Transcriptional analysis of human survivin gene expression

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The preservation of tissue and organ homoeostasis depends on the regulated expression of genes controlling apoptosis (programmed cell death). In this study, we have investigated the basal transcriptional requirements of the *survivin* gene, an IAP (inhibitor of **apoptosis**) prominently up-regulated in cancer. Analysis of the 5' flanking region of the human *survivin* gene revealed the presence of a TATA-less promoter containing a canonical CpG island of ~ 250 nt, three cell cycle dependent elements, one cell cycle homology region and numerous Sp1 sites. PCR-based analysis of human genomic DNA, digested with methylation-sensitive and -insensitive restriction enzymes, indicated that the CpG island was unmethylated in both normal and neoplastic tissues. Primer extension and S1 nuclease mapping of the human *survivin* gene identified two main transcription start sites at position -72 and within -57/-61 from the initiating ATG.

Transfection of cervical carcinoma HeLa cells with truncated or nested *survivin* promoter–luciferase constructs revealed the presence of both enhancer and repressor sequences and identified a minimal promoter region within the proximal -230 nt of the human *survivin* gene. Unbiased mutagenesis analysis of the human *survivin* promoter revealed that targeting the Sp1 sequences at position -171 and -151 abolished basal transcriptional activity by $\sim 63-82\,\%$. Electrophoretic mobility-shift assay with DNA oligonucleotides confirmed formation of a DNA–protein complex between the *survivin* Sp1 sequences and HeLa cell extracts in a reaction abolished by mutagenesis of the *survivin* Sp1 sites. These findings identify the basal transcriptional requirements of *survivin* gene expression.

Key words: apoptosis, IAP, promoter, transcription.

INTRODUCTION

The genetic control of cell death and cell survival (apoptosis or programmed cell death) plays a crucial role in embryogenesis and tissue differentiation [1]. De-regulation of this process results in aberrantly increased viability or reduced cell death and may participate in the pathogenesis of human diseases, including cancer [2]. In addition to bcl-2 proteins [3], members of the recently described IAP (inhibitor of apoptosis) gene family have emerged as unique regulators of cell death [4,5]. These molecules inhibit various forms of apoptosis induced by tumour necrosis factor- α , caspases, chemotherapeutic drugs, viral infections and oxidative stresses, potentially by directly suppressing activity of terminal caspase-3 and -7 [4,5].

Recently, a structurally unique IAP protein, designated survivin, was found abundantly expressed in many human cancers [6,7], but undetectable in normal adult tissues, *in vivo*. Consistent with a role of deregulated apoptosis in cancer [2], survivin expression correlated with unfavourable neuroblastoma [8] and abbreviated survival rates in colorectal cancer [9]. At the molecular level, survivin was expressed in mitosis in a cell cycle dependent manner and localized to mitotic spindle microtubules [10]

In this study, we sought to elucidate the molecular requirements of the basal transcriptional machinery controlling *survivin* gene expression [10]. We found that a minimal *survivin* promoter is located within a proximal unmethylated CpG island, and that basal transcriptional activity requires two critical Sp1 sites.

MATERIALS AND METHODS

Cell culture and RNA extraction

Human epithelial carcinoma HeLa cells (ATCC, Rockville, MD, U.S.A.) were grown in Dulbecco's modified Eagle's medium

(BioWhittaker, Walversville, MD, U.S.A.) with 10 % fetal bovine serum (BioWhittaker) plus antibiotics. HeLa cell total RNA was isolated with TRI reagent (106 cells/0.2 ml; Molecular Research Center, Inc., Cincinnati, OH, U.S.A.). Poly(A)+ mRNA was isolated from total RNA (1–4 mg/ml) by chromatography on oligo-dT columns (Molecular Research Center, Inc.) in 0.5 M LiCl, 40 mM sodium citrate and 0.1 % SDS, with elution in 1 mM sodium citrate/0.1 % SDS.

Primer extension and \$1 nuclease protection assay

A survPE oligonucleotide 5'GGGCTGCCAGGCAGGGGGC-AACGTCGGG3' (+36 to +9, numbering from the initiating)ATG) was synthesized, gel-purified and 5'-end-labelled (10 pmol) in 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.1 mM spermidine, 3 μ l of $[\gamma^{-32}P]ATP$ (3000 Ci/mmol, Amersham, Arlington Heights, IL) and 8–10 units of T₄ polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.) for 10 min at 37 °C. A control primer and dephosphorylated ΦX174 HinfI DNA fragments (250 ng; Promega) were also endlabelled. For primer extension, 100 fmol of labelled primer was mixed with 1–5 μ g of poly(A)⁺ RNA in 50 mM Tris/HCl/50 mM KCl/10 mM MgCl₂/10 mM DTT/1 mM dNTPs/0.5 mM spermidine for 20 min at 58 °C and annealed at room temperature for 10 min. The mixture was combined with 50 mM Tris/HCl, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT, 1 mM dNTPs, 0.5 mM spermidine, 5.6 mM sodium pyrophosphate plus 1 unit of avian myeloblastosis virus reverse transcriptase and incubated for 30 min at 42 °C. Samples were ethanol-precipitated, suspended in 5-8 µl of gel loading buffer and separated on denaturing 7 M urea/polyacrylamide gels followed by autoradiography.

