The Cancer Antiapoptosis Mouse Survivin Gene: Characterization of Locus and Transcriptional Requirements of Basal and Cell Cycle-dependent Expression

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ABSTRACT

Survivin is the first apoptosis inhibitor described to date to be expressed in G2-M in a cell cycle-dependent manner and to directly associate with mitotic spindle microtubules. To gain additional insights into this novel apoptotic checkpoint, we have now characterized the mouse survivin locus. Hybridization screening of mouse BAC libraries identified a survivin gene containing four exons and three introns, spanning >50 kb on the telomere of chromosome 11E2 and generating a 0.85-kb mRNA versus the 1.9-kb human transcript. A mouse survivin protein of 140 amino acids (M, −16,200) was 84% identical to its human orthologue and contained a structurally unique single baculovirus IAP repeat (BIR) and a −COOH-terminus coiled domain instead of a RING finger. Analysis of the 5′-flanking region of the mouse survivin gene revealed a TATA-less promoter containing a canonical Cpg island, numerous Sp1 sites, two cell cycle-dependent elements (CDEs), and one cell cycle gene homology region (CHR), typically found in G2-M-expressed genes. Primer extension and S1 nuclease mapping identified three transcription start sites at position −32, −36, and −40 from the initiating ATG. Transfection of survivin promoter-luciferase constructs identified a minimal promoter region within the most proximal 174 bp upstream of the first ATG. Transcription of survivin in human diseases, and cancer in particular, was recently suggested by the identification of the human survivin gene (14). Survivin is the smallest member of the IAP gene family, structurally characterized by a single BIR module and a −COOH-terminus α-helix coiled domain (14), substituting a canonical RING finger (4). Prominently expressed in a variety of apoptosis-regulated organs during embryonic and fetal development (15), survivin is undetectable in most normal adult tissues, and becomes abundantly reexpressed in a variety of human cancers in vivo (14).

INTRODUCTION

The genetic control of apoptosis (programmed cell death) plays a critical role in differentiation, development, and maintenance of normal homeostasis (1). Among the regulators of apoptosis (2, 3), the IAP3 proteins (4) have recently emerged as potential modulators of the terminal effector phase of cell death/survival. Evolutionarily conserved from viruses to mammalian cells, IAP molecules are structurally organized in 2–3 Cys/His BIR and a −COOH-terminus RING finger (4, 5). Counteracting apoptosis induced by chemotherapeutic drugs, factor deprivation, tumor necrosis factor α, and overexpression of Bad and FADD (6–8), certain IAP proteins have been shown to bind and inhibit effector caspase-3 and -7 (9, 10), through a BIR-dependent recognition (11). IAP molecules may also play a role in human diseases, as judged by the frequent mutations involving the neuronal apoptosis inhibitor in type I spinal muscular dystrophy (12) and the cytoprotective effect of neuronal apoptosis inhibitor overexpression on ischemic/hypoxic neuronal death (13).

Compelling experimental evidence for a broader role of IAP proteins in human diseases, and cancer in particular, was recently suggested by the identification of the human survivin gene (14). Survivin is the smallest member of the IAP gene family, structurally characterized by a single BIR module and a −COOH-terminus α-helix coiled domain (14), substituting a canonical RING finger (4). Prominently expressed in a variety of apoptosis-regulated organs during embryonic and fetal development (15), survivin is undetectable in most normal adult tissues, and becomes abundantly reexpressed in a variety of human cancers in vivo (14).

Consistent with a potential pathogenetic role of dysregulation of apoptosis in cancer (16), survivin expression correlated with unfavorable histology in neuroblastoma (17), with p53 abnormalities and coexpression of bel-2 in gastric cancer (18), and with reduced apoptotic index and abbreviated survival in colorectal cancer (19). Recent studies have demonstrated that, at variance with previously known apoptosis inhibitors, survivin is expressed in G2-M in a cell cycle-dependent manner, and binds directly to mitotic spindle microtubules (20). Consistent with a role of bel-2X (21) and p53 (22)-dependent apoptosis in controlling genetic fidelity in G2-M, these data proposed a role of survivin in a novel apoptotic checkpoint at cell division (20).

