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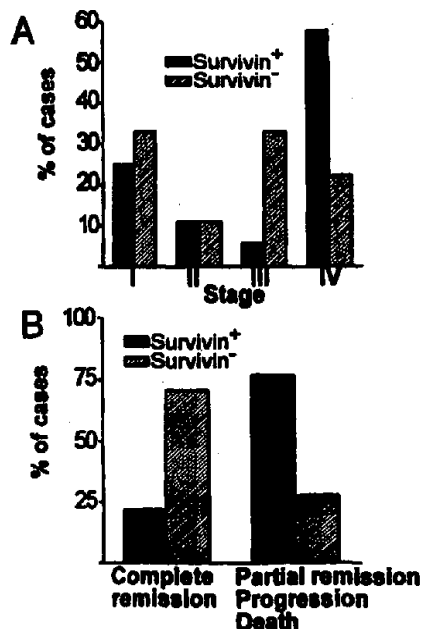
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(54) Title: SURVIVIN, A PROTEIN THAT INHIBITS CELLULAR APOPTOSIS, AND ITS MODULATION

(57) Abstract

The present invention provides the amino acid of a protein that inhibits cellular apoptosis, herein termed the Survivin protein and nucleic acid molecules that encode Survivin. Based on this disclosure, the present invention provides isolated Survivin protein, isolated Survivin encoding nucleic acid molecules, methods of isolating other members of the Survivin family of proteins, methods for identifying agent that blocks Survivin mediated inhibition of cellular apoptosis, methods of using agent that block Survivin mediated inhibition or Survivin expression to modulate biological and pathological processes, and methods of assaying Survivin activity.



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**SURVIVIN, A PROTEIN THAT INHIBITS
CELLULAR APOPTOSIS, AND ITS MODULATION**

STATEMENT OF RELATED APPLICATIONS

5 This application is based upon provisional U.S. Application Serial No. 60/031,435, filed November 20, 1996, the disclosure of which is hereby incorporated by reference.

FIELD OF THE INVENTION

10 The present invention relates to the field of modulating cell apoptosis, particularly agents useful to inhibit apoptosis, as well as to diagnostic and prognostic assays involving conditions in mediated by the expression of inhibitors of apoptosis. The invention specifically relates to the identification of a novel human gene, tentatively named *Survivin*. *Survivin* encodes a protein, Survivin, that inhibits cellular
15 apoptosis, particularly in cancer cells and embryonic cells.

BACKGROUND OF THE INVENTION

Regulation of cell proliferation by programmed cell death (apoptosis) maintains tissue homeostasis during development and differentiation (Raff, M.D.,
20 *Nature* (1992) 356:397-400; Vaux, D.L. *et al.*, *Cell* (1994) 76:777-779). This process involves an evolutionarily conserved multi-step cascade (Oltvai, Z. *et al.*, *Cell* (1994) 79:189-192), and is controlled by proteins that promote or counteract apoptotic cell death. Apoptosis also involves cell surface receptors (Smith, A. *et al.*, *Cell* (1994) 76, 959-962), and associated signal transducers (Tartaglia, L.A. *et al.*, *Immunol Today*
25 (1992) 13:151-153), protease gene families (Martin, S.J. *et al.*, *Cell* (1995) 82:349-352), intracellular second messengers (Kroemer, G. *et al.*, *FASEB J* (1995) 9:1277-1287), tumor suppressor genes (Haffner, R. *et al.*, *Curr Op Gen Dev* (1995) 5:84-90), and negative regulatory proteins that counteract apoptotic cell death (Hockenbery, D. *et al.*, *Nature* (1990) 348:334-336). Aberrantly increased apoptosis or abnormally
30 prolonged cell survival (Oltvai, Z.N. *et al.*, *Cell* (1994) 79:189-192) may both

contribute to the pathogenesis of human diseases, including autoimmune disorders, neurodegenerative processes, and cancer (Steller, H., *Science* (1995) 267:1445-1449; Thompson, C.B., *Science* (1995) 267:1456-1462).

Specifically, for example, inhibitors of apoptosis, most notably of the *bcl-2* family (Reed, J., *J Cell Biol* (1994) 124:1-6, and Yang, E., *et al.*, *Blood* (1996) 88:386-401), maintain lymphoid homeostasis and morphogenesis in adult (Hockenbery, D *et al.*, *Proc Natl Acad Sci USA* (1991) 88:6961-6965) and fetal (LeBrun, D. *et al.* (1993) 142:743-753) tissues. Deregulated expression of *bcl-2* has also been implicated in cancer, by aberrantly prolonging cell survival and facilitating the insurgence of transforming mutations.

In addition to *bcl-2*, several members of a new gene family of inhibitors of apoptosis related to the baculovirus IAP gene (Birnbau, M.J. *et al.*, *J Virology* (1994) 68:2521-2528; Clem, R.J. *et al.*, *Mol Cell Biol* (1994) 14:5212-5222) have been identified in *Drosophila* and mammalian cells (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262; Liston, P. *et al.*, *Nature* (1996) 379:349-353; Rothe, M. *et al.*, *Cell* (1995) 83:1243-1252; Roy, N. *et al.*, *Cell* (1995) 80:167-178). These molecules are highly conserved evolutionarily; they share a similar architecture organized in two or three approximately 70 amino acid amino terminus Cys/His baculovirus IAP repeats (BIR) and by a carboxy terminus zinc-binding domain, designated RING finger (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262; Liston, P. *et al.*, *Nature* (1996) 379:349-353; Rothe, M. *et al.*, *Cell* (1995) 83:1243-1252; Roy, N. *et al.*, *Cell* (1995) 80:167-178). Recombinant expression of IAP proteins blocks apoptosis induced by various stimuli *in vitro* (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Liston, P. *et al.*, *Nature* (1996) 379:349-353), and promotes abnormally prolonged cell survival in the developmentally-regulated model of the *Drosophila* eye, *in vivo* (Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262). Finally, deletions in a IAP neuronal inhibitor of apoptosis, NAIP, were reported in 75% of patients with spinal muscular atrophy, thus suggesting a potential role of this gene family in human diseases (Roy, N. *et al.*, *Cell* (1995) 80:167-178).

Therapeutic and diagnostic uses of nucleic acids that encode various inhibitors of apoptosis relating to a member of the IAP family have been described in the patent literature. See, for example, International Patent Applications No. WO 97/06255, WO 97/26331, and WO 97/32601. In particular, the uses of such genes and gene products
5 are contemplated for the novel protein and its encoding nucleic acid discussed below.

Recently, a novel gene encoding a structurally unique IAP apoptosis inhibitor, designated *Survivin* has been identified. Survivin is a ~16.5 kD cytoplasmic protein containing a single BIR, and a highly charged carboxyl-terminus coiled-coil region instead of a RING finger, which inhibits apoptosis induced by growth factor (IL-3)
10 withdrawal when transferred in B cell precursors (Ambrosini, G. *et al.*, *Nature Med.* (1997) 3:917-921). At variance with *bcl-2* or other IAP proteins, Survivin is undetectable in adult tissues, but becomes prominently expressed in all the most common human cancers of lung, colon, breast, pancreas, and prostate, and in ~50% of high-grade non-Hodgkin's lymphomas, *in vivo*. Intriguingly, the coding strand of the
15 Survivin gene was highly homologous to the sequence of Effector cell Protease Receptor-1 (EPR-1) (Altieri, D.C., *FASEB J* (1995) 9:860-865), but oriented in the opposite direction, thus suggesting the existence of two separate genes duplicated in a head-to-head configuration.

The present invention is based on the identification of a novel human gene
20 which is nearly identical to EPR-1, but oriented in the opposite direction. The antisense EPR-1 gene product, designated Survivin, is a distantly related member of the IAP family of inhibitors of apoptosis (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262; Liston, P. *et al.*, *Nature* (1996) 379:349-353; Rothe, M. *et al.*, *Cell* (1995) 83:1243-1252; Roy, N. *et al.*, *Cell*
25 (1995) 80:167-178), and is prominently expressed in actively proliferating transformed cells and in common human cancers, *in vivo*, but not in adjacent normal cells. Functionally, inhibition of Survivin expression by up-regulating its natural antisense EPR-1 transcript resulted in massive apoptosis and decreased cell growth.

SUMMARY OF THE INVENTION

The present invention is based, in part, on the isolation and identification of a protein that is expressed in most cancer cells and inhibits cellular apoptosis, hereinafter Survivin or the Survivin protein. Based on this observation, the present invention provides purified Survivin protein.

The present invention further provides nucleic acid molecules that encode the Survivin protein. Such nucleic acid molecules can be in an isolated form, or can be operably linked to expression control elements or vector sequences.

The present invention further provides methods of identifying other members of the Survivin family of proteins. Specifically, the nucleic acid sequence of *Survivin* can be used as a probe, or to generate PCR primers, in methods to identify nucleic acid molecules that encode other members of the Survivin family of proteins.

The present invention further provides antibodies that bind to Survivin. Such antibodies can be either polyclonal or monoclonal. Anti-Survivin antibodies can be used in a variety of diagnostic formats and for a variety of therapeutic methods.

The present invention further provides methods for isolating Survivin binding partners. Survivin binding partners are isolated using the Survivin protein as a capture probe. Alternatively, Survivin can be used as bait in the yeast two-hybrid system to screen an expression library and identify genes that encode proteins that bind to the Survivin protein. Binding partners isolated by these methods are useful in preparing antibodies and also serve as targets for drug development.

The present invention further provides methods to identify agents that can block or modulate the association of Survivin with a binding partner. Specifically, an agent can be tested for the ability to block, reduce or otherwise modulate the association of Survivin with a binding partner by contacting Survivin, or a fragment thereof, and a binding partner with a test agent and determining whether the test agent blocks or reduces the binding of the Survivin protein to the binding partner.

The present invention further provides methods for reducing or blocking the association of Survivin with one or more of its binding partners. Specifically, the association of Survivin with a binding partner can be blocked or reduced by

contacting Survivin, or the binding partner, with an agent that blocks the binding of Survivin to the binding partner. The method can utilize an agent that binds to Survivin or to the binding partner.

The present invention further provides methods of regulating the expression of *Survivin* within a cell. Expression of *Survivin* within a cell can be regulated so as to produce or inhibit the production of Survivin.

Blocking Survivin/binding partner associations or *Survivin* expression can be used to modulate biological and pathological processes that require Survivin. For example, methods that reduce Survivin production induce apoptosis of tumor cells. Stimulation of Survivin production can be used as a means of extending the culturability of cells or tissues.

The biological and pathological processes that require Survivin or Survivin/binding partner interactions can further be modulated using gene therapy methods. Additional genetic manipulation within an organism can be used to alter the expression of a *Survivin* gene or the production of a Survivin protein in an animal model. For example, a *Survivin* gene can be altered to correct a genetic deficiency; peptide modulators of Survivin activity can be produced within a target cell using genetic transformation methods to introduce a modulator encoding nucleic acid molecules into a target cell; etc. The use of nucleic acids for antisense and triple helix therapies and interventions are expressly contemplated.

The present invention further provides methods of reducing the severity of pathological processes that require Survivin. Since expression of *Survivin* or association of Survivin with a binding partner is required for Survivin-mediated biological processes, agents that block *Survivin* expression, Survivin activity or the association of Survivin with a binding partner, can be used in therapeutic methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the identification of a complementary EPR-1 gene. A, B. Chromosomal location. A digoxigenin-labeled human P1 genomic clone selected by hybridization with the EPR-1 cDNA, was incubated with metaphase chromosomes

isolated from phytohemagglutinin-stimulated PBMC in 50% formamide, 10% dextran sulfate and 2X SSC. The EPR-1-hybridizing gene was mapped in single-color labeling to the long arm of a group E chromosome (A, green staining), and in two-color staining with probe D17Z1, specific for the centromere of chromosome 17 (B, red staining), to the long arm of chromosome 17 (B, green staining), to band 17q25. C. Map of the antisense EPR-1 gene. A contig spanning 14796 bp was derived from two EPR-1-hybridizing P1 clones, subcloned in pBSKS⁺, and completely sequenced on both strands. Orientation of the map is 5'→3' with respect to the position of intron-exon boundaries (see below). Exons are solid boxes, a putative CpG island upstream exon 1 is an open box. The translational initiation codon (ATG) is indicated. Restriction sites are: B, *Bam*HI, H, *Hind*III; P, *Pst*I; S, *Sma*I; X, *Xba*I. D. Intron-exon boundaries of the antisense EPR-1 gene. Positions of the intron-exon boundaries in bp are indicated in parenthesis.

Figure 2 shows the complexity and evolutionary conservation of EPR-1-related sequences. A. Southern blot of human genomic DNA. Samples were digested with the indicated restriction enzymes, transferred to GeneScreen nylon membranes and hybridized with the EPR-1 cDNA, in 5X SSC, 0.5% SDS, 5X Denhardt's and 0.1% sodium pyrophosphate at 65°C. Radioactive bands indicated by an arrow (7.6 kb *Bam*HI, 7.5 kb *Xba*I and *Hind*III fragments of 15, 7.5, 6.4, and 3.7 kb) do not derive from the antisense EPR-1 gene in Figure 1C. B. Southern blot of pulsed field gel electrophoresis. High molecular weight human genomic DNA was digested with the indicated restriction enzymes, separated by pulsed field gel electrophoresis for 20 h at 200 V with a pulse time of 75 sec, transferred to nylon membrane, and hybridized with the EPR-1 cDNA, as described in A. C. Multiple species Southern blot. *Eco*RI-digested genomic DNA from the indicated species was hybridized with a 3' 548 bp fragment of the EPR-1 cDNA, as described in A. For all panels, molecular weight markers in kb are shown on the left.

Figure 3 shows the discordant tissue distribution of sense/antisense EPR-1 transcripts. Northern hybridization was carried out on a multiple tissue adult or fetal mRNA blot with single strand-specific probes in 5X SSPE, 10X Denhardt's solution,

2% SDS, 100 mg/ml denatured salmon sperm DNA at 60°C for 14 h. After washes in 2X SSC at 60°C and in 0.2X SSC at 22°C, radioactive bands were visualized by autoradiography. A. EPR-1-specific single-strand probe. B. Antisense EPR-1-specific single-strand probe. C. Control actin probe. Molecular weight markers in kb are shown on the left.

Figure 4 shows the sequence analysis of Survivin and expression in cell lines. A. Predicted translation of the antisense EPR-1 gene product (Survivin) (SEQ ID NO:34). B. Sequence alignment of the BIR in Survivin (SEQ ID NOS:8 and 21) and in other IAP proteins by the Clustal method. IAP proteins are identified by accession number, L49433 (SEQ ID NOS:9 and 22), TNFR2-TRAF signaling complex-associated IAP; L49441 (SEQ ID NOS:10 and 23), apoptosis 2 inhibitor (Drosophila); P41436 (SEQ ID NOS:11 and 24), IAP gene from *Cydia pomonella* granulosis virus; P41437 (SEQ ID NOS:12 and 25) and, IAP gene from *Orgyia pseudotsugata* nuclear polyhedrosis virus; U19251 (SEQ ID NOS:13 and 26), NAIP, neuronal inhibitor of apoptosis; U32373 (SEQ ID NOS:14 and 27), IAP-like protein ILP from *Drosophila melanogaster*; U32974 (SEQ ID NOS:15 and 28), human IAP-like protein ILP; U36842 (SEQ ID NOS:16 and 29), mouse inhibitor of apoptosis; U45878 (SEQ ID NOS:17 and 30), human inhibitor of apoptosis 1; U45879 (SEQ ID NOS:18 and 31), human inhibitor of apoptosis 2; U45880 (SEQ ID NOS:19 and 32), X-linked inhibitor of apoptosis; U45881 (SEQ ID NOS: 20 and 33), *Drosophila* inhibitor of apoptosis. Conserved residues are boxed, identities between Survivin and NAIP (U19251) (SEQ ID NOS:13 and 26) are boxed and shaded. C. Immunoblotting with anti-Survivin antibody JC700. Protein-normalized aliquots of SDS-extracts of cell lines HEL (erythroleukemia), Daudi and JY (B lymphoma), THP-1 (monocytic), Jurkat and MOLT13 (T leukemia), or non transformed human lung Lu18 fibroblasts, HUVEC or PBMC were separated by electrophoresis on a 5-20% SDS gradient gel, transferred to Immobilon and immunoblotted with control non-immune rabbit IgG (RbIgG), or anti-Survivin antibody JC700 (Survivin). Protein bands were visualized by alkaline phosphatase-conjugated goat anti-rabbit IgG and tetrazolium salts. Molecular weight markers in kDa are shown on the left.



Figure 5 shows the regulation of Survivin expression by cell growth/differentiation. HL-60 cells were terminally differentiated to a mature monocytic phenotype by a 72 h culture with 0.1 mM vitamin D₃ plus 17.8 mg/ml indomethacin. Survivin expression before or after vitamin D₃ differentiation was
5 detected by immunoblotting with JC700 antibody, or by Northern hybridization with a



AMENDED SHEET

Survivin-specific single strand probe. RbIgG, control non-immune rabbit IgG. Protein molecular weight markers in kDa and position of ribosomal bands are shown on the left of each blot.

Figure 6 shows the over-expression of Survivin in human cancer, *in vivo*. **A.** Immunohistochemical staining of human lung adenocarcinoma with affinity-purified anti-Survivin antibody JC700 (20 µg/ml). **B.** Inhibition of JC700 staining of lung adenocarcinoma by pre-absorption with the immunizing Survivin 3-19 peptide. **C.** Immunohistochemical expression of Survivin in squamous lung cell carcinoma, but not in the adjacent normal gland epithelium of the lung (**C**, arrow). **D. In-situ** hybridization of Survivin mRNA in squamous lung cell carcinoma with a Survivin-specific riboprobe. **E.** Expression of Survivin in pancreatic adenocarcinoma by immunohistochemistry with JC700. **F.** Normal pancreas, negative for Survivin expression by immunohistochemistry. **G. In situ** hybridization of Survivin mRNA expression in colon adenocarcinoma, but **H**, not in the adjacent non neoplastic colon gland epithelium (**H**, arrow). Magnifications are x200, except G, x400.

Figure 7 shows the effect of Survivin on apoptosis/proliferation. **A.** EPR-1-regulation of Survivin expression. HeLa cells were transfected with control vector pML1 or the EPR-1 cDNA (which is antisense to Survivin) by electroporation, and selected in hygromycin (0.4 mg/ml). Aliquots of vector control HeLa cells (Vector) or Survivin antisense transfectants (Antisense) were induced with 200 mM ZnSO₄ detergent-solubilized, and immunoblotted with the anti-Survivin JC700 antibody. Molecular weight markers are shown on the left. **B.** Effect of Survivin on apoptosis. Survivin antisense transfectants (1, 2), or vector control HeLa cells (3, 4) were induced with Zn²⁺ ions in 0% FBS for 24 h and stained by the AptoTag method with TdT-catalyzed dUTP labeling of 3'-OH DNA ends and immunoperoxidase (1, 3), or by hematoxylin-eosin (HE) (2, 4). 1. Prominent nuclear DNA fragmentation detected by AptoTag staining in serum-starved Survivin antisense transfectants; 2. HE staining of antisense transfectants reveals the presence of numerous apoptotic bodies (arrows); 3. AptoTag staining of vector control HeLa cells detects a few sparse apoptotic cell (arrow); 4. HE staining of vector control HeLa cells. The arrow indicates a single

apoptotic body. Magnification x400. C. Effect of Survivin on cell growth. Twenty thousands vector control HeLa cells (Vector) or Survivin antisense transfectants (Antisense) were seeded in 24-well plates, induced with ZnSO_4 , harvested at the indicated time points, and cell proliferation was determined microscopically by direct cell count. Data are the mean \pm SEM of replicates of a representative experiment out of seven independent determinations.

Figure 8 shows the expression of Survivin in HL-60 cells. HL-60 cells were examined via Western and Northern blots for Survivin expression.

Figure 9 presents a structural analysis of Survivin. The Survivin protein was analyzed using the Chou-Fasman, Garnier-Robson, Kyle-Doolittle, Eisenberg, Karplus-Schultz, Jameson-Wolf and Emini analysis methods.

Figure 10 shows the nucleotide Sequence of *Survivin*.

Figure 11 shows the expression of Survivin and the generation and characterization of anti-Survivin mAb 8E2 by ELISA and immunoblotting.

Figure 12 shows the site-directed mutagenesis of Survivin and identification of key functional residues involved in apoptosis inhibition.

Figure 13 shows the cytoprotective effect of Survivin addition on endothelial cell apoptosis.

Figure 14 shows that the presence of Survivin is a negative predictive-prognostic factor in neuroblastoma.

5 Figure 15 shows that the presence of Survivin is a negative predictive prognostic factor in high-grade non-Hodgkin's lymphoma.

Figure 16 shows the down regulation of Survivin induced by inflammatory and cytostatic cytokines.

10 Figure 17 shows the effects of Survivin constructs or XIAP on apoptosis induced in NIH3T3 cells by hydrogen peroxide.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

I. General Description

15 The present invention is based in part on identifying a novel protein that is expressed in tumor cells and inhibits cellular apoptosis, hereinafter the Survivin protein or Survivin. Survivin is also found to be expressed in embryonic tissues.

The Survivin protein can be used as an agent, or serve as a target for agents, that can be used to inhibit or stimulate Survivin mediated inhibition of cellular apoptosis, for example to block abnormal cell growth or to extend cell growth in
20 culture.

As used herein, modulation of apoptosis means increasing or decreasing the number of cells that would otherwise undergo apoptosis in a given cell population. This can be effected by increasing or decreasing the amount of Survivin present in a cell or by increasing or decreasing the activity of the Survivin. Preferably, the given
25 cell population in which apoptosis is to be modulated is found in a tumor or other tissue or group of cells in which beneficial effect results from the modulation. Also, preferably, the increase or decrease in number of cells that would otherwise undergo apoptosis in a given cell population is at least about 10 %, 20 %, 40 % or more preferably at least about 50 % of the cells in that population.

The present invention is further based on the development of methods for isolating proteins that bind to Survivin. Probes based on the Survivin protein or fragments of Survivin as discussed below are used as capture probes to isolate Survivin binding proteins. Dominant negative proteins, DNAs encoding these proteins, antibodies to these binding proteins, peptide fragments of these proteins or mimics of these proteins may be introduced into cells to affect Survivin function. Additionally, these proteins provide novel targets for screening of synthetic small molecules and combinatorial or naturally occurring compound libraries to discover novel therapeutics to regulate Survivin function.

II. Identification, General Characterization and Tissue Distribution of Survivin

The present invention is based on the identification on chromosome 17q25 of a novel member of the IAP family of inhibitors of apoptosis, designated Survivin, which may confer a selective advantage for cancer cell growth. Relevant features of the Survivin gene include its developmentally- and differentiation-regulated expression, its nearly identical and complementary DNA sequence with the factor Xa receptor EPR-1, and its abundant *in vivo* expression in common human malignancies, but not in the adjacent non-neoplastic population. As described below, targeting Survivin expression by metallothionein-induction of EPR-1 mRNA resulted in apoptosis and inhibition of proliferation of HeLa cell transfectants.

In addition to their contribution to hemostasis, cellular receptors for blood proteases have recently emerged as pleiotropic signaling molecules, playing a crucial role in embryologic development (Connolly, A.J. *et al.*, *Nature* (1996) 381:516-519), and vasculogenesis (Carmeliet, P. *et al.*, *Nature* (1996) 383:73-75). In this context, the Survivin gene was isolated by hybridization with the cDNA for EPR-1, a receptor for factor Xa contributing to procoagulant activity (Altieri, D.C., *FASEB J* (1995) 9:860-865), and T cell activation (Duchosal, M.A. *et al.*, *Nature* (1996) 380:352-356). Although the Survivin coding sequence was found to be nearly identical to the EPR-1 cDNA, its orientation was unambiguously assigned to the antisense EPR-1 strand for

the position of the consensus splice sites at intron-exon boundaries (Padgett, R.A. *et al.*, *Ann Rev Biochem* (1986) 55:1119-1150). On the other hand, the authenticity of the EPR-1 "sense" strand was demonstrated in previous studies, when mammalian cells transfected with the EPR-1 cDNA or with chimeric EPR-1 constructs
5 (Ambrosini, G.*et al.*, *J Biol Chem* (1996) 271:1243-1248 and Altieri, D.C., *FASEB J* (1995) 9:860-865), were recognized by anti-EPR-1 mAbs and bound factor Xa in a specific and saturable reaction.

These findings could be reconciled by the existence of multiple, highly homologous, EPR-1 transcripts oriented in opposite directions. The heterogeneity of
10 EPR-1 mRNA and the complex pattern of Southern hybridization support this hypothesis. Previously, double strand EPR-1 probes detected three strongly hybridizing bands of 1.9, 3.4 and ~1.5 kb in mRNA of EPR-1⁺ cells (Altieri, D.C., *FASEB J* (1995) 9:860-865). Here, single strand-specific probes confirmed the presence of multiple mature and polyadenylated EPR-1-related messages, and
15 revealed that the 1.9 and 3.4 kb bands corresponded to two highly regulated, antisense EPR-1 transcripts, while the 1.5 kb band, more accurately defined as 1.2 kb, coincided with a genuine EPR-1-encoding message. While the 1.9 kb antisense transcript clearly originated from the Survivin gene described here, a gene encoding the 1.2 kb "sense" EPR-1 message has not yet been identified.

20 However, (i) the presence of several genomic EPR-1-hybridizing bands unrelated to the Survivin gene, (ii) the different restriction pattern of EPR-1 sequences in various species, and (iii) the numerous expressed sequence tag database entries matching ($P = 0.018-7 \times 10^{-11}$) the positive (accession n. W46267), or the negative (accession n. W34764, W83810, T29149) EPR-1 strand, altogether suggest the
25 existence of at least a second, highly-related, EPR-1 gene oriented in the opposite direction to that described here, and encoding the previously characterized factor Xa receptor (Altieri, D.C., *FASEB J* (1995) 9:860-865).

A similar situation could arise from gene duplication event(s) involving EPR-1 sequences. Interestingly, the single hybridization signal detected on chromosome
30 17q25, and the single hybridizing bands identified in a Southern blot of high

molecular weight genomic DNA, suggest that EPR-1-related sequences potentially oriented in opposite directions may be adjacent in close proximity, within a physical interval of 75-130 kb.

The presence of multiple EPR-1 transcripts oriented in opposite directions implies a reciprocal regulatory mechanism by naturally occurring antisense. This is consistent with the predominantly discordant and mutually exclusive distribution of sense and antisense EPR-1 messages in developing or adult tissues *in vivo*, and during HL-60 cell terminal differentiation. While antisense regulation is common in prokaryotes (Green, P.J. *et al.*, *Annu Rev Biochem* (1986) 55:569-597), a growing number of eukaryotic gene products have been recently characterized for the occurrence of functional antisense transcripts potentially participating in gene regulation, including basic fibroblast growth factor (Kimmelman, D. *et al.*, *Cell* (1989) 59:687-696; Murphy, P.R. *et al.*, *Molecular Endocrinology* (1994) 8:852-859), $\alpha 1(I)$ collagen (Farrell, C.M. *et al.*, *J Biol Chem* (1995) 270:3400-3408 and Lukens, 1995), *n-myc* (Krystal, G.W. *et al.*, *Mol Cell Biol* (1990) 10:4180-4191), *c-myc* (Celano, P. *et al.*, *J Biol Chem* (1992) 267:15092-15096), p53 (Khochbin, S. *et al.*, *EMBO J* (1989) 8:4107-4114), *c-erbAa* (Lazar, M.A. *et al.*, *Mol Cell Biol* (1989) 9:1128-1136), and CD3 $\zeta/\eta/\theta$ locus (Lerner, A. *et al.*, *J Immunol* (1993) 151:3152-3162).

As described below, the existence of a EPR-1/Survivin gene balance regulated by functional antisense was demonstrated in HeLa cell transfectants, when metallothionein-induced transcription of the EPR-1 "sense" strand suppressed the expression of Survivin and profoundly influenced apoptosis/cell proliferation (see below). This regulatory mechanism was not due to a potential protein association between EPR-1 and Survivin, since the EPR-1 construct used for these experiments lacked a translational initiation codon. Additional experiments have evaluated the ability of a Survivin antisense to inhibit cell growth. This was done by transiently co-transfecting the Survivin antisense with a lacZ reported plasmid and making a determination of cell viability after a 48-h transfection in β -galactosidase expressing cells. The results indicated that the viability of Survivin antisense transfectants was

<20% of control cells transfected with the empty vector. A control antisense of ICAM-1 (intercellular adhesion molecule-1) similarly co-transfected in HeLa cells was ineffective.

Survivin was found to be a small protein of 142 amino acids (~ 16.5 kDa) with
5 no amino acid sequence homology to EPR-1, and designated Survivin for the presence of a BIR-homologous domain (Birnbaum, M.J. *et al.*, *J Virology* (1994) 68:2521-2528; Clem, R.J. *et al.*, *Mol Cell Biol* (1994) 14:5212-5222) found in IAP inhibitors of apoptosis (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262; Liston, P. *et al.*, *Nature* (1996) 379:349-353; Rothe, M. *et al.*, *Cell* (1995) 83:1243-1252; Roy, N. *et al.*, *Cell* (1995) 80:167-178). Based on
10 overall sequence conservation, the absence of a carboxy terminus RING finger and the presence of a single, partially conserved, BIR domain, Survivin is the most distantly related member of the IAP family, sharing the highest degree of similarity with NAIP (Roy, N. *et al.*, *Cell* (1995) 80:167-178). Thus, unlike *bcl-2* or other IAP proteins,
15 Survivin is undetectable in adult tissues, but becomes prominently expressed in all the most common human cancers of lung, colon, breast, pancreas, and prostate, and in ~50% of high-grade non-Hodgkin's lymphomas, *in vivo*. Additionally, unlike other IAP proteins (Deveraux, Q. *et al.*, *Nature* (1997) 388:300-304), Survivin does not bind caspases in a cell-free system (Roy, N. *et al.*, *Blood* (1997) 595:2645).

