THE STUDY OF THE ROLE OF CYCLIN-DEPENDENT KINASE 5 (CDK5) DURING CELL DEATH

by

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ABSTRACT

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Cell death is an important biological process that selectively eliminates cells in developing embryo as well as in adult tissues. The mechanisms underlying this selective elimination are still unclear. The aim of this work has been to study the role of cyclin-dependent kinase 5 (Cdk5) in cell death. Several established cell death models have been used in this work, which include normally developing limb and developing lens, abnormally developing cyclophosphamide (CP), an alkylating agent, treated embryo, and toxic compounds induced cell strain (COS-7) cell death.

To address the role of Cdk5 in cell death, we, first, investigated the regulation of Cdk5 from the transcription, translation, and post-translation levels and the results showed that Cdk5 was activated at the post-translation level. Second, to identify how Cdk5 was regulated by other proteins, a yeast two-hybrid system and ^{35}S-Met incorporation immunoprecipitation technique were used. From both approaches, p35 and its truncated form, p25, were identified to be associated with Cdk5. Furthermore, we investigated the pattern of p35/p25 expression, and the results showed that both p35 and p25 had similar expression pattern as Cdk5 during cell death. Further study indicates that during cell death,

p35 could be truncated to p25, and p25 might be the factor, which activates Cdk5 during cell death. These results suggested that Cdk5 and its regulatory proteins may be key players in the execution of cell death.

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Preface

Active cellular suicide plays an important role in animal development, tissue homeostasis, and a wide variety of diseases (Vaux et al.1999; Rich et al.1999; Andrew et al.2001). During the development of most metazoan animals, many more cells are produced than are eventually needed, and cell death plays a key role in removing surplus cells and sculpting the developing embryo (Golstein, et al.1991; Zakeri et al.'1997; Clarke et al.1998; Afford, 2000; Blagosklonny, et al.2000; Manolagas et al.2000). In addition, the inappropriate regulation of cell death is associated with a variety of diseases, including cancer, many neurodegenerative diseases, and ischaemic stroke (White, 1996; Geng et al.1997; Batinage, 1998; Stassi et al.1999; Nijhawan, et al.2000). Therefore, it is important to understand the mechanism of cell death and to control it eventually. Several genes have been shown to be associated with cell death. These genes include those of the caspase family (Hengartner et al. 2000; Andrew et al.2001); the bcl-2 family (Chung et al.2000, Andrew et al.2001); NF-kB (nuclear factor kappa B) (Varfolomeev et al.1996); JNK (c-JUN N-terminal kinase) (Konopleva et al.1999); p53 (Wu et al 1998, Hakem et al.1998); and the cyclin-dependent kinases family (Hakem et al. 1999; James 1999; Patirck et al. 1999; Homayouni et al. 2000; Dhavan et al. 2001; Lee et al. 2001).

Cyclin-dependent kinases (Cdks) are cell cycle-related genes. They regulate the transition between successive phases of the cell cycle in eukaryotic cells (Patrick et al.1999; Neystat et al. 2001). Some of them, such as Cdk1 and Cdk5, have been reported to be involved in cell death, but the mechanism of this

action is not clear (Lahti et al.1995; Patrick et al.1999; Lee et al.2000; Ahlijanian et al.2000). My thesis is focused on studying the role of Cdk5 during cell death.

Cdk5 was first isolated by its structural homology to the human Cdk1 (also called Cdc2) (Lew et al.1992; Meyerson et al.1992). Although Cdk5 is widely expressed, its kinase activity is detected only in the postmitotic neurons of the central nervous system (CNS) (Nikolic et al.1996). Previous studies from our laboratory suggested that Cdk5 is expressed during cell death. Therefore, the purpose of my study is to further investigate the role of Cdk5 and how it is regulated during cell death. To address these questions, I have:

a) Investigated the correlation between Cdk5 and apoptosis by studying Cdk5 expression patterns and its regulation in several embryonic cell death models, including normal *in vivo* developing mouse limbs and developing rat lens, abnormal manipulated cell death *in vivo* developing mouse whole embryo, and an *in vitro* COS-7 cell line treated with cell death-inducing reagents;

b) Using different approaches, isolated the Cdk5 interactive proteins that regulate Cdk5 during cell death in specific cell death models;

c) Illustrated the role of Cdk5's regulators during cell death in cell death models by manipulating their expression and exam their influences on cell death.

TABLE OF CONTENTS

Title.....i

Approval Page.....ii

Abstract.....iii

Acknowledgments.....v

Prologue.....vi

Table of contents.....viii

PART I. INTRODUCTION.....1

Chapter 1. The concept of cell death.....2
a. Types of programmed cell death.....3
b. The importance of programmed cell death.....5
Chapter 2. Cell death in development.....8
A. Cell Death in Limb Development.....9
B. Cell Death in Lens Development.....12
C. Cell death in Cyclophosphamide (CP) Treated Developing Embryo.....15
Chapter 3. Regulation of cell death.....18
A. Cell Death Signals and Related Genes.....19
a. The bcl-2 Family.....20
b. The caspase Family.....21
c. Cyclin-Dependent Protein Kinases.....23
B. Cdk5 and Its Activator p35/p25.....25
a. The finding of Cdk5.....25
b. The expression and activity of Cdk5.....27
c. Cdk5 associated factors.....29
d. p35/p25, the widely studied regulators of Cdk5.....32
e. Substrates of Cdk5.....34

PART II. RESULTS AND DISCUSSION.....36

Chapter I: Association of Cdk5 with both normal and induced cell death...36
1. Objective.....37
2. Material and Methods.....38
3. Results.....52
A. Analysis of the association of Cdk5 with cell death in developing mouse limb.....52
B. Analysis of the association of Cdk5 with cell death in developing rat lens.....53

C. Analysis of the association of Cdk5 with cell death in induced model of cell death.....56
D. Analysis of the association of Cdk5 with cell death in induced COS-7 cell death.....58
4. Discussion.....60

Chapter II: Identification of Cdk5 interactive proteins during cell death....63
1. Objective.....64
2. Material and Methods.....66
3. Results.....77
A. Identifying Cdk5 interactive protein(s) by the yeast two-hybrid system.....77
B. Identifying Cdk5 interactive protein(s) by ^{35}S-Methionine incorporation and immunoprecipitation.....80
4. Discussion.....83

Chapter III: Functional Analysis of p35/p25 during Cell Death.....86
1. Objective.....87
2. Material and Methods.....88
3. Results.....90
A. Expression analysis of p35/p25 during cell death in developing mouse limbs.....90
B. Expression analysis of p35/p25 during cell death in developing lens..91
C. Expression analysis of p35/p25 during cell death in CP treated mouse embryos.....92
D. Expression and characterization of p35/p25 in induced COS-7 cell death.....93
4. Discussion.....96

CONCLUSION.....99

FIGURES.....100

REFERENCES.....171

PART I. INTRODUCTION

Chapter 1. The concept of cell death

- a. Types of programmed cell death
- b. The importance of programmed cell death

Chapter 2. Cell death in development

- A. Cell Death in Limb Development
- B. Cell Death in Lens Development
- C. Cell death in Cyclophosphamide (CP) Treated Developing Embryo

Chapter 3. Regulation of cell death

- A. Cell Death Signals and Related Genes
  - a. The bcl-2 Family
  - b. The caspase Family
  - c. Cyclin-Dependent Protein Kinases
- B. Cdk5 and Its Activator p35/p25
  - a. The finding of Cdk5
  - b. The expression and activity of Cdk5
  - c. Cdk5 associated factors
  - d. p35/p25, the widely studied regulators of Cdk5
  - e. Substrates of Cdk5

Chapter 1. The concept of cell death

a. Types of programmed cell death

b. The importance of programmed cell death

"Programmed cell death" (PCD), is a term used to describe the coordinated series of events leading to cell demise. "Programmed cell death" or "apoptosis", as it is commonly called, refers to a genetically regulated form of cell suicide that is morphologically distinct. (Afford et al. 2000; Bursch et al. 2000; Bortner et al. 2002). The genetic elements that controll the executional phase of cell death, seem to be constitutively expressed in virtually every cell.

a. Types of programmed cell death

Programmed cell death is very important during development, senescence, and many diseases (Kerr et al.1972; Zakeri et al.1994; Jenner et al.1996; Wyllie 1997; Jellinger et al.1998; Zhang et al. 2002). However, the specific mechanisms and their regulations are still unknown. The primary paradigm of natural PCD is observed during normal embryogenesis. In careful morphological studies using electron microscopy, Kerr (1969); Schweichel and Merker (1973) identified three forms of PCD: the first is apoptosis, the second is necrosis, while the third type is something may have been considered to be "secondary necrosis," due to variable expression of overlapping necrotic and apoptotic processes. Recently, there is increasing evidence that apoptosis and necrosis represent only the extreme ends of a wide range of a possible morphological and biochemical death pathways (Bursch et al. 1996; Bursch et al. 2000; Albertine et al. 2002).

