



(86) Date de dépôt PCT/PCT Filing Date: 1994/11/30
(87) Date publication PCT/PCT Publication Date: 1995/06/08
(45) Date de délivrance/Issue Date: 2007/08/28
(85) Entrée phase nationale/National Entry: 1996/05/30
(86) N° demande PCT/PCT Application No.: US 1994/013930
(87) N° publication PCT/PCT Publication No.: 1995/015084
(30) Priorités/Priorities: 1993/11/30 (US08/160,067);
1994/10/07 (US08/320,157)

(51) Cl.Int./Int.Cl. *C12N 15/12* (2006.01),
A61K 31/7088 (2006.01), *A61K 35/14* (2006.01),
A61K 38/17 (2006.01), *A61K 48/00* (2006.01),
A61P 43/00 (2006.01), *C07K 14/47* (2006.01),
C07K 16/18 (2006.01), *C12Q 1/02* (2006.01),
C12Q 1/68 (2006.01), *G01N 33/53* (2006.01)

(72) Inventeurs/Inventors:
KIEFER, MICHAEL C., US;
BARR, PHILIP J., US

(73) Propriétaire/Owner:
TANOX, INC., US

(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : NOUVELLES PROTEINES MODULANT L'APOPTOSE, ADN CODANT POUR CES PROTEINES, ET LEUR
MODE D'UTILISATION
(54) Title: NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE
THEREOF

(57) Abrégé/Abstract:

The present invention provides a novel family of apoptosis-modulating proteins. Nucleotide and amino acid residue sequences, derivatives thereof and methods of use thereof are also provided.





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01N 43/04, 63/00, A61K 31/70, 37/00, C07K 16/00, 16/18, C12N 1/08, 1/21, 5/00, 5/06, 5/16, 7/00, 15/09, 15/13	A1	(11) International Publication Number: WO 95/15084 (43) International Publication Date: 8 June 1995 (08.06.95)
(21) International Application Number: PCT/US94/13930 (22) International Filing Date: 30 November 1994 (30.11.94) (30) Priority Data: 08/160,067 30 November 1993 (30.11.93) US 08/320,157 7 October 1994 (07.10.94) US (60) Parent Application or Grant (63) Related by Continuation US 08/320,157 (CIP) Filed on 7 October 1994 (07.10.94) (71) Applicant (for all designated States except US): LXR BIOTECHNOLOGY INC. [US/US]; 1401 Marina Way South, Richmond, CA 94804 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KIEFER, Michael, C. [US/US]; 401 Wright Court, Clayton, CA 94517 (US). BARR, Philip, J. [GB/US]; 152 Hillcrest Road, Berkeley, CA 94705 (US).		(74) Agents: LEHNHARDT, Susan, K. et al.; Morrison & Foerster, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US). (81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ). 2177827 Published With international search report.
(54) Title: NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF		
(57) Abstract The present invention provides a novel family of apoptosis-modulating proteins. Nucleotide and amino acid residue sequences, derivatives thereof and methods of use thereof are also provided.		

NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING
THE PROTEINS AND METHODS OF USE THEREOF

5

Field of the Invention

This invention relates to novel proteins with apoptosis-modulating activity, recombinant DNA encoding
10 the proteins, compositions containing the proteins and methods of use thereof.

Background of the Invention

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed
15 cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases
20 related to aging. Recent studies of apoptosis have implied that a common metabolic pathway leading to cell death may be initiated by a wide variety of signals, including hormones, serum growth factor deprivation, chemotherapeutic agents, ionizing radiation and infection
25 by human immunodeficiency virus (HIV). Wyllie (1980) Nature 284:555-556; Kanter et al. (1984) Biochem. Biophys. Res. Commun. 118:392-399; Duke and Cohen (1986) Lymphokine Res. 5:289-299; Tomei et al. (1988) Biochem. Biophys. Res. Commun. 155:324-331; Kruman et al. (1991)
30 J. Cell. Physiol. 148:267-273; Ameisen and Capron (1991) Immunology Today 12:102; and Sheppard and Ascher (1992) J. AIDS 5:143. Agents that modulate the biological control of apoptosis thus have therapeutic utility in a wide variety of conditions.

-2-

Apoptotic cell death is characterized by cellular shrinkage, chromatin condensation, cytoplasmic blebbing, increased membrane permeability and internucleosomal DNA cleavage. Kerr et al. (1992) FASEB J. 6:2450; and Cohen
5 and Duke (1992) Ann. Rev. Immunol. 10:267. The blebs, small, membrane-encapsulated spheres that pinch off of the surface of apoptotic cells, may continue to produce superoxide radicals which damage surrounding cell tissue and may be involved in inflammatory processes.

10 *Bcl-2* was discovered at the common chromosomal translocation site t(14:18) in follicular lymphomas which results in aberrant over-expression of *bcl-2*. Tsujimoto et al. (1984) Science 226:1097-1099; and Cleary et al. (1986) Cell 47:19-28. The normal function of *bcl-2* is
15 the prevention of apoptosis; unregulated expression of *bcl-2* in B cells is thought to lead to increased numbers of proliferating B cells which may be a critical factor in the development of lymphoma. McDonnell and Korsmeyer (1991) Nature 349:254-256; and, for review see, Edgington
20 (1993) Bio/Tech. 11:787-792. *Bcl-2* is also capable of blocking γ irradiation-induced cell death. Sentman et al. (1991) Cell 67:879-888; and Strasser (1991) Cell 67:889-899. It is now known that *bcl-2* inhibits most types of apoptotic cell death and is thought to function
25 by regulating an antioxidant pathway at sites of free radical generation and Ca^{++} flux through the endoplasmic reticulum. Lan et al. (1984) Proc. Natl. Acad. Sci. 91:6569-6573; Hockenbery et al. (1993) Cell 75:241-251.

While apoptosis is a normal cellular event, it can
30 also be induced by pathological conditions and a variety of injuries. Apoptosis is involved in a wide variety of conditions including, but not limited to, cardiovascular disease; cancer regression; immunoregulation; viral diseases; anemia; neurological disorders;
35 gastrointestinal disorders, including but not limited to, diarrhea and dysentery; diabetes; hair loss; rejection of

organ transplants; prostate hypertrophy; obesity; ocular disorders; stress; and aging.

Bcl-2 belongs to a family of proteins some of which have been cloned and sequenced. Williams and Smith

5 (1993) Cell 74:777-779.

Summary of the Invention

Substantially purified DNA encoding novel *Bcl-2*
10 homologs, termed *Cdn-1*, *Cdn-2* and *Cdn-3* and derivatives thereof, as well as recombinant cells and transgenic animals expressing the *Cdn-1* and *Cdn-2* nucleotides are provided. The substantially purified *Cdn-1* and *Cdn-2* proteins and compositions thereof are also provided.
15 Diagnostic and therapeutic methods utilizing the nucleotides and proteins are also provided. Methods of screening for pharmaceutical agents that stimulate, as well as pharmaceutical agents that modulate *Cdn-1* and *Cdn-2* expression and protein activity and interactions
20 are also provided. Methods of screening for proteins that interact with *Cdns* are also provided.

Brief description of the Drawings

Figure 1 depicts the *Bcl-2* family PCR primers used to isolate the *Cdn-1* probes.

25 Figure 2 depicts the *Cdn-1* clones obtained by the methods described in Example 1.

Figure 3 depicts the nucleotide sequence of *Cdn-1* cDNA and encoded amino acid sequence of the *Cdn-1* protein.

30 Figure 4 depicts the results of a Northern blot analysis of multiple tissues with probes specific for both *Bcl-2* and *Cdn-1*.

Figure 5 shows the sequence of the *Cdn-2* gene and flanking sequences and the corresponding predicted amino
35 acid sequence of the *Cdn-2* protein.

Figure 6 shows a comparison of the N-terminal amino acid sequences of Cdn-1, Cdn-2 and known Bcl-2 family members.

Figure 7 shows the nucleotide sequence of the Cdn-3 gene and predicted amino acid sequence of the Cdn-3 protein.

Figure 8 shows the anti-apoptotic effects of Cdn-1 and some of its derivatives in serum-deprivation induced apoptosis of WI-L2 transformants in 0.1% FBS.

Figure 9 (response of WIL-2 transformants to anti-Fas-Induced Apoptosis (50 ng/mL anti-FAS)) shows anti-apoptotic effects of Cdn-1 and some of its derivatives in FAS-induced apoptosis of WI-L2 cells.

Figure 10 shows modulation of apoptosis by Cdn-1 and Cdn-2 in FL5.12 cells.

Figure 11 depicts the Cdn-1 derivative proteins Δ1, Δ2 and Δ3. The N-terminal residues are indicated by the arrows. The remainder of the derivative proteins is the same as full-length Cdn-1.

20 Detailed Description of the Invention

The present invention encompasses substantially purified nucleotide sequences encoding the novel Bcl-2 homologs, Cdn-1 and Cdn-2; and the proteins encoded thereby; compositions comprising Cdn-1 and Cdn-2 nucleotides, and proteins and methods of use of thereof.

The invention further includes recombinant cells and transgenic animals expressing the cloned Cdn-1 or Cdn-2 genes. The nucleotide and predicted amino acid residue sequences encoded by Cdn-1 are shown in Figure 3; and those of Cdn-2 are shown in Figure 5. It has now been found that the proteins encoded by the Cdn genes are capable of

modulating apoptosis. In an Epstein-Barr Virus (EBV) transformant lymphoblastoid cell line, *Cdn-1* was shown to decrease Fas-mediated apoptosis. In a mouse progenitor B cell line, FL5.12, expression of *Cdn-2* and a derivative of *Cdn-1* decrease IL-3-induced apoptosis whereas expression of *Cdn-1* slightly increased apoptosis. Thus, depending on the cell type, the derivative or type of *Cdn* expressed and the method of induction of apoptosis, apoptosis can be modulated in a highly specific manner by controlling the expression of *Cdns* and concentration of *Cdns*.

As used herein, "*Cdns*" or "*Cdn*" refers to the nucleic acid molecules (nucleotides) described herein (*Cdn-1*, *Cdn-2*, *Cdn-3* and derivatives thereof), "*the Cdns*" or "*Cdn*" refers to the proteins encoded thereby (*Cdn-1*, *Cdn-2*, *Cdn-3* and derivatives thereof). The present invention encompasses *Cdn-1* and *Cdn-2* nucleotide sequences. The nucleotides include, but are not limited to, the *Cdn-1* cDNA, genome-derived DNA and synthetic or semi-synthetic nucleotides such as DNA, and RNA both coding and complementary to the coding region. The nucleotides may be complementary to the mRNA for at least a fragment of the *Cdns* and other nucleotides which can bind to either the DNA or mRNA encoding the *Cdns*. These complementary nucleotides include, but are not limited to, nucleotides capable of forming triple helices and antisense nucleotides. The complementary nucleotides may be expressed endogenously by one of the vectors described herein or may be added exogenously by methods known in the art of oligonucleotide therapy. Reed et al. (1990) Cancer Res. 50:6565-6570. The nucleotide sequence of the *Cdn-1* cDNA with the location of restriction endonuclease sites is shown in Figure 4. As described in the examples herein, *Cdn-1* mRNA has been detected in a variety of human organs and tissues by Northern blot analysis. These organs include liver; heart; skeletal muscle; lung; kidney; and pancreas as shown in Figure 3.

Similarly, *Cdn-2* cDNA, genomic DNA and synthetic or semi-synthetic nucleotides are additional embodiments of the present invention. The nucleotide sequence of the *Cdn-2* gene, along with the predicted amino acid sequence
5 of *Cdn-2* protein and the locations of restriction endonuclease recognition sites, is given in Figure 5.

The examples presented herein indicate that *Cdn-1* is on human chromosome 6 and that *Cdn-2* is on human chromosome 20. There is also a member of the family
10 *Cdn-3* which is on human chromosome 11. Fluorescence in situ hybridization (FISH) indicated an approximate location of *Cdn-1* to be at 6p21-23. It is possible that *Cdn-2* and *Cdn-3* are pseudogenes. While these may not be expressed endogenously, they are capable of being
15 expressed from a recombinant vector providing the appropriate promoter sequences. Thus, both *Cdn-2* and *Cdn-3* nucleotide sequences are encompassed by the present invention as are recombinant constructs thereof and proteins encoded thereby.

20 Derivatives of the genes and proteins include any portion of the protein, or nucleotide sequence encoding the protein, which retains apoptosis modulating activity. Figure 11 depicts three such derivatives of *Cdn-1* which have been shown to retain apoptosis-modulating activity.
25 The derivatives, *Cdn1-Δ1*, *Cdn1-Δ2* and *Cdn1-Δ3*, and the proteins encoded thereby are encompassed by the present invention.

The invention includes modifications to *Cdn* DNA sequences such as deletions, substitutions and additions
30 particularly in the non-coding regions of genomic DNA. Such changes are useful to facilitate cloning and modify gene expression.

The invention further encompasses various substituted nucleotides. Substitutions can be made
35 within the coding region that either do not alter the amino acid residues encoded or result in conservatively substituted amino acid residues. Nucleotide

substitutions that do not alter the amino acid residues encoded are useful for optimizing gene expression in different systems. Suitable substitutions are known to those of skill in the art and are made, for instance, to reflect preferred codon usage in the particular expression systems.

The invention encompasses functionally equivalent variants and derivatives of Cdns which may enhance, decrease or not significantly affect the properties of Cdns. For instance, changes in the DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect its properties.

Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution which does not significantly affect the properties of Cdns is encompassed by the present invention.

The invention further encompasses mutants of Cdns which, when expressed, interfere with the activity of endogenously expressed Cdns. Such mutants can be made by any method known in the art and screened for activity by their ability to affect activity of native Cdns.

Techniques for nucleic acid manipulation useful for the practice of the present invention are known in the art and described in a variety of references, including, but not limited to, Molecular Cloning: A Laboratory Manual, 2nd ed., vols. 1-3, eds. Sambrook et al. Cold Spring Harbor Laboratory Press (1989); and Current Protocols in Molecular Biology, eds. Ausubel et al.,

Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates.

The invention further embodies a variety of DNA vectors having cloned therein the *Cdn* nucleotide
5 sequences. Suitable vectors include any known in the art including, but not limited to, those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors are known in the art and need not be described in detail herein.

10 The vectors may also provide inducible promoters for expression of the *Cdn* nucleotide sequences. Inducible promoters are those which do not allow substantial constitutive expression of the gene but rather, permit expression only under certain circumstances. Such
15 promoters may be induced by a variety of stimuli including, but not limited to, exposure of a cell containing the vector to a ligand, metal ion, other chemical or change in temperature. The promoters may also be cell-specific, that is, inducible only in a
20 particular cell type and often only during a specific period of time. The promoter may further be cell cycle specific, that is, induced or inducible only during a particular stage in the cell cycle. The promoter may be both cell type specific and cell cycle specific. Any
25 inducible or noninducible promoter known in the art is suitable for use in the present invention. Preferably, the promoter used is inducible.

The invention further includes a variety of expression systems transfected with the vectors.
30 Suitable expression systems include, but are not limited to, bacterial, mammalian, yeast and insect. Specific expression systems and the use thereof are known in the art and are not described in detail herein.