20 Consistent with the anti-apoptosis properties of IAP proteins *in vitro* (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Liston, P. *et al.*, *Nature* (1996) 379:349-353), and *in vivo* (Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262), inhibition of Survivin expression by the EPR-1 transcript (which naturally is antisense to Survivin) resulted in increased apoptosis, as determined by *in situ* internucleosomal DNA fragmentation
25 in HeLa cell transfectants. The ability of a RING finger-less IAP protein to counteract apoptosis is not without a precedent, as demonstrated by the suppression of apoptosis mediated by NAIP (Liston, P. *et al.*, *Nature* (1996) 379:349-353), and by the *in vivo* gain-of-function of a *Drosophila* IAP protein following deletion of the RING finger (Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262). Although anti-apoptosis genes are
30 thought to play an indirect role in cell growth, by favoring the accumulation of

oncogenic mutations(s) in aberrantly long-living cells (Reed, J.C., *J Cell Biol* (1994) 124:1-6), down-regulation of Survivin resulted in a profound inhibition of HeLa cell proliferation. While this may derive from rapid disappearance of HeLa cells expressing the highest levels of antisense transcripts by apoptosis, a similar decrease
5 in tumor cell proliferation has been reported *in vivo* after antisense inhibition of *bcl-2* (Reed, J.C. *et al.*, *Proc Natl Acad Sci USA* (1990) 87:3660-3664).

The possibility that IAP proteins may play a more general role in cell proliferation, not exclusively restricted to apoptosis inhibition, has been proposed earlier. Rothe *et al.*, have recently demonstrated that the amino terminus BIR in two
10 IAP proteins (cIAPs) physically interacts with the signal transducers associated with the 75 kDa TNF receptor (Rothe, M. *et al.*, *Cell* (1995) 83:1243-1252), a molecule primarily implicated in cell proliferation and survival rather than apoptotic signaling (Tartaglia, L.A. *et al.*, *Immunol Today* (1992) 13:151-153). While it is not known if Survivin is physically linked to signaling molecules (Rothe, M. *et al.*, *Cell* (1995)
15 83:1243-1252), the structural divergence of its BIR as compared with other IAP proteins (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Hay, B.A. *et al.*, *Cell* (1995) 83:1253-1262; Liston, P. *et al.*, *Nature* (1996) 379:349-353; Rothe, M. *et al.*, *Cell* (1995) 83:1243-1252; Roy, N. *et al.*, *Cell* (1995) 80:167-178), may confer specificity for supramolecular interaction(s) potentially relevant to its particular
20 mechanism of apoptosis inhibition/cell growth.

Dysregulation of programmed cell death (apoptosis) has recently emerged as a primary mechanism contributing to the pathogenesis of various human diseases, including cancer (Steller, H., *Science* (1995) 267:1445-1449; Thompson, C.B., *Science* (1995) 267:1456-1462). While the impact of anti-apoptosis gene(s) in
25 neoplasia is highlighted by the role of *bcl-2* in follicular lymphoma (Korsmeyer, S.J., *Blood* (1992) 80:879-886), a potential distribution of IAP proteins in cancer had not been previously investigated. In this context, one of the most striking characteristics of Survivin was its abundant expression in actively proliferating transformed cell lines, and in all the most common human malignancies of lung, colon, pancreas, and
30 breast, *in vivo*, but not in the non-neoplastic adjacent cell population. This

distribution in multiple human cancers may signal a fundamental role of this molecule in apoptosis/cell proliferation mechanisms in neoplasia. By analogy with the paradigm of *bcl-2*, over-expression of Survivin in cancer may lead to aberrantly prolonged cell viability (Veis, D.J. *et al.*, *Cell* (1993) 75:229-240), increased
5 resistance to chemotherapy-induced apoptosis (Miyashita, T. *et al.*, *Blood* (1993) 81:151-157), and, as suggested by the *in vitro* studies reported above, in a direct advantage for transformed cell proliferation.

On the other hand, for its presence in normal PBMC and benign breast adenomas, *in vivo* (unpublished observations), Survivin expression cannot be
10 interpreted *per se* as a marker of malignant transformation but may reflect a more general, developmental- or cell type-specific response to certain stimuli. This is consistent with the presence of Survivin during normal embryonic (our unpublished observations) and fetal development, and its rapid disappearance in growth-arrested cell types (i.e. vitamin D₃-treated HL-60), and terminally-differentiated tissues, *in*
15 *vivo*. At variance with other IAP proteins which are constitutively found in adult mature tissues (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694; Liston, P. *et al.*, *Nature* (1996) 379:349-353; Rothe, M. *et al.*, *Cell* (1995) 83:1243-1252), this pattern of expression is reminiscent of the distribution of *bcl-2* in fetal tissues (LeBrun, D.P. *et al.*, *Am J Pathol* (1993) 142:743-753), and its more restricted presence in
20 differentiated cells, correlating with susceptibility to apoptosis (Hockenbery, D.M. *et al.*, *Proc Natl Acad Sci USA* (1991) 88:6961-6965).

In summary, these findings identify Survivin as a novel link between IAP proteins and cancer, *in vivo*. A key implication of the data presented below is the
25 possibility to balance the effect of this potent anti-apoptosis gene by manipulating a normal cell regulatory mechanism, centered on the expression of EPR-1 (Altieri, D.C.,

FASEB J (1995) 9:860-865). Targeting Survivin may then remove a selective advantage for transformed cell growth and be therapeutically beneficial to increase the susceptibility of cancer cells to chemotherapy-induced apoptosis. Along the same line, identification of polymorphic markers and construction of extended aplotypes within and around the EPR-1/Survivin locus may provide new insights on the population genetics of susceptibility to chemotherapy.

III. Specific Embodiments

10 A. Survivin Protein

The present invention provides isolated Survivin protein, as well as allelic variants of the Survivin protein, and conservative amino acid substitutions of the Survivin protein. As used herein, the Survivin protein (or Survivin) refers to a protein that has the amino acid sequence of human Survivin depicted in Figure 4. The term
15 "Survivin protein" also includes naturally occurring allelic variants of Survivin, naturally occurring proteins that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the requisite ability to inhibit cellular apoptosis.

20 As used herein, the Survivin family of proteins refers to Survivin proteins that have been isolated from organisms in addition to humans. The methods used to identify and isolate other members of the Survivin family of proteins are described below.

Survivin is a member of the IAP (inhibitory apoptosis proteins) family of
25 protein. However, Survivin is the first member of a unique subfamily of IAP proteins that differ from other IAP proteins in significant ways. Despite homology and sequence conservation in the BIR module between Survivin and other members of this gene family, there are important structural differences that are unique to members of the Survivin family of proteins. First unlike any other IAP protein, Survivin has only
30 one BIR module (most of the other molecules have 2-3). Further, Survivin does not

contain a carboxy-terminal RING finger but has a predicted coiled-coil instead. Only the Neuronal Apoptosis Inhibitory Protein (NAIP) in the IAP family lacks a RING finger, but does not contain a carboxy-terminus coiled coil. Finally there is no DNA sequence similarity between Survivin and other IAP proteins (PCR primers designed
5 on Survivin are unlikely to detect other IAP proteins and vice-versa).

The Survivin proteins of the present invention are preferably isolated from. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the Survivin protein from cellular constituents that are normally associated with the Survivin protein. A skilled artisan can readily
10 employ standard purification methods to obtain an isolated Survivin protein.

The Survivin proteins of the present invention further include conservative variants of the Survivin proteins herein described. As used herein, a conservative variant refers to alterations in the amino acid sequence that do not adversely affect the ability of the Survivin protein to bind to a Survivin binding partner and/or to inhibit
15 cellular apoptosis. A substitution, insertion or deletion is said to adversely affect the Survivin protein when the altered sequence prevents the Survivin protein from associating with a Survivin binding partner and/or prevents the Survivin protein from inhibiting cellular apoptosis. For example, the overall charge, structure or hydrophobic/hydrophilic properties of Survivin can be altered without adversely
20 affecting the activity of Survivin. Accordingly, the amino acid sequence of Survivin can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the activity of Survivin.

The allelic variants, the conservative substitution variants and the members of the Survivin family of proteins, will have the ability to inhibit cellular apoptosis.
25 Such proteins will ordinarily have an amino acid sequence having at least about 75% amino acid sequence identity with the human Survivin sequence, more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95%. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with
30 the known peptides, after aligning the sequences and introducing gaps, if necessary, to

achieve the maximum percent homology, and including any conservative substitutions as being homologous. N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Thus, the Survivin proteins of the present invention include molecules having
5 the amino acid sequences disclosed in Figure 1; fragments thereof having a consecutive sequence of at least about 3, 5, 10 or 15 amino acid residues of the Survivin protein; amino acid sequence variants of such sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, the disclosed Survivin sequence; amino acid sequence variants of the disclosed Survivin sequence, or their
10 fragments as defined above, that have been substituted by another residue. Contemplated variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding Survivin proteins of other animal species, including but not limited to rabbit, rat, murine, porcine, bovine, ovine, equine and non-human primate species,
15 and the alleles or other naturally occurring variants of the Survivin family of proteins; and derivatives wherein the Survivin protein has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope). The recombinant Survivin protein also can be used to solve the
20 molecular structure of Survivin by 2D-NMR, circular dichroism and X-ray crystallography, thus integrating the site-directed mutagenesis approach and the rational design of specific small molecule inhibitors.

As described below, members of the Survivin family of proteins can be used:

- 1) as a target to block Survivin mediated inhibition of cellular apoptosis, 2) to identify
25 and isolate binding partners that bind Survivin, 3) in methods to identify agents that block the association of Survivin with a Survivin binding partner, 4) as a target to assay for Survivin mediated inhibition of cellular apoptosis, 5) as an agent to block cellular apoptosis, administered alone or as part of a combination therapy, 6) as a binding partner in an assay to quantitate circulating levels of anti-Survivin antibodies,
30 7) as an antigen to elicit production of anti-Survivin antibodies that in turn can be

used in an assay to quantitate circulating levels of Survivin and or can be used for immunohistochemical purposes, and 8) as a therapeutic anti-cancer vaccine, or component of a polyvalent vaccine.

5 **B. Anti-Survivin Antibodies**

The present invention further provides antibodies that selectively bind to a Survivin protein. The anti-Survivin antibodies particularly contemplated include monoclonal and polyclonal antibodies as well as fragments containing the antigen binding domain and/or one or more complement determining regions.

10 Antibodies are generally prepared by immunizing a suitable mammalian host using a Survivin protein, or fragment, in isolated or immunoconjugated form (Harlow, Antibodies, Cold Spring Harbor Press, NY (1989)). Figure 9 provides a Jameson-Wolf plot of the antigenic index of various regions of Survivin. Such regions, in combination with the other structural analysis provided in Figure 9, provide suitable
15 fragments for use in generating Survivin specific antibodies. Methods for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be used; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be
20 effective.

Administration of the Survivin immunogen is conducted generally by injection over a suitable time period and with use of a suitable adjuvant, as is generally understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

25 While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen
30 cells, as is generally known. The immortalized cell lines secreting the desired

antibodies are screened by immunoassay in which the antigen is the Survivin peptide. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture
5 supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of F(ab')₂ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the
10 whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of receptor can also be produced in the context of chimeras or CDR grafted antibodies of multiple species origin.

15 The antibodies thus produced are useful not only as modulators of the association of Survivin with a Survivin binding partner, but are also useful in immunoassays for detecting Survivin expression/activity and for the purification of Survivin and associated binding partners.

20 C. Survivin Encoding Nucleic Acid Molecules

The present invention further provides nucleic acid molecules that encode Survivin, and the related Survivin proteins herein described, preferably in isolated form. For convenience, all Survivin encoding nucleic acid molecules will be referred to as the Survivin encoding nucleic acid molecule, the *Survivin* gene, or *Survivin*. As
25 used herein, "nucleic acid" is defined as RNA or DNA that encodes a peptide as defined above, or is complementary to a nucleic acid sequence encoding such peptides, or hybridizes to such a nucleic acid and remains stably bound to it under stringent conditions, or encodes a polypeptide sharing at least 75% sequence identity, preferably at least 80%, and more preferably at least 85%, with the peptide sequences.
30 Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules,

as well as nucleic acids based on an alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such hybridizing or complementary nucleic acid, however, is defined further as being novel and unobvious over any prior art nucleic acid including that which encodes, hybridizes
5 under appropriate stringency conditions, or is complementary to a nucleic acid encoding a Survivin protein according to the present invention.

As used herein, "stringent conditions" are conditions in which hybridization yields a clear and detectable sequence. Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl,
10 0.0015 M sodium citrate, 0.1% SDS at 50°C; or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium
15 citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

20 As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid.

The present invention further provides fragments of the Survivin encoding nucleic acid molecule. As used herein, a fragment of a Survivin encoding nucleic acid
25 molecule refers to a small portion of the entire protein encoding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the Survivin protein, such as the C-terminal β coils or the IAP motif, the fragment will need to be large enough to encode the functional region(s) of the Survivin protein. If the fragment is to be used as a nucleic
30 acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively



small number of false positives during probing/priming. Figure 1 identifies fragments of the *Survivin* gene that are particularly useful as selective hybridization probes or PCR primers.

Fragments of the *Survivin* encoding nucleic acid molecules of the present invention (i.e., synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding *Survivin* proteins can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*, *J Am Chem Soc* (1981) 103:3185-3191 or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the *Survivin* gene, followed by ligation of oligonucleotides to build the complete modified *Survivin* gene.

The *Survivin* encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. As described above such probes can be used to identify other members of the *Survivin* family of proteins and as described below, such probes can be used to detect *Survivin* expression and tumor growth potential. A variety of such labels are known in the art and can readily be employed with the *Survivin* encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides and the like. A skilled artisan can employ any of the art known labels to obtain a labeled *Survivin* encoding nucleic acid molecule.

Since the *Survivin* gene is an antisense or reverse orientation of the *EPR-1* gene, particularly preferred are single-stranded probes for use in diagnostic purposes. Specifically, single-stranded diagnostic probes can be used to selectively hybridize to mRNA that encodes *Survivin*. Single-stranded probes can be generated using known methods in which one strand of a double-stranded probe is isolated or in which a single stranded RNA probe is generated.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations

result in proteins having an amino acid sequence encoded by DNA falling within the contemplated scope of the present invention.

D. Isolation of Other Survivin Encoding Nucleic Acid Molecules

5 As described above, the identification of the human Survivin encoding nucleic acid molecule allows a skilled artisan to isolate nucleic acid molecules that encode other members of the Survivin family of proteins in addition to the human sequence herein described.

Essentially, a skilled artisan can readily use the amino acid sequence of Survivin
10 to generate antibody probes to screen expression libraries prepared from cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified Survivin protein (as described below) or monoclonal antibodies can be used to probe a mammalian cDNA or genomic expression library, such as lambda gtl1 library, to obtain the appropriate coding sequence for Survivin, or other members of the Survivin
15 family of proteins. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme. Figure 1 identifies important antigenic and/or putative operative domains found in the Survivin protein sequence. Such regions are preferred sources of antigenic portions of
20 the Survivin protein for the production of probe, diagnostic, and therapeutic antibodies.

Alternatively, a portion of the Survivin encoding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the Survivin family of proteins from any mammalian organisms that contains such a protein.
25 Oligomers containing approximately 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives.

Additionally, pairs of oligonucleotide primers can be prepared for use in a
30 polymerase chain reaction (PCR) to selectively clone a Survivin-encoding nucleic acid

molecule. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other Survivin encoding nucleic acid molecules. Figure 1 identifies regions of the human *Survivin* gene that are particularly well suited for use as a probe or as primers.

5

E. rDNA Molecules Containing a Survivin Encoding Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain a Survivin encoding sequence. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in vitro*. Methods for
10 generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, Molecular Cloning (1989). In the preferred rDNA molecules, a Survivin encoding DNA sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the
15 Survivin encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the *Survivin* gene included in the rDNA
20 molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as
25 being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a Survivin encoding nucleic acid molecule will include a prokaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed
30 therewith. Such replicons are well known in the art. In addition, vectors that include a

prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or
5 viral promoter capable of directing the expression (transcription and translation) of the Survivin encoding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for
10 insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, NJ.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form rDNA molecules that contain
15 a Survivin encoding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector
20 pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the
25 neomycin phosphotransferase (*neo*) gene. Southern *et al.*, *J Mol Anal Genet* (1982) 1:327-341. Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker.

**F. Host Cells Containing an Exogenously Supplied
Survivin Encoding Nucleic Acid Molecule**

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a Survivin protein of the present invention. The host cell can be
5 either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a Survivin protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the *Survivin* gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a
10 mouse, rat, monkey or human fibroblastic cell line, the most preferred being cells that do not naturally express a Survivin protein. Preferred eukaryotic host cells include the murine IL-3 dependent cell line BaF3, and the like eukaryotic tissue culture cell lines.

Any prokaryotic host can be used to express a Survivin-encoding rDNA molecule. The preferred prokaryotic host is *E. coli*.

15 Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc Natl Acad Sci USA* (1972) 69:2110; and Maniatis *et al.*,
20 Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virology* (1973) 52:456; Wigler *et al.*, *Proc Natl Acad Sci USA* (1979) 76:1373-76.

25 Successfully transformed cells, i.e., cells that contain a rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that

described by Southern, *J Mol Biol* (1975) 98:503, or Berent *et al.*, *Biotech* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method.

G. Production of Survivin Using a rDNA Molecule Encoding a Survivin Protein

The present invention further provides methods for producing a Survivin protein that uses one of the Survivin encoding nucleic acid molecules herein described. In general terms, the production of a recombinant form of a Survivin protein typically involves the following steps.

First, a nucleic acid molecule is obtained that encodes a Survivin protein, such as the nucleic acid molecule depicted in Figure 1. If the Survivin encoding sequence is uninterrupted by introns, it is directly suitable for expression in any host. If not, then a spliced form of the Survivin encoding nucleic acid molecule can be generated and used or the intron containing nucleic acid molecule can be used in a compatible eukaryotic expression system.

The Survivin encoding nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the Survivin encoding sequences. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the Survivin protein. Optionally the Survivin protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A

skilled artisan can readily adapt any host/expression system known in the art for use with Survivin encoding sequences to produce a Survivin protein.

H. Inhibition of Cell Death Using Survivin

5 As provided above, Survivin has been shown to inhibit cellular apoptosis. Accordingly, Survivin can be used in methods to extend the life of cells. In general, cellular apoptosis can be inhibited by contacting a cell with Survivin.

The are a number of situation in which it is desirable to inhibit cellular apoptosis. For example, the death of cells in tissues and organs being prepared for
10 transport and transplant can be inhibited using the Survivin protein. Alternatively, cells lines can be established for long term culture using Survivin encoding nucleic acid molecules expressed in the cell line.

Hence, Survivin protein or *Survivin* gene expression can be used as a means to inhibit cellular apoptosis. In cell culture systems, the Survivin protein can be
15 introduced into a cell, for example via liposomal, Penetrin-1 delivery, or inclusion in the cell growth media, to inhibit apoptosis. Alternatively, the *Survivin* gene can be introduced and expressed in cells to increase the longevity of cells in culture. These provide means and methods for increasing the ability of cultured cells to produce desired compounds as well as provide methods of establishing long-term culture of
20 primary explants of cells and tissues.

In tissue transplant, typically tissues and organs are stored and transported prior to transplant. Cell death, by mechanisms similar to apoptosis, can lead to the loss of viability of the tissues or organs. In this setting, infusion with Survivin protein can be used as a method to inhibit cell death in such tissues and organs.

25 There are pathological conditions characterized by premature and unwanted cellular apoptosis, for example in accelerated aging disorders. It is already known that inactivating mutations in a LAP protein may cause human diseases. The example is for the NAIP (see above). Studies of patients with SMA (Spinal muscular atrophy, a neurodegenerative disease that is thought to be caused by aberrantly increased
30 apoptosis) has demonstrated that the NAIP gene is inactivated and deleted in 75% of

these patients (Roy *et al.*, 1995, *Cell* 80:167). By extension, inactivating mutations in Survivin can result in degenerative diseases characterized by aberrantly increased cell death. Haplotypic markers within and around the Survivin locus on chromosome 17q25 can be used in studies of population genetics to determine if that locus has already been implicated in diseases with increased apoptosis. In such cases, the Survivin gene or the Survivin protein can be used to treat the conditions. Accordingly, the Survivin protein, or a Survivin encoding nucleic acid molecule is administered to an individual as a means of treating abnormal apoptosis.

10 I. Methods to Identify Survivin Binding Partners

Another embodiment of the present invention provides methods for use in isolating and identifying binding partners of Survivin. Specifically, the Survivin protein can be used as a capture probe to identify Survivin binding partners. As used herein, a Survivin binding partner is a biomolecule (such as a protein, DNA or other
15 cofactor) that binds to Survivin and mediates Survivin inhibition of cellular apoptosis.

In detail, a Survivin protein is mixed with an extract or fraction of a cell that expresses Survivin under conditions that allow the association of a binding partner with Survivin. After mixing, peptides that have become associated with Survivin are separated from the mixture. The binding partner that bound Survivin can then be removed and further analyzed.

To identify and isolate a binding partner, the entire Survivin protein can be used. Alternatively, a fragment of a Survivin protein can be used.

As used herein, a cellular extract refers to a preparation or fraction that is made from a lysed or disrupted cell. The preferred source of cellular extracts will be cells that naturally express Survivin. Examples of such cells include, but are not limited to tumor cells and embryonic tissues.

A variety of methods can be used to obtain an extract of a cell. Cells can be disrupted using either physical or chemical disruption methods. Examples of physical disruption methods include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to,



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detergent lysis and the enzyme lysis. In addition, the cellular extract can be prepared from cells that have been freshly isolated from a subject or from cells or cell lines which have been cultured. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

5 Once an extract of a cell is prepared, the extract is mixed with the Survivin protein under conditions in which association of Survivin with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that closely resemble conditions found in the cytoplasm of a Survivin-expressing cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract
10 used, can be varied to optimize the association of the Survivin with the binding partner.

 After mixing under appropriate conditions, Survivin is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to Survivin can be used to immunoprecipitate the
15 Survivin and associated binding partner. Alternatively, standard chemical separation techniques such as chromatography and density/sediment centrifugation can be used.

 After removal of nonassociated cellular constituents found in the extract, the binding partner can be dissociated from the Survivin protein using conventional methods. For example, dissociation can be accomplished by altering the salt
20 concentration or pH of the mixture.

 To aid in separating associated Survivin/binding partner pairs from the mixed extract, the Survivin protein can be immobilized on a solid support. For example, Survivin can be attached to a nitrocellulose matrix or acrylic beads. Attachment of Survivin to a solid support further aids in separating peptide/binding partner pair from
25 other constituents found in the extract.

 Alternatively, the Survivin-encoding nucleic acid molecule can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the Survivin encoding molecules herein described.

30

J. Use of Survivin Binding Partners

Once isolated, the Survivin binding partners obtained using the above described methods can be used for a variety of purposes. The binding partners can be used to generate antibodies that bind to the Survivin binding partner using techniques known in the art. Antibodies that bind a Survivin binding partner can be used to assay Survivin activity, as a therapeutic agent to modulate a biological or pathological process mediated by Survivin, or to purify the binding partner. These uses are described in detail below.

**K. Methods to Identify Agents that Block
Survivin/Binding Partner Interactions**

Another embodiment of the present invention provides methods for identifying agents that reduce or block the association of Survivin with a Survivin binding partner. Specifically, Survivin is mixed with a Survivin binding partner in the presence and absence of an agent to be tested. After mixing under conditions that allow association of Survivin with the Survivin binding partner, the two mixtures are analyzed and compared to determine if the agent reduced or blocked the association of Survivin with the Survivin binding partner. Agents that block or reduce the association of Survivin with the Survivin binding partner will be identified as decreasing the amount of association present in the sample containing the tested agent.

As used herein, an agent is said to reduce or block Survivin/Survivin binding partner association when the presence of the agent decreases the extent to which or prevents the Survivin binding partner from becoming associated with Survivin. One class of agents will reduce or block the association by binding to the Survivin binding partner while another class of agents will reduce or block the association by binding to Survivin.

The Survivin binding partner used in the above assay can either be an isolated and fully characterized protein or can be a partially characterized protein that binds to Survivin or a Survivin binding partner that has been identified as being present in a

cellular extract. It will be apparent to one of ordinary skill in the art that so long as the Survivin binding partner has been characterized by an identifiable property, e.g., molecular weight, the present assay can be used.

Agents that are assayed in the above method can be randomly selected or
5 rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the Survivin with the Survivin binding partner. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

10 As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. As described above, there are two sites of action for agents that block Survivin/Survivin binding partner interaction: the binding partner contact site on Survivin and the Survivin
15 contact site on the Survivin binding partner. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up the contact sites of the Survivin/Survivin binding partner pair. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to the Survivin contact site on the Survivin binding partner. Such an agent will reduce or block the
20 association of Survivin with the binding partner by binding to the Survivin binding partner.

The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present
25 invention. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the Survivin protein.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the
30 DNA encoding these peptides may be synthesized using commercially available

oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive
5 with critical positions of the Survivin or Survivin binding partner. As described above, antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the Survivin or binding partner, intended to be targeted by the antibodies. Critical regions include the contact sites involved in the association of the Survivin with the Survivin binding partner.

10 As discussed below, the important minimal sequence of residues involved in Survivin activity define a functional linear domain that can be effectively used as a bait for two-hybrid screening and identification of potential Survivin-associated molecules. Use of such Survivin fragments will significantly increase the specificity of the screening as opposed to using the full length molecule or the entire BIR domain
15 and is therefore preferred. Similarly, this linear sequence can be also used as an affinity matrix also to isolate Survivin binding proteins using a biochemical affinity purification strategy.

**L. Uses for Agents that Block the Association of
20 Survivin with a Survivin Binding Partner**

As provided in the Background section, Survivin inhibits cellular apoptosis. Agents that reduce or block the interactions of Survivin with a Survivin binding partner can be used to modulate biological and pathologic processes associated with Survivin function and activity.

25 In detail, a biological or pathological process mediated by Survivin can be modulated by administering to a subject an agent that blocks the interaction of Survivin with a Survivin binding partner.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by Survivin. The

term "mammal" is meant an individual belonging to the class *Mammalia*. The invention is particularly useful in the treatment of human subjects.

As used herein, a biological or pathological process mediated by Survivin or Survivin binding to a Survivin binding partner refers to the wide variety of cellular events mediated by Survivin. Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, a pathological process mediated by Survivin is the inhibition of cellular apoptosis in tumor cells. This pathological process can be modulated using agents that reduce or block Survivin/Survivin binding partner association or block Survivin expression.

As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For example, an agent is said to modulate tumor cell proliferation when the agent decrease the rate or extent of cell division.

M. Administration of Survivin or Agents that Affect Survivin Activity

The agents of the present invention, whether they be agents that block Survivin/binding partner association or the Survivin protein, can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For example, to treat tumor cells as a means of blocking Survivin inhibition of apoptosis, an agent that blocks Survivin expression or the interaction of Survivin with a binding partner, is administered systemically or locally to the individual being treated. As described below, there are many methods that can readily be adapted to administer such agents.

The present invention further provides compositions containing Survivin or one or more agents that block Survivin/binding partner association. While individual needs vary, a determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 $\mu\text{g/kg}$ body wt. The

preferred dosages comprise 0.1 to 10 $\mu\text{g/kg}$ body wt. The most preferred dosages comprise 0.1 to 1 $\mu\text{g/kg}$ body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be coadministered along with other compounds typically prescribed for these conditions according to generally accepted medical practice, such as chemotherapeutic agents.

N. Combination Therapy

Survivin, as well as agents of the present invention that modulate Survivin activity, can be provided alone, or in combination with another agents that modulate a particular biological or pathological process. For example, an agent of the present invention that reduces Survivin inhibited apoptosis can be administered in combination with other anti-cancer agents in methods to control cancer cell growth. Alternatively, Survivin can be administered with other protective agents as a means for reducing cellular apoptosis. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

Inhibition of Survivin activity/expression can be used in combination with conventional chemotherapies. The timing for using a chemotherapeutic agent in combination with inhibiting Survivin activity/expression depends upon chemotherapeutic agent used and the tumor cell type treated. Examples of chemotherapeutic agents that can be used in combination with agents the effect Survivin activity/expression, includes, but is not limited to alkylating agents, such as cyclophosphamide (CTX; cytoxan), chlorambucil (CHL; leukeran), cisplatin (CisP; platinol) busulfan (myleran), melphalan, carmustine (BCNU), streptozotocin, triethylenemelamine (TEM), mitomycin C, and the like alkylating agents; anti-metabolites, such as methotrexate (MTX), etoposide (VP16; vepesid) 6-mercaptopurine (6MP), 6-thioguanine (6TG), cytarabine (Ara-C), 5-fluorouracil (5FU), dacarbazine (DTIC), and the like anti-metabolites; antibiotics, such as actinomycin D, doxorubicin (DXR; adriamycin), daunorubicin (daunomycin), bleomycin, mithramycin and the like antibiotics; alkaloids, such as vinca alkaloids such as vincristine (VCR), vinblastine, and the like; and other antitumor agents, such as taxol and taxol derivatives, the cytostatic agents glucocorticoids such as dexamethasone (DEX; decadron) and corticosteroids such as prednisone, nucleoside enzyme inhibitors such as hydroxyurea, amino acid depleting enzymes such as asparaginase, and the like diverse antitumor agents.

The use of the cytotoxic agents described above in chemotherapeutic regimens is generally well characterized in the cancer therapy arts, and their use herein falls under

the same considerations for monitoring tolerance and effectiveness and for controlling administration routes and dosages, with some adjustments. For example, the actual dosages of the cytotoxic agents may vary depending upon the patient's cultured cell response determined by using the present histoculture methods. Generally, the dosage
5 will be reduced compared to the amount used in the absence of agents the effect Survivin activity/expression.

Typical dosages of an effective cytotoxic agent can be in the ranges recommended by the manufacturer, and where indicated by *in vitro* responses or responses in animal models, can be reduced by up to about one order of magnitude
10 concentration or amount. Thus, the actual dosage will depend upon the judgment of the physician, the condition of the patient, and the effectiveness of the therapeutic method based on the *in vitro* responsiveness of the primary cultured malignant cells or histocultured tissue sample, or the responses observed in the appropriate animal models.

