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The Internal Ribosome Entry Site-Mediated Translation of Antiapoptotic Protein XIAP Is Modulated by the Heterogeneous Nuclear Ribonucleoproteins C1 and C2

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The X-chromosome-linked inhibitor of apoptosis, XIAP, is the most powerful and ubiquitous intrinsic inhibitor of apoptosis. We have shown previously that the translation of XIAP is controlled by a potent internal ribosome entry site (IRES) element. IRES-mediated translation of XIAP is increased in response to cellular stress, suggesting the critical role for IRES translation during cellular stress. Here, we demonstrate that heterogeneous nuclear ribonucleoproteins C1 and C2 (hnRNPC1 and -C2) are part of the RNP complex that forms on XIAP IRES. Furthermore, the cellular levels of hnRNPC1 and -C2 parallel the activity of XIAP IRES and the overexpression of hnRNPC1 and -C2 specifically enhanced translation of XIAP IRES, suggesting that hnRNPC1 and -C2 may modulate XIAP expression. Given the central role of XIAP in the regulation of apoptosis these results are important for our understanding of the control of apoptosis.

Programmed cell death, apoptosis, is an organized, genetically determined process that is unique to multicellular organisms. Apoptosis is thought to have evolved specifically to regulate cellular homeostasis by removing damaged or redundant cells that could otherwise jeopardize survival of the whole organism. The study of mechanisms of apoptosis has been at the forefront of molecular biology since it was recognized that the misregulation of the apoptotic process has devastating consequences. Indeed, the aberrant regulation of apoptosis has been implicated in various disorders ranging from cancer to autoimmunity to neurodegeneration. Control of apoptosis has therefore emerged as a key pharmacological target (29). One group of genes involved in the regulation of apoptosis, the inhibitor of apoptosis (IAP) family of genes, block cysteine proteases known as caspases. Since the activation and amplification of the caspase cascade are essential to the execution of apoptosis, the IAP proteins are considered to be the key regulators of apoptosis by virtue of their ability to directly bind and inhibit distinct caspases (21). The X-linked inhibitor of apoptosis, XIAP (GenBank accession no. U45880), is the prototype of the IAP family (reviewed in reference 12). XIAP is the most potent inhibitor of both the initiator (caspase-9) and effector (caspase-3 and -7) caspases. Furthermore, XIAP has a number of other physiologically important cellular functions, including receptor-mediated signaling, ubiquitination, and cell cycle control (reviewed in reference 10).

We previously found that the expression of XIAP is controlled at the level of translation initiation; XIAP mRNA is translated by internal ribosome initiation mediated by a potent internal ribosome entry site (IRES) element located in its 5′ untranslated region (UTR) (13). Significantly, the XIAP IRES is active under conditions of cellular stress such as serum starvation and low-dose gamma irradiation-induced apoptosis, conditions that lead to the inhibition of cellular protein synthesis. This suggests that the function of XIAP IRES is to maintain or increase the synthesis of the XIAP protein during cellular stress. Furthermore, we discovered that IRES-mediated upregulation of XIAP in response to irradiation enhances the survival of some cancer cell lines, suggesting that enhanced IRES translation may, in fact, be critically involved in the progression of cancer (16). XIAP IRES-mediated regulation, which is the first example of the regulation of an antiapoptotic gene at the translational level, may therefore confer important survival activity to the cell under acute but transient apoptotic situations.

Deletion and mutation analyses showed that the XIAP IRES was 162 nucleotides (nt) long (13). We began to identify proteins that specifically interact with the RNA sequence of XIAP IRES and could therefore function as the regulators of XIAP IRES activity. A sequence-specific RNA-protein complex that consists of at least four cellular proteins assembles on the XIAP IRES (11). UV cross-linking experiments further revealed that the autoantigen La is an essential part of the XIAP IRES RNP complex but excluded two other known IRES-binding proteins, PTB and PCBP, from binding to XIAP IRES (11). In the present paper we identify heterogeneous nuclear ribonucleoproteins C1 and C2 (hnRNPC1 and -C2) as part of the XIAP IRES RNP complex. Significantly, we found that the cellular levels of hnRNPC1 and -C2 correlate with the XIAP IRES activity in vivo. Furthermore, overexpression of hnRNPC1 and -C2 specifically increased XIAP IRES translation. Our data therefore suggest that hnRNPC1 and -C2 bind to the XIAP IRES element and are involved in the modulation of XIAP IRES translation.

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MATERIALS AND METHODS

Cell culture and reagents. Human embryonic kidney (293T), human squamous lung carcinoma cells (H520), human non-small-lung-cell carcinoma cells (H661), and human ovary adenocarcinoma cells (SKOV3) were cultured in standard conditions as described previously (16). Transient DNA transfections were conducted by using Lipofectamine 2000 reagent (Invitrogen) in accordance with the protocol provided by the manufacturer. The expression plasmids pFLAG-hnRNPC1 and pFLAG-hnRNPC2 were constructed by inserting reverse transcription-PCR (RT-PCR)-generated hnRNPC1 or hnRNPC2 cDNAs into the pcDNA3 vector (Invitrogen) containing the coding sequence for the FLAG epitope sequence (generous gift from A. Kimchi). The glutathione *S*-transferase (GST) fusion expression plasmids pGEX-hnRNPC1 and pGEX-hnRNPC2 were constructed by inserting RT-PCR-generated hnRNPC1 or hnRNPC2 cDNAs into the pGEX-KG vector (Amersham Pharmacia). The bicistronic vector pβgal/hUTR/CAT containing the human XIAP IRES element was described previously (13). The IRES element of apoptotic protease-activating factor 1 (Apaf-1; GenBank accession no. NM013229) was amplified by RT-PCR from total RNA isolated from BJAB cells with primers 5′-CTCGAGAAGAAGAAGTAGCGA GTG and 5′-CTCGAGCTTCCCTCAGATCTTTCTCTCTC and inserted into the linker region of the bicistronic vector pβgal/CAT (13).

