The Human Caspase-8 Promoter Sustains Basal Activity through SP1 and ETS-like Transcription Factors and Can Be Up-regulated by a p53-dependent Mechanism*

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Caspase-8, also known as MACH/FLICE/Mch5, is the most upstream-located cysteine-aspartyl-protease (caspase) in a caspase cascade involved in apoptosis triggered by members of the tumor necrosis factor receptor superfamily or other stimuli such as chemotherapeutic agents. Regulation of caspase-8 expression on a post-translational level has been studied in detail, whereas only little information is available on its control by gene transcription. We identified and cloned the human caspase-8 promoter, determined the transcriptional start site of the caspase-8 gene, and examined the regulatory mechanisms of the promoter with respect to its basal activity as well as to its inducibility upon apoptotic stimuli in human hepatoma cells. We identified two minimal sequences essential for basal transcription of caspase-8 and demonstrate that a single SP1 and an ETS-like binding motif mediate this effect. We further show that the caspase-8 promoter is inducible and demonstrate that adenoviral infection increases caspase-8 mRNA levels. However, the increase in caspase-8 gene transcription after adenoviral infection absolutely depends on the p53 status of the hepatoma cell line, implying that caspase-8 is a target gene of p53. We show that delivery of exogenous p53 alone is sufficient to induce the caspase-8 promoter even in p53-deficient Hep3B hepatoma cells. Subsequent promoter deletion analysis in combination with luciferase reporter assays identified a p53-responsive element downstream of the transcriptional start site. We demonstrate that this p55-responsive sequence overlaps with the ETS-like binding site and suggest that an additional p53-inducible, yet unknown factor interacts with this region of the caspase-8 promoter. In summary, our study contributes to the understanding of the transcriptional regulation of the caspase-8 gene by basal (SP1- and ETS-dependent) and inducible (p53-dependent) mechanisms.

Caspase-8 is the most upstream-located cysteine-aspartyl-protease (caspase) involved in apoptosis mediated by Fas, TNF, and related death receptors of the TNF superfamily (1–3). Caspase-8 is highly regulated on a posttranslational level. Upon apoptotic stimulation and subsequent death-inducing signaling complex (DISC) formation, an unprocessed procaspase-8 is coupled to the adapter molecule Fas-associated death domain protein (FADD) via two death effector domains, which is a prerequisite for processing of procaspase-8 to the active caspase by proteolytic cleavage leading to the active subunits p18 and p10 (2). These subunits are released to the cytosol and mediate caspase activity via a C-terminal protease domain. Caspase-8-dependent apoptosis can be modulated by expression of c-FLIP, a molecule sharing homology to caspase-8 but lacking a functional protease domain (4).

For downstream signaling of caspase-8 leading to apoptosis two different pathways have been described (5), one via activation of Bid involving mitochondria and cytochrome c release and the other via direct activation of effector caspases as caspase-3. Details of apoptosis signaling and caspase-8 activation are reviewed in detail elsewhere (6, 7).

At present, the transcriptional regulation of the caspase-8 gene has not been examined in detail. However, it has been demonstrated in vitro that ectopic overexpression of caspase-8 is sufficient to induce apoptosis (3). This implies that a self-processing mechanism might be activated if a critical threshold of pro-caspase-8 is expressed. In addition it has been shown that in certain childhood neuroblastomas and neuroectodermal brain tumors as well as in some types of lung cancers, caspase-8 expression is rather low or even abolished, which was explained by both somatic gene mutations and deletions as well as silencing due to hypermethylation of genomic caspase-8 sequences (8–11).

The aim of this study was to examine the regulation of the caspase-8 promoter. Because acute liver failure and viral hepatitis are often associated with Fas/TNF-dependent apoptotic mechanisms (12), we focused our analysis on the transcriptional mechanisms that are involved in controlling caspase-8 expression in hepatoma cells.