The invention encompasses *ex vivo* transfection with
35 *Cdn* nucleotide sequences, in which cells removed from animals including man are transfected with vectors containing *Cdn* nucleotides and reintroduced into animals.

Suitable transfected cells include individual cells or cells contained within whole tissues. In addition, *ex vivo* transfection can include the transfection of cells derived from an animal other than the animal or human subject into which the cells are ultimately introduced. Such grafts include, but are not limited to, allografts, xenografts, and fetal tissue transplantation. In addition, *in vivo* transfection such as by pulmonary administration of suitable vectors can be used.

Essentially any cell or tissue type can be treated in this manner. Suitable cells include, but are not limited to, cardiomyocytes and lymphocytes. For instance, lymphocytes, removed, transfected with the recombinant DNA and reintroduced into an HIV-positive patient may increase the half-life of the reintroduced T cells.

As an example, in treatment of HIV-infected patients by the above-described method, the white blood cells are removed from the patient and sorted to yield the CD4⁺ cells. The CD4⁺ cells are then transfected with a vector containing a *Cdn* nucleotide and reintroduced into the patient. Alternatively, the unsorted lymphocytes can be transfected with a recombinant vector having at least one *Cdn* nucleotide under the control of a cell-specific promoter such that only CD4⁺ cells express the nucleotides. In this case, an ideal promoter would be the CD4 promoter; however, any suitable CD4⁺ T cell-specific promoter can be used.

Further, the invention encompasses cells transfected *in vivo* by the vectors. Suitable methods of *in vivo* transfection are known in the art and include, but are not limited to, that described by Zhu et al. (1993) Science 261:209-211. *in vivo* transfection may be particularly useful as a prophylactic treatment for patients suffering from atherosclerosis. Modulation of the levels of *Cdn* could serve as prophylaxis for the apoptosis-associated reperfusion damage that results from

-10-

cerebral and myocardial infarctions. In these patients with a high risk of stroke and heart attack, the apoptosis and reperfusion damage associated with arterial obstruction could be prevented or at least mitigated.

5 Infarctions are caused by a sudden insufficiency of arterial or venous blood supply due to emboli, thrombi, or pressure that produces a macroscopic area of necrosis; the heart, brain, spleen, kidney, intestine, lung and testes are likely to be affected. Apoptosis occurs to
10 tissues surrounding the infarct upon reperfusion of blood to the area; thus, modulation of Cdn levels, achieved by a biological modifier-induced change in endogenous production, by *in vivo* transfection or by anti-sense therapy, could be effective at reducing the severity of
15 damage caused by heart attacks and stroke.

 Transgenic animals containing the recombinant DNA vectors containing Cdn nucleotide sequences are also encompassed by the invention. Methods of making transgenic animals are known in the art and need not be
20 described in detail herein. For a review of methods used to make transgenic animals, see, e.g., PCT publication no. WO 93/04169. Preferably, such animals express recombinant Cdns under control of a cell-specific and, even more preferably, a cell cycle-specific promoter.

25 In another embodiment, diagnostic methods are provided to detect the expression of Cdns either at the protein level or the mRNA level. Any antibody that specifically recognizes Cdns is suitable for use in Cdn diagnostics. Abnormal levels of Cdns are likely to be
30 found in the tissues of patients with diseases associated with inappropriate apoptosis; diagnostic methods are therefore useful for detecting and monitoring biological conditions associated with such apoptosis defects. Detection methods are also useful for monitoring the
35 success of Cdn-related therapies.

 Purification or isolation of Cdns expressed either by the recombinant DNA or from biological sources such as

tissues can be accomplished by any method known in the art. Protein purification methods are known in the art. Generally, substantially purified proteins are those which are free of other, contaminating cellular substances, particularly proteins. Preferably, the purified Cdns are more than eighty percent pure and most preferably more than ninety-five percent pure. For clinical use as described below, the Cdns are preferably highly purified, at least about ninety-nine percent pure, and free of pyrogens and other contaminants.

Suitable methods of protein purification are known in the art and include, but are not limited to, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, HPLC and FPLC. Any purification scheme that does not result in substantial degradation of the protein is suitable for use in the present invention.

The invention also includes the substantially purified Cdns having the amino acid residue sequences depicted in Figures 3 and 5, respectively. The invention encompasses functionally equivalent variants of Cdns which do not significantly affect their properties and variants which retain the same overall amino acid sequence but which have enhanced or decreased activity. For instance, conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are within the scope of the invention. Any conservative amino acid substitution which does not significantly affect the properties of Cdns is encompassed by the present invention.

Suitable antibodies are generated by using the Cdns as an antigen or, preferably, peptides encompassing the Cdn regions that lack substantial homology to the other gene products of the *Bcl-2* family. Methods of detecting proteins using antibodies and of generating antibodies

-12-

using proteins or synthetic peptides are known in the art and are not described in detail herein.

Cdn protein expression can also be monitored by measuring the level of Cdn mRNA. Any method for
5 detecting specific mRNA species is suitable for use in this method. This is easily accomplished using the polymerase chain reaction (PCR). Preferably, the primers chosen for PCR correspond to the regions of the Cdn genes which lack substantial homology to other members of the
10 Bcl gene family. Alternatively, Northern blots can be utilized to detect Cdn mRNA by using probes specific to Cdns. Methods of utilizing PCR and Northern blots are known in the art and are not described in detail herein.

Methods of treatment with Cdns also include
15 modulating cellular expression of Cdns by increasing or decreasing levels of mRNA or protein. Suitable methods of modulating cellular expression of Cdn include, but are not limited to, increasing endogenous expression with biological modifiers; transfecting the cells with vectors
20 encoding Cdn nucleotides so that either a Cdn gene is overexpressed or an anti-sense nucleotide is expressed; and expressing mutant Cdns which interfere with the interaction of endogenous Cdn with other proteins such as other members of the Bcl-2 family. Cellular transfection
25 is discussed above and is known in the art. Suitable indications for modulating endogenous levels of Cdn include, but are not limited to, malignancies and cardiac-specific expression. Cardiac specific expression is particularly suitable for use in indications
30 including, but not limited to, patients susceptible to heart disease and in advance of cardiotoxic therapies including, but not limited to, chemotherapies such as adriamycin, so as to offer cardioprotection.

Modulating endogenous expression of Cdns can be
35 accomplished by exposing the cells to biological modifiers that directly or indirectly change levels of Cdns either by modulating expression of Cdns or by

modulating degradation of *Cdn* mRNA. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of interleukins, lectins and other stimulating agents e.g., PMA, LPS, bispecific antibodies and other agents which modify cellular functions or protein expression. Preferably, a suitable biological modifier is γ IFN which increases *Cdn* expression levels in HT-29 cells. Further, biological modifiers include *Cdn* nucleotides which modify expression of endogenous *Cdn* and mutant *Cdns* which interfere with the activity of endogenous *Cdns*. Cells are exposed to such biological modifiers at physiologically effective concentrations, and the expression of *Cdns* is measured relative to a control not exposed to the biological modifiers. Those biological modifiers which change expression of *Cdns* relative to the control are selected for further study.

The methods of decreasing endogenous levels of *Cdns* include, but are not limited to, antisense nucleotide therapy and methods to deliver the sense or antisense construct and down-regulation of expression by biological modifiers. Antisense therapy is known in the art and its application will be apparent to one of skill in the art.

Screening for therapeutically effective biological modifiers is done either by exposing the cells to biological modifiers which may directly or indirectly modulate levels of *Cdns* either by changing expression or by altering the half-life of *Cdn* mRNA or *Cdns*. The biological modifiers may also interfere with *Cdn*-1 interactions with both other *Bcl-2* family members, and other gene products, e.g., proteases. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of interleukins, lectins and other stimulating agents, e.g., PMA, LPS,

bispecific antibodies, *Cdn* nucleotides, *Cdn* mutants and other agents which modify cellular functions or protein expression. Cells are grown under conditions known to elicit expression of at least one *Cdn* (preferably *Cdn-1*),
5 exposed to such biological modifiers at physiologically effective concentrations, and the expression of *Cdns* is measured relative to a control not exposed to biological modifiers. Those biological modifiers which modulate the expression of *Cdns* relative to a control are selected for
10 further study. Cell viability is also monitored to ensure that altered *Cdn* expression is not due to cell death.

In determining the ability of biological modifiers to modulate (increase or decrease) *Cdn* expression, the
15 levels of endogenous expression may be measured or the levels of recombinant fusion proteins under control of *Cdn*-specific promoter sequences may be measured. The fusion proteins are encoded by reporter genes.

Reporter genes are known in the art and include, but
20 are not limited to chloramphenicol acetyl transferase (CAT) and β -galactosidase. Expression of *Cdn-1* and *Cdn-2* can be monitored as described above either by protein or mRNA levels. Expression of the reporter genes can be monitored by enzymatic assays, or antibody-based assays,
25 like ELISAs and RIAs, also known in the art. Potential pharmaceutical agents can be any therapeutic agent or chemical known to the art, or any uncharacterized compounds derived from natural sources such as fungal broths and plant extracts. Preferably, suitable
30 pharmaceutical agents are those lacking substantial cytotoxicity and carcinogenicity.

Suitable indications for modulating endogenous levels of *Cdns* are any in which *Cdn*-mediated apoptosis is involved. These include, but are not limited to, various
35 types of malignancies and other disorders resulting in uncontrolled cell growth such as eczema, or deficiencies

in normal programmed cell death such as malignancies, including, but not limited to, B cell lymphomas.

The invention also encompasses therapeutic methods and compositions involving treatment of patients with biological modifiers to modulate expression of Cdns. Effective concentrations and dosage regimens may be empirically derived. Such derivations are within the skill of those in the art and depend on, for instance, age, weight and gender of the patient and type and severity of the disease. Alternatively, patients may be directly treated with either native or recombinant Cdns. The Cdns should be substantially pure and free of pyrogens. It is preferred that the recombinant Cdns be produced in a mammalian cell line so as to ensure proper glycosylation. Cdns may also be produced in an insect cell line.

For therapeutic compositions, a therapeutically effective amount of substantially pure Cdn or biological modifier or oligonucleotide that modulates the expression or activity thereof is suspended in a physiologically accepted buffer including, but not limited to, saline and phosphate buffered saline (PBS) and administered to the patient. Preferably administration is intravenous. Other methods of administration include but are not limited to, subcutaneous, intraperitoneal, gastrointestinal and directly to a specific organ, such as intracardiac, for instance, to treat cell death related to myocardial infarction.

Suitable buffers and methods of administration are known in the art. The effective concentration of a Cdn or biological modifier therefor will need to be determined empirically and will depend on the type and severity of the disease, disease progression and health of the patient. Such determinations are within the skill of one in the art.

Bcl-2 is thought to function in an antioxidant pathway. Veis et al. (1993) Cell 75:229-240. Therefore,

therapy involving Cdns is suitable for use in conditions in which superoxide is involved. Administration of modulators of Cdn expression results in an increased extracellular concentration of Cdns, which is thought to provide a method of directly inhibiting superoxide accumulation that may be produced by the blebs associated with apoptosis. The therapeutic method thus includes, but is not limited to, inhibiting superoxide mediated cell injury.

10 Suitable indications for therapeutic use of Cdns or biological modifier therefor are those involving free radical mediated cell death and include, but are not limited to, conditions previously thought to be treatable by superoxide dismutase. Such indications include, but are not limited to, HIV infection, autoimmune diseases, 15 cardiomyopathies, neuronal disorders, hepatitis and other liver diseases, osteoporosis, and shock syndromes, including, but not limited to, septicemia.

Hybridization of cloned Cdn DNA to messenger mRNA from various regions of the brain indicated high levels of expression of Cdn-1 in each of the regions studied (Figure 8). Therefore, neurological disorders are another area in which therapeutic applications of Cdns are indicated.

25 The invention further encompasses methods of assaying for interactions between Cdns and proteins which bind specifically to Cdns. The assays entail contacting purified Cdns with cell lysates containing a protein which may bind to Cdns under conditions sufficient for the protein to bind and assaying for the presence of the protein.

Typically the assay step involves contacting the protein with a specific binding partner such as an antibody which may be directly or indirectly labeled. 35 Suitable assays include an ELISA that provides antibodies directed against the protein, in vitro translated Cdn and cell lysates containing the protein. Yeast genetic

-17-

systems such as the MatchmakerTM (Clontech) are also suitable for use in the assay.

The following examples are provided to illustrate but not limit the present invention. Unless otherwise specified, all cloning techniques were essentially as described by Sambrook et al. (1989) and all reagents were used according to the manufacturer's instructions.

Example 1

Identification and Cloning of Cdn-1 cDNA

10 An amino acid sequence comparison of the six known Bcl-2 family members (Figure 6) revealed two regions with considerable sequence identity, namely amino acids 144-150 and 191-199. In an attempt to identify new Bcl-2 family members, degenerate PCR primers based on sequences
15 in these regions were designed (Figure 1) and PCR was performed using human heart cDNA and human B lymphoblastoid cell line (WI-L2) cDNA. PCR was performed using the Hot Start/AmpliwaxTM technique (Perkin Elmer Cetus). The final concentration of the PCR primers and
20 the template cDNA were 4 μ M and 0.1-0.2 ng/ml, respectively. The conditions for cDNA synthesis were identical to those for first strand cDNA synthesis of the cDNA library as described below. PCR was performed in a Perkin Elmer Cetus DNA Thermal CyclerTM according to the
25 method described by Kiefer et al. (1991) Biochem. Biophys. Res. Commun. 176:219-225, except that the annealing and extension temperatures during the first 10 cycles were 36°C. Following PCR, samples were treated with 5 units of DNA polymerase I, Klenow fragment for 30
30 min at 37°C and then fractionated by electrophoresis on a 7% polyacrylamide, 1 X TBE (Tris/borate/EDTA) gel. DNA migrating between 170-210 base pairs was excised from the gel, passively eluted for 16 hours with gentle shaking in 10 mM Tris-HCl pH 7.5, 1 mM EDTA (TE), purified by
35 passage over an Elutip-DTM column (Schleicher and Schuell), ligated to the pCR-Script vector (Stratagene) and

-18-

transformed into *Escherichia coli* strain XL1-Blue MRF (Stratagene). Plasmid DNA from transformants (white colonies) containing both the heart and WI-L2 PCR products was isolated using the Magic Miniprep DNA Purification SystemTM (Promega), and the DNA inserts were sequenced by the dideoxy chain termination method according to Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467 (USB, SequenaseTM version 2.0). DNA sequence analysis of the eleven heart PCR products revealed two sequences identical to *Bcl-x* (Boise et al. (1993) Cell 74:597-608) and ten other sequences unrelated to the *Bcl-2* family.

DNA sequence analyses of the eleven WI-L2 PCR products yielded one *Bcl-x* sequence, five sequences identical to another *Bcl-2* family member, *bax* (Oldvai et al. (1993) Cell 74:609-619), four unrelated sequences and one novel *Bcl-2* related sequence, termed *Cdn-1*. The unique *Cdn-1* amino acid sequence encoded by the PCR product is shown in Figure 6 from amino acid 151-190 (top row).

