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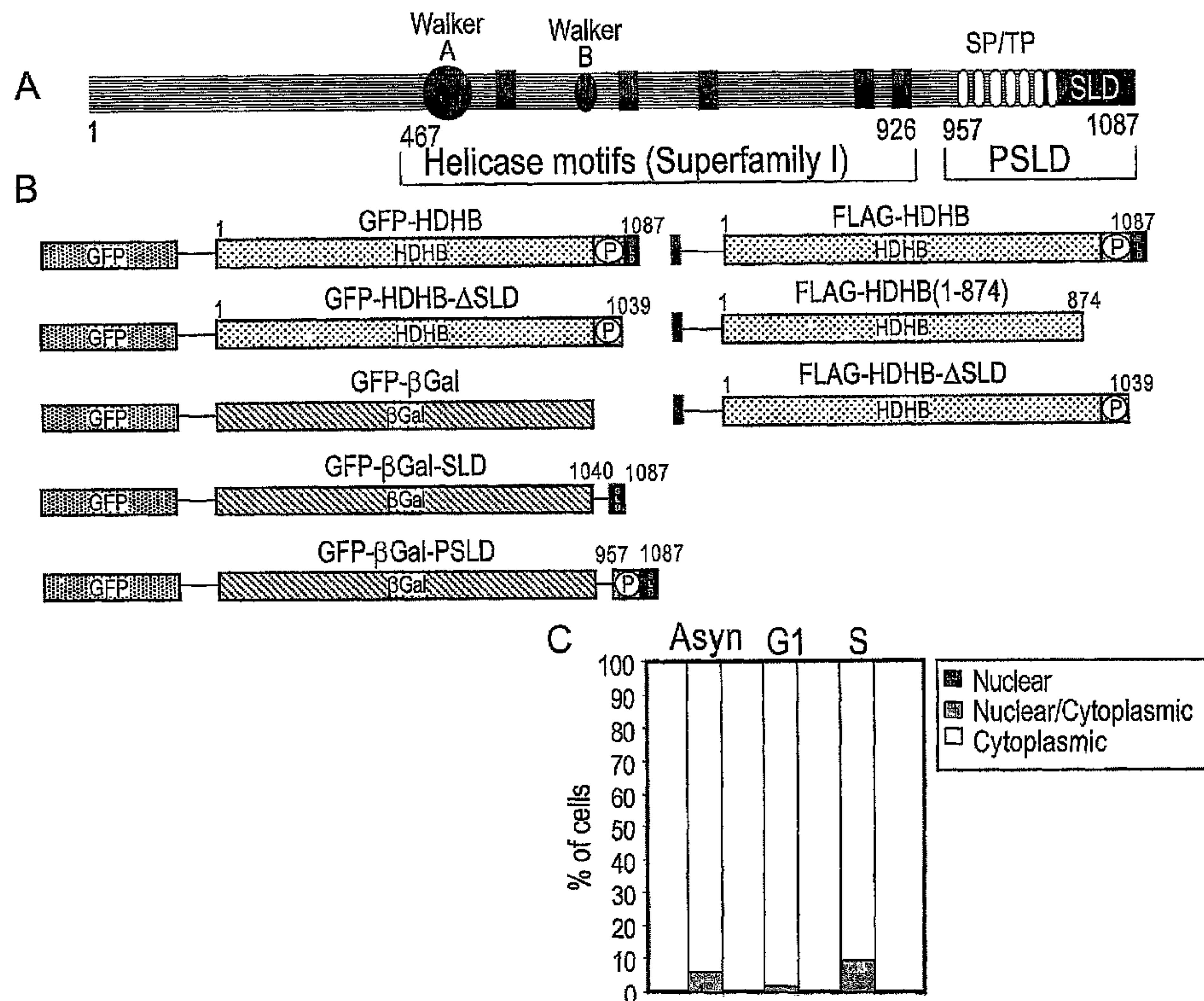
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(54) Title: CELL CYCLE PHASE MARKERS



(57) **Abrégé/Abstract:**

The present invention relates to polypeptide and nucleic acids constructs which are useful for determining the cell cycle status of a mammalian cell. Host cells transfected with these nucleic acid constructs can be used to determine the effects that test agents have upon the mammalian cell cycle.

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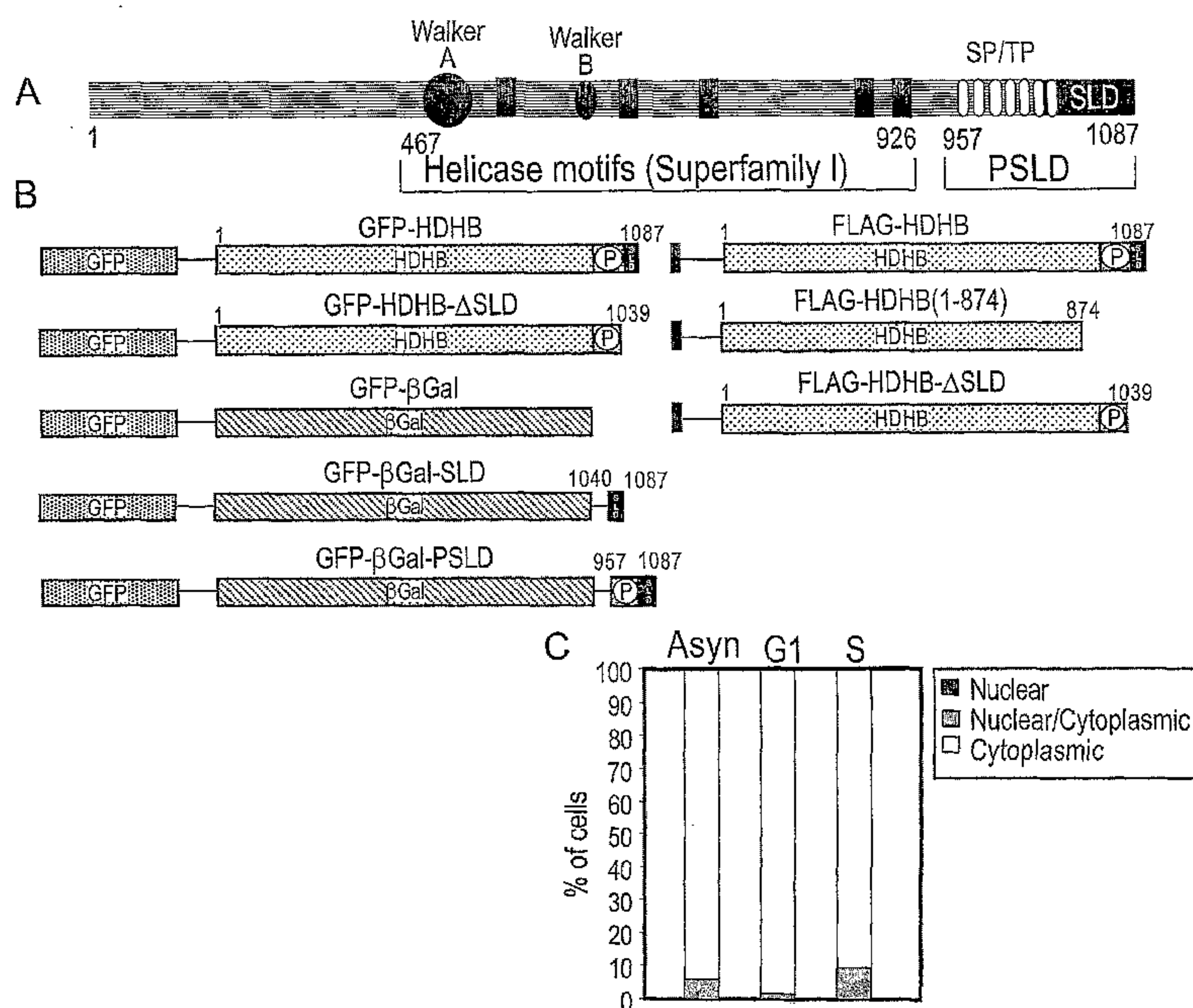
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Cell Cycle Phase Markers

Technical Field

The present invention relates to cell cycle phase-specific markers and methods for determining the transition between different phases of the cell cycle in mammalian cells.

Background of the Invention

Eukaryotic cell division proceeds through a highly regulated cell cycle comprising consecutive phases termed G1, S, G2 and M. Disruption of the cell cycle or cell cycle control can result in cellular abnormalities or disease states such as cancer which arise from multiple genetic changes that transform growth-limited cells into highly invasive cells that are unresponsive to normal control of growth. Transition of normal cells into cancer cells can arise through loss of correct function in DNA replication and DNA repair mechanisms. All dividing cells are subject to a number of control mechanisms, known as cell-cycle checkpoints, which maintain genomic integrity by arresting or inducing destruction of aberrant cells. Investigation of cell cycle progression and control is consequently of significant interest in designing anticancer drugs (Flatt, P.M. and Pieterpol, J.A. *Drug Metab. Rev.*, (2000), 32(3-4), 283-305; Buolamwini, J.K. *Current Pharmaceutical Design*, (2000), 6, 379-392).

Cell cycle progression is tightly regulated by defined temporal and spatial expression, localisation and destruction of a number of cell cycle regulators which exhibit highly dynamic behaviour during the cell cycle (Pines, J., *Nature Cell Biology*, (1999), 1, E73-E79). For example, at specific cell cycle stages some proteins translocate from the nucleus to the cytoplasm, or vice versa, and some are rapidly degraded. For details of known cell cycle control components and interactions, see Kohn, *Molecular Biology of the Cell* (1999), 10, 2703-2734.

Accurate determination of cell cycle status is a key requirement for investigating cellular processes that affect the cell cycle or are dependent on cell

cycle position. Such measurements are particularly vital in drug screening applications where:

- i) substances which directly or indirectly modify cell cycle progression are desired, for example, for investigation as potential anti-cancer treatments;
- ii) drug candidates are to be checked for unwanted effects on cell cycle progression; and/or
- iii) it is suspected that an agent is active or inactive towards cells in a particular phase of the cell cycle.

Traditionally, cell cycle status for cell populations has been determined by flow cytometry using fluorescent dyes which stain the DNA content of cell nuclei (Barlogie, B. et al, Cancer Res., (1983), 43(9), 3982-97). Flow cytometry yields quantitative information on the DNA content of cells and hence allows determination of the relative numbers of cells in the G1, S and G2+M phases of the cell cycle. However, this analysis is a destructive non-dynamic process and requires serial sampling of a population to determine cell cycle status with time. A further disadvantage of flow cytometry techniques relates to the indirect and inferred assignment of cell cycle position of cells based on DNA content. Since the DNA content of cell nuclei varies through the cell cycle in a reasonably predictable fashion, ie. cells in G2 or M have twice the DNA content of cells in G1, and cells undergoing DNA synthesis in S phase have an intermediate amount of DNA, it is possible to monitor the relative distribution of cells between different phases of the cell cycle. However, the technique does not allow precision in determining the cell cycle position of any individual cell due to ambiguity in assigning cells to G2 or M phases and to further imprecision arising from inherent variation in DNA content from cell to cell within a population which can preclude precise discrimination between cells which are close to the boundary between adjacent phases of the cell cycle. Additionally, variations in DNA content and DNA staining between different cell types from different tissues or organisms require that the technique is optimised for each cell type, and can complicate direct comparisons of data between cell types or between experiments (Herman, Cancer (1992), 69(6), 1553-1556). Flow cytometry is therefore suitable for examining the overall cell cycle distribution of cells within a

population, but cannot be used to monitor the precise cell cycle status of an individual cell over time.

EP 798386 describes a method for the analysis of the cell cycle of cell sub-populations present in heterogeneous cell samples. This method uses sequential incubation of the sample with fluorescently labelled monoclonal antibodies to identify specific cell types and a fluorochrome that specifically binds to nucleic acids. This permits determination of the cell cycle distribution of sub-populations of cells present in the sample. However, as this method utilises flow cytometry, it yields only non-dynamic data and requires serial measurements to be performed on separate samples of cells to determine variations in the cell cycle status of a cell population with time following exposure to an agent under investigation for effects on cell cycle progression.

A number of researchers have studied the cell cycle using traditional reporter enzymes that require the cells to be fixed or lysed. For example Hauser & Bauer (Plant and Soil, (2000), 226, 1-10) used β -glucuronidase (GUS) to study cell division in a plant meristem and Brandeis & Hunt (EMBO J., (1996), 15, 5280-5289) used chloramphenicol acetyl transferase (CAT) fusion proteins to study variations in cyclin levels. US 6048693 describes a method for screening for compounds affecting cell cycle regulatory proteins, wherein expression of a reporter gene is linked to control elements which are acted on by cyclins or other cell cycle control proteins. In this method, temporal expression of a reporter gene product is driven in a cell cycle specific fashion and compounds acting on one or more cell cycle control components may increase or decrease expression levels.

US 6159691 describes nuclear localisation signals (NLS) derived from the cell cycle phase-specific transcription factors DP-3 and E2F-1 and claims a method for assaying for putative regulators of cell cycle progression. In this method, nuclear localisation signals (NLS) derived from the cell cycle phase specific transcription factors DP-3 and E2F-1 may be used to assay the activity

of compounds which act to increase or decrease nuclear localisation of specific NLS sequences from DP-3 and E2F-1 fused to a detectable marker.

Jones et al (Nat Biotech., (2004), 23, 306-312) describe a fluorescent biosensor of mitosis based on a plasma membrane targeting signal and an SV40 large T antigen NLS fused to EYFP. Throughout the cell cycle the reporter resides in the nucleus but translocates to the plasma membrane during mitosis, between nuclear envelope breakdown and re-formation.

WO 03/031612 describes DNA reporter constructs and methods for determining the cell cycle position of living mammalian cells by means of cell cycle phase-specific expression control elements and destruction control elements.

Gu et al. (Mol Biol Cell., 2004, 15, 3320-3332) have recently investigated the function of human DNA helicase B (HDHB) and shown that it is primarily nuclear in G1 and cytoplasmic in S and G2 phases, that it resides in nuclear foci induced by DNA damage, that the focal pattern requires HDHB activity, and that HDHB localization is regulated by CDK phosphorylation.

None of the preceding methods specifically describe sensors which can be stably integrated into the genome and used to indicate G1, S and G2 phases of the cell cycle. Consequently, methods are required that enable these phases of the cell cycle to be determined non-destructively in a single living mammalian cell, allowing the same cell to be repeatedly interrogated over time, and which enable the study of the effects of agents having potentially desired or undesired effects on the cell cycle. Methods are also required that permit the parallel assessment of these effects for a plurality of agents.

Summary of the Invention

The present invention describes a method which utilises key components of the cell cycle regulatory machinery in defined combinations to provide novel

means of determining cell cycle status for individual living cells in a non-destructive process providing dynamic read out.

The present invention further provides proteins, DNA constructs, vectors, and stable cell lines expressing such proteins, that exhibit translocation of a detectable reporter molecule in a cell cycle phase specific manner, by direct linkage of the reporter signal to a G1/S cell cycle phase dependent location control sequence. This greatly improves the precision of determination of cell cycle phase status and allows continuous monitoring of cell cycle progression in individual cells. Furthermore, it has been found that key control elements can be isolated and abstracted from functional elements of the cell cycle control mechanism to permit design of cell cycle phase reporters which are dynamically regulated and operate in concert with, but independently of, endogenous cell cycle control components, and hence provide means for monitoring cell cycle position without influencing or interfering with the natural progression of the cell cycle.

According to a first aspect of the present invention, there is provided a polypeptide construct comprising a detectable live-cell reporter molecule linked via a group having a molecular mass of less than 112,000 Daltons to at least one cell cycle phase-dependent location control element, the location of which said element changes during G1 and S phase, wherein the translocation of said construct within a mammalian cell is indicative of the cell cycle position.

It will be understood that translocation is defined as the detectable movement of the reporter from one sub-cellular location to another, typically from the nucleus to the cytoplasm or *vice versa*. It will be further understood that the term 'live cell', as it relates to a reporter molecule, defines a reporter molecule which produces a detectable signal in living cells, or a reporter, such as an antigenic tag, that is expressed in living cells and can be detected after fixation through immunological methods, and is thus suitable for use in imaging systems, such as the IN Cell Analyzer (GE Healthcare).

Suitably, said group has a molecular mass of less than 100,000 Daltons.

Suitably, the group has a molecular mass of less than 50,000 Daltons.

Suitably, the group has a molecular mass of less than 25,000 Daltons.

Suitably, the group has a molecular mass of less than 10,000 Daltons.

Suitably, the group has a molecular mass of less than 1,000 Daltons.

Suitably, the group has a molecular mass of less than 700 Daltons.

Suitably, the group has a molecular mass of less than 500 Daltons.

Preferably, the group is a polypeptide. The polypeptide group should be relatively small and comprise amino acids that allow flexibility and/or rotation of the reporter molecule relative to the cell cycle phase-dependent location control element. More preferably, the polypeptide group is a heptapeptide. Most preferably, said heptapeptide group is Glycine-Asparagine- Glycine-Glycine-Asparagine-Alanine-Serine (GNGGNAS). As stated above, any amino acids which allow flexibility and/or rotation of the reporter molecule relative to the location control element may be used in the polypeptide.

