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(54) Title: A NOVEL NUCLEAR MITOTIC PHOSPHOPROTEIN: MITOSIN

(57) Abstract

A novel purified phosphoprotein designated mitosin is provided by this invention. Also provided is the amino acid sequence and nucleic acid molecule corresponding to mitosin protein. Diagnostic and therapeutic methods using the protein and nucleic acid molecule also are provided. A nucleic acid molecule encoding mitosin also is provided by this invention, as well as active fragments thereof. The nucleic acid molecules are useful to recombinantly produce mitosin and for use as probes. The compositions and methods of this invention are based on the instant discovery that the intracellular presence of mitosin is necessary for a eukaryotic cell to enter into the M phase of mitosis, and that the degradation of mitosin is necessary for the cell to advance to the next stage. Thus, an anti-mitosin antibody, a mutant or a non-functional analog of mitosin would inhibit the mitotic cell cycle by preventing cells from entering the M phase, and overexpression of mitosin, or a functional equivalent thereof, would inhibit the cycle by preventing cells from leaving the M phase. Such overexpression could be achieved either by addition of the protein or through gene therapy, i.e. delivery of a gene encoding the protein or a functional equivalent thereof.

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A NOVEL NUCLEAR MITOTIC PHOSPHOPROTEIN

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of U.S. Serial No. 08/141,239, filed October 22, 1993, the contents of which are hereby incorporated by reference into the present disclosure.

This invention made in part with government support under a grant from the National Institute of Health (EY05758). Accordingly, the United States Government has certain rights to this invention.

Throughout this application, various publications are referred to within parentheses. The disclosures of these references are hereby incorporated by reference into the specification.

The events that occur from one cell division to the next are deemed the cell cycle. The cell cycle is comprised of the mitotic phase (M-phase), cytokinesis (cell separation), G_1 or gap phase, the synthesis or S-phase and finally G_2.

The control of cell division is one of the most basic aspects of multicellular existence. Uncontrolled cell growth and division, which produces cells that divide when they should not, produces contiguous cellular masses called tumors that are the basis for many cancers.

Thus, information concerning the mechanisms to control or promote cell division and proliferation is important to understand and conquer many diseases including cancer. This invention provides this information and provides related advantages as well.

SUMMARY OF THE INVENTION

A novel purified protein designated mitosin is provided by this invention. Also provided are biologically active fragments of mitosin. Methods of using the mitosin protein and fragment, e.g., for the generation of monoclonal antibodies, also is provided.

A nucleic acid molecule encoding mitosin also is provided by this invention, as well as active fragments thereof. The nucleic acid molecules are useful to recombinantly produce mitosin and for use as probes.

The compositions and methods of this invention are based on the instant discovery that the intracellular presence of Mitosin is necessary for a eukaryotic cell to enter into to the M phase of mitosis, and that the degradation of Mitosin is necessary for the cell to advance to the next stage. Thus, an anti-mitosin antibody, a mutant or a non-functional analog of mitosin would inhibit the mitotic cell cycle by preventing cells from entering the M phase, and overexpression of mitosin, or a functional equivalent thereof, would inhibit the cycle by preventing cells from leaving the M phase. Such overexpression could be achieved either addition of the protein or through gene therapy, i.e. delivery of a gene encoding the protein or a functional equivalent thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Mitosin mRNA is Expressed in a Cell-cycle Dependent Manner.

(A) RNA blotting analysis using mitosin, RB, E2F-1 and Gß-like cDNA as probes. Monkey kidney CV1 cells were synchronized as described in the Experimental Procedures. 10 μg of total RNA extracted from each sample was subjected

to Northern blotting with radioactively labeled cDNAs as indicated. The level of Gß-like RNA varies very little during the cell-cycle, and thus served as an internal control. Lane 1 is RNA from cells arrested in early G1 by lovastatin (marked as 'GO/G1'). Lane 2 is cells in late G1 after removing lovastatin for 8 hours. Lane 3 is cells arrested at the G1/S boundary by hydroxyurea. Lane 4 is cells in the S phase, after removing the hydroxyurea and nocodazole double block. Lane 5 is mitotic cells collected after the hydroxyurea and nocodazole double block. Lane 6 is G0 cells after 4 days of serum starvation (0.5% fetal calf serum). (B) Quantitation of relative mRNA levels of each gene by densitometry. Each individual mRNA band was normalized to the amount of Gß-like mRNA to show the expression pattern during the cell-cycle.

Figure 2: Mitosin Migrates as a 350 Kd Cellular Protein.

$1x10^6$ actively growing HeLa cells were immunoprecipitated with each antibody to mitosin (α10Bgl, α10Stu, or α10C). After 3-12% gradient SDS-PAGE, the immunoprecipitates were immunoblotted with the same antibody in the absence (-) or presence (+) of the corresponding antigen competitor or maltose binding protein (MBP) (10 μg/ml) to demonstrate the specificity. Antigens used for production of antibodies and also as competitors are shown in the figure. A sample prepared from rabbit backbone muscles was also loaded side by side. The position of nebulin in this muscle sample, serving as a 770 Kd marker, was visualized by immunoblotting with mAb to nebulin (Kruger et al., J. Cell Biol. 115:97-107 (1991)).

Figure 3: Mitosin Redistributes from the Nucleus to the Centromere, Spindle, and Midbody during M phase Progression.

Monkey kidney CV1 cells were grown directly on glass coverslips. After methanol fixation and labeling with α10C and FITC-conjugated anti-rabbit IgG antibody, indirect immunofluorescence microscopy was performed with a laser-scanning confocal microscope. Digitized optical sections (red) and Normarski differential interference contrast (DIC) images (green) were superimposed or recorded separately. (A1-A4) demonstrates that immunostaining of mitosin was not affected by MBP (10 μg/ml) (A1), but was abolished by the same amount of antigen MBP-10 (A3). (A2) and (A4) represent the corresponding DIC images. (B1-B8) A representative cell from different stages of the cell-cycle: (B1) S or G2 phase; (B2) late G2/ early prophase; (B3) prophase; (B4) metaphase; (B5, B6) anaphase; (B7, B8) telophase; (C1-C8) depicts the centromeric staining of mitosin. The centromeric staining was not affected by MBP (10 μg/ml) (C1), but was abolished by MBP-10 (C3). (C2) and (C4) are the corresponding DIC images. Immunostaining of mitosin (C5) with mitotic cells spun onto cover slips was superimposed with the corresponding chromosome staining by propidium iodine (C6) and DIC image (C7) to confirm its centromeric localization (C8). The non-centromeric background in (C5) and (C8) was due to the cytoplasmic portion of mitosin.

Figure 4: Expression and Modification of Mitosin during the Cell Cycle.

Cell lysates prepared from synchronized CV1 as described in Experimental Procedures were analyzed by Western blotting. The appropriate portions of a blot (according to the MW of proteins to be probed) were separately probed with anti-mitosin α10C (A), anti-Rb mAb 11D7 (B), or anti-Gß-like protein antibody (C). The phosphorylation status of Rb serves as an internal control for the quality of synchronization. Gß-like protein expressed constantly

during the cell cycle was used as an internal control for quantitation of cell lysates. (D) Cell-cycle distribution of the corresponding samples analyzed by flow cytometry to show the status of cell cycle progression.

Figure 5: Modification of Mitosin by Phosphorylation.

(A-B) The results from immunoblotting and autoradiography of the same blot, respectively. Lanes 1 and 4 are mitosin prepared from cells arrested at G1/S by hydroxyurea. Lane 2 is mitosin immunoprecipitated from cells in late S phase, labeled with (32P)-orthophosphate. Lane 3 is the same sample that was treated with calf intestinal alkaline phosphatase (CIAP). (C) The slowest migrating form of mitosin (lane 5) can be converted to the fastest migrating form by CIAP treatment (lane 6).

The results shown in Figure 5A through 5C show that regulation of mitosin phosphorylation is critical to its function, as is the case for $p110^{RB}$ (Ludlow et al., Cell 56:57-65 (1989)). These results also suggest that mutants of mitosin that cannot be phosphorylated are useful as cell growth inhibitors; and that reagents which block phosphorylation of mitosin would have similar activity. Either protein or gene therapy can be used to utilize these agents to inhibit cell growth. Because of the importance of mitosin in chromosome segregation, inhibitors of mitosin are useful to block gametogenesis.

Figure 6: Mitosin Interacts with Rb in M Phase.

$3x10^6$ CV1 cells synchronized at prometaphase by nocodazole was lysed and coimmunoprecipitated with either α10Bgl (lane 1), anti-Rb mAb 11D7 (lanes 3 and 5), or a control antibody anti-GST (lanes 2 and 4). The immunoprecipitates were analyzed by immunoblotting with α10Bgl (A) and anti-Rb mAb

11D7 (B). Immunoprecipitates in lanes 2 and 3 were washed three times, while those in lane 4 and 5 were washed five times.

Figure 7: Determination of the Rb-binding Region of Mitosin.

(A) Two identical blots containing seven purified MBP fusion proteins were probed with either the "Rb-sandwich" (lanes 1-7) or with an antibody to MBP (New England Biolabs) (lanes 8-14). Only those fusion proteins sharing the extreme C-terminal region of 211 amino acid residues of mitosin bound to the "Rb sandwich". The faint band in lane 3 (MBP-10/H) is an artifact because it reproducibly migrates faster than the full-length product (lane 10) does. Lanes 1 and 8 were MBP-T antigen served as positive control, lanes 2 and 9 were MBP-10, lanes 3 and 10 were MBP-10/H, lanes 4 and 11 were MBP-10/NB, lanes 5 and 12 were MBP-10/KN, lanes 6 and 13 were MBP-10/NI, and lanes 7 and 14 were MBP alone. (B) Similarly, two identical blots of the purified GST-fusion proteins were probed with either the "Rb-sandwich" (lanes 15-17) or with an antibody to GST (lanes 18-20). Lanes 15 and 18 were GST-T antigen, lanes 16 and 19 were GST alone and lanes 17 and 20 were GST-1045 which bound to the Rb-sandwich. (C) A diagram of the different constructs used in this experiment. The Rb-binding ability for each fusion protein is also included. (D) Sequence comparison of the Rb-binding region of mitosin with the Rb-binding domain of E2F-1 (in bold letters) and neighboring residues. A 51% homology between these two sequences was found. Dashed lines indicate conserved residues and solid lines indicate identical residues.

Figure 8: Cloning and Sequence Analysis of Mitosin.

(A) A schematic diagram for overlapping cDNA clones and the full-length cDNA. Solid bars represent untranslated regions. Clones AP10 and AP1 were isolated by the "Rb-sandwich" method. Clone ClaI, BlaI and 100 were isolated from a Y79 cDNA library. The rest were from a K562 cDNA library. (B) Nucleotide and deduced amino acid sequence of mitosin. The putative leucine heptad repeats are underlined; conserved leucines are boxed. The basic residues, presumably representing a bipartite nuclear targeting motif, are circled. The polyadenylation signal at the end of the cDNA is boxed. (D) Alignment for the internal repeats. Identical residues are connected by bars, similar ones by dots.

Figure 9: Schematic Diagram of Mitosin Expression Constructs.

The episomal vector pCEP4 (Invitrogen) was used for initial construction. Each construct is assigned with a letter (A-E) for easier description in text. Both the predicted subcellular location (N- Nuclear; C- Cytoplasmic) and the predicted reactivity of expressed proteins to α10C antibody are listed.

Figure 10: Transient Expression of Mitosin in CV1 Cells.

Cells growing in 100 mm Petri dishes were methanol-fixed three days after transfection, and subjected to triple fluorescence staining. The flag epitope was stained red (by Texas red; panel A1-E1), the extreme C-terminus of mitosin stained green (by FITC; panel A2-E2), and nuclear DNA stained blue (by DAPI; panel A3-E3). Panels (A)-(E) are representative results from cells expressing the

constructs "A"-"E" sequentially. The abnormally divided cells with chromatin bridges in panel (B3), (C3) and (D3) are indicated by arrows. Scale bar: 20 μm.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a novel, purified mammalian protein designated mitosin. Mitosin has a molecular weight of about 350 kD as determined by Western blot analysis. Mitosin is a cellular protein which has been found to interact with the retinoblastoma protein. It also is cell cycle dependent, that is, it is first synthesized at the G1/S boundary, phosphorylated from S through M phase, and during mitosis, is intimately associated with centromeres/kinetochores and the spindle poles. Mitosin has many of the properties previously described which characterize transcriptional regulatory proteins (Buchkovich et al., Cell 58:1097-1105 (1989); Chen et al., Cell 58:1193-1198 (1989); deCaprio et al., Cell 58:1085-1095 (1989); Ludlow et al., Cell 60:387-396 (1990); and Mihara et al., Science 246:1300-1303 (1989)).

The compositions and methods of this invention are based on the instant discovery that the intracellular presence of Mitosin is necessary for a eukaryotic cell to enter into to the M phase of mitosis, and that the degradation of Mitosin is necessary for the cell to advance to the next stage. Thus, an anti-mitosin antibody, a mutant or a non-functional analog of mitosin would inhibit the mitotic cell cycle by preventing cells from entering the M phase, and overexpression of mitosin, or a functional equivalent thereof, would inhibit the cycle by preventing cells from leaving the M phase. Such overexpression could be achieved either addition of the protein or through gene therapy, i.e. delivery of a gene encoding the protein or a functional equivalent thereof.

This protein has been purified from both simian and human sources. "Purified", when used to describe the state of mitosin, denotes the protein free of a portion of the other proteins and molecules normally associated with or occurring with mitosin in its native environment. As used herein the term "native" refers to the form of a protein, polypeptide, antibody or a fragment thereof that is isolated from nature or that which is without an intentional amino acid substitution. Generally, antagonists of mitosin function would be expected to block cell growth; and the presence of mitosin in a cell is an index of proliferation--an important indicator of hyperproliferative diseases, such as cancer.

Thus, antagonists of the novel protein is useful to control pathologic hyperproliferative cells. As used herein, the term "hyperproliferative cells" includes but is not limited to cells having the capacity for autonomous growth, i.e., existing and reproducing independently of normal regulatory mechanisms. Hyperproliferative diseases may be categorized as pathologic, i.e., deviating from normal cells, characterizing or constituting disease, or may be categorized as non-pathologic, i.e., deviation from normal but not associated with a disease state. Pathologic hyperproliferative cells are characteristic of the following disease states, thyroid hyperplasia - Grave's Disease, psoriasis, benign prostatic hypertrophy, Li-Fraumeni syndrome including breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, various leukemias and lymphomas. Examples of non-pathologic hyperproliferative cells are found, for instance, in mammary ductal epithelial cells during development of lactation and also in cells associated with wound repair. Pathologic hyperproliferative cells characteristically exhibit loss of contact inhibition and a decline in their ability to selectively adhere which implies a change in the surface properties of the cell and

a further breakdown in intercellular communication. These changes include stimulation to divide and the ability to secrete proteolytic enzymes. Moreover, reintroduction or supplementation of lost mitosin function by introduction of the protein or nucleic acid encoding the protein into a cell can restore defective chromosome segregation, which is a marker of progressing malignancy. Malignant proliferation of cells can then be halted.

