[CANCER RESEARCH 59, 3143–3151, July 1, 1999]

The Cancer Antiapoptosis Mouse *Survivin* Gene: Characterization of Locus and Transcriptional Requirements of Basal and Cell Cycle-dependent Expression[1]

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ABSTRACT

Survivin is the first apoptosis inhibitor described to date to be expressed in G_2-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_r ~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 G_2-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. Mutagenesis of the CDE/CHR elements and Sp1 sites in this region, alone or in combination, reduced transcriptional activity by 40–60% in asynchronously growing cells and abolished cell cycle periodicity in G_2-M-synchronized cells. These data demonstrate that cell cycle expression of survivin requires integration of typical CDE/CHR G_1 repressor elements and basal transcriptional activity by Sp1. Disruption of these transcriptional requirements may provide an alternative strategy to block the overexpression of survivin in cancer.

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 IAP[3] 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 *bcl*-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 G_2-M in a cell cycle-dependent manner, and binds directly to mitotic spindle microtubules (20). Consistent with a role of bcl-X_L (21) and p53 (22)-dependent apoptosis in controlling genetic fidelity in G_2-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 G_2-M-regulated genes and is transcriptionally controlled by the integration of multiple Sp1 sites and cell cycle-dependent G_1 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 (10^6 cells/0.2 ml; Molecular Research Center, Inc., Cincinnati, OH) in the presence of 0.2 ml chloroform/ml of TRI Reagent. After centrifugation × 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′-GTACCTCAAGAACTACCGCATC-3′) and msg-P4 (exon II, 5′-GTCATCGGGTTCCCAGCCTTCC-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. 1*A*).

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

Received 12/7/98; accepted 4/27/99.

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[1] Supported by NIH Grants RO1 HL54131 and CA78810. This work was done during the tenure of an Established Investigatorship Award from the American Heart Association (to D. C. A.).

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[3] The abbreviations used are: IAP, inhibitor of apoptosis; BIR, baculovirus iap repeats; CDE, cell cycle-dependent element; CHR, cell cycle gene homology region; EST, expressed-sequence tag.

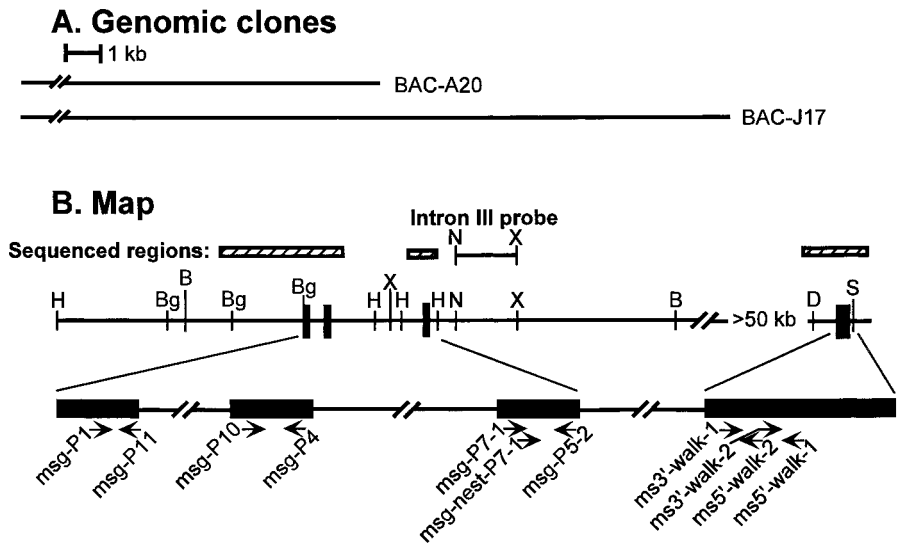

Fig. 1. Organization of the mouse *survivin* gene. *A*, map of overlapping mouse BAC genomic clones BAC-A20 and BAC-J17. *B*, map of the mouse *survivin* gene; ■, exons; *B*, *Bam*HI; *Bg*, *Bgl*II; *D*, *Dra*I; *H*, *Hin*dIII; *N*, *Not*I; *S*, *Sca*I; *X*, *Xba*I. The positions of *sequenced regions*, of an intron III-specific probe (*Intron III probe*), and of primers used for PCR amplification, sequencing, and genome walking are indicated. *C*, DNA sequence analysis of intron-exon boundaries in the mouse *survivin* gene. *D*, structural organization of human and mouse *survivin* genes. Exon sizes are indicated in bp; intron sizes are in kb.

C. Intron-exon boundaries

3'-Exon I — Intron I (0.28kb) — 5'-Exon II
CCCAGAGCGA/gtgagtcccagcttccagcgactcc--cccgctcacgagctgtcatttttcag/ATGGCGGAGG

3'-Exon II — Intron II (2.87kb) — 5'-Exon III
ACAACCCGAT/gtaagtcccacaggctactctcggt--------agtttaatttttttttttttattag/AGAGGAGCAT

3'-Exon III — Intron III (>50kb) — 5'-Exon IV
GAACAAAATT/gtatgtatgattgagaataaggact------ccttcgtgtctgttcatcattccag/GCAAAGGAGA

D. Human-mouse *survivin* gene structure

	Exon I	Intron I	Exon II	Intron I	Exon III	Intron III	Exon IV
Mouse	111	0.28	110	2.87	118	>50	513
Human	111	0.24	110	1.86	119	6.67	1230

