# Gene promoter of apoptosis inhibitory protein IAP2: identification of enhancer elements and activation by severe hypoxia

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Inhibitors of apoptosis (IAPs) antagonize cell death and regulate the cell cycle. One mechanism controlling IAP expression is translation initiation through the internal ribosome entry sites. Alternatively, IAP expression can be regulated at the transcription level. We showed recently the activation of IAP2 transcription by severe hypoxia. To pursue this regulation, we have cloned the full-length cDNA of rat IAP2, and have isolated and analysed the promoter regions of this gene. The cDNA encodes a protein of 589 amino acids, exhibiting structural features of IAP. In rat tissues, a major IAP2 transcript of $\approx$ 3.5 kb was detected. We subsequently isolated 3.3 kb of the proximal 5′-flanking regions of this gene, which showed significant promoter activity. Of interest, 5′ sequential deletion of the promoter sequence identified an enhancer of $\approx$ 200 bp. Deletion of cAMP-response-element-binding protein (CREB) sites in the enhancer sequence diminished its activity. Finally, the IAP2 gene promoter was activated significantly by severe hypoxia and not by $CoCl_2$ or desferrioxamine, pharmacological inducers of hypoxia-inducible factor-1. In conclusion, in this study we have cloned the full-length cDNA of rat IAP2, and for the first time we have isolated and analysed promoter sequences of this gene, leading to the identification of enhancer elements. Moreover, we have demonstrated activation of the gene promoter by severe hypoxia, a condition shown to induce IAP2. These findings provide a basis for further investigation of gene regulation of IAP2, a protein with multiple functions.

Key words: cell death, gene regulation, hypoxia-inducible factor-1.

## INTRODUCTION

The inhibitors of apoptosis (IAPs) are a novel family of proteins that suppress apoptosis triggered by a variety of stimuli [1–5]. Recent studies suggest that IAPs may have other functions as well. For example, XIAP, the prototype of mammalian IAP, interacts and modulates the signalling cascade initiated by transforming growth factor $\beta$ [6,7]. The newly discovered IAP family member Survivin has been shown to participate in cell-cycle control, specifically cytokinesis [8–11]. In addition, ubiquitin ligase activity has been shown for several IAPs, which may determine proteosome-mediated turnover of regulatory molecules for cell death or survival [12,13]. Thus, this family of proteins may play important roles in various physiological and pathogenic processes, including oncogenesis, neurodegeneration and ischaemic disease [14–16].

Originally identified in baculovirus, IAP has been cloned from human, mouse, pig, chicken, *Drosophila* and lately from rat [2,17–24]. In human, at least seven kinds of IAP have been identified, including XIAP, human IAPs (HIAPs) 1 and 2, neuronal IAP (NIAP), Survivin, BIR-repeat-containing ubiquitin-conjugating enzyme (BRUCE) and melanoma IAP (ML-IAP) [2,25]. Structural features of these proteins include the presence at their N-termini of up to three imperfect amino acid repeats approx. 70 residues in length, termed baculovirus IAP repeat (BIR). BIR is a sequence motif that is unique to IAP and, despite controversy [26–28], might be critical for their anti-apoptotic property under certain conditions [29,30]. In addition, a RING finger is usually present near the IAP C-termini, which might be involved in protein–DNA as well as protein–protein interactions [31], and has been shown to be responsible for the ubiquitin-protein ligase activity of IAP [12,32].

Gene regulation of IAP has been implicated in cancer, neurodegenerative disorders and ischaemic diseases [14–16]. Recent studies have demonstrated an important mechanism governing XIAP expression at the level of translation initiation [33]. A sequence of 162 nucleotides has been identified in the 5′-untranslated region of XIAP mRNA, which acts as an internal ribosome entry site and facilitates protein expression even under cellular stresses such as serum deprivation and $\gamma$-irradiation. On the other hand, expression of specific IAPs can be regulated at the transcriptional level as well. IAPs 1 and 2 and XIAP are transcribed under the direction of the transcription factor nuclear factor $\kappa$B, in response to cytokine stimulation [24,34]. Our recent observations showed a striking induction of IAP2 by severe hypoxia [35]. The induction depends on gene transcription, and yet can be dissociated from the ubiquitous hypoxia-responsive transcription factor hypoxia-inducible factor 1 (HIF-1). Investigation of IAP gene regulation at the transcription level has been hindered, mainly due to the lack of information on promoters of these genes. In the current study, we have initially isolated the full-length cDNA of IAP2 from the rat kidney proximal tubular cells. With the cDNA sequence, we have subsequently isolated 3.3 kb proximal 5′-flanking regions of the rat IAP2 gene, by the technique of genomic walking. Analysis of the 3.3 kb genomic sequence demonstrated significant promoter activities. Within

Abbreviations used: EMSA, electrophoretic mobility-shift assay; HIF-1, hypoxia-inducible factor 1; IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; RACE, rapid amplification of cDNA ends; RPTC, rat kidney proximal tubular cell; CREB, cAMP-response-element-binding protein; ATF, activating transcription factor.

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The nucleotide sequences of rat IAP2 cDNA and promoter regions reported in this paper have been submitted to the GenBank Nucleotide Sequence Database under the accession numbers AF190020 and AY027668.

the promoter regions, we have identified an enhancer sequence with critical cAMP-response-element-binding protein (CREB) elements that may regulate IAP2 gene expression. Of significance, the current study has provided the first evidence that the IAP2 promoter is activated by severe hypoxia, a condition triggering IAP2 gene expression. These results have laid the basis for further investigation of IAP2 gene regulation under various pathophysiological situations.

## MATERIALS AND METHODS

### Cell culture

Rat kidney proximal tubular cells (RPTCs) were obtained from Dr U. Hopfer at Case Western Reserve University (Cleveland, OH, U.S.A.) and maintained as described in our previous studies [35,36]. Other cells, including PC12, 3T3 and HEK-293 cells, were purchased from the A.T.C.C. (Manassas, VA, U.S.A.) and maintained following the specified procedures. Cell-culture media were purchased from Gibco Life Technologies (Rockville, MD, U.S.A.).