As is known to those of skill in the art, the term "protein" means a linear polymer of amino acids joined in a specific sequence by peptide bonds. As used herein, the term "amino acid" refers to either the D or L stereoisomer form of the amino acid, unless otherwise specifically designated. Also encompassed within the scope of this invention are equivalent mitosin proteins or equivalent mitosin peptides, having the biological activity of purified mitosin. "Equivalent proteins" and "equivalent polypeptides" refer to compounds that depart from the linear sequence of the naturally occurring proteins or polypeptides, but which have amino acid substitutions that do not change its biologically activity. "Biological activity" shall mean having the ability to bind to the retinoblastoma protein under native conditions. These equivalents can differ from the native sequences by the replacement of one or more amino acids with related amino acids, for example, similarly charged amino acids, or the substitution or modification of side chains or functional groups.

It is further understood that limited modifications may be made to the primary sequence of mitosin without destroying its biological function, and that only a portion of the entire primary structure may be required in order to effect activity, one aspect of which is the ability to bind $p110^{RB}$. One such biologically active fragment is a molecule having substantially the C-terminal

region of about 600 amino acid residues of the molecule, the sequence of which is shown in Figure 1. Another biologically active fragment is a molecule having substantially the C-terminal region of about 200 amino acid residues of the molecule, the sequence of which is shown in Figure 1. As is understood by those of skill in the art, any fragment having at least the C-terminal 200 amino acids up to about the C-terminal 600 amino acids are biologically active fragments of mitosin. Minor modifications of this sequence which do not destroy the activity of the protein also fall within the definition of mitosin and within the definition of the protein claimed as such. Moreover, fragments of the amino acid sequence shown in Figure 1, but not including the previously described 600 to 200 amino acid fragments, which retain the function of the entire protein are included within the definition. These fragments can be generated by restriction enzyme digestion of the nucleic acid molecule of Figure 1 and recombinant expression of the resulting fragments. It is understood that minor modifications of primary amino acid sequence can result in proteins which have substantially equivalent or enhanced function as compared to the sequence set forth in Figure 1. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental such as through mutation in hosts which are mitosin producers. All of these modifications are included as long as mitosin biological function is retained.

"Inhibitively active" also shall mean fragments and mutants of the mitosin protein ("muteins") that act in a dominant negative fashion thereby inhibiting normal function of the protein, thereby inhibiting the ability of mitosin to mediate host cell division and/or host cell proliferation. These can be, but are not limited to, non-phosphorylated proteins or proteins phosphorylated such that cell proliferation of the host cell is inhibited. These proteins and fragments can be made by expressing the

nucleic acid of the mitosin protein in a bacterial host cell that lacks the ability to phosphorylate or by chemical means well known to those of skill in the art. The muteins and inhibitively active fragments are useful therapeutically to inhibit hyperproliferation of cells and to generate diagnostic reagents such as anti-mitosin antibodies.

This invention also provides agents that inhibit phosphorylation of mitosin in a cell. These agents include, but are not limited to calf intestine alkaline phosphatase and other regulatory phosphatases. These agents are useful to inhibit the growth or proliferation of a cell by contacting the cell, in vitro or in vivo with the agent by methods described below. Accordingly, this invention also provides a method to inhibit the growth or proliferation of a cell, such as a hyperproliferative cell, by contacting the cell with the agent. Also provided are methods of treating pathologies characterized by hyperproliferative cell growth, such as cancer, by administering to a suitable subject these agents in an effective concentration such that cell proliferation is inhibited. A suitable subject for this method includes but is not limited to vertebrates, simians, murines, and human patients.

These agents also are useful to block gametogenesis of an immature gamete by contacting the cell, in vitro or in vivo with the agent by methods described below.

Pharmaceutical compositions comprising any of the compositions of matter described above and one or more pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are well known in the art and include aqueous solutions such as physiologically buffered saline or other solvents or vehicles such as glycols, glycerol,

vegetable oils (eg., olive oil) or injectable organic esters. A pharmaceutically acceptable carrier can be used to administer the mitosin or its equivalent proteins, fragments or mutants thereof to a cell *in vitro* or to a subject *in vivo*.

A pharmaceutically acceptable carrier can contain a physiologically acceptable compound that acts, for example, to stabilize the polypeptide or to increase or decrease the absorption of the agent. A physiologically acceptable compound can include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives, which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, for example, phenol and ascorbic acid. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the polypeptide and on the particular physio-chemical characteristics of the specific polypeptide. For example, a physiologically acceptable compound such as aluminum monosterate or gelatin is particularly useful as a delaying agent, which prolongs the rate of absorption of a pharmaceutical composition administered to a subject. Further examples of carriers, stabilizers or adjutants can be found in Martin, Remington's Pharm. Sci., 15th Ed. (Mack Publ. Co., Easton, 1975), incorporated herein by reference. The pharmaceutical composition also can be incorporated, if desired, into liposomes, microspheres or other polymer matrices (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, Florida 1984), which is incorporated

herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

Purified mitosin or mitosin pharmaceutical compositions are useful to control the growth of a cell by contacting the cell with the purified mitosin, active fragment or composition, containing these polypeptides or proteins.

For the purposes of this invention, the contacting can be effected in vitro, ex vivo or in vivo. When the cells are inhibited in vitro, the contacting is effected by mixing the composition or protein of this invention with the cell culture medium and then feeding the cells or by directly adding the composition or protein to the culture medium. Methods of determining an effective amount are well known to those of skill in the art.

This method also is useful to treat or prevent pathologies associated with abnormally proliferative cells in a subject in vivo. Thus, when the contacting is effected in vivo, an effective amount of the composition of this invention is administered to the subject in an amount effective to inhibit the proliferation of the cells in the subject. An effective amount of the pharmaceutical composition comprising described above is generally in the range of about 0.01 to 100 mg/kg body weight. An effective amount can be determined using methods known to those in the art. The total effective amount can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which the multiple doses are administered over a more prolonged period of time. One skilled in the art would know that the amount of compositions of this invention

required to obtain an effective dose in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. For the purpose of this invention, "subject" means any vertebrate, such as an animal, mammal, human, or rat.

Methods of administering a pharmaceutical are well known in the art and include but are not limited to administration orally, intravenously, intramuscularly or intraperitoneal. Administration can be effected continuously or intermittently and will vary with the subject as is the case with other therapeutic recombinant proteins (Landmann et al., J. Interferon Res. 12(2):103-111 (1992); Aulitzky et al., Eur. J. Cancer 27(4):462-467 (1991); Lantz et al., Cytokine 2(6):402-406 (1990); Supersaxo et al., Pharm. Res. 5(8):472-476 (1988); Demetri et al., J. Clin. Oncol. 7(10:1545-1553 (1989); and LeMaistre et al., Lancet 337:1124-1125 (1991)).

Isolated nucleic acid molecules which encode amino acid sequences corresponding to the purified mammalian mitosin protein, mutein, active fragments thereof, otherwise referred herein as "equivalent proteins" or "equivalent polypeptides" and anti-mitosin antibody are further provided by this invention. As used herein, "nucleic acid" shall mean single and double stranded DNA, cDNA and mRNA. In one embodiment, this nucleic acid molecule encoding mitosin protein and fragments has the sequence or parts thereof shown in Figure 1. Also included within the scope of this invention are nucleic acid molecules that hybridize under stringent conditions to the nucleic acid molecule or its complement, for example, the sequence of which is shown in Figure 1. Such hybridizing nucleic acid molecules or probes, can by prepared, for example, by nick translation of the nucleic acid molecule of Figure 1, in which case the hybridizing nucleic acid

molecules can be random fragments of the molecule, the sequence of which is shown in Figure 1. For methodology for the preparation of such fragments, see Sambrook et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), incorporated herein by reference. Nucleic acid fragments of at least 10 nucleotides are useful as hybridization probes. Isolated nucleic acid fragments also are useful to generate novel peptides. These peptides, in turn, are useful as immunogens for the generation of polyclonal and monoclonal antibodies. Methods of preparing and using the probes and immunogens are well known in the art.

The nucleic acid sequences also are useful to promote cell division and proliferation of a cell. The nucleic acid molecule is inserted into the cell, the cell is grown under conditions such that the nucleic acid is encoded to mitosin protein in an effective concentration so that the growth of the cell is inhibited. For the purposes of this invention, the nucleic acid can be inserted by liposomes or lipidated DNA or by other gene carriers such as viral vectors as disclosed in Sambrook et al., supra, incorporated herein by reference.

For the purpose of illustration only, a delivery system for insertion of a nucleic acid is a replication-incompetent retroviral vector. As used herein, the term "retroviral" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. As used herein, the terms "replication-incompetent" is defined as the inability to produce viral proteins, precluding spread of the vector in the infected host cell.

Another example of a replication-incompetent retroviral vector is LNL6 (Miller, A.D. et al., BioTechniques 7:980-990 (1989)). The methodology of using

replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll, P.H. et al., PNAS USA 86:8912 (1989); Bordignon, C. et al., PNAS USA 86:8912-52 (1989); Culver, K. et al., PNAS USA 88:3155 (1991); Rill, D.R. et al., Blood 79(10):2694-700 (1991)). Clinical investigations have shown that there are few or no adverse effects associated with the viral vectors (43: Anderson, Science 256:808-13 (1992)).

Other vectors are suitable for use in this invention and will be selected for efficient delivery of the nucleic acid encoding the mitosin genes, or the fragments or mutants thereof. The nucleic acid can be DNA, cDNA or RNA. Such vectors include adenovirus vectors, specifically replication-deficient recombinant adenovirus vectors as described in Siegfried, W., Exp. Clin. Endocrinol., 101:7-11 (1993); Rosenfeld, M.A. et al., Cell 68:143-155 (1992); Rich, D.P. et al., Human Gene Therapy, 4:460-476 (1993); and Lemarchand, P., et al., Proc. Natl. Acad. Sci. USA 89:6482-6486 (1992).

In a separate embodiment, an isolated nucleic acid molecule of this invention is operatively linked to a promoter of RNA transcription. These nucleic acid molecules are useful for the recombinant production of mitosin proteins and polypeptides or as vectors for use in gene therapy.

This invention also provides a vector having inserted therein an isolated nucleic acid molecule described above. For example, suitable vectors can be, but are not limited to a plasmid, a cosmid, or a viral vector. For examples of suitable vectors, see Sambrook et al., supra, and Zhu et al., Science 261:209-211 (1993), each incorporated herein by reference. When inserted into a suitable host cell, e.g., a procaryotic or a eucaryotic

cell, mitosin can be recombinantly produced. Suitable host cells can include mammalian cells, insect cells, yeast cells, and bacterial cells. See Sambrook et al., supra., incorporated herein by reference.

A method of producing recombinant mitosin or mitosin fragments, by growing the host cells described above under suitable conditions such that the nucleic acid encoding mitosin or its fragment, is expressed, is provided by this invention. Suitable conditions can be determined using methods well known to those of skill in the art, see for example, Sambrook et al., supra, incorporated herein by reference. Proteins and polypeptide produced in this manner also are provided by this invention.

Also provided by this invention is an antibody capable of specifically forming a complex with mitosin protein or a fragment thereof. The term "antibody" includes polyclonal antibodies and monoclonal antibodies. The antibodies include, but are not limited to mouse, rat, rabbit or human monoclonal antibodies.

As used herein, a "antibody or polyclonal antibody" means a protein that is produced in response to immunization with an antigen or receptor. The term "monoclonal antibody" means an immunoglobulin derived from a single clone of cells. All monoclonal antibodies derived from the clone are chemically and structurally identical, and specific for a single antigenic determinant.

Laboratory methods for producing polyclonal antibodies and monoclonal antibodies are known in the art, see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988), incorporated herein by reference. The monoclonal antibodies of this invention can be biologically produced by introducing mitosin or a fragment thereof into an animal, e.g., a mouse

or a rabbit. The details of this process are well known and will not be repeated here. However, basically it involves injecting a mouse, or other suitable animal, with an immunogen. The mouse is subsequently sacrificed and cells taken from its spleen are fused with myeloma cells. The results is a hybrid cell, referred to as a "hybridoma," that reproduces in vitro. The population of hybridomas is screened and manipulated so as to isolate individual clones each of which secretes a single antibody species to the antigen. Each individual antibody species obtained in this way is the product of a single B cell from the immune animal generated in response to a specific antigenic site recognized on the immunogenic substance. When an immunogenic substance is introduced into a living host, the host's immune system responds by producing antibodies to all the recognizable sites on the substance. This "shotgun" approach to producing antibodies to combat the invader results in the production of antibodies of differing affinities and specificities for the immunogenic substance. After the different hybridoma cell lines are screened to identify those that produce antibody to the desired antigen, the antibodies produced by the individual hybridoma cell lines are preferably screened to identify those having the highest affinity for the immunogenic substance stimulating their original production before selection for use in the present invention.The hybridoma cells producing the monoclonal antibodies of this invention also are provided. Monoclonal antibodies produced in this manner include, but are not limited to the monoclonal antibodies described below.

Thus, using the mitosin protein or fragment thereof, and well known methods, one of skill in the art can produce and screen the hybridoma cells and antibodies of this invention for antibodies having the ability to bind mitosin.

This invention also provides biological active fragments of the polyclonal and monoclonal antibodies described above. These "antibody fragments" retain some ability to selectively bind with its antigen or immunogen. Such antibody fragments can include, but are not limited to:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule obtained by treating with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $(Fab')_2$, the fragment of the antibody that is obtained by treating with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) SCA, defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art, see for example, Harlow and Lane, supra, incorporated herein by reference.

Specific examples of "biologically active antibody fragment" include the CDR regions of the antibodies.

Anti-idiotypic peptides specifically reactive with the antibodies or biologically active fragments thereof also are provided by this invention. As used herein, "anti-idiotyptic peptides" are purified antibodies from one species that are injected into a distant species and recognized as foreign antigens and elicit a strong humoral immune response. For a discussion of general methodology, see Harlow and Lane, supra, incorporated herein by reference.

Also encompassed by this invention are proteins or polypeptides that have been recombinantly produced, biochemically synthesized, chemically synthesized or chemically modified, that retain the ability to bind mitosin or a fragment thereof, as the corresponding native polyclonal or monoclonal antibody. The ability to bind with an antigen or immunogen is determined by antigen-binding assays known in the art such as antibody capture assays. See for example, Harlow and Lane, supra, incorporated herein by reference.

In one embodiment, the antibody is linked to a detectable agent, useful to detect the mitosin protein and fragments in a sample using standard immunochemical techniques such as immunohistochemistry as described by Harlow and Lane, supra, incorporated herein by reference.

In a separate embodiment, the antibody is administered to bind to mitosin and thereby inhibit its function within the cell. The antibody is administered by methods well known to those of skill in the art and in an effective concentration such that mitosin function is inhibited. The antibody also can be used therapeutically

to inhibit cell growth or proliferation as described above.

Another aspect of this invention is a diagnostic one, utilizing the antibodies and nucleic acid molecules of this invention are useful to detect and determine the presence of mitosin in a cell or a sample taken from a patient. Because the presence of mitosin in a cell is an index of proliferation, and thus, an important indicator of hyperproliferative disease, such as cancer, an excessive amount of mitosin is indicative of a hyperproliferative and/or pre-malignant state of the cell. Examples of the types of immunoassay such as EIA and RIA utilizing the antibodies of the instant invention are known in the art. Examples of these formats can be found, for example, in C.P. Prince, D.J. Newman, editors, Principals and Practice of Immunoassay, Stockton Press, New York, 1991, herein incorporated by reference. Nucleic acid hybridization assays utilizing the instant nucleic acids can be found in B.D. Hames and S.J. Higgins, editors, Nucleic Acid Hybridization, IRL Press, Oxford, 1991, and Larry J. Kricka, editor, Nonisotopic DNA Probe Techniques, San Diego, California, 1992.