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 were 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 membrane was hybridized with a ^{32}P-labeled human survivin cDNA probe or a mouse genomic PCR probe, as described previously (14). Radioactive bands identified by autoradiography were excised, inserted in pBluescript KS (Stratagene, San Diego, CA), and analyzed for DNA sequence using an 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 EST 34764:

(*a*) Ms-5′walk-1 (5′-CTCAGCATTAGGCAGCCAGC-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′-AAAGGAGACCAACAACAAGC-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 (*Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Ssp*I) 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-1/AP-1, followed by a second round of amplification with nested primers, ms-5′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). For extra-long PCR reactions, aliquots of WEHI genomic DNA were mixed with primers msg-P7–1 5′-GAGGAGCATAGAAAGCACTCCC-3′ (reverse primer in exon III) and ms-5′walk-1 (forward primer in exon IV, Fig. 1*B*) in the presence of increasing concentrations of magnesium. After a 2 min-denaturation at 94°C, 40 cycles of amplification were carried out in a Perkin-Elmer 480 thermal cycler with denaturation at 94°C for 30 s and annealing/extension at 68°C for 20 min, followed by one cycle at 72°C for 20 min. PCR products were subjected to a second round of amplification with the same protocol using nested primers msg-nestP7–1 (5′-CTGGCTGCGCGTTCCTCACT-3′) and ms-5′walk-2 (Fig. 1*B*). PCR products were separated on 0.5% agarose gels, transferred onto nylon membranes, and hybridized with a DNA probe specific for intron III in the mouse *survivin* gene (Fig. 1*B*).

Primer Extension and S1 Nuclease Protection Assay. A msg-PE oligonucleotide 5′GATGCGGTAGTTCTTGAGGTACAGCTGC-3′ (+57 to +30, numbering from the initiating ATG) was synthesized, gel-purified, and 5′ end-labeled (10 pmol) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 3 μl of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham, Arlington

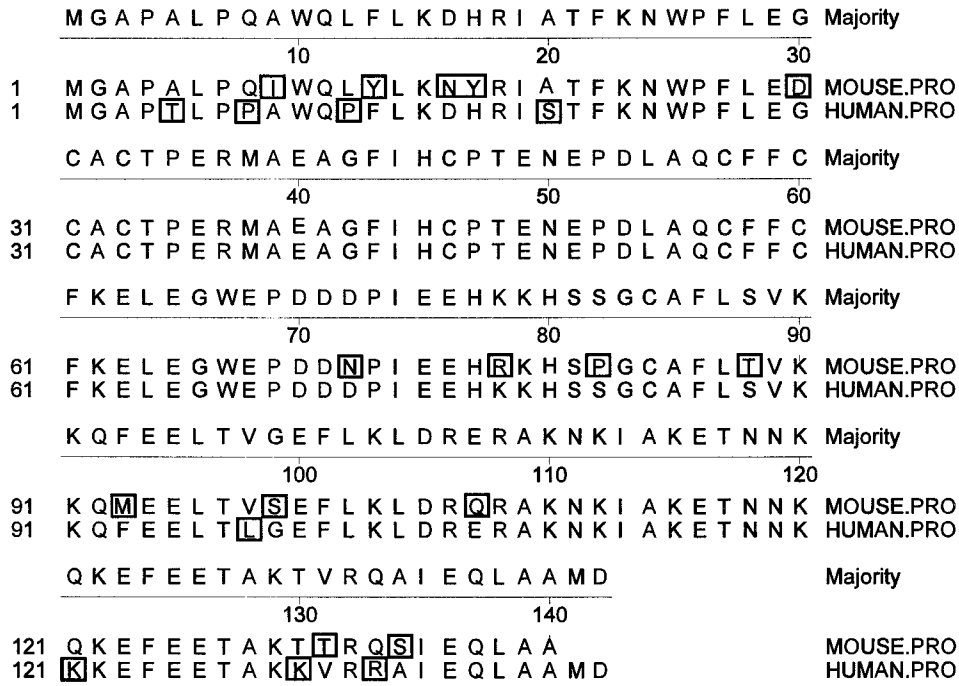

Fig. 2. Mouse survivin protein. Clustal-based alignment of human and mouse survivin proteins. *Boxed letters*, nonconserved residues.