Western blot analysis. Cells were harvested in ice-cold phosphate-buffered saline (PBS), lysed in radioimmunoprecipitation assay buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 0.01 M sodium phosphate [pH 7.2], 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C, followed by centrifugation at 14,000 × *g* for 10 min. Protein concentration was assayed with a protein assay kit (Bradford assay; Bio-Rad Laboratories), and equal amounts of protein samples were separated by SDS–10% polyacrylamide gel electrophoresis (PAGE). Samples were analyzed by Western blotting using mouse monoclonal anti-hnRNPC antibody 4F4 (generous gift from G. Dreyfuss), a mouse monoclonal antiactin antibody (Sigma), or a mouse monoclonal anti-FLAG M2 antibody (Stratagene) at 1:10,000 dilution followed by a secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G; Amersham). Antibody complexes were detected by using the ECL system (Amersham).

Cell extracts and EMSAs. The cellular extracts (S10) were prepared as previously described (17). The GST fusion proteins were purified as described previously (11). For electrophoretic mobility shift assays (EMSAs), DNA templates for synthesis of the XIAP or Apaf-1 IRES RNA probes were generated by PCR using XIAP IRES-specific primers described previously (11) or the Apaf-1-specific primers described above. The 5′ primers also incorporated the T7 promoter sequence (14). Internally labeled RNA probes were synthesized by in vitro transcription with T7 polymerase (MAXIScript T7 RNA polymerase kit; Ambion) in the presence of [α-^{32}P]UTP (Amersham). The XIAP IRES and non-IRES probes were described previously (11). EMSA analyses were carried out as detailed previously (11). Briefly, 15,000 cpm of labeled RNA was mixed with 30 μg of 293T S10 extract in a total volume of 15 μl at room temperature for 30 min, followed by the addition of 1 μl of RNase T_1 (1 U/μl) and incubation for an additional 10 min at room temperature; heparin was added to a final concentration of 5 mg/ml. The ribonucleoprotein complex was electrophoresed on a 3% native acrylamide gel at 4°C.

UV cross-linking and immunoprecipitation. RNA-protein complexes for UV cross-linking and immunoprecipitation were prepared as described above. Before RNase T_1 was added, the samples were transferred into a 96-well dish and irradiated on ice with a 254-nm UV light source at 400,000 μJ/cm^2. UV-irradiated RNA-protein complexes were treated with RNase T_1 as described above and diluted in 50 μl of PBS–NP-40 buffer (1× PBS, 2 mM EDTA, 2 mM EGTA, 0.05% NP-40), and 3 μl of monoclonal anti-hnRNPC antibody 4F4 and 20 μl of protein A plus G agarose (Calbiochem) were added. The samples were incubated at room temperature for 30 min and then washed five times in HEPES–NP-40 buffer (15 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40). The immunoprecipitated RNA-protein complexes were released from the agarose beads by boiling in SDS loading buffer (100 mM Tris [pH 6.8], 2.5% SDS, 10% glycerol, 0.025% β-mercaptoethanol, 0.1% bromophenol blue), resolved on an SDS–10% PAGE gel, and visualized by autoradiography. Alternatively, the RNA-protein complexes were formed with the purified GST, GST-hnRNPC1, or GST-hnRNPC2 fusion proteins and UV cross-linked as described above. The RNA-protein complexes were resolved on an SDS–10% PAGE gel and visualized by autoradiography.

XIAP mRNA was coimmunoprecipitated from whole-cell extracts by using the modified method of Seto et al. (31) as described previously (11). Briefly, transfected 293T cells from a 35-mm-diameter dish were harvested in 1 ml of cold PBS and collected by low-speed centrifugation at 4°C. The cell pellets were resuspended in 100 μl of RNA binding buffer (above) supplemented with 10 U of RNase inhibitor (5 Prime-3 Prime), and cell extracts were prepared by the freeze-thaw method. To whole-cell extracts, 5 μl of monoclonal anti-hnRNPC antibody 4F4, anti-La antibody A1 (3), or the antiactin antibody (Amersham), 20 μl of protein A plus G agarose beads (Calbiochem), and 5 U of RNase inhibitor were added, and the samples were incubated for 60 min at room temperature. The beads were then washed extensively with RNA binding buffer supplemented with RNase inhibitor. RNA associated with the antibody-antigen complexes was isolated by repeated phenol-chloroform extraction and precipitation with 2 M ammonium acetate and 3 volumes of cold ethanol. RNA was then analyzed by RT-PCR using XIAP-specific primers (5′ oligonucleotide, 5′-ATGACTTTTAA CAGTTTTGAAGG; 3′ oligonucleotide, 5′-GCTCGTGCCAGTGTTGATGCT G).

Northern blot analysis. Total RNA was isolated from transiently transfected cells by guanidine isothiocyanate–phenol-chloroform extraction with the TRIzol reagent (Gibco) and the protocol provided by the manufacturer. RNA was denatured in formamide and separated on 0.8% agarose gel. RNA was then transferred onto a nylon membrane (Biodyne) and hybridized sequentially with [^{32}P]dCTP (Amersham)-labeled, randomly primed (Amersham Rediprime) DNA probes derived from the coding regions of the chloramphenicol acetyltransferase (CAT) and β-galactosidase (β-Gal) genes. Blots were hybridized overnight in hybridization buffer (5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA], 10× Denhardt's solution, 100 μg of salmon sperm DNA/ml, 50% formamide, 2% SDS) and then washed with 0.2× SSC (1× SSC is 0.15 mM NaCl plus 0.015 mM sodium citrate)–0.1% SDS at 50°C. Membranes were exposed to an X-ray film (Kodak) by using an intensifying screen (Amersham).

β-Gal and CAT analysis. Transiently transfected cells were harvested 24 h posttransfection in CAT enzyme-linked immunosorbent assay (ELISA) kit lysis buffer (Roche), and cell extracts were prepared by using the protocol provided by the manufacturer. β-Gal enzymatic activity in cell extracts was determined by the spectrophotometric assay using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (22), and the CAT levels were determined by using the CAT ELISA kit (Roche) and the protocol provided by the manufacturer.

RESULTS

The 5′ UTR of XIAP mRNA is unusually long and contains a very efficient IRES element that is responsive to cellular stress (13, 16). In an attempt to identify cellular proteins that specifically modulate XIAP IRES activity we characterized a sequence-specific RNP complex that assembles on the XIAP IRES and whose level parallels IRES activity (11). The XIAP IRES RNP complex consists of at least four proteins of which we identified the 50-kDa species as the autoantigen La (11). The identities of the remaining proteins remained unknown although we have excluded two of the known IRES-binding proteins, PTB and PCBP.