EXPERIMENTAL PROCEDURES

Plasmids and Recombinant DNA Techniques—Standard recombinant DNA techniques were carried out as described elsewhere (13). For intermediate cloning steps the vectors pBlueScript SK+ (Stratagene) and pCR2.1-TOPO (Invitrogen) were used. For construction of luciferase-reporter plasmids we amplified appropriate genomic fragments of the caspase-8 promoter using a PCR approach and oligonucleotides that are flanked by KpnI and HindIII restriction sites. The resulting fragment

* This work was supported by a Deutsche Forschungsgemeinschaft Grant DFG Tr 285/4-1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TNF, tumor necrosis factor; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; wt, wild type; STAT, signal transducers and activators of transcription.
Transcriptional Control of Caspase-8 Promoter

Adenoviral Vectors Used in This Study and Infection of Hepatoma Cells—Recombinant adenoviral vectors carrying GFP (adv-GFP) and p53 (adv-p53) were kindly provided by Dr. B. Mundt and Dr. L. Zender (Hannover, Germany) and have been described in Mundt et al. (16) and Zender et al. (17). For adenoviral infection, hepatoma cells were seeded to a density of 2 × 10^5 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Before infecting the cells at a multiplicity of infection of 50, the medium was changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum.

Nuclear Extracts and Gel Retardation Assays—HepG2 and HuH7 nuclear extracts were prepared using the modified Dignam C method (18). For gel retardation assays, nuclear extracts were used as indicated. Binding buffer for SP1 EMSA consisted of 25 mM HEPES, pH 7.5, 0.5 mM MgCl₂, 50 mM KCl, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml poly(dI-dC), and 2 μg/ml bovine serum albumin. As probes, 32P-end-labeled oligonucleotides were used at an activity of 3000 cpm/μl. The oligonucleotides used for SP1 and ETS EMSA are shown under Results. The binding reaction was performed for 30 min at 30°C. Free DNA and DNA-protein complexes were resolved on a 6% polyacrylamide gel. Supershift experiments were performed with a specific SP1 antibody or an antibody directed against a broad range of ETS family members, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). For p53- and ETS-specific EMSA experiments, respectively, modified protocols were used (19, 20).

Primer Extension Analysis—For mapping the transcriptional start site of the caspase-8 promoter by primer extension we used the commercial available Primer Extension System (Promega) and performed primer extension analysis according to the manufacturer's protocol. Briefly, 10 pmol of the oligo 5'-TGGCGGGGTACAGCGAGGAGGAAGCTCTC-3' (compare Fig. 1A) was used as well as a DNA marker mix were end-labeled using 10 units of T4 polynucleotide kinase and 3000 Ci/mmol [γ-32P]ATP. The primer extension reaction was performed using 10 μg of total RNA from HepG2 or HuH7 cells and 0.2 pmol of labeled oligo. The reaction was analyzed on a denaturing polyacrylamide gel containing 8% acrylamide, 7 μM urea in 1X Tris-buffered EDTA. After autoradiography overnight, the size of the primer extension product was determined by direct comparison to the provided DNA size marker.

Cloning and Analysis of the 5' Untranslated Region of the Human Caspase-8 Gene—To isolate the human caspase-8 promoter we performed a BLAST search with the human genome using the longest isoform of the caspase-8 cDNA (variant A, accession number NM 001228) as a query. For a prediction of putative binding sites for transcription factors we used the MatInspector professional software (Genomatix) as described in Quandt et al. (22).

RESULTS

Cloning and Analysis of the 5' Upstream Region of the Human Caspase-8 Gene—To isolate the human caspase-8 promoter we performed a BLAST search with the human genome using the longest isoform of the caspase-8 cDNA (variant A, accession number NM 001228). The 5' sequence of the caspase-8 cDNA matched perfectly to a genomic sequence located on chromosome 2 (accession number NT 005403). This is in agreement with recent reports showing that the caspase-8 gene maps at chromosome 2 (band 2q35-q34) (23, 24). Therefore we concluded that the 5' upstream region of the match may contain the caspase-8 promoter. The sequence containing the putative caspase-8 promoter as well as the noncoding exons 1 and 2 was deposited to the GenBank™ database under accession number AF 291598 and is displayed in Fig. 1A. Performing an initial computer analysis of the promoter sequence using MatInspector software (22), binding sites for transcription factors including NFκB, STAT1, SP1, and ETS were predicted and are shown in Fig. 1A.