For the S1 nuclease protection assay, a survS1 oligonucleotide, 5'CCACCTCTGCCAACGGGTCCCGCGATTC3' (-11 to

Abbreviations used: CDE, cell cycle dependent element; CHR, cell cycle genes homology region; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; IAP, inhibitor of apoptosis.

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-38) was synthesized and gel-purified. A 250 nt probe of the 5' flanking region of the survivin gene was generated by incubating 0.5–2 μg of MluI-digested template DNA with 30 pmol of primer, 50 mM Tris/HCl, 10 mM MgCl₂, 200 μ M dNTPs, 10 μ l of [α -³²PldCTP (3000 Ci/mmol; Amersham) and 4–5 units of Klenow fragment (Ambion, Austin, TX, U.S.A.) for 30–60 min at 37 °C. The probe was purified on a 7 M urea denaturing polyacrylamide gel and eluted in 0.5 M ammonium acetate/1 mM EDTA/0.2 % SDS at 35 °C overnight. HeLa cell total RNA (20–50 µg) was mixed with $(2-5) \times 10^5$ c.p.m. of gel-purified probe in $10 \mu l$ of hybridization solution (80 % formamide/100 mM sodium citrate, pH 6.4/300 mM sodium acetate/1 mM EDTA), denatured for 5 min at 90 °C and incubated at 42 °C overnight. The hybridization mixture was incubated with 50 mM sodium acetate (pH 4.5), 200 mM NaCl, 1 mM ZnCl₂, 0.5 % glycerol and 1000-1500 units of S1 nuclease/ml for 30 min at 37 °C. After addition of 4 M ammonium acetate/20 mM EDTA (pH 8.0), samples were ethanol-precipitated, washed, separated by electrophoresis on a denaturing 7 M urea polyacrylamide gel and visualized by autoradiography.

Isolation of genomic DNA and PCR

Three partially overlapping human P1 genomic clones were isolated, characterized by restriction-enzyme digestion and a contig of 14796 nt was subcloned and sequenced as described [6]. Genomic DNA was isolated by proteinase K digestion and sequential phenol extraction from fresh or snap-frozen tissues, obtained from the archives of the Department of Pathology. Yale University, or as a gift from Dr. J. Diebold (Hospital Dieu, Paris, France). Genomic DNA samples from various tissues were digested with methylation-sensitive restriction enzymes SacII and Eco0109I, or methylation-insensitive DraI (England Biol., Beverly, MA, U.S.A.), and amplified by PCR with oligonucleotides 5'GTGCAGTCAACGATGTACTC3' (-705 to -686, forward primer) and 5'GCTGGCCAGAGAAGACTT-AC3' (+493 to +474, reverse primer). Thirty-five cycles of amplification were carried out in a Perkin-Elmer 480 thermal cycler with denaturation for 45 s at 94 °C, annealing for 30 s at 55 °C and extension for 1 min at 72 °C. PCR products were analysed on 1% agarose gels by ethidium bromide staining.

Generation of survivin promoter-luciferase constructs

Fragments of the 5' flanking region of the survivin gene were generated by digestion (6270 bp SalI-BsrBRI, 2840 bp BamHI-BsrBRI, 1430 bp DraI-BRI, 649 bp DrdI-BsrBRI, 441 bp SmaI-BsrBRI, 230 bp MluI-BsrBRI and 42 bp DNA oligomer-BsrBRI). All fragments share the same 3' BsrBRI site downstream of the *survivin* transcription start sites (see below) and upstream of the initiating ATG. The survivin fragments were inserted in the forward orientation upstream of a luciferase reporter gene to generate pLuc-6270c, pLuc-2840c, pLuc-1430c, pLuc-649c, pLuc-441c, pLuc-230c and pLuc-42c, using SalI-SmaI, BamHI-SmaI, SmaI, SmaI, SmaI and SmaI sites respectively. A set of control constructs with the survivin promoter fragments in the reverse orientation, pLuc-1430t, pLuc-649t, pLuc-441t, pLuc-230t and pLuc-42t, was also generated, using the SmaI site. Nested deletion of the proximal 441 nt region of the survivin promoter was carried out by exonuclease III (Promega) digestion. Briefly, pLuc-441c (100–150 μg/ml) was entirely digested with SalI, filled-in with 50 units of Klenow DNA polymerase/ml (Promega) in 20 mM Tris/HCl (pH 8.0)/100 mM MgCl, and blocked with 40 μ M α -phosphorothioate dNTP/ 1 mM DTT for 10 min at 37 °C. After heat inactivation of the