A number of different cellular proteins that are recruited to the IRES elements of viral or cellular RNAs have been identified (reviewed in references 9 and 23). Among these, hnRNPC1 and -C2 were shown to bind the differentiation-activated IRES element of platelet-derived growth factor (PDGF) (30). One of the XIAP IRES cross-linked proteins that we observed had an apparent molecular mass (45 kDa) close to those of hnRNPC1 and -C2 (Fig. 1A). We therefore examined whether the hnRNPC proteins are also components of the XIAP IRES RNP complex. The 162-nt IRES probe was mixed with the S10 cellular extracts, UV cross-linked, and immunoprecipitated with the anti-hnRNPC antibody 4F4 (Fig. 1B). The XIAP mRNA 5′ UTR segment that is located upstream of the IRES element was used as a negative control. As shown in Fig. 1B, the hnRNPC band was clearly detectable following immunoprecipitation of XIAP IRES, but not the non-IRES RNA-cross-linked proteins, with the anti-hnRNPC antibody. The appearance of a higher-molecular-mass band,

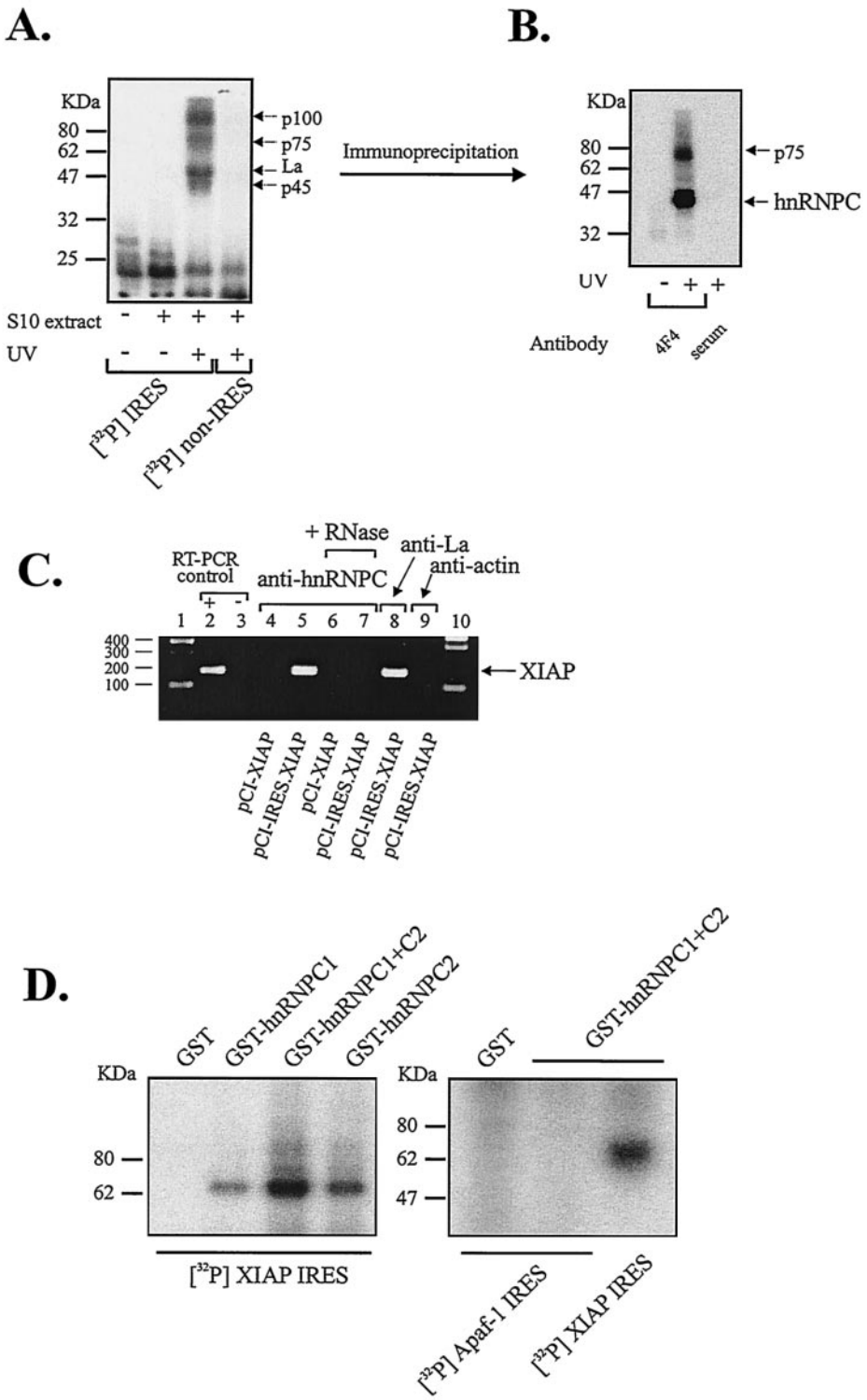

FIG. 1. hnRNPC1 and -C2 are components of the XIAP IRES RNP complex in vivo and in vitro. (A) S10 extracts from 293T cells were incubated with the XIAP IRES or control RNA probes, UV irradiated, and separated on SDS-PAGE gel as described in Materials and Methods. The positions of the molecular mass markers are indicated on the left. (B) S10 extracts were incubated with the XIAP IRES RNA probe, UV cross-linked, immunoprecipitated with the anti-hnRNPC antibody 4F4, and then separated on SDS-PAGE gel as described in Materials and Methods. + and −, UV cross-linking or the absence of UV cross-linking before immunoprecipitation, respectively. (C) hnRNPC is associated with XIAP RNA in vivo. 293T cells were transfected with plasmid pCI-XIAP or pCI-IRES.XIAP, and whole-cell extracts were prepared 24 h later as described in Materials and Methods. Following coimmunoprecipitation with the indicated antibodies (anti-hnRNPC, anti-La, antiactin) the XIAP RNA was detected by RT-PCR analysis. The positive (cDNA; lane 2) and negative (no template; lane 3) controls for RT-PCR are shown on the left. The cell lysates in lanes 6 and 7 were treated with RNase A prior to immunoprecipitation. The molecular weights of DNA marker bands (lanes 1 and 10) are indicated on the left. (D) Equal amounts of purified GST, GST-hnRNPC1, or GST-hnRNPC2 fusion proteins (100 ng of total protein per lane) were incubated with the XIAP IRES or Apaf-1 IRES RNA probe, UV-cross-linked, and then separated on SDS-PAGE gel as described in Materials and Methods. The identities of fusion proteins are indicated above the lanes; the positions of the molecular mass markers are indicated on the left.

approximately 75 kDa, was also noted, but the identity of this protein remains to be determined.