Next we were interested to map the transcriptional start site of the caspase-8 gene. For this experiment we used an anti-sense primer (see Fig. 1A, arrow) located within the noncoding exon 1. Primer extension analysis with total RNA isolated from both HepG2 and HuH7 cells revealed a 78-bp primer extension product (Fig. 1B, see lanes 2 and 3, respectively). Accordingly, we identified the sequence 7TTCG as the transcriptional start site of the caspase-8 gene. This site is located 29 bp upstream of the 5' end of the published cDNA. The position of the transcriptional start site was arbitrarily set to +1.
FIG. 1. Organization and start site of transcription of the caspase-8 promoter. A, nucleotide sequence of the caspase-8 promoter. The 5′-flanking region of the caspase-8 gene was identified by performing a BLAST search with the caspase-8 cDNA as a query sequence. The transcriptional start site was identified as described in B. It was arbitrarily set to +1 and is indicated by an arrow. The numbering of the sequence is related accordingly. Distinct DNA binding sites of transcription factors as predicted by the MatInspector software (Genomatix) are indicated (underlined sequence). The first two (noncoding) exons are highlighted in bold. The translational start site of caspase-8 (ATG) is located at position +78272, and the sequence between exons 2 and 3 is symbolized by dots. B, the transcriptional start site of the caspase-8 gene was determined using primer extension analysis in combination with a primer as indicated by an arrow in A. Lane 1, DNA length standard as provided from Promega. To exclude cell-line specific effects, we determined the start site with RNA derived from two hepatoma cell lines, HepG2 (lane 2) and HuH7 cells (lane 3). The caspase-8 signal at −78 bp is indicated by an arrow.
A Sequence Located between −121 and −71 Contributes to the Basal Activity of the Caspase-8 Promoter—For further experiments, we amplified an −1000-bp fragment containing the putative caspase-8 promoter region and a small part of the first, noncoding exon 1 using primers −972 and −76. The resulting fragment was cloned into the HindIII and KpnI sites of the pGL2 luciferase vector (Promega) and was termed −972/−76 with respect to the 5’ and 3’ positions related to the transcriptional start site.

To map the core region of the caspase-8 promoter we first introduced 5’ deletions (Fig. 2A) into the −972/−76 construct using a PCR approach (respective primers are listed under “Experimental Procedures”). All constructs were transfected in HuH7 cells including the empty pGL2 vector as a control.

Luciferase activity of the −972/−76 construct was nearly 300-fold increased compared with the pGL2 control, whereas the strongest activity was found with the −121/−76 construct (Fig. 2B). A further 5’ deletion of 50 bp (−71/−76 construct) resulted in a 8-fold reduction in luciferase activity, indicating that this region is involved in controlling basal caspase-8 promoter activity.

Because our data suggested that the region located between bp −121 and −71 is essential for basal caspase-8 promoter activity, we further concentrated on the role of this sequence. We performed a detailed analysis of this area by introducing increasing 10-bp 5’ deletions in the −121/−76 construct (Fig. 2C). Transfection experiments in HuH7 cells using these constructs revealed that the region located between position −101 and −91 increases 5’ deletions as introduced in the caspase-8 promoter are shown. The 5’ sequences of the deletion constructs used for luciferase assays are depicted. D, the 5’ deletion constructs as shown in C were transfected into HuH7 cells, and the relative luciferase activity of the respective reporter constructs is depicted. The activity of −121/+76 construct was arbitrarily set to 100%, and the relative luciferase activity of the other constructs was calculated accordingly.

A Unique SP1 Binding Motif Is Essential for Basal Activity of the Caspase-8 Promoter—MatInspector software analysis identified a potential SP1 binding site between position −101 and −91 (Fig. 2C, underlined sequence). To confirm SP1 DNA binding to this motif, we next performed gel retardation analysis with 32P-labeled oligonucleotides representing the putative SP1 site found in the caspase-8 promoter and nuclear extracts isolated from HuH7 cells. A SP1 consensus oligonucleotide (Fig. 3A) served as a control. Complex formation with the SP1 consensus oligonucleotide revealed three SP1-specific complexes, where only the upper slow-migrating complex could be supershifted by anti-SP1 (Fig. 3B, lanes 3 and 4).