Heights, IL) and 8–10 units of T_4 polynucleotide kinase (New England Biolabs, Beverly, MA) for 10 min at 37°C. A control primer and dephosphorylated ϕX174 *Hin*fI DNA fragments (250 ng, DNA markers, Promega) were also 5′ end-labeled. For primer extension, 100 fmol of labeled primer were mixed with 1–2 μg of poly(A)$^+$ RNA, 50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, and 0.5 mM spermidine for 20 min at 58°C and annealed at room temperature for 10 min. The annealed mixture was combined with a master mixture containing 50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 0.5 mM spermidine, 5.6 mM sodium PP_i, and 1 unit of AMV reverse transcriptase for 30 min at 42°C. Samples were ethanol-precipitated, suspended in 5–8 μl of gel loading buffer and separated on a 7-M urea-denaturing polyacrylamide gel. For S1 nuclease protection assay, a msg-P11 oligonucleotide 5′-CTCCAGGAAGGGCCAGTTCTTG-3′, (+66 to −87) was synthesized and gel-purified. An antisense DNA probe duplicating 271 nt of the 5′ flanking region of the mouse *survivin* gene was generated with Klenow fragment of DNA polymerase I. Briefly, 0.5–2 μg aliquots of *Bgl*I-digested *Hin*dIII fragment of mouse *survivin* gene in pBluescript in 25 μl were incubated with 30 pmol msg-P11 primer, 50 mM Tris-HCl, 10 mM $MgCl_2$, 200 μM dNTPs, 10 μl of [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml, Amersham) and 4–5 units of Klenow fragment (Ambion, Austin, TX) 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, and 0.2% SDS at 35°C overnight. The amount of 0.2–0.5 μg of WEHI cell poly(A)$^+$ RNA mixed with 1–2 × 10^5 cpm of gel-purified probe was ethanol-precipitated, resuspended in 10 μl of hybridization solution [80% formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate, and 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 $ZnCl_2$, 0.5% glycerol, and 1000–1500 units/ml of S1 nuclease for 30 min at 37°C. Samples were ethanol-precipitated, washed, and separated by electrophoresis on a 7-M urea denaturing polyacrylamide gel.

Generation of Mouse Survivin Promoter/Luciferase Constructs. Progressive deletion fragments of the 5′ flanking region of the mouse *survivin* gene of 1342 bp, 830 bp, 539 bp, 379 bp, 194 bp, and 158 bp were generated by PCR amplification. A *Hin*dIII-generated DNA fragment of the mouse *survivin* gene was used as a template for amplification with a reverse mspc-3 (5′-CCC*AAGCTT*CCTCCGCCAAGACGACTCAAAC-3′, −17 to −38) primer and each of the following forward primers:

(*a*) mspn-1 (5′-CGCGGATCCTGAAGTTGGCAAAAATCAC-3′, −1358 to −1337);

(*b*) mspn-2 (5′-CGCGGATCCGAATTATCAGGGCACTCG-3′, −846 to −827);

(*c*) mspn-3 (5′-CGCGGATCCAGCGCTGGGACATACAGAC-3′, −555 to −534);

(*d*) mspn-4 (5′-CGCGGATCCTGCAAACCTTGAGGCAGG-3′, −395 to −374);

(*e*) mspc-1 (5′-CGCGGATCCATGCCCTGCGCCCGCCACG-3′, −210 to −191); and

(*f*) mspc-2 (5′-CGCGGATCCAGATGGGCGTGGGGCGGGAC-3′, −174 to −154).

*Bam*HI and *Hin*dIII sites are underlined in each oligonucleotide. PCR products were inserted upstream of a luciferase reporter gene in pLuc at *Bam*HI and *Hin*dIII 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′-AATCCTGCGTTTGAGTCGTCTTGGCGGAGG3′, −46 to −17, and 5′-CCTCCGCCAAGACGACTCAAACGCAGGATT3′, −17 to −46) and then was subcloned upstream of the luciferase reporter gene in pLuc at *Sma*I sites to obtain pLuc-30. The constructs of pLuc-1342d, pLuc-830d, and pLuc-379d 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 using mutagenized oligonucleotides. The following constructs were generated:

(*a*) 158m1 with oligonucleotides mspc-2m1 (5′-CGCGGATCCAGATGGGCGTGGtGCGGGAC3′, −174 to −154) and mspc-3 (5′-CCCAAGCTTCCTCCGCCAAGACGACTCAAAC3′, −17 to −38);

(*b*) 158m2 with oligonucleotides mspc-2m2 (5′-CGCGGATCCAGATGGtCGTGGtGCGGGAC-3′, −174 to −154) and mspc-3 (5′-CCCAAGCTTCCTCCGCCAAGACGACTCAAAC-3′, −17 to −38);

(*c*) 158m3 with oligonucleotides mspc-2 (5′-CGCGGATCCAGATGGGCGTGGGGCGGGAC-3′, −174 to −154) and mspc-3m1 (5′-CCCAAGCTTCCTCCGaCAAGACGACTCAAAC-3′, −17 to −38);

(*d*) 158m4 with oligonucleotides mspc-2 (5′-CGC*GGATCC*AGATGGGCGTGGGGCGGGAC-3′, −174 to −154) and mspc-3m2 (5′-CCCAAGCTTCCTCCGaCAAGACGACTCAAACGCAGGATTCAcATCG-3′, −17 to −53);

(*e*) 158m5 with oligonucleotides mspc-2 (5′-CGCGGATCCAGATGGGCGTGGGGCGGGAC-3′, −174 to −154) and mspc-3m3 (5′- CCCAAGCTTCCTCCGaCAAGACGACTCAAACGCAGGATTCAcATCGCGCtGGCGG-3′, −17 to −62); and

(*f*) 158m6 with oligonucleotides mspc-2m2 (5′-CGCGGATCCAGATGGtCGTGGtGCGGGAC-3′, −174 to −154) and mspc-3m3 (5′- CCCAAGCT-