Apoptosis is differentiated from necrosis. Because in necrosis large groups of cells are involved and cellular contents are lost early into the extracellular space, necrotic tissues evoke inflammatory responses. In contrast, during apoptosis, membrane damage

occurs later, and dead cells are engulfed by neighboring cells or phagocytes *in vivo* with minimal lysosomal involvement or toxic lysis (Ahuja and Zakeri 1994; Bursch et al. 2000). These changes lead to little or no inflammation. Characteristic apoptotic morphology is observed in many situations, such as deletion of cells during development, homeostatic regulation of cell populations, and aging (Nijhawan et al. 2000). Metabolic stress induced by specific genetic defects also leads to cell death with "typical" apoptotic morphology.

Necrosis

Necrotic cells and their organelles are characteristically swollen. There is early membrane damage with loss of plasma membrane integrity and leakage of cytosol into extracellular space. Despite early clumping, the nuclear chromatin undergoes breakdown, not condensation (Cotran et al. 1999; Bursch et al. 2000). This cell death may occur in response to injury by toxins, physical stimuli, or ischemia (Ferri et al. 2000).

Apoptosis

The morphologic characteristics of typical apoptotic cells are cell shrinkage, nuclear condensation, membrane blebbing, fragmentation of cells into membrane-bound apoptotic bodies.Finally, apoptotic cells and their fragments are engulfed by phagocytes or surrounding cells (Blagosklonny et al. 2000). Apoptosis is a regulated event of a pre-existing death program; it is genetically regulated and evolutionarily conserved. This process is energy dependent and the synthesis of macromolecules, or *de novo* gene transcription is required (Bursch et al. 2000).

Other

Although most dying cells fall into one of the previous two categories, not all

follow the same morphology or biochemistry. Some cells die by a regulated mechanism that does not involve nuclease activation (Clark et al. 1990). Recently, a kind of non-apoptotic death of chondrocytes was studied (Roach et al. 2000). When these chondrocytes die, they exhibit convoluted nuclei and patchy chromatin condesations, but with an expanded endoplasmic reticular lumen and both cytoplasm and organelles reduced to dark, worm-like inclusions. Roach et al. (2000) suggested that there are also apoptotic cells that could not be phagocytosed due to their confinement within their lacunae *in vivo*.

Yet, the classical description of the apoptotic phenotype does not necessarily apply to all circumstances during which apoptosis prevails (Wyllie, 1997; Ferlini, et al. 1999; Blagosklonny et al. 2000; Song et al. 2000). The mode of cell death—whether apoptotic, necrotic, or other—can be specified by characteristic morphological and biochemical changes, but subroutines of the cell degradation program may not be active in all cases of cell death. Their differences lead to different morphologies and biochemistries of dying cells. Furthermore, since all forms of cell death overlap to some extent, the same stimulation may give rise to varying forms. For example, in circumstances involving cell stress imposed by toxins, physical agents, or ischemia, cells may die by apoptosis as well as by necrosis (Papasotiropoulos et al. 1996; Wyllie 1997; Ferlini et al. 1999; Nijhawan et al. 2000).

b. The importance of programmed cell death

Programmed cell death (PCD) is critical to the health of almost all organisms and is needed to help sculpt parts of the body, to eliminate vestigial structures during

development, and to maintain normal functioning in aging and senescence (Blagosklonny, et al. 2000; Pollack et al. 2002). When apoptosis is deregulated, the results may be dire: cancer when there is too little apoptosis, and possible stroke damage or the neurodegeneration of Alzheimer's disease when there is too much. In sum, PCD is essential for normal development and survival of animals (White 1996; Nijhawan, et al. 2000).

Importance in normal development

Cell death is detected in many organs during normal development. For example, cell death is important in developing tissues such as the tadpole tail and interdigital regions of the mouse limb (Kerr et al. 1974; Lockshin et al. 1981; Steller 1995; Jacobson et al. 1997; Zakeri et al. 1997; Blagosklonny, et al. 2000). In developing lens, lens fiber cells lose their nuclei (Gao et al. 1997). During vertebrate development, more nerve cells are generated than are needed, and the death of 20% to 80% of neurons is a regular feature (Saikumar et al. 1995; Jacobson et al. 1997; Clarke et al. 1998; Manolagas et al. 2000).

Importance in abnormal development

Excessive cell death contributes to abnormal embryonic development. For example, massive cell death occurs in the embryo when treated with cyclophosphamide (CP), an alkylating agent, (Chen et al. 1994; Moallem et al. 1995; Zhu et al. 2002). Retinoic acid (RA), another teratogenic agent, can increase cell death in the interdigital regions of the mouse limb as well as affect the pattern of limb development (Lammer et al. 1985; Alles et al. 1989; Zakeri et al. 1993; Zakeri et al. 1994; Ahuja et al. 1997).

Importance in diseases

In adult tissues, PCD is correlated with senescence and many diseases (Cohen et al. 1991; Golstein et al. 1991; Afford, 2000). Decreased apoptosis, proliferation and activation of autoreactive immune cells play an important role in the development of autoimmunity and determination of health tissues. In the acquired immunodeficiency syndrome (AIDS), the human immunodeficiency virus (HIV) causes the death of T-helper cells (Weiss et al. 1993). T-helper cells are necessary for the activation of cytotoxic T lymphocytes (Kalams et al. 1998). T lymphocytes are involved in host defense against viruses and other pathogenis. Similarly, during ischemic diseases such as myocardial infarction (Geng et al. 1997) and stroke (Rubin et al. 1997), cells die by apoptosis as well as by necrosis (Li et al. 1998; Taimor et al. 1998). Likewise, excessive heat (Nakano et al. 1997), radiation, cytotoxic drugs (Belka et al. 1998; Boesen-de et al. 1999), and hypoxia (Saikumar et al. 1998) cause extensive cell death by both apoptosis and necrosis. Apoptosis also plays a significant role in the pathology of degenerative diseases of the central nervous system. For example, abnormal neuronal disruption plays a key role in Parkinson's and Alzheimer's disease (Jenner et al. 1996; Jellinger et al. 1998), as well as in peripheral nervous system disorders such as diverse neuropathies and retinal degenerations (Erdem et al. 1998).

Thus, cell death is important in development, senescence, and disease. An understanding of the mechanisms controlling cell death has a great value. In my thesis, I intend to illustrate the function and importance of one gene (cyclin-dependent kinase 5) during cell death. Before discussing this gene, I will first discuss several developmental cell death models that I used during my study.

Chapter 2. Cell death in development

A. Cell Death in Limb Development

a. The pattern of limb formation

b. Genetic regulation during limb development

B. Cell Death in Lens Development

a. The pattern of lens formation

b. Genetic regulation during lens development

C. Cell Death in Cyclophosphamide (CP) Treated Developing Embryos

A fundamental concept of embryonic development is selective well-regulated cell death, which can be found as early as the 8 cell stage of the embryo. During normal development, cells in both parts of the blastocyst (the inner cell mass and trophoectoderm) undergo apoptosis. However, each has a different sensitivity to apoptosis-inducing factors (Afford, 2000). Distortions of apoptosis in the blastocyst result in the compromise of future maturation and may lead either to early embryonic death or the formation of anomalies in the fetus (Brill et al.1999).

At the later stages of normal embryonic development, apoptosis plays a key role in the formation of the extra-embryonic structures and of the embryo itself. For example, apoptosis has been demonstrated in fetal membranes (Nijhawan et al. 2000). The use of an electron microscope has revealed ultrastructural changes in the amniotic epithelium and chorionic trophoblast cells, consistent with apoptosis, such as the condensation of chromatin along the periphery of the nucleus and nuclear shrinkage (Wyllie et al. 1997).