In this study, we sought to dissect the molecular requirements and evolutionary conservation of this novel antiapoptotic mechanism by elucidating the structure of the mouse survivin locus. We found that the mouse survivin gene has features typical of G2-M-regulated genes and is transcriptionally controlled by the integration of multiple Sp1 sites and cell cycle-dependent G1 repressor elements (23).

MATERIALS AND METHODS

Cell Culture and RNA Extraction. Epithelial carcinoma HeLa, mouse lymphoma WEHI164, and normal mouse liver NMU2Li cells (American Type Culture Collection, Rockville, MD) were grown in DMEM (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (BioWhittaker) plus antibiotics. For total RNA extraction, mouse WEHI cells were washed in PBS (pH 7.4) and solubilized in TRI Reagent (106 cells/0.2 ml; Molecular Research Center, Inc., Cincinnati, OH) in the presence of 0.2 ml chloroform/ml of TRI Reagent. After centrifugation versus 12,000 g for 15 min at 4°C, total RNA was recovered by isopropanol precipitation. 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, 50 mM sodium citrate, and 0.1% SDS, with elution in 1 mM sodium citrate and 0.1% SDS and followed by isopropanol precipitation. Isolation of Mouse survivin Genomic DNA Clones. Genomic DNA was isolated from WEHI164 cells by proteinase K digestion and sequential phenol extraction, according to published protocols. A PCR product of 400 bp was amplified from WEHI genomic DNA with primers msg-P1 (exon I, 5′-GTACCTCGAAGACTACCGCATTCT-3′) and msg-P2 (exon II, 5′-GTATCGGTTCTCCAGGCTCTCC-3′) based on the region of high homology between human survivin and a mouse survivin EST sequence 34764. DNA sequence analysis of this PCR product demonstrated identity in putative exon regions with the mouse EST sequence and strong conservation with the human survivin sequence (14). A mouse genomic DNA BAC library (Genome Systems, St. Louis, MO) was screened by hybridization with this PCR probe with the isolation of two positive clones, BAC-A20 and BAC-J17 (Fig. 1A).

Chromosomal Location of the Mouse Survivin Locus. Aliquots of purified plasmid DNA from BAC clone J17 were labeled with digoxigenin dUTP by nick translation. The labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts in 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized DNA with antidigoxigenin antibody conjugated to alkaline phosphatase.
slides in the presence of fluoresceinated antidigoxigenin antibodies followed by counterstaining with 4,6 diamidino-2-phenylindole. In dual labeling experiments, a probe specific for the centromeric region of mouse chromosome 11 was cohybridized with BAC clone J17. A total of 80 metaphase chromosomes was analyzed, with 72 exhibiting specific labeling.

Southern Blotting, Subcloning, and DNA Sequence Analysis. Mouse survivin genomic clones BAC-A20 and BAC-J17 were digested with various restriction enzymes, separated on 1% agarose gels and transferred to GeneScreen (DuPont de Nemours, Wilmington, DE) nylon membranes. The membranes were hybridized with a 32P-labeled human survivin cDNA probe or a probe specific for the centromeric region of mouse chromosome 11 by counterstaining with 4,6 diamidino-2-phenylindole. In dual labeling experiments, aliquots of WEHI genomic DNA were mixed with primers msg-nestP7–1 (forward primer in exon IV, Fig. 1) and ms-5’walk-1 (reverse primer in exon III) in the presence of increasing concentrations of magnesium. After a 2 min-denaturation at 94°C, 40 cycles of amplification were carried out with primer combination ms-5’walk-1/AP-1 or ms-3’walk-2/AP-2 or ms-3’walk-2/AP-2, PCR products were separated on 1% agarose gels, purified by GeneClean II (Molecular Research Center, Cincinnati, OH), and sequenced with the Applied Biosystems model 373A DNA sequencing system (Foster City, CA).

