 nt) (21). In addition, the secondary structure within element II, consisting of two stem-loop structures upstream of the cleavage site, is well conserved in these species (21).

On the basis of the putative secondary structure that can be formed between elements I and II in human IGF-II mRNAs, we have aligned the analogous sequences of rat and mouse RNA (Fig. 2B). The figure shows the region of the interactions that is predicted irrespective of flanking sequences or sequences between the elements. The primary-sequence conservation of the interacting nucleotides in element I is limited to

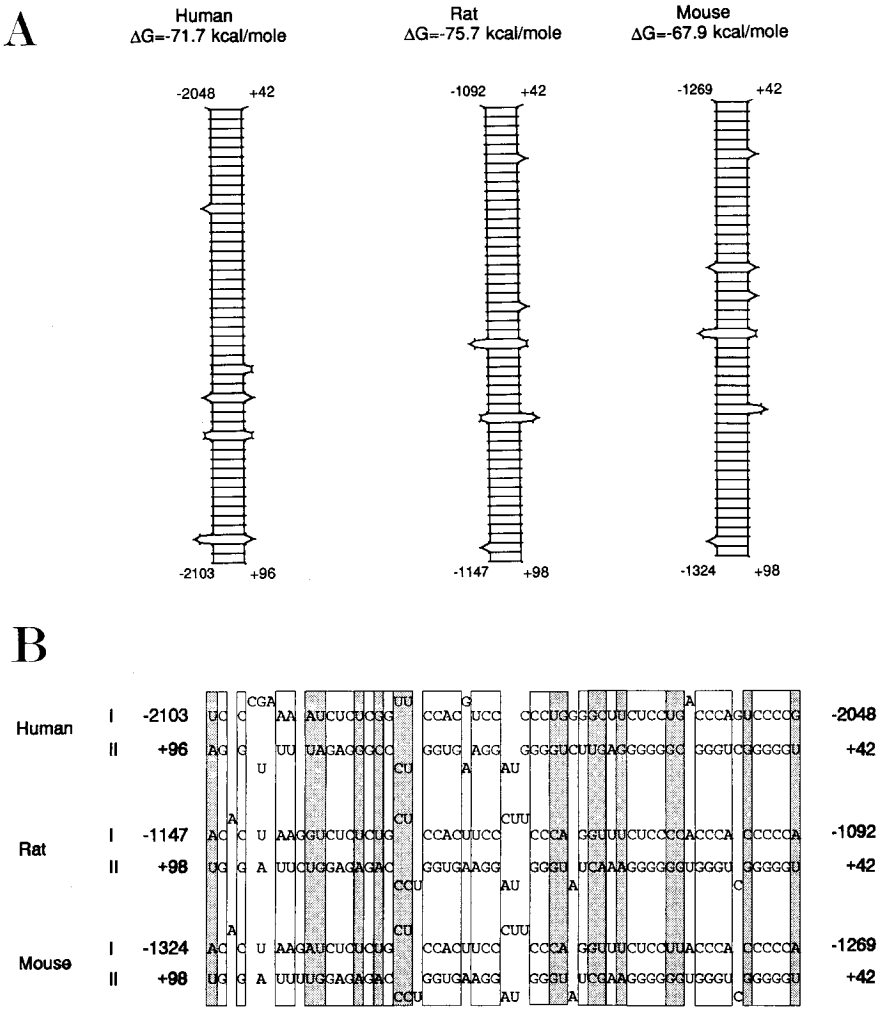


FIG. 2. Comparison of the secondary structure, formed between elements I and II of human, rat, and mouse IGF-II mRNAs. (A) Computer predictions performed by the algorithm of Zuker and Stiegler (49). Shown are analogous regions of the interaction between elements I and II in the three species. The ΔG values are indicated. (B) Alignment of the sequence of the secondary structures shown in panel A. Positions in white boxes represent conservation of the primary sequence in both elements in all three species. Conservation of secondary (paired or not paired) but not primary structure is depicted by shaded boxes.

52%. In contrast, the secondary structure (loops or base pairs) is much better conserved (78%) among the three species. This is achieved either by base changes that do not affect the secondary structure (e.g., A · U to G · U or changes in loops) or by compensating base changes in the other element (e.g., G · C to U · A). In addition, the changes are arranged in such a manner that they result in comparable energy values (ΔG_{human} = −71.7 kcal/mol, ΔG_{rat} = −75.7 kcal/mol, and ΔG_{mouse} = −67.9 kcal/mol). This shows that the secondary structure that was predicted for elements I and II is highly conserved among human, rat, and mouse mRNAs. These findings provide phylogenetic evidence for the actual existence and physiological importance of this structure in IGF-II mRNAs.

Fine mapping of elements I and II. Two widely separated regions that are essential for cleavage of IGF-II mRNAs, elements I and II, were identified by an in vivo cleavage assay (22). In this assay, deletion constructs derived from the IGF-II expression plasmid EP7-9 (Fig. 3A) were tested for their ability to yield mRNAs that could be cleaved in vivo. After expression of the different constructs in human 293 cells, the levels of IGF-II mRNAs and cleavage products were determined by hybridization of Northern blots of total RNA with a 3′-end-specific probe that detects both the 1.8-kb RNA and full-length IGF-II mRNAs. These RNAs are all derived from the introduced IGF-II constructs, since 293 cells do not express IGF-II mRNAs endogenously. For each construct, the cleavage efficiency was determined as the amount of 3′-end-specific cleavage products relative to the total amount of full-length plus 3′-end-specific IGF-II RNAs. The cleavage efficiency obtained with construct EP7-9 was set at 100%. Element II contains two conserved stem-loop structures immediately upstream of the cleavage site (−139 to −3). The 5′ border of element II was mapped within −173 to −139, showing that for efficient cleavage, sequences upstream of the stem-loop structures are required as well (22). The extent of the 3′ end of element II has not yet been determined precisely. We have shown that a fragment encompassing nt −2290 to +1051 contains all the information needed to confer the cleavage reaction to a heterologous gene, indicating that sequences downstream of +1051 are not required for cleavage (22). To map the 3′ border of element II two deletion clones were constructed. In clone Δ+84/+644 (Fig. 3B), the region from +84 to +644 was deleted from EP7-9, resulting in a construct that contains the entire conserved G-rich region but lacks approximately 20 nt