To isolate the *Cdn-1* cDNA, a human heart cDNA library (Clontech) and a WI-L2 cDNA library, constructed as described by Zapf et al. (1990) J. Biol. Chem. 265:14892-14898 were screened using the *Cdn-1* PCR DNA insert as a probe. The DNA was ³²P-labeled according to the method described by Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267 and used to screen 150,000 recombinant clones from both libraries according to the method described by Kiefer et al. (1991). Eight positive clones were obtained from the WI-L2 cDNA library. Four clones from the WI-L2 cDNA library and two from the heart cDNA library were further purified and plasmid DNA containing the cDNA inserts was excised from the λ ZAPII vector (Stratagene) (Figure 2). The two longest clones, W7 (2.1 kb) and W5 (2.0 kb) were sequenced and shown to contain the *Cdn-1* probe sequence, thus confirming their authenticity. Two clones from the heart cDNA library

were purified. The cDNA was subcloned into pBlsc and sequenced. The heart cDNAs also encoded *Cdn-1*.

The W7 DNA sequence along with the deduced amino acid residue sequence is shown in Figure 3. The deduced amino acid sequence of *Cdn-1* was also aligned for maximum sequence identity with the other *Bcl-2* family members and is shown in Figure 6. As can be seen, there is considerable sequence identity between *Cdn-1* and other family members between amino acids 100 and 200. Beyond this central region, sequence conservation falls off sharply. Like *Bcl-2*, *Cdn-1* appears to be an intracellular protein in that it does not contain either a hydrophobic signal peptide or N-linked glycosylation sites. *Cdn-1* does contain a hydrophobic C-terminus that is also observed with all *Bcl-2* family members except LMW5-HL, suggesting its site of anti-apoptotic activity, like that of *Bcl-2*, is localized to a membrane bound organelle such as the mitochondrial membrane, the endoplasmic reticulum or the nuclear membrane.

Hockenbery et al. (1990); Chen-Levy et al. (1989) Mol. Cell. Biol. 9:701-710; Jacobsen et al. (1993) Nature 361:365-369; and Monighan et al. (1992) J. Histochem. Cytochem. 40:1819-1825.

Example 2

Northern Blot Analysis of cDNA Clones

Northern blot analysis was performed according to the method described by Lehrach et al. (1977) Biochem. 16:4743-4651 and Thomas (1980) Proc. Natl. Acad. Sci. USA 77:5201-5205. In addition, a human multiple tissue Northern blot was purchased from Clontech. The coding regions of *Bcl-2* and *Cdn-1* cDNAs were labeled by the random priming method described by Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267. Hybridization and washing conditions were performed according to the methods described by Kiefer et al. (1991). Specifically, Klenow-labeled fragments of *Bcl-2*

and *Cdn-1* clones were hybridized to a multiple human tissue Northern blot (Clontech 7760-1), at a final concentration of 1×10^6 cpm/milliliter for each probe. The blot was washed at high stringency.

5 The results, presented in Figure 4 indicate that *Cdn-1* is expressed in all organs tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas) whereas *Bcl-2* is not expressed or expressed at only low levels in heart, brain, lung, and liver. Thus,
10 *Cdn-1* appears to be more widely expressed throughout human organs than *Bcl-2* and may be more important in regulating apoptosis in these tissues.

Example 3

Expression of Recombinant *Cdn-1*

15 In order to express recombinant *Cdn-1* in the baculovirus system, the *Cdn-1* cDNA generated in Example 1 was used to generate a novel *Cdn-1* vector, by a PCR methodology as described in Example 1, using primers from the 3' and 5' flanking regions of the gene which contain
20 restriction sites to facilitate cloning. The plasmids were sequenced by the dideoxy terminator method (Sanger et al., 1977) using sequencing kits (USB, Sequenase version 2.0) and internal primers. This was to confirm that no mutations resulted from PCR.

25 A clone was used to generate recombinant viruses by *in vivo* homologous recombination between the overlapping sequences of the plasmid and AcNPV wild type baculovirus. After 48 hours post-transfection in insect *Spodoptera frugiperda* clone 9 (SF9) cells, the recombinant viruses
30 were collected, identified by PCR and further purified. Standard procedures for selection, screening and propagation of recombinant baculovirus were performed (Invitrogen). The molecular mass, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of
35 the protein produced in the baculovirus system was

compared with the predicted molecular mass of Cdn-1 according to the amino-acid sequence.

In addition, similar clones can be expressed preferably in a yeast intracellular expression system by any method known in the art, including the method described by Barr et al. (1992) Transgenesis ed. JAH Murray, (Wiley and Sons) pp. 55-79.

Example 4

Expression of Cdn-1 in Mammalian Systems

10 The Cdn-1 coding sequence was excised from the plasmid generated in Example 1, and introduced into plasmids pCEP7, pREP7 and pCDNA3 (Invitrogen) at compatible restriction enzyme sites. pCEP7 was generated by removing the RSV 3'-LTR of pREP7 with XbaI/Asp718, and
15 substituting the CMV promoter from pCEP4 (Invitrogen). 25 µg of each Cdn-1-containing plasmid was electroporated into the B lymphoblastoid cell line WI-L2, and stable hygromycin resistant transformants or G418 resistant transformants (pCDNA3 constructs, Fig. 8) expressing
20 Cdn-1 were selected.

The coding region of Cdns can also be ligated into expression vectors capable of stably integrating into other cell types including, but not limited to, cardiomyocytes, neural cell lines such as GTI-7 and TNF
25 sensitive cells such as the human colon adenocarcinoma cell line HT29 so as to provide a variety of assay systems to monitor the regulation of apoptosis by Cdn-1.

Example 5

30 Effect of the Anti-Apoptotic Activity of Cdn-1 and its Derivatives in the Wild Type B Lymphoblastoid Cell Line WI-L2-729 HF2
and the Transformed Cell Expressing Excess Cdn-1

2x10⁵ WI-L2, and WI-L2 cells transformed with a vector encoding Cdn-1 as described in Example 4 were
35 grown in RPMI supplemented with 10% fetal bovine serum

-22-

(FBS) for the anti-fas experiment or 0.1% FBS for serum deprivation experiments. In the case of the anti-fas experiment, after washing with fresh medium, the cells were suspended in RPMI supplemented with 10% FBS, exposed to anti-fas antibodies and the kinetics of cell death in response to an apoptosis inducing agent were analyzed by flow cytometry with FACScanTM. In the case of the serum deprivation experiment, the WI-L2 cells were resuspended in RPMI supplemented with 0.1% FBS and apoptosis was monitored according to the method described by Henderson et al. (1993) Proc. Natl. Acad. Sci. USA 90:8479-8483. Other methods of inducing apoptosis include, but are not limited to, oxygen deprivation in primary cardiac myocytes, NGF withdrawal, glutathione depletion in the neural cell line GTI-7 or TNF addition to the HT29 cell line. Apoptosis was assessed by measuring cell shrinkage and permeability to propidium iodide (PI) during their death. In addition, any other method of assessing apoptotic cell death may be used.

Figure 9 shows the anti-apoptotic response of various WI-L2 transformants to anti-Fas treatment. Figure 8 shows the anti-apoptotic response of various WI-L2 transformants to serum deprivation. In Figure 9, duplicate wells containing 3×10^5 cells were incubated with 50 ng/ml of the cytotoxic anti-Fas antibody for 24 hours. Cell death was then analyzed by flow cytometry with FACScan. The proteins expressed from each construct are shown beneath the columns. Since many of the constructs are truncation or deletion variants, the exact amino acids expressed are also indicated. As can be seen, all of the transformants had some protective effect when compared to the control transformant containing the pREP7 vector alone. The most apoptosis-resistant transformant was the Cdn-1A2 expressing cell line, in which over 90% of the cells survived anti-fas treatment. Significant protection was also observed in transformants expressing

full length *Cdn-1* (1-211) and *Cdn-1Δ1*, followed by *Bcl-2Δ* and *Bcl-2* expressing cell lines.

Cdn-1Δ1 and *Cdn-1Δ2* are lacking the nucleotides encoding the N-terminal 59 and 70 amino acids, respectively, of full length *Cdn-1*. The observation that expression of *Cdn-1Δ2* is more effective at blocking apoptosis than full length *Cdn-1* suggests that smaller, truncated *Cdn-1* molecules may be potent therapeutics.

Example 6

10 Determination of other *Cdn* genes and Cloning of the *Cdn-2* Gene

Southern blot analyses of human genome DNA and a panel of human/rodent somatic cell DNAs indicated that at least 3 *Cdn* related genes and that they resided in
15 chromosomes 6, 11 and 20. PCR/sequence analysis of the three hybrid DNAs showed that *Cdn-1* was on chromosome 6 and that two closely related sequences were on chromosome 20 (designated *Cdn-2*) and chromosome 11 (designated *Cdn-3*). We have cloned the *Cdn-2* and *Cdn-3* genes and
20 sequenced them. Interestingly, both *Cdn-2* and *Cdn-3* do not contain introns and have all of the features of processed genes that have returned to the genome. *Cdn-3* has a nucleotide deletion, causing a frame shift and early termination and thus is probably a pseudogene.
25 Both, however, have promoter elements [CCAAT, TATAAA boxes] but are probably not transcribed as determined by Northern blot analyses with *Cdn-2* and *Cdn-3* specified probes.

900,000 clones from a human placenta genomic library
30 in the cosmid vector pWE15 (Stratagene, La Jolla, CA) were screened with a 950 bp *BglIII-HindIII* cDNA probe containing the entire coding region of *Cdn-1*. The probe was ³²P-labeled according to the method of Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267. The
35 library was processed and screened under high stringency hybridization and washing conditions as described by

-24-

Sambrook et al. (1989) Molecular Cloning, 2nd edition, Cold Spring Harbor Laboratory Press. Ten double positive clones were further purified by replating and screening as above. Plasmid DNA was purified using the Wizard
5 Maxiprep DNA Purification SystemTM as described by the supplier (Promega Corp., Madison, WI) and analyzed by *EcoRI* restriction enzyme mapping and Southern blotting. The probe used for Southern blotting and hybridization conditions was the same as above.

10 The cosmid clones fell into two groups as judged by *EcoRI* restriction analysis and Southern blotting. Cosmid clones (cos) 1-4 and 7 displayed one distinct pattern of *EcoRI* generated DNA fragments and contained a single 6.5 kb hybridizing *EcoRI* DNA fragment. Cos2 and Cos9 fell
15 into the second group that was characterized by a 5.5 kb hybridizing *EcoRI* DNA fragment. The 6.5 kb DNA fragment from cos2 and the 5.5 kb DNA fragment from cos9 were subcloned into pBluescriptTM SK⁺ (Stratagene, La Jolla, CA) using standard molecular biological techniques (Sambrook
20 et al. as above). Plasmid DNA was isolated and the DNA inserts from two subclones, A4 (from cos2) and C5 (from cos9) were mapped with *Bam*HI, *Hind*III and *EcoRI* and analyzed by Southern blotting as described above. Smaller restriction fragments from both clones were
25 subcloned into M13 sequencing vectors and the DNA sequence was determined.

The sequence of A4 contains an open reading frame that displays 97% amino acid sequence identity with *Cdn-1*. (Figure 5) The high degree of sequence identity
30 of this gene with *Cdn-1* indicates that it is a new *Cdn-1* related gene and therefore will be called *Cdn-2*. A sequence comparison of the encoded *Cdn-2* protein and the other members of the *Bcl-2* family is shown in Figure 6. *Cdn-2* contains the conserved regions, BH1 and BH2, that
35 are hallmarks of the *Bcl-2* family, and displays a lower overall sequence identity (~20-30%) to other members, which is also characteristic of the *Bcl-2* family. *Cdn-3*

-25-

has a frame shift resulting in a shorter, unrelated polypeptide and therefore does not contain the structural features of *Cdn-1*, *Cdn-2* or other *Bcl-2* family members.

Example 7

5 Chromosomal Localization of the *Cdn-1*, *Cdn-2* and *Cdn-3* Genes

Southern blot analysis of a panel of human/rodent somatic cell hybrid DNAs (Panel #2 DNA from the NIGMS, Camden, NJ) and fluorescent in situ hybridization (FISH) of metaphase chromosomes were used to map the *Cdn* genes to human chromosomes. For Southern blotting, 5 µg of hybrid panel DNA was digested with *EcoRI* or *BamHI/HindIII*, fractionated on 0.8% or 1% agarose gels, transferred to nitrocellulose and hybridized with the *Cdn-1* probe. Hybridization and washing conditions were as described above. For FISH, the *Cdn-2* subclone, A4, was biotinylated using the BionickTM Labeling System (Gibco BRL, Gaithersburg, MD) and hybridized to metaphase chromosomes from normal human fibroblasts according to the method described by Viegas-Pequignot in *In Situ Hybridization, A Practical Approach*, 1992, ed. D.G. Wilkinson, pp. 137-158, IRL Press, Oxford. Probe detection using FITC-conjugated avidin and biotinylated goat anti-avidin was according to the method described by Pinkel et al. (1988) Proc. Natl. Acad. Sci. USA 85:9138-9142.

Southern blot analysis showed three hybridizing *EcoRI* bands in the human DNA control that were approximately 12 kb, 11 kb and 5.5 kb in length. Analysis of the somatic cell hybrid DNA indicated that the 12 kb band was in two different samples, NA10629, which contained only human chromosome 6, and NA07299, which contained both human chromosomes 1 and X and, importantly, a portion of chromosome 6 telomeric to p21. The 11 kb band was in NA13140, which contains human chromosome 20. The 5.5 kb hybridizing band was found

only in sample NA10927A, which contained human chromosome 11. PCR/DNA sequencing analysis of these hybrid DNA samples using primers for *Cdn-1*, *Cdn-2* or *Cdn-3* showed *Cdn-1* sequences in NA10629 (the chromosome 6-containing hybrid DNA) and NA07299 (the chromosome 1, X and 6pter >p21-containing hybrid DNA), indicating that the *Cdn-1* gene resides on chromosome 6, telomeric to p21. *Cdn-2* sequences were found in NA13140, indicating the *Cdn-2* gene resides on chromosome 20, and *Cdn-3* sequences were found in NA10927A, indicating the *Cdn-3* gene resides on chromosome 11.

Example 8

Modulation of apoptosis by *Cdn-1* and *Cdn-2* in FL5.12 cells

FL5.12 is an IL-3-dependent lymphoid progenitor cell line (McKearn et al. (1985) Proc. Natl. Acad. Sci USA 82:7414-7418) that has been shown to undergo apoptosis following withdrawal of IL-3 but is protected from cell death by overexpression of *Bcl-2*. Nunez et al. (1990) J. Immunol. 144:3602-3610; and Hockenbery et al. (1990) Nature 348:334-336. To assess the ability of *Cdn-1* and *Cdn-2* to modulate apoptosis, cDNAs encoding *Cdn-1*, *Cdn-2*, two truncated forms of *Cdn-1* (described below) and *Bcl-2* were ligated into the mammalian expression vector, pCDNA3 (Invitrogen, San Diego, CA) and stably introduced into the mouse progenitor B lymphocyte cell line FL5.12 by electroporation and selection in media containing the antibiotic G418. Assays were then performed on bulk transformants as described below.