Suitably, the cell cycle phase-specific dependent location control element is selected from the group of peptides consisting of Rag2, Chaf1B, Fen1, PPP1R2, helicase B, sgk, CDC6 or motifs therein such as the phosphorylation-dependent subcellular localization domain of the C-terminal special control region of helicase B (PSLD). Helicase B is known to cause uncontrolled DNA licensing and may be detrimental to cell survival when over-expressed. Therefore, preferably, the cell cycle phase-dependent location control element is the phosphorylation-dependent subcellular localization domain of the C-terminal spacial control region of helicase B (PSLD).

A human helicase B homolog has been reported and characterised ((Taneja et al J. Biol. Chem., (2002), 277, 40853-40861); the nucleic acid sequence (NM 033647) and the corresponding protein sequence are given in SEQ ID No. 1 and SEQ ID No. 2, respectively. The report demonstrates that helicase activity is needed during G1 to promote the G1/S transition. Gu et al (Mol. Biol. Cell., (2004), 15, 3320-3332) have shown that a small C-terminal region of the helicase B gene termed the phosphorylation-dependent subcellular localization domain (PSLD) is phosphorylated by Cdk2/cyclin E and contains NLS and NES sequences. Gu et al (Mol. Biol. Cell., (2004), 15, 3320-3332) carried out studies on cells that had been transiently transfected with plasmid encoding an EGFP- β Gal-PSLD fusion (beta-galactosidase (β Gal) was included in the construct as an inert group to make the whole fusion protein similar in size to the complete helicase B) expressed from a CMV promoter. Cells in G1 exhibited EGFP signal predominantly in the nucleus, whilst cells in other phases of the cell cycle exhibited predominantly cytoplasmic EGFP signal. These researchers concluded that the PSLD was directing translocation of the reporter from the nucleus to the cytoplasm around the G1/S phase transition of the cell cycle.

Suitably, the live-cell reporter molecule is selected from the group consisting of fluorescent protein, enzyme and antigenic tag. Preferably, the fluorescent protein is derived from *Aequoria Victoria*, *Renilla reniformis* or other members of the classes *Hydrozoa* and *Anthozoa* (Labas et al., Proc.Natl.Acad. Sci, (2002), 99, 4256-4261). More preferably, the fluorescent protein is EGFP (BD Clontech), Emerald (Tsien, Annu. Revs. Biochem., (1998), 67, 509-544) or J-Red (Evrogen). Most preferably, the fluorescent protein is selected from the group consisting of Green Fluorescent Protein (GFP), Enhanced Green Fluorescent Protein (EGFP), Emerald and J-Red.

Suitably, the reporter is an enzyme reporter such as halo-tag (Promega).

Suitably, the reporter molecule is EGFP or J-Red and the cell cycle phase-dependent location control element is PSLD.

Suitably, the reporter molecule is tandemized (i.e. present as a tandem repeat).

A polypeptide construct comprising the amino acid sequence of SEQ ID No. 5.

According to a second aspect of the present invention, there is provided a nucleic acid construct encoding any of the polypeptide constructs as hereinbefore described.

Suitably, said nucleic acid construct additionally comprises and is operably linked to and under the control of at least one cell cycle independent expression control element.

The term, 'operably linked' indicates that the elements are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the reporter molecule of the invention.

Suitably, the expression control element controls transcription over an extended time period with limited variability in levels of transcription throughout the cell cycle. Preferably, the expression control element is the ubiquitin C or CMV I/E promoter which provide transcription over an extended period which is required for the production of stable cell lines.

Preferably, the nucleic acid construct comprises a Ubiquitin C promoter, and sequences encoding PSLD and EGFP or J-Red.

Optionally, the nucleic acid construct comprises a CMV promoter, and sequences encoding PSLD and EGFP or J-Red.

In a third aspect of the present invention, there is provided a vector comprising any of the nucleic acid constructs as hereinbefore described. Suitably, said vector is either a viral vector or a plasmid. Suitably, said viral vector is an adenoviral vector or a lentiviral vector.

Optionally, the vector additionally contains a drug resistance gene that is functional in eukaryotic cells, preferably a drug resistance gene that is functional in mammalian cells.

Expression vectors may also contain other nucleic acid sequences, such as polyadenylation signals, splice donor/splice acceptor signals, intervening sequences, transcriptional enhancer sequences, translational enhancer sequences and the like. Optionally, the drug resistance gene and reporter gene may be operably linked by an internal ribosome entry site (IRES), (Jang et al., J. Virology, (1988), 62, 2636-2643) rather than the two genes being driven by separate promoters. The pIRES-neo and pIRES vectors commercially available from Clontech may be used.

In a fourth aspect of the present invention, there is provided a host cell transfected with a nucleic acid construct as hereinbefore described. The host cell into which the construct or the expression vector containing such a construct is introduced may be any mammalian cell which is capable of expressing the construct.

The prepared DNA reporter construct may be transfected into a host cell using techniques well known to the skilled person. These techniques may include: electroporation (Tur-Kaspa et al, Mol. Cell Biol. (1986), 6, 716-718), calcium phosphate based methods (eg. Graham and Van der Eb, Virology, (1973), 52, 456-467), direct microinjection, cationic lipid based methods (eg. the use of Superfect (Qiagen) or Eugene6 (Roche) and the use of bombardment mediated gene transfer (Jiao et al, Biotechnology, (1993), 11, 497-502). A further alternative method for transfecting the DNA construct into cells, utilises the natural ability of viruses to enter cells. Such methods include vectors and

transfection protocols based on, for example, Herpes simplex virus (U.S. Pat 5288641), cytomegalovirus (Miller, Curr. Top. Microbiol. Immunol., (1992), 158, 1), vaccinia virus (Baichwal and Sugden, 1986, in Gene Transfer, ed. R. Kucherlapati, New York, Plenum Press, p117-148), and adenovirus and adeno-associated virus (Muzyczka, Curr. Top. Microbiol. Immunol., (1992), 158, 97-129).

Examples of suitable recombinant host cells include HeLa cells, Vero cells, Chinese Hamster ovary (CHO), U2OS, COS, BHK, HepG2, NIH 3T3 MDCK, RIN, HEK293 and other mammalian cell lines that are grown *in vitro*. Preferably the host cell is a human cell. Such cell lines are available from the American Tissue Culture Collection (ATCC), Bethesda, Maryland, U.S.A. Cells from primary cell lines that have been established after removing cells from a mammal followed by culturing the cells for a limited period of time are also intended to be included in the present invention.

In a preferred embodiment, the cell line is a stable cell line comprising a plurality of host cells according to the fourth aspect.

Cell lines which exhibit stable expression of a cell cycle position reporter may also be used in establishing xenografts of engineered cells in host animals using standard methods. (Krasagakis, K.J et al, Cell Physiol., (2001), 187(3), 386-91; Paris, S. et al, Clin.Exp.Metastasis, (1999), 17(10), 817-22). Xenografts of tumour cell lines engineered to express cell cycle position reporters will enable establishment of model systems to study tumour cell division, stasis and metastasis and to screen new anticancer drugs.

In a fifth aspect of the present invention, there is provided the use of a polypeptide as hereinbefore described for determining the cell cycle position of a mammalian cell.

Use of engineered cell lines or transgenic tissues expressing a cell cycle position reporter as allografts in a host animal will permit study of mechanisms

affecting tolerance or rejection of tissue transplants (Pye & Watt, J. Anat., (2001), 198 (Pt 2), 163-73; Brod, S.A. et al, Transplantation (2000), 69(10), 2162-6).

According to a sixth aspect of the present invention, there is provided a method for determining the cell cycle position of a mammalian cell, said method comprising:

- a) expressing in a cell a nucleic acid construct as hereinbefore described; and
- b) determining the cell cycle position by monitoring signals emitted by the reporter molecule.

To perform the method for determining the cell cycle position of a cell according to the sixth aspect, cells transfected with the DNA reporter construct may be cultured under conditions and for a period of time sufficient to allow expression of the reporter molecule at a specific stage of the cell cycle. Typically, expression of the reporter molecule will occur between 16 and 72 hours post transfection, but may vary depending on the culture conditions. If the reporter molecule is based on a green fluorescent protein sequence the reporter may take a defined time to fold into a conformation that is fluorescent. This time is dependent upon the primary sequence of the green fluorescent protein derivative being used. The fluorescent reporter protein may also change colour with time (see for example, Tersikh, Science, (2000), 290, 1585-8) in which case imaging is required at specified time intervals following transfection.

If the reporter molecule produces a fluorescent signal in the method of the sixth aspect, either a conventional fluorescence microscope, or a confocal based fluorescence microscope may be used to monitor the emitted signal. Using these techniques, the proportion of cells expressing the reporter molecule, and the location of the reporter can be determined. In the method according to the present invention, the fluorescence of cells transformed or transfected with the DNA construct may suitably be measured by optical means in for example; a spectrophotometer, a fluorimeter, a fluorescence microscope, a cooled charge-

coupled device (CCD) imager (such as a scanning imager or an area imager), a fluorescence activated cell sorter, a confocal microscope or a scanning confocal device, where the spectral properties of the cells in culture may be determined as scans of light excitation and emission.

In the embodiment of the invention wherein the nucleic acid reporter construct comprises a drug resistance gene, following transfection and expression of the drug resistance gene (usually 1 – 2 days), cells expressing the modified reporter gene may be selected by growing the cells in the presence of an antibiotic for which transfected cells are resistant due to the presence of a selectable marker gene. The purpose of adding the antibiotic is to select for cells that express the reporter gene and that have, in some cases, integrated the reporter gene, with its associated promoter, into the genome of the cell line. Following selection, a clonal cell line expressing the construct can be isolated using standard techniques. The clonal cell line may then be grown under standard conditions and will express reporter molecule and produce a detectable signal at a specific point in the cell cycle.

Cells transfected with the nucleic acid reporter construct according to the present invention may be grown in the absence and/or the presence of a test agent to be studied and whose effect on the cell cycle of a cell is to be determined. By determining the proportion of cells expressing the reporter molecule and the localisation of the signal within the cell, it is possible to determine the effect of a test agent on the cell cycle of the cells, for example, whether the test system arrests the cells in a particular stage of the cell cycle, or whether the effect is to speed up or slow down cell division.

Thus, according to a seventh aspect of the present invention, there is provided a method of determining the effect of a test agent on the cell cycle position of a mammalian cell, the method comprising:

- a) expressing in the cell in the absence and in the presence of the test agent a nucleic acid reporter construct as hereinbefore described; and

- b) determining the cell cycle position by monitoring signals emitted by the reporter molecule wherein a difference between the emitted signals measured in the absence and in the presence of the test agent is indicative of the effect of the test agent on the cell cycle position of the cell.

The term 'test agent' should be construed as a form of electromagnetic radiation or as a chemical entity. Preferably, the test agent is a chemical entity selected from the group consisting of drug, nucleic acid, hormone, protein and peptide. The test agent may be applied exogenously to the cell or may be a peptide or protein that is expressed in the cell under study.

In an eighth aspect of the present invention, there is provided a method of determining the effect of a test agent on the cell cycle position of a mammalian cell, the method comprising:

- a) expressing in said cell in the presence of said test agent a nucleic acid reporter construct as hereinbefore described;
- b) determining the cell cycle position by monitoring signals emitted by the reporter molecule, and
- c) comparing the emitted signal in the presence of the test agent with a known value for the emitted signal in the absence of the test agent;

wherein a difference between the emitted signal measured in the presence of the test agent and the known value in the absence of the test agent is indicative of the effect of the test agent on the cell cycle position of the cell.

In a ninth aspect of the present invention, there is provided a method of determining the effect of a test agent on the cell cycle position of a mammalian cell, the method comprising:

- a) providing cells containing a nucleic acid reporter construct as hereinbefore described;
- b) culturing first and second populations of the cells respectively in the presence and absence of a test agent and under conditions permitting expression of the nucleic acid reporter construct; and

- c) measuring the signals emitted by the reporter molecule in the first and second cell populations;

wherein a difference between the emitted signals measured in the first and second cell populations is indicative of the effect of the test agent on the cell cycle position of the cell.

According to a tenth aspect of the present invention, there is provided a method of determining the effect of the mammalian cell cycle on a cellular process measurable by a first detectable reporter which is known to vary in response to a test agent, the method comprising:

- a) expressing in the cell in the presence of the test agent a second nucleic acid reporter construct as hereinbefore described;
- b) determining the cell cycle position by monitoring signals emitted by the second reporter molecule; and
- c) monitoring the signals emitted by the first detectable reporter ,

wherein the relationship between cell cycle position determined by step b) and the signal emitted by the first detectable reporter is indicative of whether or not said cellular process is cell cycle dependent.

In an eleventh aspect of the present invention, there is provided the use of a polypeptide as hereinbefore described for measuring CDK2 activity in a cell.

According to a twelfth aspect of the present invention, there is provided a method for measuring CDK2 activity in a cell, said method comprising the steps of

- a) expressing a nucleic acid construct in a cell as hereinbefore described' and
- b) determining CDK2 activity by monitoring signals emitted by the reporter molecule.

According to a thirteenth aspect of the present invention, there is provided a method for determining the effect of a test agent on CDK2 activity of a mammalian cell, said method comprising:

- a) expressing in said cell in the absence and in the presence of said test agent a nucleic acid construct as hereinbefore described; and
- b) determining CDK2 activity by monitoring signals emitted by the reporter molecule wherein a difference between the emitted signals measured in the absence and in the presence of said test agent is indicative of the effect of the test agent on the activity of CDK2.

In a fourteenth aspect of the present invention, there is provided a method of determining the effect of a test agent on CDK2 activity of a mammalian cell, said method comprising:

- a) expressing in said cell in the presence of said test agent a nucleic acid construct as hereinbefore described; and
 - b) determining the cell cycle position by monitoring signals emitted by the reporter molecule,
 - c) comparing the emitted signal in the presence of the test agent with a known value for the emitted signal in the absence of the test agent;
- wherein a difference between the emitted signal measured in the presence of the test agent and said known value in the absence of the test agent is indicative of the effect of the test agent on the CDK2 activity of the cell.

Brief Description of the Drawings

The invention is further illustrated by reference to the following examples and figures in which:

Figure 1 – Localisation of HDHB in the nucleus or cytoplasm.

(A) Cytoplasmic and nuclear extracts of U2OS cells were analyzed by denaturing gel electrophoresis and western blotting with antibody against recombinant HDHB, α -tubulin, and PCNA. Immunoreactive proteins were detected by chemiluminescence.

(B) GFP-tagged HDHB microinjected and transiently expressed in U2OS cells were visualized by fluorescence microscopy. Nuclei were stained with Hoechst dye. Bar, 10 μ m.

(C) FLAG-tagged HDHB microinjected and transiently expressed in U2OS cells were visualized by fluorescence microscopy.

Figure 2 – The subcellular localization of GFP-HDHB is cell cycle-dependent.

(A) Subcellular localization of transiently expressed GFP-tagged HDHB in asynchronous, G1, and S phase U2OS cells was quantified. The number of GFP-positive cells with a given distribution pattern was expressed as a percentage of the total number of GFP-positive cells (>100 cells).

(B) Cytoplasmic and nuclear extracts of synchronized U2OS cells (G1 and S phase) were analyzed by denaturing gel electrophoresis and western blotting with antibody against recombinant HDHB, α -tubulin, and PCNA.

Immunoreactive proteins were detected by chemiluminescence.

Figure 3 – Identification of a domain required for nuclear localization of HDHB.

(A) Schematic representation of the HDHB protein showing seven potential phosphorylation sites for CDK (SP or TP), the putative subcellular localization domain (SLD) and phosphorylated SLD (PSLD), the Walker A and Walker B helicase motifs. Amino acid residue numbers are indicated below protein.

(B) GFP- and FLAG-tagged HDHB and C-terminal truncation mutants generated in study. The C terminus of HDHB SLD (residues 1040-1087) and PSLD (residues 957-1087) was fused to a GFP- β Gal reporter to create GFP- β Gal-SLD and GFP- β Gal-PSLD respectively.

(C) The subcellular localization of transiently expressed GFP-HDHB- Δ SLD in asynchronous, G1, and S phase U2OS cells was quantified and expressed as a percentage of the total number of GFP-positive cells.

Figure 4 – GFP- β Gal-PSLD subcellular localization pattern varies with the cell cycle.

(A) The subcellular localization of transiently expressed GFP- β Gal, GFP- β Gal-SLD, and GFP- β Gal-PSLD in asynchronous, G1, and S phase U2OS cells was quantified and expressed as a percentage of the total number of GFP-positive cells.

Figure 5 – Identification of a functional rev-type nuclear export signal (NES) in SLD of HDHB.

(A) Alignment of the putative NES in HDHB with those identified in other cell cycle-related proteins (Henderson and Eleftheriou, 2000; Fabbro and Henderson, 2003). Superscripts above the amino acid sequence indicate residue numbers. Thick arrows point to the conserved aliphatic residues in the NES. Two pairs of residues in the putative NES in HDHB were mutated to alanine as indicated by the thin arrows to create Mut1 and Mut2.

