(21) International Application Number: PCT/US97/11728
(22) International Filing Date: 16 July 1997 (16.07.97)
(30) Priority Data:
60/022,338 24 July 1996 (24.07.96) US

(71) Applicant (for all designated States except US): PHARMACIA & UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US).

(72) Inventors; and


(54) Title: CONSTRUCTS AND COMPLEXES OF CYCLIN E

(57) Abstract

This invention discloses special constructs of human cyclin E. Cyclin E, and the special constructs disclosed herein are related to the field of kinases and cyclins. Kinases are important enzymes involved in regulating the cell cycle.
FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Albania</td>
<td>ES</td>
<td>Spain</td>
<td>LS</td>
<td>Lesotho</td>
</tr>
<tr>
<td>AM</td>
<td>Armenia</td>
<td>FI</td>
<td>Finland</td>
<td>LT</td>
<td>Lithuania</td>
</tr>
<tr>
<td>AT</td>
<td>Austria</td>
<td>FR</td>
<td>France</td>
<td>LU</td>
<td>Luxembourg</td>
</tr>
<tr>
<td>AU</td>
<td>Australia</td>
<td>GB</td>
<td>United Kingdom</td>
<td>LV</td>
<td>Latvia</td>
</tr>
<tr>
<td>AZ</td>
<td>Azerbaijan</td>
<td>GE</td>
<td>Georgia</td>
<td>MC</td>
<td>Monaco</td>
</tr>
<tr>
<td>BA</td>
<td>Bosnia and Herzegovina</td>
<td>GG</td>
<td>Guinea</td>
<td>MD</td>
<td>Republic of Moldova</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
<td>GH</td>
<td>Ghana</td>
<td>MG</td>
<td>Madagascar</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
<td>GN</td>
<td>Guinea</td>
<td>MK</td>
<td>The former Yugoslav</td>
</tr>
<tr>
<td>BF</td>
<td>Burkina Faso</td>
<td>GR</td>
<td>Greece</td>
<td>ML</td>
<td>Mali</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
<td>HU</td>
<td>Hungary</td>
<td>MN</td>
<td>Mongolia</td>
</tr>
<tr>
<td>BJ</td>
<td>Benin</td>
<td>IE</td>
<td>Ireland</td>
<td>MR</td>
<td>Mauritania</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
<td>IL</td>
<td>Israel</td>
<td>MW</td>
<td>Malawi</td>
</tr>
<tr>
<td>BY</td>
<td>Belarus</td>
<td>IS</td>
<td>Iceland</td>
<td>MX</td>
<td>Mexico</td>
</tr>
<tr>
<td>CA</td>
<td>Canada</td>
<td>IT</td>
<td>Italy</td>
<td>NE</td>
<td>Niger</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
<td>JP</td>
<td>Japan</td>
<td>NL</td>
<td>Netherlands</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
<td>KE</td>
<td>Kenya</td>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
<td>KG</td>
<td>Kyrgyzstan</td>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>CI</td>
<td>Côte d'Ivoire</td>
<td>KP</td>
<td>Democratic People's Republic of Korea</td>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
<td>KR</td>
<td>Republic of Korea</td>
<td>PL</td>
<td>Poland</td>
</tr>
<tr>
<td>CN</td>
<td>China</td>
<td>KZ</td>
<td>Kazakhstan</td>
<td>PT</td>
<td>Portugal</td>
</tr>
<tr>
<td>CU</td>
<td>Cuba</td>
<td>LC</td>
<td>Saint Lucia</td>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td>CZ</td>
<td>Czech Republic</td>
<td>LC</td>
<td>Saint Lucia</td>
<td>RU</td>
<td>Russian Federation</td>
</tr>
<tr>
<td>DE</td>
<td>Germany</td>
<td>LI</td>
<td>Liechtenstein</td>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td>DK</td>
<td>Denmark</td>
<td>LK</td>
<td>Sri Lanka</td>
<td>SE</td>
<td>Sweden</td>
</tr>
<tr>
<td>EE</td>
<td>Estonia</td>
<td>LR</td>
<td>Liberia</td>
<td>SG</td>
<td>Singapore</td>
</tr>
<tr>
<td>SI</td>
<td>Slovenia</td>
<td>SK</td>
<td>Slovakia</td>
<td>SN</td>
<td>Senegal</td>
</tr>
<tr>
<td>SZ</td>
<td>Swaziland</td>
<td>TD</td>
<td>Chad</td>
<td>TG</td>
<td>Togo</td>
</tr>
<tr>
<td>TJ</td>
<td>Tajikistan</td>
<td>TM</td>
<td>Turkmenistan</td>
<td>TR</td>
<td>Turkey</td>
</tr>
<tr>
<td>TT</td>
<td>Trinidad and Tobago</td>
<td>UA</td>
<td>Ukraine</td>
<td>UG</td>
<td>Uganda</td>
</tr>
<tr>
<td>US</td>
<td>United States of America</td>
<td>UZ</td>
<td>Uzbekistan</td>
<td>VN</td>
<td>Viet Nam</td>
</tr>
<tr>
<td>YU</td>
<td>Yugoslavia</td>
<td>ZW</td>
<td>Zimbabwe</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CONSTRUCTS AND COMPLEXES OF CYCLIN E

Field of the Invention

This invention relates to field of kinases and cyclins, specifically, cyclin E and its complexes with cdk 2. Kinases are important enzymes involved in regulating the cell cycle.

Information Disclosure

1) U. S. Patent Number 5,449,755 issued September 12, 1995 to Roberts et al specifically discloses a full length wild type cyclin E polypeptide containing amino acid Met-1 to Ala-395. Incorporated by reference. Cyclin E background references and related art may be found in U.S. 5,449,755.

2) Hochuli et al., J. Chromatography, vol. 411, pp. 177-184 (1987), discloses a nitrilotriacetic acid (NTA) adsorbent useful for Immobilized Metal Affinity Chromatography (IMAC). It is reported that the disclosed adsorbent when charged with Ni$^{2+}$ is useful in binding to peptides and proteins containing neighboring histidine residues.

3) Schmidt and Skera, Protein Engineering, vol. 6, pp.109-122 (1993), and J. Chromatography 676:337-345 (1994)] disclose the use of a genetically engineered C-terminal “Strep tag” (SAWRHPQFGG) for detection, immobilization, and purification of recombinant proteins from host cell extracts by immobilized streptavidin and streptavidin-conjugated antibodies.


Background

The cell cycle is usually divided into four phases: DNA synthesis (S phase) and mitosis (M phase) separated by gaps called G1 and G2. The cell cycle is coordinated by several Ser/Thr protein kinases that are activated in a regulated
manner. It is this progressive activation and inactivation of a family of Cyclin-dependent kinases (Cdk's) that regulates the cell cycle. This enzyme engine is subject to careful control in order to ensure that each event (DNA replication, nuclear envelope breakdown, spindle formation, and chromosome segregation etc) is performed correctly and in proper sequence.

Each protein kinase consists of a catalytic cyclin-dependent kinase (Cdk) subunit and a regulatory cyclin subunit. Cdk's are proteins that contain the catalytic subunit (~ 34 kDa) and are inactive as monomers. The best characterized Cdk's, in terms of their temporal activation and cognate cyclins, are: Cdc2, Cdk2, Cdk4, and Cdk6.

Cdk activation requires their specific association with the regulatory subunits called cyclins. In human cells different types of cyclins have been identified and these include types A, B, C, D, and E. Cyclins may be further grouped into "Mitotic Cyclins," which include cyclins A and B and "G1 Cyclins" which include cyclins C, D, and E. The cyclins D, E, A, and B are required in G1, G1/S, S, and G2/M phases of the cell cycle, respectively.

Cdk activation requires both cyclin binding and phosphorylation by a Cdk-activating kinase (CAK) on Thr161 of the Cdk catalytic domain. Negative regulation of Cdk is carried out through phosphorylation of Thr14 and Tyr15. The phosphorylation and dephosphorylation at these residues is mediated by several enzymes including the Wee1 kinase and Cdc25 phosphatase.

Inactivation of the cyclin-dependent kinases (Cdk's) is potentially a promising route to cancer therapy and has been the subject of immense interest for the last few years. These kinases belong to a family of enzymes involved in the events that control the eukaryotic cell cycle.

Cdc2 and Cdk2 are two particularly well understood Cdk's. The major partner for Cdc2 in the cell is cyclin B. Cyclin B levels peak at the G2/M transition and the protein is involved in the induction of mitosis. Cdk2 is active in late G1 and remains active until the end of G2. The levels of cyclin E peak around the G1/S transition during the cell cycle.

The diminution of cyclin protein levels is an important aspect of the regulation of the cell cycle in normal cells. This reduction is thought to occur by targeted protein degradation. Indeed, most of the known cyclins contain one or the other of two classes of putative degradation motifs. These motifs are regions of the proteins containing a defined amino acid sequence. Mitotic cyclins, which include
cyclins A, and B, contain a so-called "destruction box," whereas G1 cyclins, which include cyclins C, D and E, contain potential "PEST" motifs.

The PEST motif is a stretch of amino acids having a specific composition, and its presence in some proteins is known to cause the rapid degradation of eukaryotic cells. The mutation of these motifs in a cyclin would thus result in the abnormal persistence of high levels of cyclin, and this may contribute to tumorigenesis.

Protein inhibitors of Cdk/cyclin complexes mediating cell cycle progression have been discovered. The mammalian Cdk protein inhibitors fall into two categories based on sequence homology. One class is related to p16, and includes specific inhibitors of Cdk4 and Cdk6. The other class, related to p21, includes p21, p27 and p57.

The inhibitors related to p21 are known as the cyclin-dependent kinase inhibitory proteins (Kips). "Kips" used without a specific number identification usually refer to a family of proteins. Some proteins within that family may be given a more specific "name" that includes the letters kip or even cip, such as, p27-KIP1 or p21-CIP1. Capitalization may vary, e.g. kip, Kip, KIP or cip, Cip, CIP. Kips inhibit a wide variety of complexes including Cdk4/Cdk6-cyclin D, Cdk2-Cyclin A/Cyclin E, and cdc2/cyclin B. Human p21 (Cip1) encodes a protein of 164 amino acids and human p27 (Kip1) encodes a protein of 198 amino acids.

The Cdk2/cyclin E complex and associated cell cycle regulatory functions is an important research area and the search for inhibitors of this complex is an important research goal. If soluble, active complexes of Cdk2/cyclin E could be created, they would fill a critical need for a tool to create in vitro screens designed to detect inhibitors of the kinase activity of this complex and they might facilitate studies designed to characterize the complex and elucidate its properties.

The invention disclosed herein discloses various forms of cyclin E that create such soluble and active Cdk2/cyclin E complexes.

**Brief Description of the Drawings and Figures**

**Figure 1.** DNA sequence of the GST-cyclin E-PEST*-His6 gene. Note that sequence numbering differs from that used in Figure 2 of Koff et al (1991) Cell 66:1217-1228. This is sequence listing number 1 or SEQUENCE IDENTIFICATION NUMBER 1, or SEQ. ID. NO. 1.

**Figure 2.** Amino acid sequence of the GST-cyclin E-PEST*-His6. This is SEQ. ID. NO. 2.

**Figure 3.** Comparison of the C-termini of GST-cyclin E-PEST*-His6 and
wild type cyclin E. Above the dotted lines, wild type cyclin E; and below the dotted lines, GST-cyclin E-PEST*-His6. Numbering is the same as used in Figure 2 of Koff et al (1991) Cell 66:1217-1228 for wild type cyclin E. Differences in the GST-cyclin E-PEST*-His6 from the wild type are indicated by bold type. The putative PEST region of wild type cyclin E is underlined. (The portion of full length wild type amino acid shown is provided in SEQ. ID. NO. 3, and the full length wild type DNA corresponding to SEQ. ID. NO. 3, is provided in SEQ. ID. NO. 4). The full length GST-cyclin E-PEST*-His6, which includes the C-terminus is shown in Figure 3, is provided as SEQ. ID. NOS. 1 and 2.

Figure 4. Affinity purified GST-cyclin E-PEST*-His6/ck2 complexes. Protein preparations were visualized by SDS-polyacrylamide gel electrophoresis and Coomassie staining. Lane 1, GST-cyclin E (wild type)/cdk2-HA purified by glutathione affinity chromatography. Lane 2, GST-cyclin E-PEST*-His6/ck2-HA purified by glutathione affinity chromatography. Lane 3, GST-cyclin E-PEST*.

His6/ck2-HA purified by immobilized metal affinity chromatography (IMAC). Lane 4, GST-cyclin E-PEST*-His6/ck2-HA purified by glutathione affinity chromatography and biotinylated with NHS-LC-biotin (Pierce). The markers to the left of the Figure, "GST-cycE" and "ck2HA" show the expected position of the various GST-cyclin E and ck2-HA species.

Figure 5. Kinase activity of GST-cyclin E-PEST*-His6/ck2 complexes. Kinase assays were performed by incubating cyclin E/ck2 complexes in 50 mM Tris-HCl pH 7.4, 10 mM MgCl2, 1 mM DTT, 0.2 mM ATP, 0.25 mCi/ml [γ32P]-ATP and 10 ng/μl purified RB protein (QED, Inc., San Diego). Reaction products were then analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImaging. Lane 1, purified ck2-HA only. Lane 2, GST-cyclin E (wild type)/cdk2-HA purified by glutathione affinity chromatography. Lane 3, GST-cyclin E-PEST*-His6/ck2-HA purified by glutathione affinity chromatography. Lane 4, GST-cyclin E-PEST*-His6/ck2-HA purified by immobilized metal affinity chromatography (IMAC). Lane 5, GST-cyclin E-PEST*-His6/ck2-HA purified by glutathione affinity chromatography and biotinylated with NHS-LC-biotin.