Although we have observed cell death during normal embryonic development, the details of activation, regulation and selection of particular cells for elimination is still under investigation. Several pathways have been explored (Afford, 2000). Our studies focus on the mechanism of cell death, and the role of cell death related genes, such as Cdk5, which is attracting many attentions recently, during cell death. We first discuss cell death pattern and the regulation of these cell death models.

A. Cell Death in Limb Development:

The limb digits derive from an initial foot or hand palette. At day 10.5 of the mouse embryo, the areas of death first arise in the anterior and posterior marginal zones

(AMZ and PMZ) of the bud. As the embryo develops, the interdigital mesenchymal tissue (IMT) also undergoes cell death (Hinchliffe, et al. 1981; Zakeri, et al. 1994). In the areas where cells survive, the cells condense and give rise to digits. In the regions where cells will die, the cells initially separate and then disappear (Saunders, et al. 1967). Cell death in the interdigital area starts at day 12.5, peaks at day 14.5, and is completed at day 15.5 (Zakeri, et al. 1994). Using specific markers, several investigations showed that cell death in the developing digits is apoptotic (Kochar, et al. 1973; Hurle, et al. 1988; Ingham, et al. 1992; Zakeri, et al. 1994). These markers include morphological analysis by light and electron microscopy of condensing nuclei; identification of macrophage infiltration; and an elevation in the lysosomal activity in the phagocytic cells in the interdigital regions. In addition, *in situ* DNA fragmentation analysis showed that DNA was fragmented during cell death of the normal limb (Zakeri, et al. 1994; Ahuja et al. 1997).

The activity of several genes are closely associated with cell death:

BMP genes (Bone Morphogenetic Protein genes) are one of the widely studied gene families associated with limb formation. In developing limbs, BMPs have been implicated in the control of anterior-posterior patterning, outgrowth, chondrogenesis, and apoptosis. The diverse roles of BMPs in limb development are mediated by different BMP receptors (BMPR) (Zhang et al. 2000; Pizette et al. 2000). Transgenic mice that mis-express a constitutively active BMP receptor-1B (BMPR-1B) have the bifurcation in the hind-limb early in the limb development (E10.5) associated with extensive cell death in the mesenchyme and occasionally in the apical ectodermal ridge (AER). This result suggests that BMPR-1B is the endogenous receptor mediates the role of BMPs in

anterior-posterior patterning and apoptosis in the developing mouse limb (Merino et al. 1999; Zhang et al. 2000)

The homeobox-containing gene Msx-2 is expressed in the mesoderm at the anterior margin of the limb bud and in a discrete group of mesodermal cells at the midproximal posterior margin during early stages of chick limb development. Later in development, Msx-2 is expressed in several regions of programmed cell death, including the anterior and posterior zones and the interdigital mesenchyme (Ahulja et al. 1997). Msx-2 impairs limb morphogenesis by reducing cell proliferation and promoting apoptosis in the regions of the posterior mesoderm in which it is ectopically expressed (Ferrari et al. 1998).

Gas2 (growth-arrest-specific 2) is expressed in the interdigital tissues, the chondrogenic regions, and the myogenic regions in the developing day 11.5 to 14.5 mouse embryonic limbs (Schneider et al. 1988). Gas2 is involved in the execution of the apoptotic program in hindlimb interdigital tissues by acting as a substrate for caspase enzymes. TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated biotin-dUTP nick end labeling) analysis demonstrated that the interdigital tissues undergo apoptosis between 13.5 and 15.5 days, and pro-caspase-3 (an enzyme that can process Gas2) is cleaved into its active form in the interdigital tissues. This suggests that Gas2 might be involved in the execution of apoptosis. (Lee et al. 1999)

A number of mutant mice in which limb deformity correlates with cell death have been examined (Johnson, et al. 1969; Hinchliffe, et al. 1974; Ingham, et al. 1992; Zakeri, et al. 1994; Ahuja et al. 1997). For example, Hammertoe mutant mice show webbing between digits 2-5 due to a decreased number of dying cells (Zakeri, et al. 1994). Several

factors may directly or indirectly regulate cell death and rescue this phenotype. For example, retinoic acid (RA) can increase cell death in the interdigital regions of the mouse limb as well as lay down the pattern of limb development (Lammer, et al. 1985; Alles, et al. 1989; Zakeri, et al. 1993). The cell death due to treatment by RA is also apoptotic (Zakeri, et al. 1994; Ahuja et al. 1997). RA can rescue the mutant phenotype by activating cell death in specific regions of the limbs in the Hammertoe mutant mice (Zakeri, et al. 1994). However, neither the effect of RA on cell death nor the defect of cell death in Hammertoe correlates with an altered expression pattern of the pattern forming gene, Msx genes, the RA receptors, or the ability of endogenous RA to bind its receptors. These results suggest that RA may influence pattern formation and cell death through an indirect mechanism (Ahuja et al. 1997).

The developing mouse limb provides an ideal model to study programmed cell death. Although several genes are related to cell death during limb formation, such as BMPs, gas2, and pattern forming Msx genes, the mechanisms of normal cell death in the limb are still unknown. In addition to these genes, we have shown associations of the cell cycle dependent kinase 5 (Cdk5, a cell cycle related gene, see next section) with cell death in the developing limb. The relationship of Cdk5 to the activation and regulation of cell death is the focus of this study.

B. Cell Death in Lens Development:

Developing lens provides another ideal model for studying the mechanism and regulation of programmed cell death.

The development of the mouse eye begins with evagination of the optic vesicle from the forebrain at about day 9 of gestation. It reaches the lateral surface ectoderm about a half day later, and a firm contact forms between the optic vesicle and the surface of ectoderm. At about day 10, the ectodermal cells in this area elongate to form a lens placode. At day 10.5, the lens rudiment invaginates and becomes the lens cup, and the optic vesicle invaginates to form the optic cup. The lens cup changes to a lens vesicle by pinching off from the surface ectoderm at day 11 (Robison, et al. 1977). During lens development, fiber cells differentiate, and undifferentiated epithelial cells acquire the capacity to divide, migrate, and differentiate, depending on their position in the lens (McAvoy, et al. 1978; Steinbach et al. 2002). When the proliferating epithelial cells begin their terminal differentiation, they withdraw from the cell-division cycle and form highly elongated fiber cells that move toward the center of the lens. These differentiating fiber cells lose their nuclei and other cytoplasmic organelles (Kuwabara, et al. 1974), and eventually, they degradated their DNA into small fragments (Appleby, et al. 1977), resembling apoptosis (Wyllie, et al. 1980; Gao et al. 1997; Wride et al. 1998). DNA fragmentation in lens fiber cell nuclei is identified by the TUNEL assay (Wride et al. 1998).

In contrast to classical apoptosis, lens cells remain intact, do not transmit into apoptotic bodies, and are not engulfed by neighboring phagocytes (Pan, et al. 1996). The organelles disappear 2-3 days before DNA fragmentation, while in apoptotic cells, including lens epithelial cells (Li et al. 1995), the organelles are still present after DNA has been degraded. The population of fiber cells in the center of the lens (called primary fiber cells) undergoes organelle elimination in day 3 chickens, when cell elongation

begins, and ends about day 12, when denucleation occurs (Modak, et al. 1970; Sanwal, et al.1986; Bassnett, et al. 1992). Chromatin disappearance is characterized by the same changes as in most apoptotic cells, condensation of chromatin and cleavage into high molecular weight fragments and oligonucleosomes (Counis et al. 1998; Dahm 1999). During the differentiation of the primary fiber cells, successive rounds of differentiation occur at the lens equator, forming new fiber cells (called the secondary fibers) which are laid down as concentric shells surrounding the primary fibers. These shells represent cells in different stages of differentiation arranged in order, where the least mature cells are on the surface (Gao et al. 1995).

As in the developing limbs, the formation of lens fiber is under a genetic control. Wride (1996) highlighted the possible role of TNFα in the loss of the lens cell nucleus and the role of ICE, bax and bcl-2, which are clues known to active and regulate apoptosis (Wu et al. 2002). Using immuno-fluorescence and confocal microscopy, Wride et al. (1996) have associated TNFα immunoreactivity with the lens epithelium and lens fibers. *In vitro* study shows that treatment of TNFα can increase the number of degenerating nuclei. A neutralizing antibody to TNFα significantly reduces the number of TUNEL-positive nuclei. This study suggests a potential role for TNFα-like factors and their receptors in the degeneration of lens fiber cell nuclei (Wride et al. 1998). In transgenic mice, overexpression of bcl-2 in lens was sufficient to interfere with normal lens differentiation. Morphological alterations included cell disorganization as well as inhibition of nucleus degeneration (Fromm et al. 1997; Gupta 2002).