We next wished to determine if the hnRNPC interaction with XIAP IRES also takes place in vivo by immunoprecipitating the XIAP mRNA with the anti-hnRNPC antibody from whole-cell extracts. The endogenous XIAP mRNA is not very abundant in 293T cells. We therefore first enriched XIAP mRNA levels by transfecting 293T cells with a construct expressing only the XIAP mRNA coding region (pCI-XIAP) or the plasmid expressing the XIAP mRNA coding region with 1 kb of 5′ UTR that contains the XIAP IRES element (pCI-IRES.XIAP). Twenty-four hours after transfection, whole-cell extracts were prepared and RNA associated with the hnRNPC was immunoprecipitated with monoclonal anti-hnRNPC antibody 4F4 and analyzed by RT-PCR using XIAP mRNA-coding-region-specific primers. As a control we used either an antiactin or antiautoantigen La antibody since we have shown previously that XIAP IRES associates with the autoantigen La in vivo (11). The results of the coimmunoprecipitation experiments are summarized in Fig. 1C. The specific XIAP RT-PCR signal was obtained only if the expression plasmid contained the XIAP mRNA 5′ UTR sequence (lanes 3 versus 4). Furthermore, XIAP RNA was not detected if the cell extracts were pretreated with RNase prior to immunoprecipitation (lanes 5 and 6) or if we used a nonspecific antibody for immunoprecipitation (antiactin; lane 8). These results indicate that the hnRNPC proteins associate with the XIAP RNA in vivo and that this interaction is mediated by the 5′ UTR sequence of XIAP RNA.

There exist two isoforms of the hnRNPC, hnRNPC1 and hnRNPC2, which differ only by the insertion of 13 amino acids into the hnRNPC2 isoform. We wished to elucidate which of the hnRNPC isoforms interacts with the XIAP IRES sequence or whether both do. To this end, the XIAP IRES RNA probe was incubated with affinity-purified GST, GST-hnRNPC1, or GST-hnRNPC2 proteins, cross-linked, and separated on SDS–10% PAGE gel (Fig. 1C). Although both the GST-hnRNPC1 and GST-hnRNPC2 fusion proteins were found to bind the XIAP IRES element alone, the binding of hnRNPC1 and -C2 together was the most efficient. To further demonstrate the specificity of XIAP IRES-hnRNPC binding, we used, as an RNA probe, an unrelated IRES element that was recently discovered in the 5′ UTR of the mRNA for apoptotic protease activating factor 1 (Apaf-1) (5). In contrast to what was found for the XIAP IRES, the hnRNPC proteins did not exhibit any affinity for Apaf-1 IRES (Fig. 1D), further confirming that the binding of the hnRNPC proteins to the XIAP IRES is sequence specific.

We next reasoned that if the XIAP IRES element requires auxiliary proteins for its activity (e.g., hnRNPC1 and -C2), this activity could therefore vary among different cell lines, reflecting the presence or abundance of protein factors required for IRES function. We therefore initiated experiments to determine if the XIAP IRES activity is cell line specific. Several cell lines were transiently transfected with the bicistronic reporter plasmid that contains XIAP IRES (pβgal/hUTR/CAT). While we observed insignificant differences in XIAP IRES activity among three cell lines (293T, H520, H661), one cell line, SKOV3, showed a marked reduction (fivefold) in the XIAP IRES activity (Fig. 2A; Table 1). To confirm that the observed changes were not due to a cryptic promoter activity in the XIAP mRNA 5′ UTR, we transfected the promoterless construct pCATbasic/hUTR (13) into all four cell lines but detected no CAT activity (data not shown). Alternatively, the lack of XIAP IRES activity could be due to a splicing event in the SKOV3 cells. However, there were no observed differences in the integrity of the bicistronic RNA between SKOV3 and H661 cells, excluding this possibility (Fig. 2B). In contrast, Western blot analysis of the four cell lines revealed a correlation between the activity of XIAP IRES and the levels of hnRNPC1 and -C2, since only the SKOV3 cell line had substantially less hnRNPC1 and -C2 than the other three cell lines tested (Fig. 2A, top). We wished to determine if the reduced levels of hnRNPC1 and -C2 could be responsible for the observed reduction in XIAP IRES activity. To this end, we cotransfected the SKOV3 cell line with the expression vectors encoding either hnRNPC1 or hnRNPC2 cDNAs and the bicistronic reporter construct pβgal/hUTR/CAT, which harbors the XIAP IRES element. We reasoned that, if the reduced levels of hnRNPC proteins in SKOV3 are responsible for the reduction in XIAP IRES translation, then the overexpression of hnRNPCs should increase the activity of XIAP IRES in this cell line. Indeed, transient overexpression of hnRNPC1 or hnRNPC2 increased the translation of XIAP IRES about twofold (Fig. 2C, Table 1). Moreover, the cotransfection of hnRNPC1 and hnRNPC2 increased the translation of XIAP IRES about 3.5-fold, suggesting that both isoforms of hnRNPC participate in the modulation of XIAP IRES activity in vivo. This translational activation was dose dependent, as increasing the amount of hnRNPC-expressing plasmid DNA resulted in an increase of XIAP IRES translation (Fig. 2C). Significantly, transient overexpression of hnRNPC1 and -C2 had no effect on the activity of Apaf-1 IRES. These tissue culture results thus corroborated our biochemical evidence that hnRNPC1 and hnRNCP2 bind specifically to the XIAP IRES element and modulate its activity.