Figure 6. Binding of Kinase Inhibitor Proteins (p27KIP1) to GST-cyclin E-PEST*-His6/ck2 complexes. The Y - axis (cpm) is counts per minute and the X - axis (r) is the Reaction number. KIP scintillation
proximity assays were performed as described below. **Reaction 1**, background (°H-mini-p27, such as the minimal domains disclosed in Polyak, et al., *Cell* (1994) Vol. 78, pp. 59-66, or 125I-GST-p21 + SPA beads, no cyclin E/cdk2 complex present). The 125I-GST-p21 construct is the radioactive 125I-form of a p21 construct. For preparations of non-radioactive constructs otherwise similar to 125I-GST-p21, see El-Deiry *et al.*, "WAF1, a Potential Mediator of p53 Tumor Suppression," *Cell* (1993) Vol. 75, pp. 817-825.


**Figure 7.** Stability of GST-cyclin E-PEST*-His6 vs. wild type GST-cyclin E in insect cells. Sf 9 insect cells were infected with recombinant baculoviruses encoding either GST-cyclin E-PEST*-His6 or wild type GST-cyclin E. The Y - axis (v) is the volume integration of radioactive signal and the X - axis (s) is sample type. Infected cells were pulsed for 2 hours with 35S-amino acids and then chased with cold amino acids. At the indicated time points (time 0 = end of pulse), cells were harvested and proteins analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImaging®.

Histograms represent volume integration of radioactive signal within bands corresponding to GST-cyclin E species. "wt," indicates GST-cyclin E (wild type); "PEST," indicates GST-cyclin E-PEST*-His6.

**Figure 8.** DNA sequence of special cyclin E (29E-395A). This is **SEQ. ID. NO. 5.**

**Figure 9.** Amino acid sequence of special cyclin E (29E-395A). This **SEQ. ID. NO. 6.**

**Figure 10.** IMAC purification of special cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (**SEQ. ID. NO. 7**) in the presence of NaCl which favors the association with GroEL. **SEQ. ID. NO. 8,** lists the DNA sequence that corresponds to the amino acid sequence of **SEQ. ID. NO. 7.** **SEQ. ID. NO. 9,** lists the amino acid sequence of (MRHHHHHHHK). **SEQ. ID. NO. 10,** lists the DNA sequence that corresponds to the amino acid...
sequence of **SEQ. ID. NO. 9.** **SEQ. ID. NO. 11,** lists the amino acid sequence of (SAWRHPQFGG). **SEQ. ID. NO. 12,** lists the DNA sequence that corresponds to the amino acid sequence of **SEQ. ID. NO. 11.** The cell pellets are resuspended in 20 mM Tris, pH 8.0, 0.5 M NaCl, processed using a French Pressure Cell and the extract centrifuged. The supernatant is then loaded onto an IMAC column equilibrated in 20 mM Tris, pH 8.0, 0.5 M NaCl. The column is washed with 20 mM Tris, pH 8.0, 0.5 M NaCl followed by buffer plus 75 mM imidazole. The special cyclin E (29E-395A)/GroEL complex is eluted using buffer plus 300 mM imidazole and fractions are collected and analyzed using 12% SDS-PAGE. Lane 1, total cell lysate after processing with French Press; Lane 2, supernatant loaded onto IMAC; Lane 3, IMAC flow through material; Lane 4, 75 mM imidazole wash; Lanes 5-14, fractions from 300 mM imidazole elution of cyclin E (29E-395A); Lane 15, MW marker. The major protein band between 43 Kd and 68 Kd was identified to be GroEL. The major protein band slightly below the 43 Kd band was identified to be special cyclin E (MRHRRRRHKK)-(29E-395A)-(SAWRHPQFGG), **SEQ. ID. NO. 7.**

**Figure 11.** IMAC purification of special cyclin E (MRHRRRRHKK)-(29E-395A)-(SAWRHPQFGG), **SEQ. ID. NO. 7,** in the absence of NaCl which favors the dissociation with GroEL. The cyclin E (29E-395A) clone is grown and expressed as before. The cell pellets are resuspended in 50 mM Tris, pH 8.0, processed using a French Pressure Cell and the extract centrifuged. The supernatant is then loaded onto an IMAC column equilibrated in 50 mM Tris, pH 8.0. The column is washed with 50 mM Tris, pH 8.0, followed by buffer plus 75 mM imidazole. The cyclin E (29E-395A) is eluted using buffer plus 300 mM imidazole. The cyclin E thus eluted was insoluble and cyclin E containing fractions are collected and analyzed using 12% SDS-PAGE. Lane 1, total cell lysate after processing with French Press; Lane 2, supernatant loaded onto IMAC; Lane 3, IMAC flow through material; Lanes 4-13, fractions from 300 mM imidazole elution of cyclin E (29E-395A); Lane 15, MW marker. Notably, the GroEL band between 43 Kd and 68 Kd is not observed.
**Figure 12.** IMAC purification of human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 complex (SEQ. ID. NO. 7/cdk2 complex). The cell pellets are resuspended in 50 mM Tris, pH 8.0, processed using a French Pressure Cell and the extract centrifuged. The supernatant is then loaded onto an IMAC column equilibrated in 50 mM Tris, pH 8.0. The column is washed with 50 mM Tris, pH 8.0, followed by buffer plus 50 mM imidazole. Cdk2 is equilibrated to 50 mM imidazole and loaded onto the column. The column is washed with 50 mM imidazole followed by 75 mM imidazole until the absorbance is approximately 0.25 A\textsubscript{280}. The cyclin E (29E-395A)/cdk2 complex is eluted using buffer plus 300 mM imidazole and fractions are collected and analyzed using 12\% SDS-PAGE. Lane 1, IMAC purified cyclin E (29E-395A) isolated in the absence of NaCl; Lanes 3-10, fractions from 300 mM imidazole elution of cyclin E (29E-395A)/cdk2 complex; Lane 11, cdk2; Lane 14, MW markers.

**Figure 13.** Purification of (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 complex (SEQ. ID. NO. 7/cdk2 complex) using immobilized streptavidin. SDS-PAGE (12\%) of (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 complex (SEQ. ID. NO. 7/cdk2 complex) purified by using immobilized streptavidin. Lane 1 IMAC purified complex; Lane 2, unbound fraction, Lanes 3-5, fractions eluted with 10 mM biotin; Lane 6, molecular weight markers.

**Figure 14.** Kinase activity of (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 complex (SEQ. ID. NO. 7/cdk2 complex). The complex (2\u00b5l) at a final concentration of 50 \mu g/ml is incubated for 30 min at 37\textdegree C in 15 \u00b5l buffer containing 40 mM Tris/pH 7.5, 10 mM MgCl\textsubscript{2}, 1 mM DTT, 0.1 mg/ml BSA, and 0.068mg/ml histone and [\textsuperscript{33}P] ATP (approximately 1 \mu Ci.). After 30 min at 37\textdegree C, 10 \mu l of the sample is spotted on a phosphocellulose paper, washed with 1\% phosphoric acid for 10 min and repeated two more times. The paper is washed with acetone, dry heated for 5 min, and counted for radioactivity. The Y-axis is Cpm's observed. The X-axis shows the concentration (\mu g/ml) of the cyclinE(29E-395A)/Cdk-2 complex.

**Figure 15.** Schematic for screening mimetics of the p27 kinase.
inhibitory protein (Kip) using scintillation proximity assay (SPA).

**Figure 16.** Binding of \(^{3}H\) Ub-p27 minimal domain to (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 complex (SEQ. ID. NO. 7/cdk2 complex) in SPA. SPA counts (Y-axis, CPM observed) as a function of (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 complex (SEQ. ID. NO. 7/cdk2 complex) (X-axis, nM of complex) in the presence of 200 nM of \(^{3}H\) Kip. Streptavidin coated SPA beads were resuspended in assay buffer (30 mM HEPES, pH 7.5, 7.5 mM MgCl2, 1 mM DTT) to a 10X concentration of 20 mg/ml. Varying amounts of (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 complex (SEQ. ID. NO. 7/cdk2 complex) and 0.58 \(\mu\)g of \(^{3}H\) labeled Ub-p27 minimal domain (such as the minimal domains disclosed in Polyak et al., Cell, (1994) 78:59-66, were added in a total volume of 100 \(\mu\)l in fresh assay buffer. SPA beads (20 mg/ml) were diluted to 2 mg/ml in assay buffer and 100 \(\mu\)l was added to the samples. The assay was incubated on ice for 30 min. and the solution transferred to 3 ml scintillation vials and counted on a Packard Tricarb 1900. The SPA activity was expressed as counts per minute (CPM). The Y-axis is counts per minute (cpm). The X-axis is the concentration of cyclinE (29E-395A)/cdk-2 complex in nanomolar (nM).

**Summary of the Invention**

This invention comprises various cyclin e constructs, complexes, the processes of making them, all the intermediates and the products produced from those processes.

**PEST claims, PEST** - DNA, such as, a nucleic acid polymer, designated cyclin E-PEST*-His6, coding for the polypeptide shown in Figure 2, and related or redundant polymers coding for substantially the same polypeptide, the DNA sequence designated cyclin E-PEST*-His6, comprising the DNA sequence shown in Figure 1, a biological composition, designated GST-Cyclin E-PEST*-His6, comprising the nucleic acid polymer of cyclin E-PEST*-His6, or Figure 2, having its 5' end bound to nucleic acid coding for glutathione S-transferase (GST). A biological composition, designated His6-Cyclin E-PEST*-His6, comprising the nucleic acid polymer of cyclin E-PEST*-
His6, or Figure 2 having its 5' end bound to nucleic acids coding for His6. Amino acids transcribed from PEST DNA are disclosed. Such as, a polypeptide, designated cyclin E-PEST*-His6, comprising the amino acid sequence shown in Figure 2, and equivalent substitutions thereof. A polypeptide comprising the amino acid sequence shown in Figure 2, designated GST-Cyclin E-PEST*-His6, having its N-terminus bound to glutathione S-transferase (GST). A PEST polypeptide in close association with Cdk2. A polypeptide designated His6-Cyclin E-PEST*-His6, having its N-terminus bound to His6 and optionally formed in a complex or in close association with Cdk2.

A PEST - PCR oligonucleotide primer is disclosed, that being 5'-GCAGATCTTCAGTGTTGGTGTTGGCTCCTGCTTCTTACCGCCCTGTGCCGCAGTGGAGGCC-3'. (SEQ. ID. NO. 13)

PEST plasmids such as cyclin E-PEST*-His6 fusion plasmids. A fusion plasmid, designated cyclin E-PEST*-His6 fusion plasmid, that codes for cyclin E-PEST*-His6 polypeptide, that is made from a plasmid produced from a PCR reaction where there is a PCR oligonucleotide 3' primer and a 5' primer and the 3' primer is 5'-GCAGATCTTCAGTGTTGGTG
GTGGTGTTGGCTCCTGCTTCTTACCGCCCTGTGCCGCAGTGGAGGCC-3' (SEQ. ID. NO. 13) and the 5' primer is an oligonucleotide having a cloning site and a site having nucleic acids identical to the 5' end of cyclin E and the PCR reaction amplifies native cyclin E. A fusion plasmid as above where the site having nucleic acids identical to the 5' end of cyclin E are the following nucleic acids, ATGAAGGAGGACGCAGCCGCG (SEQ. ID. NO. 19). A fusion plasmid as above where the 5' primer has a cloning site that is GATCAGATCTC (SEQ. ID. NO. 20). A fusion plasmid as above where the primer is an oligonucleotide having a cloning site and a site having nucleic acids identical to the 5' end of cyclin E, where the nucleic acids sequence is GATCAGATCTCATGAAGGAGGACGCAGCCGCG, (SEQ. ID. NO. 14) where the underlined nucleic acids are the cloning site and the nonunderlined nucleic acids are the nucleic acids identical to the 5' end of cyclin E.

Several GST/his6-cyclin E-PEST*-His6 fusion plasmids are described,
such as a fusion plasmid, designated GST/his6-cyclin E-PEST*-His6 fusion plasmid, that codes for either glutathione S-transferase (GST)-cyclin E-PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide that is made from a plasmid produced from a PCR reaction where there is a PCR oligonucleotide 3' primer and a 5' primer and the 3' primer is 5'-GCAGATCTTCAGTGGTGGTTGGTGGTGGTGCTGCTTCTTACCGCCCTGTGCGCGATGAGGAGGCC-3' (SEQ. ID. NO. 13) and the 5' primer is an oligonucleotide having a cloning site and a site having nucleic acids identical to the 5' end of cyclin E and the PCR reaction amplifies native cyclin E and this product is then subcloned into a fusion plasmid where the fusion plasmid contains either a GST or a His6 coding region at the 5' end, that codes for either the (GST)-cyclin E-PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide. A fusion plasmid where the fusion plasmid is derived from pRSET-A(Invitrogen) that codes for the His6-Cyclin E-PEST*-His6 polypeptide is described as well as a fusion plasmid where the fusion plasmid is derived from pGEX-2T(Pharmacia) that codes for the (GST)-cyclin E-PEST*-His6 polypeptide.