One caspase-3 like member of the caspase family is activated when rodent lens epithelial cells terminally differentiate into enucleate lens fibers *in vivo*. A peptide

inhibitor of this protease blocks the denucleation process in an *in vitro* model of lens fiber differentiation (Ishizaki et al. 1998). In similar studies, other investigations used synthetic peptide inhibitors of the caspases -1, -2, -4, -6, and -9 on epithelia cell cultures. The inhibition reduces nuclei degeneration at about 50-70%. On the other hand, inhibitors of caspases-3 and -8 are not effective in significantly reducing the number of TUNEL-labelled nuclei. Cleavage of the caspase substrates poly (ADP-ribose) polymerase (PARP) and a 45-kDa subunit of DNA fragmentation factor (DFF 45) have also been observed in the developing lens. These results suggest that members of the caspase family play a role in the degeneration of lens fiber cell nuclei (Wride et al. 1999).

Other evidence supports the idea of a biochemical link between apoptosis and loss of the fiber cell nucleus, such as the accumulation of G1 cyclins without initiating DNA synthesis; the expression of cyclin B/p34^{cdc2} complexes and p34^{cdc2} in fiber cells (Gao, et al.'1995; Zelenka et al. 1996; Bassnett et al. 1997; Gao et al. 1997); the induction of apoptosis in lenses from p53-/- mice (Pan et al. 1995).

Since lens has a different type of cell death from that of limb, it would be of great interest to investigate if similar genes, such as cell cycle related genes (eg. Cdks) play a role in both type of cell death.

C. Cell Death in Cyclophosphamide (CP) Treated Developing Embryos

Cyclophosphamide (CP), an alkylating agent, is commonly used in the treatment of cancer and of progressive autoimmune diseases (Fotiou et al. 2000; Janssen et al. 2000). CP disrupts normal embryonic development due to the induction of excessive cell

death and thus leads to many birth defects in animals and humans. Exposure to CP during organogenesis in mice and rats leads to malformations including cleft palate, encephaly, open eyes, kinky tail, oligodactyly, polydactyly, syndactyly, adactyly, phocomelia, and defects in skeletal ossification (Gibson and Becker, 1968; Hales, 1981; Nomura et al., 1996). CP has been frequently studied as a model teratogen (Mirkes et al. 1985).

CP is activated by cytochrome p-450 mono-oxygenase in the liver to yield 4-hydroxycyclophosphamide, which then spontaneously decomposes to phosphoramide mustard and acrolein in target cells (Foley et al., 1961; Hales, 1982; Mirkes, 1985; Slott and Hales, 1988). Acrolein binds preferentially to proteins, thus limiting its cytotoxicity, but phosphoramide mustard exerts most cytotoxic effects by covalently binding to DNA, predominantly in the S phase of the cell cycle, and causing DNA interstrand cross-links (Ojwang et al., 1989; Little and Mirkes, 1990, 1992). At higher concentrations, phosphoramide mustard may break DNA strands as a result of the destabilization of purine bonds with consequent loss of purine bases (Verly, 1974; Colvin, 1993; Pette et. al., 1995). The chromosome damaging effects of CP lead to cell death during organogenesis of murine embryos (Chen et al., 1994; Moallem and Hales, 1995).

The cell death induced by CP is considered to be apoptosis, with concurrent induction of DNA fragmentation, activation of caspase-3 and cleavage of PARP (Trasler et al., 1986; Francis et al., 1990; Hales et al., 1992; Piccart et al. 2000). CP-induced cell death may depend on p53 (Moallem et al. 1998). CP-treated embryonic mouse limbs from p53-knockout mice have much less apoptotic cell death than those of wild type mice. *In vitro,* when cells are treated with CP, they become very sensitive to anti-Fas-

induced apoptosis. This suggests that CP may kill cells by a Fas dependent pathway (Lee et al. 1997; Timmer et al. 2002).

In sum, CP treated embryos undergo massive cell death in an *in vivo* environment. This cell death has been shown in our lab to be apoptosis. The CP treated embryos represents a good *in vivo* model for the study of cell death mechanisms and the role of cell cycle related genes in cell death.

Chapter 3. Regulation of cell death

A. Cell Death Signals and Related Genes

- **a. The bcl-2 Family**
- **b. The caspase Family**
- **c. Cyclin-Dependent Protein Kinases**

B. Cdk5 and Its Activator p35/p25

- **a. The structure of Cdk5**
- **b. The function and expression of Cdk5**
- **c. Cdk5 associated factors**
- **d. p35/p25, one of the regulators of Cdk5**
- **e. Substrates of Cdk5**

A. Cell Death Signals and Related Genes:

Cell decisions to initiate apoptotic programs may be made following the cessation of survival signals that negatively regulate apoptosis, or inducers of cell death through so-called "death receptors" (Ruoslahti et al. 1994; Matsumura et al. 2000).

Cell death initiated by receptors

One of the best-characterized apoptotic signaling pathways involves the binding of proteins, such as, Fas ligand (FasL) to their cognate cell surface receptors(Magnusson et al. 1999; Marsters et al. 1999; Wang et al. 1999; Sperandio et al. 2000; Wyllie et al. 2001). FasL is a 40Kd cell surface protein that induces apoptosis by binding to Fas. Fas is a glycosylated 45 kD transmembrane receptor that belongs to the TNF/nerve growth factor (NGF) receptor family (Wallach et al. 1996; Gupta 2002; Timmer et al. 2002). Following the binding of FasL, Fas oligomerizes and recruits of adapt protein, Fas-associated death domain protein (FADD), to form death-inducing signaling complexes (DISCs). DISCs activate the caspase pathways, resulting in the activation of cell death (Nagata et al. 1997; Krammer et al. 2000; Budd 2002).

The mitochondrial pathway

This cell death pathway is activated in response to extracellular cues and internal insults such as DNA damage. ATM (ataxia telangiectasia mutated), a checkpoint activator, is very sensitive to the presence of free double-stranded DNA (Rotman et al. 1998). The activated ATM leads to p53 phosphorylation, which alters its conformation and greatly increases its stability (Sionov et al. 1999). Stabilized p53 can increase the concentration of BAX (which will be discussed later), while diminishing the level of Bcl-2. This process favors the disruption of the mitochondrial membranes and the ion

potential of mitochondrial cell membrane (Review from Rich et al. 2000; Hengartner 2000; Wu et al. 2002).

Although there are gaps in the knowledge of the mechanisms of cell death signal transduction remain, it seems certain that most of these pathways involve the sequential activation of several apoptosis related genes, such as those of the bcl-2 family and the caspase family. And then, we will illustrate another gene family, Cyclin-dependent kinases, which regulates cell cycle, as well as cell death.

a. The Bcl-2 Family:

Bcl-2 (B-cell leukaemia/lymphoma-2) was initially described as a proto-oncogene located at the chromosomal break point of t (14:18) in B-cell lymphomas (Bakhshi et.al. 1985; Neuez et al. 1990; Silvemma et al. 1991; Tsijimate et al. 1995). It is expressed in a number of proliferating and differentiating cells in cultured cell lines and during development (Hockenbery, 1991; LeBrun, 1993). Most members of the family have been associated with apoptosis.

Structural and functional analysis of the Bcl-2 family revealed genes include both apoptosis-inhibiting (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, Nrl3, and Al/ Bfl-1) and apoptosis-promoting (Bax, Bak, Bok, Diva, Bcl-Xs., Bik, Bim, Hrk, Nip3, Nix, Bad, and Bid) members (Adams et al.1998; Reed et al.1998; Wu et al. 2002). Bcl-2 family members share modular homology. Sequence comparisons have revealed up to four conserved areas in the molecules, the so-called BH (Bcl-2 homology) domains: BHI, BH2, BH3, and BH4. Among pro-apoptotic members of the Bcl-2 family, the BH3 domain is critical for both dimerization with other members and for induction of apoptosis (Kelekar et al. 1998;

Desagher et al. 1999).