It has been shown previously that hnRNPC proteins bind, with high affinity, RNA sequences that contain several uridine residues (7). More specifically, RNA sequence AGUAUUUU UGUGGA was identified through the SELEX procedure as having a high affinity for the hnRNPC proteins (32). Interestingly, there are several uridine-rich motifs in the XIAP IRES that could potentially mediate hnRNPC binding (13). We therefore used UV cross-linking combined with sequence-specific competitors to map the binding site of hnRNPC1 and -C2 on the XIAP IRES element. Out of seven different nonoverlapping competitors only one was able to block the binding of hnRNPC1 and -C2 to the XIAP IRES (Fig. 3A). This competitor spanned the sequence −114 to −135 of XIAP IRES (GU UAUUUUUAUGUCAUAAGUGGAUAA), which has a striking similarity to the SELEX-identified hnRNPC binding site. Indeed, the deletion derivative of the XIAP IRES that lacks this sequence [pβgal/5′(−102)/CAT] had significantly reduced IRES activity in transfected 293T cells (Fig. 3B). Furthermore, translation of this construct could no longer be enhanced in SKOV3 cells by the overexpression of hnRNPC1 and -C2 (Fig. 3C), confirming that the sequence −114 to −135 is the binding site of hnRNPC1 and -C2.

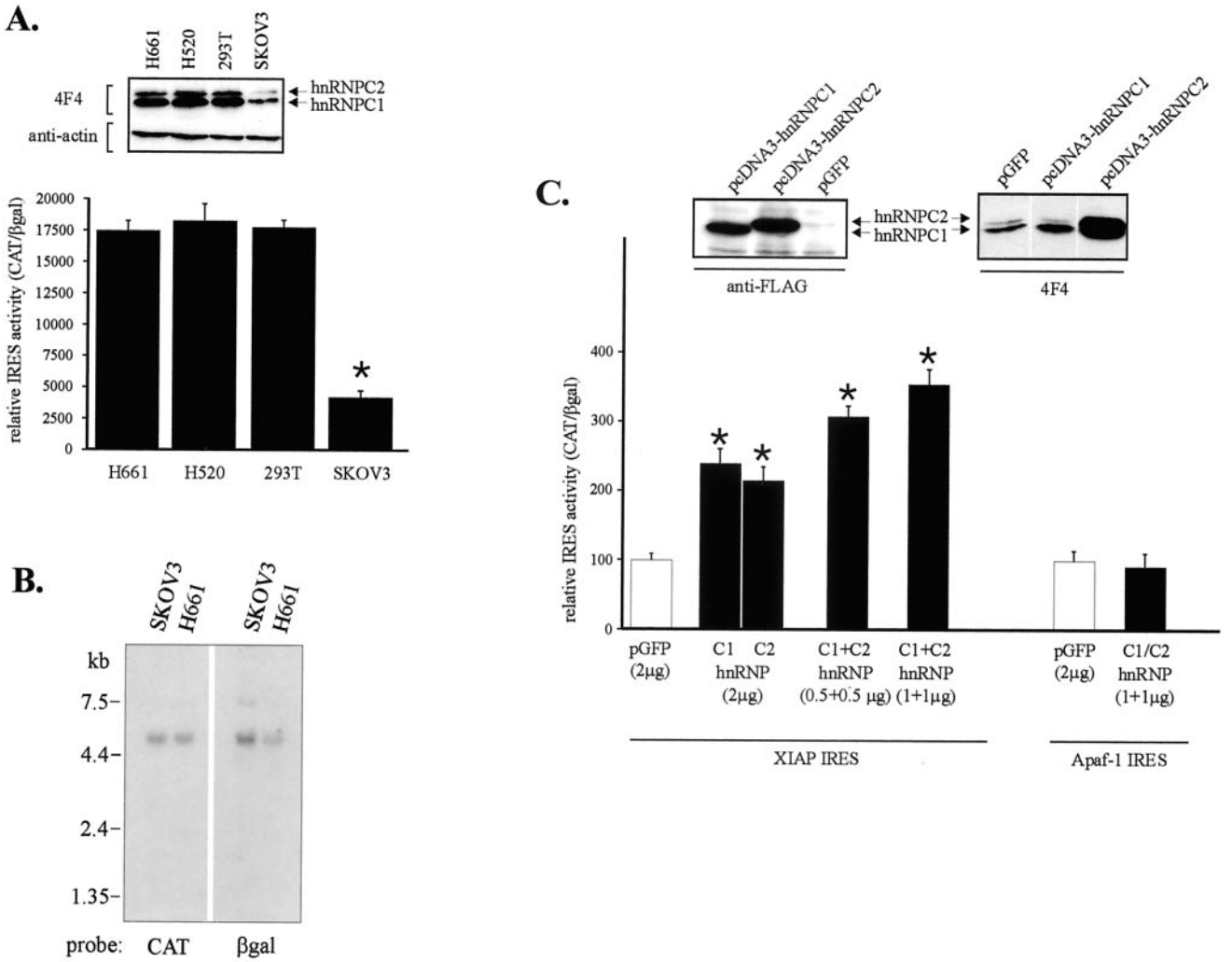

FIG. 2. hnRNPC1 and -C2 enhance XIAP IRES translation in cultured cells. (A) 293T, H520, H661, and SKOV3 cells were transiently transfected with the pβgal/hUTR/CAT construct (13), and the relative IRES activity was determined as described in Materials and Methods. Bars, averages ± standard deviations (SD) of three independent transfections performed in triplicate (*, $P < 0.05$ by one-way analysis of variance [ANOVA]). The levels of endogenous hnRNPC1, hnRNPC2, and actin in the same cell lines were determined by Western blot analysis using the anti-hnRNPC 4F4 (for hnRNPC1 and -C2) and antiactin (for actin) antibodies (top). (B) The integrity of the βgal/hUTR/CAT bicistronic RNA (~5.5 kb) in SKOV3 and H661 cells was examined by Northern blot analysis using either CAT (left) or β-Gal (right) probes. The positions of the molecular weight markers are indicated on the left. (C) SKOV3 cells were cotransfected with bicistronic plasmid pβgal/hUTR/CAT or pβgal/Apaf-1/CAT and 1 or 2 μg of pcDNA3-GFP (pGFP), pcDNA3-hnRNPC1, or pcDNA3-hnRNPC2 DNA, and the relative IRES activity was determined 24 h posttransfection. The activity of each IRES in cells cotransfected with pGFP was set as 100. Bars, average ± SD of three independent transfections performed in triplicate (*, $P < 0.05$ by one-way ANOVA, relative to pGFP-transfected cells). The levels of expression of FLAG-tagged overexpressed proteins were assessed by Western blot analysis using either an anti-hnRNPC (4F4) or anti-FLAG antibody (top).