enzyme for 10 min at 70 °C, the end-blocked DNA was phenolextracted, ethanol-precipitated and digested with BamHI, followed by phenol extraction and quantification. Aliquots of DNA (5 μg) in 60 μl were incubated at 25 °C in 66 mM Tris/HCl (pH 8.0), 0.66 mM MgCl₂ and 400 units of exonuclease III. Samples (2.5 μ l) were mixed with 7.5 μ l of 40 mM potassium acetate (pH 4.6), 340 mM NaCl, 1.35 mM ZnSO₃, 7 % glycerol and 300 units/ml S1 nuclease with a 30 s interval for the first eight time-points and a 15 s interval for the subsequent timepoints. The S1 reaction mixtures were incubated for 30 min at 22 °C, stopped with 1 μ l of 0.3 M Tris base and 0.05 M EDTA and heat-inactivated for 10 min at 70 °C. Nested DNA samples were blunted with 100-150 units of Klenow/ml for 10 min at 37 °C, re-ligated in 50 mM Tris/HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 5% poly(ethylene glycol), 1 mM DTT and 5 units of T4 ligase/ml at 16 °C overnight, and transformed in NM522 Escherichia coli. Nested clones carrying progressive deletions from the 5'-end were identified by PCR amplifications of 10–20 colonies from each time point.

Transient transfections and *survivin* promoter—luciferase reporter expression

HeLa cells were transiently transfected with the various survivin promoter-luciferase constructs by Lipofectamine (Gibco BRL). Briefly, cells were seeded in a 12-well plate $[(1-2) \times 10^5]$ cells/ well] in 1 ml of complete growth medium and grown at 50–80 % confluence before transfection. Opti-MEM-1 (50 µl; Gibco BRL) containing 1 µg of various plasmid DNAs was mixed with 50 μl of Opti-MEM-1 containing 4 μl of LipofectAMINE reagent for 30–45 min at 22 °C. The mixtures containing 350 µl of Opti-MEM-1 were overlaid onto monolayers of the various cell lines pre-incubated under serum-free conditions for 20-30 min. After 4-6 h incubation at 37 °C, the DNA-liposome complex was replaced with complete medium and cultivated for an additional 36-48 h at 37 °C. Cells were washed in PBS, pH 7.4, solubilized in 1 × lysis buffer (Promega) and scraped with a rubber policeman, then 5 μ l aliquots of the supernatant were mixed with 10 μ l of luciferase assay reagent (Promega) and analysed on a Lumat luminometer (LB9510). Alternatively, 10 µl of sample was incubated with 10 μ l of 2 × assay buffer (Promega) in a 96-well plate for 30–60 min at 37 °C and β -galactosidase activity was determined by absorbance at 405 nm. Luciferase activity under the various conditions tested was normalized to β -galactosidase activity used as an internal control.

Mutation analysis of the survivin minimal promoter

An unbiased PCR-based strategy was used to generate a mutational library targeting the survivin GC-rich proximal promoter region. Two oligonucleotides containing the underlined restriction sites 5'CGCGGATCCGCGTTCTTTGAAAGCAG-TC3' (cyc-1, -268 to -249) and 5'CCCAAGCTTTGCCGC-CGCCGCCACCTCTG3' (cyc-2, +1 to -19) were used (200 nM) for PCR amplification of survivin DNA (10-20 ng), with 50–200 μM dNTPs and 1.25 units of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD, U.S.A.). This amplification strategy for the survivin CG-rich region favours Taq-based incorporation of a single nt transition at sub-optimal concentrations of exogenous dNTPs. After pre-heating at 94 °C for 3 min, 35 cycles of amplification were carried out at 94 °C for 45 s, 55 °C for 30 s and 72 °C for 1 min, followed by a 10 min incubation at 72 °C. Purified PCR products were digested with BamHI and HindIII and directionally cloned upstream of the luciferase reporter gene in pLuc to obtain a degenerate pLuc-cyc1.2 (269 nt)

plasmid pool, containing both wild type and various *survivin* mutants. Following transformation in competent *E. coli*, the various plasmids from independent positive clones were transfected in HeLa cells and analysed for luciferase activity as described above. Relevant plasmids expressing reduced luciferase activity in mammalian cells were further characterized by DNA sequencing.