The predominant mode of action of Bcl-2 and Bcl-X_L appears to be mitochondrial. In most cases, Bcl-2 and Bcl- X_L seem to act by preventing mitochondrial permeabilization and the release of cytochrome c (Kluck et al. 1997; Yang et al. 1997). The mitochondrial actions of Bcl-2, Bcl- X_L, and Bax depend on their ability to form ion channels (Antonsson et al. 1997; Schlesinger et al. 1997; Schendel et al. 1998; Wu et al. 2002). Bax and Bid can initiate cell killing by forming pores in the outer membranes of mitochondria, thereby causing release of cytochrome c into the cytosol (Saikumar et al. 1998; Shimizu et al. 1999). Bax can permeabilize pure lipid membranes at low concentrations. Both Bcl-2 and Bcl-X_L protect against Bax-induced cytochrome c leaks from mitochondria, but only Bcl-2 can interact directly with Bax (Basanez et al.1999).

Most members of the Bcl-2 family possess carboxy-terminal hydrophobic membrane anchoring domains that allow them to localize in the outer membranes of mitochondria (Bossy-Wetzel et al. 1998; Priault et al. 1999). Members that lack these domains (such as Bid) and that are cytosolic could still migrate to mitochondria by virtue of their ability to associate and form heterodimers with other Bcl-2 family members (Shimizu et al. 1999; Priault et al. 1999; Basabez et al. 1999). These heteromeric associations and translocations are regulated by phosphorylation. Phosphorylation inhibits Bid association with Bcl- X_L and Bcl-2 (Wang et al. 1999;Saikumar et al. 1998; Finucane et al. 1999; Desagher et al. 1999).

b. The Caspase Family:

Caspases are one family of cysteine proteases involved in various cell death

pathways. Currently, there are 14 members of this family (Miura et al. 1993; Alnemri et al. 1996; Song et al. 2000). Caspases share homology with CED-3 ("CED" stands for "cell death abnormal") and generally are synthesized as inactive precursors. Based on substrate specificity, caspases are grouped into three categories (Thornberry et al. 1997). Members of group I have a substrate preference for the WEHD amino acid sequence with high promiscuity (caspase-1, -4, and -5); group II members prefer the DEXD sequence (caspase-2, -3, -7, and CED-3); and group III members recognize (I/L/V) EXD sequences (caspase-6, -8, -9). Group I caspases are primarily involved in inflammation, whereas group II and group III caspases are important for the execution and signaling events of apoptosis (Alnemri et al. 1996; Song et al. 2000).

Genetic studies involving the knockout of several caspase genes have revealed the roles of these caspases in apoptotic pathways as well as their relative importance for animal development. For example, functional caspase-3 is essential for the formation of apoptotic bodies, chromatin condensation, and DNA fragmentation in most cell types (Janicke et al. 1996; Kuida et al. 1996; Woo et al. 1998; Song et al. 2000). Caspase-8 is required for cell death during mammalian development; some studies have reported that caspase-8 may be required for FasL or TNF-A induced JNK activation (Juo et al. 1998; Varfolomeev et al. 1998; Chaudhary et al. 1999; Song et al. 2000; Timmer et al. 2002). Caspase 9 has a key role in apoptosis induced by intracellular activators, particularly those that cause DNA damage (Hakem et al. 1998; Kuida et al. 1998; Chautan et al. 1999; Sun et al. 1999; Shi 2002). In caspase-9 deficient cells, caspase-3 was not activated, suggesting that caspase-9 is upstream of caspase-3 in the apoptotic cascade. As a consequence, caspase-9 deficient cells are resistant to dexamethasone or irradiation,

while they retain their sensitivity to TNF-α (Hakem et al. 1998; Kuida et al. 1998). In this latter case, sensitivity can be explained by the fact that caspase-9 deficient cells still retain caspase-8, the initiator caspase involved in death receptor signaling that can also activate caspase-3 (Hakem et al. 1998; Kuida et al. 1998). In sum, these observations support the idea that different death-signaling pathways converge on downstream effective caspases (Sun et al. 1999).

c. Cyclin-Dependent Protein Kinases:

In addition to specific genes that regulate cell death, a number of genes have been found directly or indirectly regulate cell death. Some cell cycle genes have been illustrated in the regulation of apoptosis. The cell cycle consists of four phases: G1, S, G_2, and M. Cell cycle progression is a conserved mechanism that is controlled at several transition points. Some of these transition points reflect the action of extracellular growth factors and hormones and serve to integrate cell proliferation with cell growth, differentiation, and apoptosis (Muller, et al. 1993; Murray, et al. 1993; Hind, et al. 1994; James 1999).

Cyclin-dependent kinases (Cdks) are proteins that control the transition between successive phases of the cell cycle in all eukaryotic cells (Elledge, et al. 1991; Koff, et al. 1991; Meyerson, et al. 1992; Norbury, et al. 1992; Reed, et al. 1992; Murray, et al. 1993; Nasmyth, et al. 1993; Pine, et al. 1993; Sherr, et al. 1993; 1994; King, et al. 1994). This family consists of at least 15 Cdks (Morgan 1997; Homayouni et al 2000; Gupta et al. 2002). Activation of most Cdks requires the interaction with another group of proteins called cyclins. Cyclins are synthesized at specific stages of the cell cycle in response to

mitogenic stimuli and to certain cytokines (James 1999). For example, Cdk2 interacts with cyclin E at the beginning of S phase to induce the initiation of DNA synthesis, and then binds to cyclin A through S phase. The Cdk1-cyclin B complex (Morgan 1997) then initiates mitosis.

The Cdk family members have similar sizes (about 35-40kD) and show approximately 74% homology to each other (Morgan, et al. 1995). They are related in structure and function to the yeast cell division control kinase, Cdc2 (Cdk1), and allow eukaryotic cells to progress through DNA replication and cell division (Elledge et al. 1991; Koff et al. 1991; Meyerson et al. 1992). Some of the Cdks have a well-established role in regulating the transition between successive phases of the eukaryotic cell cycle (Norbury et al 1992; Reed et al 1992; Murray et al 1993; Pines et al 1993; Nurse et al 1994; Nigg, et al. 1995; James 1999), while others may be involved in apoptosis or differentiation (Lee et al. 2001).

Apoptotic stimuli cause an increase in the activity of certain cyclin-Cdk complexes. For example: Premature activation of p34cdc2 was shown to be required for apoptosis induced by a lymphocyte granule protease (Shi et al. 1994). Moreover, experiments with synthetic inhibitors of Cdks have shown that some other Cdks are essential for apoptosis triggered by a diverse range of death-inducers, including DNA damage, glucocorticoids, and ionomycin. For example, inhibition of Cdk2 completely protected thymocytes from apoptosis, mitochondrial changes, and caspase activation (Shi et al 1994; Hakem et al. 1999).

Many links between cell death and cell cycle control have been discovered, more and more reports have indicated that the cyclins and Cdks can be central effectors of

apoptosis. However, the specific mechanism of the association between apoptosis and Cdks is presently unclear. Among the genes that can regulate apoptosis, recent reports suggest that Cdk5 and its regulator, p25/p35, are involved in apoptosis by disrupting the cell cytoskeleton (Patrick et al.1999; Homayouni et al. 2000; Lee et al. 2000). Our laboratory has obtained results that are consistent with these reports.

B. CDK5 and its activator p35/p25:

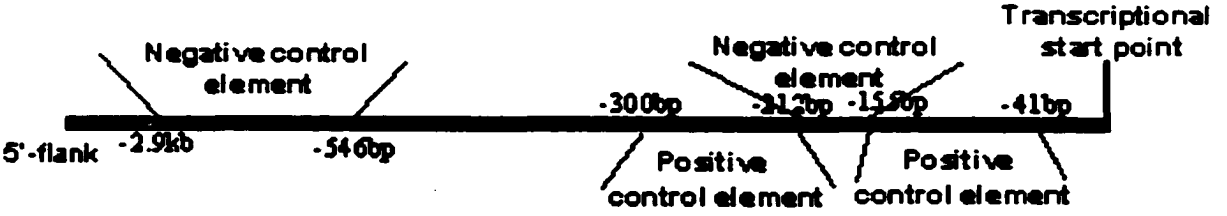
a. The finding of Cdk5

Cdk5 was originally identified from bovine brain extract. It is homologous to Cdc2 kinase (Lew et.al. 1992; Meyerson, et al. 1992), a key regulator of cell-cycle progression (Hunt, et al. 1989; Murray, et al. 1989; Draetta, et al. 1990; Nurse, et al. 1990; Pine, et al. 1993). It was also isolated as a Tau protein kinase since it phosphorylates Tau *in vitro*, a brain microtubule-associated protein (Ishiguro et. al. 1992; Dhavan et al. 2001).