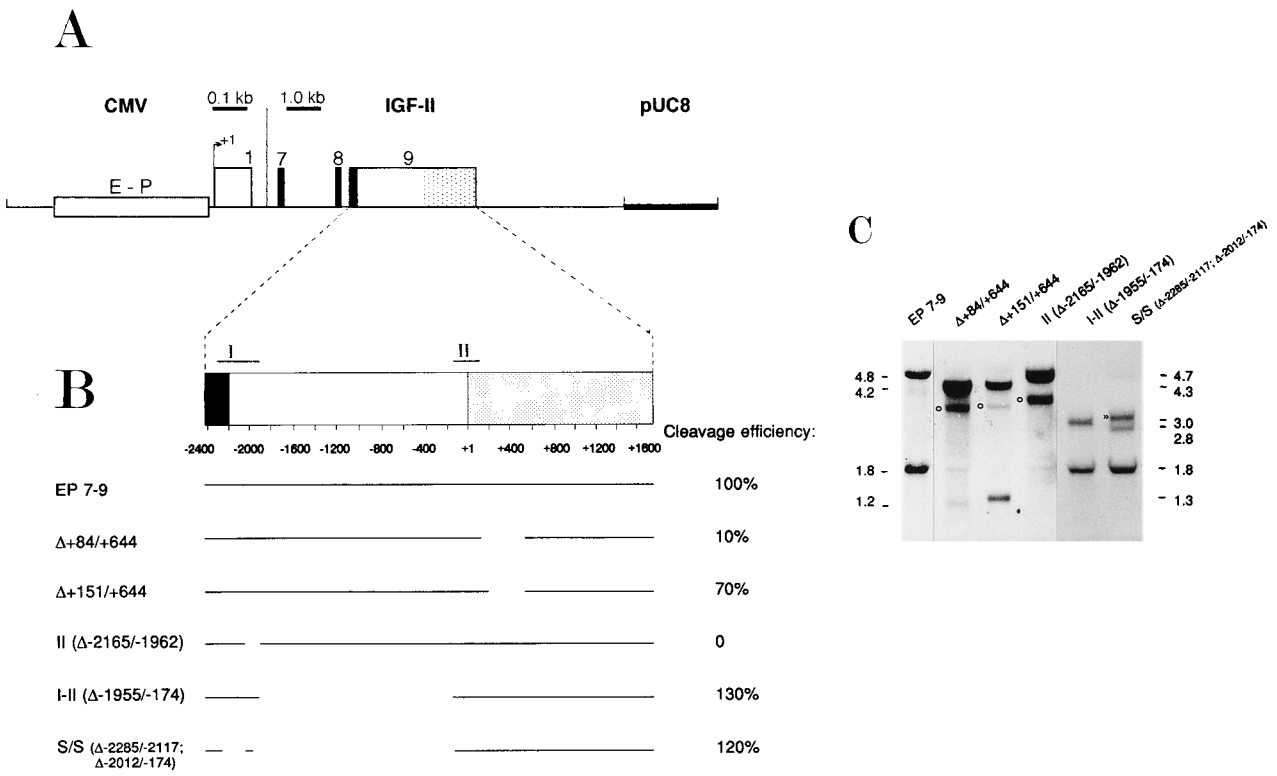
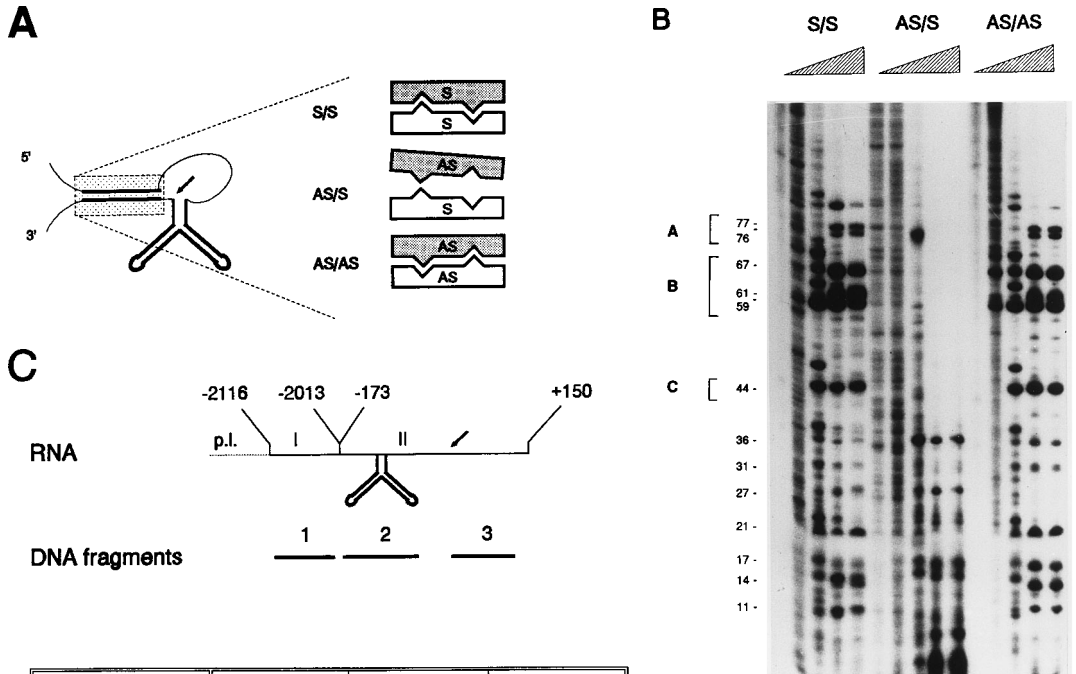


FIG. 3. Fine mapping of elements I and II. (A) Schematic representation of the expression plasmids used. Construct EP7-9 consists of the enhancer/promoter regions and exon 1 of cytomegalovirus (CMV) linked to a genomic fragment of the human IGF-II gene containing exons 7, 8, and 9. Translated and untranslated IGF-II regions are depicted by solid and open boxes, respectively. Sequences downstream of the cleavage site are stippled. (B) Enlarged representation of human IGF-II exon 9. Positions (base pairs) relative to the cleavage site (+1) are indicated. Bars marked with I and II designate the two elements required for RNA cleavage. Constructs are referred to as Δ with numbers indicating the 5′ and 3′ positions of the deleted fragments. In some cases the construct has, in addition to the numbers designating the deletion, another name. (C) Northern blot analysis of total RNA isolated from 293 cells transiently transfected with the constructs indicated. The blot was hybridized to a probe that detects only sequences 3′ of the cleavage site (+84 to +1096). Sizes of the RNA species are indicated in kilobases. Symbols: ○, bands that are probably formed through additional cleavage downstream of element I; >>, incompletely spliced mRNA that still contains the intron between exons 8 and 9.

involved in the putative formation of a stem structure with element I. Clone Δ+151/+644 (Fig. 3B) contains an additional 67 nt, including the full region involved in the putative secondary structure. After transient transfection of these constructs into 293 cells, it was shown that for Δ+84/+644 a cleavage product of 1.2 kb can be detected, albeit at very low levels, in addition to the full-length 4.2 kb transcript (Fig. 3C). However, when the extended construct (Δ+151/+644) was used, making it possible to form the full stable secondary structure, the 1.3-kb cleavage product was formed with almost normal efficiency (Fig. 3B and C). This indicates that the 3′-most border of element II is located at around position +150. Element II is therefore located within the region ranging from −173 to +150.