### Hypoxic incubation

Cells were exposed to severe hypoxia as described previously [35,36]. Briefly, cells were washed with PBS and transferred to an anaerobic chamber with 85% $N_2$/10% $H_2$/5% $CO_2$. Incubation medium was then changed to Krebs/Ringer bicarbonate buffer that had been pre-equilibrated with 95% $N_2$/5% $CO_2$. EC Oxyrase, a biocatalytic oxygen-reducing agent, was added at 1.2 units/ml to the incubation medium to consume residual $O_2$ and maximize the degree of hypoxia.

### Isolation of full-length cDNA of rat IAP2 by rapid amplification of cDNA ends (RACE)

RACE is a PCR-based technique for isolation of full-length cDNA of specific genes [37,38]. For RACE, a short stretch of sequence in the gene's cDNA should be available. Thus we initially isolated a cDNA fragment of rat IAP2 by reverse transcriptase PCR. Total RNA was isolated from RPTCs with TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). Using the RNA as templates, reverse transcriptase PCR was performed with primers (F1 and R1 in Table 1) designed according to the conservations between human and mouse IAP2 (GenBank accession numbers U88909 and U45879). This reverse transcriptase PCR amplified a cDNA fragment of $\approx$ 470 bp with significant homology to mouse and human IAP2. Based on the sequence of the 470 bp fragment, rat IAP2 gene-specific primers were designed for RACE reactions (see Table 1 for primer sequences). For 3′-RACE, the oligo-dT adaptor primer was used for reverse transcription of total RNA isolated from RPTCs. The synthesized cDNA was then subjected to a first round of PCR amplification using the primer F2 and the adaptor primer R2. An aliquot of the PCR products was used subsequently as templates for the second round of (nested) PCR with primers F3 and R2.

After these two rounds of PCR, a major cDNA fragment of $\approx$ 1.5 kb was amplified. 5′-RACE of rat IAP2 was accomplished with the Marathon™ cDNA amplification kit (Clontech, Palo Alto, CA, U.S.A.), following the manufacturer's instructions. Briefly, total RNA was isolated from RPTCs, and poly $(A)^+$ RNA purified with PolyAtract mRNA isolation system (Promega, Madison, WI, U.S.A.). First-strand DNA was synthesized through reverse transcription of poly $(A)^+$ RNA using avian myeloblastosis virus reverse transcriptase and a docking oligo-dT primer. Double-stranded DNA was synthesized subsequently using a cocktail of *Escherichia coli* DNA polymerase I, RNase H and DNA ligase. The double-stranded DNA was then ligated to an adaptor sequence (5′-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3′). The ligated double-stranded DNA was used as a template for 5′-RACE reactions.

The first-round PCR of 5′-RACE was carried out using the adaptor primer F4 and the rat IAP2 gene-specific primer R1. An aliquot of the PCR products was then used as a template for the second-round PCR using the nested adaptor primer F5 and the nested gene-specific primer R5. The 5′-RACE reactions led to the amplification of a major cDNA fragment of $\approx$ 1.7 kb. Products from 3′- and 5′-RACE were gel-purified and sequenced, showing significant homology to human and mouse IAP2. Sequences of the RACE products were aligned with the 470 bp fragment to obtain the sequence of full-length cDNA for rat IAP2.

**Table 1 Primers for RACE reactions**

F1–F6, forward (sense) primers; R1–R6, reverse (antisense) primers. Primers F1 and R1 were designed on the basis of conservations between human and mouse IAP2 to amplify a 470 bp fragment of rat IAP2 cDNA by reverse transcriptase PCR. The oligo-dT adaptor primer was used for reverse transcription of RNA to generate cDNA templates for 3′-RACE. Primer pairs F2/R2 and F3/R2 were used for the primary and nested PCR reactions in 3′-RACE. Primer pairs F4/R1 and F5/R5 were used for the primary and nested PCR reactions in 5′-RACE. Primers F6 and R6 were designed according to the full-length cDNA isolated by RACE to amplify the coding region of rat IAP2.

| Primer | Sequence |
|---|---|
| F1 | 5′-GCCCTCTTAATTCTAGAGCAG-3′ |
| R1 | 5′-ACATCTCAAGCCACCATCACA-3′ |
| Oligo-dT | 5′GGCCACGCGTCGACTAGTAC $(dT)_{17}$-3′ |
| F2 | 5′-GAGCAGCTTGCAAGTGCTGGA-3′ |
| R2 | 5′-GGCCACGCGTCGACTAGTAC-3′ |
| F3 | 5′-GTTGTGATGGTGGCTTGAGATG-3′ |
| F4 | 5′-CCATCCTAATACGACTCACTATAGGGC-3′ |
| F5 | 5′-ACTCACTATAGGGCTCGAGCGGC-3′ |
| R5 | 5′-CTCCAGGCCCTGTGTAGTAGA-3′ |
| F6 | 5′-GAAGTAGTGAGGAGCTTCAT-3′ |
| R6 | 5′-CTGCGTAGCGTGAATCAGTA-3′ |

### Isolation of the proximal 5′-flanking sequence of rat IAP2 by genomic walking

Genomic walking is a PCR-based technique for cloning of unknown genomic DNA from a known sequence, which has been used widely to isolate promoters of genes for which only the cDNA sequence is available [39,40]. GenomeWalker kits were purchased from Clontech to isolate the proximal 5′-flanking regions of rat IAP2, following the manufacturer's instructions. Briefly, five aliquots of rat genomic DNA were digested separately by five restriction enzymes (*Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I) to generate five genomic DNA pools (DL-1–DL-5). Fragments in the DNA pools were then ligated to an adaptor sequence (5′-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT-3′). The ligated DNA pools were used as templates for two rounds of PCR (primary and nested) with gene-specific primers and adaptor primers (see Table 2 for primers used in genomic walking). For the first genomic walking, gene-specific primers were designed according to the 5′-end sequence of rat IAP2 cDNA. The DNA fragments amplified by the PCR reactions were gel-purified, cloned into pGEM-T-easy vectors and sequenced. Whether the fragments were immediately upstream of the cDNA was verified by sequence

**Table 2 Primers for genomic walking**

Gene-specific primers (GSPs) for the first genomic walking were designed on the basis of the rat caspase-9 cDNA sequence isolated by RACE reactions. Other GSPs were designed based on the sequences of DNA fragments amplified by earlier genomic walking. Adaptor primers (AP1, AP2) were designed according to the adaptor sequence ligated to the digested genomic DNA.

