cDNA cloning and promoter analysis of rat caspase-9

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Caspase-9 is the apex caspase of the mitochondrial pathway of apoptosis, which plays a critical role in apoptotic initiation and progression. However, gene regulation of caspase-9 is largely unknown. This is in part due to the lack of information on the gene promoter. Here we have cloned the full-length cDNA of rat caspase-9 and have isolated promoter regions of this gene. The rat caspase-9 cDNA of 2058 bp predicts a protein of 454 amino acids, which contains a caspase-recruitment domain ('CARD') at the N-terminus and enzymic domains at the C-terminus. The enzyme's active site, with a characteristic motif of QACGG, was also identified. Overall, rat and human caspase-9 have 71 % identity. With the cDNA sequence, we subsequently isolated the proximal 5'-flanking regions of rat caspase-9 by the procedure of genomic walking. The 2270 bp genomic segment is 'TATA-less',

but contains several GC boxes. Elements binding known transcription factors such as Sp-1, Pit-1, CCAAT-enhancer-binding protein (C/EBP), glucocorticoid receptor and hypoxia-inducible factor 1 (HIF-1) were also identified. When cloned into reporter gene vectors, the genomic segment showed significant promoter activity, indicating that the 5'-flanking regions isolated by genomic walking contain the gene promoter of rat caspase-9. Of significance is that the cloned promoter segments were activated by severe hypoxia, conditions inducing caspase-9 transcription. Thus, the genomic sequences reported here contain not only the basal promoter of rat caspase-9 but also regulatory elements responsive to pathophysiological stimuli including hypoxia.

Key words: apoptosis, gene regulation, hypoxia.

INTRODUCTION

Caspases are a family of unique cysteine proteases, which cleave their substrates after aspartate residues and play a fundamental role in apoptosis [1–5]. To date, over a dozen caspases have been discovered [6,7]. Depending on their roles in apoptosis, caspases can be classified roughly into subgroups of apoptotic initiators and executioners [1–5]. Caspase-9 is the initiator caspase in the mitochondrial pathway of apoptosis [8]. In this pathway, mitochondrial cytochrome *c* is released into the cytosol under cellular stress, where it binds to the adaptor protein apoptotic protease-activating factor 1 (Apaf-1). The association of cytochrome *c* and Apaf-1 leads to the recruitment and activation of caspase-9. Upon activation, caspase-9 cleaves executioner caspases including caspase-3 at specific sites, leading to the activation of the downstream caspases and the ultimate disassembly of the committed cell [9]. Recent studies have further identified the amplification of death-receptor-mediated apoptosis by the mitochondrial pathway [10,11]. Under certain situations, ligation of death receptors leads to the activation of caspase-8, which in turn cleaves and activates BID, a pro-apoptotic Bcl-2-family protein [12]. Truncated BID targets mitochondria to release cytochrome *c*, resulting in the activation of caspase-9 and subsequent activation of executioner caspases. Thus, caspase-9 is not only critical for the mitochondrial pathway of apoptosis but may have a significant role in apoptosis triggered by death-receptor ligation in some systems.

Caspase regulation by post-translational mechanisms has been well-recognized. Proteolytic cleavage at specific sites appears to

be a common mechanism for the activation of several caspases [1–5]. Caspase translocation within different intracellular compartments has been shown under certain situations [13–15]. In addition, regulation of caspase-9 by serine phosphorylation has been reported [16]. On the other hand, compelling evidence garnered recently indicates regulation of caspases at the level of gene expression as well [17]. Up-regulation of caspase expression was shown during organ ischaemia, including in the brain and kidneys [18,19]. Caspase-3 neosynthesis is activated by fatal axotomy [20]. In acute pancreatitis, caspase expression decreases, which might be responsible for delayed neutrophil apoptosis [21]. Moreover, cellular expression of caspase-3 may play a central role in follicular atresia and luteal regression [22]. Finally, our recent studies have revealed a striking induction of caspase-9 by hypoxia in rat kidney proximal tubular cells (RPTCs) [23].