Electrophoretic mobility-shift assay (EMSA)

Complementary oligonucleotides of canonical Sp1 (5'ATTCG-ATCGGGGCGGGCGAGC3' 5'GCTCGCCCCGCCCCGA-TCGAAT3') and AP-2 (5'GATCGAACTGACCGCCCGCG-GCCCGT3′, 5′ACGGGCCGCGGGCGGTCAGTTCGATC3′) sites were obtained from Promega. Complementary oligonucleotides encompassing the wild type (5'AGCCACGC-GGCGGGAGGACT3', 5'AGTCCTCCCGCCGCGTGGCT3', Sp1), and mutated (Mut-2) survivin Sp1 site ($G^{171} \rightarrow A$, Sp1m2, 5'AGCCACGCaGCGGGAGGACT3', 5'AGTCCTCCCGCt-GCGTGGCT3') were synthesized. A control mutated (Mut-1) sequence upstream of the survivin Sp1 site $(G^{173} \rightarrow A, Sp1m1,$ 5'AGCCACaCGGGGAGGACT3', 5'AGTCCTCCCGCC-GtGTGGCT3') was also synthesized. Complementary oligonucleotides (3.5 pmol) were annealed in equimolar concentrations, 5'-end-labelled with 1 μ l of $[\gamma^{-32}P]ATP$ (3000 Ci/mmol) and 10 units of T4 polynucleotide kinase for 10 min at 37 °C, and recovered by ethanol precipitation. For EMSA, total reaction mixtures (9 µl) containing 10 mM Tris/HCl (pH 7.5), 1 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA, 50 mM NaCl, 4% glycerol and 50 μg of poly(dI-dC)–poly(dI-C)/ml, were incubated with and without HeLa cell nuclear extracts (Promega) and various unlabelled competing oligonucleotides for 10 min at 22 °C, followed by addition of 1 μ l [(0.5–2) × 10⁵ c.p.m.] of the various ³²P-end-labelled oligonucleotides. After a 20 min incubation at 22 °C, samples were separated by electrophoresis on a 4 % non-denaturing polyacrylamide gel, with detection of radioactive bands by autoradiography for 16-24 h at $-80 \,^{\circ}\text{C}$.

RESULTS

Sequence analysis of putative survivin promoter region

The entire human survivin locus spanning 14796 nt on chromosome 17q25 was assembled in contigs, subcloned and completely sequenced, as described elsewhere [6,11]. The DNA sequence of ~ 1.4 kb of the *survivin* gene upstream of the initiating ATG is shown in Figure 1(A). Sequence analysis of the 5' flanking region of the survivin gene using the TFsearch and Signal Scan algorithms revealed the absence of a TATA box and the presence of a GC-rich region extending up to -254 nt (numbering from the initiating methionine), with several potential Sp1 sites (Figure 1A). The percentage of GC dinucleotides in this region ranged between 65 and 80 %, with a CG:GC ratio of 0.94, thus fulfilling the length and base composition criteria of a canonical CpG island [12]. Clustal alignment of the human and mouse [13] survivin genes revealed a high degree of conservation of proximal putative promoter regions, including the CpG island (Figure 1B). The proximal 5' flanking region of the human survivin gene also contained three cell cycle dependent elements (CDE) (GGCGG at -6, -12, -171) and one cell cycle homology region (CHR) (ATTTGAA at -42), implicated in G1 transcriptional repression in several S/G2-regulated genes [14] (Figure 1A). In previous experiments, transfection of *survivin* promoter luciferase constructs containing all four CDE/CHR elements directed a 5-10-fold increased promoter activity in G2/Msynchronized HeLa cells, whereas deletion of the proximal CDE

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CCTGCCTAGG CCTCTCAAAG TGTTGGGATT ACAGGCGTGA GCCACTGCAC CCGGCCTGCA
Sp1

GEGGTGGGT GAAAGAGAGTC GAGGGGGGGC TAGGTGTGGG CAGGGACGAG CTGGCGCGGC

Sp1

GUE/Sp1

GCGCTGGGT GCACCGCGAC CAUGGGCAGA GCCCGGG GGGAGGACTA CAAC
                                                 -209
В
HUMPROM.SEQ
          ACGCGTTCTTTGAAAGCAGTCGAGGGGGGGGCGCTAGGTGTGGGCAGGACGAGCTGGCGCG
MOUSPROM.SEQ
          AAGATTCGAGTCAGAATAG-CAAGAAGGAACCGCA-GCAGA-AGGTAC-AACTCCCAGC
CCGGCACACCCCGCCCCCCCCCCTCTACTCCCAGAAGGCCGCGGGGGGGTGGACCGCC
HUMPROM.SEO
          CCGGCTCGCCTCGCGCCGTCC------ACTCCCAGAAGGCAGCGGGCGAGGG--CGT-
MOUSPROM. SEO
HUMPROM.SEQ
          AAGAGGGCGTGCGCTCCCGACATGCCCCGCGGCGCGCGCATTAACCGCCAGATTTGAATC
          -GGGGCCGGGGCTCTCCCGGCATGCTCTGCGGCGCGCCTCCGCCCGGGGGATTTGAATC
MOUSPROM.SED
CCCCTGCCTGGCAGCCCTTTCTCAAGGACCACCGCATCTCTACATTCAAGAACTGGCCC
HUMPROM.SEQ
                        HIIII
          CCCAGATCTGGCAGCTGTACCTCAAGAACTACCGCATCGCCACCTTCAAGAACTGGCCC
MOUSPROM. SEQ
          HUMPROM.SEQ
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Figure 1 Promoter sequence analysis of survivin genes