Recombinant PEST - bacteria are described, such as, bacteria that express either the glutathione S-transferase (GST)-cyclin E-PEST*-His6 fusion plasmid, or the His6-Cyclin E-PEST*-His6 fusion plasmid comprising, either the GST-cyclin E-PEST*-His6 polypeptide, or the His6-cyclin E-PEST*-His6 polypeptide. Bacteria where the bacteria contain GST-cyclin E fusion plasmids. Bacteria where the bacteria contain His6-cyclin E fusion plasmids. Bacteria where the bacteria is of the type E. coli.

Recombinant PEST - baculovirus, direct type, are described, such as a baculovirus, created by subcloning a linear GST/his6-cyclin E-PEST*-His6 nucleic acid fragment into a baculoviral transfer vector, and then co-transfecting this product into a baculovirus host cell with baculoviral DNA. Specific examples of this such as a baculovirus where the Baculoviral DNA is BaculoGold crippled baculoviral DNA is described and also a few of the many suitable baculovirus transfer vectors and their use is described such as where the baculovirus transfer vector is pAcGHLT-C or pVL1392.
Recombinant PEST - baculovirus, made from products of PCT reactions are described, such as a baculovirus, created by subcloning a PCR product, created from a PCR reaction starting with DNA that codes for either His6-cyclin E-PEST*-His6, or GST-cyclin E-PEST*-His6, where the PCR product is subcloned into a baculoviral transfer vector, where the baculoviral transfer vector comprising the PCR product is co-transfected into a baculovirus host cell with baculoviral DNA. The baculovirus where the PCR product is from linear GST-cyclin E-PEST*-His6 nucleic acid. The baculovirus where the PCR product is linear His6-cyclin E-PEST*-His6 nucleic acid. Baculovirus baculovirus transfer vectors are pAcGHLT-C or pVL1392. Baculovirus where the baculovirus host cell DNA is BaculoGold crippled baculoviral DNA from PharMingen.

Cells expressing (GST)-cyclin E-PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide are also described, such as, cells containing a baculovirus that express either the glutathione S-transferase (GST)-cyclin E-PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide. Various cell types are described, including, prokaryotes, eukaryotes, cells that are derived from insect cells, cells are created from a PCR reaction beginning with linear (GST)-cyclin E-PEST*-His6 DNA, or His6-Cyclin E-PEST*-His6 DNA, cells where the PCR reaction begins with linear (GST)-cyclin E-PEST*-His6 DNA, cells of the insect type cells known as Sf9 or High Five type cells, cells derived from mammals and cells derived from yeast.

Various complexes or close associations of two or more types of proteins or molecules are also described, such as, the protein complex comprising glutathione S-transferase (GST)-cyclin E-PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide with cdk2.

The methods of producing any of the cyclin E-PEST*-His6, GST-cyclin E-PEST*-His6, His6-cyclin E-PEST*-His6; DNA, amino acids, oligonucleotide primers, fusion plasmids, bacteria or baculovirus as described in any of the claims herein are also described. The method of using glutathione affinity chromatography to produce purified glutathione S-transferase (GST)-cyclin E-PEST*-His6 polypeptide/cdk2 complexes, or His6-Cyclin E-PEST*-His6
polypeptide/cdk2 complex is also described.

Any of the cyclin E-PEST*-His6, GST-cyclin E-PEST*-His6, His6-cyclin E-PEST*-His6; DNA, amino acids, oligonucleotide primers, fusion plasmids, bacteria or baculovirus as products themselves are described as being produced or created from the methods and processes described herein.

In addition to the PEST constructs this application discloses various constructs that are sometimes herein referred to as the MSG constructs. The MSG constructs are also labeled or named after unique amino acids and reference to previously published sequences of amino acids related to the cyclin E protein. Thus, "MSG" may be (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) and the DNA that codes for it.

Described herein are nucleic acid polymers, coding for the polypeptide designated 29E-395A, shown in Figure 9 or SEQ. ID. NO. 6, and related or redundant polymers coding for substantially the same polypeptide, where MSG is the DNA that codes for (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7). A nucleic acid polymer comprising the DNA sequence shown in Figure 8, or SEQ. ID. NO. 5, and equivalents thereof. The nucleic acids that code for the polypeptide, (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG), (SEQ. ID. NO. 7), attached to the nucleic acids that code for the N-terminal sequence (MRHHHHHHHK) (SEQ. ID. NO. 9) and the C-terminal sequence (SAWRHPQFGG) (SEQ. ID. NO. 11). A nucleic acid polymer comprising the DNA sequence shown in Sequence Listing number 8, (SEQ. ID. NO. 8) and equivalents thereof.

Various MSG amino acids and complexes are described, such as, a polypeptide, designated 29E-395A, shown in Figure 9 or Sequence Listing number 6, (SEQ. ID. NO. 6). A polypeptide with following amino acids attached to the N-terminal end, (MRHHHHHHHK) (SEQ. ID. NO. 9) and the following amino acids attached to the C-terminal end, (SAWRHPQFGG), (SEQ. ID. NO. 11), thus providing the polypeptide designated, (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG), (SEQ. ID. NO. 7). Any of these MSG polypeptides in close association with Cdk2 or GroEL.

The MSG/GroEL complex, by itself, and produced from a process is
described. A complex of MSG with GroEL, when the complex is purified after the expression of MSG from bacterial cells, when NaCl is present during cell lysis and purification. The complex where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin. The complex where the complex is purified using immobilized metal affinity chromatography (IMAC). Any of these GroEL complexes where the NaCl concentration is about 0.5 M.

The MSG/cdk 2 complex, by itself, and produced from a process is described. A complex of MSG with cdk2, when the complex is produced from the expression of MSG from bacterial cells, when cdk2 is present during subsequent purification. The complex where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin. The complex where the MSG is first immobilized on a column and the complex then created by exposing the immobilized MSG to cdk2.

The complex where the cdk2 is recombinant cdk2 expressed from baculovirus infected insect cells.

The process of making the MSG / GroEL complex is described. The process of preparing a complex of MSG with GroEL, by expressing MSG from bacterial cells, followed by purification of the MSG, with NaCl present during cell lysis and purification. The complex where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin. The process where the NaCl concentration is about 0.5 M is described.

The complex itself and the process of making the MSG /cdk2 complex is described. The process of preparing a complex of MSG with cdk2, by expressing and purifying the MSG from bacterial cells, and having cdk2 present during the purification. The process where the MSG expressed from bacterial cells is first immobilized on a column and the complex of MSG with cdk2 is created by exposing the immobilized MSG to cdk2. The complex and process where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin and where the cdk2 is recombinant cdk2 expressed from baculovirus infected insect cells is
described.

In addition, MSG; DNA, amino acids, oligonucleotide primers, fusion plasmids, bacteria or baculovirus related to MSG are described herein as being produced or created from the methods and processes described herein.

Additional Details and Description of the Invention

Definitions.

In this document, names, acronyms, descriptions etc. may be in either UPPER or lower case with no distinction in meaning. Alternatively UPPER and lower case letters may be used interchangably for any reason, unless indicated otherwise.

His6 refers to 6 sequential histidine amino acids, or the nucleic acids that code for these molecules.

M.o.i. is multiplicity of infection.

The term "native conditions" refers to proteins not denatured.

PCR primers or oligonucleotides described herein may be constructed one skilled in the art or they may even be custom ordered from vendors. For example, most of the primers used herein are first designed by the inventors then ordered and purchased from Genosys, Texas. Genosys Biotechnologies Inc., 1442 Lakefront Circle, Suite 185, The Woodlands, TX 77380.

Specific embodiments or descriptions of general items, procedures or descriptions should be considered to illuminate and not limit the invention in any way.

General Description.

The PEST motif is a stretch of amino acids having a specific composition, and its presence in some proteins is known to cause the rapid degradation of eukaryotic cells. The mutation of these motifs in a cyclin might therefore result in the abnormal persistance of high levels of cyclin, and this may contribute to tumorigenesis.

The human cyclin E protein contains a region (amino acids 369-385) that resembles the PEST motif. Demonstration that this region is a bona fide PEST motif would be valuable in dissecting the function of cyclin E. A complex lacking the PEST motif would simulate potentially tumorigenic
mutations. Furthermore, such a cyclin E complex might also produce higher levels of recombinant cyclin E protein in eukaryotic cells, thus facilitating production and purification.

Comparison of p21 and p27 suggest a highly conserved N-terminal region that retains cdk inhibitory activity. Specifically, p21 cdk inhibitory activity appears in a region that corresponds to amino acids 22 to 71. Likewise, amino acids 28 to 79 of p27 are sufficient to inhibit Cdk activity in vitro. The inhibitory activity of these small polypeptides supports the notion that inhibitory activity of these KIPs might be mimicked by small organic molecules.

Recent studies suggest that increased expression of cyclin E and Cdk2 may be important in the oncogenic transformation of HUT 12 cells. Increased expression of the p21 and p27 cell cycle inhibitors resulted in inhibition of cyclin E/cdk2 activity, resulting in cell cycle arrest. These results support the hypothesis that Cdk2/cyclin E is a rate-limiting target of the complex regulatory pathway during eukaryotic cell cycle. Taken together, these results suggest that cdk2/cyclin E may be an important therapeutic target.

The present invention relates to novel special constructs and complexes of human cyclin E with cdk2. According to the present invention, active protein kinase complexes including GST-cyclin E-PEST*-His6 and cdk2 can be produced by either mixing components that have been purified separately, or by co-purifying from cells that co-express the genes for GST-cyclin E-PEST*-His6 and cdk2. These complexes are said to be in close association with each other. The close association complexes have special enzymatic properties.

In another embodiment, active protein kinase complexes may also be produced using E. coli derived human cyclin E (MRHHHHHHHK-29E-395A-SAWRHPQFGG) (SEQ. ID. NO. 7). Cyclin E (MRHHHHHHHK-29E-395A-SAWRHPQFGG) (SEQ. ID. NO. 7) is immobilized to an affinity matrix and is eluted as a soluble complex in the presence of cdk2. Alternatively, a special complex of human cyclin E (MRHHHHHHHK-29E-395A-SAWRHPQFGG) (SEQ. ID. NO. 7) may be coexpressed with cdk2 in
baculovirus infected insect cells and purified from crude extract as a complex.

The special constructs of cyclin E/cdk2 complexes described herein are active kinases and can be used in screening for inhibitors of kinase activity. The present invention also relates to a method of detecting mimetics of the p21/p27 family of cyclin-dependent kinase inhibitors (also known as the KIPs). According to another embodiment of the present invention, biotinylated special complex cyclin E/cdk2 complexes are adsorbed to streptavidin-coated SPA beads, and a radiolabeled KIP species is added. Alternatively, the special cyclin E (MRHHHHHHHK-29E-395A-SAWRHPQFGG)/cdk2 (SEQ. ID. NO. 7/cdk2) is adsorbed to streptavidin-coated SPA beads by virtue of the presence of a strep tag at the C-terminus of cyclin E of the special construct. When a radiolabeled KIP, such as p27, binds to cyclin E/cdk2 special complex on the SPA beads, it produces scintillation registered as counts/min or CPM. In the presence of a small molecular weight KIP mimetic, an inhibition in scintillation signal would result in a decrease in CPM.

The present invention comprises novel special constructs and complexes of human cyclin E and methods for obtaining these special cyclin E/cdk2 complexes. Specifically, the invention includes the production of complexes between GST-cyclin E-PEST*-His6 and cdk2; the identification of complexes between E. coli expressed human cyclin E (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) and the bacterial chaperon GroEL; and E. coli expressed special complex of human cyclin E (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) and recombinant cdk2 from baculovirus infected insect cells. The present invention also provides a method for obtaining soluble, active complexes of special complex cyclin E with Cdk2 for identifying inhibitors of kinase activity of the complex. The present invention relates to the construction of special complexes of human cyclin E. This disclosure describes two unique forms of recombinant human cyclin E. The first of these, herein referred to as GST-cyclin E-PEST*-His6, contains amino acid differences at four positions.
compared to the wild type gene, resulting in the destruction of the putative "PEST" degradation targeting motif (Figure 4). It also includes a glutathione S-transferase (GST) tag on the N-terminus to facilitate purification by glutathione affinity chromatography and a hexahistidine (His6) tag on the C-terminus of the polypeptide to facilitate purification by immobilized metal affinity chromatography (IMAC).

The second cyclin E construct, herein referred to as cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG), (SEQ. ID. NO. 7) contains a truncated version of recombinant human cyclin E that contains the amino acid sequence from 29E to 395A and is capable of being recognized by cdk2. The cyclin E construct (29E-395A) is equipped with an N-terminal histidine tag (MRHHHHHHHK) (SEQ. ID. NO. 9) to facilitate purification by immobilized metal affinity chromatography (IMAC) and a 10 amino acid C-terminal tag (SAWRHPQFGG) (SEQ. ID. NO. 11) with binding affinity for streptavidin.

The present invention relates to methods for generating large amounts of soluble, active complex of GST-cyclin E-PEST*-His6/cdk2 or cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 (SEQ. ID. NO. 7/cdk2) useful for research and in vitro screening inhibitors of its kinase activity. The present invention also relates to a method for generating complexes of GST-cyclin E-PEST*-His6/cdk2 or cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG)/cdk2 (SEQ. ID. NO. 7/cdk2) with a radiolabeled minimal domain of the kinase inhibitory protein (KIP) p27, and usefulness of this assay system for screening KIP mimetics.