Despite these observations, investigation of gene regulation of caspases has been hindered, mainly due to the lack of information on the gene promoters. In the current study, we have cloned the full-length cDNA of caspase-9 from the RPTCs. 5'-Flanking regions of this gene have also been isolated. Analysis of the genomic segments using reporter-gene vectors demonstrated significant promoter activity. Of interest is that the promoter sequences were shown to be activated by hypoxia, conditions inducing caspase-9 expression. These results point to the presence of critical regulatory elements of the gene promoter that are responsive to pathophysiological stimuli. Identification of promoter sequences of caspase-9 would provide the basis for an investigation of the gene regulation of this important apoptotic molecule.

Abbreviations used: CARD, caspase-recruitment domain; HIF-1, hypoxia-inducible factor 1; RACE, rapid amplification of cDNA ends; RPTC, rat kidney proximal tubular cell; C/EBP, CCAAT-enhancer-binding protein; Apaf-1, apoptotic protease-activating factor 1; RT-PCR, reverse transcriptase PCR; NF-1, nuclear factor-1.

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The nucleotide sequences for rat caspase-9 cDNA and promoter regions have been submitted to the GenBank Nucleotide Sequence Database under the accession numbers AY027667 and AY027666.

MATERIALS AND METHODS

Cell culture

RPTCs were provided by Dr U. Hopfer (Case Western Reserve University, Cleveland, OH, U.S.A.). PC12, NIH-3T3 and HEK-293 cells were obtained from the A.T.C.C. Cell culture media were as follows: RPTCs, Ham's F-12/Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; PC12 cells, RPMI 1640 supplemented with 10% horse serum and 5% fetal bovine serum; NIH-3T3 cells, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; HEK-293 cells, minimal essential medium supplemented with 10% horse serum. The media were purchased from Life Technologies (Rockville, MD, U.S.A.). PC12, NIH-3T3 and HEK-293 cells were maintained by standard methods, and RPTCs by procedures described previously [23–25].

Isolation of rat caspase-9 cDNA by rapid amplification of cDNA ends (RACE)

RACE is a PCR-based technique for isolation of full-length cDNA of specific genes [26]. Total RNA was isolated from RPTCs with TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). Using this RNA preparation as a template, a reverse transcriptase PCR (RT-PCR), was first performed to amplify a cDNA fragment of rat caspase-9. Primers for this RT-PCR were designed based on the reported sequence of human caspase-9 cDNA (GenBank accession number U60521; Table 1). With the sequence of this RT-PCR product, rat caspase-9 gene-specific primers were designed for RACE reactions (Table 1). RACE was carried out using the Marathon cDNA amplification kit from Clontech (Palo Alto, CA, U.S.A.), following the manufacturer's instructions. Briefly, poly(A) RNA was purified from total RNA and reverse-transcribed with an oligo(dT) adaptor primer to generate the first-strand DNA. Then, second-strand DNA was synthesized using a cocktail of *Escherichia coli* DNA polymerase I, RNase H and DNA ligase. Adaptor sequences were subsequently ligated to both ends of the prepared double-strand cDNA. The ligated DNA was used as the template for two rounds of PCR (primary and nested) with gene-specific primers and adaptor primers. The PCR reactions led to the amplification of cDNA fragments covering regions to the 5' and 3' ends.

Table 1 Primers used for RT-PCR and RACE reactions

Primers for RT-PCR were designed on the basis of human caspase-9 sequence (GenBank accession number U60521). Based on the sequence of the rat caspase-9 cDNA fragment amplified by RT-PCR, gene-specific primers (GSPs) for RACE were designed. Adaptor primers (APs) were designed according to the adaptor sequence ligated to the ends of double-strand DNA in RACE reactions.