Gel shift experiments using the putative SP1 binding site of the caspase-8 promoter (Fig. 3A) revealed complex formation comparable with the pattern found with the SP1 consensus oligonucleotide. The slower migrating complex also could be supershifted with an SP1 antibody. In contrast, introducing two G → T mutations within the core sequence of the putative
A unique SP1 binding motif located between nucleotide −85 and −97 contributes to the basal activity of the caspase-8 promoter. A, alignment of the SP1 oligonucleotides used for EMSA analysis. Oligonucleotides were aligned for the core sequence (bold). The upper sequence shows the SP1 consensus site as provided by Santa Cruz. The middle sequence depicts the putative SP1 site as found in the caspase-8 promoter (see Fig 2C, underlined sequence). The lower sequence shows the introduction of two G → T mutations within the putative SP1 core sequence. B, in EMSA experiments HuH7 nuclear extracts were incubated with the p32-labeled double-stranded oligonucleotides as shown in A. Oligonucleotides used for each experiment are shown on the bottom of the panel. Except for lane 1, 5, and 9, the oligonucleotides were incubated with HuH7 nuclear extract. In lanes 3, 4, 7, 8, 11, and 12, additionally, two different concentrations (2 and 4 μg) of a SP1 antibody (SP1 ab) for supershift experiment was added to the incubation mix. Signals specific for SP1 are depicted by arrows. C, competition experiments with cold SP1-specific and mutated oligonucleotides were performed. The putative SP1 oligonucleotide was incubated with HuH7 nuclear extract (lanes 2 and 6), and increasing amounts (5-, 25-, and 100-fold excess) of cold SP1 putative (lanes 3–5, respectively) or cold mutated SP1 putative oligonucleotide (lane 6–8, respectively) were added as competitor. Arrows in accordance with B highlight the SP1 specific signals. D, the putative SP1 mutation (see A) was introduced in the −101/+76 luciferase construct (−101SP1mut/+76). The resulting −101SP1mut/+76, the −101/+76, and the −91/+76 constructs were transfected in HuH7 cells, and luciferase activity was determined. The luciferase activity of −101/+76 was arbitrarily set to 100%, and the activities of other constructs were calculated accordingly.
SP1 binding motif (Fig. 3A) in the caspase-8 promoter abolished SP1-dependent complex formation (Fig 3B, lane 10).

To further support our data, we performed competition experiments using the SP1 consensus motif as a 32P-labeled probe and competed for complex formation by using either the wt or the mutant putative binding site as derived from the caspase-8 promoter (Fig. 3A). A 5, 25, and 100 μM excess of the respective cold oligonucleotide was used for competition analysis. When the wt sequence was used as competitor, a 5 μM excess was already able to compete for binding of the three putative SP1-specific complexes (Fig. 3C, lane 3–5), whereas the mutant oligonucleotide was not able to inhibit formation of these specific complexes in the same molar range (lane 6–8).

These data together with the transfection experiments indicated that SP1 is involved in controlling the basal activity of the caspase-8 promoter. To further support our findings, we introduced the mutated SP1 motif in the caspase-8 promoter of reporter-construct −101/+76, resulting in −101SP1mut/+76. Transfection experiments using the −101/+76, the −101/+76mut, and the −91/+76 caspase-8 construct and subsequent luciferase assays revealed a significant reduction (−60%) in promoter activity of the mutant compared with the respective wt construct (Fig. 3D). However, the activity of the −91/+76 caspase-8 construct was still lower compared with −101/+76mut.

An ETS-like Element Is Located in the 3' End of the Caspase-8 Promoter and Contributes to Basal Activity—To identify further elements involved in controlling basal caspase-8 promoter activity, we introduced several 3’ deletions in the −470/+76 construct and compared the respective luciferase activities after transfection into HuH7 cells. Luciferase activity completely dropped when the deletion contained the −470/+76 construct. Because this sequence contains a predicted ETS-like binding motif, we analyzed this sequence in more detail. By introducing subsequent 8–10 bp 3’ deletions in this region we could demonstrate that luciferase activity was diminished when the predicted ETS-like motif and the surrounding sequences were missing, indicating that this site might be involved in controlling basal promoter activity (Fig. 4, C and D).