15 **O. Methods for Identifying Survivin Expression and Survivin-Mediated Inhibition of Apoptosis**

The present invention further provides methods for identifying cells involved in Survivin-mediated inhibition of apoptosis as well as techniques that can be applied to diagnose biological and pathological processes associated with Survivin activity,
20 the progression of such conditions, the susceptibility of such conditions to treatment and the effectiveness of treatment for such conditions. Specifically, Survivin-mediated inhibition of apoptosis can be identified by determining whether the Survivin protein is expressed in a cell. Cells expressing Survivin are considered to be inhibited from natural cellular apoptosis.

25 A variety of immunological and nucleic acid techniques can be used to determine if the Survivin protein, or a Survivin encoding mRNA, is produced in a particular cell. In one example, an extract of cells is prepared. The extract is then assayed to determine whether Survivin is expressed in the cell. The degree of expression provides a measurement of the degree of inhibition of apoptosis. An
30 increase in expression is a measurement of an increased inhibition of apoptosis.

The measurement of Survivin expression can be used as a marker for a variety of purposes. In tumors, the presence of Survivin expression correlates with the proliferative potential of the tumor. In the Examples, it is shown that lymphomas display varying levels of Survivin expression; lymphomas showing little to no Survivin expression are low grade lymphomas that can be effectively treated while lymphomas showing high levels of Survivin expression are high grade aggressive lymphomas that typically cannot be effectively treated. Accordingly, the level of Survivin expression in a lymphoma, or other tumor, can be used as a predictive measurement of the aggressiveness and treatability of the tumor: the higher the level of Survivin expression, the higher the aggressiveness of the tumor and the more difficult the treatment will be.

For example, to determine a tumor's proliferative potential or ease/prognosis of treatment, an extract is made of the tumor cells and the extract is then analyzed, for example, by gel electrophoresis, to determine whether a Survivin protein is present. The presence and level of Survivin correlates with the proliferative potential of the cancer and the ease of treatment. Alternatively, as described above, single-strand probes can be used to identify Survivin-encoding mRNA in the cellular extracts.

In addition to being a marker of tumor aggressiveness and treatment potential, Survivin expression can be used as a measurement of the effectiveness of anti-tumor therapy. In the Examples, it is shown that HL-60, a promyelocytic cell line, had high levels of Survivin expression. Treatment of HL-60 cells with retinoic acid, and anti-cancer agent that acts by causing the differentiation of tumor cells, resulted in a reduction and elimination of Survivin expression. The reduction in expression correlated with the degree of differentiation, the greater the differentiation, the lower the level of Survivin expression. Accordingly, Survivin expression can be used to measure the effectiveness of anti-tumor treatment: if Survivin expression decreases during treatment, the treatment protocol is effective and can be continued, whereas if Survivin expression remains unaltered, a different therapeutic regime or protocol needs to be performed.

P. Other Methods to Control Survivin Expression

The present invention further provides additional methods that can be used to control *Survivin* expression in a cell. As discussed above and below, the *Survivin* promoter has a CPG island upstream from its promoter. CPG islands are known
5 targets for DNA methylation. The DNA methylation sites in the CPG island serves as a means for regulating *Survivin* expression: methylation of CPG islands results in the suppression of transcription of the gene found downstream from the promoter. Accordingly, agents that methylate DNA, such as DNA methylase, and agents that
10 stimulate the production of endogenous methylases, can be used to control *Survivin* expression. Specifically, *Survivin* expression in a cell can be reduced or eliminated by causing the cell to increase the level of DNA methylation, particularly at the CPG island found upstream from the *Survivin* gene.

In another method, *Survivin* expression can be reduced by increasing the level of EPR-1 expression. As shown in the Examples, *Survivin* expression and EPR-1
15 expression are generally mutually exclusive, expression of EPR-1 results in a decrease or elimination of *Survivin* expression and visa-a-versa. Accordingly, *Survivin* expression can be reduced by causing a cell to increase EPR-1 expression.

Q. Animal Models

20 We have isolated almost the complete structure of the mouse *Survivin* gene. The gene is very conserved with its human counterpart including sizes of introns, exons and intron-exon boundaries. The coding regions of the mouse *Survivin* gene are 88% , to the extent sequenced, identical to the human protein, thereby
25 demonstrating strong evolutionary conservation. We have also determined the differential and developmentally-regulated distribution of *Survivin* during both human and mouse development. The availability of the complete structure of the mouse *Survivin* gene and protein will allow the preparation of targeting vectors for gene knockout experiments and a more rational approach for the generation of transgenic mice expressing *Survivin* under the control of tissue-specific promoters.

The *Survivin* gene and the Survivin protein can serve as a target for gene therapy in a variety of contexts. For example, in one application, Survivin-deficient non-human animals can be generated using standard knock-out procedures to inactivate a *Survivin* gene or, if such animals are non-viable, inducible Survivin antisense molecules can be used to regulate Survivin activity/expression. Alternatively, an animal can be altered so as to contain a Survivin or antisense-Survivin expression unit that directs the expression of Survivin or the antisense molecule in a tissue specific fashion. In such a uses, a non-human mammal, for example a mouse or a rat, is generated in which the expression of the *Survivin* gene is altered by inactivated or activation. This can be accomplished using a variety of art-known procedures such as targeted recombination. Once generated, the Survivin-deficient animal, the animal that expresses Survivin in a tissue specific manner, or an animal that expresses an antisense molecule can be used to 1) identify biological and pathological processes mediated by Survivin, 2) identify proteins and other genes that interact with Survivin, 3) identify agents that can be exogenously supplied to overcome Survivin deficiency and 4) serve as an appropriate screen for identifying mutations within *Survivin* that increase or decrease activity.

For example, it is possible to generate transgenic mice expressing the human minigene for Survivin in a tissue specific-fashion and test the effect of over-expression of the protein in district that normally do not contain Survivin. This strategy has been successfully used for another family of apoptosis inhibitors, namely bcl-2 (Veis *et al.*, *Cell* (1993) 75:229). Such an approach can readily be applied to the Survivin protein and can be used to address the issue of a potential beneficial effect of Survivin in a specific tissue area to protect cells from apoptosis (transplant).

R. Survivin Gene Therapy

In another embodiment, genetic therapy can be used as a means for modulating a Survivin-mediated biological or pathological processes. For example, in tumor therapy, it may be desirable to introduce into the subject being treated a genetic expression unit that encodes a modulator of Survivin expression, such as an antisense

encoding nucleic acid molecule. Such a modulator can either be constitutively produced or inducible within a cell or specific target cell. This allows a continual or inducible supply of a modulator of Survivin expression within the subject. Blocking Survivin expression allows for the control of tumor cell growth. Similarly, cells may
5 be genetically engineered to express Survivin, *e.g.*, in allograft pancreatic β cells for transplantation.

The level of Survivin gene expression may correlate with the level of resistance to apoptosis. Thus, Survivin genes also find use in anti-apoptosis gene therapy. In particular, a functional Survivin gene may be used to sustain neuronal
10 cells that undergo apoptosis in the course of a neurodegenerative disease, lymphocytes (*i.e.*, T cells and B cells), or cells that have been injured by ischemia.

Retroviral vectors, adenoviral vectors, adeno-associated viral vectors, or other viral vectors with the appropriate tropism for cells likely to be involved in apoptosis (for example, epithelial cells) may be used as a gene transfer delivery system for a
15 therapeutic Survivin gene construct. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, current opinion in biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta *et al.*, Nucleic Acid Research and Molecular
20 Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, blood Cells 17:407-416, 1991; Miller *et al.*, Biotechniques 7:980-990, 1989; Le Gal La Salle *et al.*, Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg *et al.*, N. Engl. J. Med 323:370, 1990; Anderson *et al.*, U.S. Patent No.
25 5,399,346).

Non-viral approaches may also be employed for the introduction of therapeutic DNA into cells otherwise predicted to undergo apoptosis. For example, Survivin may be introduced into a neuron or a T cell by lipofection (Feigner *et al.*, Proc. Natl. Acad. Sci. USA 84:7413, 1987; Ono *et al.*, Neurosci. Lett. 117:259, 1990; Brigham *et al.*,
30 Meth. Enz. 101:512, 1983), asialorosonucoid-polylysine conjugation (Wu *et al.*, J.

Biol. Chem. 263:14621, 1988; Wu *et al.*, J. Biol. Chem. 264:16985, 1989); or, less preferably, microinjection under surgical conditions (Wolff *et al.*, Science 247:1465, 1990).

For any of the methods of application described above, the therapeutic
5 Survivin nucleic acid construct is preferably applied to the site of the predicted apoptosis event (for example, by injection). However, it may also be applied to tissue in the vicinity of the predicted apoptosis event or to a blood vessel supplying the cells predicted to undergo apoptosis.

In the constructs described, Survivin cDNA expression can be directed from
10 any suitable promoter (*e.g.*, the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in neural cells, T cells, or B cells may be used to direct Survivin expression. The enhancers used could include, without limitation, those that are
15 characterized as tissue- or cell-specific in their expression. Alternatively, if a Survivin genomic clone is used as a therapeutic construct (for example, following its isolation by hybridization with the Survivin cDNA described above), regulation may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory
20 elements described above.

S. Use of the Survivin Promoter to Direct Gene Expression

The present invention further provides the promoter of the *Survivin* gene in a form that can be used in generating expression vectors. Specifically, the *Survivin*
25 promoter, identified as being 5' from the ATG start codon in of Survivin, can be used to direct the expression of an operably linked protein encoding DNA sequence. Since the Survivin promoter does not have a TATA box, a skilled artisan would use a 5' fragment, such as nucleotides 2560-2920 (including exon 1). The *Survivin* promoter is expressed in fetal tissues and can therefore be used to target protein expression in
30 specific cell types during specific stages of development. As discussed below,

transfection of 3T3 cells with the c-myc oncogene results in the up-regulation of Survivin mRNA as detected by Northern blots. Accordingly, DNA encoding anti-tumor polypeptides under the control of the *Survivin* promoter could be used to transfect tumor cell where they would be expressed. A skilled artisan can readily use
5 the *Survivin* promoter in expression vectors using methods known in the art.

T. Preventative Anti-Apoptotic Therapy

In a patient diagnosed to be heterozygous for a *Survivin* mutation or to be susceptible to *Survivin* mutations (even if those mutations do not yet result in
10 alteration or loss of *Survivin* biological activity), or a patient diagnosed with a degenerative disease (e.g., motor neuron degenerative diseases such as SMA or ALS diseases), or diagnosed as HIV positive, any of the disclosed therapies may be administered before the occurrence of the disease phenotype. For example, the therapies may be provided to a patient who is HIV positive but does not yet show a
15 diminished T cell count or other overt signs of AIDS. In particular, compounds shown to increase *Survivin* expression or *Survivin* biological activity may be administered by any standard dosage and route of administration. Alternatively, gene therapy using a *Survivin* expression construct may be undertaken to reverse or prevent the cell defect prior to the development of the degenerative disease.

20 The methods of the instant invention may be used to reduce or diagnose the disorders described herein in any mammal, for example, humans, domestics pets, or livestock. Where a non-human mammal is treated or diagnosed, the *Survivin* polypeptide, nucleic acid, or antibody employed is preferably specific for that species.

25 U. Examples of Additional Apoptosis Assays

In addition to the foregoing discussion, specific examples of apoptosis assays are also provided in the following references. Assays for apoptosis in lymphocytes are disclosed by: Li *et al.*, "Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein", *Science* 268:429-431, 1995; Gibellini *et al.*, "Tat-expressing Jurkat cells show an increased resistance to different apoptotic stimuli, including acute



human immunodeficiency virus-type 1 (HIV-1) infection", *Br. J. Haematol.* 89:24-33, 1995; Martin *et al.*, "HIV-1 infection of human CD4⁺ T cells *in vitro*. Differential induction of apoptosis in these cells." *J. Immunol.* 152:330-42, 1994; Terai *et al.*, "Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1", *J. Clin Invest.* 87:1710-5, 1991; Dhein *et al.*, "Autocrine T-cell suicide mediated by APO-1/(Fas/CD95) 11, *Nature* 373:438-441, 1995; Katsikis *et al.*, "Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals", *J. Exp. Med.* 181:2029-2036, 1995; Westendorp *et al.*, Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120", *Nature* 375:497, 1995; DeRossi *et al.*, *Virology* 198:234-44, 1994.

Assays for apoptosis in fibroblasts are disclosed by: Vossbeck *et al.*, "Direct transforming activity of TGF-beta on rat fibroblasts", *Int. J. Cancer* 61:92-97, 1995; Goruppi *et al.*, "Dissection of c-myc domains involved in S phase induction of HIH3T3 fibroblasts", *Oncogene* 9:1537-44, 1994; Fernandez *et al.*, "Differential sensitivity of normal and Ha-ras transformed C3H mouse embryo fibroblasts tumor necrosis factor; induction of bcl-2, c-myc, and manganese superoxide dismutase in resistant cells", *Oncogene* 9:2009-17, 1994; Harrington *et al.*, "c Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines", *EMBO J.*, 13:3286-3295, 1994; Itoh *et al.*, "A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen", *J. Biol. Chem.* 268:10932-7, 1993.

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15 **V. Use of Survivin in Tissue and Organ Transplantation** The present invention includes methods of inhibiting or preventing tissue or organ transplant rejection in a subject, comprising the local administration of a Survivin polypeptide, Survivin polypeptide fragment, an apoptosis-inhibiting peptidomimetic thereof, a transgene encoding a Survivin polypeptide or a transgene encoding a Survivin
20 polypeptide fragment to the tissue, organ or to a site proximal to the transplant. Local delivery of the polypeptides, peptidomimetics to the tissue, organ or to a site proximal to the transplant is accomplished by any means commonly available, including but not limited to direct local perfusion, injection, microsponges, microcapsules, liposomes or time-released delivery vehicles.

25 Local delivery of a transgene encoding a Survivin polypeptide or a transgene encoding a Survivin polypeptide fragment to the tissue, organ or to a site proximal to the transplant may be accomplished with any available vector, via lipofection or via direct plasmid DNA injection. See Qin *et al.* (1995) *Transplantation* 59(6): 809-816; Le Coultre *et al.* (1997) *Eur. J. Pediatr. Surg.* 7(4):221-226; Wang *et al.* (1992) *Transplantation* 53(3):703-705; Wang *et al.* (1996) *Transplantation* 61(12):1726-



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1729; Schmid *et al.*, (1997) *Eur. J. Cardiothorac. Surg.* 11(6):1023-28; and Boasquevisque, C. *et al.* (1997) *Ann. Thorac. Surg.* 63(6):1556-1561. Vectors encoding the transgene include both replicable and replication-defective vectors, such as retroviral vectors, adenovirus vectors or other vectors with the appropriate tropism for the cells likely to be involved in apoptosis or cells proximal to the site of apoptosis. In the transgene constructs, expression can be directed from any suitable promoter, including tissue specific promoters which direct gene expression in specific cell types, such as the human insulin promoter. Local delivery of the transgene to the tissue, organ or to a site proximal to the transplant is accomplished by any means commonly available, including but not limited to direct local perfusion, injection, microsponges, microcapsules, liposomes or time-released delivery vehicles.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure. Other generic configurations will be apparent to one skilled in the art. All journal articles and other published documents such as patents and patent applications are hereby incorporated by reference in their entireties.

EXAMPLES

Example 1 EXPERIMENTAL PROCEDURES AND CLONING

Cells and cell culture. The following cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD), erythroleukemia HEL, B-lymphoma Daudi and JY, monocytic THP-1, T leukemia Jurkat, epithelial carcinoma HeLa, promyelocytic HL-60, and non-transformed human lung fibroblast Lu18. The T leukemia cell line MOLT13 was characterized previously (Altieri, D.C., *FASEB J*

(1995) 9:860-865). Cells were maintained in culture in complete medium RPMI 1640 or DMEM (HeLa, Lu18) (BioWhittaker, Walkersville, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Whittaker), 2 mM L-glutamine, and 10 mM HEPES. Human umbilical vein endothelial cells (HUVEC) were isolated by
5 collagenase treatment and maintained in culture in DMEM medium supplemented with 20% FBS, 2 mM L-glutamine and endothelial cell growth factor (Biomedical Technologies, Stoughton, MA).

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood collected from normal informed volunteers by differential centrifugation on
10 Ficoll-Hypaque (Pharmacia, Piscataway, NJ) at 400g for 22°C, and washed in phosphate buffered saline (PBS), pH 7.4. In some experiments, HL-60 cells were terminally differentiated to a mature monocytic phenotype by a 72 h culture in the presence of 0.1 μ M 1, 25-dihydroxy-vitamin D₃ and 17.8 μ g/ml indomethacin (Sigma Chemical Co., St. Louis, MO). *De novo* induction of differentiation-dependent
15 markers on vitamin D₃-treated HL-60 cells, including CD11b/CD18 integrin (Hickstein, D.D. *et al.*, *J Immunol* (1987) 138:513-519) was determined by flow cytometry with anti-CD11b mAb LM2/1.

Genomic and cDNA cloning, chromosomal localization and Southern blots. A human P1 genomic library (Genome Systems, St. Louis, MO) was screened by
20 hybridization with a 1.6 kb fragment containing the complete human EPR-1 cDNA (Altieri, D.C., *FASEB J* (1995) 9:860-865). Three overlapping clones were isolated, purified and confirmed by Southern hybridization with the EPR-1 cDNA. Hybridizing fragments generated by restriction digest with *Bam*HI, *Hind*III and *Xba*I (Boehringer Mannheim, Indianapolis, IN) were cloned in pBluescript (pBSKS⁺,
25 Stratagene, San Diego, CA) for further analysis. An overlapping contig spanning 14796 bp from two EPR-1-hybridizing P1 clones was arrayed, characterized by restriction analysis, and completely sequenced on both strands by Taq FS polymerase-based automated sequencing using a Applied BioSystem Prism 377 automated
sequencer (Foster City, CA). In some experiments, 10 mg of total RNA extracted
30 from HeLa cells by the guanidinium isothiocyanate method was primed with EPR-1

forward "sense" oligonucleotide C3/27 (bp 80-102) and reverse transcribed in the presence of 200 U of Superscript II (Life Science, Grand Island, NY) for 50 min at 42°C.

The resulting cDNA was amplified by PCR in the presence of 0.5 mg of EPR-
5 1-derived primers T5/27 (bp 161-184) and G11/16 (1124-1098, numbering from the EPR-1 coding sequence), 200 mM dNTPs (New England Biolabs, Beverly, MA) and 2 U Vent DNA polymerase (New England Biolabs) in a total volume of 50 µl. After 35 cycles of amplification with annealing at 58°C for 1 min, denaturation at 94°C for 1 min and extension at 72°C for 1 min, the product was analyzed by agarose gel
10 electrophoresis, subcloned in pCRII (Invitrogen Corp., San Diego, CA), and completely sequenced on both strands. Contig assembly, and DNA and protein sequence analyses were performed using Lasergene (DNASTAR, Madison, WI) and MacVector (Eastman Kodak, Rochester, NY) software packages. Chromosomal location of the EPR-1-hybridizing gene was carried out by fluorescence *in situ*
15 hybridization. Purified DNA from a EPR-1-hybridizing P1 clone was labeled with digoxigenin dUTP (Amersham Corp., Arlington Heights, IL) by nick translation.

The labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated PBMC in a solution containing 50% formamide, 10% dextran sulfate and 2X SSC. For two-
20 color staining, biotin-conjugated probe D17Z1, specific for the centromere of chromosome 17, was co-hybridized with the digoxigenin-labeled P1 clone. Specific staining was detected by incubating the hybridized slides with fluoresceinated anti-digoxigenin antibodies and Texas red avidin. Slides were counterstained with propidium iodide for one color labeling, or with DAPI for two color labeling. A total
25 of 80 metaphase cells were analyzed with 69 cells exhibiting specific labeling. For Southern hybridization, human genomic DNA was extracted from HeLa cells according to published protocols, digested with *EcoRI*, *BamHI*, *XbaI* or *HindIII*, separated on a 0.8% agarose gel and transferred to GeneScreen nylon membranes (New England Nuclear, Boston, MA).

After UV cross-linking (Stratalinker, Stratagene, San Diego, CA), the membrane was prehybridized with 100 mg/ml of denatured salmon sperm DNA (Promega Corp. Madison, WI) in 5X SSC, 0.5% SDS, 5X Denhardt's solution and 0.1% sodium pyrophosphate at 65°C in a roller hybridization oven (Hoefer Scientific, San Francisco, CA). Hybridization was carried out with gel-purified (GeneClean Bio101, Vista, CA), ³²P-dCTP (Amersham) random-primed labeled (Boehringer-Mannheim, Indianapolis, IN) 1.6 kb EPR-1 cDNA for 16 h at 65°C.

After two washes in 2X SSC, 1% SDS for 30 min at 65°C, and 0.2X SSC at 22°C, radioactive bands were visualized by autoradiography using a Kodak X-Omat AR X-ray film and intensifying screens (DuPont de Nemours, Wilmington, DE). In other experiments, cultured lymphoblastoid cells were embedded in LMP agarose (Bio Rad, Richmond, CA) at the concentration of 2x10⁶/220 µl block and DNA was extracted by standard procedures. After block digestion with *Mlu*I or *Not*I, samples were separated by pulsed field gel electrophoresis on a 1% agarose gel for 20 h at 200 V with a pulse time of 75 sec using a Bio-Rad CHEF DRII apparatus (Hercules, CA). After transfer to nylon membranes, and UV cross-linking, hybridization with the EPR-1 cDNA and washes were carried out as described above.

In another series of experiments, a blot containing aliquots of genomic DNA isolated from several species (Clontech, San Francisco, CA) was hybridized with a 3' 548 bp fragment of the EPR-1 cDNA, as described above.

Northern blots. Single strand probes specific for sense or antisense EPR-1 sequences were generated by asymmetric PCR amplification of a 301 bp fragment of the EPR-1 cDNA. The template, comprising the first 5' 226 bp of the EPR-1 coding sequence plus 75 bp of the retained regulatory intron (Altieri, D.C., *FASEB J* (1995) 9:860-865), was generated by restriction digest of the EPR-1 cDNA with *Eco*RI (cloning site) and *Sac*II, gel-purified, and mixed in a total volume of 10 µl with 15 pmol dNTP (New England Biolabs), 7.5 pmol dCTP, and 25 mCi ³²P-dCTP (Amersham), in the presence of 20 mM Tris HCl, 50 mM KCl, pH 8.4, 1.5 mM MgCl₂, and 2.5 U of *Taq* DNA polymerase (Life Science).

Generation of a EPR-1-specific antisense probe was carried out by addition of 0.2 mg/ml of a "SacII" reverse oligonucleotide 5'TGCTGGCCGCTCCTCCCTC3', while extension of the EPR-1 positive strand and generation of a Survivin-specific probe was performed using 0.2 mg/ml of forward F11 oligonucleotide 5'ATGACCTCCAGAGGTTTC3'. Twenty-five cycles of amplification were carried with denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. The EPR-1 sense or antisense probes were centrifuged through a Sephadex G-50 spin column (Worthington Biochemical Corp., Freehold, NJ) at 14,000g for 5 min to separate free from incorporated radioactivity, heated at 100°C for 2 min, and immediately added to the hybridization reaction.

Identical strand-specific probes were used for hybridization of multiple tissue blots of adult or fetal human mRNA (Clontech), in 5X SSPE, 10X Denhardt's solution, 2% SDS, 100 mg/ml denatured salmon sperm DNA at 60°C for 14 h, and washes at 60°C, as described above. Aliquots of total RNA extracted from undifferentiated or vitamin D₃ terminally differentiated HL-60 cells, were processed for Northern hybridization with Survivin-specific single strand probe, as described above.

Example 2 PRODUCTION OF ANTI-SURVIVIN ANTIBODIES

A Survivin sequence-specific antibody, called JC700, was produced and characterized as follows. A seventeenmer peptide corresponding to the Survivin sequence A³PTLPPAWQPFLKDHRI¹⁹, was synthesized and characterized by mass spectrometry. One hundred mg of the Survivin peptide were coupled in a 1:1 ratio to Keyhole Limpet Hemocyanin and injected s. c. into a rabbit in complete Freund's adjuvant. After a 4-week interval, animals were boosted with s. c. injection of 100 mg of peptide in incomplete Freund's adjuvant and sequentially boosted and bled at alternate weeks.

Purification of the anti-Survivin antibody was carried out by affinity chromatography on a peptide-Sepharose matrix (5 mg/ml of peptide) with elution of the specific IgG fraction in 1 mM glycine, pH 2.5. Specificity of the affinity-purified

anti-Survivin antibody, designated JC700, was determined by ELISA against the immobilized Survivin peptide or a control EPR-1 peptide by absorbance at OD₄₀₅.

**Example 3 PRODUCTION OF A MONOCLONAL
ANTIBODY AGAINST A SURVIVIN FUSION PROTEIN**

5 The Survivin cDNA was expressed as a GST-fusion protein in E.Coli BL21 strain and purified to homogeneity with removal of the GST frame. The purified protein was used to inject mice and generate monoclonal antibodies using standard hybridoma technology. Three independent mAbs were isolated, cloned twice by
10 limiting dilution and further characterized. One of the new anti-Survivin mAbs, designated 8E2, recognized the immobilized, purified recombinant Survivin by ELISA and bound to Survivin in immunoblots, as shown in Figure 11.

Example 4 IMMUNOBLOTTING AND IN SITU HYBRIDIZATION

15 For immunoblotting, aliquots of SDS-solubilized extracts of various transformed cell lines, non-transformed HUVEC, PBMC or Lu18, or undifferentiated or vitamin D₃-differentiated HL-60 cells, were normalized for protein content by absorbance at OD₂₈₀, separated by electrophoresis on a 5-20% SDS polyacrylamide gradient gel under non reducing conditions, and electroblotted to Immobilon
20 membranes (Millipore Corp., New Bedford, MA) at 1.1 A for 30 min at 22°C. The membrane was blocked in TBS, pH 7.4, plus 5% milk, and incubated with 20 mg/ml of control non-immune rabbit IgG or anti-Survivin antibody JC700 for 1 h at 22°C, followed by washes in TBS, pH 7.4, and addition of a 1:7500 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) for 30 min at 22°C. Binding
25 of the primary antibody was revealed by addition of 75 mg/ml nitro blue tetrazolium in 70% dimethylformamide (Sigma Chemical Co., St. Louis, MO) plus 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 100% dimethylformamide.

Tissue samples, immunohistochemistry and *in situ* hybridization. Tissue
30 samples from colon adenocarcinoma (6 cases), lung squamous cell carcinoma (6 cases), lung adenocarcinoma (9 cases), pancreas adenocarcinoma (2 cases), invasive

breast adenocarcinoma (7 cases), were obtained from the archives of Yale-New Haven Hospital and used in the present study. Samples of 44 high grade lymphoma tissues and 7 low grade lymphoma tissue was also obtained. Tissue samples were fixed in formalin, embedded in paraffin, cut in 5 μ m sections, deparaffinized in xylene, and rehydrated in graded alcohol followed by quenching of endogenous peroxidase activity by treatment with 2% H₂O₂ in methanol.

For immunostaining, the slides were boiled for 5 min in a standard pressure cooker, blocked in 10% normal goat serum, and incubated with affinity-purified anti-Survivin antibody JC700 (20 μ g/ml) for 14 h at 4°C. After washes in PBS, pH 7.4, slides were further incubated with biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 30 min at 22°C, and washed in PBS, pH 7.4. After addition of streptavidin-biotin conjugated peroxidase (Boehringer Mannheim) for 30 min at 22°C, slides were washed, and binding of the primary mAbs was revealed by addition of 3'-3'-diamino-benzidine (DAB) and counterstaining with hematoxylin.

Negative controls were carried out by replacing the primary antibody with normal goat serum, under the same experimental conditions. In some experiments, aliquots of JC700 antibody were pre-adsorbed with 25 mg/ml of the Survivin 3-19 peptide before tissue staining. For *in situ* hybridization, 1 μ g of the Survivin cDNA containing the entire coding sequence plus 271 bp of 3' untranslated region in pcDNA3 (Invitrogen), was completely digested with *Eco*RI and transcribed in the antisense orientation using T7 RNA polymerase in the presence of digoxigenin 11-uridine-5' triphosphate (Boehringer Mannheim). Tissue slides were coated with 1% gelatin, 0.1% chrome-alum, baked at 120°C for 2 h, and stored dust-free at 22°C. Sections were deparaffinized and rehydrated through graded alcohol, digested with proteinase K (1 μ g/ml in 100 mM Tris HCl pH 8.7, 50 mM EDTA) for 30 min at 37°C, and acetylated in 0.25% acetic anhydride acid and 100 mM triethanolamine pH 8.0 for 10 min at 22°C.

Detection of Survivin mRNA in human tissues was carried out by *in situ* hybridization of the Survivin antisense riboprobe in a buffer containing 4X SSC, 1X Denhardt's solution, 50% deionized formamide, 250 μ g/ml yeast tRNA, 500 μ g/ml

salmon sperm DNA and 5% dextran for 16 h at 50°C. After washes in 2X SSC for 90 min at 48°C, immobilized digoxigenin was detected using an anti-digoxigenin mAb (Boehringer Mannheim) at a 1:3000 dilution, and revealed by alkaline phosphatase staining with NBT/BCIP cytochemical stain.

5

Example 5 EXPRESSION OF SURVIVIN IN HUMAN CANCERS

Survivin is prominently expressed in human cancer. For its abundant distribution in transformed cell types, a potential expression of Survivin in neoplasia was investigated, *in vivo*. Immunohistochemical analysis of formalin-fixed, paraffin
10 embedded tissue sections with the affinity-purified anti-Survivin JC700 antibody demonstrated abundant expression of Survivin in all cases examined of human lung cancer, including adenocarcinoma (Figure 6A), and squamous cell carcinoma (Figure 6C). Consistent with the topography of other IAP proteins (Duckett, C.S. *et al.*, *EMBO J* (1996) 15:2685-2694), expression of the protein was exclusively localized to
15 the cytoplasm of tumor cells, while the adjacent normal gland epithelium of the lung did not express Survivin (Figure 6C, arrow). No staining was observed when the anti-Survivin antibody was substituted with control goat serum (not shown), or after pre-adsorption with the immunizing Survivin 3-19 peptide (Figure 6B), thus confirming the specificity of the observed recognition.