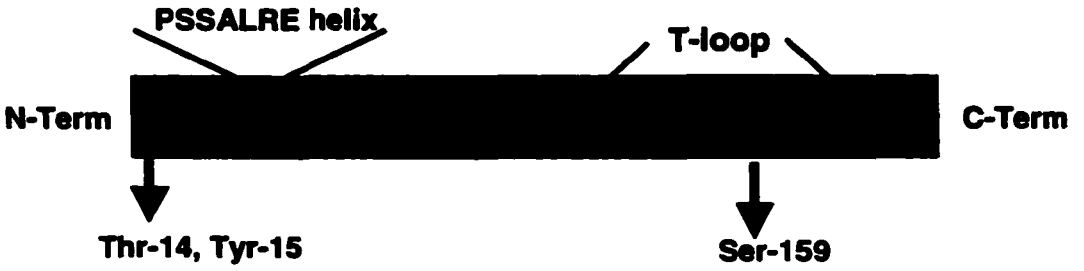
Chromosomal loci mapping using fluorescence *in situ* hybridization on human lymphocytes located Cdk5 to 7q36 (Demetrick et. al. 1994). Among vertebrates, Cdk5 has high homology to Cdc2 (58%) and Cdk2 (59%) from sequence analysis (Lee et.al. 1997), with over 99% homology at the protein level (Hellmich, et al. 1992; Lew, et al. 1992). Among different species, Cdk5 also has high homology (Tarricone et al. 2001; Smith et al. 2002). Both Tyr-15 and Thr-14 are conserved in Cdk5, and the amino acid residue of Cdk5 corresponding to Thr-161 of Cdc2 is conservatively substituted by a serine residue. This conservation suggests that Cdk5 is potentially regulated by a similar

kinase-phosphatase network. However, sequence alignment of Cdk5, Cdc2, and Cdk2, as well as Cdk3, reveals a few notable sequence divergences in Cdk5. For instance, the sequence, "EGVPSTAREIS," which is highly conserved in Cdc2, Cdk2, and Cdk3, is not in Cdk5, suggesting that Cdk5 may have some different functions; and these divergent sequences contribute to the enzymological and functional uniqueness of Cdk5 kinase (Lee et al. 2001).

The regulation of transcription of Cdk5 is under complex control. The 5'-region of the mouse Cdk5 gene was isolated and sequenced. The 5'-flanking region has a high G+C content. There is no TATA box around the transcriptional start points. One CCAAT box, one AP-1-binding site, two AP-2-binding sites, and one c-AMP-responsive element are located upstream from the transcriptional start points. The deletion of different region of promoter shows that two negative control elements are located from –2.9kb to –546bp, and from –212bp to –155bp. Two positive elements from –300bp to –212bp, and from –155 to –41 are also detected. In sum, the Cdk5 promoter region contains multiple positive and negative cis-acting regulatory elements. The Cdk5 promoter region is as follow:



Structure analysis of Cdk5 protein with its regulator (p25) shows that p25 can interact with Cdk5 at PSSALRE helix, which is equivalent to PSTAIRE helix in Cdk2. This interaction can stabilize T-loop of Cdk5, which includes its active site, Ser-159 (Tarricone et al. 2001). The Cdk5 structure is as follow:



b. The expression and activity of Cdk5

RNA analysis has shown that Cdk5 mRNA is expressed at high levels in all cultured proliferating cells examined (Tsai et al. 1993), and ubiquitously expressed in mammalian tissues (Damu et.al. 1996). The highest levels are detected in the brain and testis. Lower levels are detected in ovary and kidney (Meyerson et al. 1992; Tsai, et al. 1993; Ino et al. 1994; Lazaro et al. 1997 Smith et al. 2002).

Cdk5 expression and activity in different tissues

Initially, Cdk5 funtion was thought to be restricted to the nervous system because its kinas activity was only correlated with the proliferation and differentiation of neurons in the developing mouse neocortex (Ino, et al. 1991; Lew et al. 1992; Tsai, et al. 1993; Lazaro, et al.1997, Dhavan and Tsai, 2002). Reduction of Cdk5 kinase activity by expression of dominant-negative Cdk5 mutant inhibits neurite outgrowth during its neuronal differentiation (Nikolic et al. 1996; Xiong et al. 1997). Suppression of Cdk5 in cerebellar macroneurons results in reduced axonal elongation, further indicating that Cdk5 has a stimulatory role in axonal extension (Pigino et al. 1997; Paglini et al. 1998).

Recently, several groups have demonstrated that active Cdk5 is presented in many non-neuronal systems. For example: During the development of the rat lens, Cdk5 is expressed in lens epithelial cells and in differentiation lens fibers. The immunicipitation

of Cdk5 with its activator (p35) indicates that Cdk5 might have a role in the differentiation of lens fiber cells (Gao et al. 1997). In differentiate human leukaemia HL60 haematopoietic cells, active Cdk5 was also detected, the activated Cdk5 induces expression of CD14 and other markers of differentiation (Chen et al. 2001; Smith et al. 2002). A low level of active Cdk5 can also be detected in adult mouse prostate and embryonic limb buds, as well as Leydig cells, Sertoli cells, spermatogonia and peritubular cells of the developing rat testis, which links Cdk5 function to regulation of cytoskeleton, axon guidance, membrance transport, synaptic function, dopamine signaling and drug addition (Zhang et al. 1997; Musa et al. 1998; 2000; Session et al 2001).

All the studies of Cdk5 in neuron and non-neuron cells indicate that the role of this kinase extends to more developmental processes than was originally suspected.

Cdk5 in cell death

Recently, others and we have reported that Cdk5 is upregulated in apoptotic mouse embryonic tissue, as well as adult tissues (Ahuja et al, 1997; Zhang et al, 1997; Lee et al, 2000; Alvarez et al. 2001; Gao et al. 2001; Maccioni et al. 2001). It has also been seen to be upregulated in chicken neurons (Patrick et al, 1999) and in induced cell death in an *in vitro* model of human kidney cells (Saito et al. 2001; Gupta et al. 2002).

Cdk5 protein is upregulated when dopaminergic substantia nigra neurons die during normal development or are killed by quinolinic acid (Henchcliffe et al, 1998; Catamia et al. 2001). In apoptosis induced by oxidative stress in chick post-mitotic sympathetic neurons, the induction of Cdk5 protein coincides with the time when neurons are irreversibly committed to die, while, other cell cycle mediators such as cyclin D1,

Cdc2, and Cdk2 are undetected and not induced by exposure to oxidative stress (Shirvan et al. 1998). Cdk5 protein levels are increased in HCSMA (hereditary canine spinal muscular atrophy) dogs, where degeneration is confined to motor neurons with an accumulation of phosphorylated neurofilaments in axonal internodes (Henchcliffe et al, 1998).

Cdk5 phosphorylates neurofilaments and regulates neurofilament dynamics. It is strongly expressed in nuclei, cytoplasm, and axons where neurofilaments have accumulated. Active Cdk5 protein also accumulated (20-40X increases) in neuronal tangles of Alzheimer patients' brains, and in amyotrophic lateral sclerosis (ALS) (Patrick et al, 1999; Lee et al 2000; Smith et al. 2002). These findings suggest a novel role for Cdk5 during neuron apoptosis.

In sum, Cdk5 is mainly expressed in neuron cells, however, more and more studies have indicated that active Cdk5 is also expressed in other tissues, such as developing lens, adult mouse prostate and embryonic limb buds limbs. It would be reasonable to surmise that under different conditions, Cdk5 has different functions, such as the cytoarchitecture of the CNS, differentiation of lens fiber cells, and apoptosis in adult mouse prostate and embryonic limb buds (Dhavan et al. 2001). Overall, it might be closely related with cell differentiation and cell death. This possibility needs to be further examined.

c. Cdk5 associated factors

To function, most Cdks must associate with proteins called cyclins, which are synthesized at specific stages of the cell cycle in response to mitogenic stimuli and certain cytokines (James 1999). Like other Cdks, Ckd5 associates with several factors.