(B) GFP- and FLAG-tagged HDHB were transiently expressed in asynchronously growing U2OS cells with (+) or without (-) LMB to inhibit CRM1-mediated nuclear export. The subcellular localization of GFP-HDHB and FLAG-HDHB in asynchronous, G1, and S phase cells was quantified and expressed as a percentage of the total number of GFP-positive cells in that sample.

(C) The subcellular localization of wild type and mutant GFP-HDHB and GFP- β Gal-PSLD in asynchronous U2OS cells was quantified and expressed as a percentage of the total number of GFP-positive cells in that sample.

Figure 6 – Cell cycle-dependent phosphorylation of FLAG-HDHB in vivo.

(A) U2OS cells transiently expressing FLAG-HDHB (lane 1) and its truncation mutants 1-1039 (lane 2) and 1-874 (lane 3) were labeled with [^{32} P] orthophosphate. Cell extracts were immunoprecipitated with anti-FLAG resin. The precipitated proteins were separated by 7.5% SDS-PAGE, transferred to a PVDF membrane, and detected by autoradiography (top) or western blotting (bottom). The positions of marker proteins of known molecular mass are indicated at the left.

(B) FLAG-HDHB expressed in U2OS cells was immunoprecipitated with anti-FLAG resin, incubated with (+) or without (-) λ -phosphatase (λ -PPase) in the presence (+) or absence (-) of phosphatase inhibitors, as indicated, and analyzed by SDS-PAGE and immunoblotting with anti-HDHB antibody.

(C) U2OS cells expressing FLAG-HDHB were arrested at G1/S (top) or at G2/M(bottom), and then released from the block. FLAG-HDHB was harvested at the indicated time points, immunoprecipitated with anti-FLAG resin, treated with (+) or without (-) λ -PPase, and analyzed as in (B).

Figure 7 – Identification of S967 as a major in vivo phosphorylation site in HDHB.

(A) Phosphoamino acid markers (left) and phosphoamino acids from in vivo ³²P-labeled FLAG-HDHB (right) were separated in two dimensions and visualized by autoradiography. Some incompletely hydrolyzed phosphopeptides remained near the origin (+).

(B) Wild type and mutant FLAG-HDHB proteins were radiolabeled with orthophosphate in vivo, immunoprecipitated, separated by SDS-PAGE, and analyzed by autoradiography (top) and immunoblotting with anti-HDHB (bottom).

(C) Tryptic phosphopeptides of ³²P-labeled wild type and S967A mutant FLAG-HDHB were separated in two dimensions and visualized by autoradiography.

Figure 8 – Identification of cyclin E/CDK2 as the potential G1/S kinase of HDHB S967.

(A) Tryptic phosphopeptides from FLAG-HDHB phosphorylated in vivo as in Fig. 7C, or recombinant HDHB phosphorylated in vitro by purified cyclin E/CDK2 or cyclin A/CDK2, were separated in two dimensions, either individually or as a mixture, and visualized by autoradiography.

(B) Proteins that co-immunoprecipitated with FLAG vector (lanes 1, 4) or FLAG-HDHB (lanes 2, 5) expressed in U2OS cells were analyzed by immunoblotting with antibodies against HDHB (lanes 1-6), cyclin E (lanes 1-3), or cyclin A (lanes 4-6). One tenth of the cell lysate used for immunoprecipitation was analyzed in parallel as a positive control (lanes 3, 6).

Figure 9 – The subcellular localization of HDHB is regulated by phosphorylation of S967.

(A) Subcellular localization of GFP-HDHB S967A and S967D expressed in asynchronous, G1, and S phase U2OS cells was quantified.

Figure 10 – Localisation of EGFP-PSLD in asynchronous U2OS cells exhibiting stable expression of the pCORON1002-EGFP-C1-PSLD vector is cell cycle

dependent. Fluorescence microscopy of the same partial field of cells in which (A) nuclei were stained with Hoechst dye, (B) EGFP-PSLD was visualised, (C) nuclei were exposed to BrdU for 1 hour exposure prior to fixation and detection with Cy-5 labelled antibody to indicate cells in S-phase. (D) A graph of nuclear fluorescent intensity in both the red (Cy-5 immunofluorescent detection of BrdU) and green (EGFP-PSLD) for individual cells present in a full field of view.

Figure 11 – Vector map of pCORON1002-EGFP-C1-PSLD.

Figure 12 - Vector map of pCORON1002-EGFP-C1- β Gal-PSLD

Figure 13 – Flow cytometry data comparing brightness and homogeneity of signal for representative stable cell lines developed with pCORON1002-EGFP-C1-PSLD, pCORON1002-EGFP-C1- β Gal-PSLD and the parental U2OS cell line.

Detailed Description of the Invention

Methods

Plasmids

pGFP-HDHB and mutant derivatives (see Figs 4 and 6) were created by inserting full-length HDHB cDNA as a BglII/NotI fragment (Taneja et al., J. Biol. Chem., (2002) 277, 40853-40861) into the NotI site of the pEGFP-C1 vector (Clontech, Palo Alto, CA). pFLAG-HDHB was constructed by inserting a HindIII/NotI fragment containing full-length HDHB cDNA into the NotI site of pFlag-CMV2 vector (Eastman Kodak Co., Rochester, NY). Tagged HDHB-SLD (1-1039) was constructed by cleaving the tagged HDHB plasmid with NruI following the coding sequence for residue 1034 and with NotI in the polylinker and replacing the small fragment by a duplex adaptor oligonucleotide with a blunt end encoding residues 1035 to 1039, a stop codon, and an overhanging NotI-compatible 5' end. To create pFLAG-HDHB (1-874), StuI-digested pFLAG-HDHB DNA was treated with Klenow polymerase to generate blunt ends and ligated into the pFLAG-CMV2 vector. To generate pEGFP- β Gal, a DNA

fragment encoding *E. coli* β -galactosidase (β Gal) was amplified by PCR from p β Gal-control (Clontech) and inserted at the 3' end of the GFP coding sequence in pEGFP-C1, using the HindIII site. The HDHB sequence for amino acid residues 1040-1087(SLD) and 957-1087(PSLD) were PCR amplified and inserted at the 3' end of the β Gal cDNA in pEGFP- β Gal to create pGFP- β Gal-SLD and pGFP- β Gal-PSLD respectively. The NES mutants and phosphorylation site mutants were created in the HDHB cDNA by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA).

pCORON1002-EGFP-C1-PSLD was constructed by PCR amplification of the 390 bp PSLD region from the DNA construct pGFP-C1- β Gal-PSLD. Introduction of 5' NheI and 3' SalI restriction enzyme sites to the PSLD fragment allowed sub-cloning into the vector pCORON1002-EGFP-C1 (GE Healthcare, Amersham, UK). The resulting 6704 bp DNA construct pCORON1002-EGFP-C1-PSLD, contains an ubiquitin C promoter, a bacterial ampicillin resistance gene and a mammalian neomycin resistance gene (Figure 11). The nucleic acid sequence of the vector is shown in SEQ ID No. 3. Three further versions of this vector were created using standard cloning techniques (Sambrook, J. et al (1989)); the EGFP gene was first replaced with J-Red (Evrogen), the neomycin resistance gene was replaced with hygromycin resistance gene and the ubiquitin C promoter was replaced with the CMV I/E promoter.

pCORON1002-EGFP-C1- β Gal-PSLD was constructed by NheI and XmaI restriction enzyme digest of pEGFP-C1- β Gal-PSLD and insertion of the 4242 bp EGFP- β Gal-PSLD fragment into pCORON1002 vector (GE Healthcare). The resulting 9937 bp DNA construct pCORON1002-EGFP-C1- β Gal-PSLD (Figure 12) contains an ubiquitin C promoter, a bacterial ampicillin resistance gene and a mammalian neomycin resistance gene. The nucleic acid sequence of the vector is shown in SEQ ID No. 4.

The protein and nucleic acid sequence for the EGFP-PSLD fusion protein are shown in SEQ ID No. 5 and 6, respectively.

The correct DNA sequence of all constructs and substitution mutations was confirmed by DNA sequencing.

Antibodies

Anti-HDHB antibody was generated against purified recombinant HDHB (Bethyl Laboratories, Montgomery, TX) and affinity-purified on immobilized HDHB (Harlow & Lane, Antibodies: A laboratory manual. Cold Spring Harbor Laboratory).

Cell culture, synchronization, microinjection, electroporation, transfection and stable cell line generation

U2OS cells were cultured as exponentially growing monolayers in Dulbecco-modified Eagle medium (DMEM) (Gibco BRL Lifetechnologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) at 37°C. Exponentially growing U2OS cells were arrested at G1/S by incubation in DMEM containing 5 mM thymidine (Sigma-Aldrich, St. Louis, MO), for 24 h. To release the cells into S phase, the medium was aspirated and the cells washed three times with warm DMEM plus 10% FBS, and incubated in fresh DMEM plus 10% FBS. Exponentially growing U2OS cells were arrested in G2/M for 16 h in DMEM containing 30 ng/ml nocodazole (Sigma-Aldrich). To release cells into G1, mitotic cells were collected by gently shaking them off, washed three times with DMEM plus 10% FBS, and then plated on glass coverslips for microinjection, or in culture dishes for further manipulation.

Cell cycle synchronization was verified by flow cytometry as described previously (Taneja et al., J. Biol. Chem., (2002) 277, 40853-40861). In experiments to block nuclear protein export, cells were cultured for 3 h in DMEM containing 10 ng/ml of leptomycin B (LMB) and 10 µM cycloheximide (Calbiochem, San Diego, CA) to prevent new protein synthesis. Cells plated on glass coverslips were microinjected as described (Herbig et al., 1999) except that plasmid DNA rather than protein was injected.

For electroporation, asynchronously growing U2OS cells (5×10^6) were trypsinized, collected by centrifugation, and resuspended in 800 μ l of 20 mM HEPES (pH 7.4), 0.7 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 137 mM NaCl, 5 mM KCl, 6 mM glucose at a final pH of 7.4. Ten μ g of DNA was added, transferred to a 0.4 cm electroporation cuvette (BioRad, Hercules, CA) and electroporation performed using Gene Pulser II apparatus (BioRad). Cells were plated in tissue culture dishes for 1 h, washed with fresh medium and cultured for another 23 h.

Working with transiently transfected cells proved difficult in multiwell plate format due to low transfection efficiency, heterogeneity of expression and problems arising from the high throughput analysis of such data. Screening for the effects of large numbers of siRNA or agents upon the cell cycle therefore required production of a homogenous stable cell line. Due to the toxic effects of HDHB when overexpressed for long periods a stable cell line was generated with the PSLD region linked to a reporter. U-2OS cells were transiently transfected with pCORON1002-EGFP-C1-PSLD (Figure 11), pCORON1002-EGFP-C1- β Gal-PSLD (Figure 12) or J-Red derivatives of the above vectors. Stable clones expressing the recombinant fusion proteins were selected using 1 mg/ml G418 (Sigma) or hygromycin, where appropriate. Isolated primary clones (~60 per construct) were analysed by flow cytometry to confirm the level and homogeneity of expression of the sensor and where appropriate secondary clones were developed using methods above.

Fluorescence microscopy

For indirect immunofluorescence staining, cells were washed three times with phosphate buffered saline (PBS), fixed with 3.7% formaldehyde in PBS for 20 min, permeabilized for 5 min in 0.2% Triton X-100, and incubated with 10% FBS in PBS for 45 min. FLAG-HDHB was detected with mouse monoclonal anti-FLAG antibody (Sigma-Aldrich), 1:100 in PBS plus 10% FBS for 2 h at room temperature. After washing, cells were incubated with Texas Red-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:100 in PBS plus 10% FBS for 1 h at room temperature.

After three washes, cells were incubated for 10 min with Hoechst 33258 (2 μ M in PBS). Coverslips were mounted in ProLong Antifade (Molecular Probes, Eugene, OR). Images were obtained with a Hamamatsu digital camera using the Openlab 3.0 software (Improvision, Lexington, MA) on the Zeiss Axioplan 2 Imaging system (Carl Zeiss Inc.). The number of cells that exhibited each pattern of subcellular localization was counted and expressed as a percentage of the total number of cells scored (100 to 150 cells in each experiment). The subcellular distribution of each protein was quantitatively evaluated in at least two independent experiments.

For GFP fluorescence, cells were washed three times with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde containing 2 μ M Hoechst 33258 for 20 min and imaged and evaluated as above.

For Triton X-100 extraction, cells were washed twice with cold cytoskeleton buffer (CSK, 10 mM HEPES [pH 7.4], 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂), and extracted for 5 min on ice with 0.5% Triton X-100 in CSK buffer (supplemented with 1X protease inhibitors) and then fixed as described above.

Where appropriate, for high throughput imaging, kinetic imaging (24 hr) and analysis in multiwell plate format of stable cell lines fluorescence microscopy was conducted using a high throughput confocal imaging system (IN Cell Analyzer 1000 or IN Cell Analyzer 3000, GE Healthcare, Amersham, UK) on cells transfected with pCORON1002-EGFP-C1-PSLD, pCORON1002-EGFP-C1- β Gal-PSLD or redFP derivatives of these vectors. Images were analysed using the cell cycle phase marker algorithm (GE Health Care).

Metabolic phosphate labeling

U2OS cells (2.5x10⁶) were transiently transfected with wild type or mutant FLAGHDHB. After 24 h, cells were incubated in phosphate-depleted DMEM (Gibco BRL Lifetechnologies) for 15 min and radiolabeled with ³²P-H₃PO₄

(0.35 mCi/ml of medium; ICN Pharmaceuticals Inc., Costa Mesa, CA) for 4 h. Phosphate-labeled FLAG-HDHB was immunoprecipitated from extracts, separated by 7.5% SDS/PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane as described below.

Cell extracts, immunoprecipitation, and western blotting

At 24 h after transfection, FLAG-HDHB-transfected cultures to be analyzed by immunoprecipitation and immunoblotting were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 10% glycerol, 0.1% NP-40, 1 mM DTT, 25 mM NaF, 100 µg/ml PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin) (0.5 ml per 35 mm or 1 ml per 60 mm dish or 75 cm flask). The extract was scraped off the dish, incubated for 5 min on ice, and centrifuged for 10 min at 14 000 g. Samples of the supernatant (0.5 to 1 mg of protein) were incubated with 10 µl anti-FLAG agarose (Sigma) on a rotator for 2 h at 4°C. The agarose beads were washed three times with lysis buffer. Immunoprecipitated proteins were transferred to a PVDF membrane and analyzed by western blotting with anti-HDHB-peptide serum (1:5000), anti-cyclin E antibody (1:1000), and anticyclin A antibody (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and chemiluminescence (SuperSignal, Pierce Biotechnology Inc., Rockford, IL).

For selective nuclear and cytoplasmic protein extraction, 80-90% confluent U2OS cells were harvested by trypsinization and washed with PBS. They were resuspended and lysed in 10 mM Tris-HCl [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 0.25 M sucrose, 10% glycerol, 75 µg/ml digitonin, 1 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 100 µg/ml PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin for 10 min on ice, and centrifuged at 1000 x g for 5 min. The supernatant fraction was collected as the cytosolic extract. The pellet was washed, resuspended in high salt buffer (10 mM Tris-HCl [pH 7.5], 400 mM NaCl 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% NP-40, 100 µg/ml PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin), and rocked for 10 min at 4°C. After sonication, the suspended material, containing both soluble and chromatin-bound protein, was analyzed as nuclear extract. Proteins in the nuclear and cytoplasmic extracts were analyzed by 8.5% SDS-PAGE, followed by western

blotting with antibodies against α -tubulin, PCNA (both Santa Cruz Biotechnology), and recombinant HDHB.

Protein phosphatase reactions

FLAG-HDHB bound to anti-FLAG beads was incubated with 100 U of λ -phosphatase (New England Biolabs, Beverly, MA) in phosphatase buffer (50 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 0.01% NP-40) for 1 h at 30°C. The reaction was carried out in the presence or absence of phosphatase inhibitors (5 mM Na₃VO₄, 50 mM NaF). The proteins were separated by 7.5% SDS-PAGE (acrylamide-bisacrylamide ratio, 30:0.36) and HDHB was detected by western blotting with anti-HDHB-peptide serum and chemiluminescence.

Tryptic peptide mapping and phosphoamino acid analysis

At 24 h after transfection, radiolabeled FLAG-HDHB-transfected cultures to be used for immunoprecipitation and phosphoamino acid or phosphopeptide mapping were processed as above, except that lysis buffer was substituted by RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1% SDS, 50 mM NaF, 1 mM EDTA, 5 mM Na₃VO₄, 100 μ g/ml PMSF, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin). Immunoprecipitated proteins were separated by 7.5% SDS-PAGE and transferred to PVDF membranes. The membranes containing radiolabeled HDHB were rinsed well with deionized H₂O twice before visualization of phosphoproteins by autoradiography. The phosphoproteins were then excised, and the membrane pieces were re-wet with methanol followed by water. The membranes were blocked with 50 mM NH₄HCO₃ containing 0.1% Tween 20 (Sigma-Aldrich) for 30 min at room temperature and washed three times with 50 mM NH₄HCO₃ before enzymatic cleavage of phosphoproteins from the PVDF with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated bovine pancreatic trypsin (Worthington, Lakewood, NJ). The peptides were then subjected to two-dimensional phosphopeptide mapping or phosphoamino acid analysis as described in detail elsewhere (Boyle et al., Meth. Enzymology, (1991), 201, 110-149).