Genomic DNA Walking and Intron III Amplification of the Mouse survivin Gene. Genomic DNA walking for mouse survivin exon IV was carried out with a Genome walking kit (Clontech, Palo Alto, CA), according to the manufacturer’s specifications. Briefly, two sets of nested primer were synthesized based on the deduced exon IV sequence of human survivin and mouse survivin locus (Fig. 1). MS-5’walk-1 (5’-CTCAGCATTAGGCACGACG-3’) and ms-5’walk-2 (5’-GACTGACGGGTAGTCTTTGC-3’) were used for 5’ nested-termini of exon IV walking; and

(b) Ms-3’walk-1 (5’-AAAGGAGACCAACAACAGC-3’) and ms-3’walk-2 (5’-CTACCCGTCAGTCAATTGAG-3’) were used for 3’ nested-termini of exon IV walking.

The template for DNA walking comprised mouse genomic DNA digested with five restriction enzymes (EcoRV, Scal, DraI, PvuII, and SphI) and ligated to a linker containing two nested primers, AP-1 and AP-2. A first round of PCR amplification was carried out with primer combination ms-5’walk-1/AP-1 or ms-3’walk-2/AP-2 or ms-3’walk-2/AP-2, PCR products were separated on 1% agarose gel, purified by GeneClean II (Molecular Research Center, Cincinnati, OH), and sequenced with the Applied Biosystems model 373A DNA sequencing system (Foster City, CA).

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Primer Extension and S1 Nuclease Protection Assay. A msg-PE oligonucleotide 5’-GATGCCTGGTGTTGCTCTT-3’ (+57 to +30, numbering from the initiating ATG) was synthesized, gel-purified, and 5’ end-labeled (10 pmol) in 50 m M Tris-HCl (pH 7.5), 10 m M MgCl2, 5 m M DTT, 0.1 m M spermidine, 3 m M of [γ-32P]ATP (3000 Ci/mmol, Amersham, Arlington
The Mouse Survivin Locus

MGAPALPQAWOLFLKDHRIATFKNWPFLASMOSER

30

10

20

Majority

1 MGAPALPQAWOLFLKDHRIATFK0WPLFELD0038231

MOUSE.PRO

1 MGAPALPQAWOLFLKDHRIATFK0WPLFELD0038231

HUMAN.PRO

CACPETRMAEAGFIHCPTENEDLALACFFCMajority

40

50

60

31 CACPETRMAEAGFIHCPTENEDLALACFFCMajority

MOUSE.PRO

HUMAN.PRO

FKELEGWEPPDDPILIEEHEKHS2GCAFLVSMajority

70

80

90

61 FKELEGWEPPDDPILIEEHEKHS2GCAFLVSMajority

MOUSE.PRO

HUMAN.PRO

KQFEETLVEFLKLDREERAKNIKAKETNKMajority

100

110

120

91 KQFEETLVEFLKLDREERAKNIKAKETNKMajority

HUMAN.PRO

QKEFEETAKTVQAIEQLAMDMajority

130

140

121 QKEFEETAKTVQAIEQLAMDMajority

MOUSE.PRO

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(c) mspn-3 (5'-CGCGGATCCAGCCTGGGACCATACACGAC-3', −555 to −534);
(d) mspn-4 (5'-CGCGGATCCTGGAAACCTTGAGGCAGG-3', −395 to −374);
(e) mspn-1 (5'-CGCGGATCCATGGGACACGAC-3', −210 to −191);
and
mspn-2 (5'-CGCGGATCCAGTGGGACGAC-3', −174 to −158).

BamHI and HindIII sites are underlined in each oligonucleotide. PCR products were inserted upstream of a luciferase reporter gene in pLuc at BamHI and HindIII sites to obtain pLuc-1342, pLuc-830, pLuc-539, pLuc-379, pLuc-194, and pLuc-158. A 30-nt mouse survivin promoter fragment was generated by annealing two 30-nt DNA oligomers (5'-AATCTCGTGTTTGGATGCTGCTTGGCTGGGGAGG-3', −46 to −17, and 5'-CTCCCGCGAAGACTCAACCGGAGAATG-3', −17 to −46) and then was subcloned upstream of the luciferase reporter gene in pLuc at Smal sites to obtain pLuc-30. The constructs of pLuc-1342, pLuc-830, and pLuc-379 were generated by deleting the proximal DNA sequence from −143 to −68, which contains two contiguous Sp1-like motifs. All of the plasmids were confirmed by DNA sequencing.