DISCUSSION

The translation of mRNA of the intrinsic inhibitor of apoptosis XIAP is mediated by a potent IRES element. This mode of translation appears to be essential for proper XIAP function during early stages of apoptosis because it allows continuous expression of XIAP when the majority of the cellular protein synthesis is compromised (13). We have postulated that IRES-mediated translation of selected cellular mRNAs could be selectively regulated to achieve a balance between the pro- and antiapoptotic signals, safeguarding the cellular homeostasis (15). Identification of specific proteins that can selectively bind to and modulate distinct IRES elements is therefore an im-

TABLE 1. Overexpression of hnRNPC enhances XIAP IRES- but not cap-dependent translation in SKOV3 cells

Plasmid[a]	Avg activity (±SD) of:		Relative IRES activity[c]
	β-Gal[b]	CAT[b]	
pGFP	0.245 (±0.031)	1,120.1 (±24.3)	4,571.83
hnRNPC1	0.229 (±0.016)	2,229.9 (±52.6)	9,737.55
hnRNPC2	0.231 (±0.019)	2,194.6 (±61.1)	9,500.43
hnRNPC1+C2	0.239 (±0.029)	3,933.6 (±103.2)	16,458.57

[a] 2 μg of total plasmid DNA per transfection.
[b] β-Gal and CAT activities were determined as described in Materials and Methods.
[c] Relative IRES activity was determined as CAT/β-Gal.

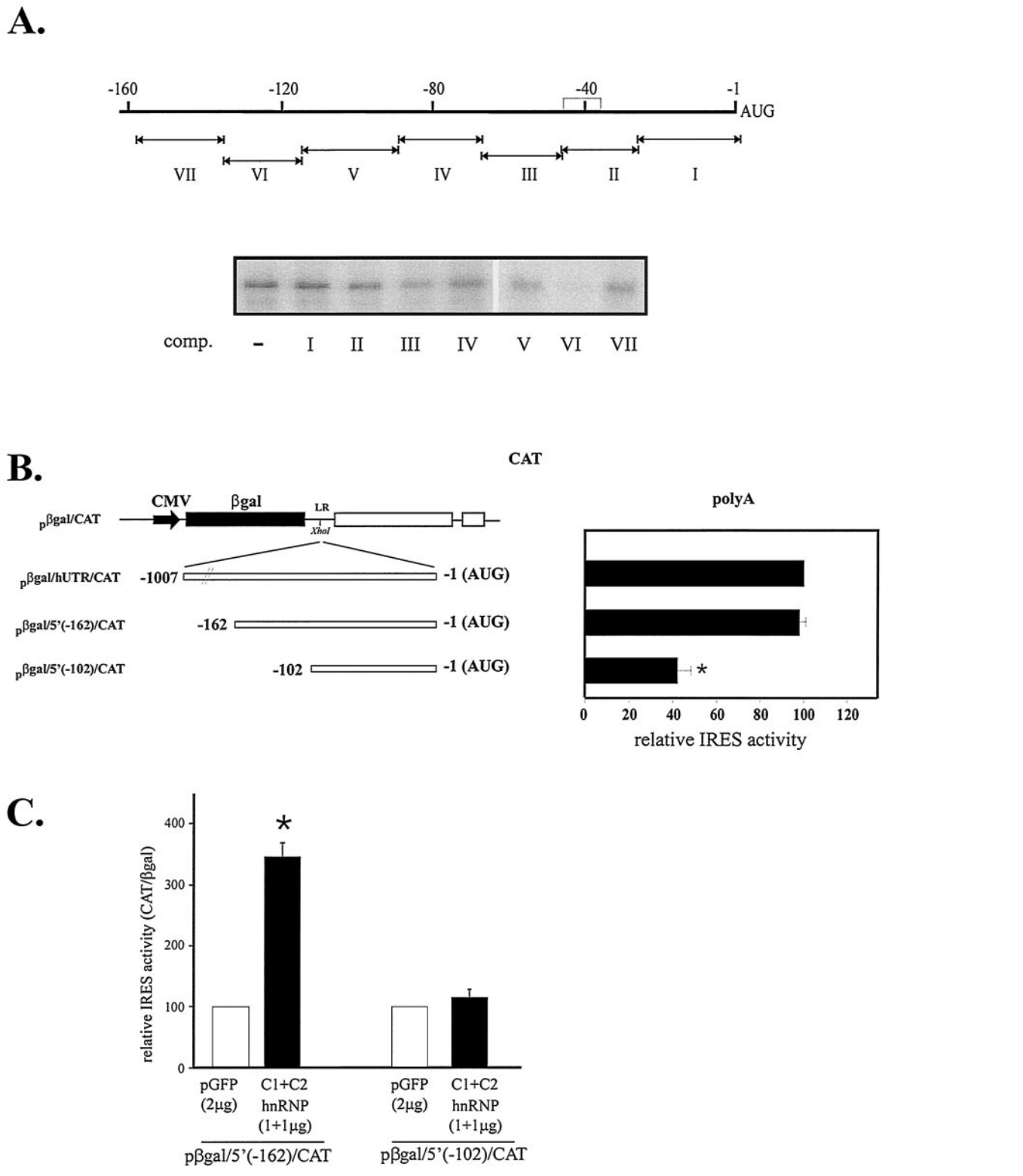

FIG. 3. Mapping of the hnRNPC binding site. (A) Schematic representation of the 162-nt portion of XIAP IRES. Arrows, positions of competitor oligonucleotides; gray box, polypyrimidine tract. Purified GST-hnRNPC1 and GST-hnRNPC2 fusion proteins were mixed and incubated with the XIAP IRES RNA probe in the presence of the indicated cold competitors (comp.; 100-fold excess), UV-cross-linked, and then separated on SDS-PAGE gel as described in Materials and Methods. (B) 293T cells were transfected with the indicated plasmids as described in Materials and Methods. The levels of β-Gal and CAT were determined 24 h posttransfection. The relative IRES activity (CAT normalized with β-Gal) from the pβgal/hUTR/CAT construct was set at 100%. Bars, averages ± standard deviations (SD) of three independent transfections performed in triplicate; *, $P < 0.05$ by one-way analysis of variance (ANOVA). CMV, cytomegalovirus; LR, linker region. (C) SKOV3 cells were cotransfected with bicistronic plasmid pβgal/5′(−162)/CAT or pβgal/5′(−102)/CAT and either 2 μg of pcDNA3-GFP (pGFP) or 2 μg of pcDNA3-hnRNPC1 and pcDNA3-hnRNPC2 DNA, and the relative IRES activity was determined 24 h posttransfection. The activity of each IRES in cells cotransfected with pcDNA3 was set as 100. Bars, averages ± SD of three independent transfections performed in triplicate; *, $P < 0.05$ by one-way ANOVA.

portant step in the understanding of IRES-mediated translation of cellular mRNAs.