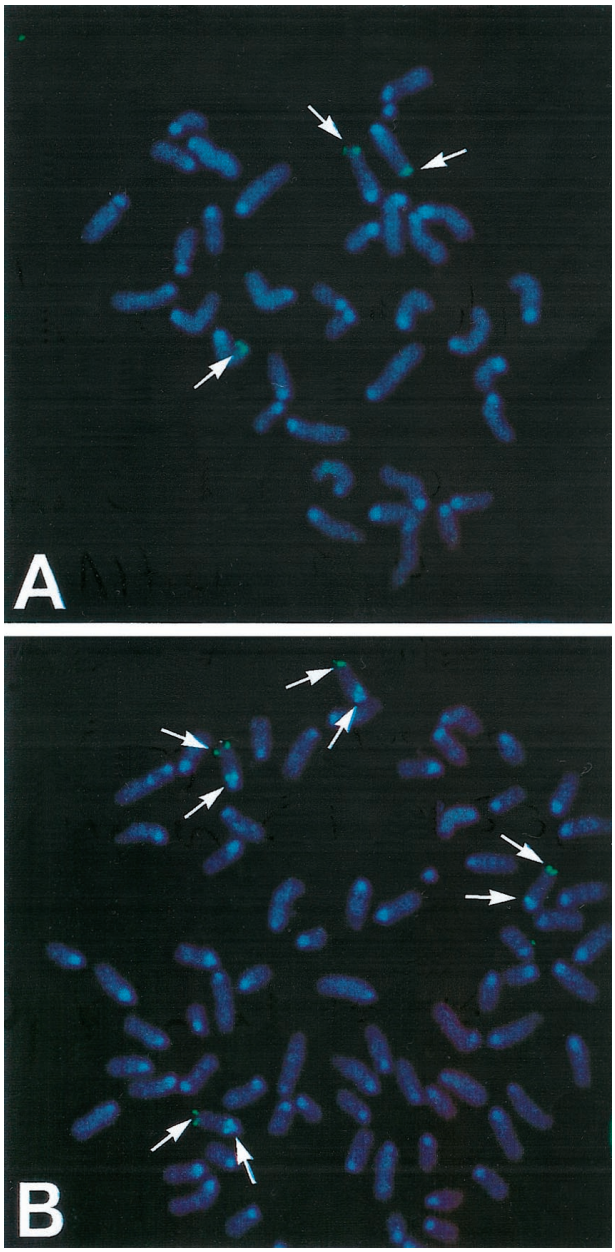

Fig. 3. Chromosomal location of the mouse *survivin* gene by single-color (*A*) and two-color (*B*) fluorescence *in situ* hybridization. *A*, a dUTP digoxigenin-labeled BAC-J17 genomic clone hybridized with a medium size chromosome from metaphase mouse chromosomes (*arrows*). *B*, in cohybridization 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 80 metaphase chromosomes were analyzed with 72 exhibiting specific labeling (*arrows*).

TCCTCCGaCAAGACGACTCAAACGCAGGATTCAcATCGCGCtGGCG-G3′, −17 to −62).

Each of the mutated 158m1–6 fragments was subcloned upstream of the luciferase reporter gene in pLuc using *Bam*HI and *Hin*dIII sites to obtain pLuc-158m1, pLuc-158m2, pLuc-158m3, pLuc-158m4, pLuc-158m5, and pLuc-158m6. The following mutagenized DNA constructs were also generated:

(*a*) 158Sp1m1 was generated with oligonucleotides mspc-2 (5′-CGCGGATCCAGATGGGCGTGGGGCGGGAC-3′, −174 to −154) and mspc-3mSp1 (5′-CCCAAGCTTCCTCCGCCAAGACGACTCAAACGCAGGATTCAAATCGCGCtGGCGG-3′, −17 to −62);

(*b*) 158Sp1m2 was generated with mspc-2mSp1 (5′-CGCGGATCCAGATGGtCGTGGGGCGGGAC-3′, −174 to −154) and mspc-3 (5′-CCCAAGCTTCCTCCGCCAAGACGACTCAAAC-3′, −17 to −38); and

(*c*) 158Sp1m3 was generated with oligonucleotides mspc-2mSp1 (5′-CGCGGATCCAGATGGtCGTGGGGCGGGAC-3′, −174 to −154) and mspc-3mSp1 (5′- CCCAAGCTTCCTCCGCCAAGACGACTCAAACGCAGGATTCAAATCGCGCtGGCGG-3′, −17 to −62).

Each of the mutated 158Sp1m1–3 fragments was also inserted in pLuc using *Bam*HI and *Hin*dIII sites to obtain pLuc-158Sp1m1, pLuc-158Sp1m2, and pLuc-158Sp1m3. All of the plasmids were independently confirmed by DNA sequencing. The indicated restriction sites *Bam*HI or *Hin*dIII 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-1 (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 DNA/liposome 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 (G_1), 2 mM thymidine (S phase) and 0.4 μg/ml nocodazole (G_2-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. 1*B*), 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. 1*A*), 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. 1*B*). 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 size of intron I (0.28 kb) and II (2.87 kb) was determined by PCR amplification from WEHI or BAC-J17 genomic DNA. The size of intron III (>50 kb) was determined by extra-long-range PCR amplification from WEHI genomic DNA with nested oligonucleotides (Fig. 1*B*) and confirmed by Southern hybridization with an intron III-specific probe (Fig. 1*B*). Sequence analysis of the intron/exon boundaries of the mouse *survivin* gene conformed to the known consensus (Fig. 1*C*). 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