20 Prominent accumulation of Survivin mRNA in squamous lung cell carcinoma was independently demonstrated by *in situ* hybridization with a Survivin-specific single strand riboprobe (Figure 6D). Survivin was also abundantly detected in all cases examined of adenocarcinoma of pancreas (Figure 6E), and breast (not shown) by immunohistochemistry, and colon (Figure 6G) by *in situ* hybridization. However,
25 consistent with its absence in non-transformed cell types HUVEC and Lu18 (Figure 4C), in mature tissues (Figure 3), and in terminally-differentiated HL-60 cells (Figure 5), no reactivity of the anti-Survivin JC700 antibody was observed with normal exocrine pancreatic epithelial cells by immunohistochemistry (Figure 6F), and no Survivin mRNA was found in the adjacent non-neoplastic colon gland epithelium by
30 *in situ* hybridization (Figure 6H).

Expression of Survivin in Lymphoma Tissue. Tissue samples were obtained from 44 patients with aggressive, high grade lymphoma and 7 samples were obtained from 7 patients with non-aggressive, low grade lymphoma. The sample were treated as described above and examined for Survivin expression. None of the low grade
5 lymphoma samples displayed Survivin expression whereas 27 samples (61%) from patients with high grade lymphoma expressed Survivin.

Example 6 EXPRESSION OF SURVIVIN IN OTHER CANCERS

In addition to the malignant forms of cancer discussed above, the expression
10 of Survivin in other types of cancers was investigated in the inventors' laboratory or collaboratively with other academic investigators. Survivin was found prominently expressed in the most aggressive and metastatic forms of malignant thymoma (-100 cases tested), in head and-neck squamous cell carcinoma (-140 cases) and in all forms of prostate cancer (15 cases), including the transition lesion of benign prostate
15 hyperplasia. The most aggressive forms of neuroblastoma are also positive for Survivin as discussed below.

Example 7 TISSUE SPECIFIC EXPRESSION OF SURVIVIN

Survivin, was recently found in all the most common human cancers but not in
20 normal, terminally differentiated adult tissues. The expression of Survivin in embryonic and fetal development was investigated. Immunohistochemistry and *in situ* hybridization studies demonstrated strong expression of Survivin in several apoptosis-regulated fetal tissues, including the stem cell layer of stratified epithelia, endocrine pancreas and thymic medulla, with a pattern non-overlapping with that of
25 another apoptosis inhibitor, i.e. *bcl-2*. A sequence-specific antibody to Survivin immunoblotted a single -16.5 kD Survivin band in human fetal lung, liver, heart, kidney and gastrointestinal tract. In mouse embryo, prominent and nearly ubiquitous distribution of Survivin was found at embryonic date (E) 11.5, whereas at E15-21, Survivin expression was restricted to the distal bronchiolar epithelium of the lung and
30 neural crest-derived cells, including dorsal root ganglion neurons, hypophysis and the

chorioid plexus. These data suggest that expression of Survivin in embryonic and fetal development may contribute to tissue homeostasis and differentiation independently of *bcl-2*.

5 **Example 8 PREPARATION OF SURVIVIN TRANSFECTANTS**

Inducible Survivin antisense transfectants and apoptosis/proliferation

experiments. A 708 bp *SmaI-EcoRI* fragment comprising nucleotides 379-1087 of the EPR-1 cDNA, was directionally cloned in the sense orientation in the mammalian cell expression vector pML1 (generously provided by Dr. R. Pytela, Cardiovascular
10 Research Institute, University of California, San Francisco). The vector is derived from the episomal mammalian cell expression vector pCEP4 by replacing the cytomegalovirus promoter cassette with the mMT1 promoter, directing Zn^{2+} -dependent expression of recombinant proteins in mammalian cells (Lukashev, M.E. *et al.*, *J Biol Chem* (1994) 269:18311-18314).

15 Ten million HeLa cells were incubated with 10 mg of pML1 DNA containing the Survivin antisense construct plus 50 mg of salmon sperm DNA for 15 min on ice, followed by a single electric pulse delivered by a Gene Pulser apparatus (Bio-Rad) at 350 V at 960 μ F. Forty-eight h after transfection, cells were diluted fifteen fold, plated onto 100 mm diameter tissue culture dishes and selected for 4 weeks in
20 complete growth medium containing 0.4 mg/ml hygromycin. Apoptosis in control cultures or Survivin antisense HeLa cell transfectants was evaluated by *in situ* detection of internucleosomal DNA degradation after Zn^{2+} -dependent induction of EPR-1 transcription under serum-starving conditions.

 Briefly, control or antisense Survivin transfectants were treated with 200 mM
25 $ZnSO_4$ in 0% FBS for 24 h at 37°C. Cells were harvested, centrifuged at 800g for 10 min at 4°C, and the pellet was fixed in 10% formalin overnight, dehydrated, embedded in paraffin blocks, and sections of 3-5 mm were put on high adhesive slides. Samples were treated with 20 mg/ml proteinase K for 15 min at 22°C, washed in distilled water, quenched of endogenous peroxidase in 2% H_2O_2 in PBS, and
30 subsequently mixed with digoxigenin-labeled dUTP in the presence of terminal

deoxynucleotidyl transferase (TdT) followed by peroxidase conjugated anti-digoxigenin antibody.

Nuclear staining in apoptotic cells was detected by DAB, according to the manufacturer's instructions (AptoTag, Oncor, Gaithersburg, MD). Control experiments were performed by omitting the enzyme incubation step. Morphologic features of apoptotic cells (apoptotic bodies) under the various conditions tested were detected by hematoxylin/eosin staining of the same slides.

For proliferation experiments, vector control HeLa cells or Survivin antisense transfectants were plated at 20×10^4 /well onto 24-well tissue culture plates (Costar), induced with 200 mM ZnSO_4 for 16 h at 37°C, harvested at 24 h intervals, and cell proliferation under the various conditions tested was determined microscopically by direct cell count. Down-regulation of Survivin expression under these experimental conditions was assessed by immunoblotting with JC700 antibody.

15 Example 9 IDENTIFICATION OF EPR-1 COMPLEMENTARY GENE

Three overlapping clones were isolated by hybridization screening of a human P1 plasmid genomic library with the EPR-1 cDNA and confirmed by Southern blot. This gene was located to the long arm of chromosome 17, to band 17q25, by fluorescence *in situ* hybridization (Figure 1A, B).

20 A contig of P1 fragments spanning 14796 bp was cloned in pBSKS⁺ and completely sequenced on both strands (Figure 1C). Three putative splice sites, matching perfectly the consensus sequences for eukaryotic intron-exon boundaries (Padgett, R.A. *et al.*, *Ann Rev Biochem* (1986) 55:1119-1150), were identified at position 2922, 3284, and 5276 (donor), and 3173, 5157, and 11954 (acceptor), thus defining a gene organization in four exons and three introns of 252, 1874, and 6678 bp, respectively (Figure 1D).

Sequence analysis of the putative coding regions demonstrated a nearly complete identity with the EPR-1 cDNA (Altieri, D.C., *FASEB J* (1995) 9:860-865), except for 5 nucleotide changes and 6 nucleotide insertions. However, the three splice sites were found on the complementary, antisense strand of the EPR-1 coding

30

sequence. Consistent with this unexpected orientation, the EPR-1 complementary gene revealed a 5' GC rich region, comprising nucleotides 2560-2920 and including exon 1 (see below), which fulfilled the base composition criteria of a CpG island (Gardiner-Garde, M. *et al.*, *J Mol Biol* (1987) 196:261-282 and Frommer, 1987).

- 5 Sequencing the 2.5 kb upstream the CpG island revealed a TATA-less promoter with numerous Sp1 sites (not shown).

Complex hybridization pattern and evolutionary conservation of EPR-1 sequences. Probing human genomic DNA with the EPR-1 cDNA revealed several hybridizing fragments (Figure 2A). Of these, a ~7.5 kb *Xba*I, a 7.6 kb *Bam*HI, and 4
10 *Hind*III fragments of ~ 15, 7.5, 6.4, and 3.7 kb, respectively (Figure 2A, arrows), could not be recapitulated by the restriction map of the antisense EPR-1 gene (Figure 1C). In contrast, other bands of comparable intensity, including a 5.15 kb *Xba*I and a 7.1 kb *Bam*HI fragment, genuinely originated from the antisense EPR-1 gene and comprised the first two, or three exons, respectively (Figure 2A).

- 15 At variance with this complex hybridization pattern, Southern blot of high molecular weight human genomic DNA digested with *Mlu*I or *Not*I and separated by pulsed field gel electrophoresis, revealed single EPR-1-hybridizing bands of ~ 75 kb and 130 kb, respectively (Figure 2B). Finally, Southern blots of multiple species genomic DNA revealed significant evolutionary conservation of EPR-1-related
20 sequences (Figure 2C), with numerous strongly hybridizing bands in mammalian species and fainter signals in rabbit or chicken genomic DNA, under high stringency hybridization conditions (Figure 2C).

- Discordant tissue distribution of sense/antisense EPR-1 transcripts. The potential expression of distinct sense or antisense EPR-1 transcripts was investigated
25 in Northern blots with single strand-specific probes. Consistent with the size of the spliced EPR-1 message (Altieri, D.C., *FASEB J* (1995) 9:860-865), an EPR-1 strand-specific probe detected a prominent ~ 1.2 kb band in mRNA extracted from all adult and terminally-differentiated human tissues examined (Figure 3A). In contrast, no specific bands hybridized with a EPR-1 antisense-specific single strand probe in adult
30 tissues, under the same experimental conditions (Figure 3B). A similar ~ 1.2 kb band

was detected by the single strand EPR-1-specific probe in fetal kidney, and, to a lesser extent, in fetal liver, lung and brain (Figure 3A). At variance with the absence of hybridization in adult tissues, the EPR-1 antisense-specific probe recognized a prominent ~ 1.9 kb band, and a larger 3.2 kb species corresponding to the size of an incompletely processed transcript, in fetal liver, while fainter hybridization bands were also seen in fetal kidney, lung and brain (Figure 3B). A control hybridization with an actin probe confirmed comparable loading of mRNA in adult or fetal samples (Figure 3C).

Characterization of the antisense EPR-1 gene product. Inspection of the 5' CpG island in the antisense EPR-1 gene revealed a putative ATG initiation codon at position 2811, surrounded by a sequence (CGGCATGG) that conformed well to the consensus for eukaryotic initiation of translation (Kozak, M., *Nucleic Acids Res* (1984) 12:857-872). Analysis of the antisense EPR-1 sequence in the 5'→3' direction dictated by the position of intron-exon boundaries revealed an open reading frame of 426 bp, spanning all four exons, and terminating with a TGA codon at position 12042 in exon 4. A canonical polyadenylation signal (AATAAA) was found at position 13166. PCR products amplified from reverse-transcribed HeLa cell RNA primed with EPR-1 "sense" oligonucleotides matched perfectly the genomic sequence and confirmed the open reading frame and the predicted intron-exon boundaries (not shown).

Two λgt11 cDNA clones isolated by hybridization of a HEL library with the EPR-1 cDNA, also matched the consensus genomic sequence and revealed a homopolymeric A tail on the antisense EPR-1 strand at position 13186, 14 bp downstream the polyadenylation signal, generating a 3' untranslated region of 1144 bp. In these clones, the 5' untranslated region upstream from the initiating ATG was of 49 bp, beginning at position 2762 in the genomic sequence, and contained an in-frame termination codon. Translation of the antisense EPR-1 open reading frame predicted a new protein of 142 amino acids, with an estimated molecular weight of 16,389 and an acidic pI of 5.74, lacking an amino-terminus signal peptide or a carboxy terminus hydrophobic stretch for membrane insertion (Figure 4A).

A coiled coil was predicted for the last 40 carboxy terminus residues (Lupas, A. *et al.*, *Science* (1991) 252:1162-1164). BLAST database searches revealed a significant degree of similarity between residues 18-88 of the antisense EPR-1 gene product and the BIR module in the IAP family of inhibitors of apoptosis (Birnbau,
5 M.J. *et al.*, *J Virology* (1994) 68:2521-2528; Clem, R.J. *et al.*, *Mol Cell Biol* (1994) 14:5212-5222). For this analogy, the antisense EPR-1 gene product was designated Survivin. At variance with other IAP proteins, Survivin contained only one BIR, encoded by the first three exons of the gene, and lacked a carboxy terminus RING finger, without additional/alternative exon(s) potentially encoding this domain (Figure
10 1C).

An alignment by the Clustal method between the Survivin BIR and that of other known IAP proteins is shown in Figure 4B. Despite the overall match of the consensus and several conservative substitutions, phylogenetic analysis suggested that Survivin is a distantly related member of the IAP family, most closely related to
15 NAIP, which also lacked a RING finger (Figure 4B, shaded boxes) (Roy, N. *et al.*, *Cell* (1995) 80:167-178).

A rabbit polyclonal antiserum designated JC700, was raised against residues A³PTLPPAWQPFLKDHRI¹⁹ (SEQ ID NO:3) of Survivin, purified by affinity chromatography on a peptide-Sepharose column, and used in Western blots.
20 Consistent with the predicted molecular weight of Survivin, JC700 antibody immunoblotted a single band of ~ 16.5 kDa from detergent-solubilized extracts of all transformed cell lines examined, including B lymphoma Daudi and JY, T leukemia Jurkat and MOLT13, monocytic THP-1, and erythroleukemia HEL (Figure 4C).

Survivin was also found in isolated peripheral blood mononuclear cells
25 (PBMC). In contrast, no expression of Survivin was detected in non-transformed Lu-18 human lung fibroblasts or human umbilical vein endothelial cells (HUVEC) (Figure 4C). No specific bands were immunoblotted by control non-immune rabbit IgG, under the same experimental conditions (Figure 4C).

Identification of agents that modulate transcription of the EPR-1 gene. Agents
30 that increase the transcription of the EPR-1 gene may be identified by conventional



techniques. Preferably, a candidate agent is brought into contact with a cell that expresses the EPR-1 gene product and the level of expression of this product or the level of transcription are determined and agents that increase or decrease EPR-1 gene transcripts may readily be identified. Alternatively, the EPR-1 transcriptional
5 regulatory elements may be placed upstream of a reporter gene such as CAT or β -galactosidase.

**Example 10 REGULATION OF SURVIVIN EXPRESSION
BY CELL GROWTH/DIFFERENTIATION**

10 Consistent with the expression of Survivin in transformed cell lines (Figure 4C), undifferentiated and actively proliferating promyelocytic HL-60 cells constitutively expressed high levels of Survivin, as demonstrated by immunoblotting of a single ~ 16.5 kDa band with JC700 antibody, and Northern hybridization of a ~ 1.9 kb transcript with a single strand-specific probe (Figure 5). In contrast, no
15 specific bands were recognized by control non-immune rabbit IgG under the same experimental conditions (Figure 5).

Vitamin D₃-induced terminal differentiation of HL-60 cells to a mature monocytic phenotype resulted in growth arrest of these cells and *de novo* induction of differentiation-specific markers, including a ~ 200-fold increased expression of
20 leukocyte CD11b/CD18 integrin detected by flow cytometry (not shown), and in agreement with previous observations (Hickstein, D.D. *et al.*, *J Immunol* (1987) 138:513-519). Under these experimental conditions, the anti-Survivin JC700 antibody failed to immunoblot specific bands from vitamin D₃-treated HL-60 extracts, and no Survivin transcript(s) were detected by Northern hybridization with a single
25 strand-specific probe (Figure 5).

In contrast, an anti-EPR-1 polyclonal antibody immunoblotted a single ~ 62 kDa band corresponding to EPR-1 in vitamin D₃-differentiated HL-60 extracts under the same experimental conditions (not shown). Moreover, down-regulation of Survivin in vitamin D₃-differentiated HL-60 cells was accompanied by a 5- to 10-fold

increased surface expression of EPR-1 in these cells, as detected by flow cytometry with anti-EPR-1 monoclonal antibodies B6 or 12H1 (Figure 8).

As shown in Figure 16, Survivin is down regulated by the combination of cytokines γ interferon and tumor necrosis factor α , but not by either cytokine alone.

- 5 Similarly, the transfection of 3T3 cells with the c-myc oncogene results in the up-regulation of Survivin mRNA as detected by Northern blots.

Example 11 PROMOTING APOPTOSIS WITH SURVIVIN

Targeting Survivin promotes apoptosis and inhibits cell proliferation.

- 10 Transfection of the Survivin cDNA in mouse or hamster cell lines (NIH 3T3, CHO) was not suitable for the presence of immunochemically indistinguishable endogenous homologues in these cells (not shown). Similarly, initial attempts to target the Survivin gene in stable antisense transfectants were unsuccessful for slow cell growth and rapid loss of viability (not shown). Therefore, Survivin⁺ HeLa cells were
- 15 transfected with the 3' end of the EPR-1 cDNA (Survivin antisense) under the control of a metallothionein-inducible promoter (Lukashev, M.E. *et al.*, *J Biol Chem* (1994) 269:18311-18314), selected in hygromycin, and analyzed for apoptosis and cell proliferation after Zn²⁺-dependent activation of transcription.

- Consistent with the expression of Survivin in transformed cell lines (Figure
- 20 4C), the JC700 antibody immunoblotted a single molecular species of ~ 16.5 kDa in extracts of control HeLa cells transfected with the vector alone (Figure 7A). In contrast, no specific bands were recognized by JC700 antibody in metallothionein-induced HeLa cells transfected with the EPR-1 cDNA (Survivin antisense) (Figure 7A). Under these experimental conditions, *in situ* analysis of internucleosomal DNA
- 25 fragmentation by AptoTag staining revealed only a few apoptotic cells in serum-starved, Zn²⁺-induced, vector control HeLa cells (Figure 7B).

- In contrast, as discussed above, inhibition of Survivin expression in Zn²⁺-induced antisense HeLa cell transfectants was associated with prominent nuclear staining in the vast majority of cells examined (Figure 7B). No nuclear staining was
- 30 detected in the absence of TdT tagging of the digoxigenin-labeled dUTP (not shown).

Typical morphologic features of apoptosis, including numerous apoptotic bodies, were also demonstrated in induced antisense HeLa cell transfectants by hematoxylin/eosin staining, while only occasional apoptotic bodies were observed in vector control HeLa cultures, under the same experimental conditions (Figure 7B).

- 5 A potential effect of Survivin on cell growth was also investigated. In these experiments, metallothionein-controlled, EPR-1-dependent, inhibition of Survivin expression caused a profound reduction of serum-dependent HeLa cell proliferation (Figure 7C). Three days after Zn^{2+} induction, the cell count in vector control HeLa cultures increased by 288%, as opposed to only a 20% increase in Survivin antisense
10 transfectants, under the same experimental conditions (Figure 7C).

Example 12 STRUCTURE - FUNCTION RELATIONSHIP OF SURVIVIN

- The minimal structural requirements involved in Survivin-mediated inhibition of apoptosis have been identified through a mutagenesis strategy of Ala substitutions
15 of the most evolutionarily conserved residues in the single Survivin BIR (baculovirus IAP repeat) module. These residues included in the *amino-terminal half* of the Survivin BIR, Arg¹⁸, Phe²², Trp²⁵, Pro²⁶, Pro³⁵, Ala³⁹, Ala⁴¹, Gly⁴², and Cys⁴⁶. In the *carboxyl-terminal half* of the Survivin BIR, Ala mutants were first targeted at the Cys⁵⁷X₂Cys⁶⁰X₁₆His⁷⁷X₆Cys⁸⁴ putative zinc binding motif. Additional conserved
20 residues targeted by mutagenesis include Asp⁵³, Leu⁶⁴, Trp⁶⁷, Pro⁶⁹, Asp⁷¹, Asp⁷² and Pro⁷³.

- The Survivin mutants are characterized in stable and transiently transfected cells, IL-3-dependent BaF3 cells and NIH3T3, respectively. In addition to these point mutants, a Survivin chimeric molecule containing a carboxyl-terminal RING finger
25 has also been generated and screened for apoptosis inhibition (the RING finger is a domain found in most other IAP proteins, but not in Survivin). Secondly, a truncated form of Survivin has also been generated, in which the last 40 carboxylterminus residues, containing a predicted coiled-coil structure, have been deleted. As shown in Figure 12, Ala mutagenesis of key conserved residues in Survivin Trp⁶⁷ - Pro⁷³ - Cys⁸⁴

produced a recombinant molecule which lacked the ability to protect BaF3 cells from apoptosis induced by IL-3 withdrawal.

Example 13 CYTOPROTECTIVE EFFECTS OF SURVIVIN

5 Classical examples of cell damage to stable cell populations mediated by apoptosis include allograft rejection by infiltrating lymphocytes, Alzheimer's disease and reperfusion injury following myocardial infarction. In addition to being expressed in cancer, thereby functioning as a growth-advantage factor for cancer cells, the targeted expression of Survivin is useful to protect stable cell populations from
10 apoptosis and other cellular insults. This application of Survivin was tested by adding increasing concentrations of purified recombinant Survivin to monolayers of human endothelial cells injured with hydrogen peroxide (H₂O₂), a classical apoptosis-inducing stimulus. The results are summarized in Figure 13. Increasing concentrations of added Survivin resulted in a significant increased viability of the
15 treated cells as opposed to control cultures treated with control protein myoglobin. Similarly, Survivin protected NIH3T3 cells from apoptosis induced by hydrogen peroxide after transient co-transfection with a lacZ reporter gene as shown in Figure 17.

Example 14 SURVIVIN AS A PREDICTIVE-PROGNOSTIC FACTOR

The presence of Survivin can be utilized as predictive-prognostic negative factor in neuroblastoma and non-Hodgkin's lymphoma, and in other cancers.

Neuroblastoma. A large series of neuroblastoma cases (72) was screened for Survivin expression in a multicentric study. As shown in Figure 14, Survivin
25 expression increased dramatically when patients contained at least one negative prognostic factor for aggressive and rapidly progressing disease. Secondly, expression of Survivin strongly correlated with a more aggressive disease and unfavorable histology. Importantly, expression of Survivin was a more sensitive prognostic index than simple histology. Survivin-positive cases with early diagnosis

of favorable histology were found to contain at least one negative prognostic factor for disease progression and dissemination.

Hodgkin's Lymphoma. A similar multicentric study has been recently completed on analysis of Survivin expression in high grade non-Hodgkin's lymphoma (n=48). The results are similar to those observed for neuroblastoma. As shown in Figure 15, expression of Survivin strongly correlated with a more widespread disease predominantly in stage IV. Clinically, Survivin-expressing patients had fewer episodes of complete remission and more episodes of incomplete remission, no remission or relapses as compared with Survivin-negative patients.

Potential implications. The demonstrated role of Survivin as a negative predictive prognostic factor in these two embryologically different types of cancer iterates the potential use of this molecules a diagnostic tool to monitor disease progression and response to the therapy. It can also be used for staging purposes and to identify populations of patients potentially susceptible to multi-drug resistance (groups with no remissions or incomplete remissions). Also, Survivin derived primers easily designed from the complete sequence of the Survivin gene can be used as a screening tool to identify potential cases of cancer in which the Survivin gene has been deleted or mutated. These cases will be very important to identify because targeted inactivation of the Survivin gene would confer a favorable prognostic factor to cancer patients, removing a potential drug-resistance gene. Inactivating mutations in the Survivin gene can target the same key residues identified in our initial screening of Ala-based mutagenesis or result in an abortive or truncated protein for premature termination of translation.

Example 15 SURVIVIN CANCER VACCINE

Vaccines directed against Survivin, as found in various types of cancer, may be developed as with other disease-related intracellular protein targets. These techniques are commonly available and representative approaches are described by the references cited below. Vaccines may also include the systemic administration of peptide fragments of Survivin and the use of vectors to deliver mini-genes encoding

Survivin peptides to tumor cell targets are contemplated. As mentioned above, Survivin is not expressed in normal cells, even in proliferating stem cells in the bone marrow. This ensures that the immune response mounted against Survivin will be highly selective and specific and will not involve normal cells.

5 Development and Administration of Polypeptide-based vaccines

Methods of the use of peptide components in a monovalent or a polyvalent cancer immunotherapy-vaccine product are described by Nardi, N. *et al.*, *Mol. Med.* (1995) 1(5):563-567. Additional references that discuss the different cancer vaccine and cancer immunotherapies currently being used include: N.P. Restifo and M. Sznol
10 "Cancer Vaccines," in DeVita's Cancer: Principles & Practice of Oncology 3023-3043 (Lippincott-Raven, Philadelphia; 1997); J. Galea-Lauri *et al.*, *Cancer Gene Ther.* (1996) 3(3): 202-214; D.C. Linehan *et al.*, *Ann. Surg. Oncol.* (1996) 3(2): 219-228; and J. Vieweg *et al.*, *Cancer Invest.* (1995) 13(2): 193-201.

Consistent with the foregoing approach, Survivin polypeptides or full length
15 Survivin are synthesized either chemically by known techniques or recombinantly by expressing appropriate cDNAs in prokaryotic or eukaryotic cells. Survivin proteins so produced are then purified as necessary to remove contaminating proteins, such as serum or bacterial proteins. Survivin can be further purified using columns containing antibodies that bind Survivin, such as the monoclonal antibody JC700 or the antibody
20 8E2 (both described above) which recognize and bind to Survivin. In purifying an antibody-based vaccine, the recombinantly produced Survivin would bind to the antibodies while other proteins and cellular debris would be washed out. Survivin polypeptides are then be isolated and concentrated to a desired strength.

Alternatively Survivin polypeptides are created by cleaving the native
25 Survivin with one or more proteases (e.g., trypsin). Proteolytic fragments are then be separated and recovered using SDS-PAGE, high-resolution/high-pressure separation techniques, or reverse-phase HPLC. See R.J. BEYNON AND J.S. BOND, *PROTEOLYTIC ENZYMES: A PRACTICAL APPROACH* (Oxford University Press, New York 1989). These isolated peptides are then be concentrated to a desired final concentration.

Once purified, Survivin polypeptides or full length Survivin molecules may then placed in an emulsion containing an adjuvant. Adjuvants contemplated for use with Survivin include aluminum adjuvants, Freund's adjuvant, oil-in-water emulsions containing tubercle bacilli, and interleukin-2 (IL-2). Additional preparations include
5 combining the Survivin polypeptides with other appropriate tumor-associated antigens and, optionally, other immunomodulatory agents such as cytokines. Other suitable carriers or excipients can be used including bovine serum albumin, coupling the Survivin polypeptide with haptens, keyhole limpet hemocyanin, ovalbumin, and purified protein derivative of tuberculin. Peptides may be coupled to carriers using
10 techniques such as those described in ED HARLOW AND DAVID LANE, ANTIBODIES: A LABORATORY MANUAL (Cold Spring Harbor Laboratory, 1988).

Vaccines in human subjects may be administered in the form of an emulsion injected subcutaneously, intradermally or intramuscularly (IM); vaccines appropriately formulated can be taken orally. With vaccines containing adjuvants, the
15 vaccine is generally preferably be given IM, *e.g.*, in the deltoid.

The amount of Survivin vaccine or Survivin peptide vaccine to be administered to a patient will correspond to values typically used in for other cancer vaccines. Dosage concentrations will range from about 0.25 g to about 1000 g per day. More preferred ranges will be from about 10 µg to about 500 µg per day.

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Example 16 DIAGNOSTIC USE OF ANTI-SURVIVIN ANTIBODIES

Frequently, tumor associated antigens (TAA) are shed from tumor cells into the surrounding plasma or into the blood. As a result, TAA often are found in the blood, and blood samples obtained from patients may be used in detecting the
25 presence of cancer, as well as used as a factor is staging cancers (*e.g.*, stage I, II, III or IV). Survivin is one such TAA, and healthy, normal individuals do not express Survivin. Results from studies of several cancers have indicated that the presence of Survivin (or Survivin fragments) correlates with and is predictive that the disease may be aggressive or may have metastasized. A similar strategy of detecting and
30 quantifying the levels of Survivin or Survivin fragments can be used to determine

residual tumor burden in patients undergoing chemotherapy or radiation therapy for cancer treatment. Elevated or increasing levels of Survivin may reflect late stage neoplastic disease.

For diagnostic uses, blood is drawn from patients, by well known techniques, who have known cancer loads or from patients suspected of having cancer. The blood sample is prepared by known techniques and is tested for binding with antibodies to Survivin that are prepared and, optionally, labeled, as discussed above. Such general antibody detection protocols and associated reagents are well established in the art. Other biological fluid samples such as semen, urine, or saliva can also be monitored for the presence of Survivin. This diagnostic technique also can be used to monitor disease progression and response to individualized therapies. This method offers a relatively non-invasive means of tracking cancer progression or regression.

Example 17 DETECTION OF SURVIVIN BY IMMUNOBIOASSAY

An illustrative example of an immunobioassay to test for the presence of Survivin in the blood of patient relies on the ability of the monoclonal antibodies to Survivin to bind Survivin and remove the detectable Survivin from solution by immunoprecipitation. Such an immunobioassay is used to detect Survivin in suspected cancer patients and in fractions eluted from fractionation columns. An aliquot of each patient sample is incubated for 2 hours at 4°C with a monoclonal antibody that specifically recognizes and binds Survivin, such as the Mab 8E2, described above. The monoclonal antibody is insolubilized on anti-mouse IgG agarose beads, which can be acquired from Sigma Chemical Co., St. Louis, Mo.

The agarose bead anti-mouse (IgG(H + L))-Survivin complex is prepared by first washing the agarose beads with binding buffer containing 0.01 M phosphate buffer, (pH 7.2), and 0.25 M NaCl and then incubating the beads with the Survivin monoclonal antibody for 18 hours at 4°C. in the same buffer. The agarose beads may then be sedimented by centrifugation for 30 seconds at 16,000 x g in a microcentrifuge and non-specific sites may be blocked by incubation with 2% non-fat dry milk in 0.5 M NaCl-TMK for 30 minutes at 4°C. After blocking, the beads may

be washed 3 times with 0.5 M NaCl-TMK and resuspended in an equal volume of the same buffer. 20 :l of the agarose bead-monoclonal antibody complex may then incubated with each 250 :l of the patient test sample for 2 hours at 4°C. Any Survivin present in the patient test sample will be found by the Survivin monoclonal antibody on the beads. The bead complex, now with Survivin bound, may be removed by centrifugation for 30 seconds at 16,000 x g. The supernatant is then assayed for Survivin activity in the bioassay as described below. Control samples are treated with blocked beads that lacked the Survivin monoclonal antibody and tested for Survivin activity in the bioassay.