The far-upstream element I was initially identified to have a size of 327 nt and to be located at positions −2286 to −1960. From the computer foldings, an interaction between nt −2108 to −2029 of element I and +18 to +101 of element II is predicted (Fig. 1), suggesting that the actual element I may be much smaller than 327 nt. To determine more precisely the size of element I, we deleted nt −2165 to −1962, which encompass the interacting region, and tested whether transcripts derived from this construct (Δ−2165/−1962) can still be cleaved. As shown on the Northern blot in Fig. 3C, this deletion completely abolishes the formation of 3′ cleavage products, again suggesting that the interacting region between nt −2108 and −2029 is important for the cleavage reaction. However, other regions within element I could be important as well. To demonstrate that the interacting region is the only essential part of element I, we constructed a clone in which the initial element I ranging from −2286 to −1960 of construct I-II was substituted by a PCR product representing the region from −2116 to −2013 (Fig. 3B, construct Δ−2285/−2117;Δ−2012/−174, also designated S/S). In a transient-transfection experiment with 293 cells, construct S/S produces a full-length mRNA of the expected size (2.8 kb; Fig. 3C). In addition, a considerable amount of 3′-end-specific cleavage product (1.8 kb) was detected (the cleavage efficiency is 120%). Apparently, the region encompassing nt −2116/−2013 is sufficient for efficient cleavage of IGF-II transcripts. This shows that within our initially identified element I, only the region predicted to interact with element II is essential, providing a strong argument for the importance of the long-range RNA-RNA interaction in the cleavage reaction.

Some of the constructs showed, in addition to full-length mRNA and cleavage products, unexpected hybridization with another RNA species. For constructs Δ+84/+644, Δ+151/+644, and Δ−2165/−1962, a prominent RNA species (indicated by open circles in Fig. 3C) approximately 0.8 kb smaller than the full-length mRNA is observed. Primer extension analysis revealed that this is a product derived from additional cleavage downstream of element I (data not shown). This process does not influence formation of 1.8-kb RNA, since both noncleavable (Δ−2165/−1962) and cleavable (Δ+84/+644 and Δ+151/+644) transcripts can give rise to the appearance of this product.



	cluster A	cluster B	cluster C
fragment 1	+++	+	+++
fragment 2	+	+++	++
fragment 3	-	-	-

FIG. 4. (A) Schematic presentation of the secondary structure of IGF-II mRNAs (not to scale). The cleavage site is indicated by an arrow. The orientations of the interacting regions in the different pBluescript constructs used as template for in vitro RNA synthesis are indicated. In construct S/S, the orientations are identical to those in the wild-type IGF-II mRNA. (B) RNase T_1 digestion pattern of labeled in vitro synthesized RNAs containing elements I and II in the S/S, AS/S, or AS/AS configuration. RNAs were incubated with increasing amounts of RNase T_1 (0, 1.9×10^{-3}, 1.9×10^{-2}, 1.9×10^{-1}, and 3.8×10^{-1} U/μl). Sizes of RNA fragments were determined by a parallel sequencing reaction. A, B, and C designate the mixtures of fragments that were used for hybridization of a Southern blot. (C) Clusters A, B, and C were isolated from RNase T_1-digested S/S RNA and hybridized to a Southern blot containing the DNA fragments specific for the various regions: fragment 1 (−2116 to −2013), fragment 2 (−173 to −23), and fragment 3 (+2 to +120). The cleavage site is indicated by an arrow; polylinker sequences from pBS are depicted by p.l.

For construct S/S, an RNA species approximately 0.3 kb larger than the full-length mRNA can be detected (indicated by the double arrowhead in Fig. 3C). We determined by hybridization with an intron-specific probe that this slower-migrating RNA is an incompletely spliced product that still contains the intron between exons 8 and 9 (data not shown). Interestingly, if a larger region (−2286 to −1960) is deleted, no incorrectly spliced products are formed (22), indicating that it is not the deletion of a primary sequence element that causes this phenomenon. Apparently, in transcripts derived from this construct, the secondary structure at the 5′ part of exon 9 is affected, resulting in impaired but not obstructed splicing of exon 8 to exon 9. Effects on splicing, caused by the secondary structure of RNA at the 5′ end of a downstream exon, have been reported previously for other systems as well (38).

Summarizing, we have mapped more precisely the sequence elements required for site-specific endonucleolytic cleavage of IGF-II mRNAs. These elements are located in exon 9 at positions −2116 to −2013 (element I) and −173 to +150 (element II). For efficient cleavage of IGF-II mRNAs, the presence of an intact cleavage unit, that is, the combination of elements I and II, is an absolute prerequisite.

Structural aspects of the interaction between elements I and II. To show that the putative stem structure between elements I and II really exists, we performed compensatory mutagenesis of the interacting regions. For this purpose, we subcloned the two elements in various configurations into pBluescript in such a way that the corresponding mRNAs could be synthesized in vitro by T7 RNA polymerase. From construct S/S (Fig. 3B), a region containing the minimal elements I and II was subcloned into pBluescript, giving construct BS-S/S. The stem structure can form in the 571-nt RNA derived from this construct, because both regions involved in stem formation are in the wild-type configuration. A second construct, BS-AS/S, was derived from S/S by inverting element I. Transcripts derived from BS-AS/S cannot form the stem structure, because elements I and II can no longer undergo base pairing (Fig. 4A). If the quintessence of interaction between elements I and II in mRNA cleavage is to provide a nonspecific stretch of dsRNA, the cleavage reaction should be retained if the base pairing is restored by compensatory mutations in the interacting region of element II. Therefore, in the third construct, BS-AS/AS, we restored the structure by also inverting the interacting region in element II (Fig. 4A). It should be noted that in the inverted region of element II of the BS-AS/AS clone, additional base changes had to be introduced to retain the secondary RNA structure. This is mainly due to the presence of G · U base pairs in the S/S RNA that become C · A pairs when inverted and thus can no longer undergo base pairing. The upstream part of element II that is also required for cleavage, including the cleavage site (−173 to +11), is unchanged in construct BS-AS/AS.