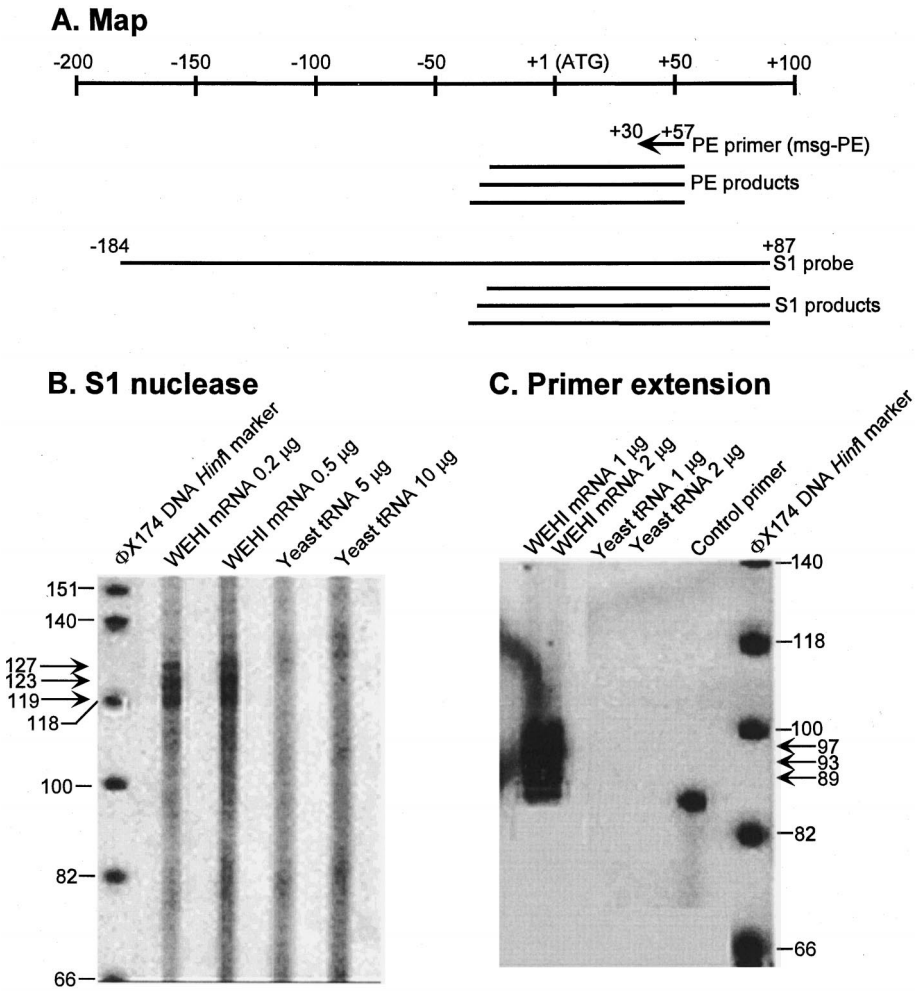

Fig. 4. Identification of transcription start sites in the mouse *survivin* gene. *A*, schematic diagram of probe/primer used in primer extension (*PE*) and S1 nuclease mapping of the mouse *survivin* gene. *B*, S1 nuclease protection. A gel-purified [α-^{32}P]dCTP-labeled mouse survivin antisense probe 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*).

survivin gene resembled that of its human horthologue (Fig. 1*D*; 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 α-helical COOH terminus, predicted to form a coiled (24) domain (Fig. 2). A second survivin-hybridizing locus, designated λ-*CCR2*, was also isolated from screening of λ phage genomic libraries, subcloned, and further characterized. DNA sequence analysis of a 3.5-kb *Xba*I fragment from λ-*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 λ-*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. 3*A*). 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. 3*B*).

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

Sequence Analysis and Promoter Activity of the Mouse *survivin* Gene. The putative regulatory sequences of the mouse *survivin* gene extending up to −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 G_2-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 −1358 from the initiating ATG (Fig. 6*A*), revealed that maximal promoter activity was contained within the proximal 158-bp region in pLuc-158 (Fig. 6*B*). A comparable level of luciferase activity was observed after transfection of pLuc-survivin promoter constructs extending up to −1358 in the mouse *survivin* gene (pLuc-1342), whereas