The effects of the overexpressed genes on FL5.12 cell viability were examined at various times following withdrawal of IL-3 and are shown in Figure 10. Cell viability was assessed by propidium iodide (PI) exclusion on a flow cytometer (Becton Dickinson FACScan). *Bcl-2* expression protected the cells significantly from cell death while *Cdn-1* appeared to enhance cell death when

compared to the vector control. *Cdn-2* expression conferred a low level of protection from cell death at earlier times but was insignificant at later time points. Interestingly, *Cdn-1Δ2* gave a moderate level of protection against cell death. *Cdn-1-112*, a molecule that contains the N-terminal 112 amino acids of *Cdn-1*, also appeared to partially protect the FL5.12 cells although at lower levels than *Bcl-2*.

As shown in Example 7, expression of *Cdn-1* and *Cdn-1Δ2* in WI-L2 cells resulted in increased cell survival in response to anti-Fas-mediated apoptosis and serum withdrawal. Taken together, these data suggest that the various *Cdn* molecules are capable of modulating apoptosis in a positive or negative manner, depending on the cell type and apoptotic stimuli. Thus, they are effective in preventing cell death such as in the post-ischemic reperfusion tissue damage in the heart or in inducing cell death in cells that have escaped apoptotic control, as is the case in various cancers.

20

Example 9

IFN- γ induces *Cdn-1* mRNA expression in HT-29 cells

It has been shown that the human colon carcinoma cell line HT-29 is sensitive to the cytotoxic effect of anti-Fas antibody or TNF only after treatment with IFN- γ . Yonehara et al. (1989) J. Exp. Med. 169: 1747-1756. These IFN- γ treated cells also show enhanced apoptosis following serum deprivation or cycloheximide treatment. This induced sensitivity of HT-29 cells to apoptotic stimuli may be partly due to the concomitant upregulation of the TNF receptor and Fas antigen that is seen following IFN- γ treatment. Yonehara et al. (1989). However, the increased cell death seen following serum deprivation or cycloheximide treatment suggests that other apoptotic mechanisms may be induced by IFN- γ . Modulation of the levels of *Bcl-2* family members by IFN- γ is another possible mechanism for the induced

sensitivity of HT-29 cells to apoptotic stimuli. An increase in *Cdn-1*, *Bax* or *Bcl-x_s* expression and/or a decrease in *Bcl-2* or *Bcl-x_L* expression could result in enhanced sensitivity of the cells to cytotoxic agents.

5 To test this possibility, the mRNA levels of *Bcl-2* family members in untreated and IFN- γ treated HT-29 cells were examined by Northern blot analysis using the methods and conditions described in Example 2. *Cdn-1* mRNA levels were increased approximately 10X following IFN- γ

10 induction whereas *Bax* and *Bcl-x* mRNA levels remained unchanged. *Bcl-2* mRNA was below detectable levels in both untreated and IFN- γ treated cells. It remained possible that the ratio of *Bcl-x_s* to *Bcl-x_L* transcripts could have increased upon IFN- γ treatment but would not

15 be detected by Northern blot analysis due to the small difference in size between the transcripts. This was the case with unstimulated versus PMA plus ionomycin stimulated thymocytes as determined by Boise et al. (1993) Cell 74:597-608. Using semiquantitative PCR, they

20 showed that the ratio of *Bcl-x_s* to *Bcl-x_L* increased following stimulation. Using similar PCR techniques it was demonstrated that the ratio of *Bcl-x_s* to *Bcl-x_L* mRNA remained unchanged following IFN- γ treatment of HT-29 cells and that the predominant transcript was *Bcl-x_L*

25 (>90%).

Thus there is a positive correlation between the upregulation of *Cdn-1* transcripts and the HT-29 tumor cells line following IFN- γ treatment and increased susceptibility to cell death. These results indicate

30 that there are positive modulators of *Cdn-1* in tumor cells and that they may be useful in treating some tumors when co-administered with appropriate apoptosis inducing agents.

Although the foregoing invention has been described

35 in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and

modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

- 30 -

CLAIMS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a Cdn selected from the group consisting of Cdn-1 depicted in the amino acid sequence in Figure 3, Cdn-2 depicted in the amino acid sequence in Figure 5, Cdn-1 Δ 1 depicted at positions 60 to 211 of the amino acid sequence in Figure 3 or Figure 11, Cdn-1 Δ 2 depicted at positions 71 to 211 of the amino acid sequence in Figure 3 or Figure 11, and Cdn-1 Δ 3 depicted at positions 96 to 211 of the amino acid sequence of Figure 3 or Figure 11.
2. The molecule according to claim 1 wherein the nucleotide sequence encodes the amino acid sequence depicted in Figure 3 or the amino acid sequence depicted in Figure 5.
3. The molecule according to claim 1 wherein the Cdn is Cdn-1 having the nucleotide sequence depicted in Figure 3.
4. The molecule according to claim 1 wherein the Cdn is Cdn-2 having the nucleotide sequence depicted in Figure 5.
5. The composition according to claim 1 wherein the Cdn is Cdn-1 Δ 1 and the nucleotide sequence has the sequence depicted in nucleotides 365 through 820 of Figure 3.
6. The composition according to claim 1 wherein the Cdn is Cdn-1 Δ 2 and the nucleotide sequence has the sequence depicted in nucleotides 398 through 820 of Figure 3.
7. The composition according to claim 1 wherein the Cdn is Cdn-1 Δ 3 and the nucleotide sequence has the sequence depicted in nucleotides 473 through 820 of Figure 3.
8. A recombinant DNA vector having the nucleotide sequence as defined in any one of claims 1 to 7.
9. The vector according to claim 8 wherein the recombinant DNA vector expresses the sequence encoding the Cdn under control of an inducible promoter.

- 31 -

10. An isolated cell transfected with a recombinant DNA vector encoding a Cdn as defined in claim 8 or 9.
11. An isolated protein comprising a substantially purified Cdn protein comprising any one of the amino acid sequences depicted in Figures 3, 5 and 11.
12. The protein according to claim 11 wherein the protein is Cdn-1 and comprises the amino acid sequence depicted in Figure 3.
13. The protein according to claim 11 wherein the Cdn is Cdn-2 and comprises the amino acid sequence depicted in Figure 5.
14. The protein according to any one of claims 11 to 13 wherein the protein is expressed by recombinant DNA.
15. The protein according to any one of claims 11 to 13 wherein the protein is a native protein.
16. A composition comprising any one of the protein of any one of claims 11 to 15, the nucleic acid molecule of any one of claims 1 to 7, the recombinant DNA vector of claim 8 or 9, and the cell of claim 10; and a pharmaceutically acceptable buffer.
17. The composition according to claim 16 wherein the protein is present and is in an amount sufficient to modulate apoptosis.
18. An antibody which specifically binds to the isolated Cdn protein of any one of claims 11 to 15 but does not specifically bind to other members of the Bcl family.
19. A method of detecting the presence of a Cdn protein comprising any one of the amino acid sequences as depicted in Figures 3, 5 and 11 in a biological sample comprising the steps of:
 - a) obtaining a sample comprising a cell;
 - b) lysing the cell or permeabilizing the cell to antibodies;
 - c) adding anti-Cdn-specific antibodies as defined in claim 18 to the sample;

- 32 -

- d) maintaining the sample under conditions that allow the antibodies to complex with the Cdn; and
 - e) detecting the antibody-Cdn complexes formed.
20. The method according to claim 19 wherein the Cdn is Cdn-1 comprising the amino acid sequence depicted in Figure 3.
21. The method according to claim 19 wherein the Cdn is Cdn-2 comprising the amino acid sequence depicted in Figure 5.
22. The method according to any one of claims 19 to 21 wherein the sample comprises a T cell.
23. A method for detecting the expression of a Cdn gene in a biological sample comprising the steps of obtaining a biological sample; and detecting the presence of RNA encoding the Cdn comprising any one of the amino acid sequences depicted in Figures 3, 5 and 11.
24. The method according to claim 23 wherein the Cdn is Cdn-1 depicted in Figure 3 or Cdn-2 depicted in Figure 5 and the method for detecting the Cdn-1 or Cdn-2 mRNA is Northern blotting.
25. A method for identifying Cdn mRNA comprising the steps of:
- a) obtaining a sample comprising a cell;
 - b) obtaining RNA from the sample;
 - c) performing a polymerase chain reaction on the RNA using primers corresponding to unique regions of the Cdn nucleotide sequence, wherein the Cdn nucleotide sequence comprises any one of the nucleotide sequences depicted in Figures 3 and 5; and
 - d) detecting the presence of products of the polymerase chain reaction.

- 33 -

26. A use of Cdn comprising any one of the amino acid sequences depicted in Figures 3, 5 and 11 for modulating apoptosis-induced cell death by the modulation of endogenous levels of the Cdn in a cell.
27. The use according to claim 26 wherein the Cdn is Cdn-1 comprising the amino acid sequence depicted in Figure 3.
28. The use according to claim 26 wherein the Cdn is Cdn-2 comprising the amino acid sequence depicted in Figure 5.
29. The use according to any one of claims 26 to 28 wherein the Cdn is increased by modulation of the expression of an endogenous Cdn gene.
30. The use according to claim 29 wherein the Cdn gene expressed is encoded by recombinant DNA.
31. The use according to claim 30 wherein expression of the DNA is under the control of an inducible promoter.
32. The use according to claim 31 wherein the cell is transfected *ex vivo* and capable of being reintroduced into an animal.
33. The use according to claim 32 wherein the cell is a T lymphocyte.
34. The use according to claim 32 wherein the recombinant gene is transfectable into cells *in vivo*.
35. A use of Cdn comprising any one of the amino acid sequences as depicted in Figures 3, 5 and 11, for modulating apoptosis in a patient in need thereof.
36. The use according to claim 35 wherein the Cdn is Cdn-1 having the nucleotide sequence depicted in Figure 3.
37. The use according to claim 35 wherein the Cdn is Cdn-2 having the nucleotide sequence depicted in Figure 5.
38. The use according to any one of claims 35 to 37 wherein the patient is afflicted with a disease characterized by free radical-mediated cell death.

- 34 -

39. A method of assaying for interactions between Cdns selected from the group consisting of Cdn-1 depicted in the amino acid sequence in Figure 3, Cdn-2 depicted in the amino acid sequence in Figure 5, Cdn-1 Δ 1 depicted at positions 60 to 211 of the amino acid sequence in Figure 3 or Figure 11, Cdn-1 Δ 2 depicted at positions 71 to 211 of the amino acid sequence in Figure 3 or Figure 11, and Cdn-1 Δ 3 depicted at positions 96 to 211 of the amino acid sequence of Figure 3 or Figure 11, and putative Cdn-binding proteins comprising the steps of:
- a) contacting purified Cdn with cell lysates containing the putative Cdn-binding protein under conditions which allow binding of the putative Cdn-binding protein and the Cdn;
 - b) isolating the purified Cdn from the cell lysate;
 - c) contacting the isolated Cdn with an antibody that is specific for the putative Cdn-binding protein, under conditions which allow binding of the antibody and the putative Cdn-binding protein; and
 - d) measuring the amount of antibody bound to the putative Cdn-binding protein to determine the amount of putative Cdn-binding protein that is bound to the isolated Cdn.
40. The method according to claim 39 wherein the cell lysate is a yeast lysate.
41. The use of a therapeutically effective amount of Cdn comprising any one of the amino acid sequences depicted in Figures 3, 5 and 11, for treating apoptosis in a patient in need of such treatment.
42. The use according to claim 41 wherein the Cdn is Cdn-1 having the nucleotide sequence depicted in Figure 3.
43. The use according to claim 41 wherein the Cdn is Cdn-2 having the nucleotide sequence depicted in Figure 5.
44. The use according to any one of claims 41 to 43 wherein the patient is afflicted with a disease characterized by free radical-mediated cell death.

- 35 -

45. An *in vitro* method of modulating the level of an apoptosis-modulating protein in a cell, comprising the steps of:
 - a) providing an isolated cell;
 - b) introducing into the cell a polynucleotide encoding a Cdn protein comprising any one of the amino acid sequences depicted in Figures 3, 5 and 11; and
 - c) expressing the Cdn protein from the polynucleotide.
46. A use of a polynucleotide encoding a Cdn protein comprising any one of the amino acid sequences depicted in Figures 3, 5 and 11 for modulating the level of an apoptosis-modulating protein in a cell by the expression of the Cdn protein from the polynucleotide.
47. The antibody according to claim 18 where the antibody is a polyclonal antibody.

1/26

5'- AGATCTGAATTC AA(C/T) TGG GGI (C/A)GI (A/G)TX GTX GC -3'

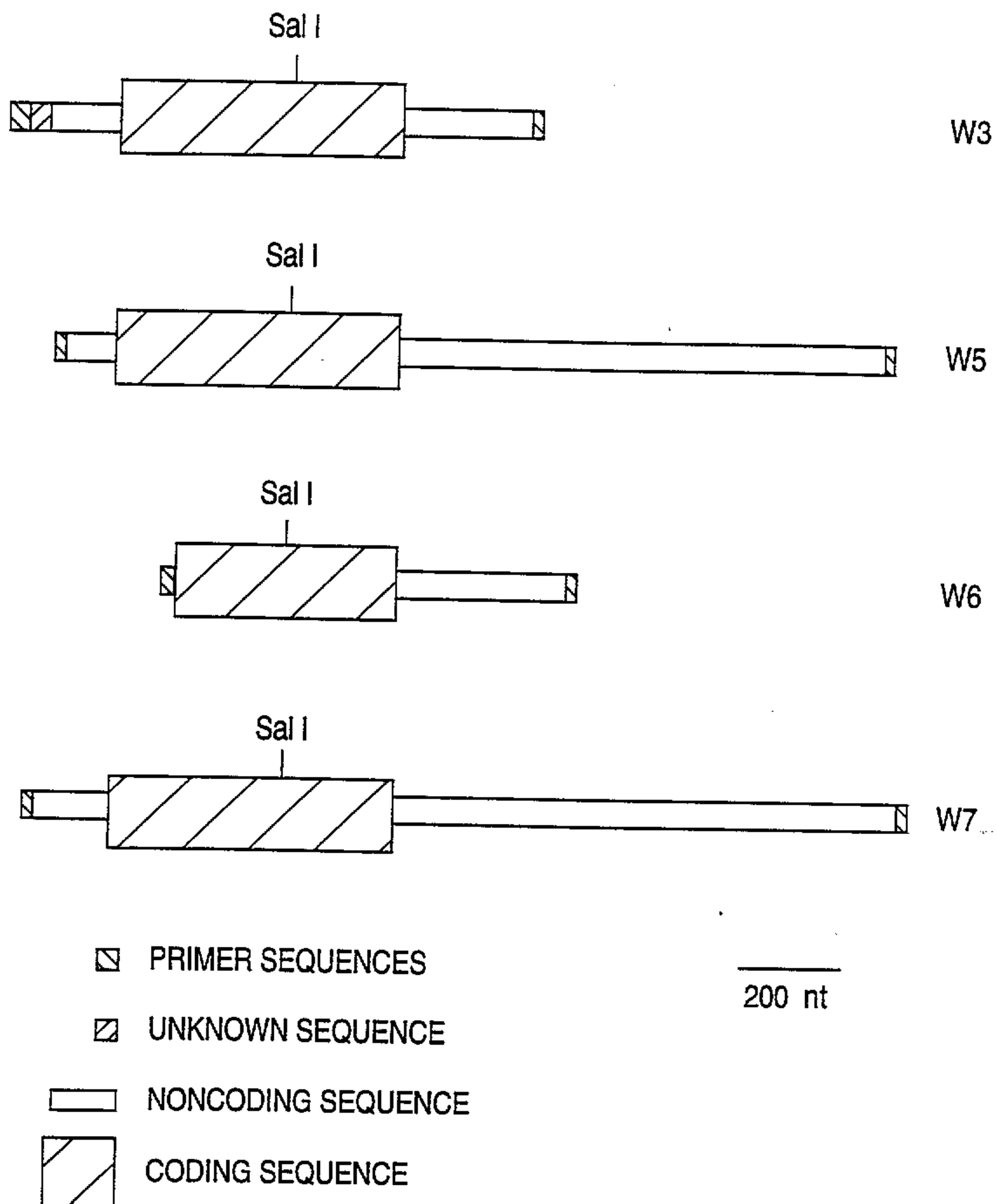
Bcl^Ix 1-32

5'- AGATCTAAGCTT GTC CCA ICC ICC XTG XTC (C/T)TG (A/T/G)AT CCA -3'