Transient protein-protein interactions are known to control a number of fundamental cellular processes such as cell cycle, cell growth, metabolic pathways, and signal transduction. It is relatively difficult to set up in vitro high volume screens for inhibitors that would interrupt such protein-protein interactions. For streptavidin-coated scintillation proximity assays (SPAs), one of the protein partners is usually biotinylated in vitro. The cdk2/human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7/cdk2) complex of the present invention can be directly recognized by
immobilized streptavidin by virtue of the presence of a 10 amino acid C-terminal tag. Alternatively, either the cdk2/human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) complex or the cdk2/GST-cyclin E-PEST*-His6 complex can be biotinylated and then bound by streptavidin. These complexes can be adsorbed to streptavidin coated SPA beads, and in the presence of a radiolabeled Kip, an increase in scintillation is observed due to specific interaction of the KIP with the complex. A disruption of interaction by inhibitors should result in a decrease in SPA counts. The present invention also relates to utility of such assays in identifying KIP mimetics.

The special complex cyclin E-PEST*-His6/cdk2 was constructed by mutation of four amino acids in the C-terminus of cyclin E. These amino acids occur within a region (amino acids 369-385) that fits the pattern of the PEST motif. PEST motifs are known to be responsible for the targeted proteolytic degradation of other proteins, and had been predicted to provide this function as part of cyclin E.

The GST-cyclin E-PEST*-His6 construct described herein may also be attached to GST and His6 affinity tags to simplify purification and detection. The protein can be produced in an expression system, for example using the baculovirus insect cell system, and can be purified by either glutathione affinity chromatography (for GST) or immobilized metal affinity chromatography (IMAC).

The special complex cyclin E gene coding for a truncated (29E-395A) form of the protein was cloned and produced in E. coli as a fusion protein. The cyclin E construct (29E-395A) is equipped with an N-terminal histidine tag (MRHHHHHHHK) (SEQ. ID. NO. 9) to facilitate purification by immobilized metal affinity chromatography (IMAC) and a 10 amino acid C-terminal tag (SAWRHPQFGG) (SEQ. ID. NO. 11) with binding affinity for streptavidin.

Under defined conditions, the fusion protein (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) isolated from crude E. coli lysates is copurified with an unexpected ~60 KD protein. This latter protein is
identified as GroEL, the bacterial equivalent of the mammalian 60 KD heat shock protein (hsp60). The cyclin E preparation thus obtained is soluble and is active in a kinase assay in the presence of recombinant cdk2 purified from baculovirus infected insect cells. The fusion protein (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) isolated without GroEL is insoluble.

Recombinant cdk2 can substitute for GroEL and, can be used to produce soluble, active cdk2-human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) complex. Thus, recombinant cdk2, produced by baculovirus-infected insect cells, is allowed to interact specifically with matrix-bound recombinant human cyclin E. This matrix-bound complex can be eluted in a soluble form, has kinase activity and binds to a radiolabeled minimal domain of the kinase inhibitory protein (KIP) p27. See Polyak et al., Cell, (1994) 78:59-66, for minimal domains of p27. Matrix-bound human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) eluted without recombinant cdk2, is insoluble.

Without intending to be inclusive, the invention described herein includes the following characteristics.

- The GST-cyclin E-PEST*-His6 protein encoded by the genetic construct described herein is capable of binding to and activating cdk2.
- The GST-cyclin E-PEST*-His6 protein has a longer half-life in mammalian cells than does full-length, wild type cyclin E protein.
- There is an association between human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) and GroEL from E. coli cells.
- Suprising, unexpected, dramatic production of a soluble human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) associated with GroEL became possible by the fortuitous use of 0.5 M NaCl, under native conditions, during E. coli cell lysis, followed by isolation by immobilized metal affinity chromatography (IMAC).
- The GroEL-associated complex of human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) is active in
binding to cdk2, resulting in kinase activity.

- Recombinant cdk2, produced by baculovirus-infected insect cells, binds specifically to matrix-bound recombinant human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG), (SEQ. ID. NO. 7) resulting in elution of a soluble complex of cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) and cdk2.

- The cdk2-human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) complex is active in a kinase activity assay.

- The cdk2-human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) complex can be directly recognized by immobilized streptavidin by virtue of the presence of a 10 amino acid C-terminal tag.

- Presence of a genetically engineered C-terminal tag, with affinity for streptavidin, obviates the need to biotinylate cdk2-cyclin E complex.

**Detailed Description of the Construction, Manipulation and uses of the Special Constructs**

The present invention relates to novel special versions of human cyclin E. According to the present invention, the desired special version of human cyclin E are produced by recombinant DNA technology and are purified as fusion proteins. The fusion proteins are produced by host cells into which the genetic information encoding the fusion proteins has been introduced. The host cells may secrete the fusion protein into the culture media or store it in the cells whereby the cells must be disrupted in order to extract the product. As hosts, any eukaryotic cells, including both *E. coli*, and baculovirus infected insect cells and yeast are possible hosts.

For purification using the His6 tag, the cell extract containing the fusion protein is passed through a column containing immobilized Ni²⁺. The immobilized metal ion chelates the protein. This impedes the movement of the protein through the column. Both the GST-cyclin E-PEST*-His6 and the cyclin E construct (29E-395A) are equipped with a terminal His6 tag to
facilitate purification by immobilized metal affinity chromatography (IMAC).

For purification or immobilization of the GST-cyclin E-PEST*-His6 protein using the GST tag, cell extract is passed over a glutathione affinity column. In addition, the cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) special version includes a 10 amino acid C-terminal tag (SAWRHPQFGG) (SEQ. ID. NO. 11) with binding affinity for streptavidin.

Under defined conditions, human recombinant human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) isolated from crude E. coli lysates is copurified with an unexpected ~60 KD protein identified as GroEL, the bacterial equivalent of the mammalian 60 KD heat shock protein (hsp60). The cyclin E preparation thus obtained is soluble and is active in a kinase assay in the presence of recombinant cdk2 from baculovirus infected insect cells. Human recombinant special cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7), isolated without GroEL, is insoluble.

In another embodiment of the present invention, recombinant cdk2 can substitute for GroEL, and can be used to produce soluble active cdk2-recombinant human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) complex. Employing this embodiment, recombinant cdk2, produced by baculovirus-infected insect cells, is allowed to interact specifically with matrix-bound recombinant human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7). This matrix-bound complex can be eluted in a soluble form, and has kinase activity which can be stimulated in the presence of a cyclin-dependent activating kinase (CAK). Matrix-bound human recombinant cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7), eluted without recombinant cdk2, is insoluble.

Native conditions that favor dissociation of GroEL, i.e., absence of 0.5M NaCl during cell lysis and IMAC purification, produces insoluble fusion protein containing truncated cyclin E. Therefore, the fortuitous use of 0.5 M NaCl, under native conditions, during cell lysis and isolation by immobilized
metal affinity chromatography (IMAC) results in the surprising and unexpected increased production, relative to conditions without NaCl, of a fusion protein containing soluble human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) associated with GroEL. The N-terminal tag (MRHHHHHHHK) (SEQ. ID. NO. 9) allows immobilization of the fusion protein. This step is essential for copurification with GroEL which in turn allows recovery of a soluble complex useful for studying kinase activity in the presence of added cdk2. Likewise, cdk2 protein can be used to interact with immobilized cyclin E and this allows recovery of a soluble complex which is active as a kinase.

In another embodiment of this invention, the C-terminal strep tag, SAWRHPQFGG, is used to facilitate immobilization of the complexes to streptavidin coated SPA beads. This eliminates the need to chemically modify the fusion protein by biotinylation prior to its use in SPA-based screening assays.

According to the present invention, under native conditions, cyclin E special construct, (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG), (SEQ. ID. NO. 7) expressed in E. coli, requires a partner protein for solubilization. We have identified these two sources as Gro EL from the E. coli host and purified recombinant cdk2 from baculovirus infected insect cells.

The present invention provides a functional complex of cdk2/GST-cyclin E-PEST*-His6 and cdk2/cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) which are suitable for anti-cdk2/cyclin E screening purposes. The method of our invention yields active complexes which contain both the regulatory (special cyclin E) and catalytic subunit (cdk2) subunits.
Description of the special GST-cyclin E-PEST*-His6 construct.

Figures 1 and 2 show the DNA and amino acid sequences for GST-cyclin E-PEST*-His6, respectively. Figure 2 shows a comparison of the GST-cyclin E-PEST*-His6 to wild type cyclin E at the C-terminus (including the putative PEST region). A schematic diagram (A) of this construct is shown below.

Schematic A

![Diagram of the GST-cyclin E-PEST*-His6 construct]

The following oligonucleotide, RH113, was used as a 3' primer for PCR mutagenesis of the cyclin E gene:

5'- GCAGATCTTCAGTGTTGTTGGTGGTTGGTGGCTCTGCTTCTTTACC

15 CCCTGTCGGCGATGAGGAGCC-3' (SEQ. ID. NO. 13)

This primer makes the following point mutations in the DNA sequence of human cyclin E: C1139→T, C1141→G, C1144→G, and A1150→G. (This numbering is according to Figure 2 in Koff, et. al., Cell 66:1217-1228. It also mutates all nucleotides 3' to C1224. These changes result in the following mutations in the amino acid sequence: T380→I, P381→A, P382→A, S384→G, and the replacement of the most C-terminal six amino acids (390-395) with six histidines. The primer also includes a BglII site to facilitate subcloning.

Once the above 3' PCR primer is selected, any suitable 5' primer may be used. The 5' primer should have a cloning site, i.e. cutting or ligating site and it should have a portion of the same sequence as cyclin E. The following...
oligonucleotide, KW133, was used as a 5' primer for PCR mutagenesis of the cyclin E gene:

5'-GATCAGATCTCATGAAGGAGGACGGCGGCGG (SEQ. ID. NO. 14)

This primer changes the noncoding DNA sequence just 5' to the codon of the cyclin E gene to a BglII site, to facilitate subcloning. The underlined portion of the primer shows the cloning site, the non-underlined portion is identical to the 5' end of cyclin E.

These oligonucleotides are then used in PCR reactions, the resulting PCR products, or special cyclin E PCR products, may then be sub-cloned into any fusion plasmid, such as, pRSET-A (Invitrogen®) or pGEX-2T (Pharmacia®) for subsequent bacterial expression (direct expression) or they may be used as templates for amplification and subcloning into baculovirus vectors.

When the PCR primers are used for amplification and subcloning of GST-cyclin E-PEST*-His6 into suitable baculovirus transfer vectors, such as, pAcGHLT-C (PharMingen®) or pVL1392 they should incorporate restriction sites and additional nucleotides to maintain the open reading frame with the GST fusion protein of the vector. Provided the open reading frame with the GST fusion protein is maintained any suitable set of PCR primers should be acceptable for amplification and subcloning.

Recombinant baculoviruses are generated by co-transfection of baculovirus host cells, commonly insect cells. One of several suitable lines of cells is Spodoptera frugiperda (Sf) and one line of Sf cells used here was Sf 9. Sf cells with the transfer plasmid constructs described above were used with BaculoGold crippled baculoviral DNA (PharMingen) according to the manufacturer's instructions. Any suitable baculovirus could be used in place of BaculoGold. Resultant viruses are purified by propagating single plaques from three consecutive plaque purification assays.

In addition to the GST-cyclin E-PEST*-His6 construct expression, cdk2 must also be expressed in order to create a special complex of GST-cyclin E-PEST*-His6/cdk2. Baculovirus which express cdk2 may be cloned and propagated according to literature references and should be known to one
skilled in the art. Sf 9 and baculovirus propagation may follow the procedures of Summers and Smith.

Co-expression of the GST-cyclin E-PEST*-His6 and the cdk2-HA baculoviruses may be performed in either Sf 9 or High Five cells (BTI-TN-5B1-4, derived from Trichoplusia ni). Sf 9 cells are grown in spinner culture at 28°C to 10^6 cells/ml in Grace's medium (GIBCO/BRL) supplemented with 10% fetal bovine serum, 100 Units/ml penicillin G sodium and 100 ug/ml streptomycin sulfate. High Five cells are grown to 10^6 cells/ml in shake flasks at 150 rpm containing InsectExpress medium (BioWhittaker). Cells are co-infected with special cyclin E baculoviruses at an m.o.i. of 20 and cdk2 baculovirus at an m.o.i. of 10. Cells are harvested by centrifugation at 48-68 hours post infection.

**Purification of GST-cyclin E-PEST*-His6/cdk2 Complexes by Glutathione Affinity Chromatography**

Cell pellets are washed with phosphate buffered saline (PBS) and resuspended in hypotonic lysis buffer (10 mM Hepes, pH 7.4/ 10 mM NaCl/ 1 mM EDTA/ 0.2 ug/ml leupeptin/ 0.2 ug/ml pepstatin/ 0.2 ug/ml aprotinin/ 0.2 mM AEBSF) at 1 ml for every 10^7 cells initially infected. Cells are lysed on ice for 1 hour, then NaCl is added to a final concentration of 150 mM. Additionally, cells are sonicated on ice for 2 minutes. Lysates are clarified by high speed centrifugation. Ten volumes of lysate is mixed with 1 packed volume (pv) of washed glutathione Sepharose 4B (Pharmacia Biotech®) for 1 hour at 4°C. The Sepharose is pelleted by centrifugation at 1500 rpm, 8 minutes and washed by addition of 10 pv PBS plus 0.2% Nonidet P-40 and rocked for 10 minutes.

The Sepharose is pelleted at 1500 rpm for 8 minutes and washes repeated 2 additional times. After the final wash, the Sepharose is resuspended in PBS without detergent, loaded into a chromatography column, and the PBS drained. To elute bound material, 3 ml Elution Buffer (15 mM reduced glutathione in 50 mM Tris-HCl, pH8) per ml of pv of glutathione Sepharose 4B is added to the column and 1 ml fractions are collected. Aliquots of each fraction are analyzed by SDS-PAGE and
Comassie or silver staining.