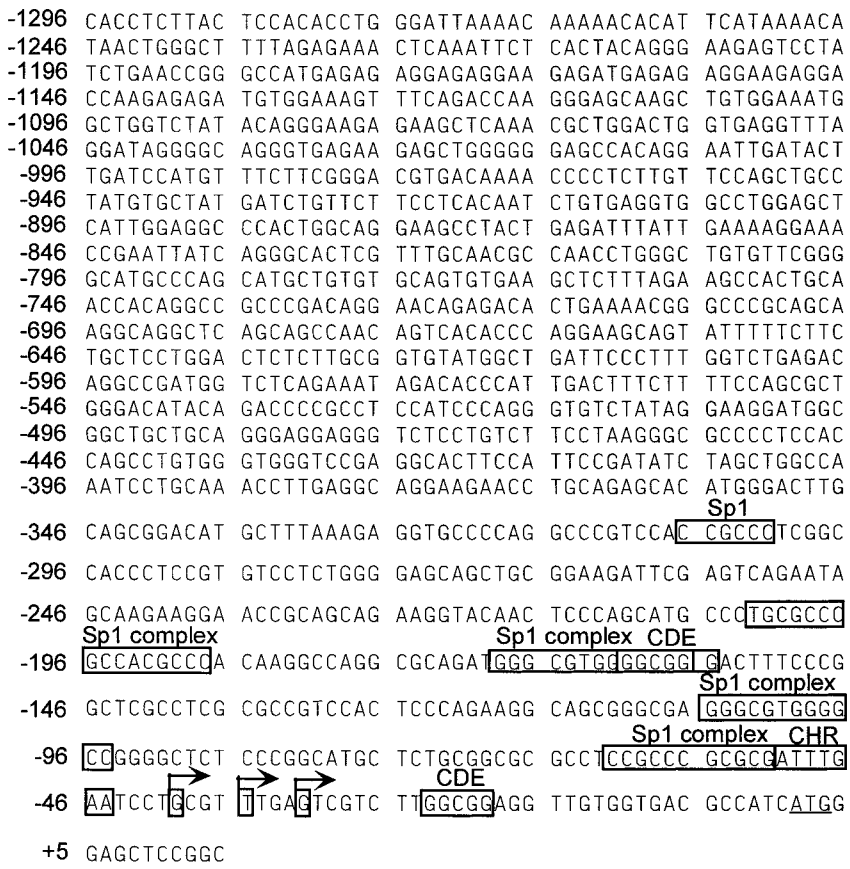

Fig. 5. DNA sequence analysis of the proximal promoter region of the mouse *survivin* gene. The +1 position corresponds to the first ATG. *Boxed letters*, Sp1/Sp1-like complexes and CDE/CHR cell cycle G_1 transcriptional repressor elements. *Arrows*, transcription start sites identified by primer extension and S1 nuclease mapping (see Fig. 4).

transfection of the first 30 bp in the survivin promoter (pLuc-30) generated only background transcriptional activity (Fig. 6*B*). 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 ± 4,003). 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. 6*C*).

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. 7*A*). 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 asynchronously growing HeLa cell transfectants (Fig. 7*B*). Simultaneous mutagenesis of all of the CDE/CHR elements and Sp1 sites did not further reduce transcriptional activity (Fig. 7, *A* and *B*). To determine the molecular requirements of cell cycle-dependence of *survivin* gene expression, similar experiments were carried out in drug-synchronized HeLa cells arrested in G_1, S phase, and G_2-M. As shown in Fig. 7*C*, transfection of the proximal survivin promoter in pLuc-158 resulted in a 3.5- to 5-fold increased transcriptional activity in G_2-M-synchronized HeLa cells, as compared with G_1- or S phase-arrested cells (Fig. 7*C*), and in agreement with previous observations (20). Transfection of the various mutants of survivin promoter-luciferase constructs abolished cell cycle periodicity and resulted in transcriptional activity in G_2-M-synchronized HeLa cells indistinguishable from G_1- or S phase-arrested cells (Fig. 7*C*). The contribution of proximal Sp1 sites, independent of CDE/CHR boxes, in basal and cell cycle-dependent transcriptional activity of the mouse *survivin* gene was also investigated (Fig. 8*A*). As shown in Fig. 8*B*, mutagenesis of Sp1 sites at position −62 and −169 resulted in a partial reduction of survivin transcriptional activity by 25 and 50%, respectively (Fig. 8*B*). Simultaneous disruption of both Sp1 sites (Fig. 8*A*) resulted in an additive 60% inhibition of luciferase activity in asynchronously growing HeLa cells (Fig. 8*B*). Furthermore, disruption of the Sp1 site at position −62 partially inhibited by ~50% *survivin* gene expression in G_2-M, whereas mutagenesis of the Sp1 site at position −169 almost completely abolished cell cycle periodicity of *survivin* gene expression in G_2-M-synchronized HeLa cells (Fig. 8*C*). The combined disruption of both Sp1 sites did not further inhibit transcriptional activity in cell cycle-synchronized cultures (Fig. 8*C*).

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 G_2-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 G_2-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 G_2-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