The above-identified proteins, polypeptides, nucleic acids, antibodies, and fragments thereof are useful for the preparation of medicaments for therapy, as outlined above.

The invention will now be described in greater detail by reference to the following examples. These examples are intended to illustrate but not limit the invention.

EXPERIMENTAL PROCEDURES

EXPERIMENT I

CLONING AND SEQUENCING OF MITOSIN

Ten (10) μg of total RNA extracted from different cell lines Y79 and K562 was subjected to Northern blotting as described by Shan, B. et al., Mol. Cell. Biol., 12:5620-5631 (1992), incorporated herein by reference, using radiolabeled mitosin cDNA as a probe. The cloning of cDNAs for RB-associated proteins was performed according to the method described previously in Shan, B. et al., supra, incorporated herein by reference. Briefly, an immunocomplex ("RB-sandwich") formed by purified p56-RB, anti-RB antibody 0.47, and an alkaline phosphatase-conjugated secondary antibody was used as a probe to screen lambda-gt11 expression human cDNA libraries, while a "sandwich" without RB was used as a negative control. Two additional libraries were used for rescreening: (1) a Y79 cDNA library prepared as described in Lee, W.-H. et al. Science 235:1394-1399 (1987), incorporated herein by reference; and (2) a K562 cDNA library (a gift from Dr. M.-L. Chu at Thomas Jefferson University, Philadelphia, PA). The orientation of internal EcoRI fragments was determined by both comparing overlapping clones in appropriate regions and directly sequencing through intact EcoRI junctions. Direct sequencing also eliminates the possibility of missing other small EcoRI fragments. DNA sequencing was performed using the dideoxynucleotide termination method (Sambrook et al., supra) and the sequences were analyzed by a computer program provided by DNASTAR (Madison, Wisconsin).

EXPERIMENT II

PREPARATION OF POLYCLONAL ANTI-MITOSIN ANTIBODIES

To prepare polyclonal antibodies against mitosin, three fusion proteins with different portions of mitosin, GST-10Bgl (comprising amino acid residues 1128-1462 of Figure 1C), GST-10Stu (comprising amino acid residues 14610-1856 of Figure 1C), or MBP-10C (comprising amino acid residues 1853-2482 of Figure 1C), were generated, using vectors (Riggs, P., in Current Protocols in Molecular Biology, Ausebel, F.M. et al. (eds.), New York (1990); Smith and Johnson, Gene 67:31-40 (1988)) capable of expressing fusion protein of either glutathione S-transferase (GST) or maltose-binding protein (MBP). Animals were immunized subcutaneously, using standard procedures, with these bacterially expressed fusion proteins. Immune sera (anti-10Bgl, anti-10Stu, and anti-10C) were preabsorbed with either GSB bound to glutathione resin or MBP bound to amylose resin, depending on the source of antigen, for 1 hour at 4°C. The flow-through was then incubated for two hours at 4°C with pre-blocked (in PBS + 1% BSA) Immobilon-P membrane (Millipore) containing 500 μg fusion protein electrophoretically transferred from an SDS-polyacrylamide gel. After extensive washing with phosphate buffered saline (PBS), specific antibodies were eluted out with aliquots of 0.2 M Glycine-Hcl, pH 2.3, and the eluent was neutralized with 3 M Tris-Hcl, pH 8.0. The specifities of these antibodies were further tested by blotting analysis using corresponding antigens.

EXPERIMENT III

IDENTIFICATION OF THE MITOSIN PROTEIN

To identify the cellular gene product of mitosin, 5 X 10^5 actively growing HeLa cells were lysed directly in

boiling SDS-loading buffer and the lysate was subjected to 3-12% gradient SDS-PAGE and electrophoretic transfer to an Immobilon-P membrane (Millipore). Three adjacent lanes with the same sample were excised and each lane probed separately with one of the three antibodies. A sample prepared from rabbit backbone muscles also was loaded side by side with the HeLa cell lysate. The position of nebulin in this sample, which served as a 770 kD marker, was visualized by probing with a monoclonal antibody provided by Dr. K. Wang (University of Texas, Austin, TX), made by the method described in Kruger, M. et al. J. Cell Biol. 115:97-107 (1991), incorporated herein by reference.

EXPERIMENT IV

DISTRIBUTION OF MITOSIN DURING THE CELL CYCLE

Monkey kidney CV1 cells were grown directly upon glass coverslips and were synchronized as described below in Example V and in the description of Figure 4. Coverslips bearing samples were washed in PBS and fixed for 10 minutes in cold absolute methanol. After hydration in TBST (0.1 M Tris, pH 7.4, 0.15 M NaCl, 0.1% Tween 20), cells were blocked with TBST containing 5% dry milk (TBST-M). A one-hour incubation with rabbit anti-10C diluted in TBST-M was followed by a 30-minute incubation with goat anti-rabbit IgG conjugated with FITC (1:100) (FisherBiotech). After washing in TBST, coverslips were mounted in Permafluor (Lipshaw-Immunonon, Inc.). Laser-scanning confocal microscopy was performed with a Zeiss LSM III, equipped with Ar and HeNe lasers. Optical sections (0.25 μm) were digitized and superimposed with Normarski differential interference contrast images and recorded directly onto Ektachrome 100 35 mm film (FocusGraphics, Inc.).

EXPERIMENT V

ANALYSIS OF CELL EXTRACTS FROM SYNCHRONIZED CELL POPULATIONS BY WESTERN BLOTTING

Normal monkey kidney CV1 cells were synchronized with lovastatin, hydroxyurea and nocodazole and released for different periods of time to obtain fairly uniform populations in different cell-cycle stages. Two plates of cells growing simultaneously under identical conditions were prepared for each sample, one for immunoblotting and the other for flow cytometry. For samples released from early G1 or G1/S, 1.5 X 10^6 cells were plated per 100 mm petri dish in fresh, complete Dulbecco's modified Eagle medium (DMEM) plus 10% serum. Lovastatin (40 μM) was added for 36 hours to arrest cells in early G1 (Keyomarsi et al., Cancer Res. 51:3602-3609 (1991)); cells were then released by adding mevalonic acid lactone to a final concentration of 4 mM. For synchronization at the G1/S boundary, hydroxyurea (0.5 mM) (Adams and Lindsay, J. Biol. Chem. 242:1314-1317 (1967)) was added for 24 hours; cells were released from the arrest by washing three times with PBS. Samples were collected at different time points as noted. For samples released from nocodazole (prometaphase) block 6 X 10^6 cells were plated per 150 mm Petri dish, in the presence of hydroxyurea for 24 hours. After washing three times with PBS, medium with nocadazole (0.4 μg/ml) was added. Mitotic cells were gently shaken off 12 hours later, spun down, and resuspended in PBS. Following three more washes with PBS, aliquots of cells (1.5 X 10^6) were replated and collected again at different time points. For immunoblotting, cells were collected into PBS from one set of dishes, using rubber policemen, then spun down and directly lysed by boiling in 100 μl SDS-loading buffer. Another set was trypsinized, spun down, washed once with PBS, and resuspended in 0.3 ml of PBS. Each sample was vortexed gently while 1 ml of methanol was added dropwise

to fix cells for flow cytometry. Fixed samples were stored at 4°C until needed.

2 x 10^6 CV1 cells, released for 4 hours from G1/S boundary (hydroxyurea block), were labeled with ^{32}P orthophosphate (0.25 mCi/ml final) in DME medium supplemented with 10% dialyzed fetal bovine serum for 2 hours. Equal amount of cells were collected by mitotic shake-off after sequential double blocking with hydroxyurea and nocodazole, as described in the legend to Figure 4. These two different cell samples were lysed in cold Ab buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholate, 0.5% SDS, plus leupeptin, aprotinin, antipain, 1 μg/ml each), sonicated briefly, clarified by centrifugation, and then subjected to immunoprecipitation using saturating amount of anti-10Bgl. Immunocomplexes were precipitated by protein A-Sepharose beads. The beads were then washed twice with RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, plus protease inhibitors), once with high salt buffer (10 mM Tris, pH 7.4, 1 M NaCl, 1% NP-40, 1% deoxycholate, plus protease inhibitors), twice with Tris-buffered saline, and three times with sterile deionized water. Immunoprecipitates from samples were divided into two equal aliquots. Twenty (20) units calf intestinal alkaline phosphatase (CIA), 20 U/μl (Boehringer Mannheim Biochemica) in 25 μl working buffer (50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA) was added to one aliquot while only 25 μl buffer (without CIAP) was added to the remaining aliquot. Both fractions were incubated at 37°C for 10 minutes. Samples were boiled in SDS-loading buffer, separated by 3-12% gradient SDS-PAGE and transferred to Immobilon-P membrane. The blots were then subject to immunoblotting and/or autoradiography.

EXPERIMENT VI

DNA CONTENT ANALYSIS BY FLOW CYTOMETRY

CV1 cells were trypsinized and washed once with PBS. After fixation in 70% methanol and RNase digestion, cells were stained with propidium iodide for DNA content analysis. For two color selection, transfected cells were fixed sequentially in 70% methanol and cold absolute methanol, stained with anti-flag mAb M2 (IBI) plus FITC-conjugated secondary antibody (Fisher Biotech) before propidium iodide staining. The DNA content of both the FITC positive cells and the FITC negative cells were analyzed.

EXPERIMENT VII

CO-IMMUNOPRECIPITATION

Co-immunoprecipitation was performed following the method previously described (Durfee et al., Genes & Development 7:555-569 (1993)) using mitotic cells collected from nocodazole-synchronized CV1 populations.

EXPERIMENT VIII

PLASMID CONSTRUCTION AND TRANSFECTION

The full-length cDNA of mitosin was obtained by ligation of appropriate cDNA fragments isolated from cDNA libraries. An artificial BamH I site was inserted immediately upstream of the first ATG. All ligated junctions of cDNAs were sequenced to ensure the correct ligation and modification.

To distinguish the exogenously expressed mitosin from the endogenous form, an ATG containing sequence

encoding the flag-tag (Hopp et al., Biotech 6:1205-1210 (1988)) was inserted between the multiple cloning sites Kpn I and Hind III of pCEP4 (Invitrogen, San Diego, CA) to create pCEP4F. The full-length cDNA of mitosin (nucleotides 543-8241) was then cloned downstream of the flag epitope to make pCF-10. Other deletion mutants (pCF-10Acc, nucleotides 543-6222; pCF-10RV, nucleotides 543-3951; pCF-10NN, nucleotides 4381-6582; and pCF10Xh, nucleotides 3397-8241) were also constructed in this way using appropriate restriction sites.

Transfection was performed with CV1 cells by calcium phosphate DNA precipitation method as previously described (Shan et al., Mol. Cell. Biol. 12:5620-5631 (1992)). 10 μg of construct DNA was mixed with 10 μg of carrier DNA (pGEM-3Z, Promega, Madison, Wisconsin) for each transfection. Cells were collected or fixed three days after transfection for immunofluorescence microscopy, or flow cytometry. When colony selection was required, cells were diluted and re-plated two days after transfection. Drug-resistant colonies were selected in the presence of hygromycin B (200 μg/ml) for two weeks.

The Rb binding domain of mitosin was located by constructing and expressing fusion proteins of various sites which encompass the C-terminal domain of the peptide. For the *in vitro* Rb-binding assay, different deletion mutants were constructed from MBP-10. The 3'-coding sequence of AP10 was deleted to either the Hind III site at nucleotide position 7,427, or Nhe I site at nucleotide position 6,582 to express MBP-10/H (amino acid residues 1,853-2,296) and MBP-10/NB (residues 1853-2041). The 5'-coding sequence of AP10 was partially deleted to express MBP-10KN (residues 2014-2482) and MBP-10NI (residues 2014-2482) and MBP-10NI (residues 2271-2482), respectively. An BspH I - Nco I fragment (nucleotides 7529-7664) containing sequences homologous to the Rb-binding domain in E2F-1 was

cloned into the unique Nco I site of pGEX-PK, a vector derived from pGEX-2T (Pharmacia Biotech, Piscataway, New Jersey) to express GST-1045 (residues 2330-2375).

Expression of fusion proteins was induced by adding IPTG to a final concentration of 0.1 mM into an exponentially growing bacterial culture at 30°C. After 1 hour of induction, bacteria were collected and lysed by mild sonication. Fusion proteins were purified either by electroelution after SDS-PAGE or by affinity chromatography.

EXPERIMENT IX

INDIRECT IMMUNOFLUORESCENCE STUDIES

CV1 cells were washed in PBS and fixed for 10 minutes in cold absolute methanol or 4% paraformaldehyde in PBS for 20 min. Both fixatives resulted in the same pattern of immunostaining. After hydration in TBST (100 mM tris-HCL, pH 7.4, 150 mM NaCl, 0.1% Tween 20), cells were clocked in TBST containing 5% dry milk (TBST-M). A one hour incubation with a suitable antibody diluted in TBST-M was followed by three washed, then by another one-hour incubation with fluorochrome-conjugated second antibody (1:100) (Fisher Biotech). Nuclear DNA was then stained by DAPI (0.5 μg/ml) to indicate different stages of M phase. Competition experiments were performed by including competitors (GST-10Bgl, GST-10Stu, MBP-10, or MBP; 10 μg/ml) to dilute antibodies. Chromosome spreads were prepared by centrifuging KCl-swollen CV1 cells onto cover slips (Earnshaw et al., J. Cell Biol. 98:352-357 (1984)) and then processed as described above, except that chromosomal DNA was stained by propidium iodide (1 μg/ml; Sigma) after RNase digestion. Samples were mounted in Permafluor (Lipshaw-Immunonon, Inc.). Laser-scanning confocal microscopy was performed with Zeiss LSM 310,

equipped with Ar and HeNe lasers. Optical sections were digitized and superimposed with Normarski differential contrast images.

EXPERIMENTAL DISCUSSION

The function of Rb in cell growth and differentiation is believed to be exerted through association with the cellular proteins (Goodrich and Lee, Biochem. Biophys. Acta. 1155:43-61 (1993); Weinberg, R.A., Science 254:1138-1146 (1991)). Mitosin cDNA was obtained using the "Rb-Sandwich" method as described previously (Shan et al., Mol. Cell. Biol. 12:5620-5631 (1992). Because Rb function is modulated in concert with the cell division cycle, the expression pattern of this gene in synchronized primate cells was examined. mRNA levels of mitosin in monkey kidney CV1 cells were low in G1, gradually increased after the G1/S boundary, and peaked in M phase (Fig. 1). This expression profile differed from those of three other genes: the transcription factor E2F-1, which is predominantly expressed at the G1/S boundary; RB, which is expressed throughout the cell cycle with 3-4 fold increase during S phase (Shan et al., Mol. Cell. Biol. 14: 299-309 (1994)), and the Gß-like gene (Gullemont et al., Proc. Natl. Acad. Sci. USA 86:4594-4598 (1989)), which is expressed uniformly throughout the cell-cycle (Shan et al., Mol. Cell. Biol. 12:5620-5631 (1992)) and served as an internal control for RNA loading. Mitosin mRNA was also detected in all human tumor cell lines tested, including HeLa (cervical tumor), Molt4 (leukemia), and Saos2 (osteosarcoma), suggesting that this gene is widely transcribed in human cells.