Additionally, samples are subjected to SDS-PAGE and Western analysis using anti-cyclin E, or anti-cdk2 antibodies (Upstate Biotechnology, Inc.). Fractions containing immunoreactive cyclin/cdk2-HA complexes are pooled. Stabilization buffer (10X: 50% glycerol/ 10 mM DTT/ 5 mM AEBSF/ 50 mM NaF/5 mM EGTA) is added to a final concentration of 1X to the pooled material and 1 ml aliquots are frozen on dry ice and stored at -80°C.

**Description of the special cyclin E** (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (**SEQ. ID. NO. 7**) construct. Also Known As (aka) the MSG construct.

This document may use the letters MSG in either upper or lower case letters as an abbreviated method of writing or referring to the special cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (**SEQ. ID. NO. 7**) construct.

**Figures 8 and 9** show the DNA sequence and amino acid sequence of special cyclin E expressed in *E. coli*, respectively. A complete schematic representation of this construct in terms of amino acid sequence is shown below. This schematic representation (B) is a short form of what is provided in full as **SEQ. ID. NO. 7**.

Schematic B

\[
\begin{align*}
29 & \quad 395 \\
MR(H)_6-\text{KE} & \quad \text{-------------------A-SAWRHPQFGG} \\
(-28)\text{Cyclin E} & \quad \text{strep tag}
\end{align*}
\]

The 5' primer contained the sequence encoding for an EcoRI restriction site as well as the region coding for the 5' end of human cyclin E starting with Glu(29).

\[
\begin{align*}
29 & \quad 30 \\
\text{MET ARG HIS HIS HIS HIS LYS GLU GLU MET} \\
30 & \quad \text{5'GGAA TTC CAT ATG CGA CAC CAT CAC CAT CAC CAT AAG GAA GAA ATG} \\
\text{ALA LYS ILE ASP ARG THR} & \quad \text{(**SEQ. ID. NO. 15**)} \\
\text{GCC AAA ATC GAC AGG ACG 3'} & \quad \text{(**SEQ. ID. NO. 16**)}
\end{align*}
\]
The 3' primer contained the anti-sense sequence coding for a HindIII site at the 3' end of the human cyclin E and a streptavidin tag.

\[
\begin{align*}
\text{Hind III} & \quad \text{GLY GLY PHE GLN PRO HIS ARG TRP ALA SER ALA} \\
5' & \quad \text{CCC AAG CTT CTA TCC AAA CTG GGG GTG TCT CCA TGC GCT CGC} \\
\text{stop} & \quad \text{---------------Strep tag---------------} \\
\text{MET GLU PRO GLY SER SER GLN LYS} & \quad \text{(SEQ. ID. NO. 17)} \\
\text{CAT TTC CGG CCC GCT GCT CTG CTT} & \quad \text{3'} \quad \text{(SEQ. ID. NO. 18)}
\end{align*}
\]

The desired DNA fragment is obtained by PCR from the human cyclin E gene and is restricted with EcoR1 and HindIII. This fragment is then inserted into EcoR1/HindIII cut pKK223-3 and transformed into E. coli strain JM-109. Colonies are selected for ampicillin resistance and analyzed by restriction enzyme digestion and expression. The clone S-18,1 was selected for expression of the special human cyclin E (MRHHHHHKK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7). Sequencing of the plasmid DNA was used to verify the construct.

Studies with \textit{E. coli} expressed special human cyclin E (MRHHHHHKK)-(29E-395A)-(SAWRHPQFGG), (SEQ. ID. NO. 7) purified by IMAC in the presence of 0.5 M NaCl, show that GroEL (\textit{E. coli} 60 kDa chaperonin) copurifies with this truncated version of human cyclin E (\textbf{Figure 10}). The fortuitous use of 0.5 M NaCl, under native conditions, during cell lysis and isolation by immobilized metal affinity chromatography (IMAC) results in the unexpected production of a fusion protein containing soluble human cyclin E (29E-395A) associated with GroEL.

These protein protein complexes may be said to be associations where one protein is in "close association" with another protein. Frequently individual proteins will have no catalytic effects without being in close association with other proteins. This was earlier described in this document with reference to the GST-cyclin E-PEST*-His/cdk2 complex, where it is the complex or the close association of the two proteins that gives the association catalytic activity. Similarly here, with the (MRHHHHHKK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) polymer, it is when the (MRHHHHHKK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) polymer is in close association with GroEL or with cdk2 that this association has its special characteristics, in this latter case the special characteristic is good activity coupled with solubility. In this document, in association or in close association may be considered synonymous with complex.

The interaction of GroEL with Cyclin E is abolished when IMAC purification
of special human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) is carried out in the absence of NaCl (Figure 11). However, native conditions that favor dissociation of GroEL, ie: absence of 0.5M NaCl during cell lysis and IMAC purification, produces insoluble protein containing special cyclin E without GroEL. These results led us to conclude that a solubilizing partner is preferred and in this regard GroEL is important in the solubilization of special complex cyclin E proteins produced in E. coli.

**Immobilization of special complex cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (aka MSG) or (SEQ. ID. NO. 7) and isolation of soluble MSG/GroEL complex and soluble MSG/Cdk2 complex**

Herein is described the use of cdk2 to substitute for GroEL in solubilizing special cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7).

The N-terminal tag (MRHHHHHHHK) (SEQ. ID. NO. 9) allows immobilization of the protein on immobilized nickel by virtue of the presence of neighboring histidines. This step is essential for copurification with GroEL which in turn allows recovery of a soluble complex useful for studying kinase activity in the presence of cdk2. The interaction of GroEL with cyclin E can be prevented by the absence of 0.5 M NaCl and this allows immobilization of special cyclin E special complex without GroEL.

When purified recombinant cdk2 from baculovirus infected insect cells is passed through a column containing immobilized special human cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7) produced in E. coli, a complex is formed on the column. This complex is eluted by using imidazole and the resulting cyclin E/cdk2 complex is soluble (Figure 12). Special cyclin E eluted in the absence of cdk2, under similar conditions, is insoluble. Therefore, cdk2 protein can also be used to interact with immobilized cyclin E and this allows recovery of a soluble complex which is active as a kinase.

**Binding of cdk2/ human cyclin E (MRHHHHHHHK-29E-395A-SAWRHPQFGG) (SEQ. ID. NO. 7) complex to streptavidin**

Figure 13 shows purification of the complex by affinity chromatography on immobilized streptavidin. As shown, the complex binds to the column and can be purified. The purified complex can be used for screening inhibitors of its kinase activity. In another embodiment, the complex can be used in assays requiring binding to streptavidin, for example, in a Scintillation Proximity Assay (SPA) based on streptavidin-coated beads.
Kinase activity of special cyclin E-cdk2 complexes.

Interestingly, neither GST-cyclin E-PEST*-Hist6/cdk2 complexes nor cyclin E (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7)/cdk2 complexes require addition of a cyclin-dependent activating kinase (CAK) for kinase activity (Figures 5 and 14). These results show that both the GST-cyclin E-PEST*-Hist6/cdk2 and cyclin E (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7)/cdk2 complexes are active. The kinase activity of the cyclin E (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7)/cdk2 complex can be further stimulated by the presence of a cyclin-dependent activating kinase (CAK).

In addition, the C-terminal strep tag may also be used for binding to immobilized streptavidin and further purification of the soluble (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7)/cdk2. This is also important because it would allow the complex to be used in assays requiring binding to streptavidin, for example, in Scintillation Proximity Assay (SPA) based on streptavidin-coated beads. Assays using special cyclin E/cdk2 complexes for detecting interaction with Kinase Inhibitor Proteins (KIPs)

The present invention includes the use of either GST-cyclin E-PEST*-Hist6/cdk2 complexes or cyclin E (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7)/cdk2 complexes in a scintillation proximity assay that detects their association with members of the p21/p27 family of Kinase Inhibitor Proteins (KIPs). Complexes are biotinylated and then are adsorbed to streptavidin-coated SPA beads. Radiolabeled KIP molecules are then added, and association with the special cyclin E/cdk2-SPA bead complexes induce scintillation. As an alternative to biotinylation, specific antibodies may be used to attach the special cyclin E/cdk2 complexes to SPA beads. As another alternative to biotinylation, the strep-tag on cyclin E (MRHHHHHHHK-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7)/cdk2 may be utilized in conjunction with streptavidin-coated SPA beads. A schematic representation of a strep tag approach for a SPA-based KIP assay for screening Kip mimetics is shown in Figure 15. The strep tag is engineered to the C-terminus of a truncated version (29E-395A) of human cyclin E which is then complexed with recombinant cdk2. This complex binds to streptavidin coated SPA beads by virtue of the presence of the strep tag at the C-terminus of human cyclin E. For the experiment shown in Figure 15, the minimal domain (amino acids 28 to 81) of the kinase inhibitory protein (Kip) p27, expressed as ubiquitin fusion in E. coli, is used and referred to as "mini p27."
A dose-response study (Figure 16) showed that the special cyclin E (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (SEQ. ID. NO. 7)/cdk2 complexes are approaching saturation with mini p27 (200 nM) at about 200 nM of the complex. The specificity of this assay is demonstrated by displacement of the $[^3H]$ labeled mini p27 in the presence of cold mini p27. Almost complete displacement of the labeled protein is observed at about 2.0 uM of the competing mini p27. Similar results have been obtained using biotinylated GST-cyclin E-PEST*-His6/cdk2 complexes (Figure 6). Recombinant cdk2 alone, or a human cyclin E/cdk2 complexes incapable of adsorption to SPA beads are used as negative controls and showed no SPA counts.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Pharmacia & Upjohn Company

(ii) TITLE OF INVENTION: Special Constructs and Complexes of
Cyclin E

(iii) NUMBER OF SEQUENCES: 21

(iv) CORRESPONDENCE ADDRESS:

15
(A) ADDRESSEE: Pharmacia & Upjohn Company
(B) STREET: 301 Henrietta Street
(C) CITY: Kalamazoo
(D) STATE: MI
(E) COUNTRY: USA
(F) ZIP: 49001

(v) COMPUTER READABLE FORM:

20
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Rel. #1.0, Ver. #1.25/WordPerfect 5.2+

(vi) CURRENT APPLICATION DATA:

30
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

35
(A) NAME: Wootton, Thomas A.
(B) REGISTRATION NUMBER: 35,004
(C) REFERENCE/DOCKET NUMBER: 6054

(ix) TELECOMMUNICATION INFORMATION:

40
(A) TELEPHONE: 616-833-7914
(B) TELEFAX: 616-833-8897

(2) INFORMATION FOR SEQ ID NO: 1:

45

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1951 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i) MOLECULE TYPE: DNA (genomic)
(ii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(iv) FRAGMENT TYPE: N-terminal

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

ATGTCCTCTA TACTAGTTA TTGGAAAATTT AAGGGCCTTG TGCAACCCAC TCGACTCTTT 60
TTGGAATATC TTGAAGAAA ATATGAAGAG CATTGTATAG AGCGCGATGA AGGTGATAAA 120
TGCGGAAACA AAAAGTTTGA ATTTGTTTG TGATTTCCCA ATCTTCTTTA TTATATGAT 180
GGTGATGTGA AATTAACACA GTCTATGGCC ATCATACGTG ATATAAGCTGA CAAGACAAC 240
ATGTGGGGTG GTGCTCCAAG AGAGCGTGCA GAGATTCTCA TGCTTGAGG AGCGGTTTTG 300
GATATTAGAT AGGTTGTTCG GAGAATTGCA TATAGTAAAG ACTTTGAAC TCTCAAGTT 360
GATTTTCTTA GCAAGCTACC TGAATTGCTG AAAATGTTCG AAGATCGTTG ATGTCAATAA 420
ACATATTTAA ATGGGTGATCA TGTAACCCAT CCTGACTTCA TGTTGATGAT CGCTCTTTGAT 480
GGTGTTTTAT ACATGGCACCT AATGGCCTG TGAGCGTTCG CAAAATTAGT TGGTTTTAAA 540
AAACGTATTG AAGCTATCCC ACAATTTGAT AATACTTGA AATCCACGCA GTATATAGCA 600
TGCCCTTGGC AGGCCGTGGCA AGCCACGTTC GTGGTGTCCG ACCATCCTCC AAAATCGGAT 660
CTCATGAAGG AGGACGCGGG CGCGAGGTTTC TGCGGCTCGCT CGAGAAGAGG GAAGGCAAC 720
GTGACCGTTT TTGGCGAGGA TCCAGATGAA GAAATGCGCA AAATCGACAG GACGCGGAGG 780
GACCAATGTCG GAGCGGACCC TTGGGACAAAT AATGCAGTCT GTGCAGACCC CTGCTCCCTG 840