Initial experiments to detect dsRNA stem formation by treatment of the in vitro synthesized RNAs with low concentrations of specific RNase, followed by primer extension analysis of the products, failed (results not shown). Apparently, the RNAs contained too much secondary structure to perform primer extension reactions with mouse mammary tumor virus reverse transcriptase. We obtained better results with a thermostable enzyme (rTth), with which extension could be performed at 72°C, but even the latter polymerase could not get through the entire stem structure, supporting the existence of the stable interaction between the elements. We then used another method to establish the structure by synthesizing internally labeled RNAs of the BS-S/S, BS-AS/S, and BS-AS/AS constructs, treating these RNAs with increasing amounts of RNase T_1, and comparing the T_1 digestion patterns of the different I/II configurations (Fig. 4B). This clearly showed that both the S/S RNA and the mutant AS/AS RNA were very resistant to RNase T_1 treatment, giving products as long as 77 nt that remained stable even at very high RNase concentrations (Fig. 4B). In contrast, the AS/S mutant did not give rise to these stable products. This strongly suggests that elements I and II do not function as separate units but interact with each other, leading to RNase T_1-resistant structures.

Three size classes of fragments that are present in the S/S and AS/AS but not the AS/S digestion reactions can be distinguished (Fig. 4B): clusters A (76 to 77 nt), B (59 to 67 nt), and C (44 nt). To prove that the large RNA fragments resulting from RNase T_1 digestion of S/S and AS/AS RNAs originate from the stem structure formed between elements I and II, the clusters A, B, and C obtained by RNase T_1 digestion of S/S RNA were eluted from the gel and used to hybridize a Southern blot containing PCR fragments spanning different regions of elements I and II (Fig. 4C). Cluster A hybridizes with fragment 1, which contains element I, indicating that this region of the RNA is protected from RNase T_1 digestion. Cluster B hybridizes mainly with fragment 2, which encompasses the region −173 to −23 and thus contains the stem-loop structures in element II upstream of the cleavage site. Cluster C hybridizes mainly with fragment 1 (element I) and to a lesser extent with fragment 2 (stem-loops), the latter indicating that regions outside the stem structure formed between element I and II are also stabilized by the presence of this interaction. Although none of the clusters hybridize with fragment 3, which consists of the region from +2 to +120 and therefore contains the part of element II that is involved in the stem structure, this is not surprising as such. Considering the extreme G richness of this region (55%), it is not hard to envision that the chance that the G-specific RNase T_1 can attack a G in a "breathing" RNA structure is much higher for this part of the interaction than for its C-rich counterpart in element I, especially at these high concentrations of RNase. None of these probes hybridized with *Alu*I-digested pBluescript.

On the basis of these experiments with in vitro synthesized RNAs, we conclude that a stable dsRNA stem can indeed be formed between elements I and II.

Functional aspects of the interaction between I and II. To obtain more insight in the functional aspects of the interaction between elements I and II, cytomegalovirus–IGF-II expression constructs containing the different I/II configurations (S/S, AS/S, and AS/AS) were used for transfection experiments. After transient transfections of 293 cells with DNA from the S/S, AS/S, AS/S expression constructs, total RNA was analyzed by Northern blotting (Fig. 5). mRNAs derived from construct S/S are cleaved very efficiently (Fig. 3C and 5; note that all these constructs show the impaired splicing, as was observed for S/S before). In contrast, cleavage does not occur in transcripts derived from AS/S (2.8 kb; Fig. 5), again suggesting that base pairing between elements I and II is essential for cleavage. For AS/AS mRNAs, however, that can form a double-stranded stem structure but also carry mutations in the primary sequence, the full-length transcript of 2.8 kb but no cleavage products could be detected, showing that restoration of the secondary structure alone is not sufficient for the mRNA to become a substrate for the cleavage reaction. Obviously, the long-range RNA-RNA interaction between elements I and II functions to create a sequence-specific dsRNA region.

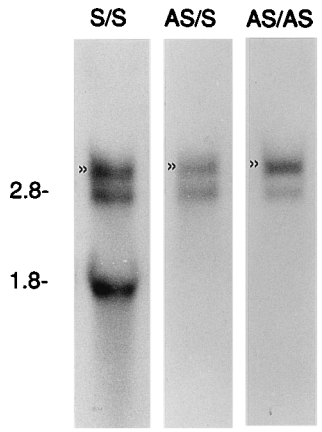


FIG. 5. Northern blot analysis of total RNA isolated from 293 cells transiently transfected with the constructs depicted above the lanes. The orientation of the interacting regions in these expression plasmids corresponds to that of the pBluescript constructs in Fig. 4A. The blot was hybridized to the 3′-end-specific probe (+84 to +1096). Sizes of the RNA species are indicated in kilobases. The hybridizing bands which migrate more slowly than the full-length transcripts in lanes S/S, AS/S, and AS/AS (designated >>) are incompletely spliced products, which still contain the intron between exons 8 and 9.

Positional aspects of the interaction between elements I and II. The results with deletion constructs, as well as previous experiments with chimeric constructs (22), show that when an mRNA contains an intact cleavage unit consisting of elements I and II, it is capable of undergoing specific endonucleolytic cleavage. The computer foldings and the RNase T_1 digestion experiments further indicate that the elements in such a cleavage unit can form a stable dsRNA stem structure. To further investigate the necessity of interaction between the two elements within a cleavage unit, we constructed a plasmid containing two intact cleavage units in tandem. This clone (I-II/I-II; Fig. 6A) was derived from construct I-II (Fig. 3B) by inserting a fragment containing a second cleavage unit 650 nt downstream of the first cleavage site. Transcripts derived from construct I-II/I-II can potentially be cleaved at two sites, designated A and B. Depending on the particular site(s) of cleavage used (site A, site B, or both sites), cleavage may result in the production of various RNA products as indicated in Fig. 6A. After transient transfection of these constructs into 293 cells, multiple RNA species were detected on a Northern blot (Fig. 6B). By differential usage of probes specific for either sequences downstream (3′-specific probe) or upstream (5′-specific probe) of the cleavage sites, the 5′ and 3′ cleavage products were identified. The initial full-length I-II/I-II mRNA of 4.1 kb is cleaved at both sites A and B, yielding products of 2.9 and 1.2 kb (cleavage at site A) or 2.3 and 1.8 kb (cleavage at site B), respectively. This indicates that mRNAs containing two cleavage units can be processed at either of the two cleavage sites. The results also show that when the mRNA is cleaved at one of the sites, the resulting RNA product, which still