| Primer | Sequence |
|---|---|
| GSP-1 for first walking | 5′-CTCTAAAGTGGGTTACATCAACTGC-3′ |
| GSP-2 for first walking | 5′-ACATCTGGTCAACAGACAGTATTTTG-3′ |
| GSP-1 for second walking | 5′-GGAACAGGCTACCAACAGGCTCCT-3′ |
| GSP-2 for second walking | 5′-CTTGACTGGAGACAAAAGGCACCTG-3′ |
| GSP-1 for third walking | 5′-GTCTCACACGACTACTGGACGCAAG-3′ |
| GSP-2 for third walking | 5′-TGCAGAAAATCTTAAGTGACAGCCG-3′ |
| GSP-1 for fourth walking | 5′-TGCAGAAAATCTTAAGTGACAGCCG-3′ |
| GSP-2 for fourth walking | 5′-CAAGGACTGGGGCAGCGAGCGG-3′ |
| AP1 | 5′-GTAATACGACTCACTATAGGGC-3′ |
| AP2 | 5′-ACTATAGGGCACGCGTGGT-3′ |

overlap. Then, rat IAP2 gene-specific primers were designed according to the 5′ end sequence of the first walking product, for the second genomic walking. Four sequential rounds of genomic walking were performed to isolate the 3293 bp proximal 5′-flanking regions of rat IAP2. Advantage genomic PCR polymerase mix was purchased from Clontech for the PCR reactions.

### 5′ Sequential deletion of the 3293 bp genomic segment

With the sequence of the 3293 bp 5′-flanking regions of rat IAP2, we generated its 5′ sequential deletion mutants by PCR. Primers for these PCR reactions are indicated in Figure 3 (see below). Extra bases of *Xho*I and *Hin*dIII digestion sites were added at the 5′ ends of forward and reverse primers, respectively. Inclusion of the digestion sites would facilitate subsequent cloning of the amplified DNA fragments into pGL3-Basic vectors (Promega, Madison, WI, U.S.A.). PCR supermix of high fidelity from Life Technologies and FailSafe PCR mix from Epicentre (Madison, WI, U.S.A.) were used for these reactions to minimize PCR errors. DNA fragments amplified by these PCR reactions were digested with *Xho*I and *Hin*dIII to prepare cohesive ends. Finally, the digested fragments were cloned into *Xho*I/*Hin*dIII-linearized pGL3-Basic by standard procedures. Cloned inserts were sequenced to verify the identity of DNA sequences.

### Deletion of CREB and Sp-1 sites from 5′-flanking regions of rat IAP2

CREB and Sp-1 elements were deleted from the 1.8 kb rat IAP2 flanking sequence by PCR-based site-directed mutagenesis [41]. This was accomplished in two steps. First, upstream and downstream DNA fragments with deletions were amplified separately by PCR, using templates of pGL3 vectors harbouring 1.8 kb IAP2 flanking sequence. PCR primers were as follows with deletions shown in lower case. Amplification of the upstream fragment for CREB site deletion, forward (5′-CTA GCA AAA TAG GCT GTC CC-3′) and reverse (CREB deletion primer 1, 5′-CCA GTA AGA CGT TAA GGtgacgtgcggtgacgtC AGC GGG CGC ACG-3′); amplification of the downstream fragment for CREB site deletion, forward (CREB deletion primer 2, 5′-CGT GCG CCC GCT GacgtcaccgcacgtcaCC TTA ACG TCT TAC TGG-3′) and reverse (5′-CTT TAT GTT TTT GGC GTC TTC-3′); amplification of the upstream fragment for Sp-1 site deletion, forward (5′-CTA GCA AAA TAG GCT GTC CC-3′) and reverse (Sp-1 deletion primer 1, 5′-GCA ATG ATG GAC GGA GgggcgggAC TGT GCG CCT GCG-3′); amplification of the downstream fragment for Sp-1 site deletion, forward (Sp-1 deletion primer 2, 5′-CGC AGG CGC ACA GTcccgcccC TCC GTC CAT CAT TGC-3′) and reverse (5′-CTT TAT GTT TTT GGC GTC TTC-3′). The amplified DNA fragments were sequenced. As expected from the primer design, the 3′-end sequence of the upstream fragments overlapped with 5′ end of the downstream fragments. Moreover, deletions were introduced in the overlapping sequences through the deletion primers. In the second step, equal numbers of moles of upstream fragments and downstream fragments were mixed. The DNA mixture was used as a template to PCR-amplify the 1.8 kb genomic fragment containing CREB or Sp-1 deletions, using the primer pair, forward (5′-CTA GCA AAA TAG GCT GTC CC-3′)/reverse (5′-CTT TAT GTT TTT GGC GTC TTC-3′). The amplified DNA fragments were cloned into the reporter gene vector pGL3-Basic, and deletions verified by sequencing.

### Measurement of promoter activity for genomic DNA with reporter-gene vectors

We determined the promoter activity of isolated 5′-flanking regions of rat IAP2, using the luciferase reporter gene vector pGL3-Basic. pGL3-Basic is promoterless but contains a coding sequence for firefly luciferase. Proper insertion of sequences with promoter activity into pGL3-Basic will lead to expression of firefly luciferase. Promoter activity of the cloned sequences was indicated by the expression of luciferase in transfected cells. Lipofectamine-Plus (Life Technologies) was used to facilitate transfection according to the manufacturer's instructions. Briefly, cells were seeded at low density and transfected with pGL3-Basic harbouring 5′-flanking sequences for rat IAP2. As an internal control, pRL-TK vector was co-transfected and led to constitutive expression of *Renilla* luciferase. Then, 24 h later, the cells were lysed and the activities of firefly and *Renilla* luciferases were measured independently with the Dual-Luciferase Reporter assay system (Promega). The activity of firefly luciferase was normalized by the activity of *Renilla* luciferase to indicate promoter activity of cloned sequences.

### Electrophoretic mobility-shift assay (EMSA)

Oligonucleotide probes of 24–26 bp were designed according to the sequence of the rat IAP2 gene promoter. The probes were $^{32}$P-labelled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase. Nuclear extracts were prepared from HEK-293 cells or RPTCs by the 'mini-extraction' method of Schreiber et al. [42]. EMSA was conducted as described previously [43]. Briefly, 6 $\mu$g of nuclear extract was incubated for 20 min at room temperature with $5 \times 10^4$ c.p.m. of labelled probes in 20 ml of binding buffer containing 10 mM Tris/HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 mg/ml BSA and 2 mg of poly-d(I-C). For supershift assays, 2 $\mu$g of specific antibodies were added and the reaction continued for 30 min. Samples were resolved by electrophoresis in 5% non-denaturing polyacrylamide gels. For competition experiments, nuclear extract was pre-incubated with competitor DNA for 10 min at 4 °C before addition of the labelled probe.