Three distinct polyclonal antibodies against three different regions of the deduced gene product were raised in mice or rabbits using GST or MBP fusion proteins as antigens (see experimental procedures). After

purification by affinity chromatography, these antibodies, termed α10Bgl, α10Stu, and α10C, all recognized a cellular protein with molecular weight approximately 350 Kd (kilodaltons) in HeLa cells by immunoblotting (Fig. 2). Detection of this protein was specifically abolished by corresponding antigen competitors (Fig. 2). The same protein was also detected in other cell lines including monkey kidney CV1, human leukemia Molt4, and osteosarcoma Saos-2. By immunostaining (Fig. 3 A1) and subcellular fractionation, this protein was located in the nucleus. The immunostaining pattern by α10C was not affected by MBP competitor (Fig. 3 A3). Similar competition results were obtained when using other anti-mitosin antibodies, suggesting that the staining pattern by these antibodies is specific to mitosin.

Interestingly, only 20-30% of unsynchronized populations were immune-positive (Fig. 3 A1), regardless of method of fixation, suggesting that the levels of mitosin protein were also cell cycle-dependent. When CV1 cells were synchronized at early G1 by lovastatin treatment (Keyomarsi et al., Cancer Res. 51:3602-3609 (1991)) and then released for 1 hour, virtually all of the cells were negative for mitosin. When hydroxyurea-treated cells were released from arrest at the G1/S boundary (Adam and Lindsay, J. Biol. Chem. 242:1314-1317 (1967)), more than 90% exhibited nuclear staining. Cell nuclei were uniformly labeled except for nucleoli (Fig. 3 B1), a pattern which remained unchanged through the rest of interphase. The localization of mitosin, however, changed dramatically during M phase. In late G2 or early prophase, brightly staining foci began to appear (Fig. 3 B2). Following chromosome condensation, more discrete parts of fluorescent spots were observed (Fig. 3 B3). During metaphase, bright and discrete dot staining was visible on the chromosomes at the midplate, in addition to some labeling in the spindle pole regions (Fig. 3 B4). The intensity of the fluorescent

dots decreased during anaphase; the staining at the spindle region became predominant (Fig. 3 B5, B6). During telophase, the midbody was labeled while the cytoplasmic staining decreased (Fig. 3 B7, B8). Following completion of cytokinesis, no immunostaining was observed. The mitotic stages of cells described above were determined by DAPI staining of nuclear DNA.

To substantiate the mitosin is located at the centromere, chromosome spreads prepared from nocodazole-arrested CV1 cells were used for immunostaining. As shown in Fig. 3 C5-C8, mitosin was unambiguously found at the centromere region. The specificity of the centromeric staining was further confirmed by competition experiments (Fig. 3, C1-C4). Identical staining patterns were obtained with either α10Bgl or α10Stu. These results confirmed that mitosin transiently associated with the centromere in M phase.

To corroborate the immunostaining observations, synchronized cell populations by Western blotting were analyzed. Using lovastatin (Keyomarsi et al., Cancer Res. 51:3602-3609 (1991)), hydroxyurea (Adams and Lindsay, J. Biol. Chem. 242:1314-1317 (1967)), and nocodazole (Zieve et al., Exp. Cell Res. 126:397-405 (1980)), CV1 cells were synchronized at various stages of the cell-cycle. The degree of synchronization was confirmed by the expression pattern of Rb (Fig. 4 B) (Buchkovich et al., Cell 58:1097-105 (1989); Chen et al., Cell 58:1193-1198 (1989)), as well as flow cytometry (Fig. 4 D). Mitosin was virtually undetectable in G1 (lane 1-5, lane 14-16), appeared when cells entered S phase, peaked in M phase (lane 6-13) and then rapidly disappeared (lane 14-15). In contrast, the level of Gß-like protein remained unchanged throughout the cell cycle (Fig. 4 C). In addition to the difference in protein quantity, the mobility of mitosin in SDS-PAGE gradually decreased, suggesting the possibility of post-

translational modification. The appearance of the multiple, slowly migrating mitosin isoforms suggested step-wise modification between S phase and prophase of mitosis. After prometaphase (the block point of nocodazole), only the slowest migrating form was present and it disappeared rapidly thereafter.

It was speculated that the mobility change of mitosin, similar to that of RB, might be due to phosphorylation. To test this hypothesis, cells synchronized in S phase were radioactively labeled with ^{32}P orthophosphate. Mitosin was then immunoprecipitated and treated with calf intestinal alkaline phosphatase (CIAP). As shown in Figure 5B, mitosin can be labeled with ^{32}P and the labeled group can be removed by incubation with CIAP. The same gel was then blotted with an anti-mitosin antibody to show the presence of unlabeled mitosin (Figure 5A). Similar experiments were performed using cells synchronized in M phase (Figure 5C); here, the isoform of mitosin with the slowest mobility can be converted into the fast-migrating isoform by treatment with CIAP, thus proving that phosphorylation is the only cause of the mobility alternation. The existence of multiple, more slowly migrating bands implicates a difference in either the extent or the specificity of phosphorylation. The temporal pattern of mitosin phosphorylation coincides directly with its spatial reorganization, suggesting that phosphorylation may be critical for these dynamic changes. It is well known that many proteins are regulated by phosphorylation through cyclin-dependent kinase (CDK) during the cell cycle progression. Although there are no typical consensus phosphorylation sites for CDKs [i.e., (ST)PX(KR)] (Shenoy et al., Cell 57:763 (1989)) in mitosin, there are four proline-derived kinase sites [i.e., (ST)P(KR)] and five cAMP or cGMP-dependent kinase sites (Feramisco et al., J. Biol. Chem. 255:4240-4245 (1980) and Glass et al., J. Biol.

Chem. 261:2987-2993 (1986)), indicating that multiple potential sites are available for phosphorylation.

Since mitosin was isolated as a candidate Rb-associated protein, the interaction of Rb with mitosin in mammalian cells was examined. Most of the effect was placed on M phase because (1) mitosin was undetectable in G1; (2) mitosin was relatively insoluble in S phase and (3) only little or no hypophosphorylated Rb, to which cellular proteins have been shown to bind, is present in S phase. Co-immunoprecipitation with anti-Rb monoclonal antibody 11D7 was performed to test such an interaction using synchronized CV1 cells at prometaphase by nocodazole treatment. As shown in Figure 6, mitosin co-immunoprecipitated with Rb protein (Fig. 6, lanes 3 and 5). Under the similar conditions, mitosin was not detected in immunoprecipitates by monoclonal antibody against bacterial GST (Fig. 6, lanes 2 and 4).

To precisely define a region of mitosin responsible for binding to Rb, the original isolated clone, AP10, containing about an approximately 60Kd portion of the C-terminal region, was fused to maltose-binding protein (MBP) and express in *E. Coli*.. Four additional constructs containing deletion fragments of AP10 (Fig. 7, panel C), and the first 300 amino acid of simian virus 40 (SV40) large T antigen, were fused to MBP and expressed. MBP alone served as a negative control. These seven MBP fusion proteins (Fig. 7, lanes 8-14) were blotted and probed with the "Rb-sandwich" (Shan et. al, Mol. Cell. Biol. 12:5620-5631 (1992)) (Fig. 7, lanes 1-7). Only the fusion proteins containing the C-terminal 211 amino acids of mitosin bound to Rb (Fig. 7, lanes 2, 5, 6) with MBP-T antigen (lane 1) serving as a positive control. The sequence comparison indicates that amino acid residues 2328-2360 of mitosin are 51% homologous and 27% identical to the surrounding region

of the known Rb-binding domain of E2F-1 (Helin et al., Cell 70:337-350 (1992)) (Fig. 7, panel D).

To further demonstrate that this region of mitosin is sufficient to bind Rb, a mitosin fragment containing amino acid residues 2,330-2,375 was fused with glutathione S-transferase (GST) to express fusion protein GST-1045. As shown in Fig. 7, both GST-T antigen (lane 15) and GST-1045 (lane 17) bound to Rb while GST alone (lane 16) did not. Thus mitosin can bind to Rb as indicated by their co-immunoprecipitation in cell lysates as well as directly probing Western blot containing mitosin with the "Rb-Sandwich".

To further characterize mitosin, the full-length cDNA was completely sequenced and its primary amino acid sequence was deduced. Four of the cDNA clones originally isolated by the "Rb-Sandwich" screening (Shan et al. Mol. Cell. Biol. 12:5620-5631 (1992)) shared identical 3' sequences of approximately 2 Kb. A series of overlapping clones spanning 8,789 bp was isolated by multiple screens of several different cDNA libraries (Fig. 8A). The longest open reading frame (ORF) of 7,446 bp encoded an acidic protein (pI 4.8) of 2,482 amino acid residues. The existence of multiple stop codons in all three reading frames upstream of the first ATG strongly suggested that the cDNA sequence defined by these clones was full-length (Fig. 8B).

The deduced amino acid sequence of mitosin exhibits its novelty. It does not share significant homology with any known proteins in GENEBANK. Interestingly, this protein is predicted to contain a pair of highly charged tandem repeats separate by two proline residues (Fig. 8C). The first repeat (residues 1,480 to 1,657) is 62% identical to the second (residues 1,662 to 1,839). This internal repeat region is flanked by two

blocks of leucine heptad repeats (Landschultz et al., Science 240:1759-1763 (1988)). Additionally, two leucine repeats are found near the N-terminal region; the other two are found closely to the C-terminal region. The secondary structure of this protein is predicted to be mostly α-helical, except for the extreme C-terminal region of 220 residues. This C-terminal region is basic (pI 10.02), proline-rich, containing a bipartite nuclear targeting signal (Dingwall and Laskey, TIBS 16:478-481 (1991)) and the Rb-binding region.

The cell-dependent expression of mitosin and its physical association with the kinetochore/centromere suggest a role for this protein in M phase. To further substantiate this notion, full-length and truncated mutants of mitosin tagged with the flag epitope (Hopp et al., Biotech 6:1205-1210 (1988)) at their N-termini (constructs "A" to "E")(Fig. 9) were expressed in CV1 cells using the pCEP4 vector. This vector utilizes a CMV promotor to drive transcription, carries a hygromycin-resistant gene for selection, and replicates episomally (Invitrogen, La Jolla, CA). Expression of the epitope-tagged proteins was confirmed by indirect immunofluorescence with (i) mouse monoclonal anti-flag antibody and Texas Red-conjugated anti-mouse IgG secondary antibody, and (ii) rabbit polyclonal anti-mitosin antibody (α10C, recognizing the C-terminus of mitosin) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibody (Fig. 10). Both "A" and "E" fusion proteins localized to the nucleus, with additional, variable staining in the cytoplasm. All three of the C-terminus truncated mutants ("B", "C", "D"), however, were exclusively cytoplasmic, consistent with the nuclear targeting signal found in the C-terminus of mitosin (Fig. 8B).

To elucidate the effect of the overexpression on cell-cycle progression, CV1 cells were analyzed by two-

parameter flow cytometry three days after transfection. As summarized in Table 1, cell fractions with 4N DNA content (G2/M phase) were largely increased and those with S phase DNA content were variably decreased in cells expressing any of the five constructs compared to non-expressing populations. Interestingly, there was no significant difference in the percentage of G0/G1 cells expressing any of the mitosin constructs. These results suggest that the inhibition of cell growth by these proteins may be at G2/M.

TABLE 1

THE EFFECT OF TRANSIENT EXPRESSION OF MITOSIN ON THE CELL DISTRIBUTION*

Samples	Cell-Cycle Stages	G0/G1 (%)		S(%)		G2/M (%)		Percentage of Expression**
		T1	T2	T1	T2	T1	T2	
A	$FITC^+$	71	74	11	11	18	15	0.03±0.01
	$FITC^-$	72	63	20	29	8	8	
B	$FITC^+$	71	67	12	16	17	17	0.6±0.1
	$FITC^-$	67	68	24	23	9	9	
C	$FITC^+$	68	61	15	17	17	22	0.6±0.1
	$FITC^-$	68	67	24	24	8	9	
D	$FITC^+$	65	60	19	15	16	25	3.8±0.7
	$FITC^-$	70	63	22	24	8	13	
E	$FITC^+$	55	47	15	14	30	39	0.6±0.1
	$FITC^-$	68	64	22	23	10	13	

* Results from two separate experiments (T1 and T2) are listed;

** $1x10^6$ CV1 cells was transfected for each construct; Samples were collected three days post-transfection;

Cells expressing mitosin proteins are labeled $FITC^+$, and *vice versa*

Detailed microscopic analysis of these transfected cells revealed that an increased number of cells expressing exogenous mitosin "A" and "E" have larger nuclei (Fig. 10 A and E), suggesting the at the arrest may be at the stage of G2/M. On the other hand, when examining cells expressing B, C, and D, signs of improper cell division were common. In addition to an increased number of multinucleated cells (Fig. 10 B, C, and D), chromatin bridges between two newly divided cells were observed with 10-40 fold higher frequency when compared with flag-negative cells.

Inhibition of cell proliferation was examined in CV1 colonies after hygromycin-selection for 14 days (Table 2). In samples transfected with "A", "B", "C" or "E" constructs, only 1-4 flag positive cells were found in some colonies regardless of colony size. These positive cells usually were well separated form each other, implying that they were not actively dividing cells. The "D" construct caused less effect on cell division; the epitope tag was expressed in a large portion of cells in each individual colony. This result served as a useful negative control for the "A", "B", "C" and "E" constructs; furthermore, it suggested that inhibition of cell division may be a specific effect of these constructs.

TABLE 2
THE EFFECT OF ECTOPIC EXPRESSION OF MITOSIN ON CELL DIVISION

CONSTRUCT	PERCENTAGE OF COLONIES WITH TR+ CELLS	AVERAGE NO. OF TR+ CELLS PER COLONY*	AVERAGE COLONY SITE
A	20	1	≈100
B	55	2	≈70
C	73	3	≈100
D	96	33	≈55
E	31	1	≈100

* Only colonies with TR+ cells are analyzed

Recently, proteins with similar properties and functions have been cloned. For example, CENP-E, a cytoplasmic protein with a kinesin-like motor, has somewhat similar patterns of cell cycle regulation. Cells in G1 and early S phases have little detectable CENP-E, but levels of the protein increase sharply during late S and G2/M. CENP-E associates with kinetochores during congression, relocates to spindle midzones at anaphase, and is discarded or degraded at the end of cell division. CENP-E is believed to serve as an organizing center, facilitating microtubule-kinetochore interaction. Whereas CENP-E is a cytoplasmic protein equipped with both kinesin and microtubule-binding domains, mitosin, however, is a nuclear protein with tandem repeats and multiple blocks of leucine heptad repeats. Based on the immunostaining data and the potential interaction sites of mitosin, it appears that mitosin can function as a bridge to link chromosomes to kinetochores and thereby allow the chromosome to move during mitosis. If so, mitosin should interact with CENP, other centromeric proteins, and/or DNA.

Based on the expression-screening data, the C-terminal one-fourth of mitosin binds to the N-terminal truncated p56-RB protein in vitro. The C-terminal 200 amino acid residues of mitosin has been further defined to be responsible for such binding.

Although the invention has been described with reference to the above embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims that follow.

What is claimed is:

1. A purified mammalian protein designated mitosin having a molecular weight of about 350 kD.

2. A biologically active fragment of the protein of claim 1.

3. The purified mammalian protein of claim 1, wherein the mammal protein is a simian protein.

4. The purified mammalian protein of claim 1, wherein the mammalian protein is a human protein.

5. A pharmaceutical composition comprising the purified mammalian protein of claim 1 and a pharmaceutically acceptable carrier.

6. An isolated nucleic acid molecule which encodes an amino acid sequence corresponding to a mammalian mitosin protein or a biologically active fragment thereof.