In the present paper we have shown that a 45-kDa protein interacts with the XIAP IRES RNA. Immunoprecipitation with a monoclonal antibody identified that this protein existed in two isoforms, hnRNPC1 and -C2. hnRNPC1 and -C2 are among the most abundant cellular proteins involved in the processing of pre-mRNA. The two isoforms, hnRNPC1 and -C2, are produced by alternative splicing and differ only by the insertion of 13 amino acids into the hnRNPC2 isoform. hnRNPC1 is more abundant than hnRNPC2, with hnRNPC2 expressed at about one-third the level of hnRNPC1 (20). The hnRNPC1 and hnRNPC2 proteins can form a stable $(C1)_3C2$ heterotetramer with high binding affinity for uridine-rich RNA sequences (32). Not unexpectedly, we found that both hnRNPC1 and hnRNPC2 proteins bind the XIAP IRES element. This result is not surprising since it was shown previously that both hnRNPC1 and hnRNPC2 have affinity for similar sequences (2). In our experiments, however, hnRNPC2 bound XIAP IRES more efficiently than hnRNPC1, although more-detailed biochemical analysis needs to be performed in order to determine the exact binding constants for both proteins. Our results also indicate that hnRNPC1 and -C2 can bind directly to the XIAP IRES element without the aid of additional cellular proteins. Additionally, both hnRNPC1 and -C2 can bind their target RNA sequences independently in vitro, demonstrating that binding is not necessarily dependent on the formation of the hnRNPC1/C2 tetramer. However, note that the combined effect of hnRNPC1 and -C2 was stronger both in vitro and in vivo. Using sequence-specific competitors we determined that the binding site for hnRNPC1 and -C2 on the XIAP IRES is located in the −114 to −135 segment of the 5′ UTR. This sequence, GUUAUUUUUAUGUCAUAAGUG GAUAA, is similar to the SELEX-identified hnRNPC1 and -C2 binding site AGUAUUUUUGUGGA (32). Importantly, the deletion of this segment both reduced the basal level of XIAP IRES activity and abolished the ability of hnRNPC1 and -C2 to enhance XIAP IRES-mediated translation in vivo. These data suggest that, while hnRNPC1 and -C2 are not essential, they positively modulate translation mediated by the XIAP IRES element.

We have shown previously that the XIAP IRES RNP core binding sequence spans the −34 to −62 region of XIAP mRNA 5′ UTR (11). In this work we mapped the hnRNPC1 and -C2 binding site to a more upstream region, −114 to −135. The inspection of the predicted secondary structure of the XIAP IRES (Fig. 4) indicates, however, that there exists a partial overlap between these two binding sites that could explain the apparent discrepancy in the mapping results. Additional biochemical information is needed, however, to fully understand the RNA-protein interactions and the proteins that participate in the assembly of XIAP IRES RNP complex.

The biochemical evidence that hnRNPC1 and -C2 are part of the XIAP IRES RNP complex was further supported by results for cultured cells. We identified one cell line, SKOV3, which exhibits reduced levels of hnRNPC1 and -C2 proteins. Significantly, the activity of the XIAP IRES element was dramatically reduced in this cell line. Transient overexpression of the expression vector encoding hnRNPC1 and -C2 in SKOV3 cells substantially enhanced XIAP IRES activity, demonstrating that the interaction between XIAP IRES and hnRNPC2 occurs in vivo.

The biochemical function of hnRNPC1 and -C2 proteins remains somewhat controversial. hnRNPC1 and -C2 were implicated in several steps of RNA biogenesis, including splicing (4), RNA turnover (8), and polyadenylation (38). The high abundance of these proteins in the cell also suggests that they may have a pleiotropic effect on unrelated RNA templates (37). Additionally, the hnRNPC1 and -C2 proteins were implicated in DNA metabolism since they can bind the telomerase holoenzyme, and this binding correlated with the ability of telomerase to access the telomere (6). It has been found that mice with targeted deletion of the hnRNPC locus exhibit an embryonically lethal phenotype consistent with the critical role of hnRNPC1 and -C2 in other cellular functions (37). Surprisingly, however, embryonic stem cells derived from hnRNPC-null mice were able to grow and differentiate in vitro.

Recently, hnRNPC1 and -C2 were also implicated in translation. It has been shown that hnRNPC1 and -C2 bind to an IRES element of PDGF/c-sis in a differentiation-induced manner although the direct involvement of hnRNPC1 and -C2 in translation of PDGF mRNA was not demonstrated (30). The hnRNPC1 and -C2 proteins were also shown to be associated with the polyuridine-rich 5′ UTR of p27 mRNA and the binding of hnRNPC1 and -C2 occurred in parallel with p27 mRNA translation (25). It is noteworthy that the 5′UTR of p27 mRNA was recently demonstrated to possess an IRES activity (26). We have shown here that hnRNPC1 and -C2 bind to the IRES element of XIAP. More importantly, the cellular levels of hnRNPC correlate with the activity of the XIAP IRES element, suggesting that hnRNPC may function as an enhancer of XIAP translation. How could hnRNPC1 and -C2, which are considered to be exclusively nuclear proteins, be involved in translation? A growing number of studies suggest that the hnRNPC1 and -C2 proteins also function in the cytoplasm (see, e.g., references 25, 27, 28, 30, 35, and 39). Furthermore, the XIAP IRES element is not active in the T7/vaccinia virus system, where the RNA is synthesized in the cytoplasm and does not enter the nucleus, suggesting that the XIAP IRES requires "nuclear experience" (G. Belsham, personal communication). We predict that this nuclear event could be provided by nuclear RNA binding proteins, such as hnRNPC1 and -C2. These proteins could interact with the XIAP IRES RNA in the nucleus and then be transported with the XIAP RNA to the cytoplasm, where they enhance XIAP mRNA translation. Alternatively, the binding of hnRNPC1 and -C2 to the XIAP IRES in the nucleus could impact the conformational state of the IRES element, which is essential for the binding of one or more auxiliary proteins, which are then involved in the translation of XIAP. The requirement for the nuclear event is not unique to the XIAP IRES. Most cellular IRES elements do not function, or function very inefficiently, in cell-free translation systems or in RNA transfection assays, suggesting that they may require a nuclear event (18, 19, 33). Although the nature of this event is yet to be understood, it is plausible that the nuclear experience of at least some IRES elements may be mediated by the hnRNPC1 and -C2 proteins.