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Example 16 DETECTING SURVIVIN USING A DIRECT ELISA TEST

Samples of normal plasma (control) and cancer patient-plasma are diluted 1:1 with phosphate buffered saline (PBS). One volume of each mixture is added to centricon-10 filter having a 10 kD molecular weight limit and centrifuged at 5000 x g (7000 rpm) for 1 hour. One volume of PBS is added to the retentate and centrifuged for 30 min. The final dilution is about 1:3. The ELISA plate wells are then coated with retentate at 1:6, 1:12, 1:24, 1:48 and 1:96 final dilution in bicarbonate coating buffer, having a pH 9.6 overnight at 4°C C. The plates are then washed 2 times with wash buffer containing 5% Tween 20 in phosphate buffered saline. Residual binding sites are blocked with 4% bovine serum albumin (BSA), 300µ/well for 2 hours. Plates are then washed 2 times with wash buffer. Next, 100 µl of a monoclonal antibody that specifically recognizes and binds to Survivin, such as Mab 8E2, is used at 1:200 dilution in 1% BSA is added to the wells and incubated for 1 hour with agitation. Plates are washed 5 times with wash buffer. Next, 100 µl horseradish peroxidase conjugated secondary antibody is added, typically at a 1:2,000 dilution to each well, and incubated for 1 hour. Plates are again washed 5 times with wash buffer. Next, 100µl/well of substrate containing 5 µg of Survivin and 5 µl H₂O₂/10 ml citrate-phosphate buffer is added to each well and incubated for 5 minutes. The enzyme reaction is stopped by adding 50 µl/well 2 M H₂SO₄. The absorbance of light is measured at 492 nm in an EIA reader. Patient samples that contain Survivin will

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produce a positive reading, whereas those samples that do not contain Survivin will be negative.

**Example 18 SURVIVIN FRAGMENTS, PEPTIDES AND
SMALL MOLECULE ANTAGONISTS**

5 As described above, key functional residues in Survivin required for apoptosis have been identified. These data provide a template upon which to produce synthetic peptides and small molecule antagonists and competitive inhibitors of Survivin function. Preferably, the peptides are produced from native Survivin or include
10 substitutions from the native Survivin peptide backbone that include the functionally relevant residues Trp⁶⁷ - Pro⁷³ - Cys⁸⁴. Peptide fragments of native Survivin can be generated by standard techniques, including protein digests. A determination of which fragments compete with Survivin can readily be made by using the apoptosis measurement systems and apoptosis assay systems described above. These results
15 provide a unique opportunity to identify a discrete linear sequence in Survivin, that is essential for inhibition of apoptosis.

Consistent with the general paradigm of IAP proteins-dependent inhibition of apoptosis, it also was predicted that a structural region in the molecule required for the anti-apoptotic function is the primary candidate for being a site of interaction with other
20 molecules (such as binding partners). The functionally relevant peptide sequence in Survivin, based on the mutagenesis data, is: EGWEPDDDPIEEHKKHSSGC (SEQ ID NO:4). Ala substitutions of the underlined residues results in a complete loss of function of Survivin in transfected cells. This linear sequence can be synthesized and used as a much more stringent and specific reagent to isolate associated molecules using
25 standard biochemical procedures of affinity chromatography or as a bait for the yeast two-hybrid system.

Also, preferably, the β COOH coiled-coil region of Survivin is included in Survivin fragments and peptides. Recent data indicates that this Survivin domain is important for the anti-apoptosis function of Survivin. We have generated a recombinant truncated form of Survivin lacking the last 40 β COOH terminus amino acids comprising



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the coiled-coil domain. This truncated form was co-transfected with a lacZ plasmid in



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NIH3T3 cells side-by-side with wild type Survivin and XIAP, another member of the IAP gene family. The results, shown in Figure 17, indicate that the truncated Survivin had lost most (~80%) of the cytoprotective effect at preventing apoptosis in transfected cells induced by hydrogen peroxide. Incidentally, in this system, Survivin was more
5 potent than NAIP at preventing apoptosis.

Agonists and antagonists of Survivin also can readily be identified through conventional techniques. Designed, synthetic peptides based on the native linear sequence also function as competitive inhibitors of Survivin's interaction with as yet unidentified partner molecules. However, this inhibition should be sufficient to block
10 the anti-apoptosis function of Survivin.

A similar peptide-based strategy has been successful to block caspase activation *in vitro* and *in vivo*, protecting cells from apoptosis. See, *e.g.*, Milligan, C.E. *et al.*, (1995) *Neuron* 15:385-393.

15 **Example 19 THERAPEUTIC USES OF ANTISENSE SURVIVIN DNA**

As described above, the transcription of a Survivin antisense sequence altered the EPR-1/Survivin gene balance. This was demonstrated in HeLa cell transfectants, in which metallothionein-induced transcription of the EPR-1 "sense" strand suppressed the expression of Survivin and profoundly influenced apoptosis/cell
20 proliferation. Additionally, transiently co-transfecting a Survivin antisense construct with a lacZ reported plasmid decreased the viability of Survivin antisense transfectants after a 48-h transfection in β -galactosidase expressing cells. Accordingly, the level of expression of Survivin in a Survivin expressing cell or tissue, such as a tumor, is decreased by transfecting the cell or tissue with the EPR-1 sense strand of DNA.
25 Alternatively, a Survivin antisense-encoding DNA is used to transfect a target cell or tissue. Such therapy effectively decreases the translation of Survivin-encoding mRNA into Survivin protein.

30 **Example 20 USE OF SURVIVIN AS A PROTECTIVE AGENT AGAINST APOPTOSIS**

Survivin has been shown to protect cells from apoptosis when administered to cells that have been exposed to hydrogen peroxide or other agents that typically induce apoptosis. It is contemplated that cellular permeability may need to be increased, preferably in a transient manner in order to facilitate delivery of Survivin, or fragments thereof effective to reduce apoptosis. Certain conditions involving transient metabolic inhibition or transient hypoxia are likely to increase cellular permeability without the need for additional, external agents. Agents that may be appropriate include, metabolic inhibitors like 2-deoxyglucose and sodium azide. However, the ability of Survivin to mediate cytoprotection during a transient increase in cellular permeability offers the possibility of using therapeutic infusion of recombinant Survivin to decrease reperfusion injury and cellular damage during myocardial infarction and stroke. It is contemplated that such processes are mediated by increased tissue damage due to apoptosis. Treatment with Survivin could reduce the extent and magnitude of the injured tissue.

The use of Survivin or allelic variants of Survivin in subjects to modulate or prevent apoptosis related cell death would be beneficial in treating or ameliorating the effects of a variety of apoptosis-related indications. These indications include, but are not limited to, dermatological effects of aging (*e.g.*, baldness that is caused by apoptosis of cells of hair follicle cells), disorders and diseases such as immunosuppression, gastrointestinal perturbations (*e.g.*, damage of lining of the gut, ulcers, and radiation or chemotherapy induced damage), cardiovascular disorders, apoptosis related to reperfusion damage (*e.g.*, coronary artery obstruction, cerebral infarction, spinal/head trauma and concomitant severe paralysis, damage due to insults such as frostbite or burns, and any indication previously thought to be treatable by superoxide dismutase), rejection of tissue transplantation (*e.g.*, graft versus host disease), and Alzheimer's disease. The administration of Survivin also may be cytoprotective against chemotherapy or radiation-induced apoptosis.

Survivin protein for administration can be produced as described above, *e.g.*, using the cDNA described herein. The protein may require purification for purposes

of pharmaceutical administration and such purification steps preferably utilize monoclonal antibody separation and purification techniques as also described above.

In a clinical setting, Survivin is administered to patients in pharmaceutically effective dosages, *i.e.*, in dosages effective to reduce the level or extent of apoptosis otherwise present, via several routes. For example, to treat dermatological ailments that involve apoptosis, Survivin can be administered in a salve, cream, ointment or powder form. Topical formulations may contain additional pharmaceutical or cosmetic compositions such as moisturizers, humectants, odor modifiers, buffer, pigment, preservatives, vitamins (such as A, C or E), emulsifiers, dispersing agents, wetting agents, stabilizers, propellants, antimicrobial agents, sunscreen, enzymes and the like. Typical dosages of Survivin that may be administered to patients will be 0.01% to 1.0% by weight. Additional topical pharmaceutical compositions are described in S. Nakai *et al.*, U.S. Patent No. 5,672,603.

Survivin may also be administered, as may be appropriate for the condition being treated, in the form of pills, solutions, suspensions, emulsions, granules or capsules. Survivin can be administered orally; injected in solutions administered intravenously either alone or in admixture with conventional fluids for parenteral infusion (*e.g.*, fluids containing glucose, amino acids *etc.*); injected intramuscularly, intradermally, subcutaneously or intraperitoneally; using suppositories; and in the form of ophthalmic solutions such as eye drops. Survivin can also be administered using delayed release carriers, such as liposomes, microsponges, microspheres or microcapsules that are deposited in close proximity to the tissue being treated for prevention of apoptosis related cell death.

Concentrations of Survivin or functional allelic variants of Survivin administered via routes other than topical administration typically would range in dose from about 10 µg per day to about 25 mg per day depending on the route of administration. Of course, it would be expected that skilled artisans, such as physicians, may alter these values on a case by case basis as required for the particular patient.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Yale University
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(ii) TITLE OF INVENTION: SURVIVIN, A PROTEIN THAT INHIBITS
CELLULAR APOPTOSIS AND ITS MODULATION

(iii) NUMBER OF SEQUENCES: 35

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US97/21880
(B) FILING DATE: 20-NOV-1997

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/031,435
(B) FILING DATE: 20-NOV-1996

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGCTGGCCGC TCCTCCCTC

19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGACCTCCA GAGGTTTC

18

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Pro Thr Leu Pro Pro Ala Trp Gln Pro Phe Leu Lys Asp His Arg
1 5 10 15
Ile

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Gly Trp Glu Pro Asp Asp Asp Pro Ile Glu Glu His Lys Lys His
1 5 10 15
Ser Ser Gly Cys
20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGGGTGAGC TGTCCTTGC AGATGGC

27

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCATGTAAGT TGATTTTCT AGAGAGG

27

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AATTGTATGT CTTTATTCC AGGCAAA

27

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu	Glu	Ala	Arg	Leu	Val	Thr	Phe	Gln	Asn	Trp	Pro	Asp	Ala	Phe	Leu
1				5					10					15	
Thr	Pro	Gln	Glu	Leu	Ala	Lys	Ala	Gly	Phe	Tyr	Tyr	Leu	Gly	Arg	Gly
		20						25					30		
Asp	Gln	Val	Gln	Cys	Phe	Ala	Cys	Gly	Gly	Lys	Leu	Ala			
		35					40					45			



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(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Glu Ala Arg Phe Leu Thr Tyr Ser Met Trp Pro Leu Ser Phe Leu
 1 5 10 15
 Ser Pro Ala Glu Leu Ala Arg Ala Gly Phe Tyr Tyr Ile Gly Pro Gly
 20 25 30
 Asp Arg Val Ala Cys Phe Ala Cys Gly Gly Lys Leu Ser
 35 40 45

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Ala Asn Arg Leu Val Thr Phe Lys Asp Trp Pro Asn Pro Asn Ile
 1 5 10 15
 Thr Pro Gln Ala Leu Ala Lys Ala Gly Phe Tyr Tyr Leu Asn Arg Leu
 20 25 30
 Asp His Val Lys Cys Val Trp Cys Asn Gly Val Ile Ala
 35 40 45

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Glu Glu Val Arg Leu Asn Thr Phe Glu Lys Trp Pro Val Ser Phe Leu
1 5 10 15
Ser Pro Glu Thr Met Ala Lys Asn Gly Phe Tyr Tyr Leu Gly Arg Ser
20 25 30
Asp Glu Val Arg Cys Ala Phe Cys Lys Val Glu Ile Met
35 40 45

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Lys Ala Ala Arg Leu Gly Thr Tyr Thr Asn Trp Pro Val Gln Phe Leu
1 5 10 15
Glu Pro Ser Arg Met Ala Ala Ser Gly Phe Tyr Tyr Leu Gly Arg Gly
20 25 30
Asp Glu Val Arg Cys Ala Phe Cys Lys Val Glu Ile Thr
35 40 45

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Glu Glu Ala Arg Leu Ala Ser Phe Arg Asn Trp Pro Phe Tyr Val Gln
1 5 10 15
Gly Ile Ser Pro Cys Val Leu Ser Glu Ala Gly Phe Val Phe Thr Gly
20 25 30
Lys Gln Asp Thr Val Gln Cys Phe Ser Cys Gly Gly Cys Leu Gly
35 40 45



(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Glu Ala Asn Arg Leu Val Thr Phe Lys Asp Trp Pro Asn Pro Asn Ile
1 5 10 15
Thr Pro Gln Ala Leu Ala Lys Ala Gly Phe Tyr Tyr Leu Asn Arg Leu
20 25 30
Asp His Val Lys Cys Val Trp Cys Asn Gly Val Ile Ala
35 40 45

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Glu Glu Ala Arg Leu Lys Ser Phe Gln Asn Trp Pro Asp Tyr Ala His
1 5 10 15
Leu Thr Pro Arg Glu Leu Ala Ser Ala Gly Leu Tyr Tyr Thr Gly Ile
20 25 30
Gly Asp Gln Val Gln Cys Phe Cys Cys Gly Gly Lys Leu Lys
35 40 45

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Glu Glu Ala Arg Leu Lys Ser Phe Gln Asn Trp Pro Asp Tyr Ala His
1 5 10 15
Leu Thr Pro Arg Glu Leu Ala Ser Ala Gly Leu Tyr Tyr Thr Gly Ala
20 25 30
Asp Asp Gln Val Gln Cys Phe Cys Cys Gly Gly Lys Leu Glu
35 40 45

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Glu Asn Ala Arg Leu Leu Thr Phe Gln Thr Trp Pro Leu Thr Phe Leu
1 5 10 15
Ser Pro Thr Asp Leu Ala Arg Ala Gly Phe Tyr Tyr Thr Gly Pro Gly
20 25 30
Asp Arg Val Ala Cys Phe Ala Cys Gly Gly Lys Leu Ser
35 40 45

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Glu Glu Ala Arg Phe Leu Thr Tyr His Met Trp Pro Leu Thr Phe Leu
1 5 10 15
Ser Pro Ser Glu Leu Ala Arg Ala Gly Phe Tyr Tyr Ile Gly Pro Gly
20 25 30
Asp Arg Val Ala Cys Phe Ala Cys Gly Gly Lys Leu Ser
35 40 45



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(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 46 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Glu Glu Ala Arg Leu Lys Ser Phe Gln Asn Trp Pro Asp Tyr Ala His
 1 5 10 15
 Leu Thr Pro Arg Glu Leu Ala Ser Ala Gly Leu Tyr Tyr Thr Gly Ile
 20 25 30
 Gly Asp Gln Val Gln Cys Phe Cys Cys Gly Gly Lys Leu Lys
 35 40 45

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Glu Ala Asn Arg Leu Val Thr Phe Lys Asp Trp Pro Asn Pro Asn Ile
 1 5 10 15
 Thr Pro Gln Ala Leu Ala Lys Ala Gly Phe Tyr Tyr Leu Asn Arg Leu
 20 25 30
 Asp His Val Lys Cys Val Trp Cys Asn Gly Val Ile Ala
 35 40 45

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Tyr Val Gly Ile Gly Asp Lys Val Lys Cys Phe His Cys Asp Gly Gly
 1 5 10 15
 Leu Arg Asp Trp Glu Pro Gly Asp Asp Pro Trp Glu Glu His Ala Lys
 20 25 30
 Trp Phe Pro Arg Cys Glu Phe Leu Leu Leu Ala Lys Gly Gln Glu Tyr
 35 40 45
 Val Ser
 50

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Tyr Val Asp Arg Asn Asp Asp Val Lys Cys Phe Cys Cys Asp Gly Gly
 1 5 10 15
 Leu Arg Cys Trp Glu Pro Gly Asp Asp Pro Trp Ile Glu His Ala Lys
 20 25 30
 Trp Phe Pro Arg Cys Glu Phe Leu Ile Arg Met Lys Gly Gln Glu Phe
 35 40 45
 Val Asp
 50

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Tyr Gln Lys Ile Gly Asp Gln Val Arg Cys Phe His Cys Asn Ile Gly
 1 5 10 15
 Leu Arg Ser Trp Gln Lys Glu Asp Glu Pro Trp Phe Glu His Ala Lys
 20 25 30



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Trp Ser Pro Lys Cys Gln Phe Val Leu Leu Ala Lys Gly Pro Ala Tyr
35 40 45
Val Ser
50

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Tyr Thr Gly Tyr Gly Asp Asn Thr Lys Cys Phe Tyr Cys Asp Gly Gly
1 5 10 15
Leu Lys Asp Trp Glu Pro Glu Asp Val Pro Trp Glu Gln His Val Arg
20 25 30
Trp Phe Asp Arg Cys Ala Tyr Val Gln Leu Val Lys Gly Arg Asp Tyr
35 40 45
Val

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Tyr Thr Gly Gln Gly Asp Lys Thr Arg Cys Phe Cys Cys Asp Gly Gly
1 5 10 15
Leu Lys Asp Trp Glu Pro Asp Asp Ala Pro Trp Gln Gln His Ala Arg
20 25 30
Trp Tyr Asp Arg Cys Glu Tyr Val Leu Leu Val Lys Gly Arg Asp Phe
35 40 45
Val



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(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Tyr Thr Gly Ile Lys Asp Ile Val Gln Cys Phe Ser Cys Gly Gly Cys
1 5 10 15
Leu Glu Lys Trp Gln Glu Gly Asp Asp Pro Leu Asp Asp His Thr Arg
20 25 30
Cys Phe Pro Asn Cys Pro Phe Leu Gln Asn Met Lys Ser Ser Ala Glu
35 40 45
Val Thr
50

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Tyr Gln Lys Ile Gly Asp Gln Val Arg Cys Phe His Cys Asn Ile Gly
1 5 10 15
Leu Arg Ser Trp Gln Lys Glu Asp Glu Pro Trp Phe Glu His Ala Lys
20 25 30
Trp Ser Pro Lys Cys Gln Phe Val Leu Leu Ala Lys Gly Pro Ser Tyr
35 40 45
Val Ser
50

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear



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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Ala Leu Gly Glu Gly Asp Lys Val Lys Cys Phe His Cys Gly Gly Gly
 1 5 10 15
 Leu Thr Asp Trp Lys Pro Ser Glu Asp Pro Trp Glu Gln His Ala Lys
 20 25 30
 Trp Tyr Pro Gly Cys Lys Tyr Leu Leu Glu Gln Lys Gly Gln Glu Tyr
 35 40 45
 Ile Asn
 50

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Ala Leu Gly Glu Gly Asp Lys Val Lys Cys Phe His Cys Gly Gly Gly
 1 5 10 15
 Leu Thr Asp Trp Lys Pro Ser Glu Asp Pro Trp Glu Gln His Ala Lys
 20 25 30
 Trp Tyr Pro Gly Cys Lys Tyr Leu Leu Asp Glu Lys Gly Gln Glu Tyr
 35 40 45
 Ile Asn
 50

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:



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Tyr Val Gly Asn Ser Asp Asp Val Lys Cys Phe Cys Cys Asp Gly Gly
1 5 10 15
Leu Arg Cys Trp Glu Ser Gly Asp Asp Pro Trp Val Gln His Ala Lys
20 25 30
Trp Phe Pro Arg Cys Glu Tyr Leu Ile Arg Ile Lys Gly Gln Glu Phe
35 40 45
Ile Arg
50

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Tyr Val Gly Arg Asn Asp Asp Val Lys Cys Phe Gly Cys Asp Gly Gly
1 5 10 15
Leu Arg Cys Trp Glu Ser Gly Asp Asp Pro Trp Val Glu His Ala Lys
20 25 30
Trp Phe Pro Arg Cys Glu Phe Leu Ile Arg Met Lys Gly Gln Glu Phe
35 40 45
Val Asp
50

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Ala Leu Gly Glu Gly Asp Lys Val Lys Cys Phe His Cys Gly Gly Gly
1 5 10 15
Leu Thr Asp Trp Lys Pro Ser Glu Asp Pro Trp Glu Gln His Ala Lys
20 25 30
Trp Tyr Pro Gly Cys Lys Tyr Leu Leu Glu Gln Lys Gly Gln Glu Tyr
35 40 45



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Ile Asn
50

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Tyr Gln Lys Ile Gly Asp Gln Val Arg Cys Phe His Cys Asn Ile Gly
1 5 10 15
Leu Arg Ser Trp Gln Lys Glu Asp Glu Pro Trp Phe Glu His Ala Lys
20 25 30
Trp Ser Pro Lys Cys Gln Phe Val Leu Leu Ala Lys Gly Pro Ala Tyr
35 40 45
Val Ser
50

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 142 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met Gly Ala Pro Thr Leu Pro Pro Ala Trp Gln Pro Phe Leu Lys Asp
1 5 10 15
His Arg Ile Ser Thr Phe Lys Asn Trp Pro Phe Leu Glu Gly Cys Ala
20 25 30
Cys Thr Pro Glu Arg Met Ala Glu Ala Gly Phe Ile His Cys Pro Thr
35 40 45
Glu Asn Glu Pro Asp Leu Ala Gln Cys Phe Phe Cys Phe Lys Glu Leu
50 55 60
Glu Gly Trp Glu Pro Asp Asp Asp Pro Ile Glu Glu His Lys Lys His
65 70 75 80



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Ser Ser Gly Cys Ala Phe Leu Ser Val Lys Lys Gln Phe Glu Glu Leu
85 90 95
Thr Leu Gly Glu Phe Leu Lys Leu Asp Arg Glu Arg Ala Lys Asn Lys
100 105 110
Ile Ala Lys Glu Thr Asn Asn Lys Lys Lys Glu Phe Glu Glu Thr Ala
115 120 125
Lys Lys Val Arg Arg Ala Ile Glu Gln Leu Ala Ala Met Asp
130 135 140

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14796 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TCTAGACATG CGGATATAT CAAGCTGGGC ACAGCACAGC AGCCCCACCC CAGGCAGCTT 60
GAAATCAGAG CTGGGGTCCA AAGGGACCAC ACCCCGAGGG ACTGTGTGGG GGTCCGGGCA 120
CACAGGCCAC TGCTTCCCC CGTCTTTCTC AGCCATTCTT GAAGTCAGCC TCACTCTGCT 180
TCTCAGGGAT TTCAAATGTG CAGAGACTCT GGCACCTTTG TAGAAGCCCC TTCTGGTCTT 240
AACTTACACC TGGATGCTGT GGGGCTGCAG CTGCTGCTCG GGCTCGGGAG GATGCTGGGG 300
GCCCCGTGCC CATGAGCTTT TGAAGCTCCT GGAAGTCGGT TTTGAGGGTG TTCAGGTCCA 360
GGTGGACACC TGGGCTGTCC TTGTCCATGC ATTTGATGAC ATTGTGTGCA GAAGTGAAAA 420
GGAGTTAGGC CGGGCATGCT GGCTTATGCC TGTAAATCCA GCACCTTGGG AGGCTGAGGC 480
GGGTGGATCA CGAGGTGAGG AGTTCAATAC CAGCCTGGCC AAGATGGTGA AACCCCTCT 540
CTACTAAAAA TACAAAAAAA TTAGCCGGGC ATGGTGGCGG GCGCATGTAA TCCAGCTAC 600
TGGGGGGGCT GAGGCAGAGA ATTGCTGGAA CCCAGGAGAT GGAGGTTGCA GTGAGCCAAG 660
ATTGTGCCAC TGCACTGCAC TCCAGCCTGG CGACAGAGCA AGACTCTGTC TCAAAAAAAA 720
AAAAAAAAG TGAAAAGGAG TTGTTCTTT CCTCCTCCT GAGGGCAGGC AACTGCTGCG 780
GTTGCCAGTG GAGGTGGTGC GTCCTTGGTC TGTGCCTGGG GGCCACCCCA GCAGAGGCCA 840
TGGTGGTGCC AGGGCCCGGT TAGCGAGCCA ATCAGCAGGA CCCAGGGGCG ACCTGCCAAA 900
GTCAACTGGA TTTGATAACT GCAGCGAAGT TAAGTTTCCT GATTTTGATG ATTGTGTTGT 960
GGTTGTGTAA GAGAATGAAG TATTCGGGG TAGTATGGTA ATGCCTTCAA CTTACAAACG 1020



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GTTCAGGTAA ACCACCCATA TACATACATA TACATGCATG TGATATATAC ACATACAGGG 1080
ATGTGTGTGT GTTCACATAT ATGAGGGGAG AGAGACTAGG GGAGAGAAAG TAGGTTGGGG 1140
AGAGGGAGAG AGAAAGGAAA ACAGGAGACA GAGAGAGAGC GGGGAGTAGA GAGAGGGAAG 1200
GGGTAAGAGA GGGAGAGGAG GAGAGAAAGG GAGGAAGAAG CAGAGAGTGA ATGTTAAAGG 1260
AAACAGGCCAA AACATAAACA GAAAATCTGG GTGAAGGGTA TATGAGTATT CTTTGTACTA 1320
TTCTTGCAAT TATCTTTTAT TTAAATTGAC ATCGGGCCGG GCGCAGTGGC TCACATCTGT 1380
AATCCAGCA CTTTGGGAGG CCGAGGCAGG CAGATCACTT GAGGTCAGGA GTTTGAGACC 1440
AGCCTGGCAA ACATGGTGAA ACCCCATCTC TACTAAAAAT AAAAAAATTA GCCTGGTGTG 1500
GTGGTGCATG CCTTTAATCT CAGCTACTCG GGAGGCTGAG GCAGGAGAAT CGCTTGAACC 1560
CGTGGCGGGG AGGAGGTTGC AGTGAGCTGA GATCATGCCA CTGCACTCCA GCCTGGGCGA 1620
TAGAGCGAGA CTCAGTTTCA AATAAATAAA TAAACATCAA AATAAAAAGT TACTGTATTA 1680
AAGAATGGGG GCGGGGTGGG AGGGGTGGGG AGAGGTTGCA AAAATAAATA AATAAATAAA 1740
TAAACCCCAA AATGAAAAG ACAGTGGAGG CACCAGGCCT GCGTGGGGCT GGAGGGCTAA 1800
TAAGGCCAGG CCTCTTATCT CTGGCCATAG AACCAGAGAA GTGAGTGGAT GTGATGCCCC 1860
GCTCCAGAAG TGACTCCAGA ACACCCTGTT CCAAAGCAGA GGACACACTG ATTTTTTTTT 1920
TAATAGGCTG CAGGACTTAC TGTTGGTGGG ACGCCCTGCT TTGCGAAGGG AAAGGAGGAG 1980
TTTGCCCTGA GCACAGGCCC CCACCCTCCA CTGGGCTTTC CCCAGCTCCC TTGTCTCTTT 2040
ATCACGGTAG TGGCCAGTC CCTGGCCCTT GACTCCAGAA GGTGGCCCTC CTGGAAACCC 2100
AGGTCGTGCA GTCAACGATG TACTCGCCGG GACAGCGATG TCTGCTGCAC TCCATCCCTC 2160
CCCTGTTCAT TTGTCTTCA TGCCCGCTG GAGTAGATGC TTTTTCAGA GGTGGCACCC 2220
TGTAAGCTC TCCTGTCTGA CTTTTTTTTT TTTTITAGAC TGAGTTTGC TCTGTGTGCC 2280
TAGGCTGGAG TGCAATGGCA CAATCTCAGC TCACTGCACC CTCTGCCTCC CGGGTTCAAG 2340
CGATTCTCCT GCCTCAGCCT CCGAGTAGT TGGGATTACA GGCATGCACC ACCACGCCCA 2400
GCTAATTTTT GTATTTTTAG TAGAGACAAG GTTTCACCGT GATGGCCAGG CTGGTCTTGA 2460
ACTCCAGGAC TCAAGTGATG CTCCTGCCTA GGCCTCTCAA AGTGTGGGA TTACAGGCGT 2520
GAGCCACTGC ACCCGGCTG CACGCGTTCT TTGAAAGCAG TCGAGGGGGC GCTAGGTGTG 2580
GGCAGGGACG AGCTGGCGCG GCGTCGCTGG GTGCACCGCG ACCACGGGCA GAGCCACGCG 2640
GCGGGAGGAC TACAACCTCC GGCACACCCC GCGCCGCCCC GCCTCTACTC CCAGAAGGCC 2700
GCGGGGGTG GACCGCCTAA GAGGGCGTGC GCTCCCGACA TGCCCCGCGG CGCGCCATTA 2760
ACCGCCAGAT TTGAATCGCG GGACCGTTG GCAGAGGTGG CGGCGGCGGC ATGGGTGCC 2820
CGACGTTGCC CCCTGCCTGG CAGCCCTTC TCAAGGACCA CCGCATCTCT ACATTCAAGA 2880



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ACTGGCCCTT CTTGGAGGGC TGGCCCTGCA CCCCAGGAGCG GGTGAGACTG CCCGGCCTCC	2940
TGGGGTCCCC CACGCCCGCC TTGCCCTGTC CCTAGCGAGG CCACTGTGAC TGGGCCTCGG	3000
GGGTACAAGC CGCCCTCCCC TCCCCGTCTT GTCCCCAGCG AGGCCACTGT GGCTGGGCCC	3060
CTTGGGTCCA GGCCGGGCTC CCCTCCCTGC TTTGTCCCA TCGAGGCCTT TGTGGCTGGG	3120
CCTCGGGGTT CCGGGCTGCC ACGTCCACTC ACGAGCTGTG CTGTCCCTTG CAGATGGCCG	3180
AGGCTGGCTT CATCCACTGC CCCACTGAGA ACGAGCCAGA CTTGGCCAG TGTTCCTTCT	3240
GCTTCAAGGA GCTGGAAGGC TGGGAGCCAG ATGACGACCC CATGTAAGTC TTCTCTGGCC	3300
AGCCTCGATG GGCTTTGTTT TGAAGTACTG TGTCAAAAGA TTTGAGTTGC AAAGACACTT	3360
AGTATGGGAG GGTGCTTTC CACCCTCATT GCTTCTTAA CAGCTGTTGT GAACGGATAC	3420
CTCTCTATAT GCTGGTGCCT TGGTGATGCT TACAACCTAA TTAATCTCA TTTGACCAA	3480
ATGCCTTGGG GTGGACGTAA GATGCCTGAT GCCTTTCATG TTCAACAGAA TACATCAGCA	3540
GACCTGTGTT TTGTGAATC CCAGGAATGT CCAAGTGCTT TTTTGTAGAT TTTTAAAAA	3600
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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method of modulating apoptosis effected by Survivin in a cell, the method including the step of administering to the cell an agent that modulates the amount or activity of Survivin in the cell, wherein the agent is selected from the group consisting of Survivin and fragments thereof, antibodies, Survivin binding partners, Survivin inhibitors, DNA methylases, antisense molecules of the nucleic acid encoding Survivin and fragments thereof, and nucleic acids encoding Survivin and fragments thereof.
2. The method of claim 1, wherein the level of apoptosis is increased by decreasing the amount or activity of Survivin in the cell.
3. The method of claim 1, wherein the level of apoptosis is decreased by increasing the amount or activity of Survivin in the cell.
4. A method of inhibiting apoptosis in a cell, the method including the step of administering to the cell a Survivin polypeptide, Survivin polypeptide fragment or an apoptosis-inhibiting peptidomimetic thereof in an amount effective to inhibit apoptosis in the cell.
5. A method of inhibiting apoptosis in a cell, the method including the step of administering to the cell a transgene encoding a Survivin polypeptide or Survivin polypeptide fragment thereof, the transgene being effective to cause expression of the Survivin polypeptide or fragment thereof in an amount effective to inhibit apoptosis in the cell.
6. A method of increasing apoptosis effected by Survivin in a cell, the method including the step of administering to the cell an agent that decreases the apoptosis-inhibiting activity of the Survivin polypeptide present in the cell, in an amount that is effective to increase the level of apoptosis in the cell, and wherein the agent is selected from the group consisting of antibodies, Survivin binding partners, Survivin inhibitors, DNA methylases, and antisense molecules of the nucleic acid encoding Survivin and fragments thereof.