Primer	Sequence
Forward primer for RT-PCR	5'-TGTGGTGGTCATCCTCTCTCA-3'
Reverse primer for RT-PCR	5'-GTCACTGGGGTAGGCAACT-3'
Oligo(dT) adaptor primer for synthesis of first-strand cDNA	5'-TTCTAGAATTCAGCGGCCGC(dT) ₃₀ NN-3'
GSP-1 for 5'-RACE	5'-GGGCCTTCCTGGTATGGGACAGCAT-3'
GSP-2 for 5'-RACE	5'-CCTGGATGAAGAACAGCTTGGGCTTCC-3'
GSP-1 for 3'-RACE	5'-CCTCCAGTTCCTGGGTGCTGTCTA-3'
GSP-2 for 3'-RACE	5'-GGAGGGAAGCCCAAGCTGTTCTTCATC-3'
AP-1	5'-CCATCCTAATACGACTCACTATAGGGC-3'
AP-2	5'-ACTCACTATAGGGCTCGAGCGGC-3'

Table 2 Primers used 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 gene-specific primers were designed based on the sequences of DNA fragments amplified by earlier genomic walking. Adaptor primers (APs) were designed according to the adaptor sequence ligated to the digested genomic DNA.

Primer	Sequence
GSP-1 for 1st and 2nd walking	5'-GCACAAGGCGTACCCTGCATCG-3'
GSP-2 for 1st walking	5'-CATGGCTAGCGGATTGCTGGAC-3'
GSP-2 for 2nd walking	5'-TCCACCTCCGACGCCACCTCAG-3'
GSP-1 for 3rd walking	5'-CAAGGCTGGAGAGATGGTTCACTGG-3'
GSP-2 for 3rd walking	5'-CCCTCTTTTGGTGTGCATGAAGATT-3'
GSP-1 for 4th walking	5'-CTCAGCTTGACCCCAACAGAGG-3'
GSP-2 for 4th walking	5'-GAGGCATCTGAGATTCAACCCGAAC-3'
GSP-1 for 5th walking	5'-CAAGTCAAGGAGATTCGCCATGTTG-3'
GSP-2 for 5th walking	5'-GTTGGCTAAACTGGCTGGTTTGG-3'
AP-1	5'-GTAATACGACTCACTATAGGGC-3'
AP-2	5'-ACTATAGGGCAGCGGTGGT-3'

Isolation of 5'-flanking regions of rat caspase-9 by genomic walking

Genomic walking is a PCR-based technique for rapid cloning of unknown genomic DNA from a known sequence [27]. It has been widely used to isolate promoters of genes for which only cDNA sequence is available [28–30]. A GenomeWalker kit from Clontech was used to isolate the proximal 5'-flanking regions of rat caspase-9, following the manufacturer's instructions. Briefly, five aliquots of rat genomic DNA were digested separately by five restriction enzymes (*EcoRV*, *ScaI*, *DraI*, *PvuII* and *SspI*) to produce five rat genomic DNA pools (DL-1, DL-2, DL-3, DL-4 and DL-5). Then, each pool of DNA fragments was ligated to an adaptor sequence. The ligated DNA pools were used as templates for two rounds of PCR (primary and nested) with gene-specific primers and adaptor primers. For the first genomic walking, gene-specific primers were designed based on the 5'-end sequence of rat caspase-9 cDNA that had been isolated by RACE reactions (Table 2). Whether the DNA fragments amplified by genomic walking were upstream sequences of the cDNA was determined by sequence overlap. Five sequential genomic walkings were performed to isolate the 2270 bp proximal 5'-flanking regions of rat caspase-9. Advantage genomic PCR polymerase mix and advantage-GC genomic PCR polymerase mix from Clontech were used for these reactions.