### Biochemical analyses

Northern hybridization was performed by the procedure described in our previous studies [35], using NorthernMax kits from Ambion (Austin, TX, U.S.A.). The rat multiple tissue blots were purchased from Clontech. Probes for the hybridization

contained a cDNA sequence of rat IAP2 and were $^{32}$P-labelled using the Ready-To-Go random-labelling beads (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Immunoblot analysis of proteins was carried out by standard procedures.

## RESULTS AND DISCUSSION

### Isolation of the full-length cDNA of rat IAP2

The full-length cDNA of rat IAP2 was isolated by the RACE technique [37,38] (refer to the Materials and methods section for technical details). The sequence of the cDNA has been deposited in GenBank under the accession number AF190020. The longest open reading frame of rat IAP2 encodes a protein of 589 amino acids, which displays the characteristic structural features of IAPs with three BIR domains at its N-terminus and one RING finger at the C-terminus (Figure 1). At the protein level, rat IAP2 shares 89 and 85% homology with mouse and human IAP2, respectively (Figure 1). Transfection of HEK-293 cells with the isolated rat IAP2 cDNA led to IAP2 protein expression (results not shown).

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                                                                  BIR1
rIAP2   --------------------MENSTVLSNWTKENKEKMKYDFSCELYRMSTYSAFPRGV 39
mIAP2   MDKTVSQRLGQGTLHQKLKRIMEKSTILSNWTKESEEKMKFDFSCELYRMSTYSAFPRGV 60
hIAP2   MHKTASQRLFPGPSYQNIKSIMEDSTILSDWTNSNKQKMKYDFSCELYRMSTYSTFPAGV 60
                          **.**:**:**:..::***:************:** **

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rIAP2   PVSERSLARAGFYYTGVNDKVKCFCCGLMLDNWKQGDSPTEKHRQFYPSCSFVQTLLSGG 99
mIAP2   PVSERSLARAGFYYTGVNDKVKCFCCGLMLDNWKQGDSPVEKHRQFYPSCSFVQTLLSAS 120
hIAP2   PVSERSLARAGFYYTGVNDKVKCFCCGLMLDNWKLGDSPIQKHKQLYPSCSFIQNLVSAS 120
        ********************************** **** :**:*:******:*.*:*..

rIAP2   LQSAAKNTSPAKSRFAHSLP--LEQG----GIHSSLPSNPLNSRAVEDFS-LRMNPCSYA 152
mIAP2   LQSPSKNMSPVKSRFAHSSP--LERG----GIHSNLCSSPLNSRAVEDFS-SRMDPCSYA 173
hIAP2   LGSTSKNTSPMRNSFAHSLSPTLEHSSLFSGSYSSLPPNPLNSRAVEDISSSRTNPYSYA 180
        * *.:** ** :. **** .  **:.    * :*.* ..*********:*  * :* ***
                                  BIR2
        ____________________________________________________________
rIAP2   MSTEEARFLSYSMWPLSFLSPAELAKAGFYYTGPGDRVACFACGGKLSNWEPNDDPLSEH 212
mIAP2   MSTEEARFLTYSMWPLSFLSPAELARAGFYYIGPGDRVACFACGGKLSNWEPKDDAMSEH 233
hIAP2   MSTEEARFLTYHMWPLTFLSPSELARAGFYYIGPGDRVACFACGGKLSNWEPKDDAMSEH 240
        *********:* ****:****:***:***** ********************:**.:***
                                                        BIR3
        _________   ________________________________________________
rIAP2   RRHFPHCPFLENTSETQRFSVSNLSMQTHSARMSTFLYWPSSVLVQPEQLASAGFYYVDH 272
mIAP2   RRHFPHCPFLENTSETQRFSISNLSMQTHSARLRTFLYWPPSVPVQPEQLASAGFYYVDR 293
hIAP2   RRHFPNCPFLENSLETLRFSISNLSMQTHAARMRTFMYWPSSVPVQPEQLASAGFYYVGR 300
        *****:******: ** ***:********:**: **:***.** **************.:

        ____________________________________
rIAP2   NDDVKCFCCDGGLRCWEPGDDPWIEHAKWFPRCEFLIRMKGQEFVDEIQARYPHLLEQLL 332
mIAP2   NDDVKCFCCDGGLRCWEPGDDPWIEHAKWFPRCEFLIRMKGQEFVDEIQARYPHLLEQLL 353
hIAP2   NDDVKCFGCDGGLRCWESGDDPWVEHAKWFPRCEFLIRMKGQEFVDEIQGRYPHLLEQLL 360
        ******* *********.*****:*************************.**********

rIAP2   STSDTSEEENADP--PVVHLGPGENW-EDAVMMNTPVVKAALDMGFSRSLVRQTVQRQIL 389
mIAP2   STSDTPGEENADPTETVVHFGPGESS-KDVVMMSTPVVKAALEMGFSRSLVRQTVQRQIL 412
hIAP2   STSDTTGEENADP--PIIHFGPGESSSEDAVMMNTPVVKSALEMGFNRDLVKQTVLSKIL 418
        *****. ******  .::*:****.  :*.***.*****:**:***.*.**:***  :**

rIAP2   ATGENYRTVSDIVSALLNAEDERREEEKERQSEETASGDLSLIRKNRMALFQQLTCVIPI 449
mIAP2   ATGENYRTVNDIVSVLLNAEDERREEEKERQTEEMASGDLSLIRKNRMALFQQLTHVLPI 472
hIAP2   TTGENYKTVNDIVSALLNAEDEKREEEKEKQAEEMASDDLSLIRKNRMALFQQLTCVLPI 478
        :*****:**.****.*******:******:*:** **.***************** *:**

rIAP2   LDDLLEASVLTKEEHDIIRQKTQIPLQARELIDTILVKGNAAASVFKNSLKEVDSTLYEH 509
mIAP2   LDNLLEASVITKQEHDIIRQKTQIPLQARELIDTVLVKGNAAANIFKNSLKEIDSTLYEN 532
hIAP2   LDNLLKANVINKQEHDIIKQKTQIPLQARELIDTIWVKGNAAANIFKNCLKEIDSTLYKN 538
        **:**:*.*:.*:*****:***************: *******.:***.***:*****::
                                                RING finger
        ____________________________________________________________
rIAP2   LFVEKTMKYIPTEDVSGLSLEEQLRRLQEERTCKVCMDREVSIVFIPCGHLVVCRECAPS 569
mIAP2   LFVEKNMKYIPTEDVSGLSLEEQLRRLQEERTCKVCMDREVSIVFIPCGHLVVCQECAPS 592
hIAP2   LFVDKNMKYIPTEDVSGLSLEEQLRRLQEERTCKVCMDKEVSVVFIPCGHLVVCQECAPS 598
        ***:*.*********************************:***:***********:*****