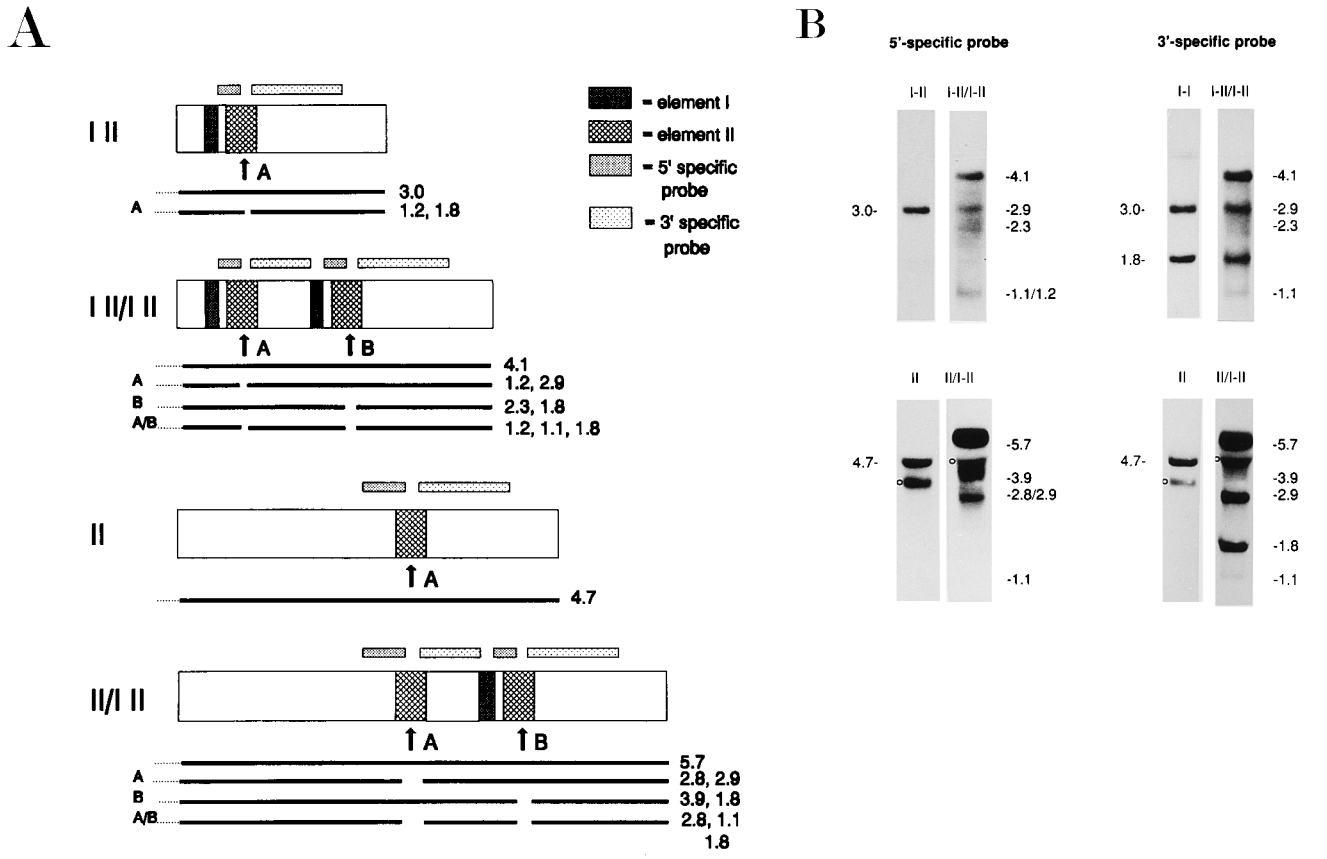


FIG. 6. Cleavage of mRNAs containing multiple copies of elements I and II. (A) Schematic representation of the constructs. Only exon 9 of the IGF-II expression plasmids (see Fig. 3A for details) is shown. The positions of elements I (dark grey) and elements II (grey) are indicated. The potential cleavage sites are indicated by arrows A and B. Below the exon, the sizes and positions of RNAs derived from the constructs after cleavage at any of the potential cleavage sites are shown. (B) Northern blot analysis of total RNA isolated from 293 cells transiently transfected with the constructs indicated. Blots were hybridized with the 5′-end-specific and 3′-end-specific probes (positions indicated in the drawing of the constructs). The sizes of the RNA species are depicted in kilobases. The hybridizing bands designated ○ are probably formed by additional cleavage downstream of element I.

contains an intact cleavage unit, can be processed again at the second site as well. The latter was concluded from the presence of the 1.1-kb RNA that contains the region between the two sites and is produced only if both cleavage sites A and B are used. In this experiment we could not establish the site where the cleavage event takes place first. However, products derived from cleavage at only one of the two sites are readily detected, suggesting that there is no absolute preference of cleavage site usage. Interestingly, the 5′ cleavage products derived from construct I-II/I-II (2.3, 1.2, and 1.1 kb) are quite easily detectable, whereas the 5′ product of I-II is barely visible (Fig. 6B). This suggests that cleavage products are more stable if they have the ability to form the stem structure between elements I and II.

Subsequently, we addressed the question whether the relative position of the elements within a cleavage unit is important. We therefore inserted the fragment containing a cleavage unit 651 nt downstream of the cleavage site of construct II (Fig. 3B). Construct II, which does not contain an intact cleavage unit, yields transcripts that cannot be cleaved. Insertion of an intact cleavage unit downstream of the cleavage site of construct II results in construct II/I-II (Fig. 6A). Similar to mRNAs derived from I-II/I-II, transcripts from II/I-II contain two potential cleavage sites, but in this case cleavage site A is not in the context of an intact cleavage unit because element I is lacking. However, on a Northern blot of RNA from a transient-transfection experiment, not only products resulting from cleavage at site B (3.9 and 1.8 kb) but also products from cleavage at site A (2.9 and 2.8 kb) are found. In addition, a 1.1-kb RNA is detected that is formed when both sites are used (Fig. 6B).

These results indicate that cleavage at site A occurs by using the downstream element I. This shows that although the presence of an intact element I is absolutely required for cleavage, its position relative to element II is not important, since it can function upstream as well as downstream of element II. This further supports the presence of the interaction between the two elements, because the stable stem structure can be formed, regardless of the positioning of the elements within a cleavage unit.

In summary, we have identified a long-range RNA-RNA interaction between two elements required for site-specific cleavage of human IGF-II mRNAs. The relative position of these elements and the spacing between the elements and the IGF-II reading frame are not important. Both the double-stranded character and the sequence of the interacting regions are essential for this endonucleolytic cleavage reaction.

DISCUSSION

A striking feature of human IGF-II mRNAs is that two widely separated elements, elements I and II, are required for

a site-specific endonucleolytic cleavage reaction. These two elements were mapped in detail, and it was shown that they can interact with each other through the formation of a dsRNA stem structure. This structure is unique because it shows a very stable interaction ($\Delta G = -100$ kcal/mol) of two elements separated by an unusually long region of RNA (approximately 2.1 kb). A complete cleavage unit is about 400 nt, consisting of element I of approximately 100 nt (−2116 to −2013) and element II of approximately 300 nt (−173 to +150). Compared with other known mRNA-processing determinants, cleavage of IGF-II mRNAs requires a large region of the mRNA. In the regulation degradation of transferrin receptor mRNAs, a structure of only 23 to 37 nt is involved in binding of the iron-responsive-element-binding protein (17).