Bcl^Ix 2-39

FIG. 1

2/26

**FIG. 2**

SUBSTITUTE SHEET (RULE 26)

3/26

>Aha2

80

GGACAAGTAA AGGCTACATC CAGATGCCGG GAATGCACTG ACGCCCATTC CTGGAAACTG GGCTCCCACT CAGCCCCTGG

>Sma1 >BamH1

160

GAGCAGCAGC CGCCAGCCCC TCGGACCTCC ATCTCCACCC TGCTGAGCCA CCCGGGTTGG GCCAGGATCC CGGCAGGCTG

ATCCCGTCCT CCACTGAGAC CTGAAAA ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC AGG CAG GAG TGC

M A S G Q G P G P P R Q E C>

240

GGA GAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG GTA GCC CAG GAC ACA GAG GAG GTT TTC CGC

G E P A L P S A S E E Q V A Q D T E E V F R>

320

AGC TAC GTT TTT TAC CGC CAT CAG CAG GAA CAG GAG GCT GAA GGG GTG GCT GCC CCT GCC GAC CCA

S Y V F Y R H Q Q E Q E A E G V A A P A D P>

>Nco1

400

GAG ATG GTC ACC TTA CCT CTG CAA CCT AGC AGC ACC ATG GGG CAG GTG GGA CGG CAG CTC GCC ATC

E M V T L P L Q P S S T M G Q V G R Q L A I>

480

>Pst1

ATC GGG GAC GAC ATC AAC CGA CGC TAT GAC TCA GAG TTC CAG ACC ATG TTG CAG CAC CTG CAG CCC

I G D D I N R R Y D S E F Q T M L Q H L Q P>

>Sca1

560

ACG GCA GAG AAT GCC TAT GAG TAC TTC ACC AAG ATT GCC ACC AGC CTG TTT GAG AGT GGC ATC AAT

T A E N A Y E Y F T K I A T S L F E S G I N>

TGG GGC CGT GTG GTG GCT CTT CTG GGC TTC GGC TAC CGT CTG GCC CTA CAC GTC TAC CAG CAT GGC

W G R V V A L L G F G Y R L A L H V Y Q H G>

FIG. 3A SUBSTITUTE SHEET (RULE 26)

4/26

>Sal1

640

CTG ACT GGC TTC CTA GGC CAG GTG ACC CGC TTC GTG GTC GAC TTC ATG CTG CAT CAC TGC ATT GCC
L T G F L G Q V T R F V V D F M L H H C I A>

720

CGG TGG ATT GCA CAG AGG GGT GGC TGG GTG GCA GCC CTG AAC TTG GGC AAT GGT CCC ATC CTG AAC
R W I A Q R G G W V A A L N L G N G P I L N>

800

GTG CTG GTG GTT CTG GGT GTG GTT CTG TTG GGC CAG TTT GTG GTA CGA AGA TTC TTC AAA TCA TGA
V L V V L G V V L L G Q F V V R R F F K S >

>Afl2

880

CTCCCAA GGGTGCCCTT TGGGTCCCGG TTCAGACCCC TGCCTGGACT TAAGCGAAGT CTTTGCCTTC TCTGTTCCCT

>Hind3

960

TGCAGGGTCC CCCCTCAAGA GTACAGAAGC TTTAGCAAGT GTGCACTCCA GCTTCGGAGG CCCTGCGTGG GGGCCAGTCA

>Pst1

>Apa1

1040

GGCTGCAGAG GCACCTCAAC ATTGCATGGT GCTAGTGCCC TCTCTCTGGG CCCAGGGCTG TGGCCGTCTC CTCCCTCAGC

1120

TCTCTGGGAC CTCCTTAGCC CTGTCTGCTA GGCGCTGGGG AGACTGATAA CTTGGGGAGG CAAGAGACTG GGAGCCACTT

1200

CTCCCCAGAA AGTGTTTAAC GGTTTTAGCT TTTTATAATA CCCTTGTGAG AGCCCATTC CACCATTCTA CCTGAGGCCA

>Aha2

1280

GGACGTCTGG GGTGTGGGA TTGGTGGGTC TATGTTCCCC AGGATTCAGC TATTCTGGAA GATCAGCACC CTAAGAGATG

1360

GGACTAGGAC CTGAGCCTGG TCCTGGCCGT CCCTAAGCAT GTGTCCAGG AGCAGGACCT ACTAGGAGAG GGGGGCCAAG

FIG. 3B

SUBSTITUTE SHEET (RULE 26)

5/26

1440
* * * * *
GTCCTGCTCA ACTCTACCCC TGCTCCCATT CCTCCCTCCG GCCATACTGC CTTTGCAGTT GGACTCTCAG GGATTCTGGG

1520
* * * * *
CTTGGGGTGT GGGGTGGGGT GGAGTCGCAG ACCAGAGCTG TCTGAACTCA CGTGTCAAGG GCCTCCAAGC CTGCCTCCCA

1600
* * * * *
AGGTCCTCTC AGTTCTCTCC CTCCTCTCT CTTATAGAC ACTTGCTCCC AACCCATTCA CTACAGGTGA AGGCTCTCAC

1680
* * * * *
CCATCCCTGG GGGCCTTGGG TGAGTGGCCT GCTAAGGCTC CTCCTTGCCC AGACTACAGG GCTTAGGACT TGGTTTGTTA

1760
* * * * *
TATCAGGGAA AAGGAGTAGG GAGTTCATCT GGAGGGTTCT AAGTGGGAGA AGGACTATCA ACACCACTAG GAATCCCAGA

>BamH1
|
1840
* * * * *
GGTGGATCCT CCCTCATGGC TCTGGCACAG TGTAATCCAG GGGTGTAGAT GGGGGAAGTGA TGAATACTTG AACTCTGTTC

1920
* * * * *
CCCCACCCTC CATGCTCCTC ACCTGTCTAG GTCTCCTCAG GGTGGGGGGT GACAGTGCCT TCTCTATTGG CACAGCCTAG

2000
* * * * *
GGTCTTGGGG GTCAGGGGGG AGAAGTTCTT GATTCAGCCA AATGCAGGGA GGGGAGGCAG ATGGAGCCCA TAGGCCACCC

* * * * *
CCTATCCTCT GAGTGTTTGG AAATAAACTG TGCAATCCCC TCAAAAAAAAA AA

FIG. 3C

6/26

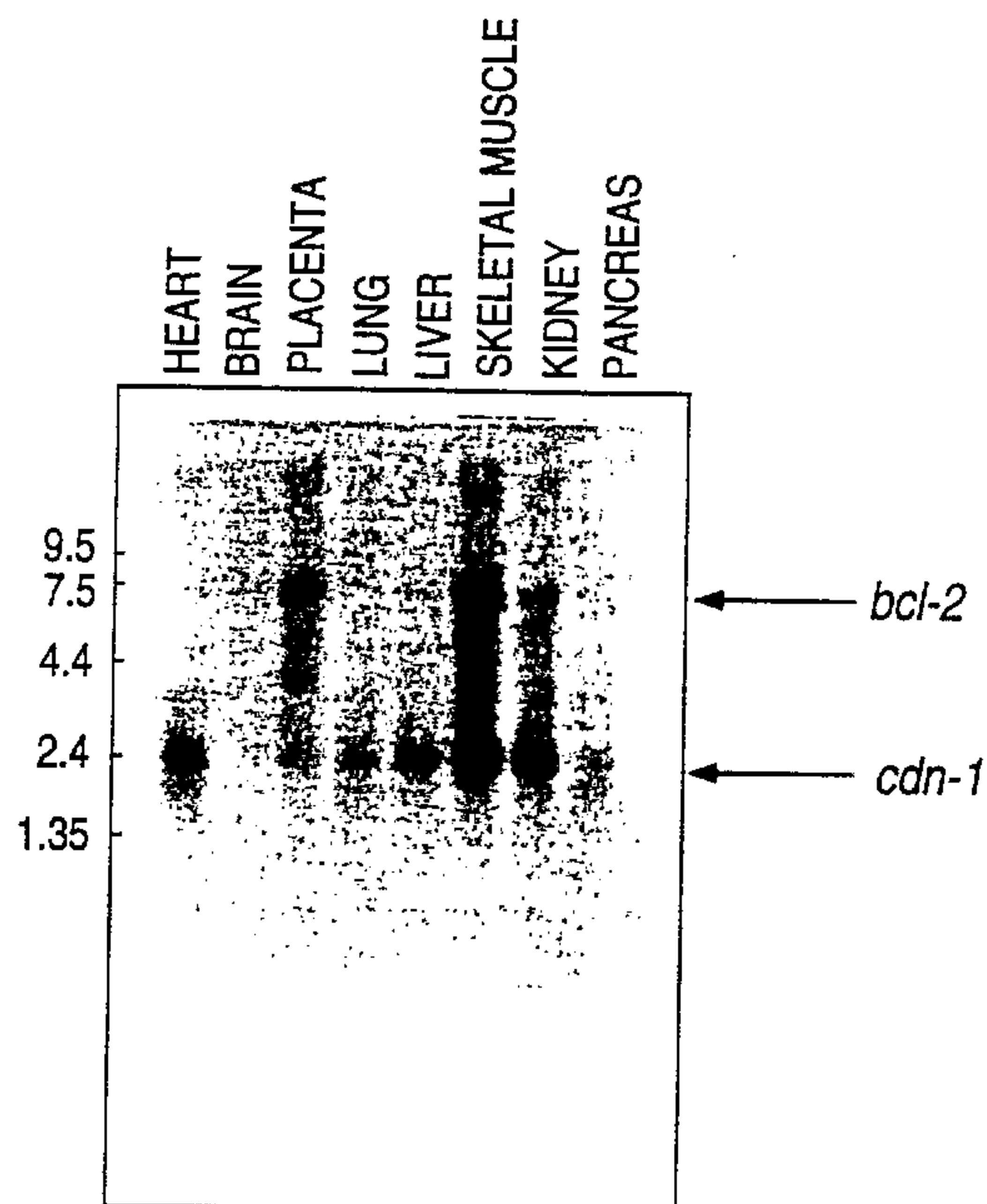


FIG. 4

7/26

>EcoR1
|
* * * * *
GAATTCCCGG CCGCAATTAA CCCTCACTAA AGGGATCCTC CTGCCTTGGT CTCCCAAAT GTTGAGATTA TAGGCATGAG
* * * * *
160
CCCACCACGC CTGGCTGGGG TTTTGT TTTT GTTTTTTAA CATTTGTCAC ATTTCAAAA GGTATTTTCAG AATCTCTGAG
* * * * *
240
AAAAGTGCTA TAATGTCTAA TGATACTTTA TATTTGGACA GCACTTTTCGT TTGTTTTTTT TGGCGGGGGG GGTGGGAGAA
* * * * *
320
GTCAAGTAAC TTACATATAG TGAAATTTAC CCTTCTTGAG TATGCAGTTC AGTGAGTTTT GATAAATGTG TAATGGTAGT
* * * * *
400
GTAATCACTA CCACAGTCAA GACATGGACA ATTTTCATTA CCCACGAAG GTCCCTCATG TGTGGTTAGA GTCAGCCCTC
* * * * *
480
CCATCAGCAC AGTCCTGGCA GCCACTGACC TGGTTTCTGT CCCTACTGTT TTGCCTTTTC CAGAATGTCA TTTAAGTGAC
* * * * *
560
ATCATTCAAT ATGGAGACTT GTTTTATTTT TTATTTT TTTTGTGAGA AGGAGTCTCG CTCTTGTTGC CCAGGCTAGA
* * * * *
>Sma1
|
* * * * *
640
GTGCAATGCT GTGATTTTCGG CTCAGTGCAA CCTCCGCCTC CCGGGTTCAA GTGATTCTCC TGCCTCAGCC TCCCGAGTAG
* * * * *
720
TTCGGGACCA CAGGCGTACA CCACCATGCC CAGCTAATTT TTTTTTTTTT TGAGATGGAG TCTCGCCCTG TCACCCAGGC
* * * * *
>Sma1
|
* * * * *
800
TAGAGTCCAG TGGCATGATC TGCCTCACT GCCAAGCTCC TGCCTCCCGG GTTTCACGCC ATTCCCCTGC CTCAGCCTCC
* * * * *
>Bcl1
|
* * * * *
880
CGAGTATGCC CGGCTAATTT TTGTATTTT AGTAGAGACG GGGTTTCCCC ATGTTGGCCA GGCTAGTCTC AAACCTCTGA
* * * * *

FIG. 5A

8/26

960
* * * * *
CCTCAAGTAA TCCGCCTGCC TTGGCTCCCA AAGTGCTGGG ATTACAGGTG TGAGCCACCG CGCCCAGCCC ATTATGTAGC

>Spe1
|
1040
* * * * *
TTTTTGTGCC CCACTTCTCC CACTTAGCAT AATGCTTTTT GAGATTCATC TGTATTACTA GTGCATCTGT AGTTCTTTCC

1120
* * * * *
TTTTTATGGC TTGGGTTGTT TTTTGTTTTT GTTTTTGTTT TTGAGATAGG GTCTCACTCT GTTGCCCAGG GTGGAGTGCA

>EcoR5
|
1200
* * * * *
CTATCGCAGC TCTCCGCAAC CTCCACCTCC CAGGCTCAAG ATATCCTCCC ACCTCAGCCT CCTGAGTAGC TGGAAATACA

>Nco1
|
1280
* * * * *
AGTGTGTGTG CCACCATGCC GGTTAATTT TTTTCTTTT TTTTTTTTTT TTTTCAATTT TTGTTGGAAG CACCATGGAG

>Bcl1
|
>Sph1
|
1360
* * * * *
CCGCCTGAGC CTGGCTGAGC CTAAAAGCCC TGTGGTGCAT GCCTGGCCAA TTTTGTATT TTTTAGTAGA GACGGGATTT

1440
* * * * *
TGCCATGTCG CCCAGGCTGG TCTGGAAGTC CTGGTCTCAG GTGATTCTCC TGCTTCGGCC TCCAAGTAG CTGGGGTTAC

1520
* * * * *
AGGCATGTGC CACCATGCTC AGCCCTCCCG TCAGCACAGT CCTGGCAGCC ACTGGCCTGG TTTCTGTCCC TACTGTTTTG

1600
* * * * *
CCTTTTACTG GTCTCCATGC TCACCTAAAT TTTTTTTTAT TTTTGTAGA GACAGATTCT CGCAATGTTG CTCAGGCTAG

1680
* * * * *
TCTCGAACTC CCGGCTTCAA GCAATCCTCC CACCTCAGTC CTCCAAAGTT CTGGAATTAC AGGCATGAAT CACTGTGCCA

FIG. 5B

RECTIFIED SHEET (RULE 91)