7. The method of claim 6, wherein said agent is a polypeptide including the sequence EGWEPDDDDPIEEHKKHSSGC, its conservatively substituted homologs or small molecule peptidomimetics thereof.

5 8. A method of increasing the level of apoptosis in a cell, the method including the step of administering to the cell an agent that increases the transcription of the sense strand of EPR-1, in an amount effective to inhibit the translation of mRNA encoding a Survivin polypeptide, thereby decreasing the transcription of the Survivin polypeptide and increasing the level of
10 apoptosis in the cell, wherein the agent is an antisense molecule of the nucleic acid encoding Survivin and fragments thereof.

9. An isolated nucleic acid molecule capable of expressing the amino acid sequence depicted in SEQ ID NO:34, allelic variants of the amino acid
15 sequence of SEQ ID NO:34, and fragments thereof that are effective to inhibit apoptosis.

10. The isolated nucleic acid molecule of claim 9, wherein said nucleic acid molecule is operably linked to one or more expression control elements.
20

11. The isolated nucleic acid molecule of claim 9, wherein said nucleic acid molecule is included in a vector.

12. An isolated nucleic acid molecule that encodes a member of the Survivin family of proteins, wherein said nucleic acid molecule hybridizes to a nucleic acid molecule of claim 9 under conditions of sufficient stringency to produce a clear signal.
25

13. A host cell transformed to contain the nucleic acid molecule of claim 9.
30

14. The host cell of claim 13, wherein said host is selected from the group consisting of prokaryotic hosts and eukaryotic hosts.

15. A method for producing a Survivin protein the method including the step of culturing a host transformed with the nucleic acid molecule of claim 11 under conditions in which the Survivin protein is expressed.
35



16. The method of claim 15, wherein said host is selected from the group consisting of prokaryotic hosts and eukaryotic hosts.
- 5 17. An isolated polypeptide including the amino acid sequence depicted in SEQ ID NO:34, allelic variants thereof and fragments thereof that retain the ability to inhibit cellular apoptosis.
18. An isolated polypeptide including the sequence
 10 EGWEPDDDDPIEEHKKHSSGC, its conservatively substituted homologs and small molecule peptidomimetics thereof.
19. A monoclonal antibody that binds to the polypeptide, allelic variants thereof and fragments thereof that retain the ability to inhibit cellular
 15 apoptosis of claim 17.
20. The monoclonal antibody of claim 19 which has been humanised.
21. A method for reducing the severity of a pathological state mediated by Survivin comprising the step of administering an agent that reduces Survivin
 20 expression or activity in a cell influencing the pathological state, wherein the agent is selected from the group consisting of antibodies, Survivin binding partners, Survivin inhibitors, DNA methylases, and antisense molecules of the nucleic acid encoding Survivin and fragments thereof.
- 25 22. The method of claim 21 wherein said pathological state is caused by abnormal cell growth.
23. The method of claim 21 wherein said Survivin expression is reduced
 30 by contacting affected cells with a RNA molecule that is complementary to a Survivin encoding mRNA molecule.
24. The method of claim 21, wherein said Survivin activity is reduced by blocking the intracellular interaction of Survivin with a Survivin binding
 35 partner the method including the step of contacting said Survivin with an agent that blocks the binding of Survivin to said binding partner.



25. The method of claim 24 wherein said agent blocks the binding of said Survivin to said binding partner by selectively binding to Survivin.

5 26. The method of claim 25 wherein said agent blocks the binding of said Survivin to said binding partner by selectively binding to the binding partner.

27. The method of claim 26 wherein said agent is a polypeptide fragment of Survivin.

10

28. The method of claim 26 wherein said agent includes the polypeptide sequence EGWEPDDDDPIEEHKKHSSGC, its conservatively substituted homologs and small molecule peptidomimetics thereof.

15

29. A method for identifying agents that block the interaction of Survivin with a Survivin binding partner the method including the steps of:

a) incubating Survivin, or a fragment thereof, or a polypeptide comprising the sequence EGWEPDDDDPIEEHKKHSSGC, and a binding partner, or a fragment thereof, with an agent to be tested, and

20

b) determining whether said agent blocks the binding of Survivin to said Survivin binding partner.

30. A method to detect Survivin in a sample, the method including the step of determining whether a Survivin protein is expressed by the sample.

25

31. The method of claim 30, wherein said sample is selected from the group consisting of a tissue biopsy, stool, blood, urine and saliva.

32. The method of claim 30 further the method including the steps of:

30

a) preparing an extract of the cells in said sample, and

b) examining the proteins of said cell extract to determine the presence of a Survivin protein.

33. The method of claim 30 further the method including the steps of:

35

a) preparing an extract of the cells in said sample, and



b) examining the mRNA of said cell extract to determine the presence of a Survivin encoding mRNA.

5 34. The method of claim 30 wherein said method is used to determine the growth potential of a tumor cell by correlating the level of Survivin expression with control samples to indicate tumor growth potential.

35. A method of detecting or monitoring the stage or progression of a cancer the method including the steps of :
10 obtaining a biological fluid sample from a subject;
contacting said sample with a monoclonal antibody that recognises and binds to Survivin; and
determining whether the monoclonal antibody recognises and binds to Survivin in said sample, the presence of Survivin thereby predicting the
15 presence of cancer.

36. The method of claim 35, wherein the presence of Survivin predicts late stage neoplastic disease.

20 37. A method for maintaining the growth of cells in culture, the method including the step of contacting the cells with an amount of Survivin that is effective to reduce apoptosis.

38. A kit for detecting the presence of Survivin in a sample, the kit
25 including an antibody that binds specifically to Survivin and reagents to detect the antibody-Survivin binding pair.

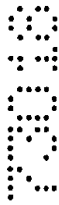
39. A therapeutic composition including an agent that modulates the level
30 of Survivin-mediated apoptosis in a host, wherein the agent is selected from the group consisting of Survivin and fragments thereof, Survivin antibodies, Survivin binding partners, Survivin inhibitors, DNA methylases, antisense molecules of the nucleic acid encoding Survivin and fragments thereof, and nucleic acids encoding Survivin and fragments thereof.



40. The composition of claim 39, the vaccine including a component that decreases the activity of Survivin in the host, selected from the group of components consisting of:

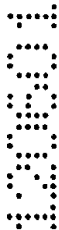
- (a) Survivin protein or fragments thereof sufficient to evoke a cellular mediated response,
- (b) a DNA molecule that is antisense to Survivin mRNA or portions thereof sufficient to inhibit translation of Survivin,
- (c) the sense DNA strand of EPR-1acid or portions thereof sufficient to inhibit translation of Survivin.

10



15

41. A method of prophylactic or preventative anti-apoptotic therapy, the method including the administration of a Survivin polypeptide, Survivin polypeptide fragment, an apoptosis-inhibiting peptidomimetic thereof, a transgene encoding a Survivin polypeptide, or a transgene encoding a Survivin polypeptide fragment to a subject before the occurrence of a disease phenotype associated with cell apoptosis.



20

42. The method of claim 41, wherein the disease phenotype is a diminished T cell count in a subject infected with HIV.

43. The method of claim 41, wherein the disease phenotype is a degenerative disease.

25

44. The method of any of claim 6 and 20 to 22, further including the administration of one or more additional agents to control cell growth.

30

45. A method to treat cancer in a patient the method including the step of increasing the level of apoptosis effected by Survivin in one or more cells of the cancer comprising the step of administering an agent that decreases the amount or activity of Survivin in said one or more cancer cells, wherein the agent is selected from the group consisting of antibodies, Survivin binding partners, Survivin inhibitors, DNA methylases, and antisense molecules of the nucleic acid encoding Survivin and fragments thereof.

35

46. The method of claim 45, further including the step of administering to the patient an agent that decreases the translation of Survivin mRNA in said



one or more cancer cells, in an amount that is effective to increase the level of apoptosis in said one or more cancer cells.

47. The method of claim 45, further the method including the step of
5 administering to the patient an agent that decreases the apoptosis-inhibiting activity of the Survivin polypeptide present in said one or more cancer cells, in an amount that is effective to increase the level of apoptosis in the cell.

48. The method of claim 47, wherein said decreased apoptosis-inhibiting
10 activity of Survivin is effected by the administration of an agent that blocks the intracellular interaction of Survivin with a Survivin binding partner.

49. The method of claim 48 wherein said agent blocks the binding of said
15 Survivin to said binding partner by selectively binding to Survivin.

50. The method of claim 48 wherein said agent blocks the binding of said
Survivin to said binding partner by selectively binding to said binding
partner.

20 51. The method of claim 50 wherein said agent is a fragment of Survivin.

52. The method of claim 50 wherein said agent includes the polypeptide
sequence EGWEPDDDDPIEEHKKHSSGC, its conservatively substituted
homologs and small molecule peptidomimetics thereof.

25 53. A method of inhibiting or reversing reperfusion injury in a subject the method including the step of administering to subject in need thereof a Survivin polypeptide, an apoptosis-inhibiting Survivin polypeptide fragment or an apoptosis-inhibiting peptidomimetic thereof.

30 54. The method of claim 53 wherein said step of administering includes local administration at the site of injury.

35 55. A method for inhibiting or preventing tissue or organ transplant rejection, the method including a local administration of a Survivin polypeptide, an apoptosis-inhibiting Survivin polypeptide fragment, an



apoptosis-inhibiting peptidomimetic thereof, a transgene encoding a Survivin polypeptide or a transgene encoding an apoptosis-inhibiting Survivin polypeptide fragment to the tissue or organ or to a site proximal to the transplant.

5

56. A method for enhancing viability of organs and tissues prior to their transplantation into a subject, the method including the perfusion of the organs or tissues with a Survivin polypeptide, an apoptosis-inhibiting Survivin polypeptide fragment, or an apoptosis-inhibiting peptidomimetic thereof.

10

57. The method of claim 56, wherein said transgene is delivered via a viral vector.

15

58. The method of claim 57 wherein said vector is replication defective.

59. The method of claim 56 wherein said transgene is delivered as a naked nucleic acid.

20

60. The use of a Survivin polypeptide, Survivin polypeptide fragment, an apoptosis-inhibiting peptidomimetic thereof, a transgene encoding a Survivin polypeptide, or a transgene encoding a Survivin polypeptide fragment for the preparation of a medicament for prophylactic or preventative anti-apoptotic therapy.

25

61. The use of an agent which decreases the translation of Survivin mRNA in one or more cancer cells for the preparation of a medicament for treatment resulting in increasing the level of apoptosis effected by Survivin in one or more cancer cells, wherein the agent is selected from the group consisting of antibodies, Survivin binding partners, Survivin inhibitors, DNA methylases, and antisense molecules of the nucleic acid encoding Survivin and fragments thereof.

30

62. The use of a Survivin polypeptide, an apoptosis-inhibiting Survivin polypeptide fragment, an apoptosis-inhibiting peptidomimetic thereof, a transgene encoding a Survivin polypeptide or a transgene encoding an

35



apoptosis-inhibiting Survivin polypeptide fragment for the preparation of a medicament for inhibiting or preventing tissue or organ transplant rejection.

5 63. A therapeutic vaccine including an agent that modulates the level of Survivin-mediated apoptosis in a host, wherein the agent is selected from the group consisting of Survivin and fragments thereof, Survivin antibodies, Survivin binding partners, Survivin inhibitors, and DNA methylases.

10 64. The vaccine of claim 39, the vaccine including a component that decreases the activity of Survivin in the host, selected from the group of components consisting of Survivin protein and fragments thereof sufficient to evoke a cellular mediated response.

Dated this 6th day of June 2001

Yale University
Patent Attorneys for the Applicant:

F B RICE & CO



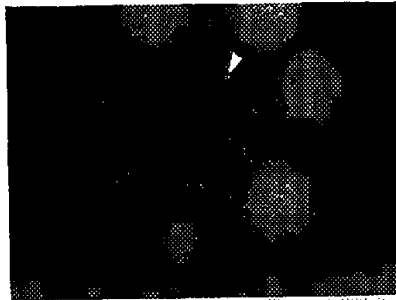


FIG. 1A

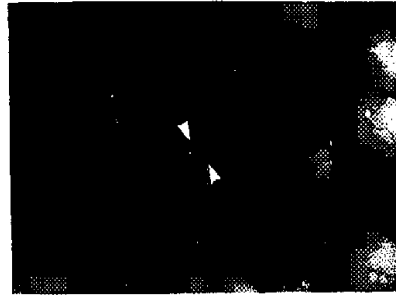


FIG. 1B

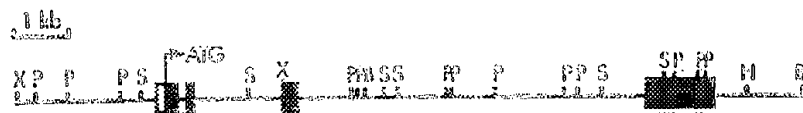
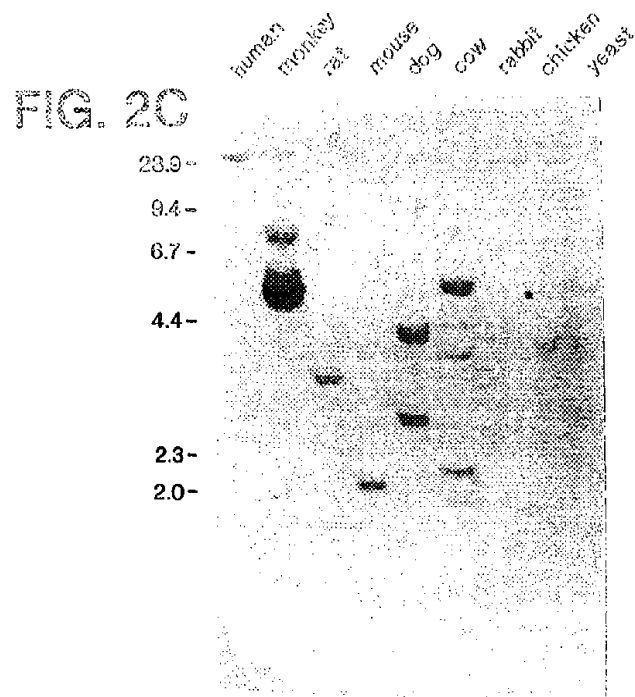
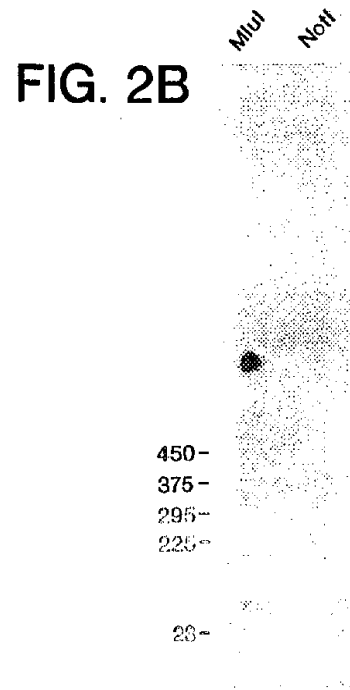
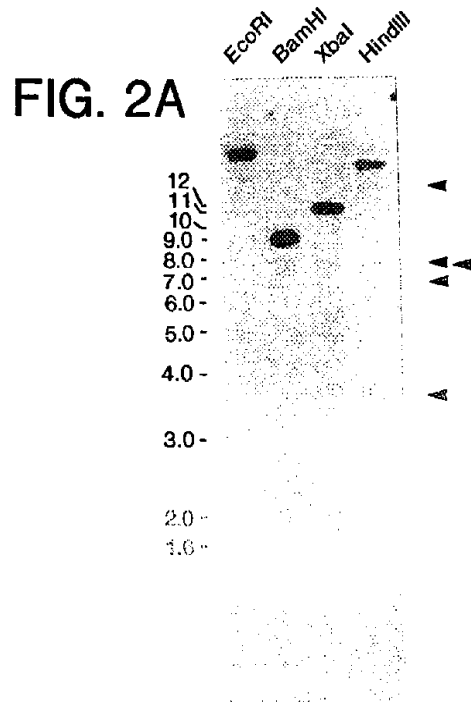


FIG. 1C

5' Exon	Intron	3' Exon
(2918)GCGG GTCAG	(3161)CTGTCCCCTGCAG	ATGCC
(3280)CCAT GTAAG	(3145)TTGATTTTCTAG	AGAGG
(5272)AATT GTATG	(11942)TCTTTATTTCCAG	GCAAA

FIG. 1D



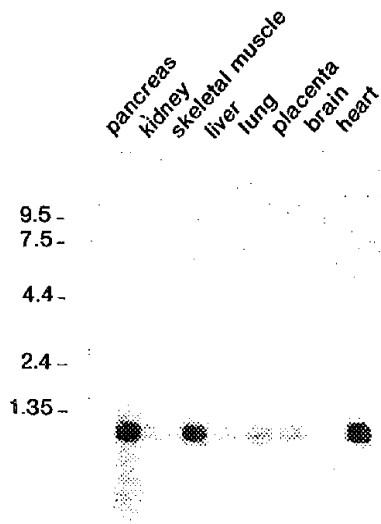


FIG. 3A

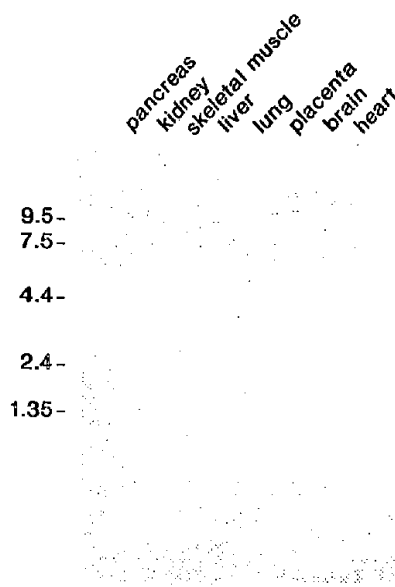


FIG. 3B

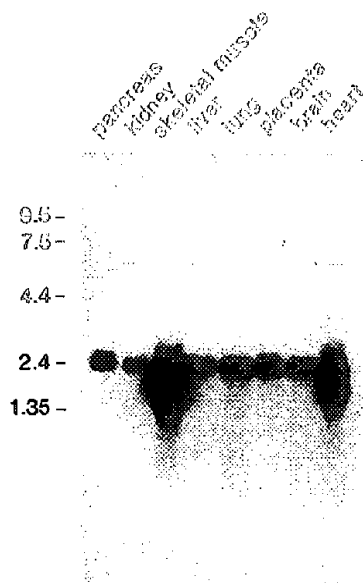


FIG. 3C

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FIG. 3D

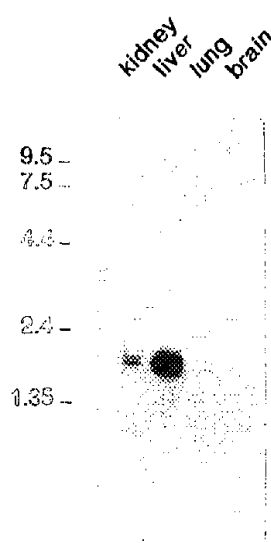


FIG. 3E

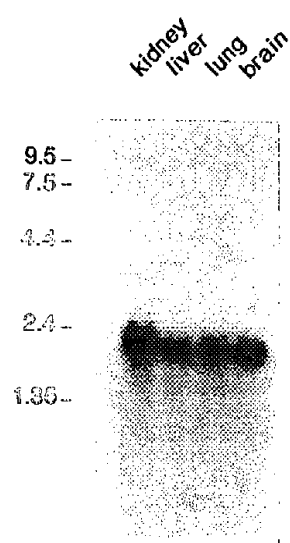


FIG. 3F

FIG. 4A

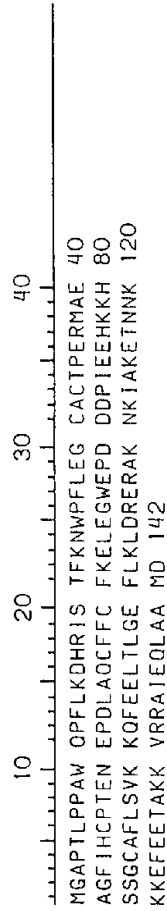


FIG. 4B-1

	210	220	230	240	250	Majority
177	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
113	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
7	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
18	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
159	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
113	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
163	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
163	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
169	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
184	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
163	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
113	EEARLVTFQNWPD-AFL	---	TPQELAKAGFY	YVLGRGDQV	QCFACGGKLA	
15	KDHRITSTHKNWPF-LFC	---	CACTPERMAEAGFI	---	---	SURVIVIN.PRO

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FIG. 4B-2

	YVIGDKVKCFHC	DGGLRDWEP	GGDDPWEEHAKW	FP	RC	FE	LLAKGQ	EYVS	Majority
290	YMDRNDVVKCF	CCDGGLR	CMWEPGGD	PPWIEHAKW	FP	RC	FE	LLAKGQ	EYVS
240	YOKIGDQVR	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
136	YTGVEDNTR	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
139	YTGVEDNTR	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
306	YTGVEDNTR	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
240	YOKIGDQVR	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
291	ALGEGDKVK	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
290	ALGEGDKVK	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
283	YVGNNDVVK	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
297	YVGNNDVVK	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
291	ALGEGDKVK	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
240	YOKIGDQVR	CFHCNIG	LRSMWKE	DEPWFEEHAKW	SPK	CC	QEVLLAK	GPAYVS	
53	-----DLAOC	FFCFKEL	EGWEPDD	DETEEHKHSS	GC	AF	LS	-----	

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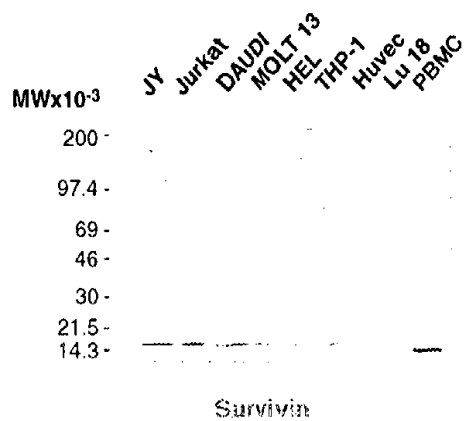


FIG. 4C-1

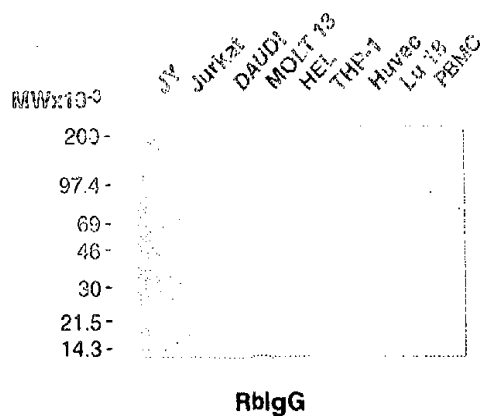


FIG. 4C-2

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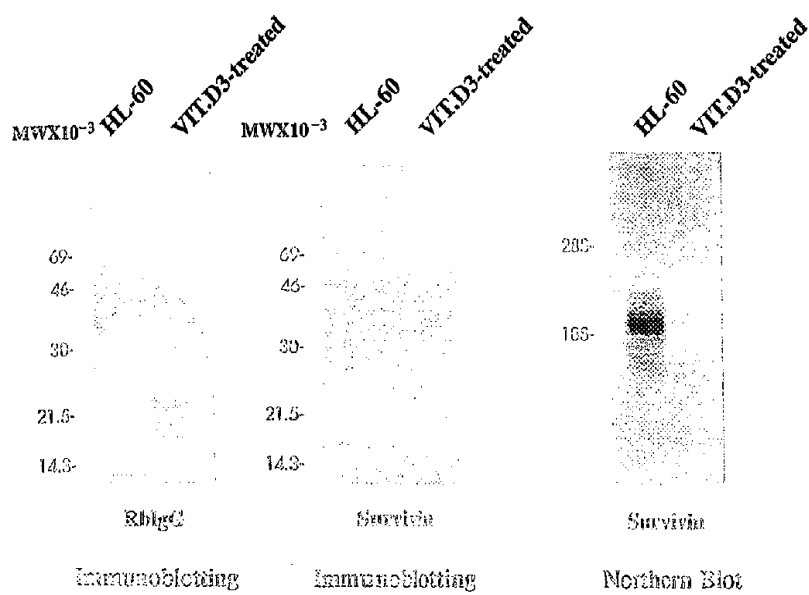


FIG. 5A

FIG. 5B

FIG. 5C



FIG. 6A

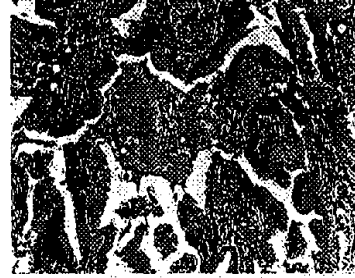


FIG. 6B

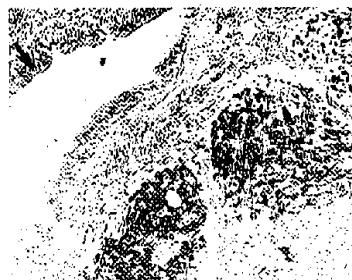


FIG. 6C

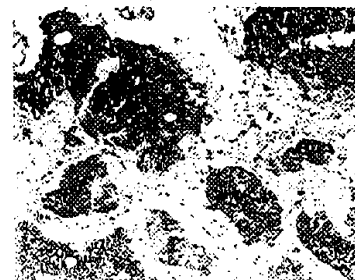


FIG. 6D



FIG. 6E

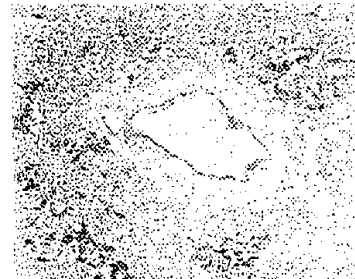


FIG. 6F



FIG. 6G



FIG. 6H

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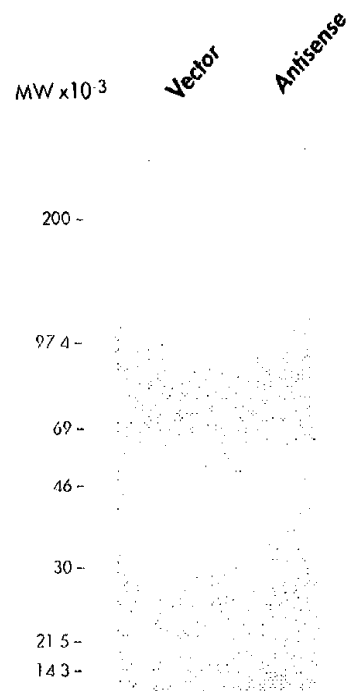


FIG. 7A

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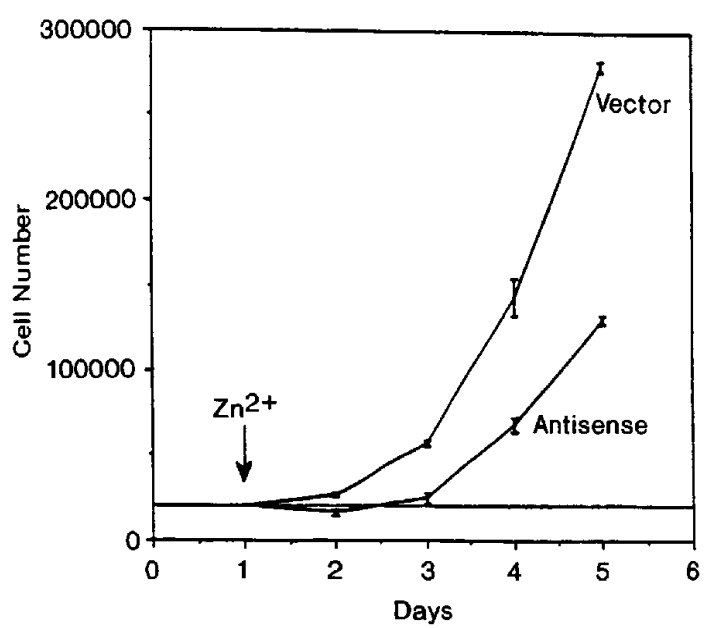