Mutagenesis of the Proximal Promoter Region of the Mouse Survivin Gene. A detailed mutagenesis analysis of the proximal mouse survivin promoter was carried out by PCR mutagenesis using oligonucleotides. The following constructs were generated:

(a) 158m1 with oligonucleotides mspc-2m1 (5'-CGCGGATCCAGCAGCGGGGACG-3', −174 to −154) and mspc-3 (5'-CCCAAGCTTCTCCGTAACAGACTCAAC-3', −17 to −38);
(b) 158m2 with oligonucleotides mspc-2m2 (5'-CGCGGATCCAGTGACTCGTGCGGGGACG-3', −174 to −154) and mspc-3 (5'-CCCAAGCTTCTCCGTAACAGACTCAAC-3', −17 to −38);
(c) 158m3 with oligonucleotides mspc-2 (5'-CGCGGATCCAGTGGGACGAC-3', −174 to −154) and mspc-3m1 (5'-CCCAAGCTTCTCCGTAACAGACTCAAC-3', −17 to −38);
(d) 158m4 with oligonucleotides mspc-2 (5'-CGCGGATCCAGTGGGACGAC-3', −174 to −154) and mspc-3m2 (5'-CCCAAGCTTCTCCGTAACAGACTCAAC-3', −17 to −38);
(e) 158m5 with oligonucleotides mspc-2 (5'-CGCGGATCCAGTGGGACGAC-3', −174 to −154) and mspc-3m3 (5'-CCCAAGCTTCTCCGTAACAGACTCAAC-3', −17 to −38);
(f) 158m6 with oligonucleotides mspc-2 (5'-CGCGGATCCAGTGGGACGAC-3', −174 to −154) and mspc-3m3 (5'-CCCAAGCTTCTCCGTAACAGACTCAAC-3', −17 to −38);
TGCTGGGGCGGGAC-3', -174 to -154) and mspc-3mSp1 (5'-CCCAAGCTTGTCCGGCAGAACGAATGCTTCGCGCGCAGG-3', -17 to -62).

Each of the mutated 158Sp1m1–3 fragments was also inserted in pLuc using BamHI and HindIII sites to obtain pLuc-158Sp1m1, pLuc-158Sp1m2, and pLuc-158Sp1m3. All of the plasmids were independently confirmed by DNA sequencing. The indicated restriction sites BamHI or HindIII are underlined in each mutagenized oligonucleotide.

Liposome-mediated Transient Transfection and Luciferase/β-Galactosidase Expression Assay. Cells were transiently transfected with the various mouse survivin promoter constructs by LipofectAMINE. Briefly, cells were seeded in a 12-well plate (1–2 × 10^5 cells/well) in 1 ml of complete DMEM growth medium and grown at 50–80% confluence. Fifty μl of Opti-MEM-I (Life Technologies, Inc.) containing 1 μg of various plasmid DNA molecules were mixed with 50 μl of Opti-MEM-1 containing 4 μl of LipofectAMINE reagent for 30–45 min at 22°C. Each of the above mixtures was supplied with 350 μl of Opti-MEM-1 and then overlaid onto the cell monolayer, which was preincubated under serum-free conditions for 20–30 min. After 4–6 h incubation at 37°C, the DNAliposome complex was replaced with complete medium, and luciferase activity was determined within 36–48 h from transfection. After two washes with PBS (pH 7.4), cells were solubilized in 60 μl of 1× lysis buffer (Promega) and scraped with a rubber policeman, and 5-μl aliquots of the supernatant were mixed with 10 μl of luciferase assay reagent (Promega) and analyzed on a Lumat luminometer (LB 9510). Alternatively, 10-μl samples were incubated with 10 μl of assay 2× buffer (Promega) in a 96-well plate for 30–60 min at 37°C and β-galactosidase activity was determined by absorbance at A_{405 nm}. Luciferase activity under the various conditions tested was normalized to the value of β-galactosidase activity used as an internal control.