-32-
ATCCCCACAC CTGACAAAGA AGATGATGAC CGGTTTACC CAAACTCAAC GTGCAAGCCT
CGGATTATGG CACCATCAG AGGCTCCCGC CTGCCCTGTAC TGAGCTGGGC AAATAGAGAG
5 GAAGTCTGGA AAATCATGTT AAACAAGGAA AAGACATACT TAAGGGATCA GCACCTTCTT
GAGCAAACACC CTCTTCTGCA GCCAAAAATG CGAGCAATTTC TTTGAGATGG GTTAATGGAG
10 GTGTGTAAGG TCTATAAACT TCAACAGGGGAC ACCTTTTACT TGGCACAAGA TTCTTTTGAC
CGGTATATGG CGACACAAGA AAATGTTGTA AAAACTCTTT TACAGCTTAT TGGGATTCTCA
TCTTTATTTA TTGCAGCCCCA ACTTGGAGAA ATCTATCCTC CAAAGTTGCA CCAGTTTGCG
15 TATGGGACAG ATGGGACTTG TTTCAAGGAT GAAATCTCA CCATGGAATT AATGATTATG
AAGGCCCTTA AGTGCGGTCTT AAGTCCCCTG ACTATTTGTT CCTGGCTGAA TGTATACATG
CAGGTTGCAT ATCTAAATGA CTTCATGAA GTGCTACTGC CCGATATCC CCAAGAATCTC
20 TTTATACAGA TGTCAGAGCT GTGTTGACTC TGTTGCTCTGG ATGTGACTCC CTTGAAATTT
CTTACATGAA GTGCTACTGC CCGATATCC CCAAGAATCTC TTTATACAGA TTGCAGAGCT
GTGGGATCTC TGTCCTCTGG ATGGGACTCG CCTTGAATTT CCTATGGTA TACTTGCTGC
25 TTCGGCCTTG TATCATTTCT CGTCATCTGA ATGTGATGCA AAAGTTTCG GGTATACGTG
GTGGACATA GAGAAGTGTG TCAAGTGTTG GGTCTCATTGC GCCATGGTTA TAAGGGAGAC
30 GGGGAAGCTCA AAACGTAGGC ACTTCAGGGG CGTGCGTGAT GAAGATGCAC ACAACATACA
GACCCACAGA GACAGCTTGG ATTTGCTGGA CAAAGCCCCGA GCAAAGAGAA CCATGTTCGT
35 TGAAACAATA GAGGCTCTTC CTCTCCCCG AGGGCTCTTC ATCCGGGCAC AGGCGGTAAA
GAAGCAGAGC CACCAACACC ACCAACCCTG A
40
(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 616 amino acids
  (B) TYPE: amino acid
  (C) STRANDDEDNESS: single
  (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro

Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu

Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu

Gly Leu Glu Phe Pro Asn Leu Pro Pro Tyr Tyr Ile Asp Gly Asp Val Lys

Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn

Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu

Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser

Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu

Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn

Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp

Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
His Gln Phe Ala Tyr Val Thr Asp Gly Ala Cys Ser Gly Asp Glu Ile
420 425 430
Leu Thr Met Glu Leu Met Ile Met Lys Ala Leu Lys Trp Arg Leu Ser
435 440 445
Pro Leu Thr Ile Val Ser Trp Leu Asn Val Tyr Met Gln Val Ala Tyr
450 455 460
Leu Asn Asp Leu His Glu Val Leu Leu Pro Gln Tyr Pro Gln Gln Ile
465 470 475 480
Phe Ile Gln Ile Ala Glu Leu Leu Asp Leu Cys Val Leu Asp Val Asp
485 490 495
Cys Leu Glu Phe Pro Tyr Gly Ile Leu Ala Ala Ser Ala Leu Tyr His
500 505 510
Phe Ser Ser Ser Glu Leu Met Gln Lys Val Ser Gly Tyr Gln Trp Cys
515 520 525
Asp Ile Glu Asn Cys Val Lys Trp Met Val Pro Phe Ala Met Val Ile
530 535 540
Arg Glu Thr Gly Ser Ser Lys Leu Lys His Phe Arg Gly Val Ala Asp
545 550 555 560
Glu Asp Ala His Asn Ile Gln Thr His Arg Asp Ser Leu Asp Leu Leu
565 570 575
Asp Lys Ala Arg Ala Lys Lys Ala Met Leu Ser Glu Gln Asn Arg Ala
580 585 590
Ser Pro Leu Pro Ser Gly Leu Leu Ile Ala Ala Gln Gly Gly Lys Lys
595 600 605
Gln Ser His His His His His
610 615

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSOR: NO

(v) FRAGMENT TYPE: N-terminal

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

Arg Ala Ser Pro Leu Pro Ser Gly Leu Leu Thr Pro Pro Gln Ser Gly
   1      5      10      15

Lys Lys Gln Ser Ser Gly Pro Glu Met Ala
   20     25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 81 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSOR: NO

(v) FRAGMENT TYPE: N-terminal

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

AGGGCTTC TCCTCCCCAG TGGGCTCCTC ACCCCGCCAC AGAGGGTAA GAAGCAGAC

AGCGGGCCGG AAATGGGGTG A

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 1101 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

GAAAGAATGG CCAAAAATCA CAGGACGGCG AGGGACCATG GTGGGAGCCA GCCTTGGGAC 60

AATAATGCAG TCTGTGCAGA CCCCTGCTCC CTGATCCCCA CACCTGACAA AGAAGATGAT 120

GACCGGGTTT ACCAAAACTC AACGTGCAAG CCTCGGATTA TCGACCATC CAGAGGCTCC 180

CCGCTGCGCTG TACTGAGCTG GGCAATAAGA GAGGAAGTCT GAAAAATCAT GTTAAACAAG 240

GAAAAAGACAT ACTTTAGGGA TCAGCACCTT CTTGAGCAAC ACCCTCTCTT GCAGCCAAAA 300

ATGCCGAGCAA TTCTTCCTGA TTGGTTAATG GAGGTGTGCTG AAGTCTTAA AACTCACAGG 360

GAGACCTTTT ACTGGGCACA AGATTTCTTT GACCGGTATA TGGCGACACA AGAAAAATGT 420

GTAAAAACTC TTTTACAGCT TATTGGGATT TCATCTTTAT TTTATGACGC CAAACTTTGAG 480

GAAATCTATC CTCCAAAGTT GCACCAGTTT GCCTATGTTGA CAGATGGGAC TTGGTCAGGA 540

GATGAAATTTC TCACCATGGA ATTAATGATT ATGAGGGCCCC TTAAGTGCGC TTTAAGTCCC 600

CTGACTATTG TGTCCTGGCT GAATGTATAC ATGCAAGGTTG CATATCTAAA TGACTTACAT 660

GAAGTGCTAC TGCCGCAGTA TCCCCAGCCTT ATTTTTATAC AGATTGCAGA GCTGTTGGGAT 720

CTCTGTGATCC TGGATGTTGA CTGCCATTGA TTTTTTATAG GATATCTTGC TGCTCAGGCC 780

TTGTATCATT TTCTGTCATC TGAATTGATG CAAAAGGTTC CAGGGTATCA GTGGTGCGAC 840
(2) INFORMATION FOR SEQ ID NO:6:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 367 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

  (iii) HYPOTHETICAL: NO

  (iv) ANTI-SENSE: NO

  (v) FRAGMENT TYPE: N-terminal

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

Glu Glu Met Ala Lys Ile Asp Arg Thr Ala Arg Asp Gln Cys Gly Ser
  1  5  10  15

Gln Pro Trp Asp Asn Ala Val Cys Ala Asp Pro Cys Ser Leu Ile
  20  25  30

Pro Thr Pro Asp Lys Glu Asp Asp Arg Val Tyr Pro Asn Ser Thr
  35  40  45

Cys Lys Pro Arg Ile Ile Ala Pro Ser Arg Gly Ser Pro Leu Pro Val
  50  55  60

Leu Ser Trp Ala Asn Arg Glu Glu Val Trp Lys Ile Met Leu Asn Lys
  65  70  75  80

-39-
Glu Lys Thr Tyr Leu Arg Asp Gln His Phe Leu Glu Gln His Pro Leu
  
85  
Leu Gln Pro Lys Met Arg Ala Ile Leu Leu Asp Trp Leu Met Glu Val
100 105 110
Cys Glu Val Tyr Lys Leu His Arg Glu Thr Phe Tyr Leu Ala Gln Asp
115 120 125
Phe Phe Asp Arg Tyr Met Ala Thr Gln Glu Asn Val Val Lys Thr Leu
130 135 140
Leu Gln Leu Ile Gly Ile Ser Ser Leu Phe Ile Ala Ala Lys Leu Glu
145 150 155 160
Glu Ile Tyr Pro Pro Lys Leu His Gln Phe Ala Tyr Val Thr Asp Gly
165 170 175
Ala Cys Ser Gly Asp Glu Ile Leu Thr Met Glu Leu Met Ile Met Lys
180 185 190
Ala Leu Lys Trp Arg Leu Ser Pro Leu Thr Ile Val Ser Trp Leu Asn
195 200 205
Val Tyr Met Gln Val Ala Tyr Leu Asn Asp Leu His Glu Val Leu Leu
210 215 220
Pro Gln Tyr Pro Gln Glu Ile Phe Ile Gln Ile Ala Glu Leu Leu Asp
225 230 235 240
Leu Cys Val Leu Asp Val Asp Cys Leu Glu Phe Pro Tyr Gly Ile Leu
245 250 255
Ala Ala Ser Ala Leu Tyr His Phe Ser Ser Ser Glu Leu Met Glu Lys
260 265 270
Val Ser Gly Tyr Gln Trp Cys Asp Ile Glu Asn Cys Val Lys Trp Met
275 280 285
Val Pro Phe Ala Met Val Ile Arg Glu Thr Gly Ser Ser Lys Leu Lys
290 295 300
His Phe Arg Gly Val Ala Asp Glu Asp Ala His Asn Ile Gln Thr His
305 310 315 320

-40-
Arg Asp Ser Leu Asp Leu Leu Asp Lys Ala Arg Ala Lys Lys Ala Met
325
330
335
Leu Ser Glu Gln Asn Arg Ala Ser Pro Leu Pro Ser Gly Leu Leu Thr
340
345
350
Pro Pro Gln Ser Gly Lys Lys Gln Ser Ser Ser Gly Pro Glu Met Ala
355
360
365

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 386 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

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

-41-
<table>
<thead>
<tr>
<th>Residue</th>
<th>Sequence</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu Val Trp Lys Ile Met Leu Asn Lys Glu Lys Thr Tyr Leu Arg Asp</td>
<td>85</td>
<td>90</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Glu His Phe Leu Glu Gln His Pro Leu Leu Gln Pro Lys Met Arg Ala</td>
<td>100</td>
<td>105</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Ile Leu Leu Asp Trp Leu Met Glu Val Cys Glu Val Tyr Lys Leu His</td>
<td>115</td>
<td>120</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Arg Glu Thr Phe Tyr Leu Ala Glu Asp Phe Phe Asp Arg Tyr Met Ala</td>
<td>130</td>
<td>135</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Thr Gln Glu Asn Val Val Lys Thr Leu Leu Gln Leu Ile Gly Ile Ser</td>
<td>145</td>
<td>150</td>
<td>155</td>
<td>160</td>
</tr>
<tr>
<td>Ser Leu Phe Ile Ala Ala Lys Leu Glu Glu Ile Tyr Pro Pro Lys Leu</td>
<td>165</td>
<td>170</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>His Gln Phe Ala Tyr Val Thr Asp Gly Ala Cys Ser Gly Asp Glu Ile</td>
<td>180</td>
<td>185</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>Leu Thr Met Glu Leu Met Ile Met Lys Ala Leu Lys Trp Arg Leu Ser</td>
<td>195</td>
<td>200</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>Pro Leu Thr Ile Val Ser Trp Leu Asn Val Tyr Met Gln Val Ala Tyr</td>
<td>210</td>
<td>215</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>Leu Asn Asp Leu His Glu Val Leu Leu Pro Gln Tyr Pro Gln Gln Ile</td>
<td>225</td>
<td>230</td>
<td>235</td>
<td>240</td>
</tr>
<tr>
<td>Phe Ile Gln Ile Ala Glu Leu Leu Asp Leu Cys Val Leu Asp Val Asp</td>
<td>245</td>
<td>250</td>
<td>255</td>
<td></td>
</tr>
<tr>
<td>Cys Leu Glu Phe Pro Tyr Gly Ile Leu Ala Ala Ser Ala Leu Tyr His</td>
<td>260</td>
<td>265</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>Phe Ser Ser Glu Leu Met Gln Lys Val Ser Gly Tyr Gln Trp Cys</td>
<td>275</td>
<td>280</td>
<td>285</td>
<td></td>
</tr>
<tr>
<td>Asp Ile Glu Asn Cys Val Lys Trp Met Val Pro Phe Ala Met Val Ile</td>
<td>290</td>
<td>295</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Arg Glu Thr Gly Ser Ser Lys Leu Lys His Phe Arg Gly Val Ala Asp</td>
<td>305</td>
<td>310</td>
<td>315</td>
<td>320</td>
</tr>
</tbody>
</table>
Glu Asp Ala His Asn Ile Gln Thr His Arg Asp Ser Leu Asp Leu Leu 325 330 335
Asp Lys Ala Arg Ala Lys Lys Ala Met Leu Ser Glu Gln Asn Arg Ala 340 345 350
Ser Pro Leu Pro Ser Gly Leu Leu Thr Pro Pro Gln Ser Gly Lys Lys 355 360 365
Gln Ser Ser Gly Pro Glu Met Ala Ser Ala Trp Arg His Pro Gln Phe 370 375 380
Gly Gly 385

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1158 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