        _________
rIAP2   LRKCPICRGTIKGTVRTFLS 589
mIAP2   LRKCPICRGTIKGTVRTFLS 612
hIAP2   LRKCPICRGIIKGTVRTFLS 618
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**Figure 1 Amino acid sequences of rat, mouse and human IAP2s**

The deduced amino acid sequence of rat (r) IAP2 was aligned with the sequences of mouse (m) and human (h) IAP2 using CLUSTAL W software [46]. Conservations are indicated below the sequence: * for fully conserved residues; : for strong conservations; . for weak conservations. N-terminal BIR domains and the C-terminal RING finger motif are also indicated. Significant homology is shown between rat, mouse and human IAP2s (GenBank accession numbers AF190020, U88909 and U45879).

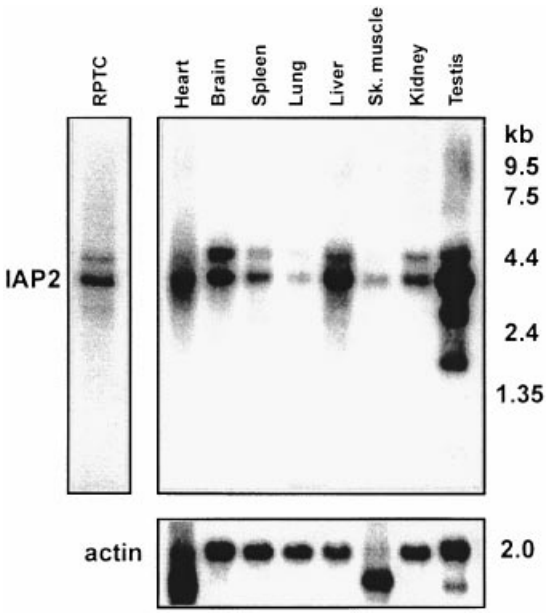


**Figure 2 Rat IAP2 expression *in vivo* shown by Northern hybridization**

Probes for Northern hybridization were prepared by $^{32}$P-labelling of a cDNA fragment within the rat IAP2 coding region using Ready-To-Go DNA-labelling beads (Amersham Pharmacia Biotech). Rat multiple tissue blots from Clontech were hybridized with the cDNA probe at high stringency. The blots were subsequently stripped and reprobed with a $\beta$-actin cDNA probe to assess sample loading. Note that actin hybridization detected $\approx$ 1.6 kb transcripts in heart and skeletal muscles. According to Clontech, manufacturer of the blot, this was not due to RNA degradation but to probe hybridization to other isoforms of actin. To examine IAP2 expression in RPTCs, mRNA was isolated, resolved by denaturing agarose-gel electrophoresis (2 $\mu$g/lane) and transferred to nylon membranes. The blots were subsequently hybridized with the rat IAP2 cDNA probe, showing a major transcript of $\approx$ 3.5 kb.

### Expression of rat IAP2 *in vivo*

By Northern hybridization, we detected a major rat IAP2 transcript of $\approx$ 3.5 kb in RPTCs (Figure 2). The size of this transcript is comparable with the full-length cDNA (3290 bp) that we have isolated. To examine IAP2 expression *in vivo*, we performed Northern hybridizations of rat multiple tissue blots. The hybridization probe was prepared by $^{32}$P-labelling of a cDNA fragment within the rat IAP2 coding region. As shown in Figure 2, the 3.5 kb IAP2 transcript was detected in all rat tissues examined, with the most in testis and abundant expression in brain and liver, moderate expression in heart, spleen and kidney, and the least in lung and skeletal muscle. In addition, a larger transcript of $\approx$ 5 kb was also shown in all tissues except the heart. Of interest, two smaller transcripts were also expressed abundantly in rat testis (Figure 2). Whether these transcripts are alternatively spliced forms of rat IAP2 remains to be determined.

### Isolation of the proximal 5′-flanking sequence of rat IAP2

With the sequence of rat IAP2 full-length cDNA, we subsequently isolated 5′-flanking genomic regions of this gene. This was accomplished by the procedure of genomic walking [39,40] (see the Materials and methods section for technical details). Four rounds of genomic walking led to the isolation of a 3293 bp proximal 5′-flanking region of rat IAP2. The sequence of this region has been submitted to GenBank under the accession number AY027668 (Figure 3, top panel). Analysis of the genomic sequence by the database SIGNAL SCAN [44] suggests the presence of the key transcriptional element TFIID within 26 bp from the transcription-initiation site (Figure 3, bottom panel). TATA boxes were also identified, although they are 600–700 bp away. In addition, potential binding sites for numerous known transcription factors, such as nuclear factor-1, glucocorticoid receptor, Pit-1, c-Myc and CREB, are present. Of interest, four core binding sites for the hypoxia-responsive transcription factor HIF-1 [45] were also found at positions $-1055$, $-1195$, $-1727$ and $-2236$ (Figure 3, bottom panel). Our recent studies have demonstrated a striking induction of IAP2 by severe hypoxia; however, this induction can be dissociated from HIF-1 [35]. When HIF-1 was activated pharmacologically or by 2% oxygen, IAP2 was not induced. On the other hand, IAP2 could be induced strongly by severe hypoxia in HIF-1-deficient cells. Thus, the HIF-1-binding elements present in the 5′-flanking regions of IAP2 could be silent under those conditions.