FIG. 7C

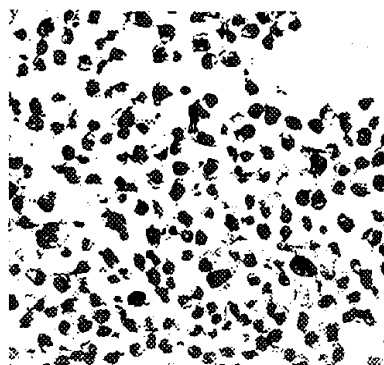


FIG. 7B-1

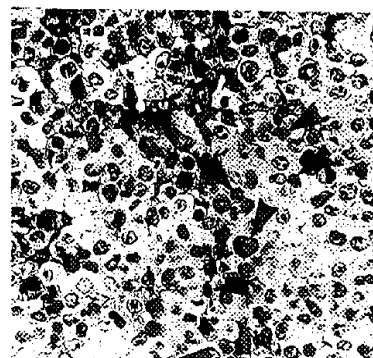


FIG. 7B-2

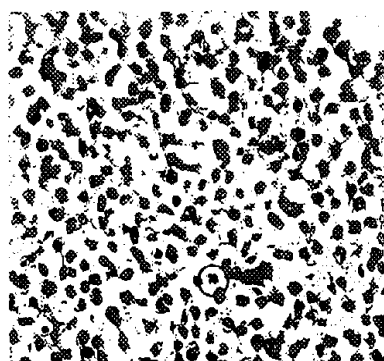


FIG. 7B-3

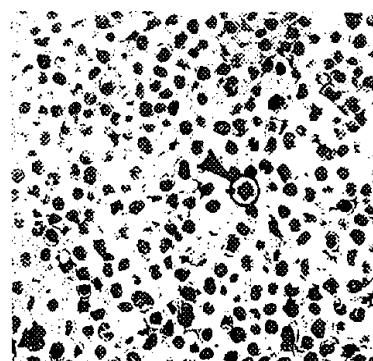


FIG. 7B-4

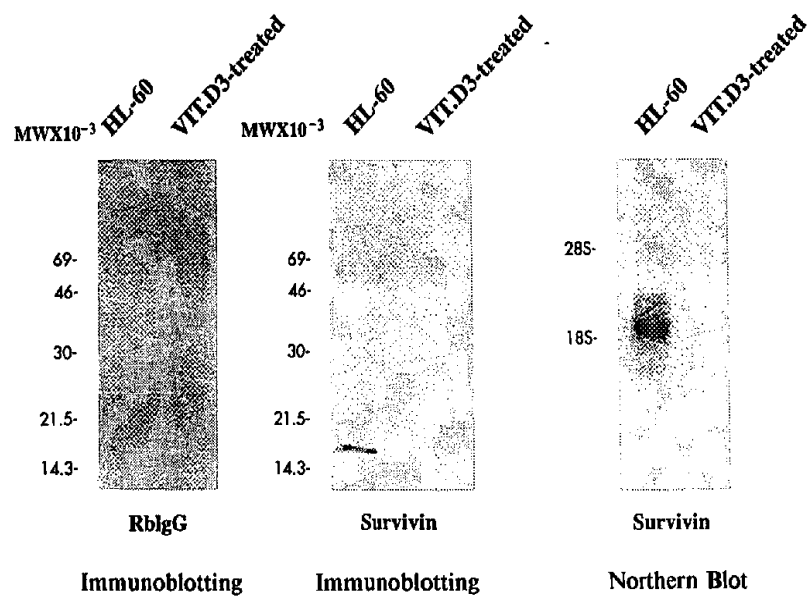


FIG. 8A

FIG. 8B

FIG. 8C

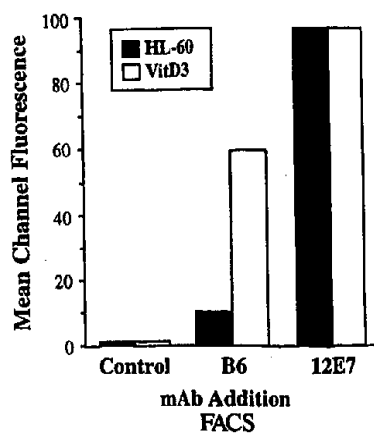


FIG. 8D

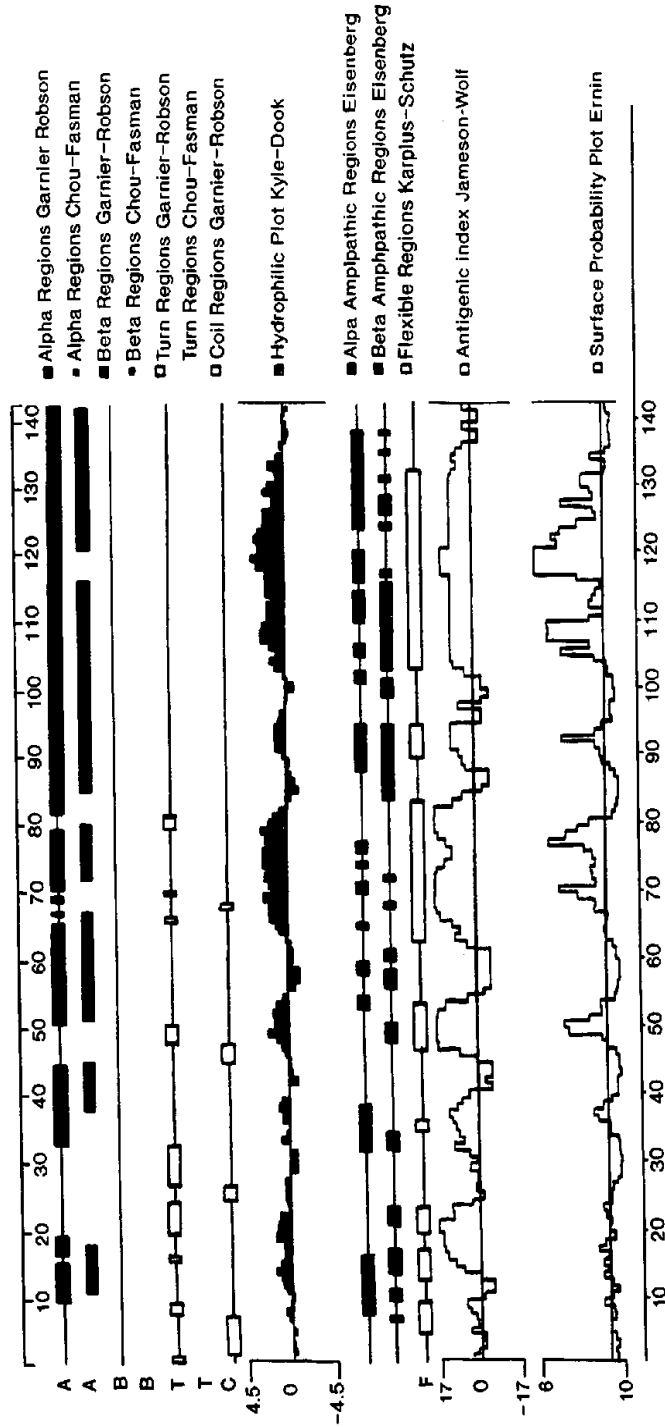


FIG. 9

translation=MGAPTLPPAWQPFLKDHRISTFKNWPFLGCACTPERMAEAGFIHCP
TENEPDLAQCFPCFKELEGWEPDDDDPIEEHKKHSSGCAFLSVKKQFEELTLGEFL
KLDREKAKNKIAKETNNKKKEFEETAKKVRRAIEQLAAMD

1	TCTAGACATG	CGGATATATT	CAAGCTGGGC	ACAGCACAGC	AGCCCCACCC
51	CAGGCAGCTT	GAAATCAGAG	CTGGGGTCCA	AAGGGACCAC	ACCCCGAGGG
101	ACTGTGTGGG	GGTCGGGGCA	CACAGGCCAC	TGCTTCCCCC	CGTCTTCTC
151	AGCCATTCCCT	GAAGTCAGCC	TCACTCTGCT	TCTCAGGGAT	TTCAAATGTG
201	CAGAGACTCT	GGCACTTTTG	TAGAAGCCCC	TTCTGGTCCT	AACCTACACC
251	TGGATGCTGT	GGGGCTGCAG	CTGCTGCTCG	GGCTCGGGAG	GATGCTGGGG
301	GCCCGGTGCC	CATGAGCTTT	TGAAGCTCCT	GGAACTCGGT	TTTGAGGGTG
351	TTCAGGTCCA	GGTGGACACC	TGGGCTGTCC	TTGTCCATGC	ATTTGATGAC
401	ATTGTGTGCA	GAAGTGAAAA	GGAGTTAGGC	CGGGCATGCT	GGCTTATGCC
451	TGTAATCCCA	GCACCTTTGGG	AGGCTGAGGC	GGGTGGATCA	CGAGGTCAGG
501	AGTTCAATAC	CAGCCTGGCC	AAGATGGTGA	AACCCCGTCT	CTACTAAAAA
551	TACAAAAAAA	TTAGCCGGGC	ATGGTGGCGG	GCGCATGTAA	TCCCAGCTAC
601	TGGGGGGGCT	GAGGCAGAGA	ATTGCTGGAA	CCCAGGAGAT	GGAGGTTGCA
651	GTGAGCCAAG	ATTGTGCCAC	TGCACTGCAC	TCCAGCCTGG	CGACAGAGCA
701	AGACTCTGTC	TCAAAAAAAA	AAAAAAAAG	TGAAAAGGAG	TTGTTCTTTT
751	CCTCCCTCCT	GAGGGCAGGC	AACTGCTGCG	GTTGCCAGTG	GAGGTGGTGC
801	GTCCTTGTC	TGTGCCTGGG	GGCCACCCCA	GCAGAGGCCA	TGGTGGTGCC
851	AGGGCCCGGT	TAGCGAGCCA	ATCAGCAGGA	CCCAGGGGCG	ACCTGCCAAA
901	GTCAACTGGA	TTTGATAACT	GCAGCGAAGT	TAAGTTTCCT	GATTTTGATG
951	ATTGTGTTGT	GGTGTGTAA	GAGAATGAAG	TATTTCCGGG	TAGTATGGTA
1001	ATGCCTTCAA	CTTACAAACG	GTTTCAGTAA	ACCACCCATA	TACATACATA
1051	TACATGCATG	TGATATATAC	ACATACAGGG	ATGTGTGTGT	GTTTACATAT
1101	ATGAGGGGAG	AGAGACTAGG	GGAGAGAAAG	TAGGTTGGGG	AGAGGGAGAG
1151	AGAAAGGAAA	ACAGGAGACA	GAGAGAGAGC	GGGGAGTAGA	GAGAGGGAAG
1201	GGGTAAGAGA	GGGAGAGGAG	GAGAGAAAGG	GAGGAAGAAG	CAGAGAGTGA
1251	ATGTTAAAGG	AAACAGGCAA	AACATAAACA	GAAAATCTGG	GTGAAGGGTA
1301	TATGAGTATT	CTTTGTACTA	TTCTTGCAAT	TATCTTTTAT	TTAAATTGAC
1351	ATCGGGCCGG	GCGCAGTGGC	TCACATCTGT	AATCCCAGCA	CTTTGGGAGG
1401	CCGAGGCAGG	CAGATCACTT	GAGGTCAGGA	GTTTGAGACC	AGCCTGGCAA
1451	ACATGGTGAA	ACCCCATCTC	TACTAAAAAT	ACAAAAATTA	GCCTGGTGTG
1501	GTGGTGCATG	CCTTTAATCT	CAGCTACTCG	GGAGGCTGAG	GCAGGAGAAT
1551	CGCTTGAACC	CGTGGCGGGG	AGGAGGTTGC	AGTGAGCTGA	GATCATGCCA
1601	CTGCACTCCA	GCCTGGGCGA	TAGAGCGAGA	CTCAGTTTCA	AATAAATAAA
1651	TAAACATCAA	AATAAAAAGT	TACTGTATTA	AAGAATGGGG	GCGGGGTGGG
1701	AGGGGTGGGG	AGAGGTTGCA	AAAATAAATA	AATAAATAAA	TAAACCCCAA
1751	AATGAAAAAG	ACAGTGGAGG	CACCAGGCCT	GCGTGGGGCT	GGAGGGCTAA
1801	TAAGGCCAGG	CCTCTTATCT	CTGGCCATAG	AACCAGAGAA	GTGAGTGGAT
1851	GTGATGCCCA	GCTCCAGAAG	TGACTCCAGA	ACACCCTGTT	CCAAAGCAGA
1901	GGACACACTG	ATTTTTTTTT	TAATAGGCTG	CAGGACTTAC	TGTTGGTGGG
1951	ACGCCCTGCT	TTGCGAAGGG	AAAGGAGGAG	TTTGCCCTGA	GCACAGGCCC
2001	CCACCCCTCA	CTGGGCTTTC	CCCAGCTCCC	TTGTCTTCTT	ATCACGGTAG
2051	TGGCCCAAGC	CCTGGCCCTC	GACTCCAGAA	GGTGGCCCTC	CTGGAACCCC
2101	AGGTCGTGCA	GTCAACGATG	TACTCGCCGG	GACAGCGATG	TCTGCTGCAC
2151	TCCATCCCTC	CCCTGTTTAT	TTGTCTTCA	TGCCCCGTCTG	GAGTAGATGC

FIG. 10A

SUBSTITUTE SHEET (RULE 26)

2201 TTTTTCGAGA GGTGGCACCC TGTAAGCTC TCCTGTCTGA CTTTTTTTTT
2251 TTTTITAGAC TGAGTTTTGC TCTTGTGGC TAGGCTGGAG TGCAATGGCA
2301 CAATCTCAGC TCACTGCACC CTCTGCCTCC CGGGTTCAAG CGATTCTCCT
2351 GCCTCAGCCT CCGAGTAGT TGGGATTACA GGCATGCACC ACCACGCCCA
2401 GCTAATTTTT GTATTTTTAG TAGAGACAAG GTTTCACCGT GATGGCCAGG
2451 CTGGTCTTGA ACTCCAGGAC TCAAGTGATG CTCCTGCCTA GGCCTCTCAA
2501 AGTGTGGGA TTACAGGCGT GAGCCACTGC ACCCGGCCCTG CACGCGTTCT
2551 TTGAAAGCAG TCGAGGGGGC GCTAGGTGTG GGCAGGGACG AGCTGGCGCG
2601 GCGTCGCTGG GTGCACCGCG ACCACGGGCA GAGCCACGCG GCGGGAGGAC
2651 TACAACTCCC GGCACACCCC GCGCGCCCC GCCTCTACTC CCAGAAGGCC
2701 GCGGGGGTG GACCGCCTAA GAGGGCGTGC GCTCCCGACA TGCCCCGCGG
2751 CGCGCCATTA ACCGCCAGAT TTGAATCGCG GGACCCGTTG GCAGAGGTGG
|----->Start
2801 CCGCGGCGGC ATGGGTGCCC CGACGTTGCC CCCTGCCTGG CAGCCCTTTC
2851 TCAAGGACCA CCGCATCTCT ACATTCAAGA ACTGGCCCTT CTGGAGGGG
2901 TCGCGCTGCA CCGCGGAGCG GGTGAGACTG CCGGCCCTCC TGGGTCCCC
2951 CACGCCCGCC TTGCCCTGTC CCTAGCGAGG CCACTGTGAC TGGGCCTCGG
3001 GGGTACAAGC CGCCCTCCCC TCCCGTCTCT GTCCCCAGCG AGGCCACTGT
3051 GGCTGGGCCC CTGGGTCCA GGCGGCGCTC CCCTCCCTGC TTTGTCCCA
3101 TCGAGGCCTT TGTGGCTGGG CCTCGGGGT CCGGGCTGCC ACGTCCACTC
3151 ACGAGCTGTG CTGTCCCTTG CAGATGGCCG AGGCTGGCTT CATCCACTGC
3201 CCCACTGAGA ACGAGCCAGA CTTGGCCAG TGTTCCTTCT GCTTCAAGGA
3251 GCTGGAAGGC TGGGAGCCAG ATGACGACCC CATGTAAGTC TTCTCTGGCC
3301 AGCCTCGATG GGCTTTGTTT TGAAGTGAGT TGTCAAAAGA TTTGAGTTGC
3351 AAAGACACTT AGTATGGGAG GGTGCTTTC CACCCTCATT GCTTCTTAAA
3401 CAGCTGTGTG GAACGGATAC CTCTCTATAT GCTGGTGCCT TGGTGATGCT
3451 TACAACCTAA TTAAATCTCA TTTGACCAAA ATGCCTTGGG GTGGACGTAA
3501 GATGCCTGAT GCCTTTTCATG TTCAACAGAA TACATCAGCA GACCCCTGTG
3551 TTGTGAATC CCAGGAATGT CCAAGTGCTT TTTTGTAGAT TTTTAAAAA
3601 ACAGTTTAAAT TGAATATAA CCTACACAGC ACAAATTA CCCTTTGAAA
3651 GTGTGCATT CACACTTTCG GAGGCTGAGG CGGGCGGATC ACCTGAGGTC
3701 AGGAGTTCAA GACCTGCCTG GCCAACTTGG CGAAACCCCG TCTCTACTAA
3751 AAATACAAAA ATTAGCCGGG CATGGTAGCG CACGCCCGTA ATCCCAGCTA
3801 CTCGGGAGGC TAAGGCAGGA GAATCGCTTG AACCTGGGAG GCGGAGGTG
3851 CAGTGAGCCG AGATTGTGCC AATGCACTCC AGCCTCGGCG ACAGAGCGAG
3901 ACTCCGTGAT AAAAATAAAA AATTGAAAA AAAAAAGAA AGAAAGCATA
3951 TACTTCAGTG TTGTTCTGGA TTTTTTCTT CAAGATGCCT AGTTAATGAC
4001 AATGAAATTC TGTACTCGGA TGGTATCTGT CTTTCCACAC TGTAATGCCA
4051 TATTCTTTTC TCACCTTTTT TTCTGTGCGA TTCAGTTGCT TCCACAGCTT
4101 TAATTTTTTT CCCCTGGAGA TCTTAGTATG TTTGCTATGG TGGTTATACT
4151 GCATCCCCGT AATCACTGGG AAAAGATCAG TGGTATTCTT CTTGAAAATG
4201 AATAAGTGTT ATGATATTTT CAGATTAGAG TTACAACCTG CTGTCTTTTT
4251 GGAATTTGTG TGGCCATGTT TTCATTGTAA TGCAGTTCTG GTAACGGTGA
4301 TAGTCAGTTA TACAGGGAGA CTCCCTAGC AGAAAATGAG AGTGTGAGCT
4351 AGGGGGTCCC TTGGGGAACC CGGGGCAATA ATGCCCTTCT CTGCCCTTAA
4401 TCCTTACAGT GGGCCGGGCA CGGTGGCTTA CGCTGTAAAT ACCAGCACTT
4451 TGGGAGGCCG AGGCGGGCGG ATCAGGAGGT CAGGAGATCG AGACCATCTT
4501 GGCTAATACG GTGAAACCCC GTCTCCACTA AAAATACAAA AAATTAGCCC

FIG. 10B

SUBSTITUTE SHEET (RULE 26)

4551 GGCCTGGTGG TGGGCGCCTG TAGTCCCAGC TACTCGGGAG GCTGAGGCAG
4601 GAGAATGGCG TGAACCCAGG AGGCGGAGCT TGCAGTGAGC CGAGATTGCA
4651 CCACTGCACT CCAGCCTGGG CGACAGAATG AGACTCCGTC TCAAAAAAAA
4701 AAAAAAAGA AAAAAATCTT TACAGTGGAT TACATAACAA TTCCAGTGAA
4751 ATGAAATTAC TTCAAACAGT TCCTTGAGAA TGTGGAGGG ATTTGACATG
4801 TAATTCCTTT GGACATATAC CATGTAACAC TTTTCCAAC TATTGCTAAG
4851 GAAGTCCAGA TAAAAATAGT ACATTAGCCA CACAGATGTG GGGGGAGATG
4901 TCCACAGGGA GAGAGAAGGT GCTAAGAGGT GCCATATGGG AATGTGGCTT
4951 GGGCAAAGCA CTGATGCCAT CAACTTCAGA CTTGACGTCT TACTCCTGAG
5001 GCAGAGCAGG GTGTGCCTGT GGAGGGCGTG GGGAGGTGGC CCGTGGGGAG
5051 TGGACTGCCG CTTTAATCCC TTCAGCTGCC TTTCCGCTGT TGTTTTGATT
5101 TTTCTAGAGA GGAACATAAA AAGCATTCTG CCGGTTGCGC TTTCTTTCT
5151 GTCAAGAAGC AGTTTGAAGA ATTAACCCTT GGTGAATTTT TGAAACTGGA
5201 CAGAGAAAGA GCCAAGAACA AAATTGTATG TATTGGGAAT AAGAACTGCT
5251 CAAACCCTGT TCAATGTCTT TAGCACTAAA CTACCTAGTC CCTCAAAGGG
5301 ACTCTGTGTT TTCCTCAGGA AGCATTTTTT TTTTTTTTCT GAGATAGAGT
5351 TTCACTCTTG TTGCCCAGGC TGGAGTGCAA TGGTGCAATC TTGGCTCACT
5401 GCAACCTCTG CCTCTCGGGT TCAAGTGATT CTCCTGCCTC AGCCTCCCAA
5451 GTAACCTGGA TTACAGGGAA GTGCCACCAC ACCCAGCTAA TTTTGTATT
5501 TTTAGTAGAG ATGGGGTTTC ACCACATTGC CCAGGCTGGT CTTGAACCTC
5551 TGACCTCGTG ATTCGCCCAC CTTGGCCTCC CAAAGTGCTG GGATTACAGG
5601 CGTGAACCAC CACGCCTGGC TTTTTTTTTT TTGTTCTGAG ACACAGTTTC
5651 ACTCTGTATC CCAGGCTGGA GTAGGGTGGC CTGATCTCGG ATCACTGCAA
5701 CCTCCGCCTC CTGGGCTCAA GTGATTTGCC TGCTTCAGCC TCCCAAGTAG
5751 CCGAGATTAC AGGCATGTGC CACCACACCC AGGTAATTTT TGTATTTTGG
5801 GTAGAGACGA GGTTCACCA TGTTGGCCAG GCTGGTTTTG AACTCCTGAC
5851 CTCAGGTGAT CCACCCGCCT CAGCCTCCCA AAGTGCTGAG ATTATAGGTG
5901 TGAGCCACCA CACCTGGCCT CAGGAAGTAT TTTTATTTT AAATTTATTT
5951 ATTTATTTGA GATGGAGTCT TGCTCTGTCTG CCCAGGCTAG AGTGCAGCGA
6001 CGGGATCTCG GCTCACTGCA AGCTCCGCC CCCAGTTCA AGCCATTCTC
6051 CTGCCTCAGC CTCCCGAGTA GCTGGGACTA CAGGCGCCCG CCACCACACC
6101 CGGCTAATTT TTTTGTATTT TTAGTAGAGA CGGGTTTTCA CCGTGTTAGC
6151 CAGGAGGGTC TTGATCTCCT GACCTCGTGA TCTGCCTGCC TCGGCCTCCC
6201 AAAGTGCTGG GATTACAGGT GTGAGCCACC ACACCCGGCT ATTTTATTT
6251 TTTTGAGACA GGGACTCACT CTGTCACCTG GGCTGCAGTG CAGTGGTACA
6301 CCATAGCTCA CTGCAGCCTC GAACTCCTGA GCTCAAGTGA TCCTCCACCC
6351 TCATCCTCAC AAGTAATTGG GACTACAGGT GCACCCACCC ATGCCACCTT
6401 AATTTATTTA TTTATTTATT TATTTATTTT CATAGAGATG AGGGTTCCTT
6451 GTGTTGTCCA GGCTGGTCTT GAACTCCTGA GCTCACGGGA TCCTTTTGCC
6501 TGGGCCTCCC AAAGTGCTGA GATTACAGGC ATGAGCCACC GTGCCACGCT
6551 AGGAATCATT TTTAAAGCCC CTAGGATGTC TGTGTGATTT TAAAGCTCCT
6601 GGAGTGTGGC CCGTATAAGT ATATACCGGT ATAAGTAAAT CCCACATTTT
6651 CTGGGCTTTA TTTATTTATT TATTTATTTA TTTATTTTAA ATTTTTTTTT
6701 TTGAGACGAG TCTCACTTTG TCACCCAGGC TGGAGTGAGG TGGCACGATC
6751 TCGGCTCACT GCAACCTCTG CCTCCCGGGG TCAAGCGATT CTCTGCCTT
6801 AGCCTCCCGA GTAGCTGGGA CTACAGGCAC GCACCACCAT GCCTGGCTAA
6851 TTTTGTATTT TTTAGTAGAC GGGGTTTCAC CATGCTGGCC AAGCTGGTCT
6901 CAAACTCCTG ACCTTGTGAT CTGCCCGCTT TAGCCTCCCA GAGTGCTGGG

FIG. 10C

SUBSTITUTE SHEET (RULE 26)

6951 ATTACAGGCA TGAGCCACCA TCGTGGTCT TTTTAAATF TTTTGATTTT
7001 TTTTTTTTTT GAGACAGAGC CTTGCTCTGT CGCCCAGGCT GGAGTGCAGT
7051 GGCACGATCT CAGCTCACTA CAAGCTCCGC CTCCCAGGTT CACGCCATTTC
7101 TTCTGCCTCA GCCTCCTGAG TAGCTGGGAC TACAGGTGCC CACCACCACG
7151 CCTGGCTAAT TTTTTTGGT ATTTTATTA GAGACAAGGT TTCATCATGT
7201 TGGCCAGGCT GGTCTCAAAC TCCTGACCTC AAGTGATCTG CCTGCCTCGG
7251 CCTCCCAAAG CGCTGAGATT ACAGGTGTGA TCTACTGCGC CAGGCCTGGG
7301 CGTCATATAT TCTTATTTGC TAAGTCTGGC AGCCCCACAC AGAATAAGTA
7351 CTGGGGGATT CCATATCCTT GTAGCAAAGC CTGGGGTGA GAGTCAGGAG
7401 ATGTTGTAGT TCTGTCTCTG CCACTTGCGC ACTTTGAGTT TAAGCCAGTC
7451 GTGCTCATGC TTTCCCTTGCT AAATAGAGGT TAGACCCCTC ATCCCATGGT
7501 TTCTCAGGTT GCTTTTCAGC TTGAAAATTG TATTCCTTTG TAGAGATCAG
7551 CGTAAATATA TTCTGTCTT ATATGTGGCT TTATTTAAT TTGAGACAGA
7601 GTGTCACTCA GTCGCCAGG CTGGAGTGTG GTGGTGGAT CTTGGCTCAC
7651 TGCACCTCC ACCTCCAGG TTCAAGCGAT TCTCGTGCT CAGGCTCCCA
7701 AGTAGCTGAG ATTATAGGTG TGTGCCACCA GGCCAGCTA ACTTTTGTAT
7751 TTTTAGTAGA GACAGGTTT TGCCATGTTG GCTAAGCTGG TCTCGAACTC
7801 CTGGCCTCAA GTGATCTGCC CGCCTTGGCA TCCCAAAGTG CTGGGATTAC
7851 AGGTGTGAAC CACCACACCT GGCCTCAATA TAGTGGCTT TAAGTGCTAA
7901 GGACTGAGAT TGTGTTTGT CAGGAAGAGG CCAGTTGTGG GTGAAGCATG
7951 CTGTGAGAGA GCTTGTCAAC TGGTTGAGGT TGTGGGAGCT GCAGCGTGGG
8001 AACTGGAAG TGGGCTGGG ATCATCTTTT TCCAGGTCAG GGGTCAGCCA
8051 GCTTTTCTGC AGCGTGCCAT AGACCATCTC TTAGCCCTCG TGGGTCAGAG
8101 TCTCTGTGTC ATATTGTCTT TTGTTGTTTT TCACAACCTT TTAGAAACAT
8151 AAAAAGCATT CTTAGCCCGT GGGCTGGACA AAAAAAGGCC ATGACGGGCT
8201 GTATGGATT TTGGCCAGCAG GCCCTTGCTT GCCAAGCCCT GTTTTAGACA
8251 AGGAGCAGCT TGTGTGCTG GAACCATCAT GGGCACAGGG GAGGAGCAGA
8301 GTGGATGTGG AGGTGTGAGC TGGAAACCAG GTCCAGAGC GCTGAGAAAG
8351 ACAGAGGGTT TTGGCCCTTG CAAGTAGAGC AACTGAAATC TGACACCATC
8401 CAGTTCCAGA AAGCCCTGAA GTGCTGGTGG ACGCTGCGGG GTGCTCCGCT
8451 CTAGGGTTAC AGGGATGAAG ATGCAGTCTG GTAGGGGGAG TCCACTCACC
8501 TGTGGAAGA TGTGATTAAG AAAAGTAGAC TTTAGGGCC GGGCATGGTG
8551 GCTCAGCCT GTAATCCCAG CACTTTGGGA GGCCGAGGCG GGTGGATCAC
8601 GAGGTCAGGA GATCGAGACC ATCCTGGCTA ACATGGTGAA ACCCCGTCTT
8651 TACTAAAAAT ACAAAAAATT AGCTGGGCGT GGTGGCGGGC GCCTGTAGTC
8701 CCAGCTACTC GGGAGGCTGA GGCAGGAGAA TGGCGTGAAC CTGGGAGGTG
8751 GAGCTTGCTG TGAGCCGAGA TCGCGCCACT GCACTCCAGC CTGGGCGACA
8801 GAGCGAGACT CCGTCTCAA AAAAAAATAA AAAGTAGGCT TTCATGATGT
8851 GTGAGCTGAA GGCGCAGTAG GCAGAAGTAG AGGCCTCAGT CCCTGCAGGA
8901 GACCCCTCGG TCTCTATCTC CTGATAGTCA GACCCAGCCA CACTGGAAGG
8951 AGGGGAGACA TTACAGCCTG CGAGAAAAGT AGGGAGATTT AAAAAGTGT
9001 TGGCTTTTAT TTTGAACGT TTTTTTTGTT TGTGTTTTT CCCCATTCA
9051 GAATACAGAA TACTTTTATG GATTTGTTTT TATTACTTTA ATTTTGAAAC
9101 AATATAATCT TTTTTTTGTT GTTTTTTTGA GACAGGGTCT TACTCTGTCA
9151 CCCAGGCTGA GTGCAGTGGT GTGATCTTGG CTCACCTCAG CCTCGACCCC
9201 CTGGGCTCAA ATGATTCTCC CACCTCAGCT TCCCAAGTAG CTGGGACCAC
9251 AGGTGCGTGT GTTGCCTAT ACAATCCTG AAGACAAGGA TGCTGTGTCT
9301 GGTGATGCTG GGGATCCCA AGATCCAGA TTTGATGGCA GGATGCCCTT