7. An isolated nucleic acid molecule of claim 6, wherein the mammalian protein is a human protein.

8. An isolated nucleic acid molecule of claim 6, wherein the nucleic acid molecule is a DNA molecule.

9. An isolated nucleic acid molecule of claim 6, wherein the nucleic acid molecule is a cDNA molecule.

10. An isolated nucleic acid molecule of claim 6 wherein the nucleic acid molecule is an RNA molecule operatively linked to a promoter of RNA transcription, which in turn is encompassed in an expression vector.

11. A host vector system for the production of an amino acid molecule which is the mitosin protein or biologically active fragment thereof which comprises the vector of claim 10 in a suitable host cell.

12. A host vector system of claim 16, wherein the eucaryotic cell is a mammalian cell.

13. An antibody capable of specifically forming a complex with mitosin protein.

14. The antibody of claim 13, wherein the antibody is a polyclonal antibody.

15. The antibody of claim 13, wherein the antibody is linked to a detectable agent.

16. An agent that binds to mitosin and inhibits cellular division or proliferation.

17. The agent of claim 16, wherein the agent binds to mitosin thereby inhibiting the ability of mitosin to mediate cell division or proliferation.

18. The agent of claim 17, wherein the agent is an anti-mitosin antibody.

19. A biologically active fragment of the antibody of claim 18.

20. The agent of claim 18, wherein the anti-mitosin antibody is a polyclonal antibody.

21. The agent of claim 16, wherein the agent is a mitosin mutein that is phosphorylated unlike native mitosin.

22. An isolated nucleic acid molecule which encodes the agent of claim 16 or 21.

23. An isolated cDNA molecule which encodes an amino acid sequence corresponding to the antibody of claim 13 or a biologically active fragment thereof.

24. An isolated nucleic acid molecule of claim 23, operatively linked to a promoter of RNA transcription.

25. An expression vector which comprises the isolated nucleic acid molecule of claim 24.

26. A vector of claim 25, wherein the vector is a plasmid, a cosmid or a virus.

27. A host vector system for the production of an amino acid molecule which is an anti-mitosin antibody or a biologically active fragment thereof which comprises the vector of claim 26 in a suitable host cell.

28. A host vector system of claim 27, wherein the eucaryotic cell is a mammalian cell.

29. A method of producing recombinant mitosin, which comprises the steps of growing the host cell of claim 11 under suitable conditions such that the nucleic acid encoding mitosin is expressed and purifying the mitosin so produced.

30. A method of controlling the growth of a cell which comprises contacting the cell with the purified mitosin of claim 1 or 2.

31. A method of controlling the growth of a cell which comprises inserting into the cell the nucleic acid of claim 8, growing the cell under conditions such that the nucleic acid is encoded to mitosin protein in an effective concentration so that the growth of the cell is controlled.

32. A method of controlling the growth of a cell which comprises inserting the vector of claim 10 into the cell, growing the cell under conditions such that the nucleic acid encoding mitosin is encoded to mitosin protein in an effective concentration so that the growth of the cell is controlled.

33. A method of inhibiting division and/or proliferation of a host cell which comprises contacting the cell with the agent of claim 16 or 21.

34. A method of inhibiting division and/or proliferation of a host cell which comprises inserting the nucleic acid molecule of claim 22.

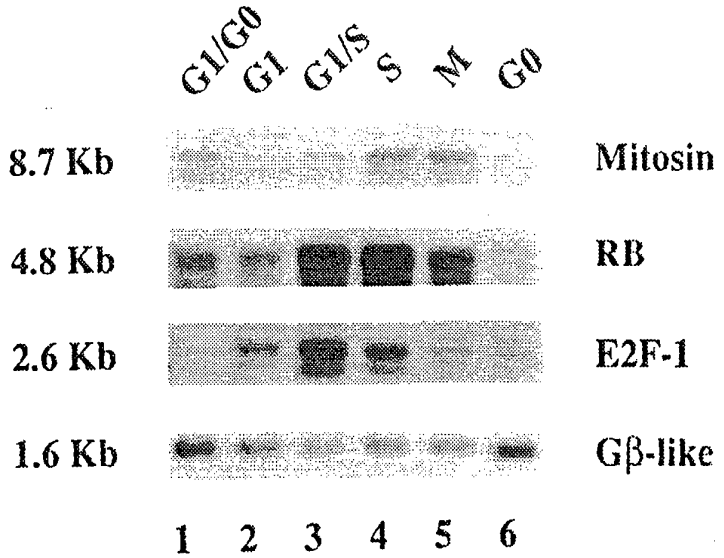

G1/G0
G1
G1/S
S
M
G0
8.7 Kb
Mitosin
4.8 Kb
RB
2.6 Kb
E2F-1
1.6 Kb
Gβ-like
1 2 3 4 5 6

FIG. IA

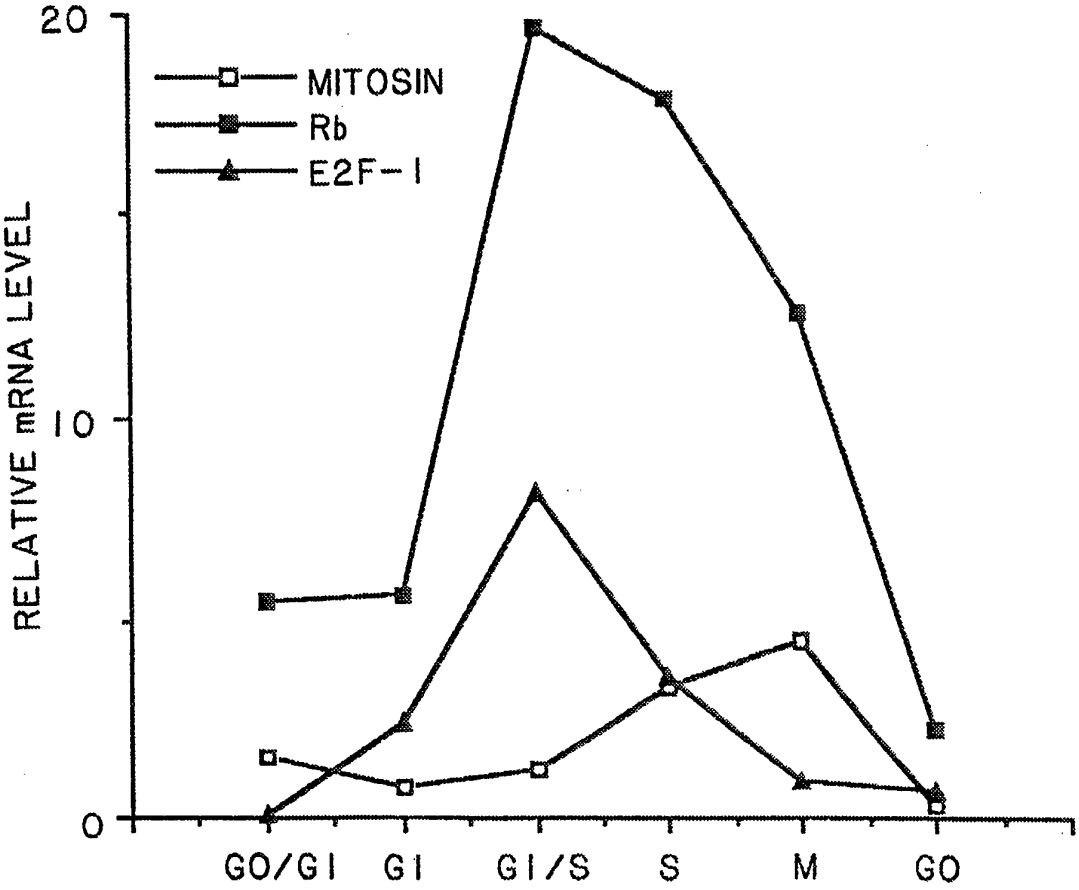
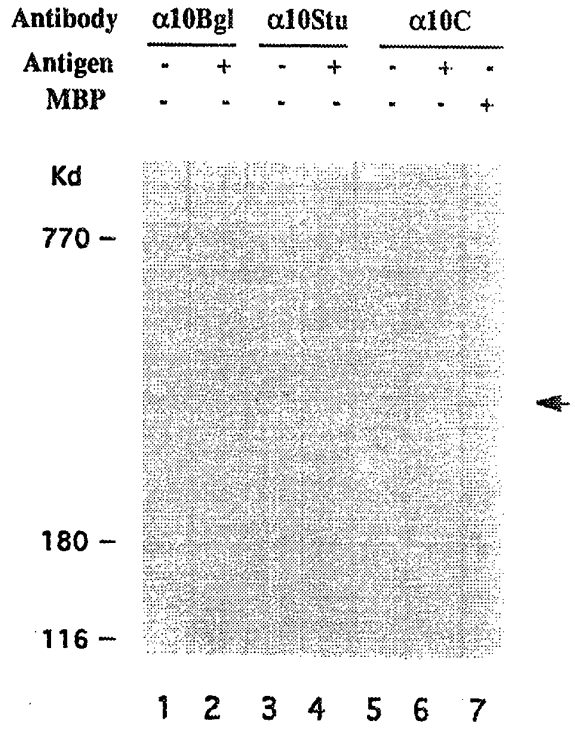