### Promoter activity of rat IAP2 5′-flanking sequence

To examine whether the 5′-flanking regions isolated by genomic walking contain the gene promoter of rat IAP2, we determined the promoter efficiency of the sequences using the luciferase reporter gene vector, pGL3-Basic. We initially dissected the IAP2-flanking region by PCR-based sequential 5′ deletions. Primers for the PCR reactions were designed according to the 3.3 kb genomic segment (Figure 3, top panel, underlined sequences). The PCR reactions amplified genomic DNA fragments immediately upstream of IAP2 cDNA of lengths 0.13, 0.29, 0.45, 0.85, 1.6, 2.0 and 3.1 kb. These fragments were subsequently cloned into pGL3-Basic for transfection (Figure 4A). Firefly luciferase activity in transfected cells was monitored to indicate promoter efficiency of the cloned genomic sequences, and the results are shown in Figure 4.Transfection of HEK-293 and 3T3 cells with pGL3-Basic harbouring the 0.13 kb genomic segment led to 13- and 38-fold increases in promoter activity, respectively (Figures 4B and 4C). Promoter efficiency decreased thereafter, up to 1.6 kb. To our surprise, when the sequence was extended from 1.6 to 2.0 kb, a remarkable increase in promoter activity was detected. In HEK-293 cells, the activity was 140-fold over control, and in 3T3 cells, 270-fold (Figures 4B and 4C). Further extension of the sequence to 3.1 kb led to decreases in promoter efficiency (Figure 4). Together, these results demonstrate significant promoter activity for the IAP2 5′-flanking regions isolated by genomic walking.

### Identification of an enhancer sequence in rat IAP2 promoter region

Our results have shown an abrupt increase in promoter activity between 1.6 and 2.0 kb within the rat IAP2 promoter region (Figure 4). To identify further the enhancer elements, we dissected the sequence by $\approx$ 100 bp using PCR-based methodology. The amplified DNA fragments were cloned into pGL3-Basic for transfection to determine their promoter activity. The results are shown in Figure 5. Significant increases in promoter activity were detected when the sequence was extended from 1.6 to 1.8 kb; further extensions to 1.9 or 2.0 kb resulted in reductions. These analyses indicate that the enhancer elements are localized within the 200 bp between 1.6 and 1.8 kb.

### Binding of CREB and Sp-1 to the enhancer sequence

The data presented in the above section have narrowed down the enhancer sequence to $\approx$ 200 bp (Figure 5). We subsequently analysed this 200 bp sequence using databases, and identified a


**Figure 3 Sequence of the 5′-flanking region of rat IAP2**

Top panel: sequence of the 3293 bp 5′-flanking region of rat IAP2 isolated by genomic walking. Numbers on the left indicate the distance from the transcription start site (+1). Underlined sequences were used to design PCR primers to generate sequential 5′ deletion mutants (0.13–3.1 kb) of this fragment. These 5′ deletion mutants were cloned into the reporter-gene vector pGL3-Basic for transfection to determine their promoter activities (see Figures 4 and 5). Bases in parentheses indicate sequences in question. For example, at base −1158 (C/T), sequencing results showed signals of both C and T. Bottom panel: organization of the 5′-flanking region of rat IAP2. Sequence of the 3293 bp was analysed by the database software SIGNAL SCAN. Binding sites for known transcription factors are listed; numbers indicate locations of these sites shown in terms of distance from the transcription start site. Boxed are elements that may be critical for the basal promoter activity of the sequence.

perfect binding site for Sp-1 and two binding sites for the CREB/activating transcription factor (ATF) family of transcription factors (Figure 6). To examine the roles played by these elements in the enhancer, we initially examined the binding of CREB, ATF and Sp-1 to the enhancer sequence by EMSA. In these experiments, nuclear extracts of HEK-293 cells were incubated with $^{32}$P-labelled probes of the enhancer sequence. The results are shown in Figure 7. Three major DNA–protein complexes were formed during the incubation of nuclear extracts with $^{32}$P-labelled oligonucleotide probe encompassing the CREB site (Figure 7, lane 2). The complexes appear to be specific, since their formation was prevented in the presence of excess unlabelled probe (Figure 7, lane 4). Unlabelled oligonucleotides with mutations in the CREB site were much less effective in preventing the complex formation (Figure 7, lane 5). Of significance, the complexes were supershifted by a CREB antibody (Figure 7, lane 3), further indicating the specific binding of CREB to the enhancer sequence. On the other hand, no obvious DNA–protein complexes were detected during the incubation of nuclear extract with $^{32}$P-labelled oligonucleotide probe encompassing the ATF site (Figure 7, lanes 6–8). For Sp-1, multiple complexes were formed between $^{32}$P-labelled oligonucleotide probe and the nuclear proteins (Figure 7, lane 9). Formation of these complexes was suppressed by excess amounts of unlabelled probe (Figure 7, lane 10), and, to a lesser extent, by a consensus Sp-1-binding sequence (Figure 7, lane 11). Addition of Sp-1 antibody to the incubation

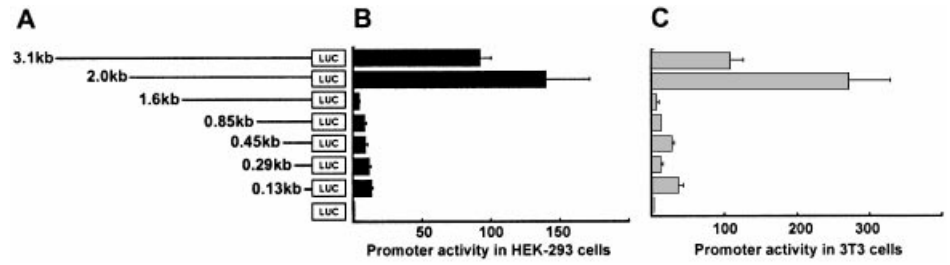


**Figure 4 Promoter activity of the 5′-flanking genomic sequence of rat IAP2**

(**A**) 5′ Sequential deletion constructs cloned into pGL3-Basic. Seven 5′ sequential deletion constructs (0.13–3.1 kb) were generated by PCR. Primers for the PCR reactions were designed according to the 3293 bp genomic sequence isolated by genomic walking, and have been underlined in Figure 3 (top panel). The deletion constructs were cloned into pGL3-Basic. (**B**, **C**) Promoter activity of rat IAP2-flanking regions in transfected HEK-293 (**B**) and 3T3 (**C**) cells. Cells were transfected with pGL3-Basic empty vector, or the vector containing 0.13–3.1 kb IAP2-flanking regions. Promoter activity was indicated by firefly luciferase expression in transfected cells (see the Materials and methods section for details). The values obtained from control transfection with empty pGL3-Basic were arbitrarily set at 1. The experiments were repeated three times with duplicates for each condition. Data points are means ± S.D. The results demonstrate significant promoter activity for the 5′-flanking regions of rat IAP2 that were isolated by genomic walking.