To further test this hypothesis we next performed EMSA experiments using modified binding conditions for ETS (20) with oligonucleotides representing either the wt promoter sequence (ETSput, Fig. 5A, upper sequence) or a modified sequence carrying a mutation in the predicted ETS site (ETSmut, Fig. 5A, lower sequence). The 32P-labeled oligonucleotides were incubated with HuH7 nuclear extracts. Complex formation
could be detected with the oligonucleotide representing the wt promoter sequence, which was not found with the mutant sequence (Fig. 5B, compare lanes 1 and 3). Complex formation with the wt oligonucleotide could be blocked by an antibody directed against a broad range of ETS-like factors (anti-ETS), indicating that an ETS-like factor binds to this sequence in the caspase-8 promoter. In contrast, the anti-ETS antibody had no effect on complex formation when the mutant oligonucleotide was used (Fig. 5B). Additionally, supershift analysis was performed with an ETS-1-specific antibody. However, no effect on complex formation was found (data not shown), indicating that this element is recognized by another ETS family member.

Because our experiments suggested that an ETS-like factor binds to this region in the caspase-8 promoter, we introduced the mutant sequence in the \( 470/76 \) construct (\( 470/76 \)mutETS). Transfection experiments of the mutant compared with the wt and \( 470/76 \) construct demonstrated that luciferase activity of \( 470/76 \)mutETS was reduced to a range as found with the \( 470/48 \) construct (Fig. 5C). These results indicate that besides the SP1, the ETS site in the promoter also contributes to the basal activity of caspase-8-dependent gene transcription.

The Caspase-8 Promoter Is Inducible upon Adenoviral Infection in p53 wt Hepatoma Cells—Adenoviral gene transfer into the liver and hepatoma cells is an established approach for effective gene delivery (19–22). However, several disadvantages have been described including strong cytotoxic effects mainly due to Fas-mediated apoptosis and induction of p53 (19, 25). To further evaluate the molecular mechanisms responsible for adenoviral-induced apoptosis in the liver, we wanted to examine if caspase-8-dependent gene transcription is increased after adenoviral challenge.

Three hepatoma cell lines (HepG2, HuH7, Hep3B) with a different p53 status (14) were transfected with the caspase-8 promoter construct. 24 h after transfection we infected the cells with an adenovirus expressing GFP at a multiplicity of infection of 50. Efficiency of infection was controlled by GFP fluorescence (data not shown). Cells were harvested before and 8 and 24 h after adenoviral infection, and luciferase activity was measured (Fig. 6A). In HepG2 cells expressing wt p53 protein, an up to 10-fold induction of the caspase-8 promoter was observed 24 h after infection. In contrast, in hepatoma cells carrying a mutant p53 allele (HuH7 cells) or a p53 deletion (Hep3B cells) no induction was found after adenoviral infection. To test if the reporter gene experiments also reflect the transcriptional up-regulation of the endogenous caspase-8
gene, we established a duplex RT-PCR analysis to study caspase-8-specific transcripts. Total RNA from the adenoviral-transduced hepatoma cells was isolated at the same time points as indicated for the luciferase assays. In HepG2 cells a more than 10-fold increase in caspase-8 transcripts was found 24 h after transduction (Fig. 6, B and C). In HuH7 and Hep3B cells, no significant increase in RNA levels could be observed. However, the basal caspase-8 mRNA level in both cell lines was higher compared with the level found in HepG2 cells.

**p53 Expression Stimulates Caspase-8 Promoter Activity**—Our data suggested that the increase in caspase-8 promoter activity after adenoviral infection is p53-dependent. Therefore, we co-transfected HuH7, HepG2, and Hep3B cells with the −470/+76 construct and increasing amounts of a p53 expression vector (pcmv-p53). 24 h after transfection cells were harvested, and luciferase activity was measured (Fig. 7, A–C). In all cell lines included we found a dose-dependent increase in caspase-8 promoter activity beginning when 10 ng of the pcmv-p53 expression vector were used. The maximum in promoter activity was 3-fold in HepG2 (Fig. 7A), 6-fold in HuH7 (Fig. 7B), and 4-fold in Hep3B cells (Fig. 7C). Because ectopic p53 is highly toxic for Hep3B cells, pcmv-p53 concentrations higher than 30 ng were excluded.