(A) DNA sequence analysis of the 5' flanking region of the human *survivin* gene. Human P1 genomic clones encoding survivin were isolated and characterized as described previously [6]. Numbering is from the initiating ATG. Canonical Sp1, Sp1-like sites and CDE/CHR elements [14,23] are boxed. Arrows indicate the position of the two main transcription start sites identified by primer extension and S1 nuclease protection experiments (see Figure 2). An upward arrow indicates the first intron—exon boundary. (B) Clustal alignment of the proximal promoter regions of the human and mouse *survivin* genes. The translational start sites are boxed. An upward arrow indicates the end of the first exon.

boxes abolished cell cycle periodicity of human *survivin* gene expression [10].

Identification of transcription start sites(s) in the human survivin gene

A schematic diagram of the 5' flanking region of the *survivin* gene and the position of primer/probe used in primer extension and S1 nuclease mapping is shown in Figure 2(A). In primer extension experiments, reverse transcription of a 32 P-end-labelled oligonucleotide (+36 to +9) annealed to poly(A)⁺ mRNA from HeLa cells generated a single product of 95 nt (Figure 2B), corresponding to a region within -57/-61 nt in the human *survivin* gene (Figure 1). In contrast, no specific products were generated in the presence of yeast tRNA (Figure 2B). In S1

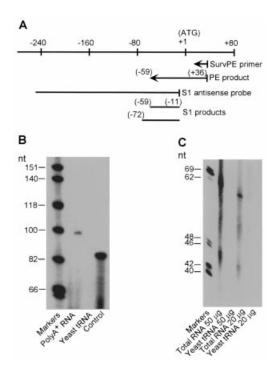


Figure 2 Identification of transcription start sites in the human $\mathit{survivin}$ gene

(A) Diagram of the human *survivin* promoter region. The position of primer/product and probe/products used in primer extension and S1 nuclease mapping is shown. (B) Primer extension. A $^{32}\text{P-labelled}$ antisense oligonucleotide comprising the *survivin* sequence +36 to +9 was annealed to HeLa cell poly(A) $^+$ RNA, reverse transcribed and the derived product of 93–97 nt separated on 7 M urea denaturing polyacrylamide gels followed by autoradiography. Control, RNA primer extension product of 87 nt. (C) S1 nuclease protection assay. A gel-purified $[\alpha^{-32}\text{P}]\text{dCTP-labelled}$ survivin antisense probe comprising a 250 nt region shown in (A) (-260 to -11) was hybridized to $20-50~\mu\text{g}$ of HeLa cell total RNA or control yeast tRNA overnight at 42 °C, followed by incubation with 1000–1500 units/ml S1 nuclease. Protected fragments were ethanol-precipitated, electrophoresed on a 7 M urea polyacrylamide gel and visualized by autoradiography.

nuclease protection experiments, a *survivin* antisense probe extending from -11 up to -240 nt at the 5'-end, protected in a template concentration-dependent manner a fragment migrating at position 46, consistent with the position of the transcription initiator within -57/-61 identified by primer extension (Figure 2C). The same probe also protected a second fragment of approx. 61 nt, which corresponds to position -72 in the *survivin* gene (Figure 2C). The fact that only S1 mapping identified this potential upstream initiator suggested the existence of strong secondary structures potentially associated with the CpG island, which required extensive denaturing conditions (i.e. 85% formaldehyde in S1 mapping).

Methylation status of the survivin CpG island

A potential role of methylation in the differential tissue distribution of the *survivin* gene [6] was next investigated. The position of the CpG island in the *survivin* gene and of methylation-sensitive restriction enzymes *SacII* and *Eco0191I*, or the methylation-insensitive restriction enzyme *DraI*, are shown in Figure 3(A). PCR amplification of *DraI*-digested genomic DNA isolated from normal and neoplastic tissues generated a single product of the expected molecular mass of 1.2 kb, consistent with the position of the amplifying forward (-705 to -686) and reverse (+474 to +493) oligonucleotides (Figure 3B). In contrast,