HeLa Cell Synchronization. HeLa cells were transiently transfected with the indicated mouse survivin promoter constructs as reported above and synchronized to the various phases of the cell cycle by treatment with 400 μM mimosine (G1), 2 mM thymidine (S phase) and 0.4 mM mimosine (G2/M) for 16 h at 37°C. Cells were harvested, lysed, and analyzed for β-galactosidase-normalized luciferase activity as described above. Drug treatment resulted in the synchronization of >75% of HeLa cells in the various phases of cell cycle, as determined by propidium iodide staining and flow cytometry and in agreement with previous observations.

RESULTS

Characterization of the Mouse survivin Gene. Mouse genomic BAC libraries were screened by hybridization with a 400-bp PCR product amplified from WEHI genomic DNA using oligonucleotides msg-P1 and msg-P4 (Fig. 1B), derived from the mouse EST sequence 34764, which is highly homologous to human survivin (14). Two partially overlapping clones, designated BAC-A20 and BAC-J17 (Fig. 1A), were isolated and further characterized by restriction digest analysis and Southern hybridization. The DNA locus partially comprised by BAC-A20 and BAC-J17 contained a four-exon/three-intron gene preceded by a CG-rich region of ~200 bp upstream of a putative exon 1 (Fig. 1B). Southern hybridization and direct DNA sequencing (see below) of the putative exons confirmed the authenticity of this gene as the mouse orthologue of survivin (14). The 3' end of exon IV contained a canonical polyadenylation signal 399 bp downstream of an in-frame TGA translational stop codon, followed by a potential polyadenylation splice/addition site and several GT-clusters, consistent with transcription termination sites (not shown). With the exception of the large size of intron III (>50 kb) and of the smaller 3' untranslated region in exon IV, the overall structure of the mouse

Fig. 3. Chromosomal location of the mouse survivin gene by single-color (A) and two-color (B) fluorescence in situ hybridization. A, a dUTP digoxigenin-labeled BAC-J17 genomic clone hybridized with a medium size chromosome from metaphase mouse chromosomes (arrows). B, in colyhybridization with a probe specific for the centromeric region of chromosome 11, the mouse survivin probe reacted with the telomeric region of chromosome 11 to band 11E2. A total of 30 metaphase chromosomes were analyzed with 72 exhibiting specific labeling (arrows).
survivin gene resembled that of its human orthologue (Fig. 1D; Ref. 14). Analysis of the putative translational product revealed an open reading frame (ORF) of 420 bp, spanning all four of the exons and encoding a cytoplasmic protein of 140 amino acids (Fig. 2). By sequence alignment, the mouse survivin protein was 84.3% identical to the human sequence (14), and contained a single BIR module, found in IAP apoptosis inhibitors (4), and a 40-amino acid $\alpha$-helical COOH terminus, predicted to form a coiled (24) domain (Fig. 2). A second survivin-hybridizing locus, designated $\lambda$-CCR2, was also isolated from screening of $\lambda$ phage genomic libraries, subcloned, and further characterized. DNA sequence analysis of a 3.5-kb $XbaI$ fragment from $\lambda$-CCR2 revealed a GAAA expansion repeated 49 times and a region of 437 bp sharing 88% identity with the contiguous sequences of exons III and IV of the survivin gene (not shown). A potential role of $\lambda$-CCR2 in gene expression and/or its relationship with the mouse survivin locus was not further investigated.

**Chromosomal Location of Mouse survivin Gene.** In single-color fluorescence in situ hybridization, a dUTP digoxigenin-labeled probe BAC-J17 specifically reacted with a medium size chromosome (Fig. 3A). In two-color cohybridization experiments with a probe to the centromeric region of chromosome 11, specific labeling of the mouse survivin probe was observed in the telomeric region of mouse chromosome 11, to an area corresponding to band 11E2 (Fig. 3B).