9/26

>Aha3
|
>Dra1
|

1760
* * * * *
GTGTTGCATT CCAAAGCATG GATACATCAC AGTTTTTAAA ATGTTTACCA ATGTAAATGG CCCGTTTTAA TATGAGAATA

1840
* * * * *
ACTAATGTTG AAGAGAGTGC TACAAAGAGG ATCATGGTCG TGATGTGTAG ACACAAAAAT GAGGTTAGAA GACAGTAAGG

1920
* * * * *
TGAGGGCCGG GCACAGTGGC TCATGCTTGT AATCCTAGCA CTTTGGGAGG TGCAGGTGGG AGGATTGCTT GAGGCCAGGA

2000
* * * * *
GCTGGAGACA AGTCCAGGCA ACACAGCGAG TCCCTGCCTT TATAAAAAAT CAGAAATTAA AAAAGCCTTG GCGGTGGCTC

>Bcl1
|

2080
* * * * *
ACGCCTGTAA TCCCAGCACT TTGGGAGGCC GAGACGGGTG GATCACGAAG TCAGGAGTTC AAGACCAGCT TGGCCAAGAT

2160
* * * * *
GGTGAAACCC TGTCTCTACT AAAAATAAAA AAAAAAATT AGCCAGTCGT GGTGGTGGCA CCTGTAATCC CAGCTACTCA

2240
* * * * *
GGAGGCTGAG GCAGGAGAAT CGCTTGAACC CAGGAGGCGG AGTTTGCAGT AAGCCAAGGT GCGCCACTGC ACTCCAGCCT

2320
* * * * *
GGGCAACAGA GTAAGACTCT GTCTCAAAAA AAAACAAAAA ACAAAAAAAC AAAAAAAAAA CAGGCCGGCG CAGTGGCTCA

>Sca1
|

>Bcl1
|

2400
* * * * *
TGCCTATAAT CCAAGTACTT TGGGAGGCCA AGGCAGGCGG ATCGCAAAGT CAGGAGTTCG AGACCAGCCT GGCCAATATG

2480
* * * * *
GTGAAACCCT GTTTCTGCTA AAAATACAAA AAATAGCCAG GTGTGGTGGG AAGCGCCTGT AGTCCCAGCT ACTCAGGAGG

>Sma1
|

2560
* * * * *
CTGAGGCAGG AGAATCGCTT GAACCCGGGA GGCAGAAGTT GCAGTGAGCT GAGATTGCGC CACTGCACTC CAGCCTGGGC

FIG. 5C

RECTIFIED SHEET (RULE 91)

10/26

```

                                     >Aha3
                                     |
                                     >Dra1
                                     |
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
AACAGAGCGA GACTCCATCT CAAAGAAAAA AAAGCCAAAA CATAGTAAGG TGAGGGTGAA ACTTCTCTTT TAAAAAATG
                                     |
                                     2640
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
TTTACATAGA AACAACTAA ATGGACAAAA TGGATATAAA CAAAAATGTT ATCGGTGGTT ATTTTGGGC AGTAGAATTA
                                     |
                                     2720
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
TAGGTTTTTA ATTTCTTTTG CTTATTTATA GTTCAAAAA TTTCAATTT TAATATAAAT TAATGTGCTC TATTTATAGA
                                     |
                                     2800
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
GACAATACAT GAAATATACT TAATAAAAAT TCAAATGTTA TAGAACTGAA AAAGATGAAA AGTAAAAACA ACCTATTCCC
                                     |
                                     2880
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
CAGAGGTAGC CACTGTCCAT AGTTTCTATT TTAGATTCTT TCCTTTATAC AAGATTATTA TAGCTTCTAT TTTTGGTGT
                                     |
                                     2960
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
ATGAACTGTA GTCCTAGAGG ATTTTATTAG TTATGAGTTC TATAACTAAG ATCCATCATC TTAGTTGCTA AGAACGTAGA
                                     |
                                     3040
                                     >Dra1
                                     |
                                     >Aha3
                                     |
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
TACTGAGAAC ATCATTTAAA AAAACATTTT TGGCTGGCAC CTCTATGATC ACTGGAGTCT CGCGGGTCCC TCAGGCTGCA
                                     |
                                     3120
                                     >Aha2
                                     |
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
CAGGGACAAG TAAAGGCTAC ATCCAGATGC TGGGAATGCA CTGACGCCCA TTCCTGGAAA CTGGGCTCCC ACTCAGCCCC
                                     |
                                     3200
                                     >Sma1      >BamH1
                                     |      |
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
TGGGAGCAGC AGCCGCCAGC CCCTCGGGAC CTCCATCTCC ACCCTGCTGA GCCACCCGGG TTGGGCCAGG ATCCCGGCAG
                                     |      |
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
GCTGATCCCG TCCTCCACTG AGACCTGAAA A ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC AGG CAG GAG
                                     M  A  S  G  Q  G  P  G  P  P  R  Q  E>

```

FIG. 5D

RECTIFIED SHEET (RULE 91)

11/26

3360
 * * * * *
 TGC GGA GAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG GTA GCC CAG GAC ACA GAG GAG GTT TTC
 C G E P A L P S A S E E Q V A Q D T E E V F>

3440
 * * * * *
 CGC AGC TAC GTT TTT TAC CAC CAT CAG CAG GAA CAG GAG GCT GAA GGG GCG GCT GCC CCT GCC GAC
 R S Y V F Y H H Q Q E Q E A E G A A A P A D>

>Nco1
 |
 3520
 * * * * *
 CCA GAG ATG GTC ACC TTA CCT CTG CAA CCT AGC AGC ACC ATG GGG CAG GTG GGA CGG CAG CTC GCC
 P E M V T L P L Q P S S T M G Q V G R Q L A>

>Pst1
 |
 3600
 * * * * *
 ATC ATT GGG GAC GAC ATC AAC CGA CGC TAT GAC TCA GAG TTC CAG ACC ATG TTG CAG CAC CTG CAG
 I I G D D I N R R Y D S E F Q T M L Q H L Q>

>Sca1
 |
 3680
 * * * * *
 CCC ACG GCA GAG AAT GCC TAT GAG TAC TTC ACC AAG ATT GCC TCC AGC CTG TTT GAG AGT GGC ATC
 P T A E N A Y E Y F T K I A S S L F E S G I>

AAT TGG GGC CGT GTG GTG GCT CTT CTG GGC TTC AGC TAC CGT CTG GCC CTA CAC ATC TAC CAG CGT
 N W G R V V A L L G F S Y R L A L H I Y Q R>

3760
 * * * * *
 GGC CTG ACT GGC TTC CTG GGC CAG GTG ACC CGC TTT GTG GTG GAC TTC ATG CTG CAT CAC TGC ATT
 G L T G F L G Q V T R F V V D F M L H H C I>

3840
 * * * * *
 GCC CGG TGG ATT GCA CAG AGG GGT GGC TGG GTG GCA GCC CTG AAC TTG GGC AAT GGT CCC ATC CTG
 A R W I A Q R G G W V A A L N L G N G P I L>

3920
 * * * * *
 AAC GTG CTG GTG GTT CTG GGT GTG GTT CTG TTG GGC CAG TTT GTG GTA CGA AGA TTC TTC AAA TCA
 N V L V V L G V V L L G Q F V V R R F F K S>

FIG. 5E

RECTIFIED SHEET (RULE 91)

12/26

```

                                >Af12
                                |
                                4000
*      *      *      *      *      *      *
TGA CTC CCAAGGGTGC CCTTTGGGGT CCCAGTTCAG ACCCCTGCCT GGACTTAAGC GAAGTCTTTG CCTTCTCTGC
*>

                                >Hind3
                                |
                                4080
*      *      *      *      *      *      *
TCCTTGCAGG GGTCCCCCT CAAGAGTACA GAAGCTTTAG CAAGTGTGCA CTCCAGCTTC GGAGGGCCCC TGTGTGGGGG

                                >Pst1
                                |
                                >Nco1
                                |
                                >Apa1
                                |
                                4160
*      *      *      *      *      *      *
CCAGTCAGGC TGCAGAGGCA CCTCAACATT CCATGGTGCT AGTGGGCCCT CTCTCTGGGC CCAGGGGCTG TGGCGTCTCC

                                >Apa1
                                |
                                >Aha2
                                |
                                4240
*      *      *      *      *      *      *
TCCCTCAGCT CTCTGGGACC TCCTTAGCCC TGTCTGCTAG GCGCTGGGGA GACTGATAAC TTGGGGAGGC AAGAGACTGG

                                4320
*      *      *      *      *      *      *
GAGCCACTTC TCCCCAGAAA GTGTTTAATG GTTTTAGCTT TTTATAATAC CCTTGTGAGA GCCCATTCCC ACCATTCTAC

                                >Aha2
                                |
                                4400
*      *      *      *      *      *      *
CTGAGGCCAG GACGTCTGGG GTGTGGGGAT TGGTGTGTCT ATGTTCCCCA GGATTCAGCT ATTCTGGAAG ATCAGCACCC

                                4480
*      *      *      *      *      *      *
TAAGAGATGG GACTAGGACC TGAGCCTGGT CCTGGCCGTC CCTAAGCATG TGTCCCAGGA GCAGGACCTA CTAGGAGAGG

                                4560
*      *      *      *      *      *      *
GGGGCCAAGG TCCTGCTCAA CTCTACCCCT GCTCCCATTC CTCCCTCCGG CCATACTGCC TTTGCAGTTG GACTCTCAGG

                                4640
*      *      *      *      *      *      *
GATTCTGGGC TTGGGGTGTG GGGTGGGGTG GAGTCGAGAC CAGAGCTGTC TGAACATG TGTCAGAAGC CCTCCAAGCC

                                4720
*      *      *      *      *      *      *
TGCCTCCCAG GGTCTCTCA GTTCTCTCCC TTCCTCTCTC CTTATAGACA CTTGCTCCCA ACCCATTCAC TACAGGTGAA

```

FIG. 5F

RECTIFIED SHEET (RULE 91)

13/26

>StuI

4800

* * * * *

GGCTCCTCAC CCCCATCCCT GGGCCTTGGG TGAGTAACCT GCTAAGGCCT CCTTGCCCAG ACTACAGGGC TTAGGACTTG

4880

* * * * *

GTTTGTATT TCAGGAAAA GGAGTAGGGA GTTCATCTGG AGGGTTCTAA GTGGGAGAAG GACTATCAAC ACCACTAGGA

>BamHI

4960

* * * * *

ATCCCAGAGG TGGGATCCTC CCTCATGGCT CTGGCACAGT GTAATCCAGG GGTGTAGATG GGGGAAGTGT GAATACTTGA

5040

* * * * *

ACTCTGTTCC CCCACCCTCC ATGCTCCTCA CCTGTCTAGG TCTCCTCAGG GTGGGGGGTG AGAGTGCCTT CTCTATTGGG

5120

* * * * *

CACAGCCTAG GGTCTTGGGG GTCGGGGGGA GAAGTTCTTG ATTCAGCCAA ATGCAGGGAG GGGAGGCAGA TGGAGCCCAT

5200

* * * * *

AGGCCACCTC CTATCCTCTG AGTGTTTGA AATAAACTGT GCAATCCCCT CAAAAAATA AAAATAAAAA AAATAAAAT

5280

* * * * *

AAAAAACAT TTTTTTCAAG CAGGGAGTGG TGGCTCCCGC CTGTAATCCC AGCACTTTGG GAGGCCAAGG CGTGCAGATT

5360

* * * * *

GCTTCAGTTC AGGAGTTCAA GACCAGCCTG GGAAACATGG TGAAACCCCA TCTCTACTAA AAATAAAAAA TTAGCCAGGC

5440

* * * * *

ATAGTGTCGC GCACCTGTAC TCCCAGCTAT TTGGGAGGCT GAGGTAGGAG AATTGCTTGA ACCCAGGAGG TGGAGGTTGC

5520

* * * * *

AGTGAGCTGA GATCAGGCCA CTGCACTCCA ACGTAGGTGA CAGAGATAGC CTCCTTCTAA AAAAACAACC TTTTTTCCAG

>XbaI

5600

* * * * *

CCAAAACAAC TGAACCTCCT CCCCACTGAC CACCTCAATT ATTTCTAGAT GCCTTGTTGC TGTCCAGACT GCGGTGATTC

5680

* * * * *

CCTGGGCTGA TCTGAGCCCG TGGCCTGAGT CATTTGCAGT TCCTCTAGCA GGTGGTCCCC CATGTCATGG CCCCTGTGAA

FIG. 5G

RECTIFIED SHEET (RULE 91)