Cyclin-dependent kinase reactions *in vitro*

Kinase reactions using purified cyclin/CDK (200 pmol/h) (provided by R. Ott and C. Voitenleitner) and purified recombinant HDHB (Taneja et al., J. Biol. Chem., (2002) 277, 40853-40861) as the substrate were performed as described previously (Voitenleitner et al., Mol. Cell. Biol., (1999), 19, 646-56).

BrdU labelling, identification of chemical cell cycle blocks and RNAi experiments on stable cell lines

Stable cells expressing the pCORON1002-EGFP-C1-PSLD construct, were seeded at 0.3×10^5 /ml in 96-well Greiner plates using antibiotic-free medium (100µl/well) and incubate for 16 hours.

To demonstrate the distribution of EGFP-PSLD in S-phase, stable cells were marked with BrdU for 1hr using the cell proliferation kit (Amersham Biosciences, GE Health Care). Cells were fixed in 2% formalin and incorporated BrdU was detected by immunofluorescence with a Cy-5 labelled secondary antibody system (Cell proliferation kit; GE Health Care). Nulcei were stained with hoechst (2 µM).

For chemical block studies (Table 1), stable cells were exposed to olomoucine, roscovitine, nocodazole, mimosine, colcemid or colchicine (Sigma). Cells were fixed in 2% formalin and nulcei stained with hoechst (2 µM).

For siRNA studies, siRNA pools (Dharmacon) against certain cyclins, MCM proteins, CDKs, polo-like kinase (PLK), and a random control duplex (Table 2) were diluted in lipofectamine/optimem I (Invitrogen) to 25 nM and added to stable cells for 4hrs. The medium was replaced and plates incubated for 48hr. Cells were fixed in 2% formalin and nulcei stained with hoechst (2 µM).

After highthroughput imaging and analysis on the IN Cell Analyzer system (GEHC), data for average nuclear intensity and N:C ratio (EGFP signal), nuclear size (hoescht signal) and, where appropriate, nuclear signal intensity (BrdU) were obtained for for the total number of individual cells in a field of view using

hoescht as a nuclear mask and the IN Cell Analyzer 3000 cell cycle phase marker algorithm (GEHC). For each well, the total number of cells per field of view were catagorised into G1-phase (predominantly nuclear EGFP distribution; high EGFP-PSLD nuclear intensity and N:C ratio), S-phase (nuclear BrdU signal >3SDs above background; EGFP-PSLD N:C ratio around 1) and G2-phase (large nuclear size; low EGFP-PSLD N:C ratio). Although it was possible to differentiate M-phase cells (based on small nuclear size and very intense EGFP signal) very few such cells were seen in wells fixed with formalin since they were removed during the washing and fixation process.

Results

HDHB resides in nuclear foci or in the cytoplasm

To determine the subcellular localization of endogenous HDHB, nuclear and cytoplasmic proteins were selectively extracted from human U2OS cells, separated by denaturing gel electrophoresis, and analyzed by western blotting (Fig. 1). The presence of PCNA and α -tubulin in each extract was first monitored to assess the extraction procedure. PCNA was enriched in the nuclear extract and not in the cytoplasmic fraction, while α -tubulin was found primarily in the cytoplasmic fraction, validating the fractionation. HDHB was detected in both the nuclear and cytoplasmic fractions (Fig. 1). The cytoplasmic HDHB migrated more slowly than the nuclear fraction (Fig. 1), suggesting the possibility of post-translational modification.

These results could indicate either that HDHB was distributed throughout the cell, or that a mixed population of cells contained HDHB in either the nucleus or the cytoplasm. To distinguish between these alternatives, HDHB was localized *in situ* in single cells; GFP- and FLAG-tagged HDHB were expressed in human U2OS cells by transient transfection. Since prolonged over-expression of tagged or untagged HDHB was cytotoxic, all experiments were conducted in the shortest time period possible (usually 24 h). Tagged HDHB localization was analyzed in individual cells by fluorescence microscopy. Both GFP-HDHB and FLAG-HDHB displayed two major patterns of localization, either in the nucleus in

discrete foci or in the cytoplasm (Fig. 1). GFP-HDHB transiently expressed in primary human fibroblasts was also observed in either the nucleus or the cytoplasm.

Identification of a cell cycle-dependent subcellular localization domain in HDHB

U2OS cells were arrested in G2/M with nocodazole, released into G1 for three hours, and then microinjected with pGFP-HDHB DNA into their nuclei. GFP-HDHB expression was easily detectable six hours later, when approximately 70% of G1 phase cells had accumulated the fusion protein primarily in the nuclei (Fig. 2). In contrast, when cells were synchronized at G1/S with thymidine, released into S phase, and then microinjected with pGFP-HDHB DNA, more than 70% of S phase cells had accumulated the fusion protein predominantly in the cytoplasm (Fig. 2). Selective extraction of U2OS cells in G1 and S phase revealed that endogenous HDHB was mostly nuclear in G1 and cytoplasmic in S phase (Fig. 2b). However, endogenous HDHB was clearly detectable in both subcellular fractions. The mobility of the S phase HDHB was slightly retarded compared to the G1 phase protein. These results indicate that the subcellular localization of HDHB is regulated in the cell cycle and that GFP-tagged HDHB reflects the localization of the endogenous untagged helicase.

Prompted by the identification of C-terminal nuclear location signals in Bloom's syndrome helicase and other RecQ-family helicases (Hickson, Nature Rev. Cancer, (2003) 3, 169-178), a possible subcellular localization domain (SLD) was identified at the extreme C-terminus of HDHB (Fig. 3). To determine whether this putative SLD was important for HDHB localization, a truncation mutant of HDHB (GFP-HDHB- Δ SLD) was generated that lacks the C-terminal 48 residues containing the SLD (Fig. 3). The expression vector was microinjected into U2OS cells in G1 or S phase and the subcellular localization of the fusion protein was examined by fluorescence microscopy six hours later. Over 95% of the cells accumulated the fusion protein in the cytoplasm, regardless of the cell cycle timing of HDHB expression (Fig. 3c). This result suggests that HDHB may carry a NLS that is impaired or abolished by the C-terminal deletion in GFP-HDHB- Δ SLD.

To determine whether the C-terminal domain of HDHB was sufficient for nuclear localization, a bacterial β -galactosidase (β Gal) was used as a reporter protein because it has a molecular mass (112 kDa) close to that of HDHB and does not contain subcellular localization signals (Kalderon et al., Cell, (1984), 39, 499-509). As a control, a GFP- β Gal expression vector (Fig. 3) was created and the subcellular localization of the fusion protein monitored after microinjection of the expression vector into U2OS cells. As expected, GFP- β Gal protein accumulated primarily in the cytoplasm (Fig. 4). In contrast, GFP- β Gal-SLD was found in both the nucleus and cytoplasm in asynchronous or synchronized U2OS cells (Fig. 4), suggesting that SLD contains a NLS, but was not sufficient for nuclear localization of the reporter protein. Reasoning that perhaps the neighboring potential CDK phosphorylation sites might affect subcellular localization in the cell cycle (Fig. 3), a GFP- β Gal-PSLD was constructed, in which the C-terminal 131 residues of HDHB, containing the putative SLD and the cluster of potential CDK phosphorylation sites, were appended to the C-terminus of GFP- β Gal (Fig. 3). When the GFP- β Gal-PSLD plasmid DNA was transiently expressed in asynchronous and synchronized U2OS cells, GFP- β Gal-PSLD was found in the nucleus in over 90% of G1 phase cells, and in the cytoplasm in more than 70% of S phase cells (Fig. 4). In contrast with the focal pattern observed for nuclear GFP-HDHB in G1, GFP- β Gal-PSLD and EGFP-PSLD proteins were distributed evenly throughout the nucleus in G1, sparing only the nucleoli. Analysis of stable cell lines expressing pCORON1002-EGFP-C1-PSLD that have been marked with BrdU emphasized that cells in S-phase (equal to approx 60% of the asynchronous population) exhibit equidistribution or predominantly cytoplasmic distribution of the EGFP-PSLD signal (Figure 10). S-phase cells do not show a predominantly nuclear distribution of EGFP-PSLD associated with G1 cells. Some cells were seen to exhibit absolute nuclear exclusion of the EGFP-PSLD reporter (Figure 10) however these cells did not incorporate BrdU. We hypothesised that cells demonstrating absolute clearance of EGFP-PSLD from the nucleus were in G2. Kinetic imaging of the EGFP-PSLD stable cell lines over 24 hours showed that

EGFP-PSLD is predominantly nuclear in G1 after mitosis, exhibits a rapid nuclear to cytoplasmic movement around the G1/S transition (~3.5 hours after cytokinesis) and further progressive translocation from the nucleus to the cytoplasm from G1/S through to the end of G2 (approx 19 hours); at this point cell rounding occurred prior to re-division. These observations seem to confirm the possibility that G2 cells exhibit an absolute cytoplasmic distribution of the EGFP-PSLD reporter. Stable expression of the EGFP-PSLD fusion was not found to affect the total length of the cell cycle (approx 24 hours) when compared to U2OS cells or the G2M cell cycle phase marker cell line (GEHC). Taken together, these data suggest that the subcellular localization of HDHB is dependent on the cell cycle, that the C-terminal PSLD domain of HDHB plays a major role in regulating the subcellular localization of the protein in a cell cycle dependent manner and that HDHB is nuclear in G1 but progressively translocates to the cytoplasm during S-phase and possibly G2.

Identification of a functional rev-type NES in HDHB

A number of proteins that shuttle between the nucleus and cytoplasm have been demonstrated to contain a NES similar to the prototype NES of HIV rev protein (Fig. 5). Proteins containing a rev-type NES require the export factor CRM1 (also called exportin 1) to bind and transport proteins from the nucleus to the cytoplasm (reviewed by Weis, Cell, (2003), 112, 441-451). Leptomycin B (LMB), specifically inhibits CRM1 activity in nuclear protein export (Wolff et al., Chem. Biol., (1997), 4, 139-147; Kudo et al., Exp. Cell. Res., (1998), 242, 540-547). Inspection of the PSLD sequence in HDHB revealed a putative rev-type NES (LxxxLxxLxL; Fig. 5). To determine whether the cytoplasmic localization of HDHB requires a functional NES, expression plasmids for GFP-HDHB or FLAG-HDHB DNA were microinjected into asynchronous, G1, and S phase cells in the presence and absence of LMB. The localization of the fusion proteins was examined by fluorescence microscopy and quantified. In the presence of LMB, both fusion proteins accumulated in the nucleus independently of the cell cycle (Fig. 5), consistent with the possibility that HDHB contains a rev-type NES that functions through CRM1. However it is also possible that HDHB may not be a direct cargo of CRM1 and that its export may be indirectly mediated through

some other protein(s). To assess whether the putative NES in HDHB was functional, we mutated Val/Leu and Leu/Leu of the NES motif to alanine to create NES mutants 1 and 2 (Fig. 5). GFP-HDHB and GFP- β Gal-PSLD harboring these NES mutations were transiently expressed in either asynchronous or synchronized U2OS cells. Both NES mutant fusion proteins accumulated in the nucleus in more than 80% of cells, no matter when they were expressed in asynchronous or synchronized cells (Fig. 5). The results indicate that the NES mutations specifically impaired the export of both GFP-HDHB and GFP- β Gal-PSLD, arguing that the PSLD region of HDHB contains a functional NES.

FLAG-HDHB is phosphorylated in a cell cycle-dependent manner *in vivo*.

The cluster of potential CDK phosphorylation sites in the PSLD domain of HDHB (Fig 3) suggested that phosphorylation of HDHB might regulate its subcellular localization in the cell cycle. If so, one would expect the PSLD region of HDHB to be phosphorylated in a cell cycle-dependent manner. To test whether HDHB undergoes phosphorylation in PSLD, U2OS cells were transiently transfected with expression plasmids for wild type and C-terminally truncated forms of FLAG-HDHB, radiolabeled with phosphate, and then FLAG-HDHB was immunoprecipitated from cell extracts. Immunoprecipitated proteins were analyzed by denaturing gel electrophoresis, immunoblotting, and autoradiography (Fig. 6). A radiolabeled band of FLAG-HDHB was detected at the same position as the immunoreactive HDHB band (Fig. 6A, lanes 1). Truncated FLAG-HDHB lacking SLD was also robustly phosphorylated *in vivo* (lanes 2), while truncated FLAG-HDHB (1-874) lacking PSLD was not significantly phosphorylated (lanes 3). These results demonstrate that SLD is not required for HDHB phosphorylation, while PSLD is required, and suggest that the phosphorylation sites probably reside in PSLD.

To examine the timing of HDHB phosphorylation in the cell cycle, it would be convenient to detect phosphorylation without the use of radiolabeling. Since phosphorylation often reduces the electrophoretic mobility of a protein in denaturing gels, transiently expressed FLAG-HDHB was immunoprecipitated

and its mobility examined before and after treatment with λ -phosphatase (λ -PPase) (Fig. 6B). Without λ -PPase treatment, FLAG-HDHB was detected in western blots in two very closely migrating bands (lane 1), while dephosphorylated FLAG-HDHB migrated as a single band at the mobility of the faster band of the doublet (lane 2). When λ -PPase inhibitors were present in the reaction, FLAG-HDHB migrated as a doublet identical to the mock-treated protein (lane 3). These data suggest that the electrophoretic mobility of FLAG-HDHB was reduced by phosphorylation and that this assay may be suitable to track HDHB phosphorylation in the cell cycle.

To determine whether HDHB is phosphorylated in a cell cycle-dependent manner, U2OS cells transiently expressing FLAG-HDHB were arrested in G1/S by adding thymidine to the medium or in G2/M by adding nocodazole to the medium. The cells were released from the blocks for different time periods, and FLAG-HDHB was immunoprecipitated from cell extracts.

The immunoprecipitated material was incubated with or without λ -PPase and then analyzed by denaturing gel electrophoresis and western blotting (Fig. 6C). The mobility of FLAG-HDHB from cells arrested at G1/S was increased by λ -PPase treatment, suggesting that the protein was phosphorylated at G1/S (Fig. 6C, upper panel). A similar mobility shift was detected after phosphatase treatment of FLAG-HDHB for at least nine hours after release from the G1/S block (upper panel), as well as in cells arrested at G2/M (Fig. 6C, lower panel). However, after the cells were released into G1 for four and eight hours, FLAG-HDHB migrated as a single band that was much less affected by phosphatase treatment (Fig. 6C, lower panel). By twelve hours after release from the G2/M block, when most of the cells were entering S phase (data not shown), the mobility of FLAG-HDHB was again increased by phosphatase treatment, restoring the pattern observed in nocodazole-arrested cells (lower panel). These results strongly suggest that phosphorylation of FLAG-HDHB is cell cycle-dependent, with maximal phosphorylation from G1/S through G2/M and minimal phosphorylation during G1.

Serine 967 is the major phosphorylation site of ectopically expressed HDHB.

To map the phosphorylation sites in FLAG-HDHB, we first wished to determine what amino acid residues were modified. Phosphoamino acid analysis of *in vivo* radiolabeled FLAG-HDHB revealed that phosphoserine(s) was the major phosphoamino acid of FLAG-HDHB *in vivo* (Fig. 7A). Assuming that the cell cycle-dependent phosphorylation sites of HDHB are located in PSLD between residues 874 and 1039 (Fig. 3A), that these sites are modified by CDKs, and that phosphoserine is the major amino acid modified (Fig. 7A), only four of the seven potential CDK sites would remain as candidate sites. To test each of these sites individually, FLAG-HDHB expression plasmids with the corresponding serine to alanine mutations were constructed. Cells transiently transfected with these plasmids were radiolabeled with orthophosphate *in vivo* and FLAG-HDHB was immunoprecipitated and analyzed by autoradiography and western blotting (Fig. 7B). The results showed that FLAG-HDHB and three of the mutant proteins were phosphorylated approximately equally, while the S967A mutant protein was only weakly phosphorylated (Fig. 7B). This result suggested that S967 might be the primary site of HDHB phosphorylation *in vivo*. Consistent with this interpretation, an electrophoretic mobility shift after phosphatase treatment of immunoprecipitated FLAG-HDHB was detected with three of the mutant proteins, but not with S967A protein.

To confirm that S967 was the major phosphorylation site in HDHB *in vivo*, tryptic phosphopeptide mapping was carried out with wild type and S967A mutant FLAG-HDHB that had been metabolically radiolabeled with orthophosphate (Fig. 7C). One predominant radiolabeled peptide and a weakly labeled peptide were observed with the wild type protein (left panel). The predominant phosphopeptide was absent in the S967A protein, but the weakly labeled peptide remained detectable (Fig. 7C, right panel). The results provide additional evidence that serine 967 is a prominent phosphorylation site in HDHB *in vivo*.