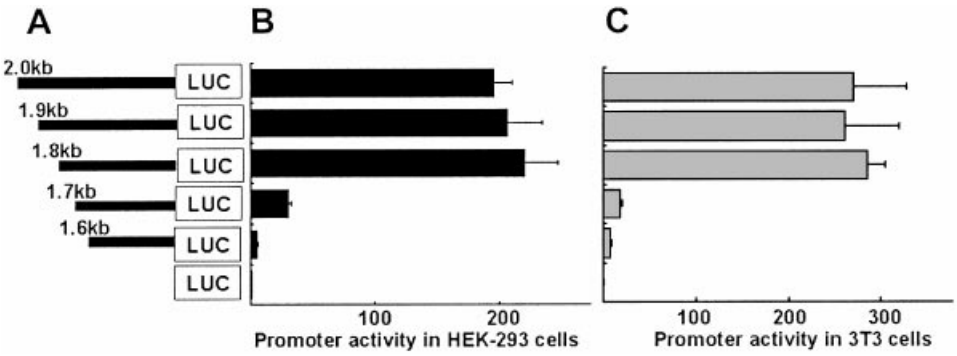


**Figure 5 Promoter activity of 1.6–2.0 kb 5′-flanking regions of rat IAP2**

The genomic fragments were PCR-amplified using primers indicated in Figure 3 (top panel), and cloned into pGL3-Basic (**A**). Empty pGL3-Basic or pGL3-Basic containing the genomic fragments were transfected into HEK-293 (**B**) or 3T3 (**C**) cells. Expression of firefly luciferase was analysed to indicate promoter activity of transfected fragments. The values obtained from control transfection with empty pGL3-Basic were arbitrarily set at 1. The experiments were repeated three times with duplicates for each condition. Data points are means ± S.D. The results show significant increases in promoter activity during the sequence extension from 1.6 to 1.8 kb, indicating the presence of enhancer elements within the 200 bp.

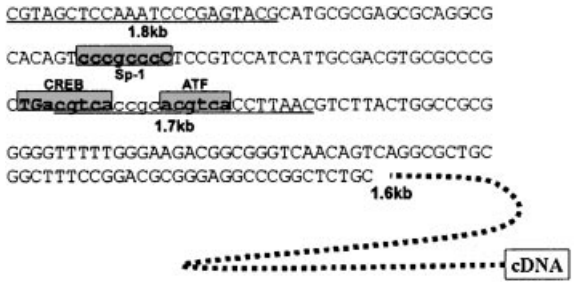


**Figure 6 Analysis of the 200 bp enhancer sequence**

The sequence was analysed for potential transcription-factor binding sites, using the database SIGNAL SCAN. Potential CREB-, ATF- and Sp-1-binding sites are shaded. Sequences written in lower-case letters were deleted to test their involvement in the enhancer activity (Figure 8). Underlined sequences were used to design PCR primers to generate 1.7 and 1.8 kb genomic fragments.

led to the supershift of a specific complex of high molecular mass (Figure 7, lane 12). The EMSA results suggest that CREB and Sp-1 can bind to the enhancer sequence and may regulate the promoter activity of IAP2.

### CREB-binding site as a critical element for the enhancer sequence

To identify further the key enhancer elements, we introduced specific deletions at the CREB/ATF and Sp-1 sites in the 1.8 kb fragment. The intact fragment and the mutants were then transfected into HEK-293 cells to measure their promoter activities. As shown in Figure 8, deletion of CREB/ATF sites led to marked decreases in promoter activity of the 1.8 kb fragment, whereas Sp-1 deletion had little or no effect. Similar results were obtained for transfection of 3T3 cells (results not shown). These experiments, along with the results showing CREB binding to the enhancer sequence (Figure 7), suggest a critical role for the CREB site in the enhancer activity.

### Activation of IAP2 gene promoter by severe hypoxia and not by $CoCl_2$ or desferrioxamine

Expression of IAP2 is induced by severe hypoxia or anoxia in a remarkable manner [35]. To a large extent, this induction depends on the activation of gene transcription. Further investigations have dissociated IAP2 induction from the ubiquitous hypoxia-responsive transcription factor HIF-1 [35]. These results suggest a novel mechanism for hypoxic regulation of the IAP2 gene,

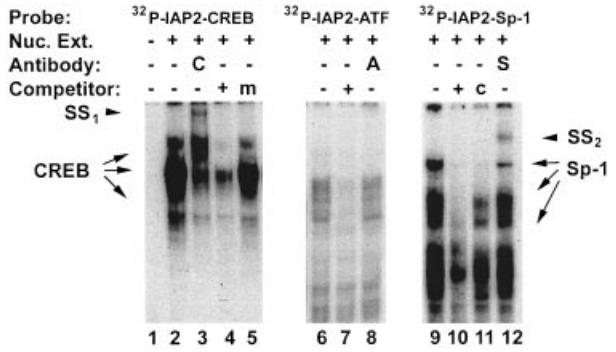


**Figure 7 EMSA analysis of CREB-, ATF- and Sp-1-binding to the enhancer sequence**

Nuclear extracts of HEK-293 cells were incubated with $^{32}$P-labelled oligonucleotide probes encompassing the CREB, ATF or Sp-1 site of the enhancer sequence. DNA sequences of the probe were $^{32}$P-IAP2-CREB (gtgcgcccgctgacgtcaccgcacg), $^{32}$P-IAP2-ATF (gtcaccgcacgtcaccttaacgtc) and $^{32}$P-IAP2-Sp-1 (cgcacagtcccgcccctccgtccatc). For supershift, antibodies to CREB (C; lane 3), ATF (A; lane 8) or Sp-1 (S; lane 12) were added during nuclear-extract incubation. For competition, excess amounts (100 ×) of unlabelled oligonucleotides were added along with the $^{32}$P-labelled probes (lanes 4, 7 and 10). An unlabelled oligonucleotide with a mutated CREB site (lane 5; m; DNA sequence, gtgcgcccgctgtggtcaccgcacg) and a consensus Sp-1-binding sequence (lane 11; c; DNA sequence, attcgatcggggcggggcgag) were also tested for competition with the $^{32}$P-labelled probes. EMSA was conducted as described in the Materials and methods section. Major DNA–protein complexes are indicated by arrows. Supershift complexes are indicated by arrowheads with $SS_1$ for CREB supershift and $SS_2$ for Sp-1 supershift. Apparent DNA–protein-complex formation was shown for the probes containing CREB or Sp-1 sites (lanes 2 and 9). Addition of CREB and Sp-1 antibodies led to supershift of the complexes (lanes 3 and 12).