In further experiments we co-transfected the different cell lines with 30 ng of pcmv-p53 and the −470/+76 construct. 24 h after transfection adenoviral infection was performed. However, the co-administration of the p53 expression plasmid and the adenoviral vector did not result in a further increase of caspase promoter activity (data not shown), indicating that the stimulating effect on caspase-8 promoter activity after adenoviral infection is mainly mediated through a p53-dependent mechanism.

**Identification of a p53-responsive Element within the Caspase-8 Promoter**—Because our results revealed a p53-dependent mechanism in stimulating the caspase-8 promoter, we next were interested in identifying the sequence responsible for this regulation. A computer search for p53 binding sites using MatInspector software (22) revealed no obvious binding motif within the promoter sequence. Therefore, we used the deletion constructs shown in Fig. 2A (5’ deletions) and Fig. 4, A and C (3’ deletions) for further evaluation of the sequence responsible for the p53-dependent induction of the caspase-8 promoter. HuH7 cells were transfected with the respective promoter constructs and stimulated with an adenovirus expressing p53 (adv-p53). Analysis of the 5’ deletions indicated that in this region there was no specific element mediating the p53-dependent effect because inducibility of all constructs varied in the range between 3- and 5-fold (Fig. 8, A and B).

In contrast, a 3’ deletion of 10 bp (plasmid −470/+66, Fig. 8, C and D) abolished the p53-dependent effect on caspase-8 promoter activity. As found with all further 3’ deletions examined,
infection of the p53-expressing adenovirus had no enhancing effect on caspase-8 promoter activity, indicating that the sequence located between position +60 and +76 of the caspase-8 promoter is involved in mediating the p53-dependent effect. Interestingly, the effect on caspase-8 promoter activity was not altered when the $470^/-76$ promoter-luciferase construct was transfected (Fig. 8, C and D). Taken together, the data suggest that besides ETS, an additional factor might bind to this region of the caspase-8 promoter in a p53-dependent manner.

Identification of a p53-dependent Binding Activity—To understand the mechanism responsible for p53-dependent up-regulation at the sequence identified, we performed EMSA analysis. To ensure that p53 is functional after adv-p53 infection we first demonstrated by EMSA that nuclear extracts from HuH7 cells infected with adv-p53 were able to bind a p53 consensus oligonucleotide using optimized p53 binding conditions (19) after treatment with a p53 activating antibody (Fig. 9A, left panel).

Because the oligonucleotide ETSput (compare Fig. 5A) covers both the ETS-like binding site and the p53-responding site, we next investigated whether p53 binds to this sequence after adv-p53 infection of HuH7 cells. A gel-shift experiment under p53-specific binding conditions (20) using HuH7 nuclear extracts did not reveal a specific signal in p53-stimulated cells (Fig. 9A, right panel). This indicates that p53 does not interact directly with the caspase-8 promoter to cause promoter induction.

However, with HuH7 nuclear extracts using standard binding conditions, we detected new complex formation that was only obvious after infection of the p53-expressing adenovirus (Fig. 9B, arrow I) and which could not be supershifted by the ETS antibody. These data indicate that the binding is caused by a not yet characterized factor that can be activated by p53.

DISCUSSION

Apoptosis is an important mechanism involved in many biological processes including development, immunity, and with respect to the liver, acute liver failure and onset of adenoviral hepatitis (12, 26). On the contrary, it has been discussed that during tumor development a mis-regulation of apoptotic genes might be important for malignant growth (27, 28).

In this context, the aim of the present study was to investigate the transcriptional regulation of the caspase-8 gene. We first cloned the human caspase-8 promoter and determined a unique start site of transcription. The impact of this site could be confirmed by additional luciferase reporter gene constructs...
lacking this sequence. Therefore, earlier experiments that indicated that the promoter and, thus, the potential start site of transcription of the caspase-8 gene are located further 3’ from the TATA box are not in agreement with our present analysis.