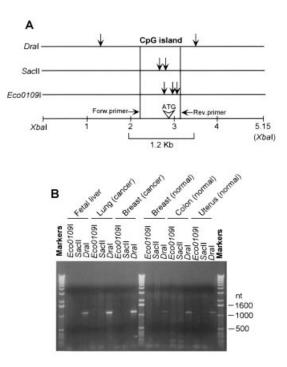


Figure 3 Methylation status of the CpG island in the human survivin gene

(A) Schematic diagram of the 5'-flanking region of the survivin gene. The positions of the CpG island, initiating ATG (arrowhead), amplifying primers and methylation-sensitive (SacII and Eco01091) and -insensitive (Dra1) restriction sites are indicated. (B) PCR amplification of digested genomic DNA. Human genomic DNA was extracted from the indicated tissues, digested with methylation-sensitive restriction enzymes SacII and Eco01091 and amplified with oligonucleotide primers indicated in (A). A control PCR product with the expected size of 1.2 kb was amplified from all genomic DNA samples analysed after digestion with methylation-insensitive restriction enzyme Dra1 under the same experimental conditions.

amplification of *SacII*- and *Eco0191I*-digested genomic DNA from the various tissues examined did not generate any specific PCR product(s) (Figure 3B), thus suggesting that these restriction sites were unmethylated. Similar results were obtained by

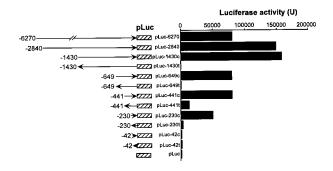


Figure 4 Identification of promoter sequences in the human survivin gene

The indicated survivin constructs were generated by restriction digestion and inserted in forward (cis, c) or reverse (trans, t) orientation upstream of a luciferase reporter gene in pLuc, transiently transfected in HeLa cells by Lipofectamine and analysed for luciferase activity at 36–48 h after transfection. Luciferase units (U) were calculated by dividing the luciferase activity (counts in 10 s) by the absorbance at 405 nm of β -galactosidase expression as an internal control. Results are the means of duplicates of a representative experiment out of at least three independent determinations.

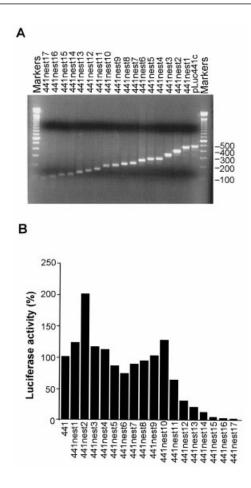


Figure 5 Deletion analysis of the minimal survivin promoter

(A) PCR analysis of nested deletion constructs. The *survivin* pLuc-441c construct shown in Figure 4 was progressively digested with exonuclease III, followed by re-ligation, transformation and direct PCR amplification of resulting colonies. The PCR products containing progressive deletions of approx. 20–25 nt from the 5' end were separated by 3% agarose gel electrophoresis and visualized by ethidium bromide staining. (B) Functional analysis of exonuclease III-generated nested deletion constructs of pLuc-441c. The nested *survivin* promoter—luciferase constructs corresponding to the fragments identified in (A) were transfected in HeLa cells and analysed for luciferase activity as described in Figure 4(B). The luciferase activity obtained from full-length pLuc-441c was considered as 100%. For (B), results are the means of duplicates of a representative experiment out of three independent determinations.

Southern hybridization of human genomic DNA (results not shown).

Identification of promoter sequences in the human survivin gene

Truncated constructs extending up to -6270 nt in the *survivin* gene were inserted in forward or reverse (control) orientation upstream of a luciferase reporter gene (pLuc) and analysed for luciferase activity in transiently transfected HeLa cells (Figure 4). Significant luciferase activity was observed after transfection of the construct containing the proximal 230 nt region upstream of the *survivin* transcription start sites (pLuc-230c), whereas transfection of the most proximal 42 nt (pLuc-42) did not generate luciferase activity (Figure 4). Transfection of the more upstream *survivin* sequences, pLuc-441c and pLuc-649c, resulted in a significant increase in promoter activity as compared with pLuc-230c, whereas none of the *survivin* constructs in the reverse orientation (pLuc-230t, pLuc-441t, pLuc-649t) was transcriptionally active in HeLa cells (Figure 4). Expression of pLuc-

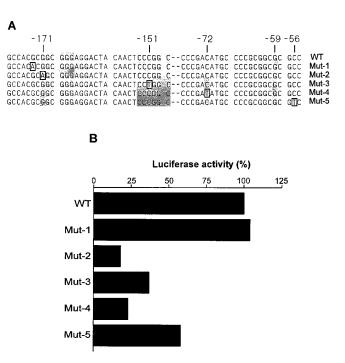


Figure 6 Unbiased mutational analysis of survivin promoter sequences

(A) DNA sequence analysis of wild-type and mutated *survivin* minimal promoter. An unbiased mutational library of the GC-rich region in the *survivin* core promoter was generated by PCR. Products were inserted in pLuc, analysed for luciferase activity and relevant plasmids were further characterized by DNA sequencing. Critical Sp1 sites at positions —171 and —151 are tinted. Single nt changes inserted by PCR amplifications are boxed in bold. (B) Luciferase activity of *survivin* promoter mutants. Wild-type or mutated constructs were transfected in HeLacells with determination of luciferase activity 36—48 h after transfection, as described in Figure 5. Results are the means of duplicates of a representative experiment out of three independent determinations.