14/26

>Hind3
|

5760
* * * * *
ACCAGTTCCT TACCATCTCT GTTCATCGCT GCTCCCTAAG TTAGGCCCTG CATGTCTTGA GGGTAGGTTA GATTCAGAAA

5840
* * * * *
AGCTTTGGTC GCATCACTGC TTTCATAAAC TCAAATGAGA GGGAGGGAGG GAAGGCAGGA AGAAGGGAGG GAGTCCTTTC

5920
* * * * *
TCTCCCACAG TGTGCATTAC CTCATGTAAC ACTTCTTGCT AATGTGGTAG AATGTGTTTG ACTTTGAATG AGACTTGGGT

6000
* * * * *
TTATTTTAT TTATTTATTT ATTTATTTAT TTATTTATTT TGAGATGGAG TTCACTCTT GTTGCCCAGG CTGGAGTGTA

6080
* * * * *
GTGGCACGAT CTCTACTCAT TGCACCCTCC GCCTTCCAGG TTCAAACGAT TCTCCTGCCT CAGCCTCCCA AGTAGCTGGG

>Sph1
|

6160
* * * * *
ATTACAGGGG CATGCCACCA TGCCCAGCTA ATTTTGTAT TTTTAGTAGG GACGGGGTTT CACCATGTTG ACCAGGCTGG

6240
* * * * *
TCTGGAAGTC CTGATCTCAG GTGATCCACC TGCCTCGGCC TCCCAAAGTG TTGGGATTAC AGGCGTGAGC CACCGTGCCT

6320
* * * * *
GGCCTGAGAC TTAAATCCAT CTCTTTTTTC TTCTTCTTTT TGAGACAGAG CCTCATTCTG TTCCCATGC TGGAGTTCAG

>Bcl1
|

6400
* * * * *
TGGCGTGATT TTGGCTCACT GCAACCTTGG CCATCTGGGT TTGAGCAATT CTCGTGCCTC AGCCTCCTGA GTAGCTGGCA

6480
* * * * *
CTATAGTCAC ATGCCACCAC GCCCGGCTAA CTTTTTGTG TTTTGTAGTAG AGACAGGGTT TCACTATGTT AGCCAGGCTG

>EcoR1
|

*
GTCTCGAATT C

FIG. 5H

cdn1	masgqgppprqecgepalpsaseeqvaqdtteevfrsyvfyrrhqqeqeaaapadpemt	
cdn2	masgqgppprqecgepalpsaseeqvaqdtteevfrsyvfyHhqqeqeaaapadpemt	
bc12	mahagrtgyDNREIVMKYIHYKLSQRGYEWdagvgaapagifssqpghtphtaasrdpvarstplqtpaapgaa	
bax	mdgsgeqprgggptsseqimktgallqgfiqdragmggeap	
bc1-x	msqSNRELVDLSYKLSQKGYSWsqfsdveenrteapegetesemetpsaingnpswhladspavngatghsssl	
mc1-1	...(+123 aa) eldgyepeplgkrpavlpillelvgesGnntstdgslpstppaeeedeelyrqsleiiisrylreqatgakdtk	
A1	maeselmhihsiaehylqyvlq	
bhrf	maystreillalalcirdsrvhngntlhpvlelaar	
LMW5-HL	megeeliyhniineilvgg	
ced9	mtrctadnsltnpayrrrtmatgemkeflgikgteptdfginsdaqlpspsrqastrmsigesidgkindweeprlDIEGFVVDYFTHRIRQNGMEWfgapg	
cdn1	lp1qpsstmgQVGRQLAIIGDDINRRYDSEFQTLQHLQPTAENAYEYFTKIATSLFESGI-NWGRVVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVDFMLHH	
cdn2	lp1qpsstmgQVGRQLAIIGDDINRRYDSEFQTLQHLQPTAENAYEYFTKIATSLFESGI-NWGRVVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVDFMLHH	
bc12	agpalpvpvVHLTLRQAGDDFSRYYRDRFAEMSRQLHLtpftargRFATVVEELFRDGV-NWGRIVAFFEFGGVMCVESVNREMSPLVDNIALNMTY-LNR ^{15/26}	
bax	eialdpvpqastkklsec1krigdelnsmlqrmiaavdtsprevFFRVAADMFSQGNFNWGRVVALFYFASKLVLKALCTKVPPELIRTIMGWTLDL-LRE	
bc1-x	darevipma-AVKQALREAGDEFELRYRRAFSDLTSQLHITPGTAYQSFEQVNVNELFRDGV-NWGRIVAFFEFGGALCVESVDKEMQVLSRIAANMATY-LND	
mc1-1	pmgrsgatsrka1ETLRRVGDGVQRNHETVFGMLRKLDIKNEDDVKSLSRVMIHVFSQGNFNWGRVVALFYFASKLVLKALCTKVPPELIRTIMGWTLDL-LNR	
A1	vpafesapsqacrvlqrva fsvqkevekn1ksylddfhvesidtar1iFNQVMEKEFEEDGIINWGRIVTIFAFGGVLLKKlpqeqialdvcaykqvssfvaei	
bhrf	etplrlspedtvvlryhvlleeiienrsetftetwnrfithtehvdldfnsvfliefhd-LINWGRICGFIVFSARMAKYCKDANN-HLESTVITTAYNF-SEG	
LMW5-HL	ikyymndihelspyqqqikkiltyydeclnkqvttitfsltnaqeiktQFTGVVTELFKrgdpslgralawmawcmhacrtlccnqstpyyvvdlsvrgmleam-	
ced9	lpcgvqpehemrmvmtifekkhafenfetfceqLlavprisfslqdvrvrtvgnadtqcpMSYGRGLIGLISFGGFVAAKMMesvelqgqvrnlfvtytslFIKT	
cdn1	CIAR--WIA-QR-GGWAAALNLGngpilnlvlvlgvllgqfvvrrffks	
cdn2	CIAR--WIA-QR-GGWAAALNLGngpilnlvlvlgvllgqfvvrrffks	
bc12	HLHT--WI--QDNGGWDVAFVELYgpsmrplfdswlskltilslalvgacitlgaylghk	
bax	RLLG--WI--QDQGGWDGLLSYfgtptwtvtifvavltastliwkkmg	
bc1-x	HLEP--WI--QENGWDTFVELYgnnaaaesrkqgerfnrwlftgmtvavvllgslfsrk	
mc1-1	TKRD--WLVKQ--RGWDGFVEFFhvedleggirnvlafagvavvgaglaylir	
A1	MNNTGEWI-RQ-NGGWEDgfikkfepksgwtflqmtgqiweimflfk	
bhrf	-LDG--WIHQ--GGWStliednipsrrfswtlflagtlslivicsylfisgrh	
LMW5-HL	KHNLPLPMWISH--GGQEEFLAFslhsqiyvifnikyflskfchhflrscvqlrkcnl1	
ced9	-RIRNNWKE-H-NRSWDDFMTLgkqmkedyeraeakvgrkrqnrrwsmigavtagaigivgvvvcgrmmfs1k	

SEQUENCE IDENTITY:

cdn1/cdn2 = 97%

FIG. 6

>EcoRI
|
* * * * *
GAATTCTGGT AATTAGTTAA ACAACCTTGA ACAAGTTGTT TCACTTCTCT GAGTCTCAGT TTCTCACTCA AAAATGGTGA
* * * * *
* * * * *
ATAATTTGTA AGACTTCGCT AATAATCTAC GACTCTACAA GAGGCAATAG GGTACTGTGG ACAGAGAGCA GGCTTTGGAA
* * * * *
* * * * *
ACACACAAGA CTGGGTTTAG ATTCCTGCAC TCCACCCAGT GTGTGACTTG GCCAAGCTTC TTCACTTCTC TAAACCCCCA
* * * * *
* * * * *
TCTGTGTATC TGTACAGGAA TGAATGAATG AGTATGTGCA GCCAAGCTAT GCAAACTCCA GGTAAAATA TTGCCTTGGG
* * * * *
* * * * *
TTTTTTAGTA AATTGTTCAA GCCCATGACA TTCTAGCAGA AAAAGCCTAG TGTCTCTTTC TTAAGGTGAT TGTGTCCATG
* * * * *
* * * * *
TGTTTTCCAG GAACTCTATG GGTTTCTCAA CCCAAATTCA CCCTGCCCTT GACCAAATGG CTCACCAGCT TCACGGATGC
* * * * *
* * * * *
TGCTCTGATG ACACACCCTG CAGTCAGCAT CTGCCCCTGC AGCTAGAATG GATTTCTGAG TGGGCATTAG CTGGGGGATA
* * * * *
* * * * *
CCACATGGGC ACCAATGTCA CAGATCTTCT GTCACAGTCC ACCCGAACC ATTGCTTCTC AAATCATAAT CCCTTAGCAG
* * * * *
* * * * *
GACAGCTAGG TGCAGCACGC ATGACACAAA CACCAGCCCT TGCCTACAAT CTCAGCCACT ATCTTGAGTC TGAGCAACTA
* * * * *
* * * * *
GTCTAGTGGC AGCCGCGCCC TTCCTTTTCA AGAGAGTTCT GGGATCAGAT CCTTTCACAA ACAGATCCCT CCCCACCCTG
* * * * *
* * * * *
CCTGTTGTCC AGGTCTGCAC ACTGAAAAGT AAGACAGCAT TTGCTAAGCC ATATTTCAAA AAGTTTGCTT ATACCTTCAT

FIG. 7A

SUBSTITUTE SHEET (RULE 26)

18/26

1680
 * * * * *
 CAA GGC CCA GGG CCT CCC AGG CAG GAG TGC GGA AAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG
 Q G P G P P R Q E C G K P A L P S A S E E Q>

1760
 * * * * *
 GTA GCC CAG GAC ATG GAG GGG TTT TCC GCA GCT ACG TTT TTT ACC ACC ATC AGC AGG AAC AGG AGG
 V A Q D M E G F S A A T F F T T I S R N R R>

>Not1
 * * * * *
 CTG AAG GGG CGG CCG CCC CTG CCG ACC CAG AGA TGG TCA CCT TGC CCC TCC AAC CTA GCA GCA CCA
 L K G R P P L P T Q R W S P C P S N L A A P>

1840
 * * * * *
 TGG GGC AGG TGG GAC GGC AGC TCG CCA TCA CCA GGA CGA CAT CAA CCG GCA CTA TGA CTTCGGAGT
 W G R W D G S S P S P G R H Q P A L *>

>Pst1 >Pst1 >Sca1
 * * * * *
 TCCAGACCAT GCTGCAGCAC CTGCAGCCCA CGGCAGAGAA CGCCTACGAG TACTTCACCA AGATCGCCTC CAGCCTGTTT

2000
 * * * * *
 GAGAGTGGCA TCAACCGGGG CCGTGTGGTG GCTCTCCTGG GCTTCGGCTA CCGTCTGGTC CTACATGTCT ACCAGCACGG

2080
 * * * * *
 CTTGACTGGC TTCCTGGGCC TGGTGACCCG CTTCGTGGTC TTCATGCTGC AACAAGGCAT CGCCCGGTGG ATCTCGCAGA

2160
 * * * * *
 GGGGCGGCTG GGTGGCAGCC CTGGACTTGG GCAATAGTCC CATCCTGAAC GTGCTGGTGG TTGTGGGTGT GGTTCGTGCTG

2240
 * * * * *
 GGCCAGTTTG TGGTAAGAAG ATTCTTCAAA TCATGACTCC CAGGGGTGTC CTTTGGGGTC CCAGCTGTGA CCCCTGCCTG

>Pvu2
 * * * * *
 GGGCAGTTTG TGGTAAGAAG ATTCTTCAAA TCATGACTCC CAGGGGTGTC CTTTGGGGTC CCAGCTGTGA CCCCTGCCTG

2320
 * * * * *
 GACTTAAGCC AAGTCTTTGC CTTCCCCACT CCCTTGCAAG GGTACCCCTT CAAAAGTACA GAAGCTCTAG CAAGTGTGCA

>Af12
 * * * * *
 GACTTAAGCC AAGTCTTTGC CTTCCCCACT CCCTTGCAAG GGTACCCCTT CAAAAGTACA GAAGCTCTAG CAAGTGTGCA

2400
 * * * * *

FIG. 7C

>Apa1
* * * * *
CCCCCGCTGC GGAGGGCCCC TCGTGGGGG CCAGTCAGGC TCGGAGGCA CCTCAACATT GCACGGTGCT AGTGGGCCCT
2480
* * * * *
>Apa1
* * * * *
CTCTCTGGGC CCAGGGGCTG TGCCCTCCTC CTTGGCTCT CTGGGACCTC CTTAGTCTTG TCTGCTAGGC GCTGCAGAGG
2560
* * * * *
>Pst1
* * * * *
CTGATAACTT GGGGAAGCAA GAGACTGGGA GCCACTCCTC CCCAGTAAGT GTTTAACGGT TTTAGCTTTT TATAATACCC
2640
* * * * *
TTGGGAGAGC CCATTCCCAC CATTCTACCC AAGGCCGGGA TGTCTGGGT GTGGGGTTG GTGGGTCGTA ACCTACGTGC
2720
* * * * *
CCCAGGATTC AGCTATTCTG GAAGATCAGA GCCTAAGAGC TAGGACTTGA TCCTGGTCCT GGCCGTCCCT AAGCATCATG
2800
* * * * *
TGTCCCAGGA GCAGGACTGA CTGGGAGAGG GGACCAAGGT CCTACCCAGC TCTCCCCGTG CCCCATTCC TCCTCCGGCC
2880
* * * * *
ATACTGCCTT TGCAGTTGGA CTCTCAGGGA TTCTGGGCTT GGGGTGTGGG GCGGCGTGGA GTAACAGGCC AGAGCTGTCT
2960
* * * * *
GAACTTATGT GTCAGAAGCC TCCAAGCCTG CCTCCAAGG TCCTCTCAGC TCTCTCCCTT CCTCTCTCCT TATAGATACT
3040
* * * * *
TGCTCCCAAC CCATTCATA CAGGTGAAGG CCCTCACCCA TCCCTGGGG CTTGGGTGA GTGATGCGCT AAGGCCCTC
3120
* * * * *
CCGCCCAGA CTACAGGGCT TGGTTTAGGG CTTGGTTTGT TATTTAGGG ATAAGGAGTA GGGAGTTCAT CTGGAAGGT
3200
* * * * *
>BamH1
* * * * *
CTAAGTGGGA GAAGGACTAT CAACACCACA GGAATCCCAG AGGTGGGATC CTCCCTCATG GCTCTGGCAC AGTGTAATCC
3280
* * * * *
AGGGGTGGAG ATAGGGAAC GTGAATACCT GAACTCTGTC CCGGACCCT CCATGCTCCT CACCTTTCTG GGTCTCTCCT
3360
* * * * *

FIG. 7D

SUBSTITUTE SHEET (RULE 26)

3440
* * * * *
CAGTGTGGGG GTGAGAGTAC CTTCTCTATC GGGCACAGCC TAGGGTGTG GGGGTGAAGG GGGAGAAGTT CTTGATTCAG

3520
* * * * *
CCAAATGCAG GGAGGGGAGG CAGAAGGAGC CCACAGGCCA CTCCTATCC TCTGAGTGT TGGAAATAAA CTGTGCAATC

3600
* * * * *
CCATCAAAAA AAAAAAGGAG AAAAAATGT AAAAAACATT CTTAGCTGTA AGCTACTTAT AGGGGGATAA AGACAGGACT

3680
* * * * *
GTTAATGGAC ACAAACATAC AGTTAGAGAG AAGAAATAAG TTCTGTCCAG GCACGGTGGC TCACACCTCT AACTCCAGCA

>Bgl2
3760
* * * * *
CTTTGGGAGA CCAAAGTGGG AAGATCATTT GAGTCCAGGA GTTCGAGACC AGCCTGGACA ACATAGCAAG ATCTTATCTC

>Dra1
|
>Aha3
|
3840
* * * * *
TACAGAAAAT TTAAAAAAA GAAAAAACT AGCCGCACAG GTCTGCAGTC CTAGCTACTC GGGAGGCTAA GGTGGGAGAA

>Pst1
|
3920
* * * * *
TCCTTGAACC CAGGGATTTA GTTTGAGGTT GCAGTGAGCT ATGATTGCAC CACTGCACTC CAGACTGGGT GACTGAGTGA

4000
* * * * *
GACCCTGTCT CAAATATAAA GAAGGAACAA GTTCTAGTTT TCAATAGCGC AATAGGGTGA GTGCAGTTAG CAACAACATA

4080
* * * * *
TTGTGTATTT CAAAATAGCT ACAAGAGAGG ATATGAAGTG TTCCCCCAA CAAGGAATGA TAACGTTCTGA GGTGACAGAT

4160
* * * * *
ACCTTAAATA CCCTGATTTG ATCATTACAC ATTCAATGTA TGTATCAAAA TATTACATGT ACCCCACAAA TTTGTGTAAA

>Dra1
|
>Aha3
|
4240
* * * * *
TATTATGTAT CCACTTTTTA AAGTTGGCAG AGCCCAAAG CACTACTATG GCTTCCAGTG GTCAGTGTGA GCACTGCCAG

FIG. 7E

SUBSTITUTE SHEET (RULE 26)