Analysis of promoter activity of the genomic sequence of rat caspase-9

We determined the promoter activity of isolated 5'-flanking regions of rat caspase-9, using the luciferase reporter-gene vector pGL3-Basic (Promega, Madison, WI, U.S.A.). pGL3-Basic is a promoterless vector but contains the coding sequence for firefly luciferase. Proper insertion of sequences with promoter activity into pGL3-Basic will lead to expression of firefly luciferase. With the sequence information of the 2270 bp 5'-flanking regions of caspase-9, we first generated 5' sequential deletion mutants of this segment by PCR, using genomic DNA isolated from RPTCs as templates. Primers used for these PCRs are indicated in Figure 4 (top panel; see below). These primers also contained extra bases including *XhoI*, *HindIII* or *MluI* restriction enzyme sites at their 5' ends to facilitate subsequent cloning of the amplified DNA fragments into pGL3-Basic vectors. PCR Supermix of high fidelity from Life Technologies was used for these reactions to minimize PCR errors. DNA fragments amplified by these

PCRs were digested by appropriate restriction enzymes to prepare cohesive ends: *Mlu*I and *Xho*I for 2.2 kb, *Xho*I and *Hind*III for other fragments. Finally, the digested fragments were cloned into pGL3-Basic by standard procedures. Cloned inserts were sequenced to verify the identity of DNA sequences, prior to transfection into mammalian cells to determine their promoter activity. Promoter activity of the cloned sequences was determined 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 various lengths of caspase-9 genomic DNA. As an internal control, the pRL-TK vector was co-transfected and led to constitutive expression of *Renilla* luciferase. The cells were lysed 24 h later and 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.

Hypoxic incubation

Cells were exposed to hypoxia as described in our previous studies [23–25]. Briefly, cells were washed with PBS and transferred to an anaerobic chamber with 95% N₂/5% CO₂. Incubation medium was then changed to Krebs–Ringer bicarbonate buffer that had been pre-equilibrated with 95% N₂/5% CO₂. EC Oxyrase, a biocatalytic oxygen-reducing agent, was added at 1.2 units/ml to the incubation medium to consume residual O₂ and maximize the degree of hypoxia.

Northern hybridization

mRNA were measured by Northern hybridization, using NorthernMax kits (Ambion, Austin, TX, U.S.A.) [25]. Total RNA was extracted from the cells with TRI reagent (Molecular Research Center), and mRNA purified with polyAtract (Promega). mRNAs (1 µg/lane) were resolved by electrophoresis on formaldehyde-containing agarose gels, and transferred to BrightStar-Plus positively charged nylon membranes. After UV cross-linking, the membranes were hybridized with a caspase-9 gene-specific probe. The probe containing a cDNA sequence of rat caspase-9 was ³²P-labelled using the Ready-To-Go random labelling beads (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). To assess variations in loading and transfer of RNA, the blots were stripped and re-probed with ³²P-labelled GAPDH cDNA. Levels of gene-specific mRNA were revealed by autoradiography.

RESULTS AND DISCUSSION

Cloning of the full-length cDNA of rat caspase-9

At first, we isolated a cDNA fragment of rat caspase-9 from RPTCs by RT-PCR. Primers for this reaction were designed based on the cDNA sequence of human caspase-9 [31] (GenBank accession number U60521). The amplified DNA fragment of 325 bp (Figure 1, lane 1) was gel-purified and sequenced, showing significant homology with its human counterpart. With sequence information for this fragment, the gene-specific primers for RACE were designed (Table 1). 5'- and 3'-RACE reactions led to the amplification of two DNA fragments of ≈ 1.0 and ≈ 1.1 kb, respectively (Figure 1, lanes 2 and 3). The fragments were gel-purified, sequenced and aligned to obtain the 2058 bp full-length cDNA of rat caspase-9. Overall, the rat cDNA shares 83% identity with human caspase-9. It consists of 25 bp of the 5'-untranslated region, 1365 bp of the coding region and 668 bp of