FIG. 10D

SUBSTITUTE SHEET (RULE 26)

9351 GTCTGCTGCC TTGCCAGGGT GCCAGGAGGG CGCTGCTGTG GAAGCTGAGG
9401 CCCGGCCATC CAGGGCGATG CATTGGGCGC TGATTCTTGT TCCTGCTGCT
9451 GCCTCGGTGC TTAGCTTTTG AAACAATGAA ATAAATTAGA ACCAGTGTGA
9501 AAATCGATCA GGGAAATAAT TTAATGTGGA AATAAACTGA ACAACTTAGT
9551 TCTTCATAAG AGTTTACTTG GTAAATACTT GTGATGAGGA CAAAACGAAG
9601 CACTAGAAGG AGAGGCGAGT TGTAGACCTG GGTGGCAGGA GTGTTTGTG
9651 TGTTTTCTTT GGCAGGGTCT TGCTCTGTG CTCAGGCTGG AGTACAGTGG
9701 CACAATCACA GCTCACTATA GCCTCGACCT CCTGGACTCA AGCAATCCTC
9751 CTGCCTCAGC CTCCCAGTAG CTGGGACTAC AGGCGCATGC CACCATGCCCT
9801 GGCTAATTTT AAATTTTTTT TTTTCTCTTT TTTGAGATGG AATCTCACTC
9851 TGTCGCCCAG GCTGGAGTGC AGTGGCGTGA TCTCGGCTGA CGGCAAGCTC
9901 CGCCTCCCAG GTTCACTCCA TTCGCCTGCC TCAGCCTCCC AAGTAGCTGG
9951 GACTACAGGC GCTGGGATTA CAAACCCAAA CCCAAAGTGC TGGGATTACA
10001 GGCGTGAGCC ACTGCACCCG GCCTGTTTTG TCTTTCAATA GCAAGAGTTG
10151 TGTTTGCTTC GCCCTACCT TTAGTGGAAT AATGTATAAA ATGGAGATAT
10201 TGACCTCCAC ATTGGGGTGG TTAAATTATA GCATGTATGC AAAGGAGCTT
10251 CGCTAATTTA AGGCTTTTTT GAAAGAGAAG AAAGTGAATA ATCCATGTGT
10301 GTATATATAT TTTAAAGGCC ATGGTCATCT TTCCATATCA GTAAAGCTGA
10351 GGCTCCCTGG GACTGCAGAG TTGTCCATCA CAGTCCATTA TAAGTGCCT
10401 GCTGGGCCAG GTGCAGTGGC TTGTGCCTGA ATCCCAGCAC TTTGGGAGGC
10451 CAAGGCAGGA GGATTCATTG AGCCCAGGAG TTTTGAGGCG AGCCTGGGCA
10501 ATGTGGCCAG ACCTCATCTC TTCAAAAAAT ACACAAAAAA TTAGCCAGGC
10551 ATGGTGGCAC GTGCCTGTAG TCTCAGCTAC TCAGGAGGCT GAGGTGGGAG
10601 GATCACTTTG AGCCTTGCAG GTCAAAGCTG CAGTAAGCCA TGATCTTGCC
10651 ACTGCATTCC AGCCTGGATG ACAGAGCGAG ACCCTGTCTC TAAAAAATAA
10701 AAAAACCAAA CGGTGCACTG TTTTCTTTTT TCTTATCAAT TTATTATTTT
10751 TAAATTAAAT TTTCTTTTAA TAATTTATAA ATTATAAATT TATATTAAAA
10801 AATGACAAAT TTTTATTACT TATACATGAG GTAAACTTAA GGATATATAA
10851 AGTACATATT GAAAAGTAAT TTTTGGCTG GCACAGTGGC TCACACCTGT
10901 AATCCCAGCA CTTTGGGAGG CCGTGGCGGG CAGATCACAT GAGATCATGA
10951 GTTCGAGACC AACCTGACCA ACATGGAGAG ACCCCATCTC TACTAAAAAT
11001 ACAAAATTAG CCGGGGTGGT GGCGCATGCC TGTAATCCCA GCTACTCGGG
11051 AGGCTGAGGC AGGAGAATCT CTGGAACCCG GGAGGCAGAG GTTGCCTGTA
11101 GCCAAGATCG TGCCTTTGCA CACCAGCCTA GGCAACAAGA GCGAAAGTCC
11151 GTCTCAAAAA AAAAGTAAT TTTTAAAGT TAACCTCTGT CAGCAAACAA
11201 ATTTAACCCA ATAAAGGTCT TTGTTTTTAA ATGTAGTAGA GGAGTTAGGG
11251 TTTATAAAAA ATATGGTAGG GAAGGGGGTC CCTGGATTG CTAAATGTGAT
11301 TGTCATTTGC CCCTTAGGAG AGAGCTCTGT TAGCAGAATG AAAAAATTGG
11351 AAGCCAGATT CAGGGAGGGA CTGGAAGCAA AAGAATTTCT GTTCGAGGAA
11401 GAGCCTGATG TTTGCCAGGG TCTGTTTAACT TGGACATGAA GAGGAAGGCT
11451 CTGGACTTTC CTCCAGGAGT TTCAGGAGAA AGGTAGGGCA GTGGTTAAGA
11501 GCAGAGCTCT GCCTAGACTA GCTGGGGTGC CTAGACTAGC TGGGGTGCCC
11551 AGACTAGCTG GGGTGCTAG ACTAGCTGGG TACTTTGAGT GGCTCCTTCA
11601 GCCTGGACCT CGGTTTCCTC ACCTGTATAG TAGAGATATG GGAGCACCCA
11651 GCGCAGGATC ACTGTGAACA TAAATCAGTT AATGGAGGAA GCAGGTAGAG
11701 TGGTGCTGGG TGCATACCAA GCACTCCGTC AGTGTTTCCT GTTATTGAT
11751 GATTAGGAGG CAGCTTAAAC TAGAGGGAGT TGAGCTGAAT CAGGATGTTT
11801 GTCCCAGGTA GCTGGGAATC TGCCTAGCCC AGTGCCAGT TTATTTAGGT

FIG. 10E

SUBSTITUTE SHEET (RULE 26)

9351 GTCTGCTGCC TTGCCAGGGT GCCAGGAGGG CGCTGCTGTG GAAGCTGAGG
9401 CCCGGCCATC CAGGGCCGATG CATTGGGCGC TGATTCTTGT TCCTGCTGCT
9451 GCCTCGGTGC TTAGCTTTTG AAACAATGAA ATAAATTAGA ACCAGTGTGA
9501 AAATCGATCA GGAATAAAT TTAATGTGGA AATAAACTGA ACAACTTAGT
9551 TCTTCATAAG AGTTTACTTG GTAAATACTT GTGATGAGGA CAAAACGAAG
9601 CACTAGAAGG AGAGGCGAGT TGTAGACCTG GGTGGCAGGA GTGTTTTGTT
9651 TGTTTTCTTT GGCAGGGTCT TGCTCTGTTG CTCAGGCTGG AGTACAGTGG
9701 CACAATCACA GCTCACTATA GCCTCGACCT CCTGGACTCA AGCAATCCTC
9751 CTGCCTCAGC CTCCCAGTAG CTGGGACTAC AGGCGCATGC CACCATGCCT
9801 GGCTAATTTT AAATTTTTTT TTTTCTCTTT TTTGAGATGG AATCTCACTC
9851 TGTCGCCCAG GCTGGAGTGC AGTGGCGTGA TCTCGGCTGA CGGCAAGCTC
9901 CGCCTCCCAG GTTCACTCCA TTCGCCTGCC TCAGCCTCCC AAGTAGCTGG
9951 GACTACAGGC GCTGGGATTA CAAACCCAAA CCCAAAGTGC TGGGATTACA
10001 GGCGTGAGCC ACTGCACCCG GCCTGTTTTG TCTTTCAATA GCAAGAGTTG
10151 TGTTTGCTTC GCCCTACCT TTAGTGGAAA AATGTATAAA ATGGAGATAT
10201 TGACCTCCAC ATTGGGGTGG TTAAATTATA GCATGTATGC AAAGGAGCTT
10251 CGCTAATTTA AGGCTTTTTT GAAAGAGAAG AAAGTGAATA ATCCATGTGT
10301 GTATATATAT TTTAAAGCC ATGGTCATCT TTCCATATCA GTAAAGCTGA
10351 GGCTCCCTGG GACTGCAGAG TTGTCCATCA CAGTCCATTA TAAGTGCCTC
10401 GCTGGGCCAG GTGCAGTGGC TTGTGCCTGA ATCCCAGCAC TTTGGGAGGC
10451 CAAGGCAGGA GGATTCATTG AGCCCAGGAG TTTTGAGGCG AGCCTGGGCA
10501 ATGTGGCCAG ACCTCATCTC TTCAAAAAAT ACACAAAAAA TTAGCCAGGC
10551 ATGGTGGCAC GTGCCTGTAG TCTCAGCTAC TCAGGAGGCT GAGGTGGGAG
10601 GATCACTTTG AGCCTTGCA GTCAAAGCTG CAGTAAGCCA TGATCTTGCC
10651 ACTGCATTCC AGCCTGGATG ACAGAGCGAG ACCCTGTCTC TAAAAAAA
10701 AAAAAACCAA CCGTGCACTG TTTTCTTTTT TCTTATCAAT TTATTATTTT
10751 TAAATTAAAT TTTCTTTTAA TAATTATATA ATTATAAATT TATATTAAAA
10801 AATGACAAAT TTTTATTACT TATACATGAG GTAAAACTTA GGATATATAA
10851 AGTACATATT GAAAAGTAAT TTTTGGGCTG GCACAGTGGC TCACACCTGT
10901 AATCCCAGCA CTTTGGGAGG CCGTGGCGGG CAGATCACAT GAGATCATGA
10951 GTTCGAGACC AACCTGACCA ACATGGAGAG ACCCCATCTC TACTAAAAAT
11001 ACAAATTAG CCGGGGTGGT GGCGCATGCC TGTAAATCCA GCTACTCGGG
11051 AGGCTGAGGC AGGAGAATCT CTTGAACCCG GGAGGCAGAG GTTGCCTGTA
11101 GCCAAGATCG TGCCTTTGCA CACCAGCCTA GGCAACAAGA GCGAAAGTCC
11151 GTCTCAAAAA AAAAGTAATT TTTTFTAAGT TAACCTCTGT CAGCAAAACA
11201 ATTTAACCCA ATAAAGGTCT TTGTTTTTTA ATGTAGTAGA GGAGTTAGGG
11251 TTTATAAAAA ATATGGTAGG GAAGGGGTC CCTGGATTG CTAATGTGAT
11301 TGTCAATTGC CCCTTAGGAG AGAGCTCTGT TAGCAGAATG AAAAAATTGG
11351 AAGCCAGATT CAGGGAGGGA CTGGAAGCAA AAGAATTTCT GTTCGAGGAA
11401 GAGCCTGATG TTTGCCAGGG TCTGTTTAAC TGGACATGAA GAGGAAGGCT
11451 CTGGACTTTC CTCCAGGAGT TTCAGGAGAA AGGTAGGGCA GTGGTTAAGA
11501 GCAGAGCTCT GCCTAGACTA GCTGGGGTGC CTAGACTAGC TGGGGTGCCC
11551 AGACTAGCTG GGGTGCCTAG ACTAGCTGGG TACTTTGAGT GGCTCCTTCA
11601 GCCTGGACCT CGGTTTCCTC ACCTGTATAG TAGAGATATG GGAGCACCCA
11651 GCGCAGGATC ACTGTGAACA TAAATCAGTT AATGGAGGAA GCAGGTAGAG
11701 TGGTGCTGGG TGCATACCAA GCACTCCGTC AGTGTTCCT GTTATTGGAT
11751 GATTAGGAGG CAGCTTAAAC TAGAGGGAGT TGAGCTGAAT CAGGATGTTT
11801 GTCCCAGGTA GCTGGGAATC TGCCTAGCCC AGTGCCAGT TTATTAGGT

FIG. 10F

SUBSTITUTE SHEET (RULE 26)

11851 GCTCTCTCAG TGTTCCCTGA TTGTTTTTTC CTTTGTGTCATC TTATCTACAG
11901 GATGTGACTG GGAAGCTCTG GTTTCAGTGT CATGTGTCTA TTCTTTATTT
11951 CCAGGCAAAG GAAACCAACA ATAAGAAGAA AGAATTGAG GAAACTGCCA
12001 AGAAAGTGCG CCGTGCCATC GAGCAGCTGG CTGCCATGGA TTGAGGCCTC
12051 TGGCCGGAGC TGCCCTGGTCC CAGAGTGGCT GCACCACTTC CAGGGTTTAT
12101 TCCCTGGTGC CACCAGCCTT CCTGTGGGCC CCTTAGCAAT GTCTTAGGAA
12151 AGGAGATCAA CATTTTCAAA TTAGATGTTT CAACTGTGCT CCTGTTTTGT
12201 CTTGAAAGTG GCACCAGAGG TGCTTCTGCC TGTGCAGCGG GTGCTGCTGG
12251 TAACAGTGGC TGCTTCTCTC TCTCTCTCTC TTTTGGGG GCTCATTTTT
12301 GCTGTTTTGA TTCCCGGGCT TACCAGGTGA GAAGTGAGGG AGGAAGAAGG
12351 CAGTGTCCCT TTTGCTAGAG CTGACAGCTT TGTTCGCGTG GGCAGAGCCT
12401 TCCACAGTGA ATGTGTCTGG ACCTCATGTT GTTGAGGCTG TCACAGTCTT
12451 GAGTGTGGAC TTGGCAGGTG CCTGTTGAAT CTGAGCTGCA GGTTCCTTAT
12501 CTGTACACAC TGTGCCTCCT CAGAGGACAG TTTTGTGTT GTTGTGTTTT
12551 TTTGTTTTTT TTTTGTGTA GATGCATGAC TTGTGTGTGA TGAGAGAATG
12601 GAGACAGAGT CCCTGGCTCC TCTACTGTTT AACACATGG CTTTCTTATT
12651 TTGTTTGAAT TGTAAATTCA CAGAATAGCA CAAACTACAA TTAAACTAA
12701 GCACAAAGCC ATTCTAAGTC ATTGGGAAA CGGGGTGAAC TTCAGGTGGA
12751 TGAGGAGACA GAATAGAGTG ATAGGAAGCG TCTGGCAGAT ACTCCTTTTG
12801 CCACTGCTGT GTGATTAGAC AGGCCAGTG AGCCGCGGG CACATGCTGG
12851 CCGCTCCTCC CTCAGAAAAA GGCAGTGGCC TAAATCCTTT TTAATGACT
12901 TGGCTCGATG CTGTGGGGGA CTGGCTGGGC TGCTGCAGGC CGTGTGTCTG
12951 TCAGCCCAAC CTTACATCT GTACAGTTCT CCACACGGGG GAGAGACGCA
13001 GTCCGCCAG GTCCCGCTT TCTTTGGAGG CAGCAGCTCC CGCAGGGCTG
13051 AAGTCTGGCG TAAGATGATG GATTTGATTG GCCCTCCTCC CTGTACATAGA
13101 GCTGCAGGGT GGATTGTTAC AGCTTCGCTG GAAACCTCTG GAGGTCTCT
13151 CGGCTGTTCC TGAGAAATAA AAAGCCTGTC ATTTCAAACA CTGCTGTGGA
13201 CCCTACTGGG TTTTAAAT ATTGTCAGTT TTTTCATGTC GTCCCTAGCC
13251 TGCCAACAGC CATCTGCCCA GACAGCCGCA GTGAGGATGA GCGTCTGGC
13301 AGAGACGCAG TTGTCTCTGG GCGCTTGCCA GAGCCACGAA CCCCAGACCT
13351 GTTTGTATCA TCCGGGCTCC TTCCGGGCAG AAACAACGAA AAATGCATT
13401 CAGACCCACT TATTTATGCC ACATCTGAGT CCGCCTGAGA TAGACTTTTC
13451 CCTCTAAACT GGGAGAAATAT CACAGTGGTT TTTGTTAGCA GAAATGCAC
13501 TCCAGCCTCT GTACTCATCT AAGCTGCTTA TTTTGTATAT TTGTGTGAGT
13551 CTGTAAATGG ATACTTCACT TTAATAACTG TTGCTTAGTA ATTGGCTTTG
13601 TAGAGAAGCT GGAATAAAT GGTTTGTCT TCAACTCCTT TGCATGCCAG
13651 GCGGTGATGT GGAATCTCGC TTCTGTGAGC CTGTGCTGTG GGCAGGGCTG
13701 AGCTGGAGCC GCCCCTCTCA GCCCGCTGC CACGGCCTTT CTTAAAGGC
13751 CATCCTTAAA ACCAGACCCT CATGGCTGCC AGCACCTGAA AGCTTCCTCG
13801 ACATCTGTTA ATAAAGCCGT AGGCCCTGT CTAAGCGCAA CCGCTAGAC
13851 TTTCTTTCAG ATACATGTCC ACATGTCCAT TTTTCAGGTT CTCTAAGTTG
13901 GAGTGGAGTC TGGGAAGGGT TGTGAATGAG GCTTCTGGGC TATGGGTGAG
13951 GTTCCAATGG CAGGTTAGAG CCCCTCGGGC CAACTGCCAT CCTGGAAAGT
14001 AGAGACAGCA GTGCCCGCTG CCCAGAAGAG ACCAGCAAGC CAACTGGAG
14051 CCCCCATTGC AGGCTGTGCG CATGTGGAAA GAGTAACTCA CAATTGCCAA
14101 TAAAGTCTCA TGTGGTTTTA TCTACTTTT TTTTCTTTT CTTTTTTTTT
14151 GAGACAAGGC CTTGCCCTCC CAGGCTGGAG TGCAGTGAA TGACCACAGC
14201 TCACCGCAAC CTCAAATCT TCGTTCAAG TGAACCTCCC ACTTTAGCCT

FIG. 10G

SUBSTITUTE SHEET (RULE 26)

14251 CCCAAGTAGC TGGGACTACA GGCGCACGCC ATCACACCCG GCTAATTGAA
14301 AAATTTTTTT TTTTGTTTAG ATGGAATCTC ACTTTGTTGC CCAGGCTGGT
14351 CTCAAACTCC TGGGCTCAAG TGATCATCCT GCTTCAGCGT CCGACTTGTT
14401 GGTATTATAG GCGTGAGCCA CTGGGCCTGA CCTAGCTACC ATTTTTTAAT
14451 GCAGAAATGA AGACTTGTAG AAATGAAATA ACTTGTCAG GATAGTCGAA
14501 TAAGTAACTT TTAGAGCTGG GATTTGAACC CAGGCAATCT GGCTCCAGAG
14551 CTGGGCCCTC ACTGCTGAAG GACACTGTCA GCTTGGGAGG GTGGCTATGG
14601 TCGGCTGTCT GATTCTAGGG AGTGAGGGCT GTCTTTAAAG CACCCCATTC
14651 CATTTTCAGA CAGCTTTGTC AGAAAGGCTG TCATATGGAG CTGACACCTG
14701 CCTCCCCAAG GCTTCCATAG ATCCTCTCTG TACATTGTAA CCTTTTATTT
14751 TGAATGAAA ATTCACAGGA AGTTGTAAGG CTAGTACAGG GGATCC

FIG. 10H

SUBSTITUTE SHEET (RULE 26)

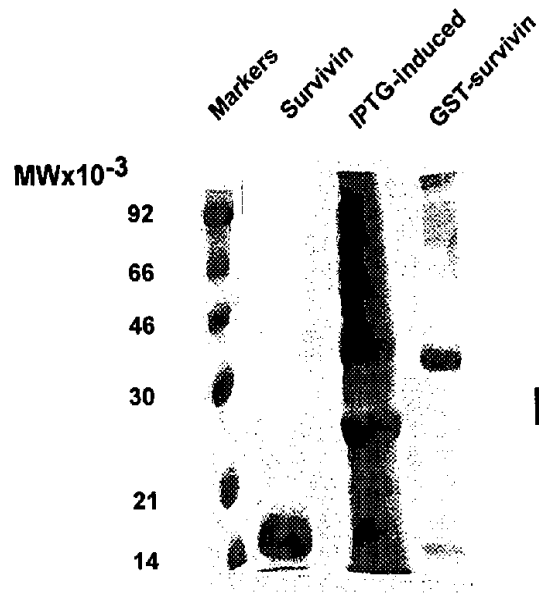


FIG. 11A

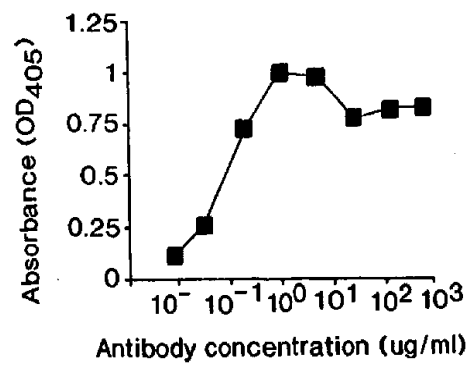


FIG. 11B

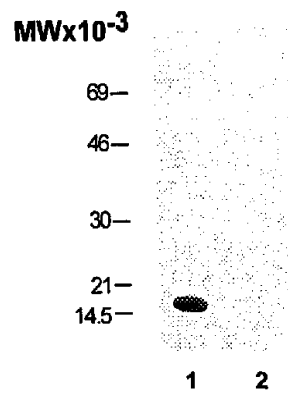


FIG. 11C

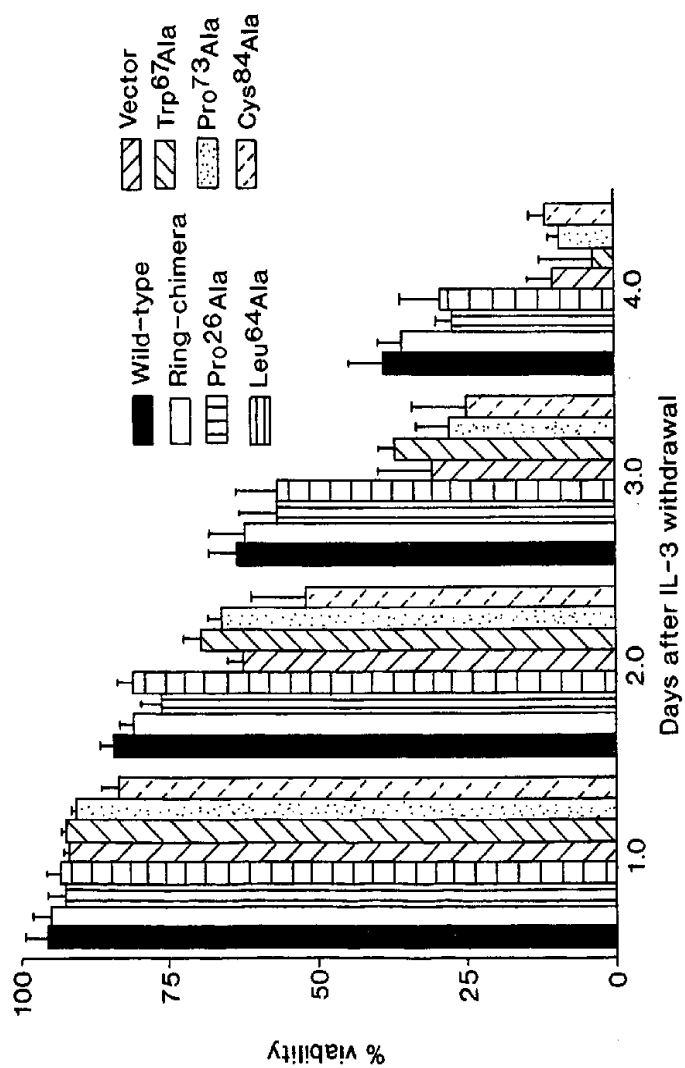


FIG. 12

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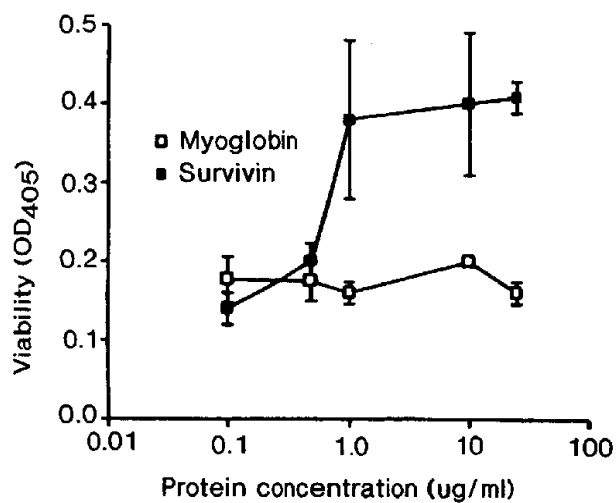


FIG. 13

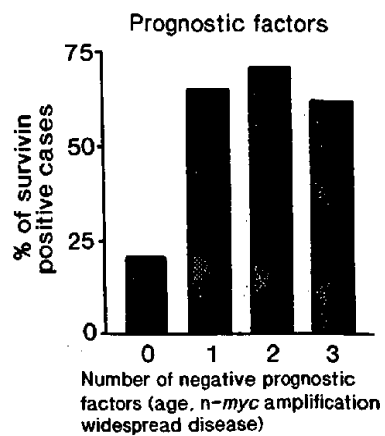


FIG. 14A

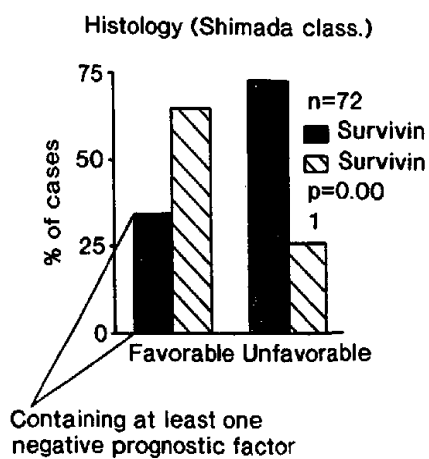


FIG. 14B

FIG. 15A

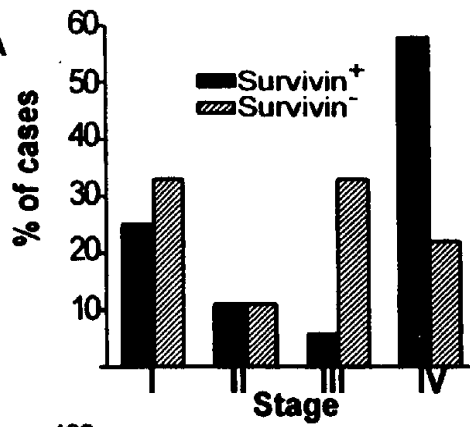
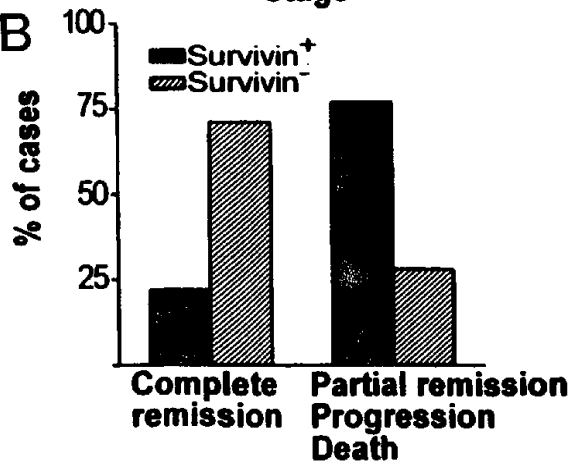


FIG. 15B



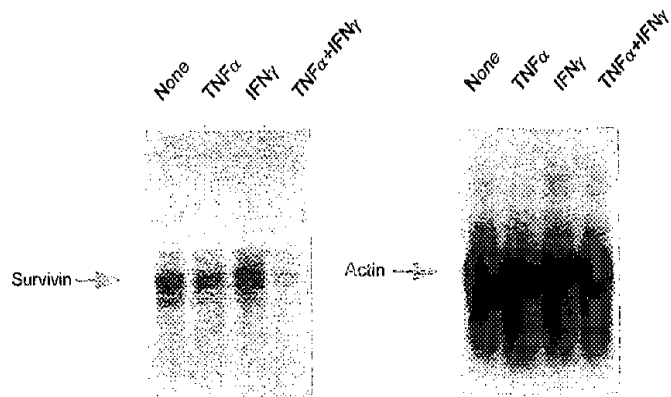
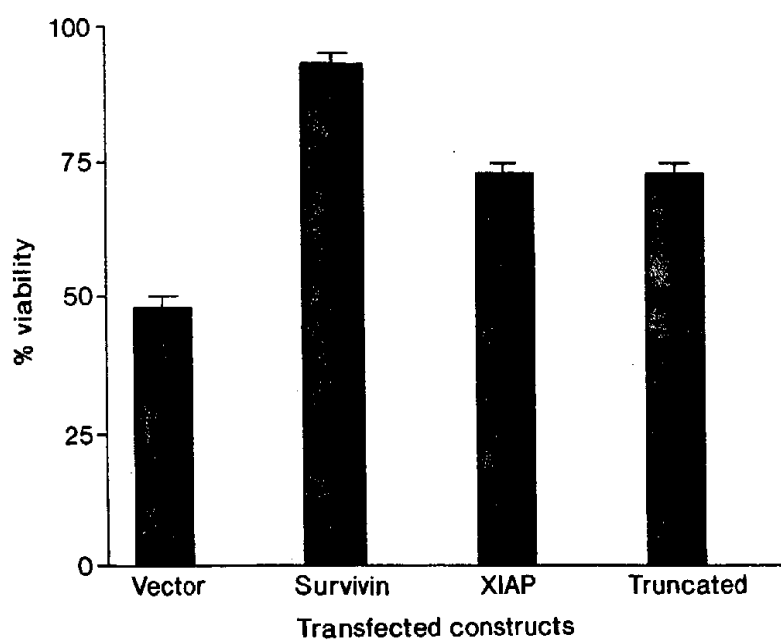


FIG. 16A

FIG. 16B

**FIG. 17**