Several regulatory factors of Cdk5 have been identified, including: p23 (Ishiguro, et al. 1994; Uchida, et al. 1994), cyclin E (Donnellan et al. 1999), p35 (Tsai, et al. 1994; Nikolic, et al. 1996; Harada et al. 2001), p25 (Ishiguro et al.1994; Lew, et al. 1994; Uchida et al.1994; Patrick et al.1999; Lee et al. 2000), p39 (Tang et al.1995; Ko et al. 2001), and p67 (Shetty, et al. 1995; Rajgopal et al. 2001). Among them, p35/Cdk5 and p25/Cdk5 complex appear to be associated with cell differentiation and cell death, while the functions of other factors remain unclear. Each of these associated factors will be discussed in the following:

p23

p23 has a partial amino acids similarity to a cyclin box important for the interaction of cyclin with Cdc2-related kinase. p23 is cleaved from the processing of the precursor protein, pre-p23. The precursor mRNA is expressed most abundantly in rat brain just before and after birth (Uchida et.al. 1994). By immunoprecipitation, p23 has been identified as a Cdk5 associated protein. The complex of p23 and Cdk5 is the Tau protein kinase II, which phosphorylates Tau protein (Lew, et al. 1992; Paudel, et al. 1993; Miyajima, et al. 1995), and may play a role in regulation of cytoskeleton dynamics.

CyclinE

Cyclin E/Cdk5 complex is also found in the nervous system. Two forms of cyclin E in the mouse nervous system have been identified, with a 56kD form mainly expressed in neurons, and a 51kD form expressed in astrocytes and oligodendrocytes (Miyajima et.al. 1995). Because cyclin E interacts with Cdk2 during the initiation of DNA synthesis during cell cycle, it is possible that Cdk5 and cyclin E complex also interact for the control of the cell cycle (Donnellan et al. 1999).

p39

p39 was cloned from rat brain as a Cdk5 activator by immunoprecipitation. In adults, the mRNA and protein expression all decreased, except in Purkinje and granule cells in the cerebellum. Northern blot analysis showed that p39 expression was low in 15-day old fetuses, and was most prominent in 1-3 week old rat brains. Tang et al. (1995) and Ko et al. (2001) found by immunohistochemical analysis that p39 expression is enriched in the hippocampus. These findings suggest that p39 functions during different states of rat brain development (Zheng et.al. 1998; Teruyuki et al. 2001; Gupta et al. 2002). However, the specific role remains undetermined.

p67

p67 (Munc-18) also increases Cdk5 kinase activity *in vivo*, and copurifies with Cdk5 from rat spinal cord homogenates. p67 mRNA and protein are expressed in E18 rat brain neural tissue, especially highly enriched in axons. Therefore, Rajgopal et al. suggest that p67/Cdk5 complex may participate in the phosphorylation of cytoskeletal proteins (Shetty et.al. 1995; Rajgopal et al. 2001).

L34 and dbpA

Moorthamer et al. (1999a) found that the human 60S ribosomal protein L34 interacts with Cdk5 in Hela cells, by using a yeast-two-hybrid system to pull out Cdk5 associated protein. Biochemical analyses reveal that L34 does not activate Cdk5 but is a potential inhibitor of the p35-activated kinase. Thus Cdk5 may play a role in translational regulation (Moorthamer et.al. 1999a) in cells.

Using a similar method, Moorthamer et al. (1999b) also demonstrated that the DNA binding protein, dbpA, can bind to Cdk5. This reflects apparent cross-talk between

the distinct signal transduction pathway controlled by dbpA on the one hand and Cdk5 on the other.

d. p35/p25, the widely studied regulators of Cdk5

Most studies indicated that p35 is a potent activator of Cdk5. p35 displays a neuronal cell-specific pattern of expression and physically associates with Cdk5 *in vivo* and activates the Cdk5 kinase (Tsai, et al. 1994; Floyd et al. 2001). Despite its regulatory role for Cdk5, p35 does not exhibit homology with any known cyclins that act as regulators of other Cdk family members (Tsai, et al. 1996). p35 appears to have unique specificity for Cdk5 as it does not activate other known members of the Cdk family (Tsai, et al. 1994; Neystat et al. 2001). The Cdk5/p35 kinase activity increases during central nervous system (CNS) neurogenesis, suggesting that this interaction plays a role in neuronal differentiation and neurite outgrowth. Mutants of Cdk5 or p35 display inhibited neurite outgrowth (Tsai, et al. 1996; Ohshima et al. 2001; Smith et al. 2002).

Cdk5/p35 may serve as an important regulatory linker between environmental signals (e.g. laminin) and constituents of the intracellular machinery (e.g. MAP1B) involved in axonal elongation. Laminin is an extracellular matrix molecule capable of selectively stimulating axonal extension and promoting MAP1B phosphorylation at a proline-directed protein kinase epitope. Laminin selectively stimulates p35 expression. It also increases the association of p35 with the subcortical cytoskeleton and accelerates its redistribution to the axonal growth cones. Suppression of p35, but not of a highly related isoform (designated as p39) reduces Cdk5 activity, laminin-enhanced axonal elongation, and MAP1B phosphorylation (Chae et.al. 1997; Paglini et al. 1998; Ohshima et al. 2001).

p35 knock-out mice display severe cortical lamination defects and suffer from seizures and sporadic adult lethality. The mutant mice lack the characteristic laminated structure of the cortex. The phenotype of p35 mutant mice thus demonstrates that the formation of cortical laminar structure depends on the action of the p35/Cdk5 kinase (Chae et al. 1997; Paglini et al. 1998).

p35 may be truncated to p25, the C-terminal fragment, by an activated calpain protease (Kusakawa et al.2000; Lee et al.2000; Nath et al.2000; Zhang et al.2000). p25, like p35, correlates with the expression of Cdk5 in developmental cell death (Zhang et al, 1997) and induced cell death in COS-7 cells. p25 accumulates in the brains of patients with Alzheimer's disease (Patrick et al. 1999; Gupta et al. 2002). The accumulation of Cdk5/p25 complex can cause neurofilament hyperphosphorylation and disrupt cytoskeleton. Cdk5/p25 can form Tau protein kinase II, which phosphorylates Tau protein, destabilizes the cytoskeleton, and leads to death (Lew et al. 1992; Ishiguro et al. 1994; Lew et al. 1994; Kusakawa et al. 2000; Lee et al. 2000; Kerokoski et al.2001). Patrick et al (1999) suggest that the translocation of the complex from the cell membrane to cell soma in neurons leads to the phosphorylation of Tau, destabilization of the cytoskeleton, and the death of neurons. Sobue et al. (2000) found that Cdk5 binds to Tau and Tau anchors Cdk5 to microtubules.

Most of the initial studies of the function and regulation of Cdk5 and its regulator were focused on understanding the role of Cdk5 during cell differentiation. Recently, several results, including data from our lab, have suggested that Cdk5 plays a role during cell death. It is essential to investigate whether or not Cdk5 is also involved in cell death.

e. Substrates of activated Cdk5

Cdk5 has several known substrates. The best substrate found to date is a synthetic peptide derived from the Cdc2 kinase phosphorylation site of histone H1, P-A-T-P-K-K-A-K-K. A number of neuronal proteins are phosphorylated by purified Cdk5. Among them are neurofilament proteins, and the neuron-specific microtubule-associated protein, Tau (Ishiguro et.al. 1992; Lew et al. 1992; Paudel et al. 1993). All of them were first purified from bovine brain through affinitylabeling using an ATP analog on SDS-PAGE gel, or immuno-blotting analyses using peptide antibodies specific for Cdk5 protein.

Neurofilaments

Neurofilaments are composed mainly of three subunits known as high (NF-H), middle (NF-M), and low (NF-L) molecular weight neurofilament proteins. Neurofilament proteins are synthesized and assembled into neurofilaments in the cell body and then transported down the axon. During axonal transport, NF-H and NF-M become heavily phosphorylated on serine and threonine residues in the carboxyl-terminal regions (Jones et al. 1982; Julien et al. 1983; Carden et al. 1985). Protein phosphorylation is important in the modulation of axonal transport and in the interaction of neurofilaments with other cytoskeletal elements. The Cdk5/p25 complex has a high V_{max} as a kinase than the Cdk5/p35 complex. The accumulation of Cdk5/p25 is associated with the hyperphosphorylation of neurofilaments and disruption of cytoskeleton (Patrick et al. 1999), which leads to apoptosis.