Identification of cyclin E/CDK2 as a kinase that potentially modifies HDHB in G1/S

To test whether CDKs can actually modify HDHB, as suggested by the timing of HDHB phosphorylation in the cell cycle and the identification of S967 as a primary site of modification, purified cyclin E/CDK2 or cyclin A/CDK2 were incubated with purified recombinant HDHB and radiolabeled ATP *in vitro*. After the kinase reactions, the proteins were separated by denaturing gel electrophoresis, transferred to a PVDF membrane, and detected by autoradiography. The results revealed that recombinant HDHB could be phosphorylated strongly by both cyclin E/CDK2 and cyclin A/CDK2. The radiolabeled HDHB bands were then further processed for tryptic phosphopeptide mapping. Peptides from each digestion were separated in two dimensions, either individually or after mixing with tryptic peptides from *in vivo* phosphorylated FLAG-HDHB, and visualized by autoradiography (Fig. 8A). HDHB peptides phosphorylated by cyclin E/CDK2 and cyclin A/CDK2 yielded patterns essentially identical to those observed in the *in vivo* labeled peptide map, with one major spot and one minor spot (Fig. 8A). When the *in vitro* and *in vivo* labeled peptides were mixed and separated on one chromatogram, they co-migrated (Fig. 8A, right). These data argue that the major phosphopeptides modified *in vitro* by cyclin E/CDK2 and cyclin A/CDK2 in purified recombinant HDHB were the same ones modified *in vivo* in FLAG-HDHB.

Since cyclin E activity in human cells rises in late G1, while cyclin A activity rises later coincident with the onset of S phase (Pines, 1999; Erlandsson et al., 2000), it was important to try to distinguish whether one of these kinases might preferentially modify HDHB. Cyclin subunits frequently form a complex with the substrate proteins that they target for phosphorylation (Endicott et al., 1999; Takeda et al., 2001). To test whether cyclin E or cyclin A could associate with HDHB, FLAG-HDHB and associated proteins were immunoprecipitated from extracts of cells transfected with either FLAG-HDHB expression vector or empty FLAG vector as a control. The cell extracts and the immunoprecipitated material were analyzed by western blotting (Fig. 8B). Cyclin E clearly co-precipitated with FLAG-HDHB, but cyclin A did not (Fig. 8B, lanes 2 and 5),

suggesting that FLAG-HDHB may interact preferentially with cyclin E *in vivo*. It is conceivable that this interaction may be required for phosphorylation of HDHB by cyclin E/CDK2 *in vivo*, and if so, mutations in HDHB that prevent its association with cyclin E would abrogate phosphorylation by cyclin E/CDK2. To test the possibility that the FLAG-HDHB mutant S967A was not phosphorylated *in vivo* (Fig. 7B, C) due to an inability to bind to cyclin E, FLAG-HDHB-S967A and associated proteins were immunoprecipitated from extracts of transfected cells and analyzed by western blotting. Co-precipitation of cyclin E with the mutant protein was as robust as with wild type FLAG-HDHB.

Phosphorylation of serine 967 is critical for regulation of HDHB localization.

The data above indicate that subcellular localization and phosphorylation of ectopically expressed HDHB were regulated in a cell cycle-dependent manner with maximal phosphorylation from G1/S to G2/M, coinciding with the period when HDHB accumulated in the cytoplasm. These results, together with the identification of S967 as the major *in vivo* phosphorylation site in HDHB, suggest that phosphorylation of S967 may regulate the subcellular localization of HDHB. To test this idea, expression plasmids for wild type GFP-HDHB and the mutants S967A, S984A, S1005A, and S1021A were microinjected into synchronized U2OS cells. Wild type GFP-HDHB accumulated in nuclear foci of cells in G1, but in the cytoplasm of cells in S phase as expected. However, regardless of cell cycle timing, GFP-HDHB-S967A localized in nuclear foci in about 70% of the fluorescent cells (Fig. 9). The other three substitution mutants localized in either the nucleus or the cytoplasm like wild type GFP-HDHB. In an attempt to mimic the phosphorylation of S967, serine 967 was mutated to aspartic acid, GFP-HDHB-S967D was expressed in asynchronous and synchronized U2OS cells, and the subcellular distribution of the mutant fusion protein was examined.

About 60% of the cells expressing GFP-HDHB-S967D displayed cytoplasmic fluorescence in asynchronous, G1 phase, and S phase cells (Fig. 9A), demonstrating that the S967D mutation mimicked phosphorylated S967. The data strongly suggest that phosphorylation of serine 967 is critical in regulating the subcellular localization of HDHB.

A C-terminal domain of HDHB confers cell cycle-dependent localization

A 131-residue domain, PSLD, is sufficient to target HDHB, EGFP or a β Gal reporter to either the nucleus or the cytoplasm in a cell cycle-dependent manner (Fig. 4 and 10). A rev-type NES resides in this domain (Fig. 5), but its activity or accessibility to the nuclear export machinery depends on phosphorylation of PSLD, primarily on serine 967, at the G1/S transition (Fig. 6-9). S967 is a perfect match to the consensus CDK substrate recognition motif (S/T)PX(K/R). Both cyclin E/CDK2 and cyclin A/CDK2 can modify HDHB *in vitro*, but the ability of cyclin E/CDK2 to complex with HDHB in cell extracts suggests that it may be the initial kinase that modifies HDHB at the G1/S transition (Fig. 8). Addition of olomoucine and roscovitin, known Cdk2 inhibitors (Table 1), or siRNA toward cyclin E (Table 2) resulted in predominantly nuclear distribution of EGFP-PSLD and arrest in G1 for EGFP-PSLD stable cell lines, further supporting the possibility that Cdk2/cyclin E is responsible for control of the observed cell-cycle based phosphorylation-dependent subcellular localisation. Phosphorylation of PSLD appears to persist through the latter part of the cell cycle, correlating well with the predominantly cytoplasmic localization of HDHB in S and G2. Kinetic imaging of stable cell lines treated with olomoucine over 24 hours showed that, for cells arrested in G2 the EGFP-PSLD signal redistributes from the cytoplasm to the nucleus over ~4-8 hours (without the cell passing through mitosis) suggesting that in the absence of cdk2 activity the EGFP-PSLD either becomes dephosphorylated and re-enters the nucleus, or is destroyed and newly synthesised protein is not phosphorylated due to cdk2 inhibition and therefore locates in the nucleus.

Table 1

Compound	%			Total cells
	S	G1	G2	
Colcemid (0.3 μ M)	41	16	43	490
Colcemid (1.2 μ M)	32	8	59	450
Colchicine (4 μ M)	36	9	55	467
Colchicine (100 μ M)	32	12	57	439
L-mimosine (2 mM)	68	6	26	1710
Olomoucine (500 μ M)	33	63	4	600
Roscovitin (100 μ M)	36	52	13	693
Nocodazole (3 μ M)	33	6	61	606
Control	61	17	22	2137

Table 2

siRNA	%			Total cells
	S	G1	G2	
PLK	53	9	38	66
MCM7	58	13	29	231
MCM6	64	14	22	166
MCM5	63	17	20	260
MCM4	56	20	24	223
MCM3	59	23	19	188
MCM2	50	24	26	266
Cyclin B1 V2	49	36	15	280
Cyclin B1 V1	60	24	17	203
CDK8	50	23	27	299
CDK7	56	18	26	354
CDK6	58	22	20	328
Cyclin A2	61	13	26	319
Cyclin A1	66	10	24	298
Cyclin T2b	57	12	31	267
Cyclin T2b	55	22	23	355
cyclinT1	60	20	20	260
cyclinE1	49	27	24	272
Control	69	10	20	262

It was not possible to distinguish whether HDHB undergoes dephosphorylation at the M/G1 transition (Fig. 6C) or is perhaps targeted for proteolysis and rapidly re-synthesized in early G1, when it would enter the

nucleus. However, kinetic imaging of stable cell lines over 24 hours showed that the EGFP-PSLD signal is not greatly reduced during M phase or at the M/G1 boundary, but becomes predominantly nuclear approximately 30 minutes after cytokinesis (this state then persists for ~3 hours during G1), coincident with nuclear membrane formation. This indicates that the EGFP-PSLD construct is dephosphorylated rather than undergoing significant destruction around the M/G1 boundary.

These data provide strong evidence that the PSLD contains active targeting signals that are independent of protein context (Fig. 2-5, 10). Since mutant HDHB with an inactivated NES is nuclear even when it is expressed during S phase and thus presumably phosphorylated (Fig. 5), it is probable that the NLS is not inactivated by phosphorylation and that the primary target of CDK regulation is the NES. Extending this reasoning, the NES may be masked during G1 when the CDK motifs in PSLD are unmodified, and that the NES is liberated when S967 becomes phosphorylated, leading to NES recognition by nuclear export factors (Fig. 3-5). Structural studies of a rev-type NES have shown that it forms an amphipathic α -helix, with the leucines aligned on one side of the helix and charged residues on the other side (Rittinger et al., Mol. Cell. Biol. (1999), 4, 153-166). Since the SLD of HDHB contains both the rev-type NES and an NLS, and the basic residues likely to serve as the NLS are interspersed through the NES, the NES and NLS may reside on opposite faces of an amphipathic helix. Additional sequences in PSLD would mask the NES intramolecularly, allowing only the NLS to be recognized. Phosphorylation of S967 would alter the conformation of the mask in PSLD to expose the NES, without affecting exposure of the NLS.

High throughput screening for inhibitors of the cell cycle with EGFP-PSLD stable cell lines

As stated above, working with transiently transfected cells proved difficult in multiwell plate format due to low transfection efficiency, heterogeneity of expression and problems arising from the high throughput analysis of such data. Screening for the effects of large numbers of siRNA or agents upon the cell

cycle therefore required production of a homogenous stable cell line. A stable cell line was generated with the PSLD region linked to a reporter (EGFP) via a flexible seven amino acid linker (using pCORON1002-EGFP-C1-PSLD). As can be seen from Figure 13, the fluorescent signal generated by the stable cell lines developed with pCORON1002-EGFP-C1- β Gal-PSLD was significantly smaller (approximately ten-fold) than that produced by cells lines having the flexible seven amino acid linker. This is probably due to the size of the β Gal protein placing large demands upon the transcriptional and translational machinery of the cell.

A stable cell line developed with pCORON1002-EGFP-C1-PSLD (see figure 13) was homogeneous (average total cell RFU 435, SD 58; n=271; see Fig 10) in nature and provided sensitive, stable and uniform assays for investigating the cell cycle and for rapidly screening the effect of agents upon the cell cycle in mutliwell plate format (Tables 1 and 2; and Figure 10).

Certain aspects of the invention disclosed hereinabove has been published in Molecular Biology of the Cell (15: 3320-3332, July 2004) and electronically published as MBC in press, 10.1091/mbc.E04-03-0227 on May 14, 2004, under the title of "Cell Cycle-dependent Regulation of a Human DNA Helicase That Localizes in DNA Damage Foci", the disclosure of which is incorporated herein by reference in its entirety.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. Although a few exemplary embodiments of this invention have been described, those skilled in the art will readily appreciate that many modifications are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the claims. Therefore, it is to be understood that the foregoing is illustrative of the present invention and is not to be construed as limited to the specific embodiments disclosed, and that modifications to the disclosed embodiments, as well as other embodiments, are intended to be

included within the scope of the appended claims. The invention is defined by the following claims, with equivalents of the claims to be included therein.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 40

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JUMBO APPLICATIONS/PATENTS

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THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 40

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NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

What is claimed is:

1. A polypeptide construct comprising a detectable live-cell reporter molecule linked via a group having a molecular mass of less than 112,000 Daltons to at least one cell cycle phase-dependent location control element, the location of which said element changes during G1 and S phase, wherein the translocation of said construct within a mammalian cell is indicative of the cell cycle position.
2. A polypeptide construct according to claim 1 wherein said group has a molecular mass of less than 100,000 Daltons.
3. A polypeptide construct according to claim 1 wherein said group has a molecular mass of less than 50,000 Daltons.
4. A polypeptide construct according to claim 1 wherein said group has a molecular mass of less than 25,000 Daltons.
5. A polypeptide construct according to claim 1 wherein said group has a molecular mass of less than 10,000 Daltons.
6. A polypeptide construct according to claim 1 wherein said group has a molecular mass of less than 1,000 Daltons.
7. A polypeptide construct according to claim 1 wherein said group has a molecular mass of less than 700 Daltons.
8. A polypeptide construct according to claim 1 wherein said group has a molecular mass of less than 500 Daltons.
9. A polypeptide construct according to claim 1 wherein the group is a polypeptide.

10. A polypeptide construct according to claim 9 wherein said polypeptide group is a heptapeptide.
11. The polypeptide construct of claim 10, wherein said heptapeptide is Glycine-Asparagine- Glycine-Glycine-Asparagine-Alanine-Serine (GNGGNAS).
12. A polypeptide construct according to any preceding claim, wherein the cell cycle phase-specific dependent location control element is selected from the group of peptides consisting of Rag2, Chaf1B, Fen1, PPP1R2, helicase B, sgk, CDC6 or motifs therein such as the phosphorylation-dependent subcellular localization domain of the C-terminal special control region of helicase B (PSLD).
13. A polypeptide construct according to any of claims 1 to 12 wherein the live-cell reporter molecule is selected from the group consisting of fluorescent protein, enzyme reporter and antigenic tag.
14. A polypeptide construct according to claim 13 wherein said fluorescent protein is selected from Green Fluorescent Protein (GFP), Enhanced Green Fluorescent Protein (EGFP), Emerald and J-Red.
15. A polypeptide construct according to claim 13 wherein said enzyme reporter is halo-tag (Promega).
16. A polypeptide construct according to any preceding claim, wherein the cell cycle phase-dependent location control element is PSLD.
17. A polypeptide construct according to any preceding claim, wherein the reporter molecule is EGFP and the cell cycle phase-dependent location control element is PSLD.

18. A polypeptide construct comprising the amino acid sequence of SEQ ID No. 5.
19. A nucleic acid construct encoding any of the polypeptide constructs according to any preceding claim.
20. The nucleic acid construct of claim 19, wherein said construct additionally comprises and is operably linked to and under the control of at least one cell cycle independent expression control element.
21. The nucleic acid construct of claim 20, wherein said expression control element is either an ubiquitin C promoter or a CMV promoter.
22. The nucleic acid construct according to any of claims 19 to 21 comprising a CMV promoter, and sequences encoding PSLD and EGFP or J-Red.
23. The nucleic acid construct according to any of claims 19 to 21 comprising a ubiquitin C promoter, and sequences encoding PSLD and EGFP or J-Red.
24. A vector comprising any of the nucleic acid constructs of any of claims 19 to 23.
25. A vector according to claim 24, wherein said vector is either a viral vector or a plasmid.
26. A vector according to claim 25, wherein said viral vector is an adenoviral vector or a lentiviral vector.
27. A host cell transfected with a nucleic acid construct according to any of claims 19 to 23.
28. The host cell according to claim 27, wherein said cell is a human cell.

29. A stable cell line comprising one or more of the host cells according to either of claims 27 or 28.
30. Use of a polypeptide construct according to any of claims 1 to 18 for determining the cell cycle position of a mammalian cell.
31. A method for determining the cell cycle position of a mammalian cell, said method comprising:
 - a) expressing in a cell a nucleic acid construct according to any of claims 19 to 23; and
 - b) determining the cell cycle position by monitoring signals emitted by the reporter molecule.
32. A method of determining the effect of a test agent on the cell cycle position of a mammalian cell, said method comprising:
 - a) expressing in said cell in the absence and in the presence of said test agent a nucleic acid construct according to any of claims 19 to 23; and
 - b) determining the cell cycle position by monitoring signals emitted by the reporter molecule wherein a difference between the emitted signals measured in the absence and in the presence of said test agent is indicative of the effect of the test agent on the cell cycle position of the cell.
33. A method of determining the effect of a test agent on the cell cycle position of a mammalian cell, said method comprising:
 - a) expressing in said cell in the presence of said test agent a nucleic acid construct according to any of claims 19 to 23; and
 - b) determining the cell cycle position by monitoring signals emitted by the reporter molecule,

- c) comparing the emitted signal in the presence of the test agent with a known value for the emitted signal in the absence of the test agent;

wherein a difference between the emitted signal measured in the presence of the test agent and said known value in the absence of the test agent is indicative of the effect of the test agent on the cell cycle position of the cell.

34. A method of determining the effect of a test agent on the cell cycle position of a mammalian cell, said method comprising:

- a) providing cells containing a nucleic acid construct according to any of claims 19 to 23;
- b) culturing first and second populations of said cells respectively in the presence and absence of a test agent and under conditions permitting expression of the nucleic acid reporter construct; and
- c) measuring the signals emitted by the reporter molecule in said first and second cell populations;

wherein a difference between the emitted signals measured in said first and second cell populations is indicative of the effect of said test agent on the cell cycle position of said cell.