1430c and pLuc-2830 generated a further $\sim 50\,\%$ increase in luciferase activity (Figure 4). In contrast, transfection of the longest *survivin* construct pLuc-6270 resulted in a level of promoter activity indistinguishable from that obtained with the more proximal sequences pLuc-441c and pLuc-649c (Figure 4), thus suggesting the presence of a potential repressor element(s) harboured upstream of -2840.

Nested constructs of pLuc-441c, carrying a series of deletions that originate from the 5'-end with progressive increments of approx. 20–25 nt as estimated by electrophoresis on 3 \% agarose gels (Figure 5A), were analysed for promoter activity in transfected HeLa cells. With the exception of the second deletion construct, 441nest2, which resulted in a \sim 2-fold increase in luciferase activity, the additional nested constructs 441nest1 to 441nest10 generated luciferase activity comparable with that obtained with full-length pLuc-441c (Figure 5B). The potential presence of negative regulatory element(s) deleted in the 441nest2 construct has not been further investigated. In contrast, transfection of the proximal deletion constructs 441nest11 to 441nest17 was associated with progressive loss of promoter activity, which was completely abolished for sequences comprising the first 90-110 nt upstream of the survivin translational initiation site (Figures 5A and 5B). These results are consistent with the initial transcriptional mapping data reported above, in which expression of pLuc-230c (equivalent to 441nest11) was sufficient to generate basal promoter activity of the human survivin gene (Figure 4).

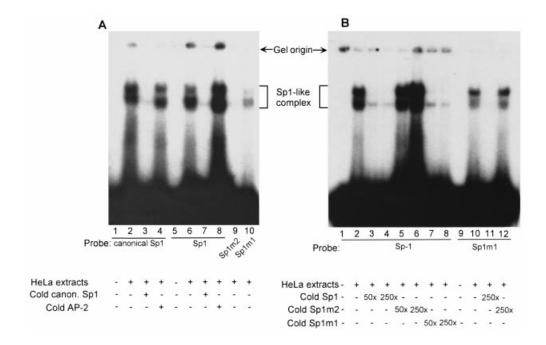


Figure 7 Physical association of Sp1/Sp1-like proteins with the *survivin* promoter region by EMSA

HeLa cell nuclear extracts were incubated with $[\gamma^{-32}P]$ ATP-labelled oligonucleotides of canonical Sp1, wild type (Sp1), or a control mutated sequence upstream of the Sp1 site, with no effect on promoter activity (Sp1m1, Mut-1, Figure 6). Formation of DNA—protein complexes was assessed in the presence and absence of the indicated concentrations of the unlabelled competitors.

Participation of functional Sp1 sites in survivin gene transcription

An unbiased mutational library was generated by PCR amplification of the proximal CG-rich region of the survivin gene (pLuc-cyc1.2). PCR products potentially carrying Taq polymerase-inserted mismatch mutations were cloned in pLuc, analysed blind for luciferase activity in HeLa cells and relevant clones were further characterized by DNA sequence analysis. As shown in Figure 6(A), a $G \rightarrow A$ transition abolishing the distal CDE/Sp1 site at position -171 resulted in loss of promoter activity in transfected HeLa cells (Mut-2, Figures 6A and 6B). Similarly, a $C \rightarrow T$ mutation involving the more proximal Sp1 site at position -151 also resulted in loss of luciferase activity in HeLa cells (Mut-3, Figures 6A and 6B). In contrast a $G \rightarrow A$ change at position -173 immediately upstream of the distal Sp1 site (Mut-1) did not affect luciferase activity (Figures 6A and 6B). Two additional mutations in survivin promoter-luciferase constructs displayed a consistently reduced luciferase activity when transfected in HeLa cells. These included a $C \rightarrow T$ change abolishing the upstream transcription start site at position -72(Mut-4, Figures 6A and 6B), and another $C \rightarrow T$ change at position -56 immediately downstream of the proximal transcription start site, which caused a $\sim 40 \%$ reduction in promoter activity (Mut-5, Figures 6A and 6B). Although not directly participating in survivin gene transcription, it seems plausible to hypothesize that mutations surrounding the putative transcription start sites (Mut-4 and Mut-5) may affect the assembly of the basal transcriptional machinery and reduce survivin gene expression (Figures 6A and 6B).