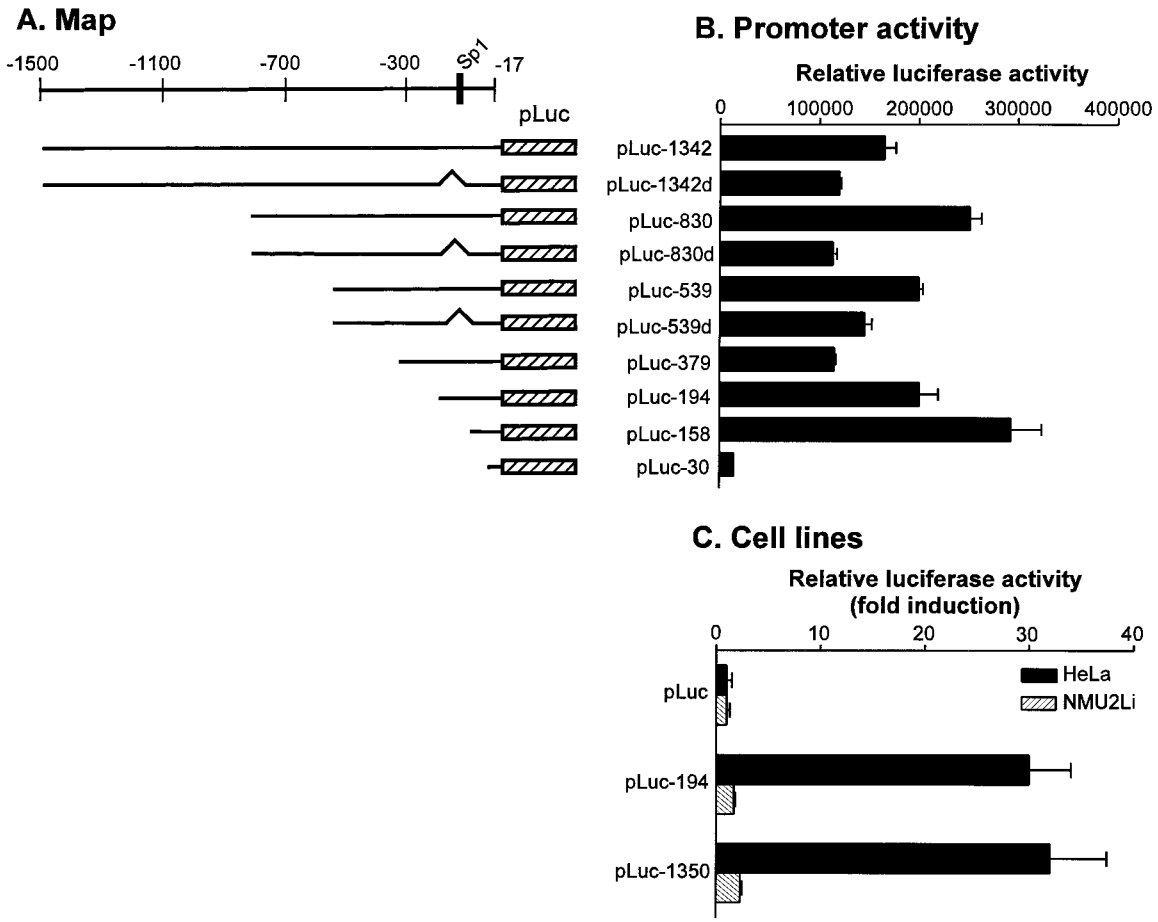

Fig. 6. Identification of promoter sequences in the mouse *survivin* gene. *A*, the indicated *survivin* wild-type or deleted mutant promoter constructs were generated by PCR and inserted in the forward orientation upstream of a luciferase reporter gene in pLuc. *B*, luciferase activity of the indicated survivin promoter-luciferase constructs in transiently transfected HeLa cells by lipofectAMINE. *C*, analysis of survivin promoter activity in HeLa cells *versus* normal mouse NMU2Li liver cells. Values were normalized to β–galactosidase expression as an internal control. Data are the mean ± SD of duplicates of a representative experiment of at least three independent determinations.

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 *Eco*RI 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 M_r of 16,200. The mouse survivin protein contained a structurally unique BIR module (4), characterized by the three-amino acid insertion Cys^{46}-Pro^{47}-Thr^{48} 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^{67}, Pro^{73}, and Cys^{84} in the BIR module required for apoptosis inhibition (Ref. 20 and unpublished observations),[4] 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 G_2-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) G_1 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 asynchronously growing HeLa cells, and a 3- to 5-fold increased promoter activity in G_2-M-synchronized cultures as compared with G_1- or S phase-arrested cells. Although the spacing of the survivin CDE/CHR boxes is different from that of other G_2-M-regulated genes (23, 32), deletion and mutagenesis studies identified two Sp1 sites at position −62 and −169, and the CDE/CHR G_1 repressor elements (23), as

[4] Unpublished observations.