35. A method of determining the effect of the mammalian cell cycle on a cellular process measurable by a first detectable reporter which is known to vary in response to a test agent, said method comprising:

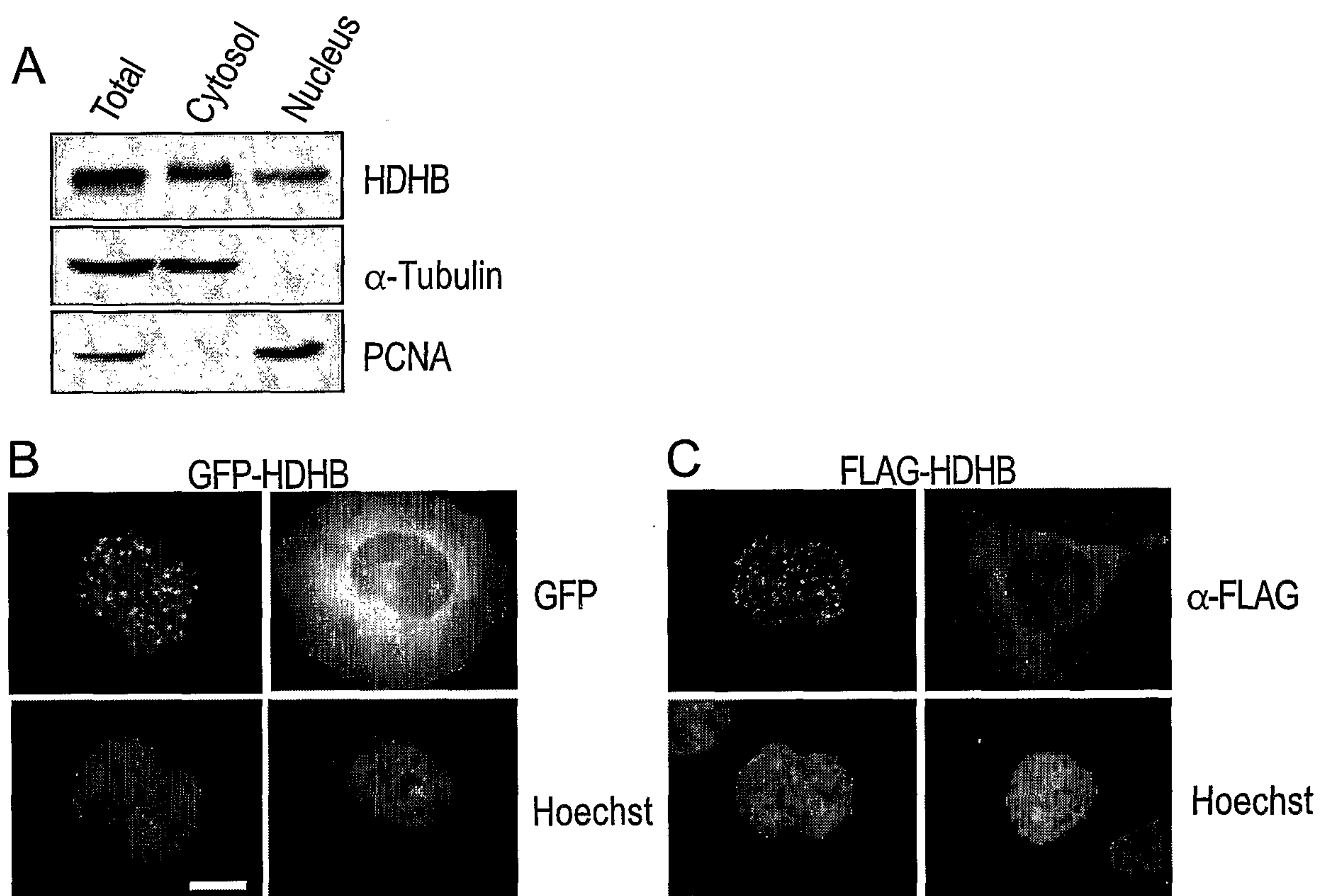
- a) expressing in said cell in the presence of said test agent a second nucleic acid reporter construct according to any of claims 19 to 23;
- b) determining the cell cycle position by monitoring signals emitted by the second reporter molecule; and
- c) monitoring the signals emitted by said first detectable reporter.

wherein the relationship between cell cycle position determined by step b) and the signal emitted by the first detectable reporter is indicative of whether or not said cellular process is cell cycle dependent.

36. Use of a polypeptide construct according to any of claims 1 to 18 for measuring CDK2 activity in a cell.
37. A method for measuring CDK2 activity in a cell, said method comprising the steps of
- a) expressing a nucleic acid construct according to any of claims 19 to 23 in a cell, and
 - b) determining CDK2 activity by monitoring signals emitted by the reporter molecule.
38. A method for determining the effect of a test agent on CDK2 activity of a mammalian cell, said method comprising:
- a) expressing in said cell in the absence and in the presence of said test agent a nucleic acid construct according to any of claims 19 to 23; and
 - b) determining CDK2 activity by monitoring signals emitted by the reporter molecule wherein a difference between the emitted signals measured in the absence and in the presence of said test agent is indicative of the effect of the test agent on the activity of CDK2.
39. A method of determining the effect of a test agent on CDK2 activity of a mammalian cell, said method comprising:
- a) expressing in said cell in the presence of said test agent a nucleic acid construct according to any of claims 19 to 23; and
 - b) determining the cell cycle position by monitoring signals emitted by the reporter molecule,
 - c) comparing the emitted signal in the presence of the test agent with a known value for the emitted signal in the absence of the test agent;
- wherein a difference between the emitted signal measured in the presence of the test agent and said known value in the absence of the

test agent is indicative of the effect of the test agent on the CDK2 activity of the cell.

40. The method according to any of claims 37 to 39, wherein said test agent is a form of electromagnetic radiation or is a chemical entity.