Computational analysis of the putative promoter region using the MatInspector software (22) revealed interesting information about the potential regulation of the caspase-8 promoter. We did not identify any obvious TATA box, and TATA-less promoters are thought to be a typical feature of many housekeeping genes (29). Additionally, potential DNA binding sequences for the transcription factors STAT1, NFκB, SP1, and ETS were predicted in close proximity to the start site of transcription of the caspase-8 gene.

Until now the role of these potential DNA binding sites in the caspase-8 promoter was not addressed in detail. A recent report (30) indicated that interferon-κ up-regulates caspase-8 expression and thereby sensitizes cells for apoptosis by activating STAT1. Additionally, while this manuscript was in preparation, Yang et al. (31) reported that both caspase-8 promoter activity and caspase-8 mRNA levels are up-regulated upon interferon-γ stimulation, which further supports a potential role of STAT1 in controlling caspase-8 promoter activity. However, at present the ultimate proof of a functional role of the predicted STAT site for regulating caspase-8 mRNA expression is still missing.

By performing promoter deletion analysis we could show that the sequence containing a potential SP1 site in the caspase-8 promoter contributes to its basal activity. Further EMSA experiments and the introduction of point mutations in reporter gene constructs demonstrated that binding of SP1 in this region controls basal caspase-8 promoter activity. However, the activity of the reporter gene construct comprising the mutated SP1 sequence was higher compared with the 5’ deletion construct where the complete SP1 site had been deleted. These results indicate that the single point mutation is not sufficient to completely block SP1 DNA binding, and some residual activity might be present. Alternatively, a second factor, which could not be identified by EMSA, might also bind to this region and, thus, contributes in part to basal activity. The potential relevance of SP1 in regulating caspase-8 activity was further supported by sequence comparison with the murine caspase-8 promoter. In the same region of the murine caspase-8 promoter two potential SP1 DNA binding sites are present, indicating that the machinery controlling the basal activity of caspase-8 gene expression might be evolutionarily conserved.

Our experiments revealed that also an ETS-like factor contributes to basal activity of the caspase-8 promoter. However, these results indicate that the single point mutation is not sufficient to completely block SP1 DNA binding, and some residual activity might be present. Alternatively, a second factor, which could not be identified by EMSA, might also bind to this region and, thus, contributes in part to basal activity. The potential relevance of SP1 in regulating caspase-8 activity was further supported by sequence comparison with the murine caspase-8 promoter. In the same region of the murine caspase-8 promoter two potential SP1 DNA binding sites are present, indicating that the machinery controlling the basal activity of caspase-8 gene expression might be evolutionarily conserved.

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transcriptional control of caspase-8 promoter.

The p53-specific signals are indicated by arrows. On the right panel a similar experiment is depicted using the ETSput oligonucleotide covering the p53-responsive element. B, the double-stranded oligonucleotide ETSput (compare Fig. 5A) derived from the caspase-8 promoter was used in EMSA experiments with nuclear extracts derived from HuH7 cells using standard conditions. Cells were either untreated (control) or infected with av-p53. Arrow I indicates additional signals after p53 stimulation. In lanes 2 and 4, 2 μg of anti-ETS (ETS ab) was added to the incubation mix.

because the family of ETS-like transcription factors contains more than 40 proteins, in the present study we did not further determine the factor having high affinity to the identified sequence in the caspase-8 promoter region. Interestingly, the three characteristics, namely a missing TATA-box and binding activities for SP1 and ETS, were also identified to control caspase-3 gene expression (32). Therefore, our present results indicate that some members of the caspase machinery might be regulated through a redundant mechanism.

In our earlier work we found that adenoviral infection of the liver results in p53 up-regulation and Fas-mediated apoptosis (23). Because caspase-8 is the most upstream located initiator caspase and its activity can be controlled through its antagonist c-FLIP, we were now interested in studying if this balance can be shifted toward pro-apoptosis through induction of caspase-8 gene expression. Adenoviral infection of hepatoma cells induced caspase-8 gene expression in a p53-dependent manner. p53 expression alone induced caspase-8 promoter activity, and by deletion analysis p53-inducibility was restricted to the earlier identified ETS site located 3' of the start site of transcription. Interestingly, p53 inducibility was not dependent on the intact ETS motif because the mutated site also confers this effect. Additionally, deletion of the SP1 site in the caspase-8 promoter allowed the p53-dependent increase in gene transcription. Therefore, our results show that the p53-dependent effect on the caspase-8 promoter is independent from the mechanisms that control the basal activity.