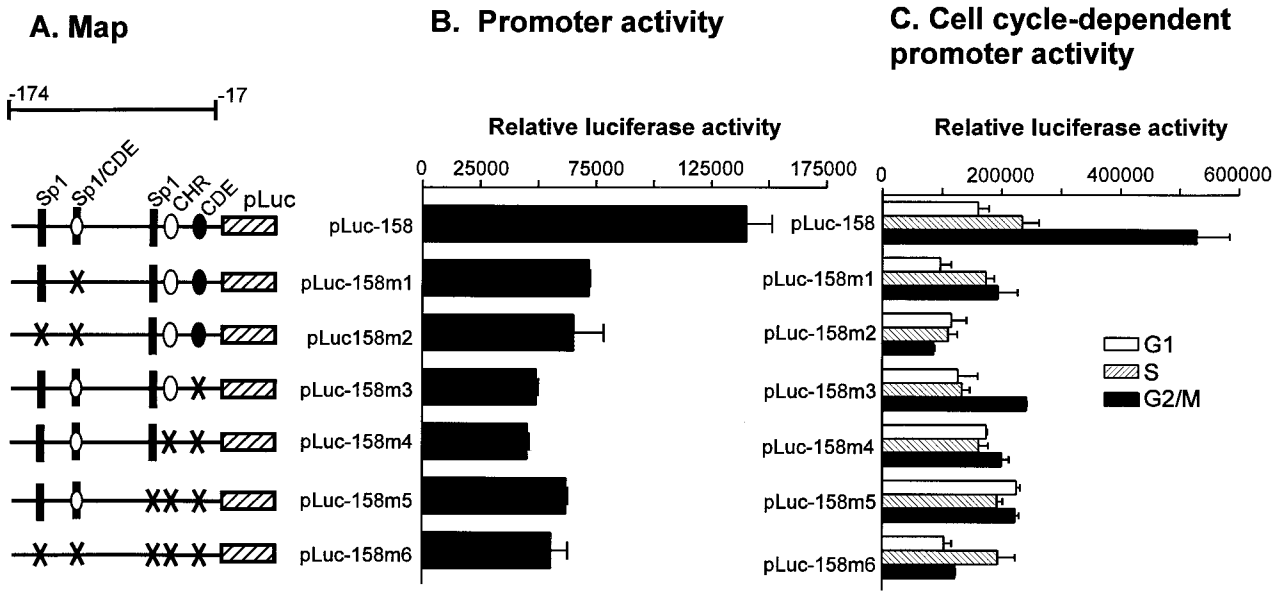

Fig. 7. Mutation analysis of mouse survivin promoter sequences. *A*, map of wild-type and mutated survivin constructs targeting Sp1 sites and CDE/CHR G_1 transcriptional repressor elements in the proximal mouse survivin promoter. *B*, requirement of CDE/CHR elements and Sp1 sites for transcriptional activity of the mouse *survivin* gene. The determination of β-galactosidase-normalized luciferase activity in asynchronously growing HeLa cells transfected with the indicated constructs is as described in Fig. 6. *C*, requirement of CDE/CHR elements and Sp1 sites for cell cycle-dependent mouse *survivin* gene transcription in G_2-M. The experimental conditions are the same as in *B*, except that HeLa cells were synchronized by a 16-h treatment with mimosine (G_1), thymidine (S), or nocodazole (G_2-*M*) 24 h after transfection and were further analyzed for β–galactosidase-normalized luciferase activity. ✕, the mutated sites. Data are the mean ± SD of duplicates of one of at least three independent experiments.

critically required for basal and cell cycle periodicity of *survivin* gene expression in G_2-M. Altogether, these data suggest that transient G_1 repression mediated by CDE/CHR boxes (23, 32) imparts cell cycle periodicity in G_2-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 $p21^{waf\text{-}1/cip1}$ and $p15^{INK4B}$ 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, chorioid plexus, hypophysis, and dorsal root ganglion (15). This is consistent with a prominent role of apoptosis inhibitors, *i.e.*, *bcl*-2,

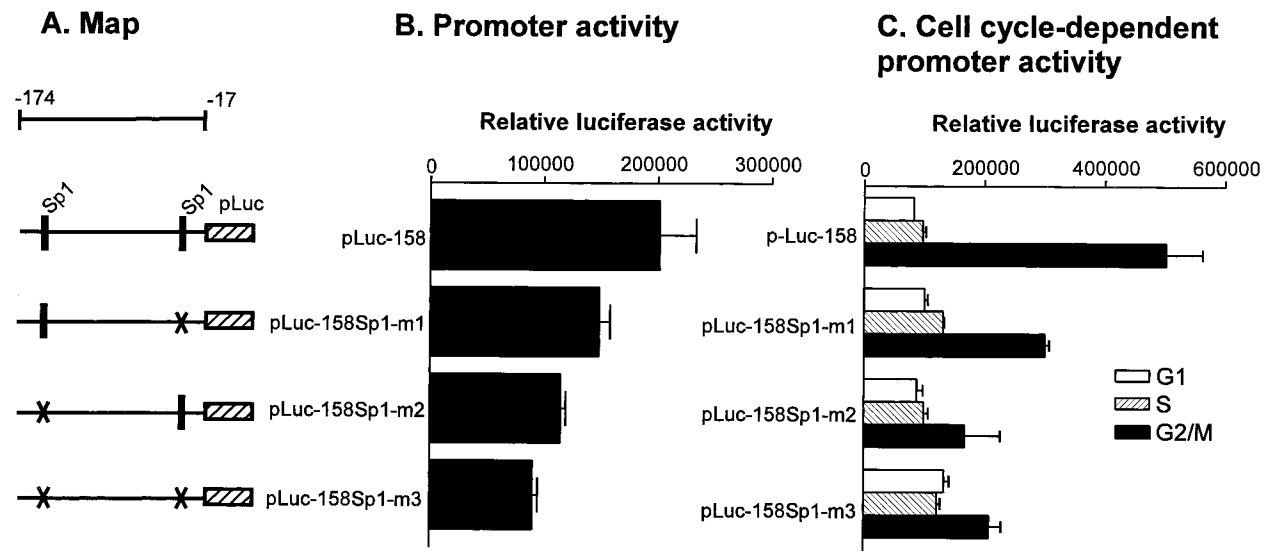

Fig. 8. Independent role of Sp1 sites in basal and cell cycle-dependent survivin promoter activity. *A*, map of wild-type and mutated mouse survivin promoter constructs targeting the indicated *Sp1 sites* independently of CDE/CHR boxes. *B*, participation of Sp1 sites in basal survivin promoter activity in asynchronously growing HeLa cell transfectants. *C*, requirement of Sp1 site at position −169 for cell cycle-dependent transcription of the mouse *survivin* gene in G_2-M. For *B* and *C*, the experimental conditions for the determination of β–galactosidase-normalized luciferase activity of wild-type or mutated mouse survivin constructs are the same as in Fig. 7. ✕, the mutated sites. Data are the mean ± SD of duplicates of one of at least three independent experiments.

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 G_2-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.[5] 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).

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[5] Unpublished observations.

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

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Cancer Res 1999;59:3143-3151.