ATGCGACACC ATCACCATCA CCATAAGGAA GAAATGCGCA AAATCGACAG GACGCGAGG 60
GACCAGTGTG GGACCACGG TGGGGACAAT AATGCAGTCT GTGCGAGCCC CTGCTCCCTG 120
ATCCCCACAC CTGCAAAAGA AGATGATGAC CGGTTTACC CAAACTCAAC GTGCAAGCCT 180
CGGATTATGG CACCATCCAG AGGCTCCCCCG CTGCTGTAC TGAGCTGGGC AAATAGAGAG 240
GAAGTCTGGA AAATCATGTT AAACAAGGAA AAGACATACT TAAGGATCA GCACCTTTCTT 300
GAGCAACACC CTTTTCTGCA GCCAAAAATG CGAGCAATTC TTCTGGATTG GTAATGGAG
360
GTGTGTGAAG TCTATAAAACT TCACAGGGAG ACCTTTTACT TGGCACAAGA TTCTTTTGAC
420
CGGTATATGG CGACACAAGA AAATGTGTA AAAACTCTTT TACAGCTTAT TGGGATTACA
480
TCTTTATTTA TTGCGAGCCAA ACTTGAGGAA ATCTATCCTC CAAAGTTGCA CCAAGTTTCG
540
TATTGTGACAG ATGGAGCCTTG TCGAGAGAT GAAATTCTCA CCATGGAATT AATGATATTG
600
AAGGCCTTTA AGTGGGTTTT AAGTCCCCCTG ACTATTGTGT CCTGCTGAA TGTTATACAG
660
CAGGTTGACAT ATCTAAATGA CTTACATGAA GTGCTACTGC CGCAGTATCC CCAGCAAAATC
720
TTTATACAGA TTGCGAGGCT TTGGATCTC TGGTTCTGGG ATGTGACTGT CCTGAATTTI
780
CTTGATGTTA TACTTGGCTG TGGCGCTTTG TATCATTTCT CGTCATCTGA ATTTGATGCAA
840
AAGGTTCAG GGTATCAAGT GTGCGACATA GAGAAGTGTG TCAAATGGAG GGTCCATTTT
900
GCCATGGTAA TAAGGGAGAC GGGGAGCTCA AAACAGAAGC ACTTCAGGGG CGTCCGTGAT
960
GAAGATGCAAC ACAACATACA GACCCCACAGA GACAGCTTTG ATTTGCTGGA CAAAGCCCGA
1020
GCAAAGAAAG CCATTTTGTG TGAACAAATA AGGCTTTCTC CTCTCCCCAG TGGCTCTCTC
1080
ACCCCGCGCAC AGACGCGTAA GAAGCAGAGC AGCGGGCGGG AAATGGCGAG CGCATGGAGA
1140
CACCCCCAGT TTGGAGGA
1158

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 9 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

1  Met Arg His His His His His Lys
5

(2) INFORMATION FOR SEQ ID NO:10:

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

15 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

ATGCGACACC ATCACCATCA CCATAAG

30 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal
(XI) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Ala Trp Arg His Pro Gln Phe Gly Gly

1  5  10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

AGCGCATGGA GACACCCCCA GTTTGGAGGA

30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 67 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCAAGATCTTC AGTGGTGTTG GTGGTGTGGG CTCTGCTTTCT TACCGCCCTG TGCCGGCATG

5 AGGAGCC

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 30 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

20 (v) FRAGMENT TYPE: N-terminal

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

GATCAGATCT CATGAAAGG GACGCGCCGGC

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 18 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Arg His His His His His Lys Glu Glu Met Ala Lys Ile Asp
1  5  10  15

Arg Thr

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 64 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

GGAATTCCAT ATGCGACACC ATCACCATCA CCATAAGGAA GAAATGGCCA AAATCGACAG
30

GACG

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 19 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

Gly Gly Phe Gln Pro His Arg Trp Ala Ser Ala Met Glu Pro Gly Ser
1  5  10  15
Ser Gln Lys

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 69 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

CCCAAGCTTC TATCCTCCAA ACTGGGGTG TCTCCATGCG CTCGCCATT T CCGGCCC GT

GCTCTGCTT

(2) INFORMATION FOR SEQ ID NO:19:

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

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

ATGAAGGAGGACGGCGCG

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 11 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

GATCAGATCTC

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

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

Arg Ala Ser Pro Leu Pro Ser Gly Leu Leu Ile Ala Ala Gln Gly Gly

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Lys Lys Gln Ser His His His His His

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>
CLAIMS

1.* A nucleic acid polymer, designated, cyclin E-PEST*-His6, coding for the polypeptide shown in Figure 2, and related or redundant polymers coding for substantially the same polypeptide.

2. A nucleic acid polymer, of claim 1, designated, cyclin E-PEST*-His6, comprising the DNA sequence shown in Figure 1, and related or redundant polymers comprising the DNA sequence shown in Figure 1, and where the nucleic acid residues in the related or redundant polymers are the same as those in Figure 1, and allowing for the deleting, adding or replacing one to several nucleic acid residues.

3. A nucleic acid polymer, of claim 1, designated, GST-Cyclin E-PEST*-His6, comprising the nucleic acid polymer of claim 1, having its 5' end bound to nucleic acid coding for glutathione S-transferase (GST).

4. A nucleic acid polymer, of claim 1, designated, His6-Cyclin E-PEST*-His6, comprising the nucleic acid polymer of claim 1, having its 5' end bound to nucleic acids coding for His6.

5.* A polypeptide, designated, cyclin E-PEST*-His6, comprising the amino acid sequence shown in Figure 2, and equivalent substitutions thereof allowing for the deleting, adding or replacing one to several amino acid residues.

6. A polypeptide of claim 5, designated, GST-Cyclin E-PEST*-His6, having its N-terminus bound to glutathione S-transferase (GST).

7. A composition comprising the polypeptide of claim 6 in close association with Cdk2.

8. A polypeptide of claim 7, designated His6-Cyclin E-PEST*-His6, having its N-terminus bound to His6.

9. A composition comprising the polypeptide of claim 8 in close association with
Cdk2.

10.*  An oligonucleotide primer comprising the following sequence, 5'-
GCAGATCTTCAGTGGTGGTGCTCGTCTCCTTACCG
5
CCCCTGCGCCGATGAGAGCC-3'  (SEQ. ID. NO. 13)

11.*  A fusion plasmid, designated cyclin E-PEST*-His6 fusion plasmid, that codes
for cyclin E-PEST*-His6 polypeptide, comprising a plasmid made from a plasmid
produced from a PCR reaction where there is a PCR oligonucleotide 3' primer and a
5' primer and the 3' primer is 5'-
GCAGATCTTCAGTGGTGGTGCTCGTCTCCTTACCG
CCCCTGCGCCGATGAGAGCC-3'  (SEQ. ID. NO. 13) allowing for the deleting,
adding or replacing one to several nucleic acid residues, and the 5' primer is an
oligonucleotide having a cloning site and a site having nucleic acids identical to the
5' end of cyclin E and the PCR reaction amplifies native cyclin E.

12.  A fusion plasmid of claim 11 where the site having nucleic acids identical to
the 5' end of cyclin E are the following nucleic acids, ATGAAGGAGGACGGCGCG
(SEQ. ID. NO. 19)

13.  A fusion plasmid of claim 12 where the 5' primer has a cloning site that is
GATCAGATCTTC  (SEQ. ID. NO. 20).

14.  A fusion plasmid of claim 11 where the 5' primer is an oligonucleotide having
a cloning site and a site having nucleic acids identical to the 5' end of cyclin E,
where the nucleic acids sequence is GATCAGATCTTCATGAAGGACGGCGCG,
(SEQ. ID. NO. 14) where the underlined nucleic acids are the cloning site and the
nonunderlined nucleic acids are the nucleic acids identical to the 5' end of cyclin E.

15.*  A fusion plasmid, designated GST/his6-cyclin E-PEST*-His6 fusion plasmid,
that codes for either glutathione S-transferase (GST)-cyclin E-PEST*-His6
polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide that is made from a
plasmid produced from a PCR reaction where there is a PCR oligonucleotide 3' primer and a 5' primer and the 3' primer is 5'-
GCAGATCTTCAGTGGTGGTGCTCGTCTCCTTACCG

-53-
CCCTGTGCCGGATGAGGAGCC-3' (SEQ. ID. NO. 13) allowing for the deleting, adding or replacing one to several nucleic acid residues and the 5' primer is an oligonucleotide having a cloning site and a site having nucleic acids identical to the 5' end of cyclin E and the PCR reaction amplifies native cyclin E and this product is then subcloned into a fusion plasmid where the fusion plasmid contains either a GST or a His6 coding region at the 5' end, that codes for either the (GST)-cyclin E-PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide.

16. A fusion plasmid of claim 15 where the fusion plasmid is derived from pRSET-A (Invitrogen) that codes for the His6-Cyclin E-PEST*-His6 polypeptide.

17. A fusion plasmid of claim 15 where the fusion plasmid is derived from pGEX-2T (Pharmacia) that codes for the (GST)-cyclin E-PEST*-His6 polypeptide.

18. A bacteria that expresses either the glutathione S-transferase (GST)-cyclin E-PEST*-His6 fusion plasmid, or the His6-Cyclin E-PEST*-His6 fusion plasmid where the expressed protein comprises, either the GST-cyclin E-PEST*-His6 polypeptide, or the His6-cyclin E-PEST*-His6 polypeptide.

19. A bacteria of claim 18 where the bacteria contains a GST-cyclin E fusion plasmid.

20. A bacteria of claim 18 where the bacteria contains a His6-cyclin E fusion plasmid.

21. A bacteria of claim 18 where the bacteria is of the type E. coli.

22.* A baculovirus, created by subcloning a linear GST/his6-cyclin E-PEST*-His6 nucleic acid fragment into a baculoviral transfer vector, and then co-transfecting this product into a baculovirus host cell with baculoviral DNA.

23. A baculovirus of claim 22 where the Baculoviral DNA is BaculoGold crippled baculoviral DNA.

24. A baculovirus of claim 23 where the baculovirus transfer vector is any of the
vectors in the specification, including pAcGHLT-C and pVL1392.

25.* A baculovirus, created by subcloning a PCR product, created from a PCR reaction starting with DNA that codes for either His6-cyclin E-PEST*-His6, or GST-cyclin E-PEST*-His6, where the PCR product is subcloned into a baculoviral transfer vector, where the baculoviral transfer vector comprising the PCR product is co-transfected into a baculovirus host cell with baculoviral DNA.

26. The baculovirus of claim 25 where the PCR product is linear GST-cyclin E-
5 PEST*-His6 nucleic acid.

27. The baculovirus of claim 25 where the PCR product is linear His6-cyclin E-
10 PEST*-His6 nucleic acid.

28. The baculovirus of claim 25 where the baculovirus transfer vector is any of the transfer vectors mentioned in the specification, including pAcGHLT-C and pVL1392.

29. The baculovirus of claim 28 where the baculovirus transfer vector is
20 pAcGHLT-C.

30. The baculovirus of claim 25 where the baculovirus host cell DNA is BaculoGold crippled baculoviral DNA from Pharmingen.

25 31.* Cells containing a baculovirus that express either the glutathione S-
transferase (GST)-cyclin E-PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide.

32. Cells of claim 31 where the cells are prokaryotes.

30

33. Cells of claim 31 where the cells are eukaryotes.

34. Cells of claim 31 where the cells are derived from insect cells.

35 35. Cells of claim 34 where the cells are created from a PCR reaction beginning
with linear (GST)-cyclin E-PEST*-His6 DNA, or His6-Cyclin E-PEST*-His6 DNA.

36. Cells of claim 35 where the PCR reaction begins with linear (GST)-cyclin E-
PEST*-His6 DNA.

37. Cells of claim 36 where the cells are insect type cells known as Sf 9 or High
Five type cells.

38. Cells of claim 33 where the cells are derived from mammals.

39. Cells of claim 33 where the cells are derived from yeast.

40.* The protein complex comprising glutathione S-transferase (GST)-cyclin E-
PEST*-His6 polypeptide, or the His6-Cyclin E-PEST*-His6 polypeptide with cdk2.

41.* The method of producing any of the the cyclin E-PEST*-His6, GST-cyclin E-
PEST*-His6, His6-cyclin E-PEST*-His6 ; DNA, amino acids, oligonucleotide
primers, fusion plasmids, bacteria or baculovirus as described in the specification.

42.* The method of using glutathione affinity chromatography to produce purified
glutathione S-transferase (GST)-cyclin E-PEST*-His6 polypeptide/cdk2 complexes, or
His6-Cyclin E-PEST*-His6 polypeptide/cdk2 complex.

43.* Any of the cyclin E-PEST*-His6, GST-cyclin E-PEST*-His6, His6-cyclin E-
PEST*-His6 ; DNA, amino acids, oligonucleotide primers, fusion plasmids, bacteria
or baculovirus as described herein produced or created from the methods and
processes described herein.

44.* A nucleic acid polymer, coding for the polypeptide designated 29E-395A,
shown in Figure 9 or **SEQ. ID. NO. 6**, and related or redundant polymers coding for
substantially the same polypeptide, where MSG is the DNA that codes for
(MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (**SEQ. ID. NO. 7**), and allowing for
the deleting, adding or replacing one to several nucleic acid residues.

45. A nucleic acid polymer of claim 44, comprising the DNA sequence shown in
Figure 8, or **SEQ. ID. NO. 5**, and allowing for the deleting, adding or replacing one to several nucleic acid residues thereof.

46.* The nucleic acids that code for the polypeptide, (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (**SEQ. ID. NO. 7**), as shown in **SEQ. ID. NO. 7**, comprising the nucleic acids of claim 1, attached to the nucleic acids that code for the N-terminal sequence (MRHHHHHHHK) (**SEQ. ID. NO. 9**) and the C-terminal sequence (SAWRHPQFGG) (**SEQ. ID. NO. 11**), and allowing for the deleting, adding or replacing one to several nucleic acid residues.