Further EMSA experiments with the ETS-like motif in the caspase-8 promoter revealed new complex formation in a p53-dependent manner. However, the complex could not be supershifted with a p53-specific antibody. Additionally the sequence does not represent an ideal or modified p53 consensus sequence. Therefore, these results indicate that a p53-regulated, yet unknown factor binds in this region of the caspase-8 promoter, overlapping with the identified ETS binding motif and confers p53-dependent inducibility.

In our experimental system a more than 10-fold up-regulation of caspase-8 mRNA expression in HepG2 cells was not associated with a significant increase in the rate of apoptosis (3). Earlier reports indicated that overexpression of caspase-8 in several cell lines can lead to massive apoptosis (1, 3). However, these experimental conditions were not physiological. Therefore, regarding our data we hypothesize that an increase in caspase-8 expression would rather sensitize cells for apoptosis by a second stimulus like TNF, FasL, or TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Further investigations, especially in vivo, will be necessary to proof this hypothesis.

Computer-assisted analysis with the p53-responsive element 5'-TCTGCCTGCTGCTG-3' revealed 100% identity to a sequence located within the murine caspase-8 promoter. This further strengthens the importance of this motif in the regulation of the caspase-8 promoter and suggests that the p53 regulation might be conserved between mice and man. At present, the functional consequences in the overlap between the ETS- and the p53-dependent binding motif is unclear. However, the close interaction of these two different transcription factors and its potential upstream regulatory pathways might be relevant during different pathophysiological conditions controlling the balance between cell growth and apoptosis. Interestingly, p53 up-regulates a variety of genes involved in the TNF-related apoptosis cascade. This has been reported for the Fas receptor (14, 33), caspase-1 (34), caspase-6 (35), Apaf-1 (36), and, finally, as shown here, for caspase-8. Especially the synergistic regulation of the Fas receptor and caspase-8 could have major implications for the liver. Earlier results identified hepatocyte in vivo as extremely sensitive for Fas-induced apoptosis (37). In contrast, hepatocellular carcinomas frequently express mutated p53 and are resistant against different chemotherapeutic agents (38–40). The Fas receptor and caspase-8 are crucial switches in determining the elimination of diseased e.g. virus-infected hepatocytes (41). However, the up-regulation of both players by p53 can be potentially eliminated through mutated p53. Therefore, this system is a good candidate to play a role in the tumorigenesis of hepatocellular carcinoma and during further tumor progression.

Therefore, further studies will be interesting as they better define the relationship between tumor growth, the selection for p53 mutations, and caspase-8 mRNA expression in hepatocellular carcinoma. The potential knowledge of these molecular mechanisms might ultimately result in a better understanding of tumor physiology and in the design of new treatment options dependent on the molecular expression profile of the tumor.

Fig. 9. p53-dependent complex formation at the ETS site in the caspase-8 promoter. A, in the left panel an EMSA experiment is depicted using a p53 consensus oligonucleotide and nuclear extracts from HuH7 cells infected with av-p53. In lane 2 a p53 activating antibody was used in the binding reaction, enabling binding to DNA. The p53-specific signals are indicated by arrows. On the right panel a similar experiment is depicted using the ETSput oligonucleotide covering the p53-responsive element. B, the double-stranded oligonucleotide ETSput (compare Fig. 5A) derived from the caspase-8 promoter was used in EMSA experiments with nuclear extracts derived from HuH7 cells using standard conditions. Cells were either untreated (control) or infected with av-p53. Arrow I indicates additional signals after p53 stimulation. In lanes 2 and 4, 2 μg of anti-ETS (ETS ab) was added to the incubation mix.

3 C. Liedtke and C. Trautwein, unpublished observations.
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J. Biol. Chem. 2003, 278:27593-27604.
doi: 10.1074/jbc.M304077200 originally published online May 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304077200

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