**Identification of Transcription Start Site(s) in the Mouse survivin Gene.** A schematic map of the probe/primer used in S1 nucleic acid mapping and primer extension experiments is shown in Fig. 4A. In S1 mapping, an antisense DNA probe comprising the mouse survivin sequence +87 to –184 (Fig. 4A), protected—in a template concentration-dependent manner—three fragments migrating at position $\sim$119, $\sim$123, $\sim$127 (Fig. 4B), corresponding to –32, –36, and –40 from the initiating ATG in the mouse survivin gene (Fig. 4A). In control experiments, no radioactive bands were protected from yeast tRNA under the same conditions (Fig. 4B). In primer extension, a $\left[{\gamma}^{32}P\right]$ATP end-labeled oligonucleotide from +57 to +30 (Fig. 4A) identified three main products migrating at position $\sim$89, $\sim$93, and $\sim$97 in reverse-transcribed WEHI poly(A)$^+$ mRNA (Fig. 4C), which coincided with the transcription start sites identified by S1 nucleic acid mapping (Fig. 4B). In contrast, no specific products were reverse-transcribed from yeast tRNA (Fig. 4C).

**Sequence Analysis and Promoter Activity of the Mouse survivin Gene.** The putative regulatory sequences of the mouse survivin gene extending up to $\sim$1396 bp upstream of the initiating ATG is shown in Fig. 5. Analysis of this region using the transcription factor search and Signal Scan algorithms revealed the absence of a TATA box and the presence of several potential Sp1/Sp1-like sites, of two CDEs, and one CHR, typically found in G2-M-expressed genes (Ref. 23; Fig. 5). The 60–80% GC content of the proximal survivin promoter and its CG:GC dinucleotide ratio of 0.87 define this region as a canonical CpG island (25). Transfection of nested survivin promoter-luciferase constructs extending up to $\sim$1358 from the initiating ATG (Fig. 6A), revealed that maximal promoter activity was contained within the proximal 158-bp region in pLuc-158 (Fig. 6B). A comparable level of luciferase activity was observed after transfection of pLuc-survivin promoter constructs extending up to $\sim$1358 in the mouse survivin gene (pLuc-1342), whereas

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**Fig. 4. Identification of transcription start sites in the mouse survivin gene.** A, schematic diagram of probe/primer used in primer extension (PE) and S1 nucleic acid mapping of the mouse survivin gene. B, S1 nucleic acid protection. A gel-purified [a-32P]dCTP-labeled mouse survivin antisense probe comprising 271 bp shown in A was hybridized with WEHI poly(A)$^+$ RNA overnight at 42°C, followed by incubation with 1000–1500 units/ml S1 nuclease. Protected fragments were ethanol-precipitated, electrophoresed on a 7% 7-M urea polyacrylamide gel, and visualized by autoradiography (arrows). C, primer extension. An antisense oligonucleotide msg-PE (A) was annealed to WEHI poly(A)$^+$ RNA and reverse-transcribed, and the extended products were separated on 7-M urea polyacrylamide gels and identified by autoradiography (arrows).
transfection of the first 30 bp in the survivin promoter (pLuc-30) generated only backgound transcriptional activity (Fig. 6B). Deletion of the region comprised between -143 to -68, which contains a Sp1 complex at position -106, inhibited luciferase activity of pLuc-1342 by 28% (119,964 ± 1,919 versus 165,155 ± 11,395), of pLuc-830 by 55% (113,786 ± 4,096 versus 251,597 ± 11,573), and of pLuc-539 by 28% (145,378 ± 7,559 versus 200,157 ± 143,903). Consistent with a potential transcriptional mechanism of survivin overexpression in transformed cells, the survivin promoter constructs pLuc-194 and pLuc-1342 directed a >10-fold increased luciferase activity in epithelial carcinoma HeLa cells as compared with normal mouse liver NMU2Li cells (Fig. 6C).