20
10
0
MITOSIN
Rb
E2F-1
RELATIVE mRNA LEVEL
GO/GI
GI
GI/S
S
M
GO

FIG. IB


Antibody
α10Bgl
α10Stu
α10C
Antigen
MBP
Kd
770 –
180 –
116 –
1 2 3 4 5 6 7

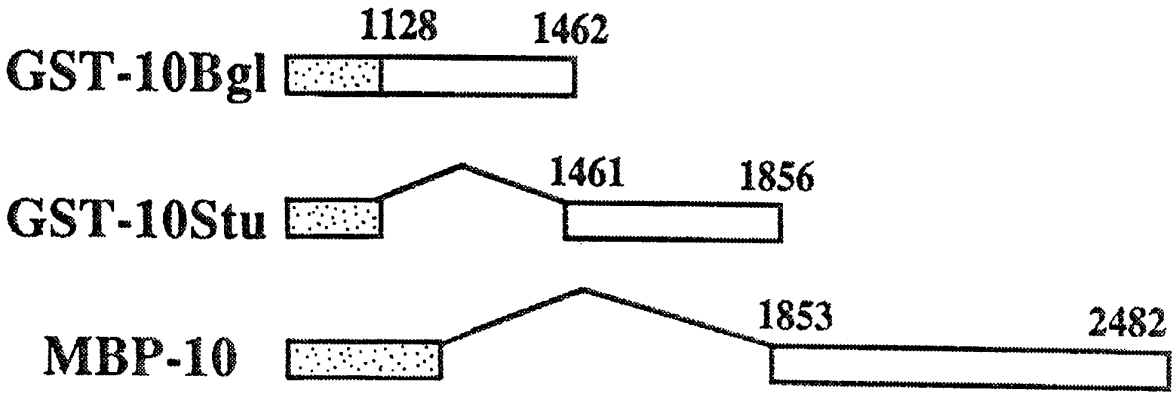

GST-10Bgl
1128
1462
GST-10Stu
1461
1856
MBP-10
1853
2482

FIG. 2

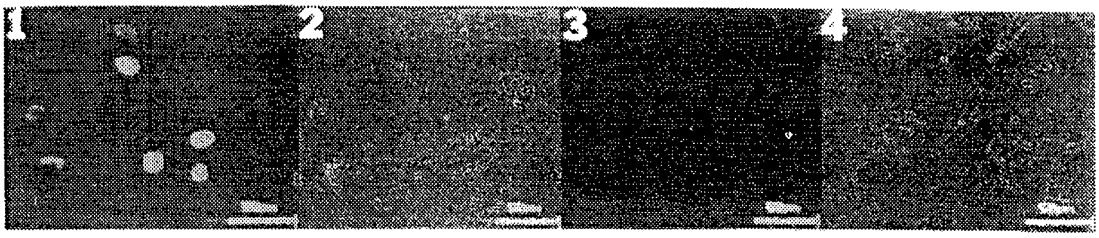


FIG. 3A

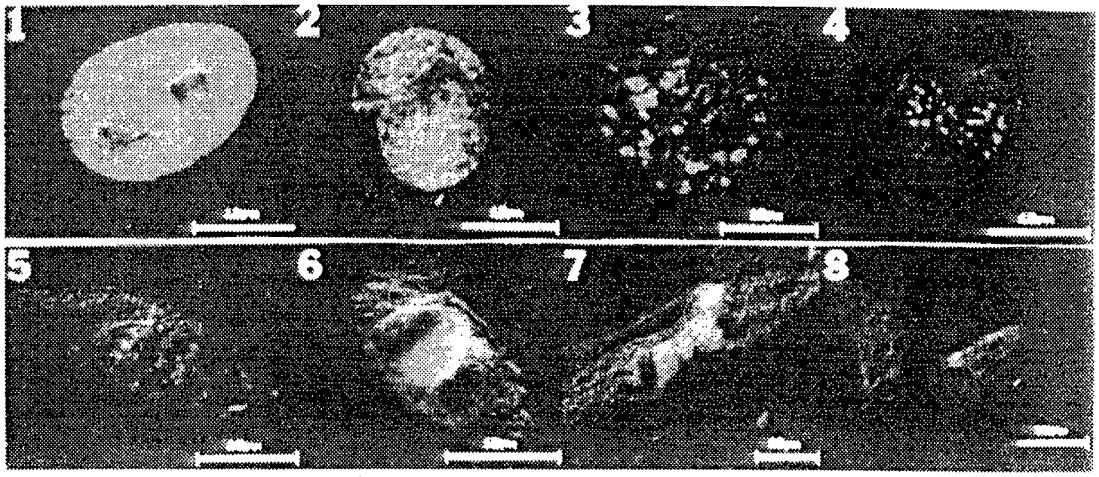


FIG. 3B

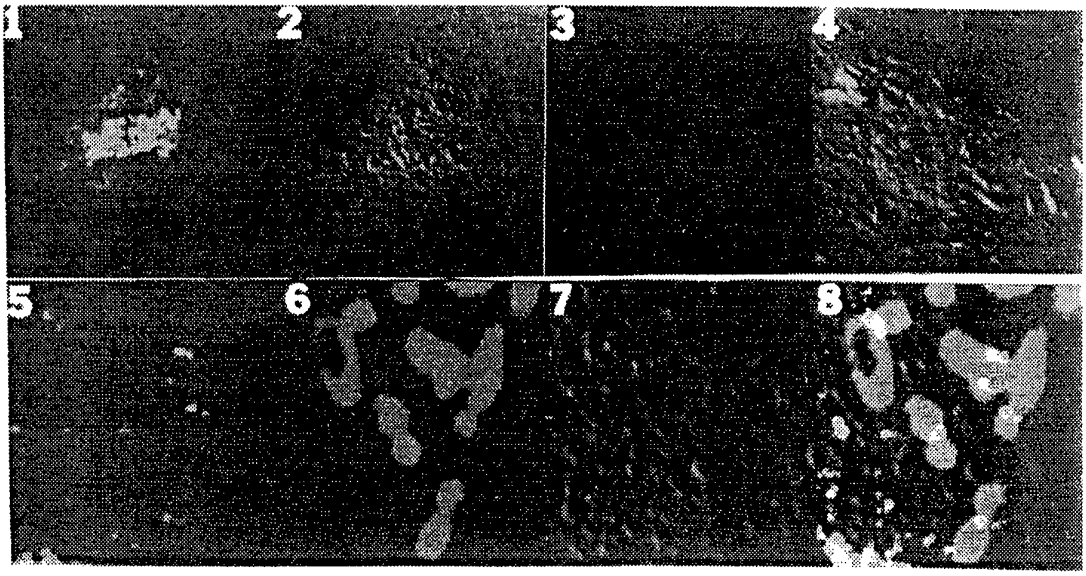


FIG. 3C

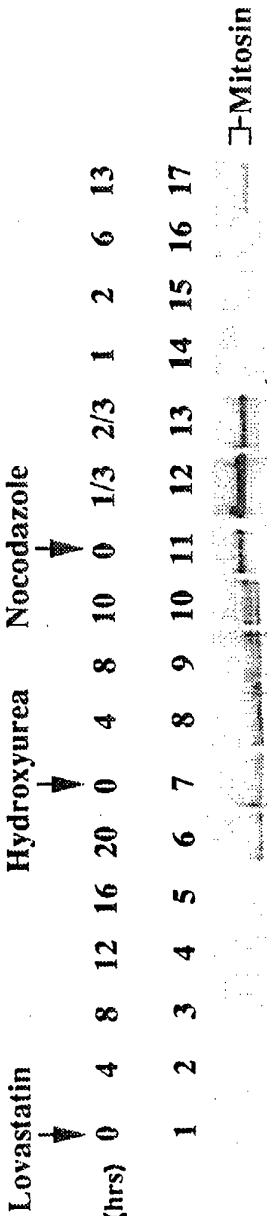


FIG. 4A

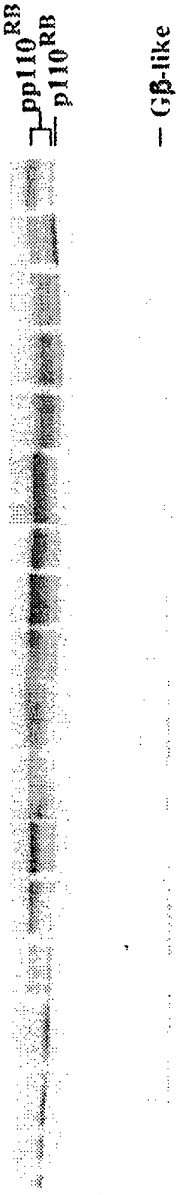


FIG. 4B

FIG. 4C

FIG. 4D

(%)																		
G0/G1	91	89	89	90	92	58	19	12	31	53	1	nd	nd	nd	nd	nd	95	54
S	2	1	1	1	1	35	78	76	18	13	16	nd	nd	nd	nd	nd	1	34
G2/M	7	10	10	9	7	7	3	12	51	34	82	nd	nd	nd	nd	nd	4	12

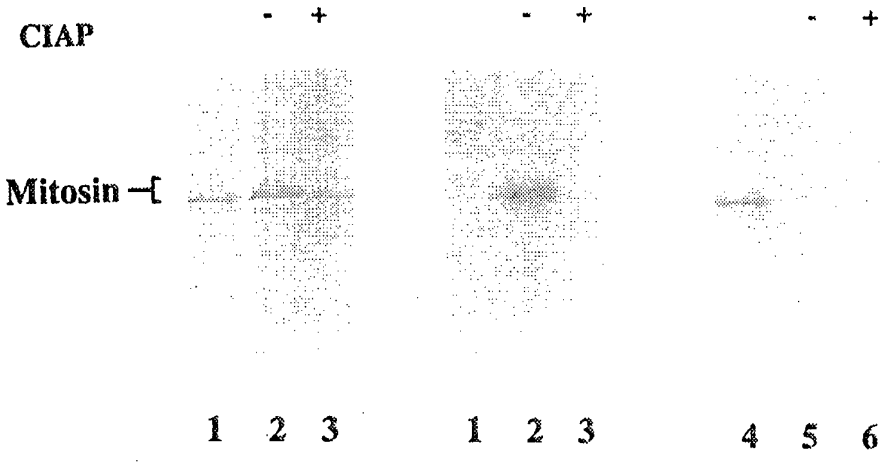


FIG. 5A FIG. 5B FIG. 5C

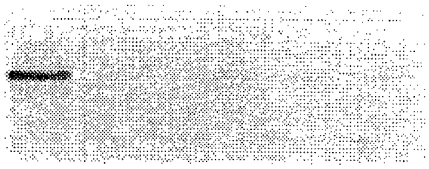


FIG. 6A

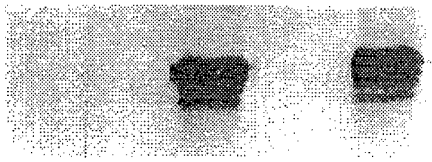


FIG. 6B

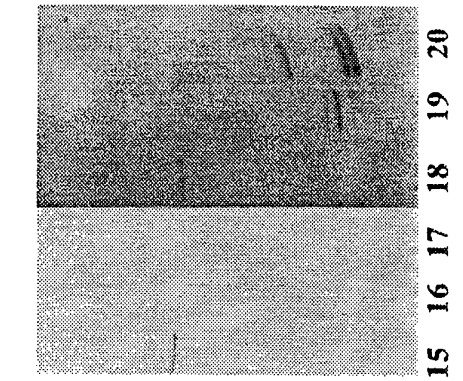

Kd
180
116
84
58
48.5
36.5
26.6
15 16 17 18 19 20

FIG. 7B

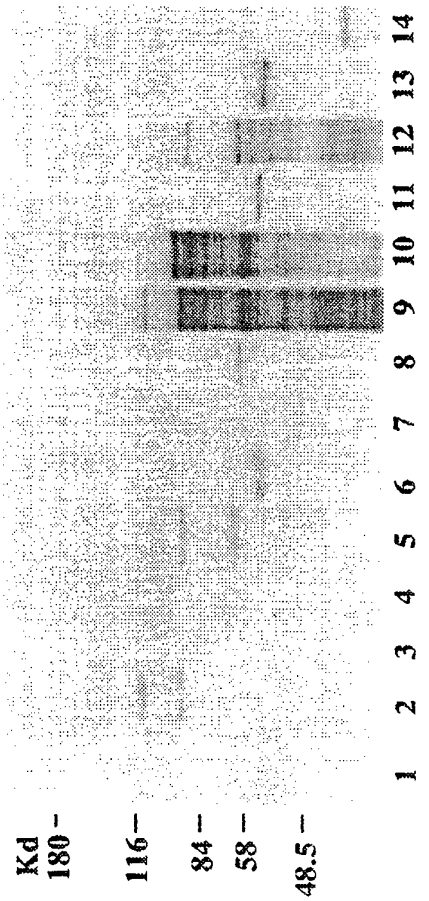
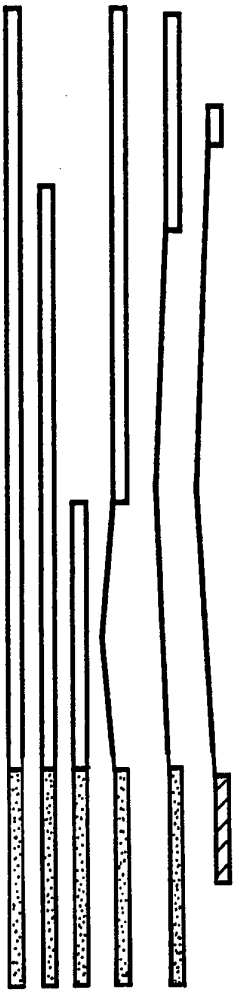

Kd
180
116
84
58
48.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 7A



Name	Amino acid residues	56K^{RB} Binding
MBP-10	1853-2482	+
MBP-10/H	1853-2296	-
MBP-10/NB	1853-2014	-
MBP-10/KN	2014-2482	+
MBP-10/NI	2271-2482	+
GST-1045	2330-2375	+

FIG. 7C

E2F-1 439 I S LSPPHEAL DYHFGLEEGE GIRDLF DCDF GDL 471

Mitosin 2328 AVMSGIHPAE DTEGTE FEPE GLPEVVKKGF ADI 2360

FIG. 7D

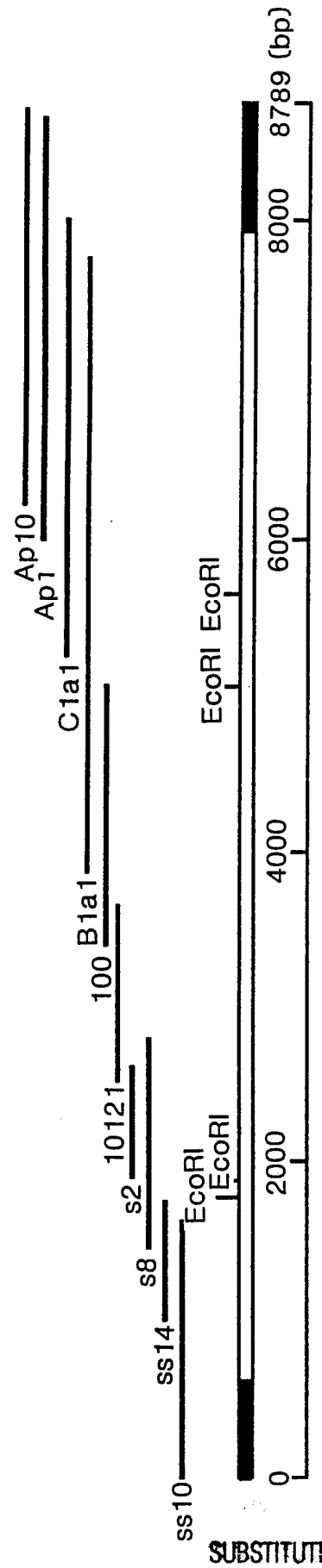

Ap10
Ap1
C1a1
B1a1
100
10121
s2
s8
ss14
ss10
EcoRI
EcoRI
EcoRI
EcoRI
0
2000
4000
6000
8000
8789 (bp)

FIG. 8A

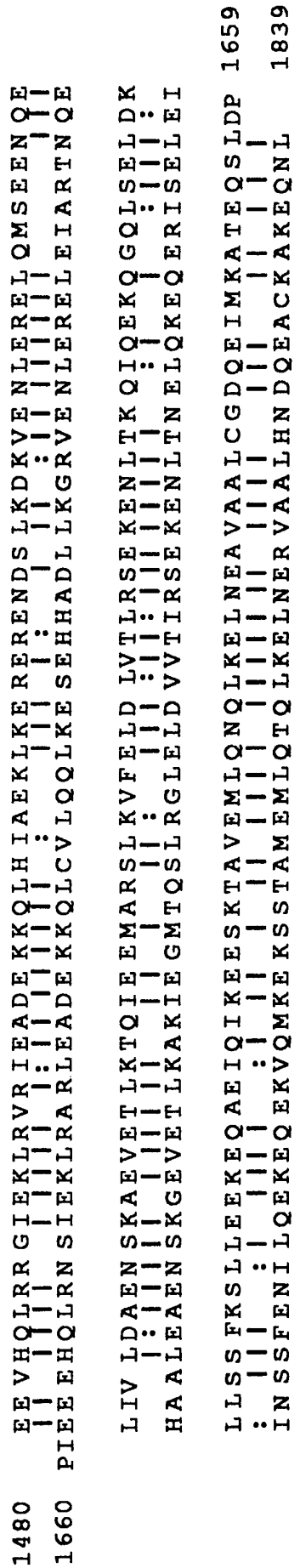

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1660 PIEEEHQLRNSIEKLRARLEADEKKQLCVLQQLKESEHHADLLKGRVENLEREL EIARTNQE
LIV LDAENSKAEVETLKTQIEEMARSLKVFELD LVTLRSEKENLTK QIQEKQGQLSELDK
HAALEAENSKGEVETLKAKIEGMTQSLRGLELD VVTIRSEKENLTN ELQKEQERISELEI
LLSSFKSLLEEKEQAEIQIKEESKTAVEMLQNQLKELNEAVAALCGDQEIMKATEQSLDP 1659
INSSFENILQEKEQEKVQMKEKSSTAMEMLQTQLKELNERVAALHNDQEACKAKEQNL 1839

FIG. 8C

SUBSTITUTE SHEET (RULE 26)