As is common in RNA recognition by *trans*-acting factors, both primary sequence and secondary structure are important. Our results show that this is also the case for IGF-II mRNAs. It is also interesting that the cleavage site is exposed in a single-stranded RNA region, as has been found for the *Xenopus* Xlhbox2B mRNA (8) and chicken apolipoprotein II mRNA (4).

We have provided evidence for the functional significance of the interaction between the elements from different perspectives. First, the double-stranded stem structure is predicted by two separate algorithms, the widely used folding program of Zuker and Stiegler (49) and the program of Abrahams et al. (1). This stem structure is highly conserved among human, rat, and mouse IGF-II mRNAs over approximately 55 nt (Fig. 2). Most base changes are compensated in the matching element, so that the base pairing is maintained, or do not affect the secondary structure, providing phylogenetic evidence for the importance of the stem structure. The second line of evidence comes from in vivo experiments. We have previously mapped the far-upstream element I to be located within positions −2286 and −1960. In this study we show that from this element, only the part that interacts with element II is necessary for efficient cleavage of the mRNA. In addition, if element II is extended from +83 to +150 (the interacting region is present at +18 to +101), the cleavage efficiency is dramatically increased. In support of the putative interaction, we have further shown that the relative position of the elements is not important for cleavage. The stem structure between the elements can be formed whether element I is situated 5′ or 3′ of element II. Interestingly, if the region between the elements is deleted, cleavage is enhanced, as was shown for both I-II (cleavage efficiency, 130%) and S/S (120%). This suggests that if formation of the stem structure is facilitated by reducing the distance between the elements, the efficiency of cleavage increases.

By RNase T_1 digestion of in vitro synthesized RNAs, we could show that RNAs that are able to form the stable stem structure between elements I and II (S/S and AS/AS [Fig. 4]) are very resistant to treatment with this single-stranded G-specific endonuclease, in contrast to a mutant RNA that is unable to fold into this conformation (AS/S [Fig. 4]). In addition to long, stable digestion products that originate from the stem structure, products derived from the stem-loops in element II upstream of the cleavage site are detected, indicating that the latter structure is stabilized by the presence of the interaction between elements I and II.

In many examples of regulated mRNA degradation, it has been demonstrated that translation is involved. Several mRNAs are stabilized in the presence of inhibitors of translation (18, 45). This can be explained by the requirement for a labile protein component that is readily depleted after translational arrest (7). Alternatively, translation of the mRNA itself can be essential for degradation. For example, the cell-cycle-dependent degradation of histone mRNAs requires translation within a certain distance from the 3′ end of the transcript (15). Quite differently, degradation of tubulin mRNA is regulated by the binding of tubulin subunits to the nascent peptide (46). Further, it has been suggested that degradation of many mRNAs takes place on the polysomes, probably because components of the decay system are ribosome associated (2). An attractive hypothesis for IGF-II mRNAs is that element I serves to bring the cleavage site in closer proximity to the IGF-II coding region by the interaction between elements I and II. The IGF-II termination codon is located 2.2 kb upstream of the cleavage site, whereas it is separated from element I by only 75 nt. However, such a mechanism does not apply to this system, because in construct II/I-II the distance between the stop codon and the first element is 3 kb but cleavage still occurs very efficiently (Fig. 6). Furthermore, in construct S/S the reading frame is extended in such a way that translation stops just downstream of element I. Apparently, cleavage is also not affected if translation proceeds through element I. This is in contrast to some other systems, in which the functioning of *cis*-acting sequences is dependent on their presence within or outside the reading frame (19, 28, 30, 35).

Using constructs I-II/I-II and II/I-II, we proved that a single transcript containing two cleavage sites can be cleaved twice, because the 1.1-kb RNA formed by double cleavage is detected for both constructs (Fig. 6). From the amounts of the various cleavage products, it appears that there is no preferential usage of cleavage sites and that both are cleaved equally well.

Several models can explain the role of the long-range interaction between elements I and II in the cleavage reaction. In the first model, the interaction serves only to position the cleavage site at a proper location. This could be an overall spatial positioning within the IGF-II mRNA. Alternatively, the positioning could be to overcome steric hindrance, by exposing the cleavage site or the upstream stem-loop structures to a *trans*-acting factor(s). This leads to a model in which the specificity of cleavage is obtained by structures other than the interaction itself, with the upstream stem-loop structures in element II as good candidates. In the second model, the interaction functions merely as a long stretch of dsRNA in the recognition of a *trans*-acting factor. In this model, the specificity of cleavage is again obtained by other regions than the long-range RNA-RNA interaction, most probably the upstream stem-loops. This can be envisaged if the endonuclease specifically recognizes the upstream stem-loops (or factor[s] binding to this structure) and requires dsRNA for its activity. In the third model, the interacting region itself is recognized by a *trans*-acting factor(s). Specificity in this case is provided by the interaction either alone or in cooperation with another region within the elements.

To obtain information about the mechanism of cleavage of IGF-II mRNAs, we compared the cleavage ability of different I/II conformations: the wild-type construct S/S and the mutant constructs AS/S and AS/AS. Of the transcripts derived from these constructs, only the S/S mRNAs were cleaved (Fig. 5). The difference between this construct and construct AS/AS is the sequence of the RNA-RNA interaction between the elements, implying that the mere presence of a similar dsRNA element does not suffice for cleavage. Although the stem structure formed in AS/AS is a little shorter than that of the actual mRNA, it is unlikely that this is the cause of the cleavage deficiency, since transfections with construct Δ+84/+644, an even shorter structure, can result in cleavage, albeit with lower efficiency (Fig. 3). From these results, the first two models described above can be excluded, because it is obvious that the

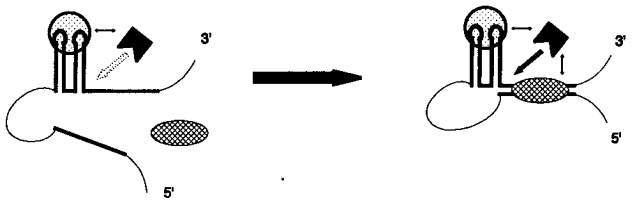


FIG. 7. Model explaining the involvement of the interaction between elements I and II in cleavage of IGF-II mRNAs. The stem structure has to be present for cleavage to occur. The interaction is recognized by a *trans*-acting factor (depicted by the hatched ellipse) in a sequence-specific manner, possibly in conjunction with other regions and factors. The factor recognizing the interaction may be the same as or interact with a factor that recognizes the stem-loops and/or the endonuclease, as illustrated by the small arrows. The large arrow indicates the cleavage site.