By EMSA, incubation of a canonical Sp1 ³²P-labelled oligonucleotide with HeLa cell nuclear extracts resulted in the formation of a specific DNA–protein complex, in a reaction competitively inhibited by unlabelled canonical Sp1 oligonucleotide, but not by control AP-2 oligonucleotide (Figure 7A). Under these

experimental conditions, a survivin-specific Sp1 oligonucleotide also bound to HeLa cell nuclear extracts with the formation of a DNA-protein complex inhibited by canonical Sp1, but not by a AP-2 oligonucleotide (Figure 7A). In contrast, a mutated Sp1 ³²P-labelled oligonucleotide corresponding to the loss-of-function sequence in Mut-2 (Sp1m2, Figure 6B) failed to form a DNAprotein complex in HeLa cell extracts (Figure 7A). Consistent with its promoter activity (Figure 6B), a mutated ³²P-labelled oligonucleotide (Sp1m1) corresponding to the Mut-1 sequence upstream of the Sp1 site at position -173 (see above), resulted in complex formation with HeLa cell extracts, albeit less efficiently than the corresponding wild-type sequence (Figure 7A). In parallel experiments, a DNA-protein complex formed between wild-type survivin Sp1 and HeLa cell extracts was competitively inhibited by excess unlabelled wild-type Sp1 or Sp1m1 oligonucleotides, whereas the loss-of-function Sp1m2 sequence was ineffective (Figure 7B). Conversely, binding of ³²P-labelled Sp1m1 sequences to HeLa cell extracts was competitively inhibited by unlabelled wild-type Sp1 oligonucleotide, but not by the Sp1m2 sequence (Figure 7B).

DISCUSSION

In this study, we have shown that basal transcription of the cancer anti-apoptosis *survivin* gene [6] requires a proximal promoter region of ~ 250 nt in an unmethylated CpG island. Secondly, mutation of two critical Sp1 sites at positions -151 and -171 resulted in a $60\text{--}80\,\%$ reduction of *survivin* gene expression.

Zinc finger transcription factors of the Sp1 family have emerged as key modulators of a variety of essential cellular genes. In addition to activating RNA polymerase II, binding of Sp1 proteins to properly positioned GC boxes [15] has been implicated

in chromatin remodelling [16], maintenance of methylation-free CpG islands [17] and the control of many cell cycle related genes. Potentially modulated by association with E2F transcription factors [18] and retinoblastoma protein-like pocket proteins [19], Sp1-dependent transcription has been implicated in expression of various cell cycle regulated genes, including dihydrofolate reductase, thymidine kinase, histone H4 [20,21], CDC25C phosphatase [22] and cyclin A1 [3]. Consistent with this view, recent data have demonstrated that survivin gene expression is cell cycle dependent and maximal in the G2/M phase, resulting in a \sim 40fold up regulation of survivin RNA in mitotic cells [10]. Similarly to other G2/M-expressed genes, including cyclins A, B, Polo-like kinase and CDC25C [14,23], cell cycle periodicity of survivin gene expression required the integrity of proximal G1 repressor CDE elements at positions -8 and -2 in the *survivin* promoter [10]. Altogether, and by analogy with other S/G2M-regulated genes [14], these data suggest that Sp1 and/or other members of the Sp1 family may regulate basal transcriptional expression of the human survivin gene. This may be further modulated by CDE/CHR elements in the survivin gene, potentially involved in imparting cell cycle periodicity of expression in G2/M [10]. Alternatively, it is also possible that the numerous E2F-like binding sites observed in the proximal survivin promoter may contribute a context-dependent inhibition of transcription, as recently demonstrated for other TATA-less, GC-rich cell cycle regulated genes [24].

Recent studies have underscored the role of Sp1 in homoeostasis and embryonic development, as reflected by the lethal phenotype of Sp1-null mice at E10.5-11 [25]. Although other members of the Sp1 family, most notably Sp3, may compensate for the null phenotype and stimulate expression of Sp1-regulated genes [25], it is intriguing that survivin is abundantly expressed during early mouse development, i.e. E11-11.5 [26]. This is in line with a critical role of apoptosis inhibitors, i.e. bcl-2, in tissue morphogenesis and differentiation [27], and suggests that Sp1 may contribute to survivin gene expression and control of apoptosis during early developmental stages [26]. In this context, other apoptosis inhibitors, including bcl-2 [28] and bcl-X [29], have been previously characterized for the presence of upstream GC-rich promoter sequences, containing numerous Sp1 and transcription initiation sites. Functionally, apoptosis inhibition by IAP molecules [4,5], or bcl-2 [3], involves non-overlapping pathways, suppressing effector caspase activity or preserving mitochondrial integrity respectively. This suggests that Sp1dependent transcription may influence expression of both classes of apoptosis inhibitors and integrate, at least in part, the control of cell survival during development and differentiation.

In summary, we have elucidated the basal transcriptional requirements of *survivin* gene expression and identified a potential role of Sp1 sites in IAP-dependent control of apoptosis. These data should be helpful in identifying the molecular basis of the

dramatic over-expression of *survivin* in all the most common human cancers [6] and its participation in an apoptotic checkpoint at mitosis [10].

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