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                                                                                                                    120
GGCCGCATAGGATGCCCAGCCATAGTTGAATCTCAGATAAACAATGAATAATATTTTCATACAGTATGCCTCAATTATTACATGGGACACACTCATACCAAAAATTATTTTAGATTCTAT
AGGGTGTCTTGTATTTTATGTGCCAATCCGATCAATGTGAAGATTCAACAGGTGCATTTCTTGGAAGAGAGAAAGTGGGGTTGGCCTTGAGCAGGAGAAGGTTGAGAAGGAAGGGCTGGT 240
GGCCACAGAAAGAGAGGTGGCCTCAAAGACATCCATGAAGATAAGCCAACAAGGACATCAGGGTGCCTGCTCCTGACCCCACTCCAGGGAGGAGGCTGGGAAGCACTGCCCTTCCTCCCC 360
GTACCCACTCACGACCCCAGATTCTGGCCTGTGCTTCAGGGGCTTTGTGAGCATGTGCAGCTTTTCTTCTTCTTCTTCCTCTTCCTCTCTTTAGAGTTAAAAAAGAAAGAATATGAAGAA 480
TTGAAAGAAGAGAAAACTCTGTTTTCTTGTTGGAAAAGTGAAAACGAAAAACTTTTAACTCAGATGGAATCAGAAAAGGAAAACTTGCAGAGTAAAATTAATCACTTGGAAACTTGTCTG 600
                                                                                                                    M  E  S  E  K  E  N  L  Q  S  K  I  N  H  L  E  T  C  L
1   AAGACACAGCAAATAAAAAGTCATGAATACAACGAGAGAGTAAGAACGCTGGAGATGGACAGAGAAAACCTAAGTGTCGAGATCAGAAACCTTCACAACGTGTTAGACAGTAAGTCAGTG 720
    K  T  Q  Q  I  K  S  H  E  Y  N  E  R  V  R  T  L  E  M  D  R  E  N  L  S  V  E  I  R  N  L  H  N  V  L  D  S  K  S  V
20  GAGGTAGAGACCCAGAAACTAGCTTATATGGAGCTACAGCAGAAAGCTGAGTTCTCAGATCAGAAACATCAGAAGGAAATAGAAAATATGTGTTTGAAGACTTCTCAGCTTACTGGGCAA 840
    E  V  E  T  Q  K  L  A  Y  M  E  L  Q  Q  K  A  E  F  S  D  Q  K  H  Q  K  E  I  E  N  M  C  L  K  T  S  Q  L  T  G  Q
60  GTTGAAGATCTAGAACACAAGCTTCAGTTACTGTCAAATGAAATAATGGACAAAGACCGGTGTTACCAAGACTTGCATGCCGAATATGAGAGCCTCAGGGATCTGCTAAAATCCAAAGAT 960
    V  E  D  L  E  H  K  L  Q  L  L  S  N  E  I  M  D  K  D  R  C  Y  Q  D  L  H  A  E  Y  E  S  L  R  D  L  L  K  S  K  D
100 GCTTCTCTGGTGACAAATGAAGATCATCAGAGAAGTCTTTTGGCTTTTGATCAGCAGCCTGCCATGCATCATTCCTTTGCAAATATAATTGGAGAACAAGGAAGCATGCCTTCAGAGAGG 1080
    A  S  L  V  T  N  E  D  H  Q  R  S  L  L  A  F  D  Q  Q  P  A  M  H  H  S  F  A  N  I  I  G  E  Q  G  S  M  P  S  E  R
140 AGTGAATGTCGTTTAGAAGCAGACCAAAGTCCGAAAAATTCTGCCATCCTACAAAATAGAGTTGATTCACTTGAATTTTCATTAGAGTCTCAAAAACAGATGAACTCAGACCTGCAAAAG 1200
    S  E  C  R  L  E  A  D  Q  S  P  K  N  S  A  I  L  Q  N  R  V  D  S  L  E  F  S  L  E  S  Q  K  Q  M  N  S  D  L  Q  K
180 CAGTGTGAAGAGTTGGTGCAAATCAAAGGAGAAATAGAAGAAAATCTCATGAAAGCAGAACAGATGCATCAAAGTTTTGTGGCTGAAACAAGTCAGCGCATTAGTAAGTTACAGGAAGAC 1320
    Q  C  E  E  L  V  Q  I  K  G  E  I  E  E  N  L  M  K  A  E  Q  M  H  Q  S  F  V  A  E  T  S  Q  R  I  S  K  L  Q  E  D
220 ACTTCTGCTCACCAGAATGTTGTTGCTGAAACCTTAAGTGCCCTTGAGAACAAGGAAAAAGAGCTGCAACTTTTAAATGATAAGGTAGAAACTGAGCAGGCAGAGATTCAAGAATTAAAA 1440
    T  S  A  H  Q  N  V  V  A  E  T  L  S  A  L  E  N  K  E  K  E  L  Q  L  L  N  D  K  V  E  T  E  Q  A  E  I  Q  E  L  K
260 AAGAGCAACCATCTACTTGAAGACTCTCTAAAGGAGCTACAACTTTTATCCGAAACCCTAAGCTTGGAGAAGAAAGAAATGAGTTCCATCATTTCTCTAAATAAAAGGGAAATTGAAGAG 1560
    K  S  N  H  L  L  E  D  S  L  K  E  L  Q  L  L  S  E  T  L  S  L  E  K  K  E  M  S  S  I  I  S  L  N  K  R  E  I  E  E
300 CTGACCCAAGAGAATGGGACTCTTAAGGAAATTAATGCATCCTTAAATCAAGAGAAGATGAACTTAATCCAGAAAAGTGAGAGTTTTGCAAACTATATAGATGAAAGGGAGAAAAGCATT 1680
    L  T  Q  E  N  G  T  L  K  E  I  N  A  S  L  N  Q  E  K  M  N  L  I  Q  K  S  E  S  F  A  N  Y  I  D  E  R  E  K  S  I
340 TCAGAGTTATCTGATCAGTACAAGCAAGAAAAACTTATTTTACTACAAAGATGTGAAGAAACCGGAAATGCATATGAGGATCTTAGTCAAAAATACAAAGCAGCACAGGAAAAGAATTCT 1800
    S  E  L  S  D  Q  Y  K  Q  E  K  L  I  L  L  Q  R  C  E  E  T  G  N  A  Y  E  D  L  S  Q  K  Y  K  A  A  Q  E  K  N  S
380 AAATTAGAATGCTTGCTAAATGAATGCACTAGTCTTTGTGAAAATAGGAAAAATGAGTTGGAACAGCTAAAGGAAGCATTTGCAAAGGAACACCAAGAATTCTTAACAAAATTAGCATTT 1920
    K  L  E  C  L  L  N  E  C  T  S  L  C  E  N  R  K  N  E  L  E  Q  L  K  E  A  F  A  K  E  H  Q  E  F  L  T  K  L  A  F
420 GCTGAAGAAAGAAATCAGAATCTGATGCTAGAGTTGGAGACAGTGCAGCAAGCTCTGAGATCTGAGATGACAGATAACCAAAACAATTCTAAGAGCGAGGCTGGTGGTTTAAAGCAAGAA 2040
    A  E  E  R  N  Q  N  L  M  L  E  L  E  T  V  Q  Q  A  L  R  S  E  M  T  D  N  Q  N  N  S  K  S  E  A  G  G  L  K  Q  E
460 ATCATGACTTTAAAGGAAGAACAAAACAAAATGCAAAAGGAAGTTAATGACTTATTACAAGAGAATGAACAGCTGATGAAGGTAATGAAGACTAAACATGAATGTCAAAATCTAGAATCA 2160
    I  M  T  L  K  E  E  Q  N  K  M  Q  K  E  V  N  D  L  L  Q  E  N  E  Q  L  M  K  V  M  K  T  K  H  E  C  Q  N  L  E  S
500 GAACCAATTAGGAACTCTGTGAAAGAAAGAGAGAGTGAGAGAAATCAATGTAATTTTAAACCTCAGATGGATCTTGAAGTTAAAGAAATTTCTCTAGATAGTTATAATGCGCAGTTGGTG 2280
    E  P  I  R  N  S  V  K  E  R  E  S  E  R  N  Q  C  N  F  K  P  Q  M  D  L  E  V  K  E  I  S  L  D  S  Y  N  A  Q  L  V
540 CAATTAGAAGCTATGCTAAGAAATAAGGAATTAAAACTTCAGGAAAGTGAGAAGGAGAAGGAGTGCCTGCAGCATGAATTACAGACAATTAGAGGAGATCTTGAAACCAGCAATTTGCAA 2400
    Q  L  E  A  M  L  R  N  K  E  L  K  L  Q  E  S  E  K  E  K  E  C  L  Q  H  E  L  Q  T  I  R  G  D  L  E  T  S  N  L  Q
580 GACATGCAGTCACAAGAAATTAGTGGCCTTAAAGACTGTGAAATAGATGCGGAAGAAAAGTATATTTCAGGGCCTCATGAGTTGTCAACAAGTCAAAACGACAATGCACACCTTCAGTGC 2520
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FIG. 8B-1