Figure 1 Isolation of caspase-9 cDNA from RPTCs by RT-PCR and RACE

A cDNA fragment of 325 bp was first RT-PCR amplified from total RNA extracted from RPTCs (lane 1). Primers for the RT-PCR were designed based on the cDNA sequence of human caspase-9. With the sequence of the 325 bp cDNA, rat caspase-9 gene-specific primers were designed for 5'- and 3'-RACE reactions. The reactions amplified major products of ≈ 1.0 and ≈ 1.1 kb in 5'- and 3'-RACE, respectively (lane 2, arrow; lane 3, arrowhead). The cDNA fragments were gel-purified, sequenced and aligned to obtain the full-length cDNA sequence of rat caspase-9. Further primers were designed with this sequence to RT-PCR-amplify the coding region of caspase-9 from RPTCs (lane 4).

the 3'-untranslated region. The longest open reading frame of this cDNA predicts a protein of 454 amino acids. Shown in lane 4 of Figure 1 is an RT-PCR-amplified DNA fragment covering the coding region. Compared with human caspase-9, the rat protein is 38 amino acids larger. Nevertheless, caspase-9 from these two species shares 71% identity. In addition, the rat caspase-9 shows high homology, 90% identity, with mouse caspase-9 [32] (GenBank accession number AB019600). As in human, the rat caspase-9 contains a caspase-recruitment domain (CARD) at the N-terminus, which is expected to mediate its interaction with Apaf-1 [8,33]. At the C-terminus, rat caspase-9 has a large subunit and a small subunit. The enzymic active site of caspase-9 (QACGG) was identified in the large subunit [31] (Figure 2).

Isolation of the 5'-flanking region of rat caspase-9

Based on the cDNA sequence, gene-specific primers of rat caspase-9 were designed for the first genomic walking experiment [27] (Table 2). This walking amplified a DNA fragment from the rat genomic DNA pool DL-4. The fragment was gel-purified and sequenced. The sequence of 238 bp showed at its 3' end a 28 bp overlap with the 5' end of rat caspase-9 cDNA (Figure 3). Based on this sequence, the second genomic walking was performed to amplify a 1.1 kb genomic fragment from the DNA pool DL-3 (Figure 3). After five sequential genomic walking steps, we isolated and sequenced 2270 bp of the proximal 5'-flanking regions of rat caspase-9 (Figure 3).

As described above, our initial approach to determine whether the DNA fragments amplified by genomic walking were upstream sequences was to analyse the sequence overlap. To substantiate these results, we designed forward primers based on the sequence obtained by genomic walking and reverse primers within the cDNA region to PCR-amplify genomic DNA extracted from RPTCs. DNA fragments amplified by these PCR reactions were sequenced and shown to contain the 5' end sequence of rat

Figure 2 Sequence and schematic diagram of rat caspase-9

The full-length cDNA of caspase-9 is 2058 bp and consists of 25 bp of 5'-untranslated region, 1365 bp of coding region and 668 bp of 3'-untranslated region. The longest open reading frame predicts a protein of 454 amino acids. The protein contains a CARD domain at the N-terminus, and a large subunit and a short subunit at the C-terminus. The enzymic active site, with a characteristic sequence of QACGG, resides in the large subunit.

Figure 3 Schematic diagram showing genomic walking of rat caspase-9

For the first genomic walking experiment, gene-specific primers were designed based on the sequence of rat caspase-9 cDNA. Two rounds of PCR (primary and nested) were performed to amplify five genomic DNA pools (DL-1–DL-5) that were digested separately by *EcoRV*, *SacI*, *DraI*, *PvuII* and *SspI*. A DNA fragment of 238 bp was amplified from the genomic DNA pool DL-4. 28 bp at 3' end of this fragment overlapped with the 5' end of caspase-9 cDNA, indicating that the 238 bp sequence was immediately upstream of the cDNA. For the second genomic walking, gene-specific primers were designed based on the 238 bp sequence to amplify a genomic fragment of 1.1 kb from the DNA pool DL-3. After five sequential genomic walkings, 2270 bp of the proximal 5'-flanking region of rat caspase-9 was isolated.

caspase-9 cDNA (results not shown). The results indicate that the genomic segments isolated by genomic walking are indeed the proximal 5'-flanking regions of rat caspase-9.