mere presence of a similar secondary structure does not suffice for cleavage. We present the third model as a working hypothesis for the mechanism of cleavage of IGF-II mRNAs. This model (Fig. 7) involves specific recognition by one or more *trans*-acting factors of at least the interaction between elements I and II and possibly also of some additional structure or sequence. Whether this *trans*-acting factor is the RNase itself is unknown. From in vitro degradation experiments, we concluded that the site-specific cleavage reaction is not due to self-cleavage, because cellular extract is absolutely required for cleavage (24, 35a). It is possible that the function of the specific binding of a factor to the structure is simply to expose the cleavage site/stem-loop structures. In any case, the long-range interaction is recognized in a sequence-specific manner. The sequence specificity of the interaction will most probably not cover the entire region, because there are several base differences among human, rat, and mouse mRNAs.

Our data suggest a different mechanism of cleavage from the one that was previously postulated by Nielsen and Christiansen (24). They identified the possible formation of a quadruplicate structure just downstream of the cleavage site. However, only sequences surrounding the cleavage site were considered, and our in vivo experiments clearly show that this region does not suffice for cleavage and that the far-upstream element I is absolutely required as well. The involvement of the region downstream of the cleavage site in the I/II interaction or the quadruplex is mutually exclusive. Therefore, the T_1 digestion experiments with the S/S RNA and the mutant AS/S and AS/AS RNAs that show the presence of the I/II stem structure (Fig. 4) exclude the formation of a quadruplex in the presence of element I. In our model such a quadruplicate structure may be formed at the 5′ end of the 1.8-kb RNA after cleavage has occurred, and this may explain the unusual stability of the 1.8-kb RNA that we have observed.

Cleavage of IGF-II mRNAs represents a previously unique mRNA processing event. Although the function of the cleavage reaction is still elusive, it is tempting to speculate that this endonucleolytic cleavage is the first step in the degradation of IGF-II mRNAs. Such a mechanism has been hypothesized for other mRNAs as well (3, 4, 8, 40). After cleavage, the 5′ product containing the coding region is very unstable, supporting a means of rapid change in IGF-II availability. Therefore, it is intriguing that under serum-free conditions the cleavage efficiency seems to increase (21). Another interesting option is that the 3′ product of the cleavage reaction has a (yet unknown) function itself. Recently, it was reported for a myogenic cell line that 3′-UTRs can promote differentiation (32) or function as tumor suppressors (33). In this respect it is interesting that IGF-II has been implicated in myogenic differentiation (42), opening new perspectives for an unconventional role of the 1.8-kb mRNA cleavage product.

ACKNOWLEDGMENTS

We thank C. W. A. Pleij and E. B. ten Dam for their help with computer-aided RNA folding and our colleagues R. J. T. Rodenburg and L. A. Nolten for stimulating discussions.

REFERENCES

1. **Abrahams, J. P., M. Van den Berg, E. Van Batenburg, and C. Pleij.** 1990. Prediction of RNA secondary structure, including pseudoknotting, by computer simulation. Nucleic Acids Res. **18:**3035–3044.
2. **Bandyopadhay, R., M. Coutts, A. Krowczynska, and G. Brawerman.** 1990. Nuclease activity associated with mammalian mRNA in its native state: basis for selectivity in mRNA decay. Mol. Cell. Biol. **10:**2060–2069.
3. **Binder, R., J. A. Horowitz, J. P. Basilion, D. M. Koeller, R. D. Klausner, and J. B. Harford.** 1994. Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3′ UTR and does not involve poly(A) tail shortening. EMBO J. **13:**1969–1980.
4. **Binder, R., S. L. Hwang, R. Ratnasabapathy, and D. L. Williams.** 1989. Degradation of apolipoprotein II mRNA occurs via endonucleolytic cleavage at 5′-AAU-3′/5′-UAA-3′ elements in single-stranded loop domains of the 3′-noncoding region. J. Biol. Chem. **264:**16910–16918.
5. **Bohjanen, P. R., B. Petryniak, C. H. June, C. B. Thompson, and T. Lindsten.** 1991. An inducible cytoplasmic factor (AU-B) binds selectively to AUUUA multimers in the 3′ untranslated region of lymphokine mRNA. Mol. Cell. Biol. **11:**3288–3295.
6. **Brewer, G., and J. Ross.** 1988. Poly(A) shortening and degradation of the 3′ A+U-rich sequences of human c-*myc* mRNA in a cell-free system. Mol. Cell. Biol. **8:**1697–1708.
7. **Brewer, G., and J. Ross.** 1989. Regulation of c-*myc* mRNA stability in vitro by a labile destabilizer with an essential nucleic acid component. Mol. Cell. Biol. **9:**1996–2006.
8. **Brown, B. D., and R. M. Harland.** 1990. Endonucleolytic cleavage of a maternal homeo box mRNA in Xenopus oocytes. Genes Dev. **4:**1925–1935.
9. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
10. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
11. **Daughaday, W. H., and P. Rotwein.** 1989. Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum and tissue concentrations. Endocr. Rev. **10:**68–91.
12. **DeChiara, T. M., E. J. Robertson, and A. Efstratiadis.** 1991. Parental imprinting of the mouse insulin-like growth factor II gene. Cell **64:**849–859.
13. **De Pagter-Holthuizen, P., M. Jansen, R. A. Van der Kammen, F. M. A. Van Schaik, and J. S. Sussenbach.** 1988. Differential expression of the human insulin-like growth factor II gene. Characterization of the IGF-II mRNAs and an mRNA encoding a putative IGF-II-associated protein. Biochim. Biophys. Acta **950:**282–295.
14. **Froesch, E. R., C. Schmidt, J. Schwander, and J. Zapf.** 1985. Actions of insulin-like growth factors. Annu. Rev. Physiol. **47:**443–467.
15. **Graves, R. A., N. B. Pandey, N. Chodchoy, and W. F. Marzluff.** 1987. Translation is required for regulation of histone mRNA degradation. Cell **48:**615–626.
16. **Holthuizen, P. E., C. B. J. M. Cleutjens, G. J. C. Veenstra, F. M. Van der Lee, A. M. C. B. Koonen-Reemst, and J. S. Sussenbach.** 1993. Differential expression of the human, mouse and rat IGF- II genes. Regul. Pept. **48:**77–89.
17. **Klausner, R. D., T. A. Rouault, and J. B. Harford.** 1993. Regulating the fate of mRNA: the control of cellular iron metabolism. Cell **72:**19–28.
18. **Koeller, D. M., J. A. Horowitz, J. L. Casey, R. D. Klausner, and J. B. Harford.** 1991. Translation and the stability of mRNAs encoding the transferrin receptor and c-fos. Proc. Natl. Acad. Sci. USA **88:**7778–7782.
19. **Lim, S.-K., C. D. Sigmund, K. W. Gross, and L. E. Maquat.** 1992. Nonsense codons in human β-globin mRNA result in the production of mRNA degradation products. Mol. Cell. Biol. **12:**1149–1161.
20. **Malter, J. S.** 1989. Identification of an AUUUA-specific messenger RNA binding protein. Science **246:**664–666.
21. **Meinsma, D., P. E. Holthuizen, J. L. Van den Brande, and J. S. Sussenbach.** 1991. Specific endonucleolytic cleavage of IGF-II mRNAs. Biochem. Biophys. Res. Commun. **179:**1509–1516.
22. **Meinsma, D., W. Scheper, P. E. Holthuizen, and J. S. Sussenbach.** 1992. Site-specific cleavage of IGF-II mRNAs requires sequence elements from two distinct regions of the IGF-II gene. Nucleic Acids Res. **20:**5003–5009.
23. **Mullner, E. W., B. Neupert, and L. C. Kuhn.** 1989. A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. Cell **58:**373–382.
24. **Nielsen, F. C., and J. Christiansen.** 1992. Endonucleolysis in the turnover of insulin-like growth factor II mRNA. J. Biol. Chem. **267:**19404–19411.
25. **Nielsen, F. C., G. Haselbacher, J. Christiansen, M. Lake, M. Gronborg, and**