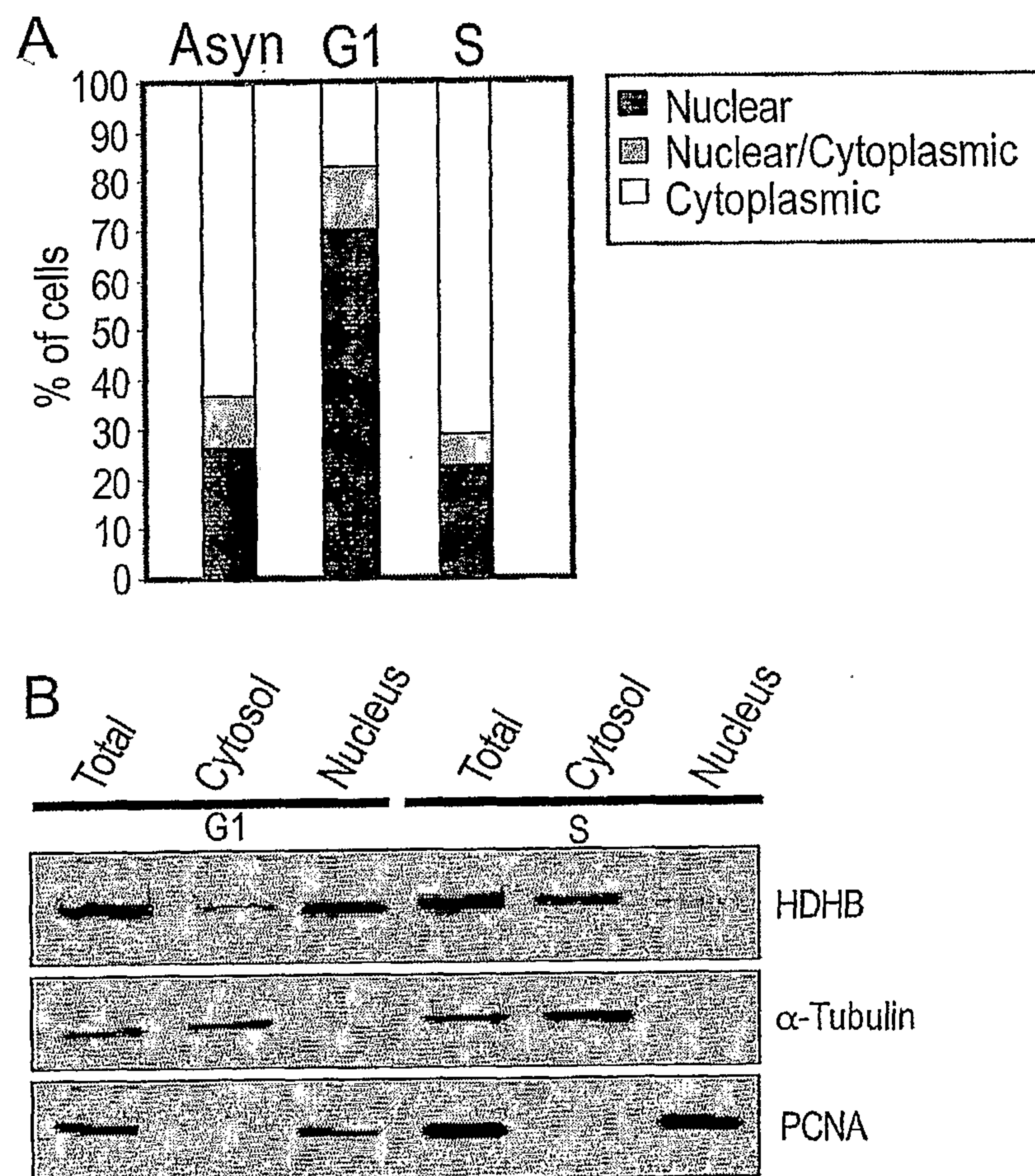


Fig. 2

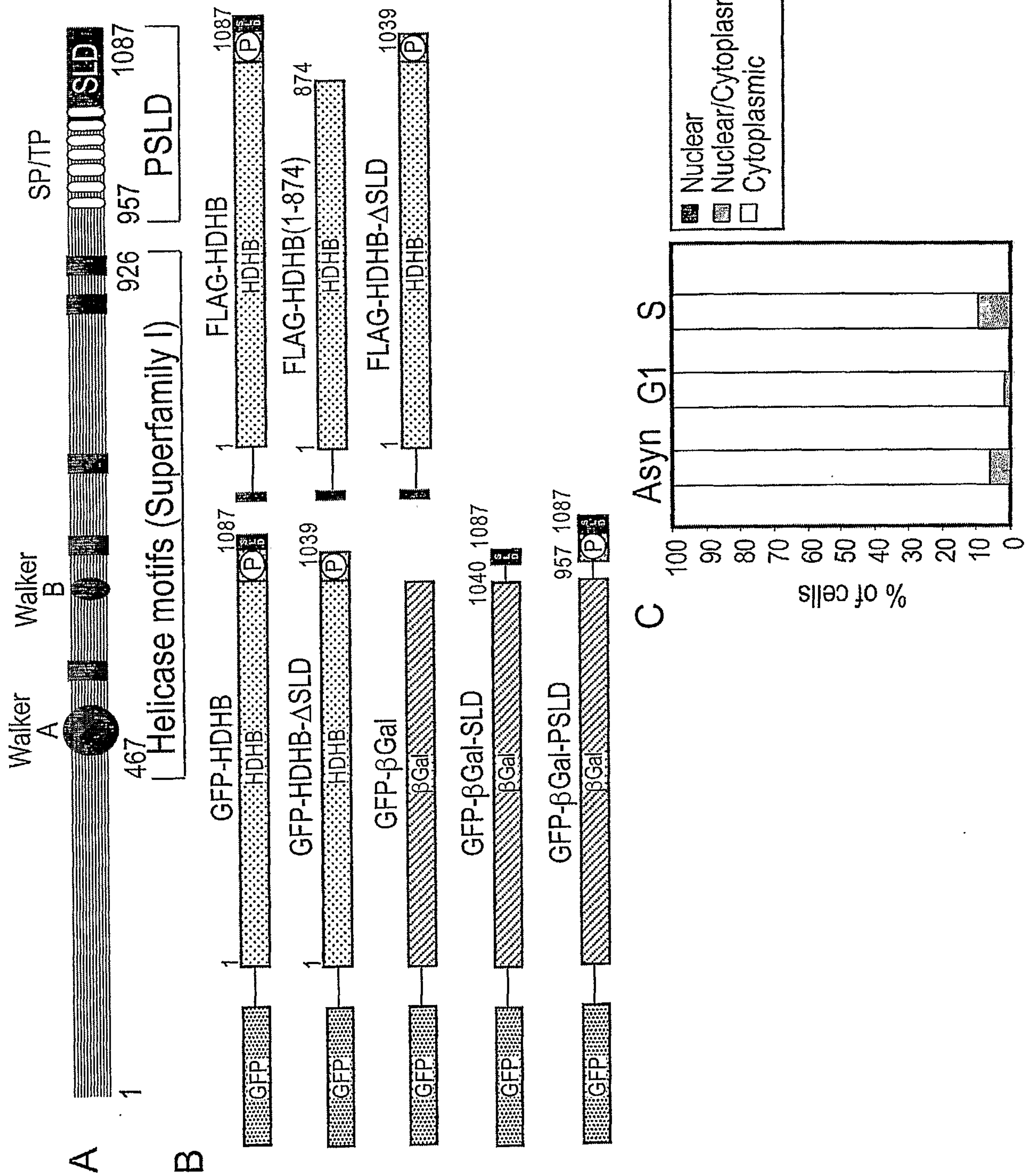


Fig. 3

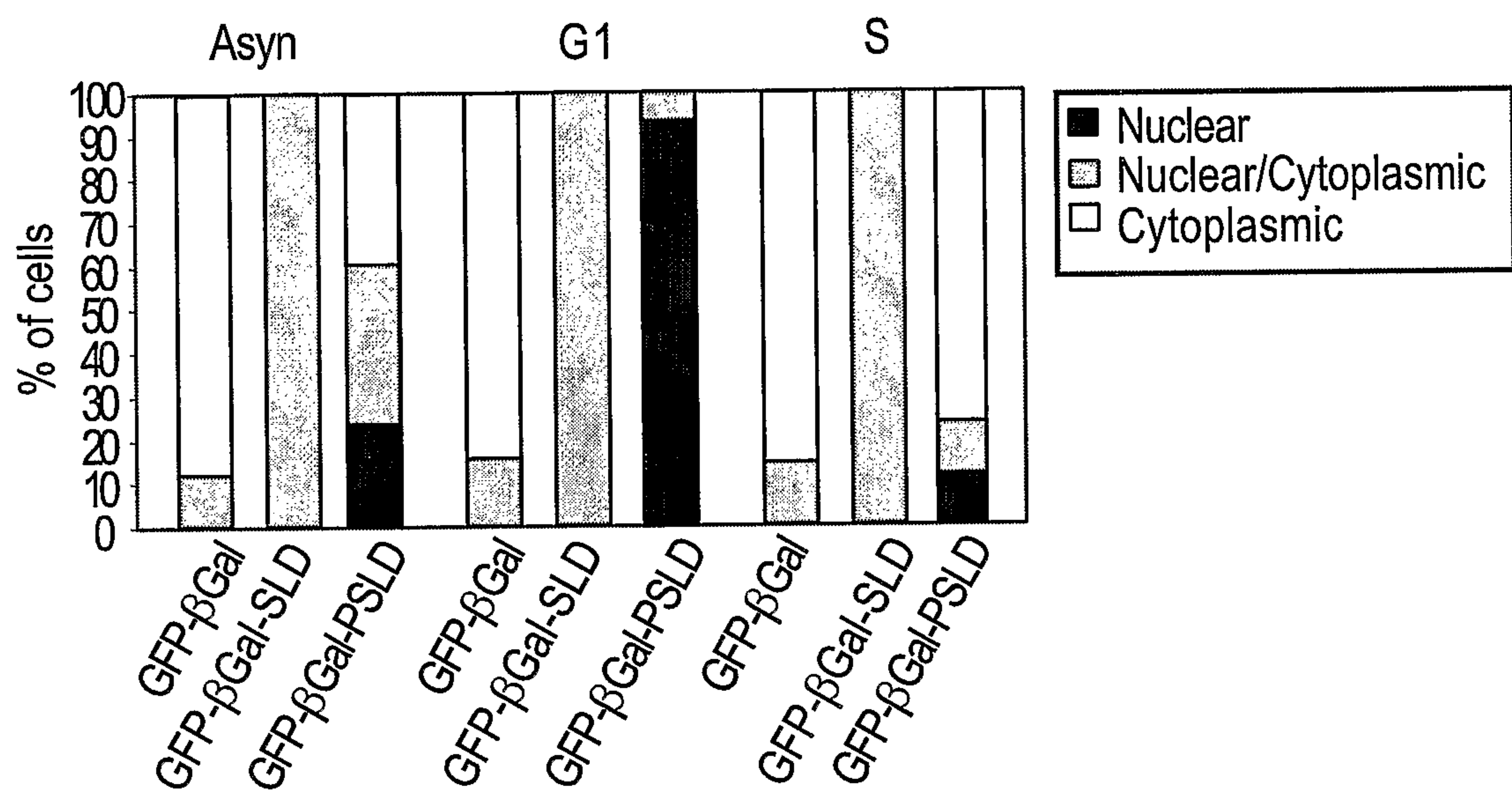


Fig. 4

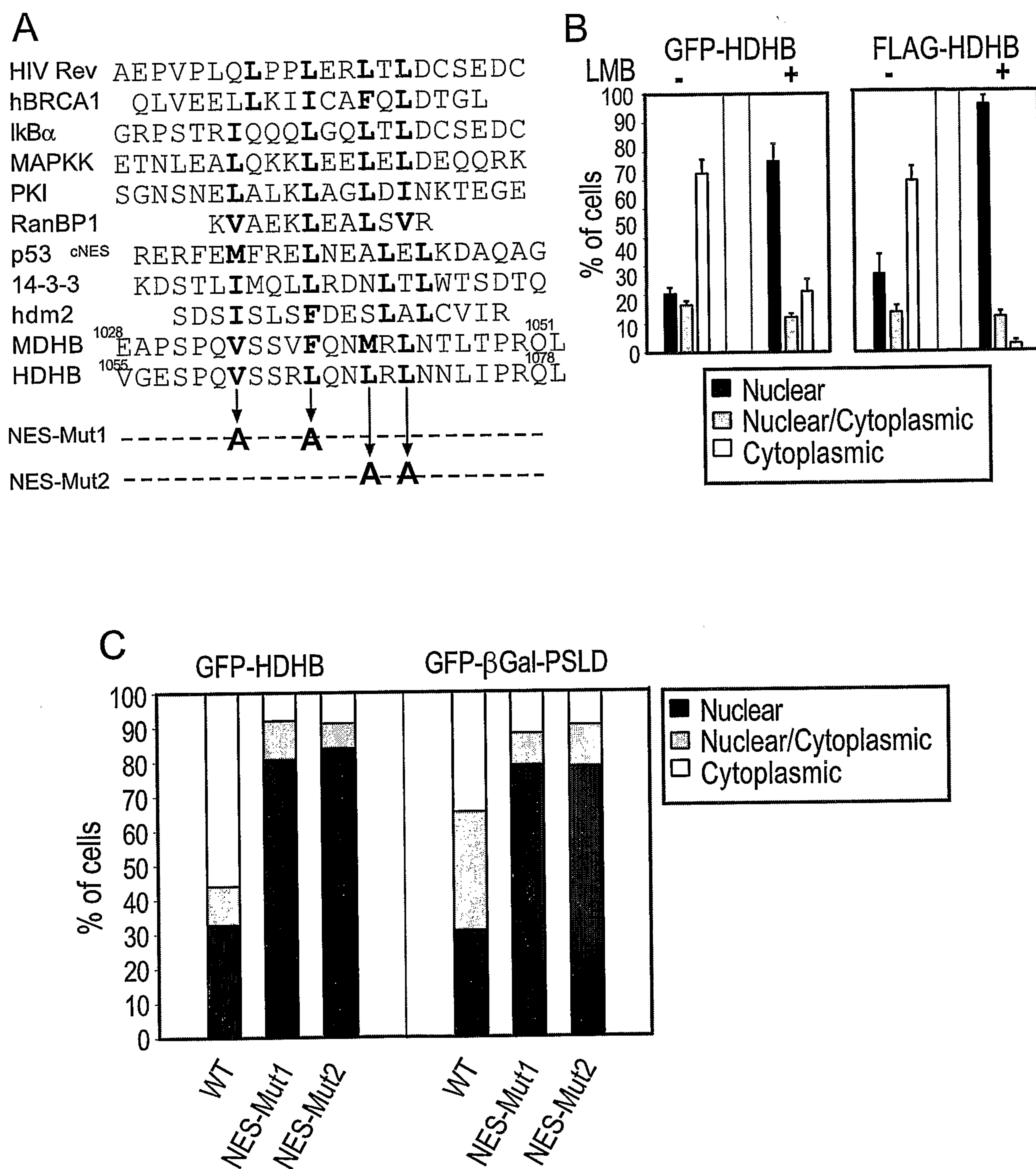


Fig. 5

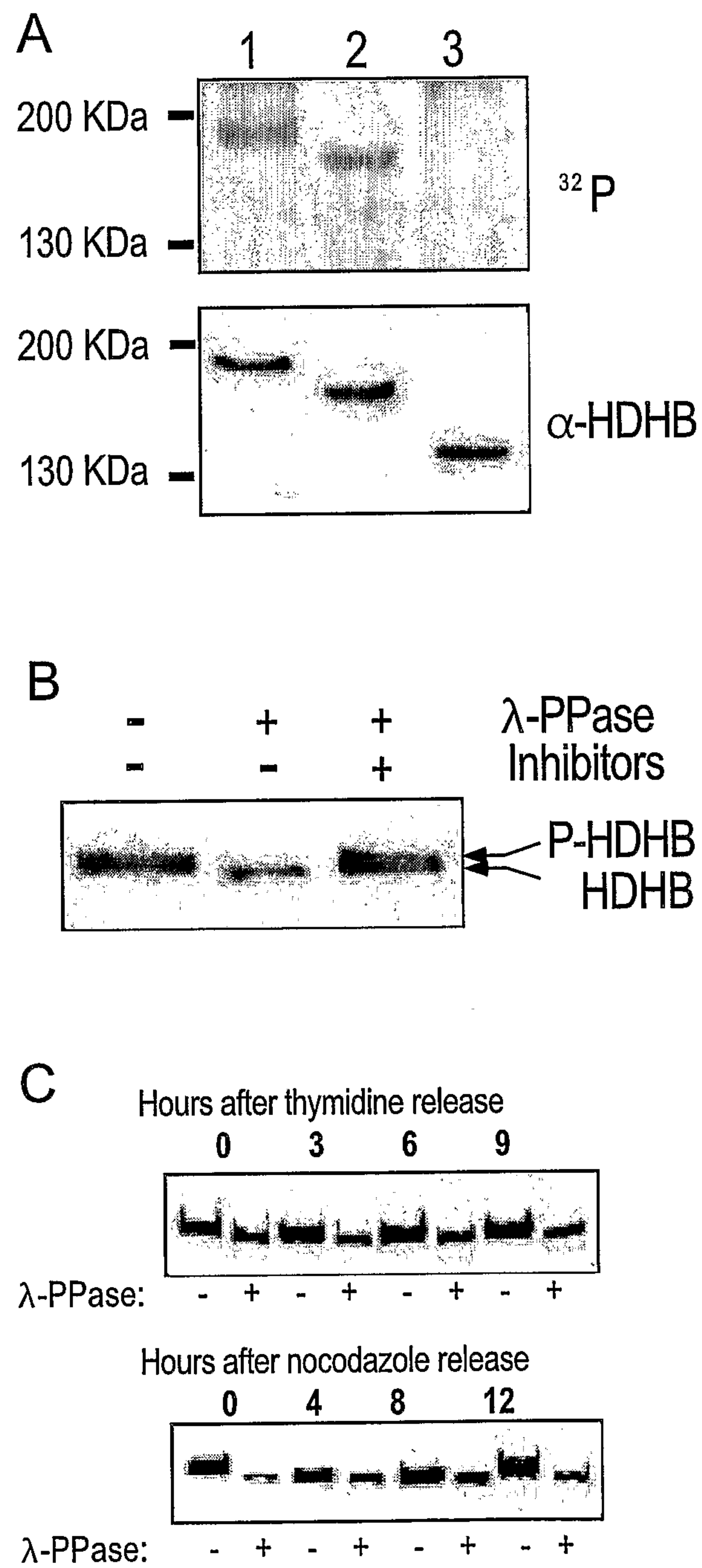


Fig. 6

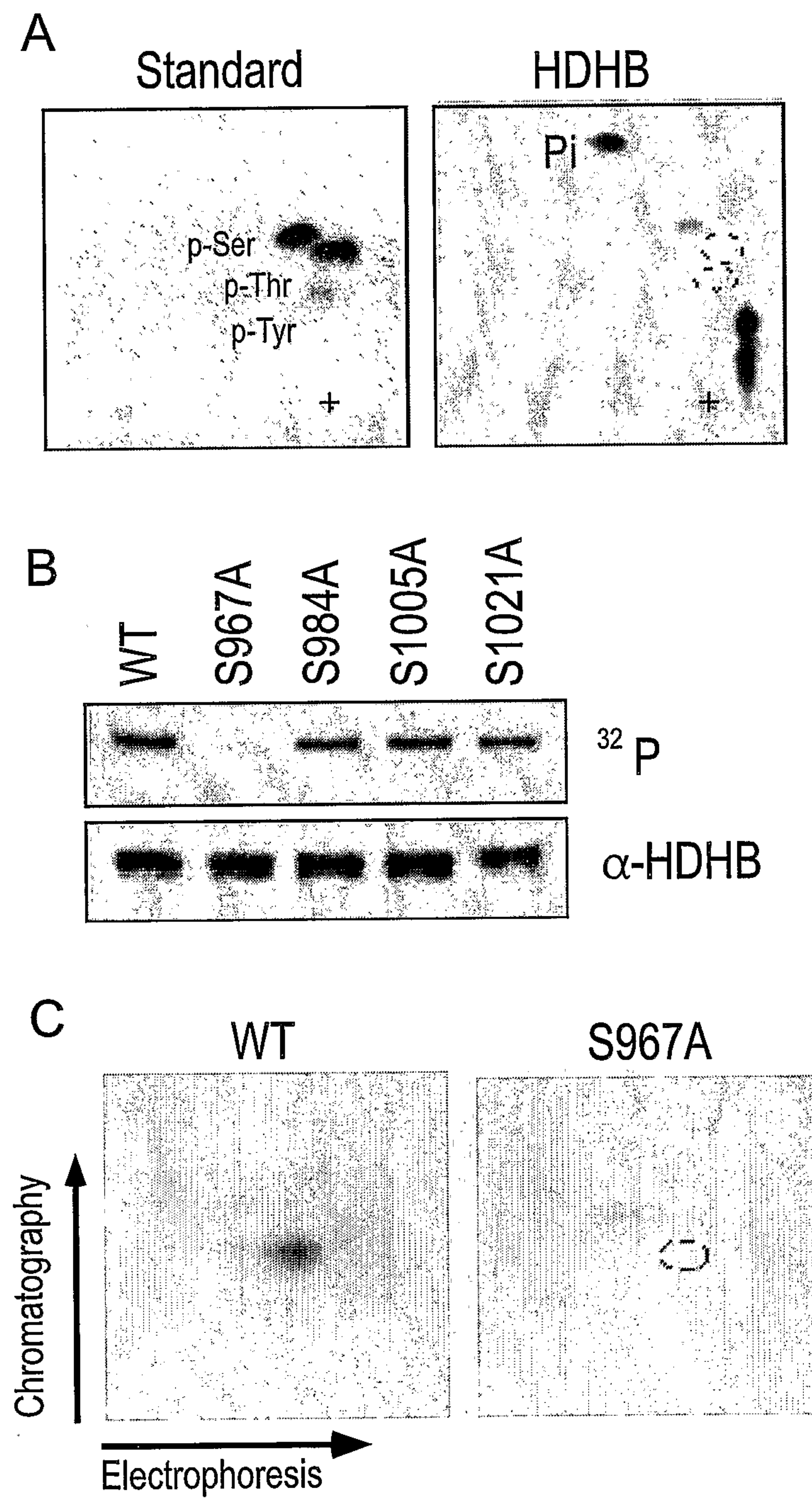


Fig. 7