47. A nucleic acid polymer of claim 46, comprising the DNA sequence shown in **SEQ. ID. NO. 8** and allowing for the deleting, adding or replacing one to several nucleic acid residues.

48.* A polypeptide, designated 29E-395A, shown in Figure 9 or **SEQ. ID. NO. 6** and allowing for the deleting, adding or replacing one to several amino acid residues.

49. A polypeptide of claim 48, with the following amino acids attached to the N-terminal end, (MRHHHHHHHK) (**SEQ. ID. NO. 9**) and the following amino acids attached to the C-terminal end, (SAWRHPQFGG) (**SEQ. ID. NO. 11**) , thus providing the polypeptide designated, (MRHHHHHHHK)-(29E-395A)-(SAWRHPQFGG) (**SEQ. ID. NO. 7**), as shown in Sequence Listing number 7.

50. A composition comprising the polypeptide of claim 49 in close association with Cdk2.

51. A composition comprising the polypeptide of claim 49 in close association with GroEL.

52.* A composition comprising the complex of MSG with GroEL, where the complex is purified after the expression of MSG from bacterial cells, and where NaCl is present during cell lysis and purification.
53. A composition comprising the complex of claim 52, where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin.

54. A composition comprising the complex of claim 53, where the complex is purified using immobilized metal affinity chromatography (IMAC).

55. A composition comprising the complex of claim 52 where the NaCl concentration is about 0.5 M.

56.* A composition comprising the complex of MSG with cdk2, when the complex is produced from the expression of MSG from bacterial cells, when cdk2 is present during subsequent purification.

57. A composition comprising the complex of claim 56 where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin.

58. A composition comprising the complex of claim 57 where the MSG is first immobilized on a column and the complex then created by exposing the immobilized MSG to cdk2.

59. A composition comprising the complex of claim 58 where the cdk2 is recombinant cdk2 expressed from baculovirus infected insect cells.

60.* The process of preparing a complex of MSG with GroEL, by expressing MSG from bacterial cells, followed by purification of the MSG, with NaCl present during cell lysis and purification.

61. The process of preparing of claim 60, where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin.

62. The process of claim 61 where the complex is purified using immobilized metal affinity chromatography (IMAC).
63. The process of claim 60 where the NaCl concentration is about 0.5 M.

64.* The process of preparing a complex of MSG with cdk2, by expressing and purifying the MSG from bacterial cells, and having cdk2 present during the purification.

65. The process of claim 64 where the MSG expressed from bacterial cells is first immobilized on a column and the complex of MSG with cdk2 is created by exposing the immobilized MSG to cdk2.

66. The complex of claim 64, where the complex is purified using either immobilized metal affinity chromatography (IMAC) or immobilized streptavidin.

67. The complex of claim 64, where the cdk2 is recombinant cdk2 expressed from baculovirus infected insect cells.

68. Any of the MSG; DNA, amino acids, oligonucleotide primers, fusion plasmids, bacteria or baculovirus as described herein produced or created from the methods and processes described herein.
FIGURE 1 - continued

1401 CTTACATGAA GTGCTACTGC GCCAGTGATCC CCAGCAAATC TTTTATACAGA
1451 TGGCAGAGCT GTTGGATCTC TGTTGCTCTGG ATGTTGACTG CCTGGAATTT
1501 CCTTATGGTA TACTTGTGTC TGCCGCCTTG TATCATTTCT CTTGATGCTGA
1551 ATGTGACAA AAGGTTTCAG GGATACAGTG GTCCGACATA GAGAACTGTG
1601 TCGAGTGGAT GTTCTCATTT GCCATGGTTA TAAGGGAGAC GGGAGCTCA
1651 AAATTGAAGC ACTTCAAGGGG CTTCGGTAT GAAGATGCAC ACAACATACA
1701 GACCCACAGA GACAGCTTTGG ATTTGCTGGA CAAAGCCCCGA GCAAAAGAAAG
1751 CCATGTTGTC TGAACAAAAAT AGGCTTTCCTC CTCTCCCAG TGCGTCCTTC
1801 ATCGCCGAC AGGCGGTTAA GAAGCAGAGC CACCACCCAC ACCACCACTG
1851 A
<table>
<thead>
<tr>
<th>10</th>
<th>30</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSPILGKYWKIGLVPTRLLLEYYEEHYERDEGDKWNNKKEFGLEFFPNLPPYYID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>90</td>
<td>110</td>
</tr>
<tr>
<td>GDVKTQSMAIRYIADKHMLGGCPKERAEMSLEGAVLDVIRGVSRIAYSDKFETLVK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>150</td>
<td>170</td>
</tr>
<tr>
<td>DFLSKLPFMIKMFEDRLCHKTYLNGDVTHPDMLYDADVLVDLYMDPCLDAFPPKLVCFK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>190</td>
<td>210</td>
<td>230</td>
</tr>
<tr>
<td>KRIEAIPQIDKYLKKSSKYIAWPLQGQATFGGVDPHPPKSDLMKEDGGAEFSRASRKRKAN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>270</td>
<td>290</td>
</tr>
<tr>
<td>VTVFLOQPDPEEAKDTARDQCGSQFWDNNAVCADECPLSILPTPKDEDDORVYPNSTCKP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>310</td>
<td>330</td>
<td>350</td>
</tr>
<tr>
<td>RIIAPSRSPLPVLSWANREEERVKIMLNEKTYLRDQHFLQHPLQPKMRAILLDWLME</td>
<td></td>
<td></td>
</tr>
<tr>
<td>370</td>
<td>390</td>
<td>410</td>
</tr>
<tr>
<td>VCEVYLHRETFLAQDDDRYMATQENFVKTLQLISLFIAAKLIEJYPKKHLQFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>430</td>
<td>450</td>
<td>470</td>
</tr>
<tr>
<td>YVTDGACSDEILTMELMIMKALKWRLSCPSTIVSWLNVMQVAYNDLHEVLLPOPYQQI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>490</td>
<td>510</td>
<td>530</td>
</tr>
<tr>
<td>FIOIAELLEDLCVLBDVCELPPYGILASAALHYFSSELMQVGYWCDIECVKVMVF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>550</td>
<td>570</td>
<td>590</td>
</tr>
<tr>
<td>AMVIRETGGSKKHFGRVADEDAHNITHRDSLLDDKARAKAMLSEQRASQLPSGGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>610</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAAQGGKQSHHHHHH*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 3

370... Arg Ala Ser Pro Leu Pro Ser Gly Leu Leu Thr Pro Pro
1108... AGG GCT TCT CTC CCC AGT GGG CTC CTC ACC CCG CCA

-----------------------------------------------

1108... AGG GCT TCT CTC CCC AGT GGG CTC CTC ATC GCG GCA
370... Arg Ala Ser Pro Leu Pro Ser Gly Leu Leu Ile Ala Ala

Sequences from above contined below.

383... Gln Ser Gly Lys Lys Gln Ser Ser Gly Pro Glu Met Ala End
1147... CAG AGC GGT AAG AAG CAG AGC AGC GGG CCG GAA ATG GCG TGA

-----------------------------------------------

1147... CAG GGC GGT AAG AAG CAG AGC CAC CAC CAC CAC CAC TGA
383... Gln Gly Gly Lys Lys Gln Ser His His His His His His End
FIGURE 7
FIGURE 8

85   GAAGAAAATG 93
94   GCCAAAATCG ACAGGACGGC GAGGGACCAG TGTGGGAGCC AGCCTTGGGA
144  CAATAATGCA GTCTGTGCAG ACCCCTGCTC CCTGATCCCC ACACCTGACA
194  AAGAGATGTA TGACCGGGTT TACCCCAACT CAACGTGCAC GCTCGAGATT
244  ATGCACCACT CCAGAGGCCT CCCCTGCCTG GTCTGAGCT GGCGAAATGAG
294  AGAGGAAAGTC TGGAAAAATCA TGTTAAGACAGA TACTTAAGGG
344  ATCTGCACTT TCTTGGCACA CACCCCTCCTG CGACGCCTAA AACAGGGGCA
394  ATTTCTCTGG ATTTGTTAAT GGAGGTGTGT GAAGTCTATA AACTCCACG
444  GGAGACCTTT TACTTGGCAC AAGATTTTCTT TGACCGGTAT ATGGCGACAC
494  AAGAAAATGT TGTAAAAACT CTTTACAGC TTATTGGGAT TTTATCTTTA
544  TTATATGCAG CCAAACTTGA GGAATCTAT CTTCCAAAGT TGCAACGATT
594  TGGGTATGCT ACAGATGGAG CTGGTTCAAG AGATGAAATCTCAGCACTGG
644  ATATTATGAT TATGAAGGCC CTTAAGTGCC GTTTAAGTCC CCTGACTTAT
694  GTGTCCCTGCG CGAATGCTA CATGCAGGTT GCATATCTAA AGACTTACA
744  TGAAGTGCTA CTGCCCCGAG ATCCCCAGCA AAATTTATA CAGATTGCAG
794  AGCTGTGGGA TCTCTGTGCAC TGGGATGTGG ACTGCGTCTA AATTTCTTAT
844  TTATACTTGG CTGCTTCGGC CTCTGTTAAT CTGGCTGTCG CTGAATTGAT
894  GCAGAAAGTGG TAGCAGGTATG CAGGGTGCGA CATAGAAACG TGTCGAAGT
944  GGTGTATCCG AATTTGGCCAATTGTTAGG AGCGGGGAG CTTCAAACCTG
994  ATGCAGTCTC GGGGCGTCGG TGATGAAGAT GCACACACAG TACAGACCCA
1044 CGAGGAGAGC TGGAGATTGC TGGGAAAGCG CGGAGCAAAG AAGCCCATGT
1094 TGCTCTGAACA AAATAGGGCT TCTCCTTAC CCAGTGGGCT CCTCACCCCCG
1144 CCACAGAGCG GTTAGAAAGCA GAGCAGCGGG CCGGAAATGG CG 1185
E. coli Extract containing cyclin E (29E-395A) with N-terminal 6His tag and C-terminal strep tag (○)

↓

IMAC

↓

Immobilized cyclin E (29E-395A)

Recombinant cdk-2

Cyclin E (29E-395A)/cdk-2 complex

SPA

Strep Tag

In vivo [3H] labeled Ub-p27 "mini klp"

Streptavidin Coated SPA Bead
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

<table>
<thead>
<tr>
<th>IPC 6</th>
<th>C12N15/12</th>
<th>C07K14/47</th>
<th>C12N15/62</th>
<th>C12N1/21</th>
<th>C12N5/10</th>
</tr>
</thead>
</table>

According to International Patent Classification (IPC) or to both national classification and IPC.

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

<table>
<thead>
<tr>
<th>IPC 6</th>
<th>C12N</th>
<th>C07K</th>
</tr>
</thead>
</table>

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic database consulted during the international search (name of database and, where practical, search terms used).

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>see page 1225, left-hand column; figure 2</td>
<td>68</td>
</tr>
<tr>
<td>Y</td>
<td>WO 93 06123 A (HUTCHINSON FRED CANCER RES :UNIV ROCKEFELLER (US)) 1 April 1993 cited in the application</td>
<td>1-6, 17-24</td>
</tr>
<tr>
<td>X</td>
<td>see page 52 - page 53</td>
<td>68</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of box C. Patent family members are listed in annex.

**Date of the actual completion of the international search**

13 November 1997

**Date of mailing of the international search report**

11/12/1997

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Espen, J
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>see the whole document</td>
<td>68</td>
</tr>
<tr>
<td>Y</td>
<td>EP 0 646 646 A (SQUIBB BRISTOL MYERS CO) 5 April 1995</td>
<td>1-6, 17-24</td>
</tr>
<tr>
<td></td>
<td>see claims 1-21; figure 1</td>
<td>68</td>
</tr>
<tr>
<td>Y</td>
<td>DAVIES A H ET AL: &quot;RECOMBINANT BACULOVIRUS VECTORS EXPRESSING GLUTATHIONE-S-TRANSFERASE FUSION PROTEINS&quot; BIO/TECHNOLOGY, vol. 11, 1 August 1993, pages 933-936, XP002000891</td>
<td>1-6, 17-24</td>
</tr>
<tr>
<td></td>
<td>see figure 1</td>
<td>68</td>
</tr>
<tr>
<td>Y</td>
<td>WO 95 09239 A (HARVARD COLLEGE) 6 April 1995</td>
<td>1-6, 17-24</td>
</tr>
<tr>
<td></td>
<td>see page 3; examples 6,8; table 1</td>
<td>68</td>
</tr>
<tr>
<td>Y</td>
<td>SALAMA SR ET AL: &quot;G1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover.&quot; MOL CELL BIOL, DEC 1994, 14 (12) P7953-66, UNITED STATES, XP002046707</td>
<td>1-6, 17-24</td>
</tr>
<tr>
<td></td>
<td>see page 7954, right-hand column - page 7956, left-hand column; figure 1</td>
<td>68</td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>WO 9306123 A</td>
<td>01-04-93</td>
<td>AU 676137 B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 2666392 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2119443 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0604560 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 7502164 T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 5645999 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 5449755 A</td>
</tr>
<tr>
<td>EP 0646646 A</td>
<td>05-04-95</td>
<td>CA 2132937 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 7184663 A</td>
</tr>
<tr>
<td>WO 9509239 A</td>
<td>06-04-95</td>
<td>US 5648244 A</td>
</tr>
</tbody>
</table>