Requirements of Basal and Cell Cycle-dependent Promoter Activity of the Mouse survivin Gene. A systematic mutation analysis of the proximal Sp1 sites and cell cycle-regulated elements in the survivin promoter (Fig. 5) was next carried out (Fig. 7A). Targeted disruption of the CDE/CHR cell cycle regulatory elements or of the proximal Sp1 sites independently resulted in a 50–65% reduction in luciferase activity in cell cycle-regulated elements or of the proximal Sp1 sites and cell cycle-regulated elements in the survivin gene. Simultaneous disruption of both Sp1 sites (Fig. 8A) resulted in an additive 60% inhibition of luciferase activity in asynchronously growing HeLa cells (Fig. 8B). Furthermore, disruption of the Sp1 site at position -62 partially inhibited by ~50% survivin gene expression in G2-M, whereas mutagenesis of the Sp1 site at position -62 completely abolished cell cycle periodicity of survivin gene expression in G2-M-synchronized HeLa cells (Fig. 8C). The combined disruption of both Sp1 sites did not further inhibit transcriptional activity in cell cycle-synchronized cultures (Fig. 8C).

DISCUSSION

In this study, we have characterized the structure of the mouse survivin gene and elucidated the molecular requirements of cell cycle-dependent transcriptional activity. At variance with apoptosis inhibitors of the bcl-2 (3) or IAP (4) family, survivin is expressed in G2-M in a cell cycle-dependent manner and directly associates with mitotic spindle microtubules (20). Disruption of the survivin-tubulin interaction or targeted mutagenesis of the caspase-binding survivin BIR module (26) resulted in increased caspase-3 activity and induction of apoptosis in G2-M-synchronized cells, thus postulating a role of survivin in a novel apoptotic checkpoint at cell division (20).

Consistent with a high degree of evolutionarily conservation of this G2-M apoptotic checkpoint, the mouse survivin gene displayed a four-exon/three-intron organization similar to the human gene (14), differing only for the size of intron III (50 kb versus 6.7 kb) and exon IV (513 bp versus 1230 bp). The mouse locus was also located to the telomeric region of the mouse survivin gene. The +1 position corresponds to the first ATG. Boxed letters, Sp1/Sp1-like complexes and CDE/CHR cell cycle gene elements. Arrows, transcription start sites identified by primer extension and S1 nuclease mapping (see Fig. 4).
A region of chromosome 11E2, reminiscent of the telomeric position of the human survivin gene at 17q25 (27). Intriguingly, a second survivin-hybridizing locus was also identified. In previous studies, Southern hybridization and Northern blots with single strand-specific probes suggested the existence of multiple survivin-related transcripts potentially originating from head-to-head duplicated gene(s) (27). Specifically, Southern blots from mouse genomic DNA identified 2 EcoRI fragments of 2 and 6.7 kb, hybridizing with survivin sequences (27). Here, the 2-kb band was identified as genuinely originating from the mouse survivin locus, whereas the 6.7-kb band comprised a nearly perfect duplication of uninterrupted survivin sequences in exons III and IV. Although consistent with postulated duplication events of survivin sequences (27), the potential participation of this second locus in gene expression and its topography with respect to the mouse survivin gene have not yet been determined.

For a shorter 3’ untranslated region in exon IV, transcription from the mouse survivin gene originates a 852-bp mature RNA as opposed to the larger 1.9-kb human transcript (14) and encodes a cytoplasmic protein of 140 amino acids with an estimated Mr of 16,200. The mouse survivin protein contained a structurally unique BIR module (4), characterized by the three-amino acid insertion Cys-Pro-Thr separating the two halves of the BIR domain, and exclusively found in human survivin (14), and the recently characterized single-BIR IAP molecule, BRUCE (28). Sharing an overall 84% identity with the human counterpart, the mouse survivin protein showed conservation of the critical residues Trp, Pro, and Cys in the BIR module required for apoptosis inhibition (Ref. 20 and unpublished observations), and potentially implicated in caspase-3 and -7 recognition (26). The second structural features of mouse survivin includes an α-helical-COOH terminus of 40 amino acids predicted to form a coiled structure (24) instead of a canonical RING finger (4). α-helical-coiled coil motifs are frequently found in microtubule-associated proteins (29, 30), potentially stabilizing charged electrostatic interactions with the acidic COOH-terminal domain of tubulin (31). Although less charged than its human counterpart, the mouse survivin COOH terminus may also mediate binding to mitotic spindle microtubules at cell division (20).