Many cellular proteins are specifically cleaved during apoptosis. Recently, identification of proteins that are modified during Fas-induced apoptosis in Jurkat cells revealed that a

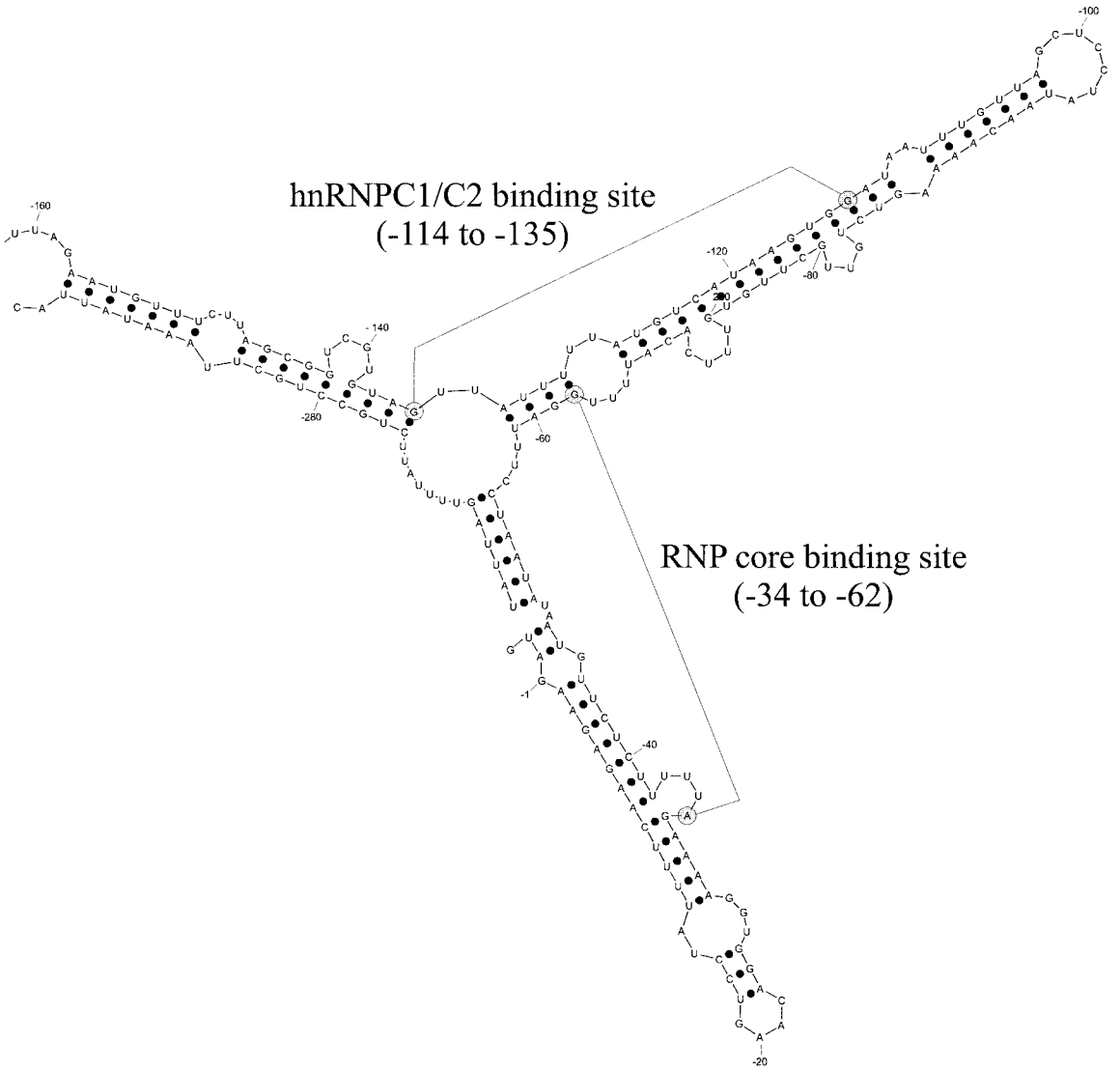

FIG. 4. Proposed secondary structure of the XIAP IRES element. The secondary structure of the 300-nt XIAP 5′ UTR sequence was predicted by using the *mfold* algorithm (version 3.1; http://bioinfo.math.rpi.edu/~mfold/rna/) (24, 40). The RNP core binding site (11) and the hnRNPC1 and -C2 binding site are bracketed, with the first and the last nucleotides of each binding site shown in gray circles. The numbering of the 5′ UTR begins with the first nucleotide (G) 5′ to the initiator AUG, designated −1 (13). For clarity, only sequence relevant to this work is shown.

large number of the apoptosis-modified proteins are, in fact, RNA-binding proteins (34). More specifically, hnRNPC1 and hnRNPC2 were shown to be cleaved by caspases in cells induced to undergo apoptosis by a variety of triggers (1, 36). The advantage of regulating apoptosis by modulating the RNA metabolism is not clear. Since the regulation of apoptosis requires rapid responses, it could be achieved by the regulation of RNA metabolism (e.g., splicing, turnover, and translation) more efficiently than by regulation of de novo mRNA synthesis (34). XIAP is a potent inhibitor of apoptosis by virtue of blocking caspase activation. It is tempting to speculate that the proteolytic cleavage of hnRNPC1 and -C2 at the onset of apoptosis is targeted to attenuate the synthesis of XIAP. This notion, and the effect of cleavage of hnRNPC1 and -C2 on XIAP expression, however, await further experiments.

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