SUBSTITUTE SHEET (RULE 26)

```
620  D M Q S Q E I S G L K D C E I D A E E K Y I S G P H E L S T S Q N D N A H L Q C
     TCTCTGCAAACAACAATGAACAAGCTGAATGAGCTAGAGAAAATATGTGAAATACTGCAGGCTGAAAAGTATGAACTCGTAACTGAGCTGAATGATTCAAGGTCAGAATGTATCACAGCA 2640
660  S L Q T T M N K L N E L E K I C E I L Q A E K Y E L V T E L N D S R S E C I T A
     ACTAGGAAAATGGCAGAAGAGGTAGGGAAACTACTAAATGAAGTTAAAATATTAAATGATGACAGTGGTCTTCTCCATGGTGAGTTAGTGGAAGACATACCAGGAGGTGAATTTGGTGAA 2760
700  T R K M A E E V G K L L N E V K I L N D D S G L L H G E L V E D I P G G E F G E
     CAACCAAATGAACAGCACCCTGTGTCTTTGGCTCCATTGGACGAGAGTAATTCCTACGAGCACTTGACATTGTCAGACAAAGAAGTTCAAATGCACTTTGCCGAATTGCAAGAGAAATTC 2880
740  Q P N E Q H P V S L A P L D E S N S Y E H L T L S D K E V Q M H F A E L Q E K F
     TTATCTTTACAAAGTGAACACAAAATTTTACATGATCAGCACTGTCAGATGAGCTCTAAAATGTCAGAGCTGCAGACCTATGTTGACTCATTAAAGGCCGAAAATTTGGTCTTGTCAACG 3000
780  L S L Q S E H K I L H D Q H C Q M S S K M S E L Q T Y V D S L K A E N L V L S T
     AATCTGAGAAACTTTCAAGGTGACTTGGTGAAGGAGATGCAGCTGGGCTTGGAGGAGGGGCTCGTTCCATCCCTGTCATCCTCTTGTGTGCCTGACAGCTCTAGTCTTAGCAGTTTGGGA 3120
820  N L R N F Q G D L V K E M Q L G L E E G L V P S L S S S C V P D S S S L S S L G
     GACTCCTCCTTTTACAGAGCTCTTTTAGAACAGACAGGAGATATGTCTCTTTTGAGTAATTTAGAAGGGGCTGTTTCAGCAAACCAGTGCAGTGTAGATGAAGTATTTTGCAGCAGTCTG 3240
860  D S S F Y R A L L E Q T G D M S L L S N L E G A V S A N Q C S V D E V F C S S L
     CAGGAGGAGAATCTGACCAGGAAAGAAACCCCTTCGGCCCCAGCGAAGGGTGTTGAAGAGCTTGAGTCCCTCTGTGAGGTGTACCGGCAGTCCCTCGAGAAGCTAGAAGAGAAAATGGAA 3360
900  Q E E N L T R K E T P S A P A K G V E E L E S L C E V Y R Q S L E K L E E K M E
     AGTCAAGGGATTATGAAAAATAAGGAAATTCAAGAGCTCGAGCAGTTATTAAGTTCTGAAAGGCAAGAGCTTGACTGCCTTAGGAAGCAGTATTTGTCAGAAAATGAACAGTGGCAACAG 3480
940  S Q G I M K N K E I Q E L E Q L L S S E R Q E L D C L R K Q Y L S E N E Q W Q Q
     AAGCTGACAAGCGTGACTCTGGAGATGGAGTCCAAGTTGGCGGCAGAAAAGAAACAGACGGAACAACTGTCACTTGAGCTGGAAGTAGCACGACTCCAGCTACAAGGTCTGGACTTAAGT 3600
980  K L T S V T L E M E S K L A A E K K Q T E Q L S L E L E V A R L Q L Q G L D L S
     TCTCGGTCTTTGCTTGGCATCGACACAGAAGATGCTATTCAAGGCCGAAATGAGAGCTGTGACATATCAAAAGAACATACTTCAGAAACTACAGAAAGAACACCAAAGCATGATGTTCAT 3720
1020 S R S L L G I D T E D A I Q G R N E S C D I S K E H T S E T T E R T P K H D V H
     CAGATTTGTGATAAAGATGCTCAGCAGGACCTCAATCTAGACATTGAGAAAATAACTGAGACTGGTGCATTGAAACCCACAGGAGAGTGCTCTGGGGAACAGTCCCCAGATACCAATTAT 3840
1060 Q I C D K D A Q Q D L N L D I E K I T E T G A L K P T G E C S G E Q S P D T N Y
     GAGCCTCCAGGGGAAGATAAAACCCAGGGCTCTTCAGAATGCATTTCTGAATTGTCATTTTCTGGTCCTAATGCTTTGGTACCTATGGATTTCCTGGGGAATCAGGAAGATATCCATAAT 3960
1100 E P P G E D K T Q G S S E C I S E L S F S G P N A L V P M D F L G N Q E D I H N
     CTTCAACTGCGGGTAAAAGAGACATCAAATGAGAATTTGAGATTACTTCATGTGATAGAGGACCGTGACAGAAAAGTTGAAAGTTTGCTAAATGAAATGAAAGAATTAGACTCAAAACTC 4080
1140 L Q L R V K E T S N E N L R L L H V I E D R D R K V E S L L N E M K E L D S K L
     CATTTACAGGAGGTACAACTAATGACCAAAATTGAAGCATGCATAGAATTGGAAAAAATAGTTGGGGAACTTAAGAAAGAAAACTCAGATTTAAGTGAAAAATTGGAATATTTTTCTTGT 4200
1180 H L Q E V Q L M T K I E A C I E L E K I V G E L K K E N S D L S E K L E Y F S C
     GATCACCAGGAGTTACTCCAGAGAGTAGAAACTTCTGAAGGCCTCAATTCTGATTTAGAAATGCATGCAGATAAATCATCACGTGAAGATATTGGAGATAATGTGGCCAAGGTGAATGAC 4320
1220 D H Q E L L Q R V E T S E G L N S D L E M H A D K S S R E D I G D N V A K V N D
     AGCTGGAAGGAGAGATTTCTTGATGTGGAAAATGAGCTGAGTAGGATCAGATCGGAGAAAGCTAGCATTGAGCATGAAGCCCTCTACCTGGAGGCTGACTTAGAGGTAGTTCAAACAGAG 4440
1260 S W K E R F L D V E N E L S R I R S E K A S I E H E A L Y L E A D L E V V Q T E
     AAGCTATGTTTAGAAAAAGACAATGAAAATAAGCAGAAGGTTATTGTCTGCCTTGAAGAAGAACTCTCAGTGGTCACAAGTGAGAGAAACCAGCTTCGTGGAGAATTAGATACTATGTCA 4560
1300 K L C L E K D N E N K Q K V I V C L E E E L S V V T S E R N Q L R G E L D T M S
     AAAAAAACCACGGCACTGGATCAGTTGTCTGAAAAAATGAAGGAGAAAACACAAGAGCTTGAGTCTCATCAAAGTGAGTGTCTCCATTGCATTCAGGTGGCAGAGGCAGAGGTGAAGGAA 4680
1340 K K T T A L D Q L S E K M K E K T Q E L E S H Q S E C L H C I Q V A E A E V K E
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FIG. 8B-2

SUBSTITUTE SHEET (RULE 26)

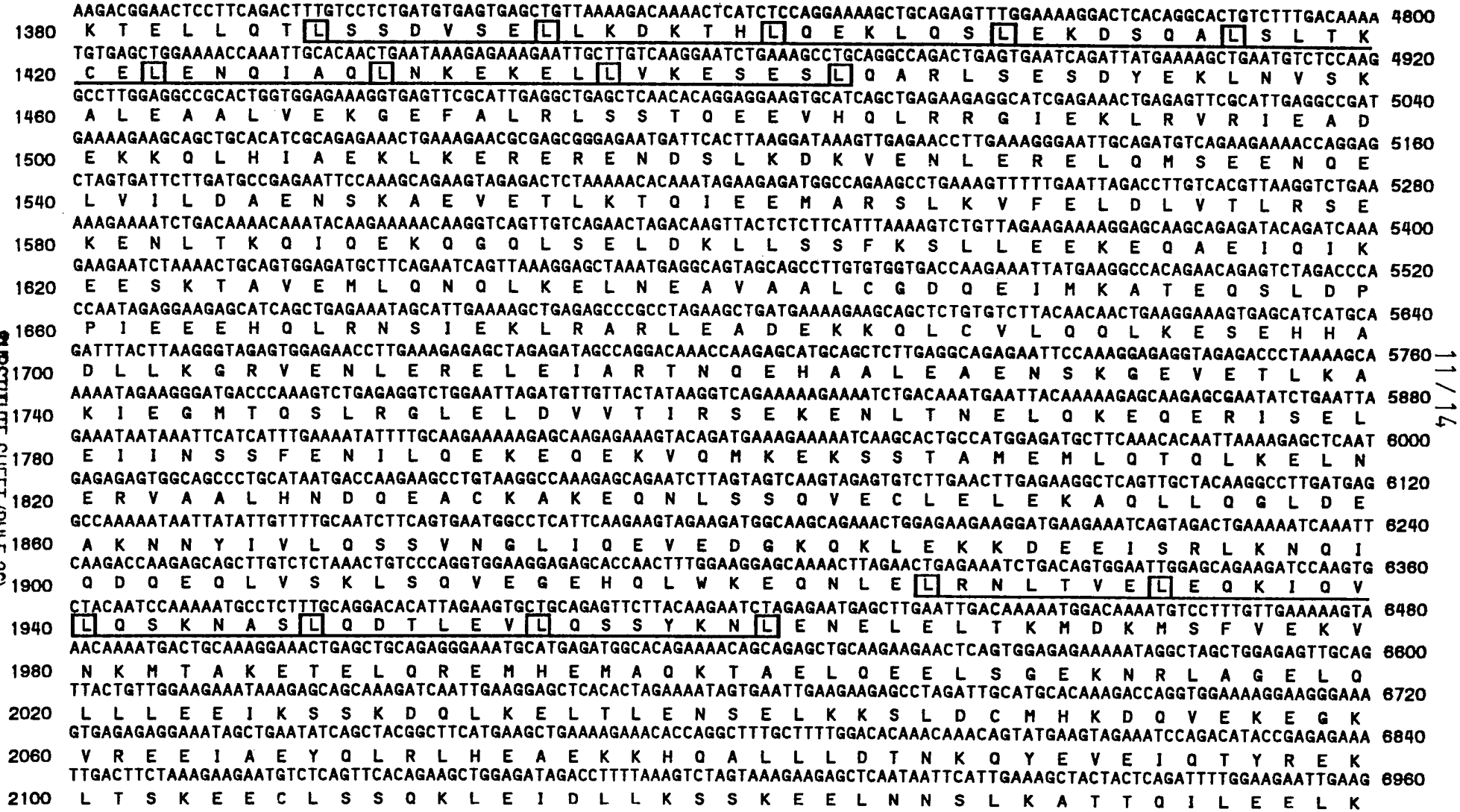


FIG. 8B-3

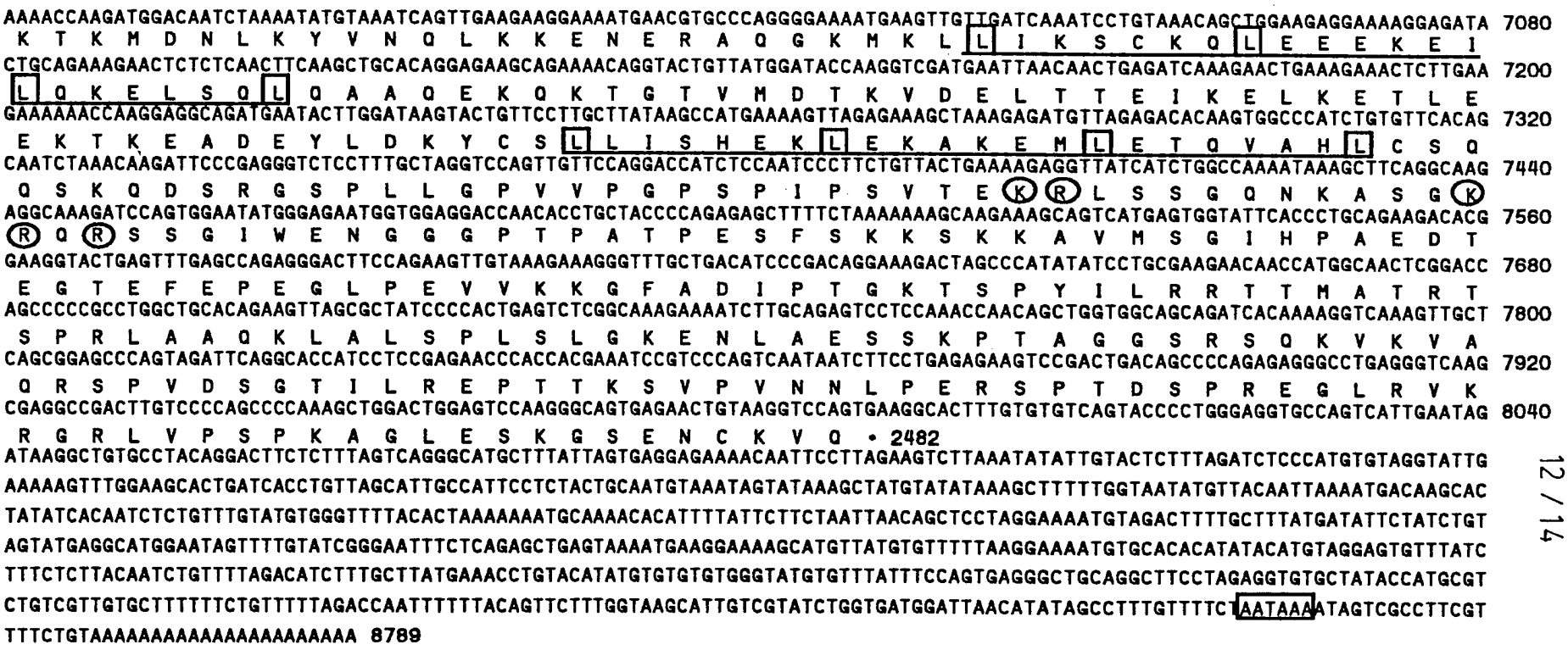

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2140 K T K M D N L K Y V N Q L K K E N E R A Q G K M K L L I K S C K Q L E E E K E I
CTGCAGAAAGAACTCTCTCAACTTCAAGCTGCACAGGAGAAGCAGAAAACAGGTACTGTTATGGATACCAAGGTCGATGAATTAACAACTGAGATCAAAGAACTGAAAGAAACTCTTGAA 7200
2180 L Q K E L S Q L Q A A Q E K Q K T G T V M D T K V D E L T T E I K E L K E T L E
GAAAAAACCAAGGAGGCAGATGAATACTTGGATAAGTACTGTTCCTTGCTTATAAGCCATGAAAAGTTAGAGAAAGCTAAAGAGATGTTAGAGACACAAGTGGCCCATCTGTGTTCACAG 7320
2220 E K T K E A D E Y L D K Y C S L L I S H E K L E K A K E M L E T Q V A H L C S Q
CAATCTAAACAAGATTCCCGAGGGTCTCCTTTGCTAGGTCCAGTTGTTCCAGGACCATCTCCAATCCCTTCTGTTACTGAAAAGAGGTTATCATCTGGCCAAAATAAAGCTTCAGGCAAG 7440
2260 Q S K Q D S R G S P L L G P V V P G P S P I P S V T E K R L S S G Q N K A S G K
AGGCAAAGATCCAGTGGAATATGGGAGAATGGTGGAGGACCAACACCTGCTACCCCAGAGAGCTTTTCTAAAAAAAGCAAGAAAGCAGTCATGAGTGGTATTCACCCTGCAGAAGACACG 7560
2300 R Q R S S G I W E N G G G P T P A T P E S F S K K S K K A V M S G I H P A E D T
GAAGGTACTGAGTTTGAGCCAGAGGGACTTCCAGAAGTTGTAAAGAAAGGGTTTGCTGACATCCCGACAGGAAAGACTAGCCCATATATCCTGCGAAGAACAACCATGGCAACTCGGACC 7680
2340 E G T E F E P E G L P E V V K K G F A D I P T G K T S P Y I L R R T T M A T R T
AGCCCCCGCCTGGCTGCACAGAAGTTAGCGCTATCCCCACTGAGTCTCGGCAAAGAAAATCTTGCAGAGTCCTCCAAACCAACAGCTGGTGGCAGCAGATCACAAAAGGTCAAAGTTGCT 7800
2380 S P R L A A Q K L A L S P L S L G K E N L A E S S K P T A G G S R S Q K V K V A
CAGCGGAGCCCAGTAGATTCAGGCACCATCCTCCGAGAACCCACCACGAAATCCGTCCCAGTCAATAATCTTCCTGAGAGAAGTCCGACTGACAGCCCCAGAGAGGGCCTGAGGGTCAAG 7920
2420 Q R S P V D S G T I L R E P T T K S V P V N N L P E R S P T D S P R E G L R V K
CGAGGCCGACTTGTCCCCAGCCCCAAAGCTGGACTGGAGTCCAAGGGCAGTGAGAACTGTAAGGTCCAGTGAAGGCACTTTGTGTGTCAGTACCCCTGGGAGGTGCCAGTCATTGAATAG 8040
2460 R G R L V P S P K A G L E S K G S E N C K V Q • 2482
ATAAGGCTGTGCCTACAGGACTTCTCTTTAGTCAGGGCATGCTTTATTAGTGAGGAGAAAACAATTCCTTAGAAGTCTTAAATATATTGTACTCTTTAGATCTCCCATGTGTAGGTATTG
AAAAAGTTTGGAAGCACTGATCACCTGTTAGCATTGCCATTCCTCTACTGCAATGTAAATAGTATAAAGCTATGTATATAAAGCTTTTTGGTAATATGTTACAATTAAAATGACAAGCAC
TATATCACAATCTCTGTTTGTATGTGGGTTTTACACTAAAAAAATGCAAAACACATTTTATTCTTCTAATTAACAGCTCCTAGGAAAATGTAGACTTTTGCTTTATGATATTCTATCTGT
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FIG. 8B-4

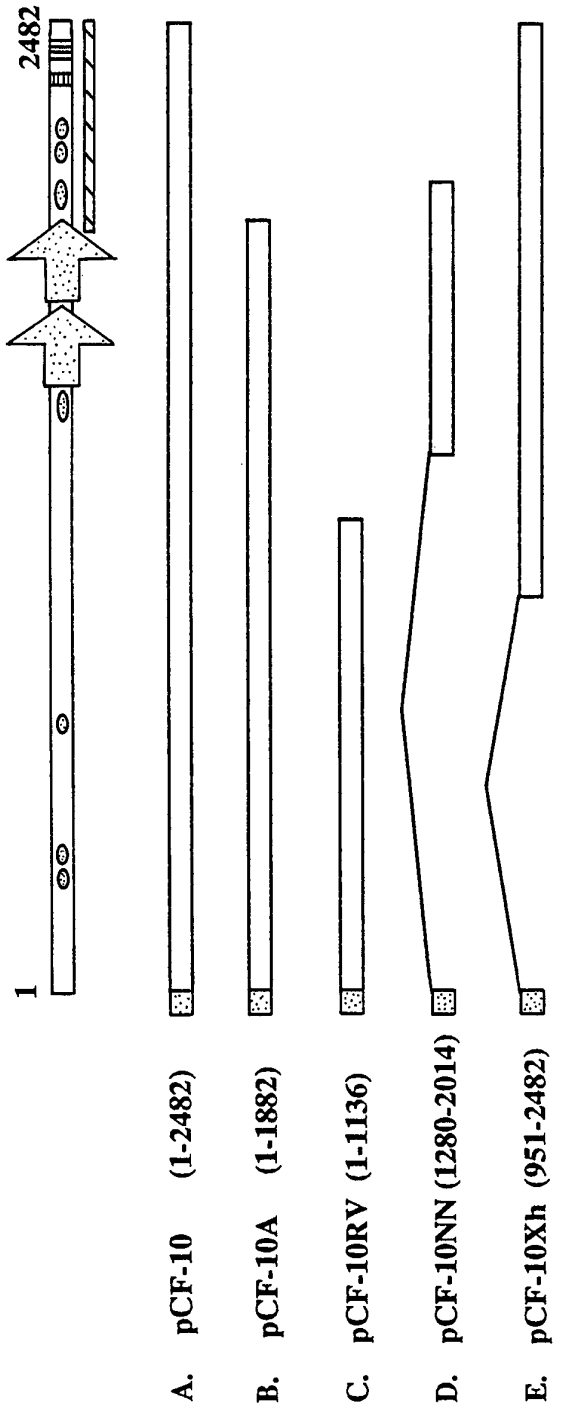

1
2482
Subcellular Location
Reactivity to Anti-10C
A. pCF-10 (1-2482) N ++++
B. pCF-10A (1-1882) C +
C. pCF-10RV (1-1136) C -
D. pCF-10NN (1280-2014) C ++
E. pCF-10Xh (951-2482) N ++++
Flag Epitope
Bipartite Nuclear Targeting Signal
Rb-binding Region
Internal Repeat
Leucine Heptad Repeat
Epitopes for Anti-10C

FIG. 9

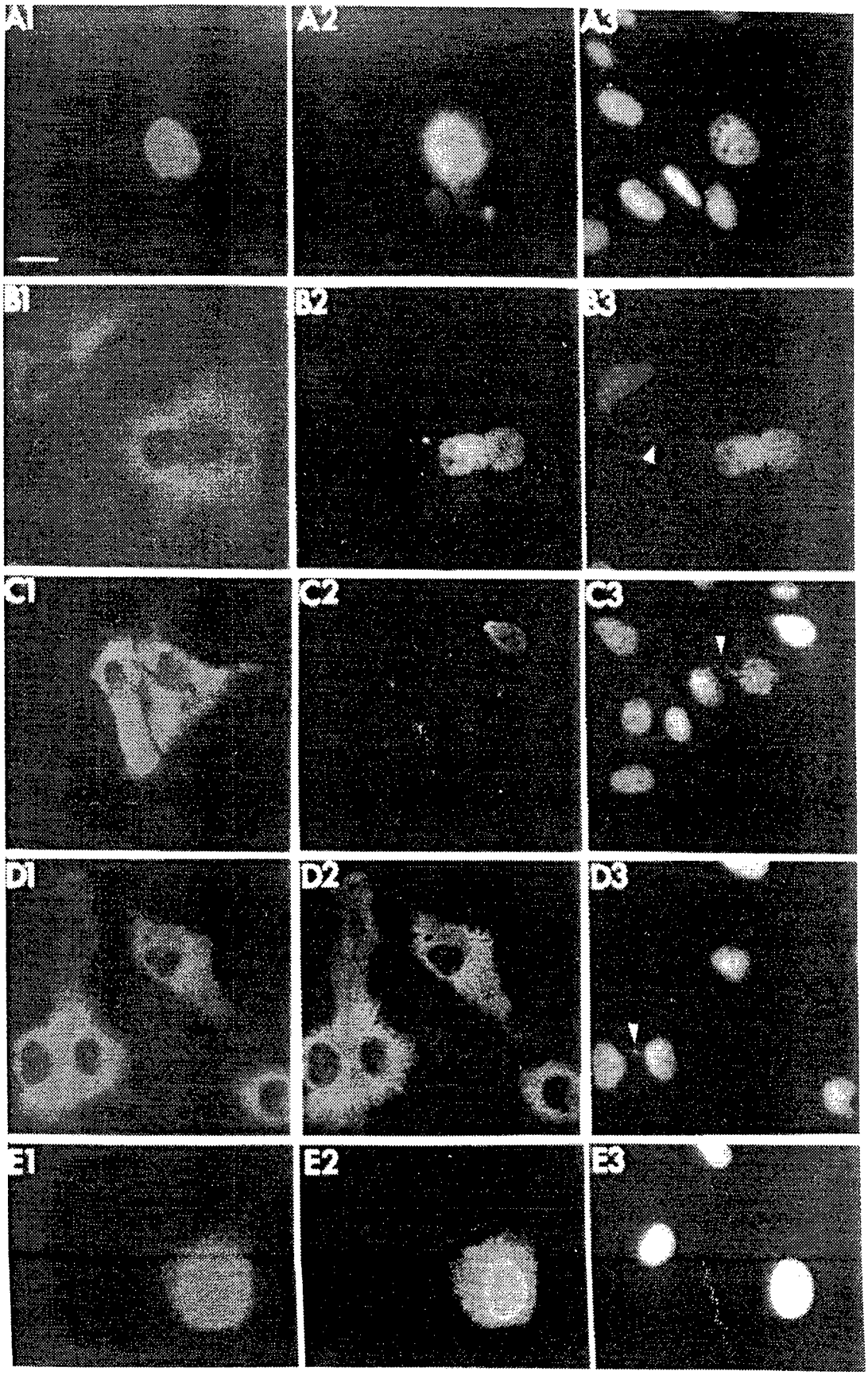


FIG. 10