We subsequently analysed the 5'-flanking sequence of rat caspase-9 by the database software SIGNAL SCAN [34] (Figure 4, top panel). This genomic segment was shown to be TATA-box-less, whereas several GC-boxes and putative Sp-1-binding elements were identified. Potential binding sites for transcription factors such as nuclear factor-1 (NF-1), glucocorticoid receptor, Pit-1 and CCAAT-enhancer-binding protein (C/EBP) were present. Seven core binding sites for hypoxia-inducible factor-1 (HIF-1) were also found [35] (Figure 4). Our recent studies have demonstrated a striking induction of caspase-9 by hypoxia [23]. Whether the HIF-1-binding elements are responsible for this inductive response remains to be elucidated. On the other hand, elements binding interferon regulatory factor-1 (IRF-1) and signal transduction and activators of transcription 1 (STAT-1) that were reported to have a role in caspase gene regulation [36,37] were not shown in the 5'-flanking region of rat caspase-9.

Recent data on the sequence of human chromosome 1 identified the gene of caspase-9, which apparently contains nine exons (GenBank accession number AL512883). We have compared the sequence immediately upstream of the first exon of this gene with the 2270 bp 5'-flanking sequence of rat caspase-9. These two sequences showed weak homology, with 47% identity and 12% gaps, as shown by the BLAST database. A noticeable feature shared by these two sequences is the high GC content. Within the 300 bp immediately upstream of their first exons, the genes for human and rat caspase-9 have 76.6% and 71% GCs, respectively. Putative binding sites for transcription factors including Sp-1, NF-1 and C/EBP were identified in the regulatory regions of these two genes. In addition, as in the rat sequence, four HIF-1 binding sites were shown in the proximal 5'-flanking region of human caspase-9.

Promoter activity of the 5'-flanking region of rat caspase-9

To determine whether the 5'-flanking region isolated by genomic walking contains the gene promoter, we measured its promoter

The results demonstrating the highest promoter activity for the 0.36 kb genomic segment were consistent with results from theoretical analysis using PROMOTER SCAN software [38].

Figure 5 Promoter activity of the 5'-flanking region of rat caspase-9

(A) 5' Sequential deletion constructs cloned into the luciferase reporter-gene vector pGL3-Basic. Six 5' sequential deletion constructs (0.2–2.2 kb) were generated by PCR, using primers designed according to the 2270 bp caspase-9 genomic sequence (underlined in Figure 4, bottom panel). The DNA fragments were cloned into the promoterless reporter-gene vector pGL3-Basic. (B–D) Promoter activity of rat caspase-9 genomic DNA cloned into pGL3-Basic analysed by transfection of HEK-293 cells (B), NIH-3T3 cells (C) and PC12 cells (D). Cells were co-transfected with 1 μ g of pGL3-Basic with or without inserts, and 0.02 μ g of pRL-TK. The transfected cells were lysed 24 h later. Activities of firefly and *Renilla* luciferase in cell lysates were measured by the Dual-Luciferase Reporter assay system. Firefly luciferase activity was normalized with *Renilla* luciferase activity. The activity ratio obtained from control empty pGL3-Basic was arbitrarily set at 1. The experiments were repeated three times with duplicates for each condition. Data are means $\pm$ S.D.

Figure 6 Expression of caspase-9 mRNA induced by hypoxia

RPTCs (control, lane 1) were subjected to 1, 3 or 5 h of hypoxic incubation (lanes 2–4), or to 5 h of hypoxia in the presence of 50 μ g/ml actinomycin D (lane 5). At the end of the incubation, cellular RNA was extracted. Northern-blot hybridization was performed using rat caspase-9 gene-specific probes. To control sample loading and RNA transfer, the blots were stripped and re-probed with 32 P-labelled GAPDH cDNA. Levels of gene-specific mRNA were revealed by autoradiography.