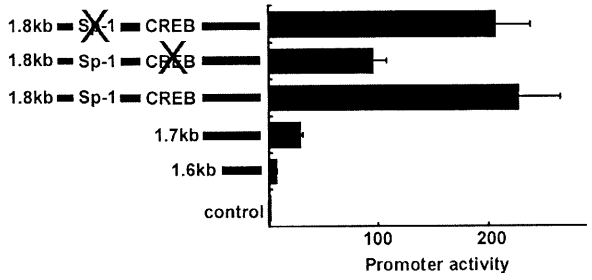


**Figure 8 Loss of enhancer activity after CREB-site deletion**

Deletions of CREB/ATF- or Sp-1-binding sites were introduced by PCR-based site-directed mutagenesis. The deleted sequences are shown in Figure 6 in lower-case letters. The deletions are indicated in this Figure by crosses. The intact fragments and deletion mutants were cloned into pGL3-Basic for transfection into HEK-293 cells. Expression of firefly luciferase in transfected cells was analysed to indicate promoter activity of the genomic DNA fragments. The values obtained from control transfection with empty pGL3-Basic were arbitrarily set at 1. The experiments were repeated three times with duplicates for each condition. Data points indicate means ± S.D. The results show that deletion of CREB/ATF- but not Sp-1-binding sites led to significant decreases in promoter activity of the 1.8 kb fragment, suggesting a role for the CREB/ATF elements in the enhancer activity of the sequence.

probably at the promoter level. Therefore, our subsequent studies examined whether the isolated IAP2 promoter contains functional *cis*-acting elements that are responsive to severe hypoxia. pGL3 vectors harbouring promoter segments of various lengths were transfected into rat adrenal phaeochromocytoma PC12 cells. The experimental groups of cells were subjected to 3 h of hypoxic incubation, whereas the control cells were incubated under normal oxygen. Luciferase expression was determined at the end of incubation to indicate promoter efficiency. Results from a

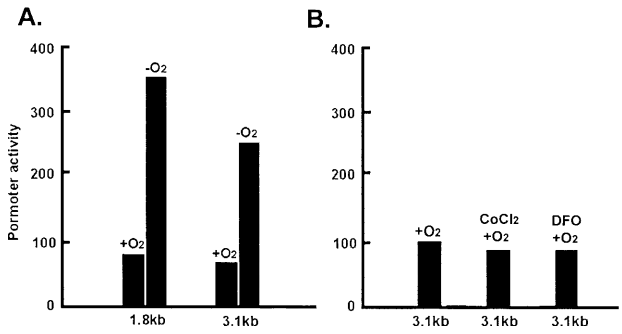


**Figure 9 Activation of IAP2 gene promoter by severe hypoxia and not by $CoCl_2$ or desferrioxamine**

(**A**) IAP2 gene promoter is activated by severe hypoxia. PC12 cells were transfected with pGL3-Basic vectors harbouring 1.8 or 3.1 kb rat IAP2 promoter sequences. One group of transfected cells was subjected to 3 h of hypoxic incubation ($-O_2$), while the other group of cells was incubated under normal oxygen as the control ($+O_2$). (**B**) IAP2 gene promoter is not activated by $CoCl_2$ or desferrioxamine. Cells were transfected with pGL3-Basic vectors harbouring 3.1 kb rat IAP2 promoter sequence. Transfected cells were incubated for 6 h under normal oxygen without ($+O_2$) or with 100 $\mu$M $CoCl_2$ ($CoCl_2+O_2$) or desferrioxamine (DFO + $O_2$). After incubation, cells were lysed and firefly luciferase activity in the lysates was measured to indicate promoter activity, as described in the Materials and methods section. Significantly higher promoter activity was shown for the genomic fragments after hypoxic incubation, indicating activation of the IAP2 gene promoter by severe hypoxia. On the other hand, the gene promoter was not activated by $CoCl_2$ or desferrioxamine, negating a role for HIF-1 in IAP2 gene regulation.

typical experiment are shown in Figure 9(A). Promoter activity within hypoxic cells was 4.6- and 3.2-fold above the control level for the 1.8 and 3.1 kb segments respectively, indicating significant activation of the IAP2 gene promoter by severe hypoxia. In sharp contrast, pharmacological inducers of HIF-1, including $CoCl_2$ and desferrioxamine, activated HIF-1 [35,45], but not the gene promoter of IAP2 (Figure 9B). These results are in support of our previous observations of hypoxic induction of IAP2 [35], and further negate the role of HIF-1 in this inductive response.

## Conclusions

The current study has cloned the full-length cDNA of rat IAP2 from kidney proximal tubular cells. With significant homology to mouse and human, rat IAP2 is characterized by its structure, including typical BIR domains and a RING motif. Expression of rat IAP2 *in vivo* varies among different tissues, with a major transcript of ≈ 3.5 kb. With the cDNA sequence, we have subsequently isolated 3293 bp of the proximal 5′-flanking region of the IAP2 gene. Analysis by reporter-gene vectors has demonstrated significant promoter activity for the genomic sequence. This is also the first promoter region isolated for this gene. The study has further identified an enhancer sequence of ≈ 200 bp in the gene promoter, which might have a significant role in IAP2 regulation. The CREB-binding element within this sequence appears to be critical for the enhancer activity. Of significance, the rat IAP2 gene promoter can be activated by severe hypoxia or anoxia, conditions shown to stimulate IAP2 expression. The findings indicate the presence of critical *cis*-acting elements in the gene promoter, which are responsible for IAP2 regulation under pathophysiological situations, including hypoxia. Certainly, identification of such elements would be the focus of further investigations.

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