Reminiscent of typical G2-M-expressed, cell cycle-regulated genes (23, 32), the mouse survivin gene contained multiple transcription start sites, a TATA-less, GC-rich promoter region with numerous Sp1 sites, and two CDE (GGCGG) and one CHR (ATTTGAA) G1 repressor elements (23). As determined in transfection experiments, a 200-bp region immediately upstream of the initiating ATG was sufficient to direct maximal survivin promoter activity in asynchronized HeLa cells, and a 3- to 5-fold increased promoter activity in G2-M-synchronized cultures as compared with G1- or S phase-arrested cells. Although the spacing of the survivin CDE/CHR boxes is different from that of other G2-M-regulated genes (23, 32), deletion and mutagenesis studies identified two Sp1 sites at position –62 and –169, and the CDE/CHR G1 repressor elements (23), as

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Unpublished observations.
critically required for basal and cell cycle periodicity of survivin gene expression in G2-M. Altogether, these data suggest that transient G1 repression mediated by CDE/CHR boxes (23, 32) imparts cell cycle periodicity in G2-M to basal transcription of the mouse survivin gene mediated by Sp1.

Through potential interactions with S1 phase-inducer E2F (33), and Rb-like proteins (34), zinc finger Sp1 transcription factors regulate expression of a variety of essential genes participating in cell cycle progression, including dihydrofolate reductase, thymidine kinase, and histone H4 (35, 36). Sp1 also negatively regulates cell cycle progression through p53-independent transcription of cyclin-dependent kinase inhibitors p21\textsuperscript{waf-1/cip1} and p15\textsuperscript{INK4B} in response to growth-arresting stimuli, progesterone (37), butyrate (38), and transforming growth factor β (39). A potential role of Sp1 in chromatin remodeling (40) and maintenance of unmethylated CpG island (41) has also been proposed, whereas disruption of the Sp1 locus in mice resulted in profound developmental abnormalities and embryonic lethality (42). In this context, it is noteworthy that survivin is abundantly expressed during early mouse embryonic development at E11–11.5 and is prominently distributed in apoptosis-regulated neuronal tissues, including brain, choroid plexus, hypophysis, and dorsal root ganglion (15). This is consistent with a prominent role of apoptosis inhibitors, i.e., bcl-2,
in developmental homeostasis and tissue and organ morphogenesis (43). Similarly to the mouse survivin gene, the bcl-2 and bcl-X genes also contained TATA-less, GC-rich promoter regions, transcriptionally controlled by Sp1 sites (44, 45). Altogether, these findings propose a novel expanded role of Sp1-dependent transcription at the interface between the control of cell proliferation and multiple antiapoptotic pathways mediated by bcl-2 molecules (3) or via cell cycle-dependent expression of survivin in G2-M (20).

As one of its most intriguing feature, survivin becomes prominently expressed in a variety of cancers but is undetectable in many normal adult tissues in vivo (14). This paradigm may also hold true for mouse tissues, in which prominent expression of survivin RNA was detected in a variety of transformed cell lines of epithelial and lymphoid lineage but not in nontransformed cells.3 Rather than chromosomal abnormalities, the overexpression of survivin in cancer may reflect deregulation of Sp1-dependent transcription of the survivin gene because, as shown here, survivin promoter activity increased by >10-fold in transformed epithelial cells versus nontransformed normal mouse liver cells. In this context, the elucidation of the molecular requirements of survivin gene transcription as detailed here may provide alternative interventional strategies to block the increased expression of survivin in cancer (14) and its potential participation in disease progression (17).

REFERENCES

The Cancer Antiapoptosis Mouse Survivin Gene: Characterization of Locus and Transcriptional Requirements of Basal and Cell Cycle-dependent Expression

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