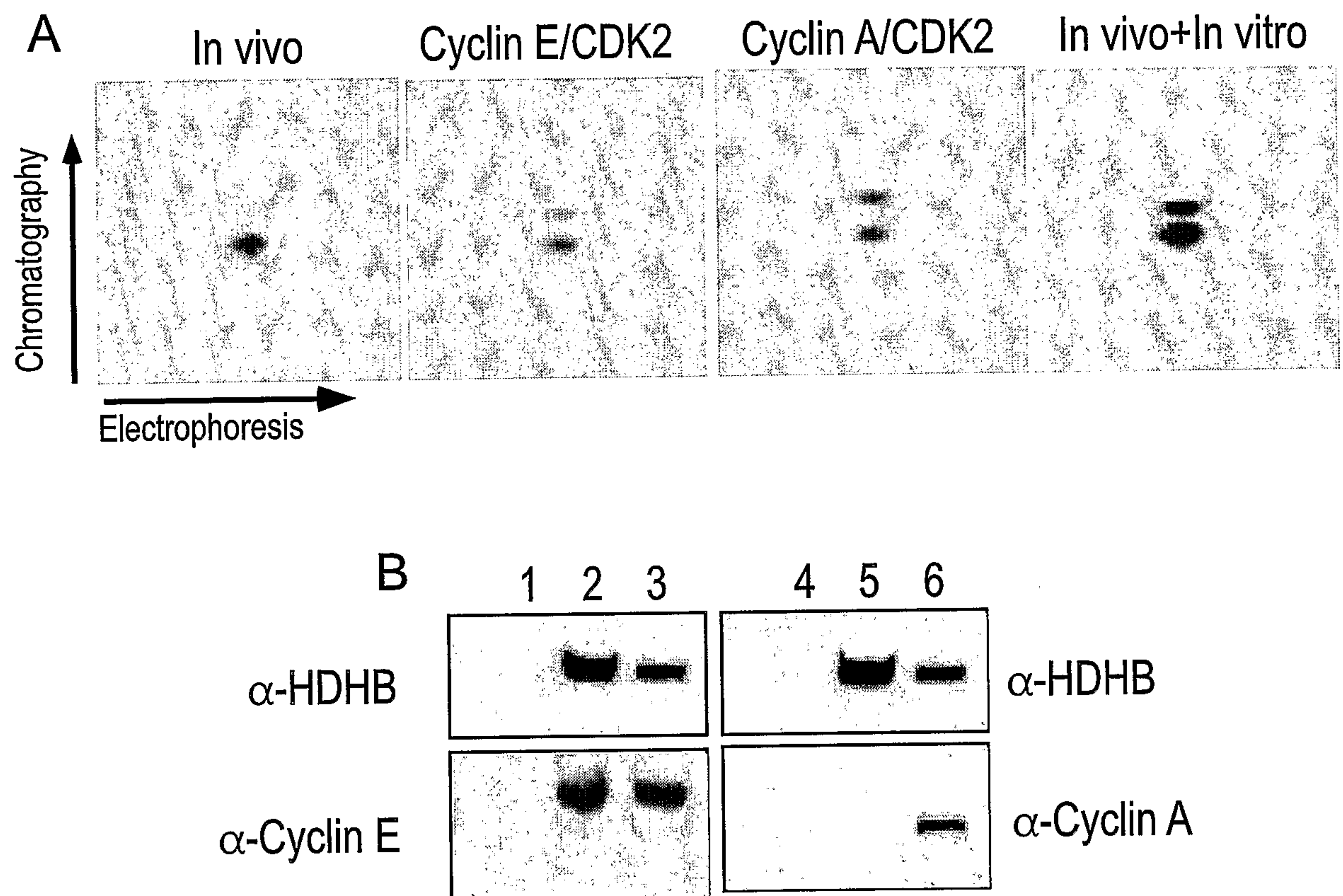


Fig. 8

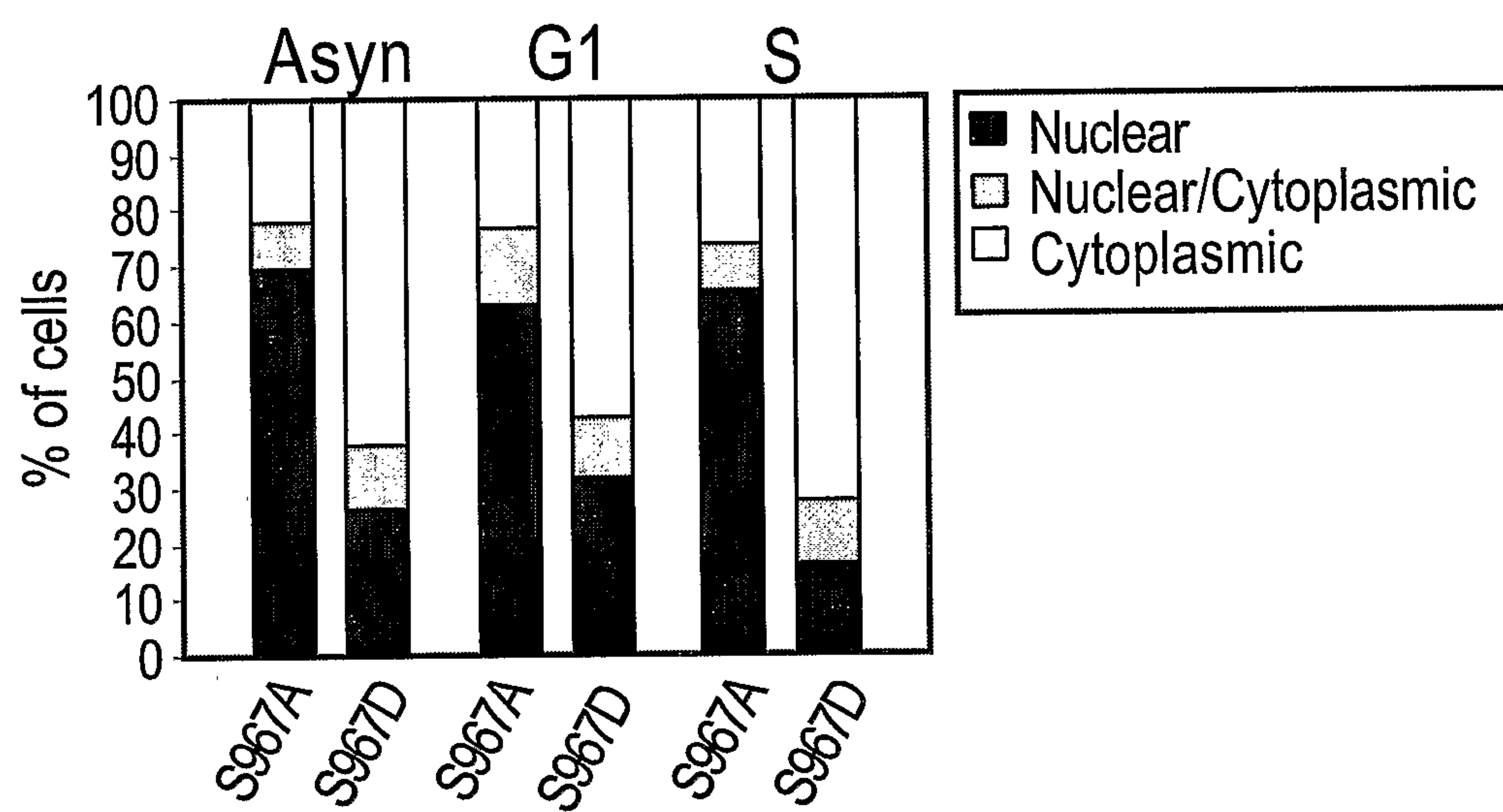


Fig. 9

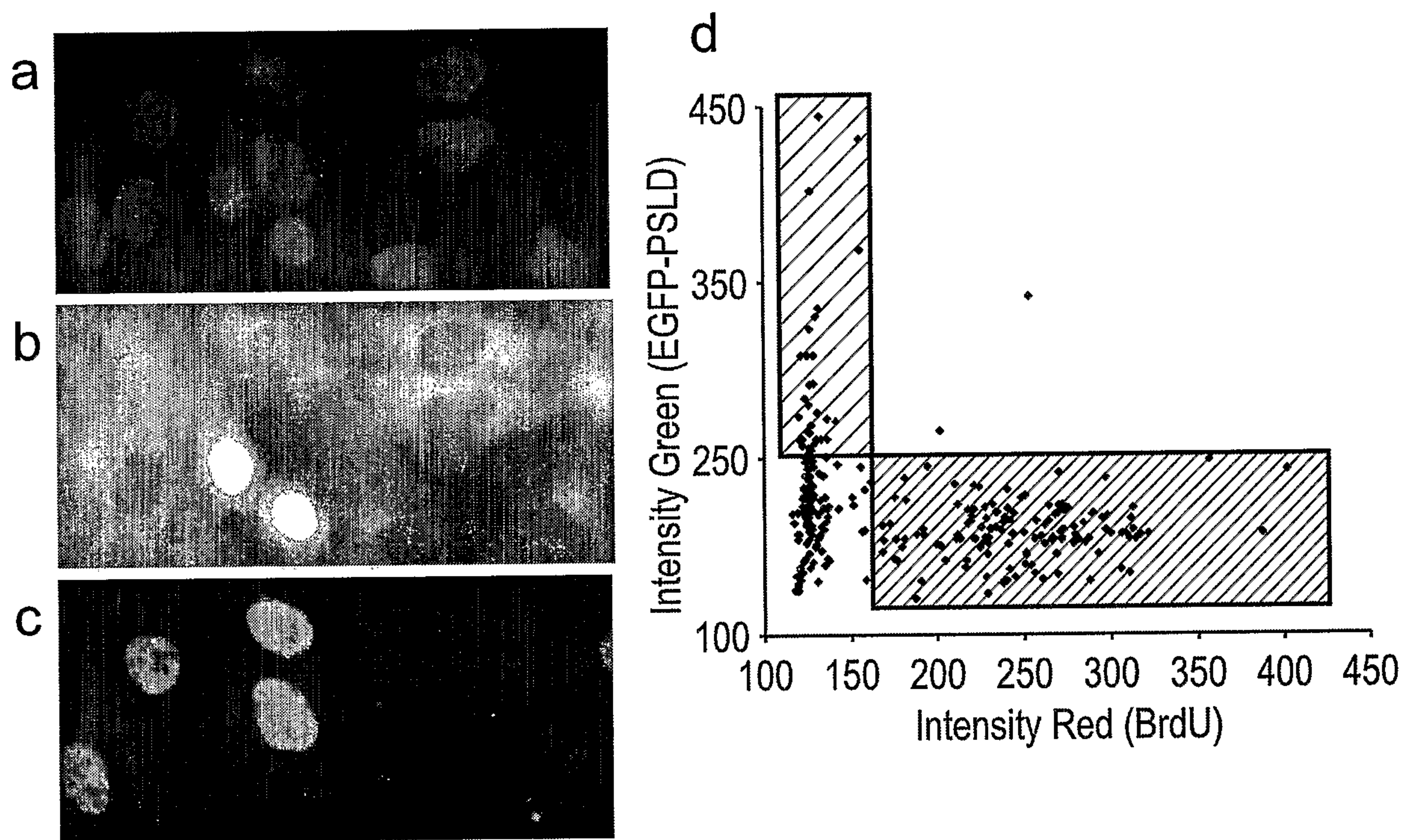


Fig. 10

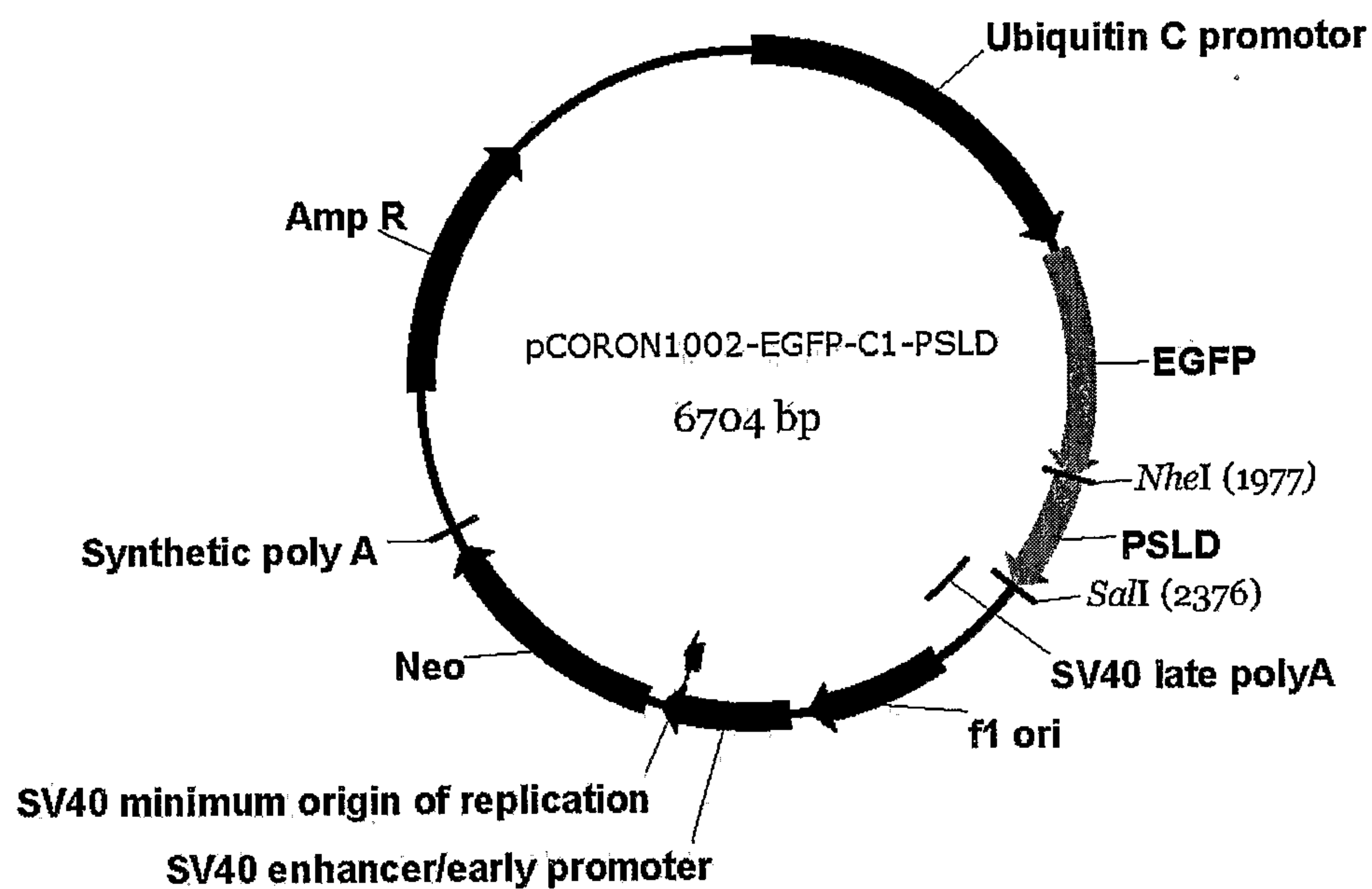


Fig. 11

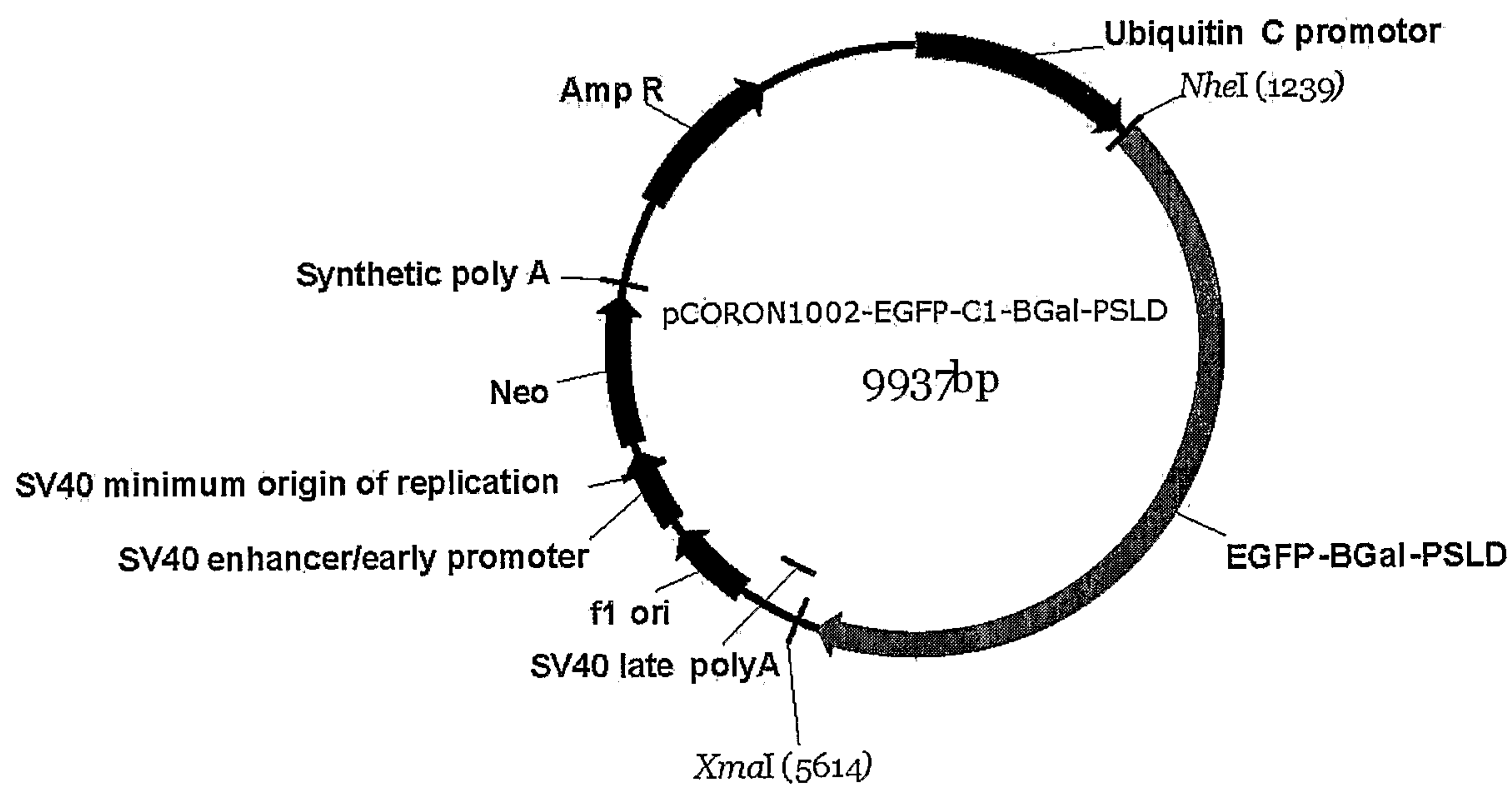


Fig. 12

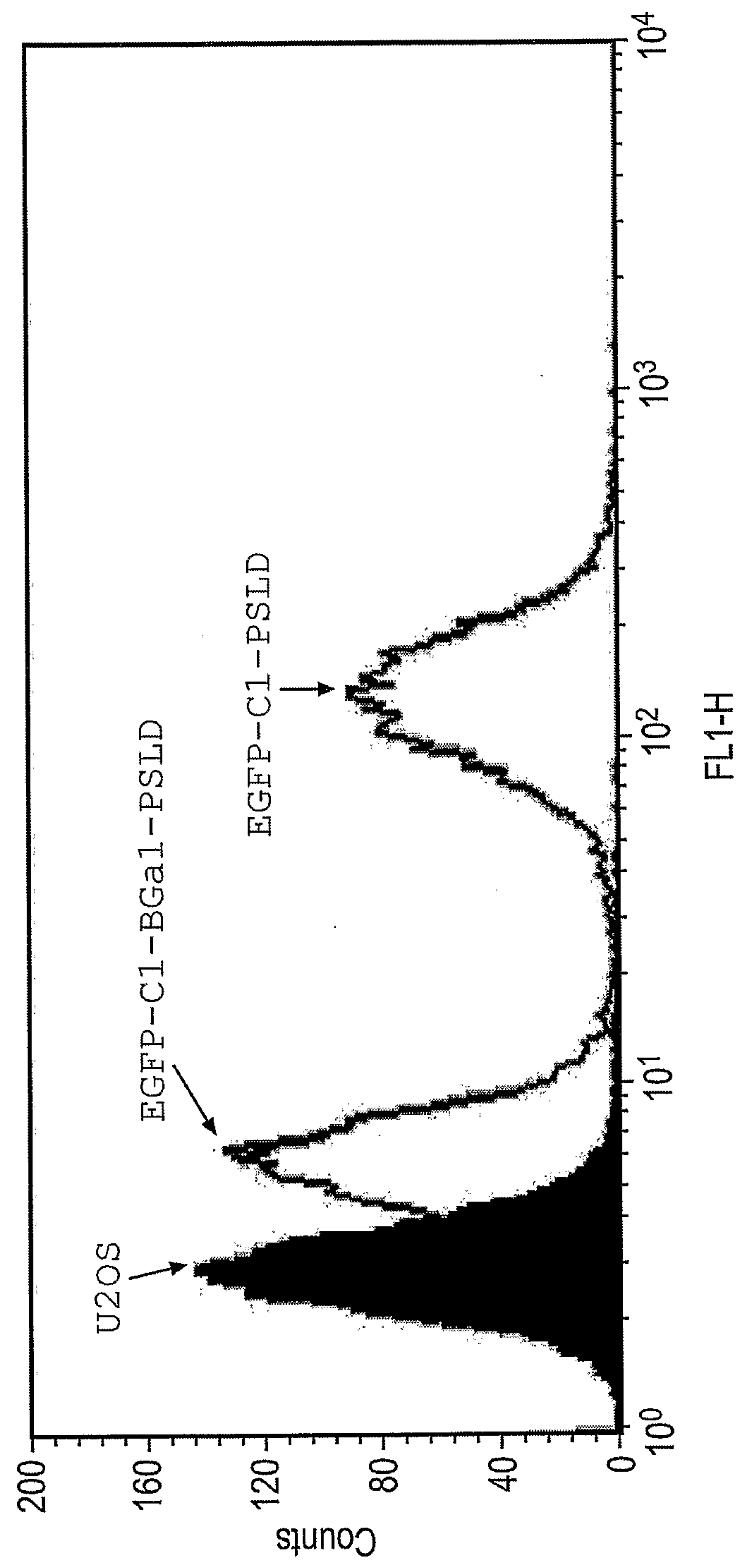


Fig. 13