S. Gammeltoft. 1993. Biosynthesis of 10 kDa and 7.5 kDa insulin-like growth factor II in a human rhabdomyosarcoma cell line. Mol. Cell. Endocrinol. **93:**87–95.

26. **Ogawa, O., M. R. Eccles, J. Szeto, L. A. McNoe, K. Yun, M. A. Maw, P. J. Smith, and A. E. Reeve.** 1993. Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. Nature (London) **362:**749–751.
27. **Ohlsson, R., L. Holmgren, A. Glaser, A. Szpecht, and S. Pfeifer-Ohlsson.** 1989. Insulin-like growth factor II and short-range stimulatory loops in control of human placental growth. EMBO J. **8:**1993–1999.
28. **Parker, R., and A. Jacobson.** 1990. Translation and a 42 nucleotide segment within the coding region of the mRNA encoded by the mat-α-1 gene are involved in promoting rapid mRNA decay in yeast. Proc. Natl. Acad. Sci. USA **87:**2780–2784.
29. **Peltz, S. W., G. Brewer, P. Bernstein, P. A. Hart, and J. Ross.** 1991. Regulation of mRNA turnover in eukaryotic cells. Crit. Rev. Eukaryotic Gene Expression **1:**99–126.
30. **Peltz, S. W., A. H. Brown, and A. Jacobson.** 1993. mRNA Destabilization triggered by premature translational termination depends on at least three *cis*-acting sequence elements and one *trans*-acting factor. Genes Dev. **7:**1737–1754.
31. **Rainier, S., L. A. Johnson, C. J. Dobry, A. J. Ping, P. E. Grundy, and A. P. Feinberg.** 1993. Relaxation of imprinted genes in human cancer. Nature (London) **362:**747–749.
32. **Rastinejad, F., and H. M. Blau.** 1993. Genetic complementation reveals a novel regulatory role for 3′ untranslated regions in growth and differentiation. Cell **72:**903–917.
33. **Rastinejad, F., M. J. Conboy, T. A. Rando, and H. M. Blau.** 1993. Tumor suppression by RNA from the 3′ untranslated region of α-tropomyosin. Cell **75:**1107–1117.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1987. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Savant-Bhonsale, S., and D. W. Cleveland.** 1992. Evidence for instability of mRNAs containing AUUUA motifs mediated through translation-dependent assembly of a >20S degradation complex. Genes Dev. **6:**1927–1939.

35a. **Scheper, W.** Unpublished data.

36. **Shaw, G., and R. Kamen.** 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:**659–667.
37. **Shyu, A. B., J. G. Belasco, and M. E. Greenberg.** 1991. Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev. **5:**221–231.
38. **Solnick, D.** 1985. Alternative splicing caused by RNA secondary structure. Cell **43:**667–676.
39. **Stoeckle, M. Y.** 1992. Removal of a 3′ non-coding sequence is an initial step in degradation of groα mRNA and is regulated by interleukin-1. Nucleic Acids Res. **20:**1123–1127.
40. **Stoeckle, M. Y., and H. Hanafusa.** 1989. Processing of 9E3 mRNA and regulation of its stability in normal and Rous sarcoma virus-transformed cells. Mol. Cell. Biol. **9:**4738–4745.
41. **Sussenbach, J. S.** 1989. The gene structure of the insulin-like growth factor family. Prog. Growth Factor Res. **1:**33–48.
42. **Tollefsen, S. E., J. Levis Sadow, and P. Rotwein.** 1989. Coordinate expression of insulin-like growth factor II and its receptor during muscle differentiation. Proc. Natl. Acad. Sci. USA **86:**1543–1547.
43. **Vakalopoulou, E., J. Schaack, and T. Shenk.** 1991. A 32-kilodalton protein binds to A+U-rich domains in the 3′ untranslated regions of rapidly degraded mRNAs. Mol. Cell. Biol. **11:**3355–3364.
44. **van Dijk, M. A., H. J. Bootsma, F. M. A. Van Schaik, P. Holthuizen, and J. S. Sussenbach.** 1991. Initial characterization of the four promoters of the human insulin-like growth factor II gene. Mol. Cell. Endocrinol. **81:**81–94.
45. **Wisdom, R., and W. Lee.** 1991. The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. Genes Dev. **5:**232–243.
46. **Yen, T. J., P. S. Machlin, and D. W. Cleveland.** 1988. Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin. Nature (London) **334:**580–585.
47. **You, Y., C.-Y. A. Chen, and A.-B. Shyu.** 1992. U-rich sequence-binding proteins (URBPs) interacting with a 20-nucleotide U-rich sequence in the 3′ untranslated region of c-*fos* mRNA may be involved in the first step of c-*fos* mRNA degradation. Mol. Cell. Biol. **12:**2931–2940.
48. **Zarrilli, R., V. Colantuoni, and C. B. Bruni.** 1992. Regulation of insulin-like growth factor II gene expression in rat liver cells. Eur. J. Biochem. **209:**445–452.
49. **Zuker, M., and P. Stiegler.** 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. **9:**133–148.