The database predicted the 0.36 kb segment to be a good promoter region. The sequence of this region, although it does not have a TATA box, is characterized by a high (69.7%) GC content and the presence of four GC boxes and four putative Sp-1-binding elements (Figure 4, bottom panel). Recent studies have shown clearly that many gene promoters are indeed 'TATA-less' [39]. Whereas transcriptional regulation of these genes is enigmatic, the interactions between initiator elements, functional TATA-box analogues, and other transcriptional factors including Sp-1, may play an important role [40,41].

mRNA expression of caspase-9 induced by hypoxia

We have shown by immunoblotting that caspase-9 is induced by severe hypoxia [23]. To examine whether the induction involves gene transcription, we determined the steady-state levels of caspase-9 mRNA by Northern-blot hybridization (Figure 6). Hybridization with rat caspase-9-specific probes revealed a major band of $\approx$ 2.0 kb, which is consistent with the size of the cloned full-length cDNA. Significantly, expression of this transcript was noticeably increased after hypoxic exposure (Figure 6, lanes 2–4). This increase was ameliorated by actinomycin D, a DNA-primed RNA polymerase inhibitor that blocks gene transcription (Figure 6, lane 5). Together, these results suggest an important role for gene transcription in caspase-9 expression under hypoxia.

Activation of the caspase-9 gene promoter by hypoxia

Transcription of caspase-9 is activated by hypoxia (Figure 6). This observation implies potential regulation at the level of the gene promoter. Thus our subsequent studies were to determine whether the isolated promoter segments contained functional *cis* elements that could be activated by hypoxia. The luciferase reporter-gene vector pGL3-Basic harbouring 0.2, 1.2 or 2.2 kb promoter segments was transfected into PC12 cells. The experimental groups of cells were subjected to 3 h of hypoxic incubation, while the control cells were kept in a normal oxygen atmosphere. Cells were lysed at the end of incubation to measure luciferase expression as an indicator of promoter efficiency. The results are summarized in Figure 7. Promoter activity within hypoxic cells was 150, 220 and 190 % of the control for the 0.2, 1.2 and 2.2 kb segments, respectively, indicating a significant activation. The promoter activation was in the range of $\approx$ 2-fold (Figure 7), and appeared to be less than the mRNA accumulation within hypoxic cells (Figure 6). One possibility is that transcription/promoter activation is not the only factor responsible for mRNA accumulation: stabilization of the messengers may have a role as well. Alternatively, there might be *cis*-acting elements in sequences beyond the 2.2 kb region isolated in this study, which may enhance the hypoxic response. Nevertheless, activation of the promoter segments by hypoxia suggests

Figure 7 Activation of the caspase-9 gene promoter by hypoxia

PC12 cells were transfected with pGL3-Basic vectors containing 0.2, 1.2 or 2.2 kb caspase-9 promoter sequences. One group of transfected cells (experimental) was subjected to 3 h of hypoxia, whereas the other (control) was incubated under normal oxygen. At the end of incubation, cells were lysed. Luciferase activities in cell lysates were measured to indicate promoter efficiency. Changes in promoter activity induced by hypoxia were calculated as a percentage of the activity in control cells. The experiments were repeated three times with duplicates for each condition. Data are means $\pm$ S.D.

the presence of functional regulatory elements for caspase-9. In addition, the results provide further support for our observation of caspase-9 gene regulation under hypoxia.

Conclusions

In summary, we have cloned the full-length cDNA of caspase-9 from RPTCs. Moreover, we have isolated for the first time 2270 bp of the proximal 5'-flanking region of this gene. Analysis using reporter-gene vectors has demonstrated the promoter activity of isolated genomic segments. Of significance was that the promoter regions can be activated by severe hypoxia, conditions shown to induce caspase-9 gene transcription. These findings indicate the presence of critical *cis*-acting elements in the cloned sequences, which may participate in caspase-9 gene regulation under certain pathophysiological situations.

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