4320
* * * * *
CTCAGCAAAT GTATCACCCA AAATCTGGGC AATGTGGGAA ATTGGCTTCA TGGCAGCTAT GGCTTTGCCA CTGATAGGAA
>Bcl1
|
4400
* * * * *
TGATTTCCAG AGATACTTAA TCCTCAATTC GGGACTCTTT GCTTCAGGAG TTTGGCTGGC CAGGAACATG AGTGACAGTG
>Pvu2 >Xba1
| |
4480
* * * * *
ACCTCTTGGC ACTTCAGCTG GGGGTGTAGC CAAGCAGACA AATGGAATCT TGTGCTGAAC CCAAACCTTC TAGAAACAGA
>Pvu2
|
4560
* * * * *
GCCTGTGAGC ATCACAAGAT ATGCCCTGAT GGAAGCTGAA GTTTAATTCA GCTGAGCGCT TGCCCCTTTC CAACCTGGTT
>Bcl1
|
4640
* * * * *
TCTTTTGTGTT CCTTGAGTCC AGTCAGAATG CCATTCCCTG GCCAGCAGCC AGCCTTTAGT GACTGTCTCT GTTCTGCAAA
>Pst1
|
4720
* * * * *
GCTCTGTATA TAGTTACTGA GTTTCTGCAG GGGGTGATCT TTGCTCTTGT CCTAAGAAAT AACTACAGTG TTTTAAGAAA
4800
* * * * *
TATTTGAGGC CGGGTGCAGT GGTTCACACC TGTAATCCAG CACTTTGGGA GGCCAAGGCA GGTGGATCAT GAGGTCAAGA
>Bcl1
|
4880
* * * * *
GTTTGAGACC ATCATGGCCA ACATGGTGAA ACCCCATCTC TACTAAAAAT AAAAAATTA GCTGGGTGTG GTGGCGGGCA
>EcoR5
|
4960
* * * * *
CCTGTAGTCC CAGCTACTCG GGAGGCTGAG GCAGGAGAAT CGCTTGAGCC TGGGAGGCGG AGGTTGCACT GAGCCGATAT

FIG. 7F

22/26 ,

5040
* * * * *
CACGCCACTG CACTCCAGCC TGGCGACAGA GCGAGACTCC ATCTCAAAAA AAAGAAAAAA TAAATAGTTG AAATAAAGAC

>Apa1
5120
* * * * *
TGCACATAAA GACAAAAAAA AAGTTTATAA AGTTAAAAAA TAAATAAAAA AACAGGCTCC AGGCTGGATT GGGCCCAGAG

5200
* * * * *
GCTGTAGGAC ACAGACCCCC AGCCAATGAC TTCATAAATC CGGATGTTAA TCAGCCTCAC CTGGGAATTT GGGGAGGGGA

>Aha3
|
>Dra1
|
5280
* * * * *
CTCATTTTAA AACAGTTTCC TGGATTCTAA CCCAACCCAG AAAATCAGAC TCTTTGAGCT AAATTCTTAA GCTCCCTGGT

>Af12
|
5360
* * * * *
GATGATGATG GAACCAGTTT ATGGCTGACC CCAGAGTACC GTCTGAAAGA CGTGCCACAT CCCTCTCTCT CCAGCCTCCC

>EcoR1
|
* *
CTTCTCCTCC ATTCCCCAGG GAGAATTC

FIG. 7G

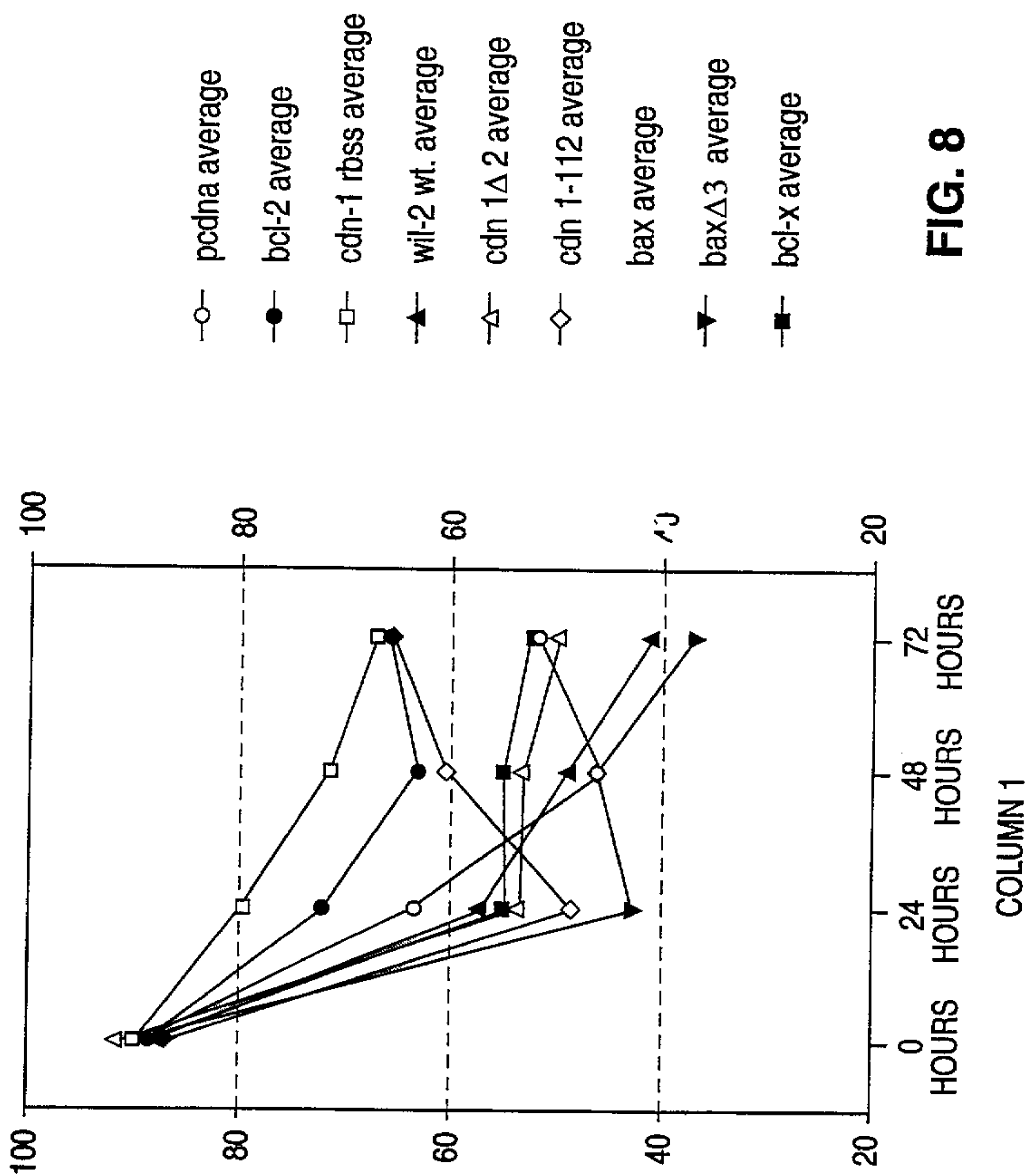


FIG. 8

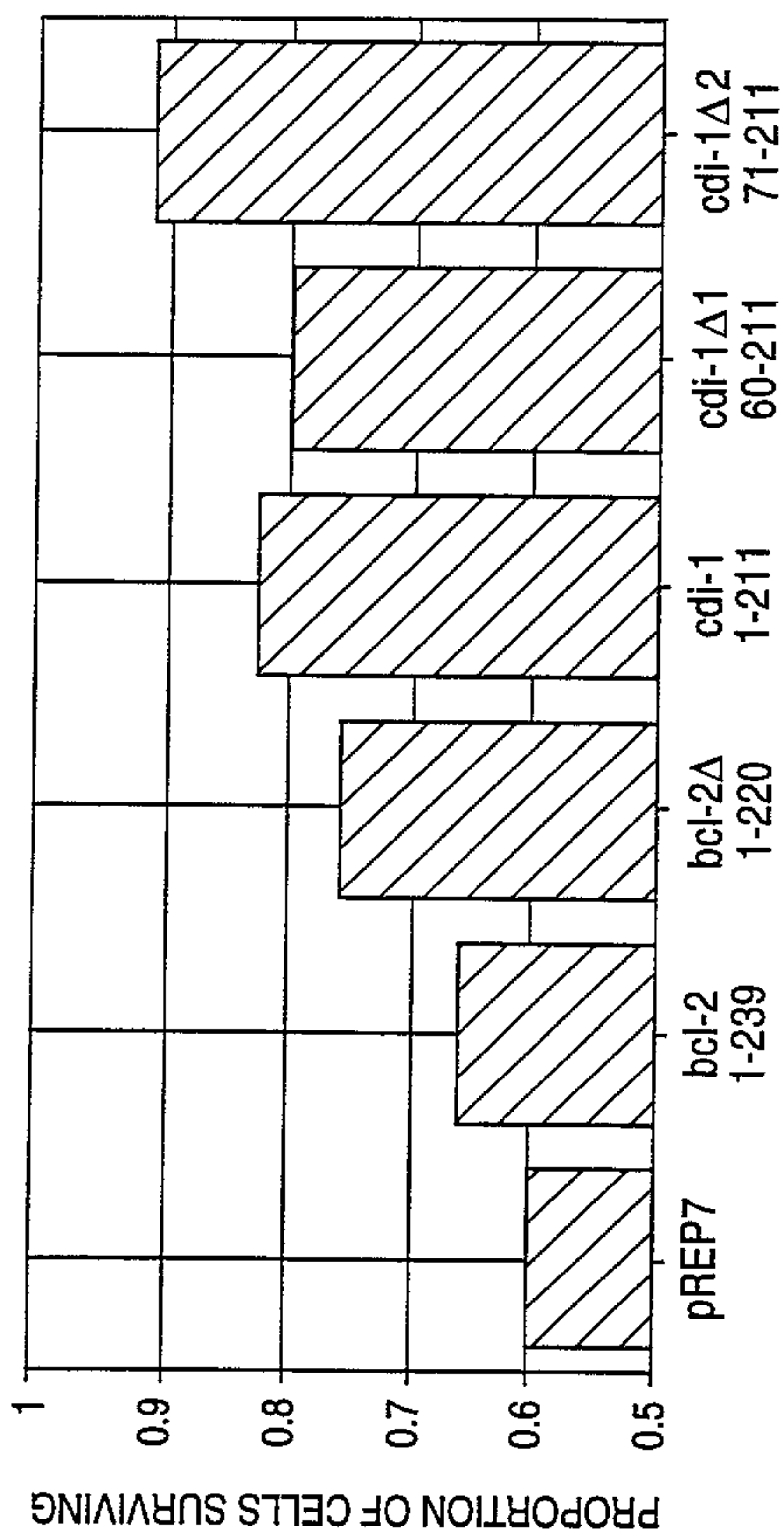
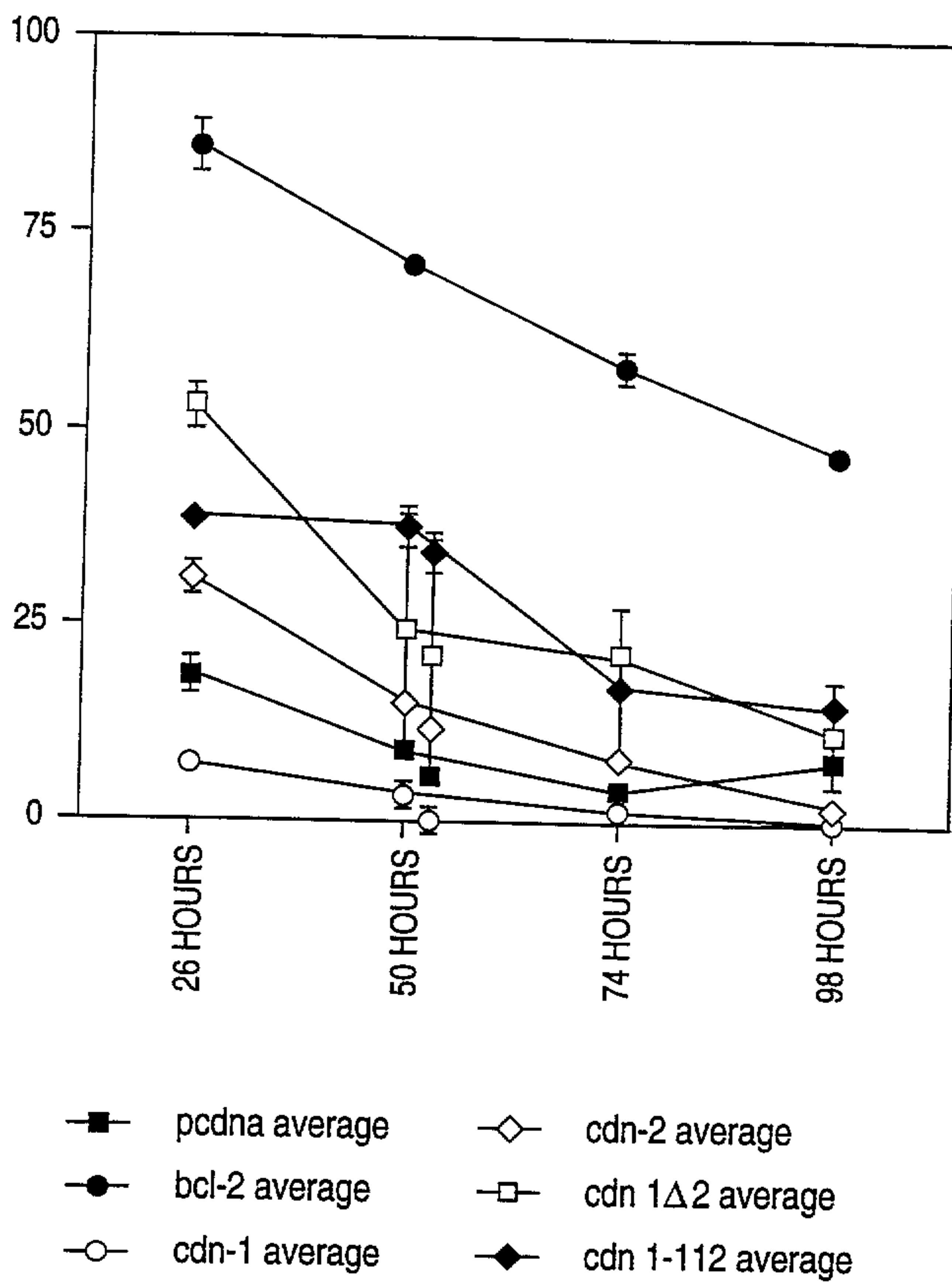


FIG. 9

**FIG. 10**

MASGQGP GPPRQECGEPALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEGVAAPADPEMVT^{Δ1}
LPLQPSSTMGQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESGNWGR^{Δ2}
VVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVDFMLHHCIARWIAQRGGWVAALNLGNGPILN^{Δ3}
VLVVLGVVLLGQFVVRFFKS

FIG. 11