Tau protein

Tau, the other known protein substrate of Cdk5, exists in the adult human brain (Goedert et al. 1991). This protein possesses *in vitro* microtubule assembly promoting activity. Tau proteins are the substrates of many protein kinases. The ability of Tau proteins to promote microtubule assembly is dependent on the phosphorylation states of the protein. Purified bovine Tau proteins are phosphorylated by Cdk5 at seven sites (Paudel et al. 1993). Cdk5/p25, however, can hyperphosphorylate Tau and prevent its binding to cytoskeleton (Patrick et al.1999). The phosphorylation of Tau proteins by Cdk5 probably represents an important mechanism in the regulation of Tau function in CNS neurons (Hosoi et al. 1995).

Overall, all Cdk5 substrates are associated with cytoskeleton, and this indicates that Cdk5 may affect cell differentiation and cell death through phosphoration of cytoskeletal proteins.

PART II. RESULTS AND DISCUSSION

Chapter I: Association of Cdk5 with both normal and induced cell death

1. **Objective**

2. **Material and Methods**

3. **Results**
   - **A. Analysis of the association of Cdk5 with cell death in developing mouse limb**
   - **B. Analysis of the association of Cdk5 with cell death in developing rat lens**
   - **C. Analysis of the association of Cdk5 with cell death in induced model of cell death**
   - **D. Analysis of the association of Cdk5 with cell death in induced COS-7 cell death**

4. **Discussion: Cdk5 expression is upregulated during apoptosis, this upregulation is at post-translational level.**

Results and discussion:

Chapter I: Association of Cdk5 with both normal and induced cell death

1. Objective

In the past three years, several reports have identified the activation of cyclin-dependent kinase (Cdk) 5 in apoptosis, but the mechanisms are unclear (Lahti et al.1995; Patrick et al.1999; Lee et al.2000; Ahlijanian et al.2000; Gupta et al. 2002; Smith et al. 2002). Previous data from our lab have showed that the expression of Cyclin-dependent kinase 5 (Cdk5) is upregulated in the inter-digital areas of developing limb, where cell death has occurred (Zhang et al.1997). However, it is not clear what is the relation between Cdk5 expression and apoptosis; how Cdk5 is regulated during apoptosis in the developing limb; whether or not this correlation is unique in limb development or common in apoptosis in other systems.

Therefore, I used several cell death models, including: two models of normal development (developing mouse limbs and rat lens), one model of abnormal development (cyclophosphamide-treated mouse embryo), and one model of *in vitro* induced cell death (COS-7 cell line) to investigate the expression of the Cdk5 at mRNA level, protein level, and kinase activity level during cell death in these models.

2. Materials and Methods

a. Materials used in the experiments:

Animals

Swiss Webster mice from Jackson Laboratories (Bar Harbor, ME) were mated overnight and females were checked for the presence of a vaginal plug. The time of plug detection was designated gestational day 0.5. At different days of gestation, the mice were sacrificed by cervical dislocation, embryos were removed from pregnant female embryos or embryonic limbs were excised in cold 1X phosphate buffered saline (PBS, 0.85% NaCl, 0.02% KCl; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The whole embryos or limbs were either frozen in liquid nitrogen for RNA isolation or fixed in 4% paraformaldehyde for frozen sections.

CP (Cyclophosphamide) treatment of mouse embryo

Swiss Webster mice were mated overnight, and females were checked for a vaginal plug. Positive females were designated as gestational day 0.5. Females of gestational day 9.5 were treated with 10mg/kg bodyweight of CP (Cyclophosphamide) (Sigma, St. Louis, MO) in 0.7% saline by intra-peritoneal injections (Francis et. al., 1990). The treated mice were sacrificed by cervical dislocation at 24 hr after injection, and the embryos were removed. Embryos were washed with 1X Phosphate-buffered saline (PBS, 0.85% NaCl, 0.02% KCl, 10 mM Na2HPO4, 1.8mM KH2PO4, pH 7.4) and fixed in 4% paraformaldehyde by shaking at 4^0C for 18-24hr.

COS-7 cell culture and treatment

COS7 cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM (GIBCO BRL Inc.) supplemented with 10% fetal calf serum,

50U/ml penicillin and 100ug/ml streptomycin. The cultures were maintained in a humidified atmosphere in 95% air and 5% CO_2 at 37^{0}C. The cells were cultured to 80% coverage. In order to induce cell death and collect cells under different condition, cells were treated with Ceramide (CER) (10uM); Cycloheximide (CHX) (100ug/ml); or Camptothecin (CPT) (15uM); and Ethanol (5% v/v) for 8 hours (for immunoprecipitation and western studies) or 18 hours (for cell count). Cells were collected in lysis buffer (50mM Tris, pH7.5, 150mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.15% SDS, 0.1mM sodium vanadate, 1mM DTT, 20mM β–glycerophosphate, 2mM EDTA, 1X protease cocktail tablet (Roche Inc.). Sample can be saved at -20^{0}C for further study.

Slide preparation

Slides were placed in autoclaved metal racks and dipped in acetone for 5 minutes. Slides were allowed to dry for 2 hours in a hood, dipped in 350ml VECTORBOND reagent (Vector Laboratories, Burlingame, CA) for 5 minutes. Racks of slides were moved for drying, and gently agitated or tapped, and air-dried thoroughly at room temperature.

Tissue fixation and preparation for frozen sections

The embryos or limbs were fixed overnight in 4% paraformaldehyde (Fisher). Paraformaldehyde was prepared fresh and used the same day. After overnight fixation, tissues were placed in 20% sucrose in 1X PBS overnight (filtered and stored at 4°C), embedded in OCT (Tissue Tek; Miles Inc.) embedding compound, and frozen in isopropanol/liquid N_2. Special care was used in the orientation of the mouse embryo or limb samples during the embedding procedure to make sure the cutting section represents a good map of the tissue. Frozen sections (5μm) were cut under –20^{0}C and placed on VECTORBOND coated slides and stored at -70°C prior to use.

Probes and antibodies

***In situ* probe:** Cdk5 constructs were gifts from Dr. Zelenka at the National Institutes of Health. It is a 270bp insert at BamHI (for anti-sense probe) and NotI (for sense probe).

Antibodies: Anti-Cdk2 (M-2), anti-Cdk3 (Y-20), anti-Cdk-4 (H-303), anti-Cdk5 (C-8), anti-Cdk5 (N-20),anti-Cdk6 (C-21) are from Santa Cruz Inc. Anti-Cdc2 (Cdk1) is from Upstate Biotechnology Inc. Anti-Cdk7, or anti-Cdk8 are gifts from Dr. Zalenka (NIH).

b. Cell biology technologies:

Cell transfection:

COS-7 cells are cultured in optimized medium (DMEM medium with 10% fetal bovine serum, 1X L-glutamine, and 1X antibiotic) to 80% coverage on a 10cm plate. Wash cells by DMEM medium only twice, then, add 3.2ml DMEM medium on the plate.

Dissolve 5ug plasmid DNA in 400ul DMEM medium, dissolve 25ul Lipofectamine (GIBCO BRL Life Technology Co.) in 400ul DMEM medium. Gently mix two solutions, incubate at 25^{0}C for 45 min. and add onto plate with 3.2ml DMEM medium. Shake gently to mix, and incubate at 37^{0}C overnight. After an overnight incubation, change medium to growth medium (DMEM with 10% FBS, 1X L-glutamine) to let cells recover overnight. Change to minimum medium for further treatment as mentioned above.

Trypan Blue staining to characterize the percentage of cell death:

Cells are washed with 1XPBS, and resuspended in 1XPBS. Take 30ul of cells and mix with equal volume of 0.4% Trypan Blue (GIBCO BRL Inc.). Incubate at 25^{0}C for 5min. Count cells under the microscope. White cells show cells are alive, while blue cells show cells are dead.

Immunohistochemistry

Frozen sections were rehydrated in 1X PBST (phosphate buffered saline with 0.1% Tween) twice for 10 minutes each. Paraffin embedded sections were rehydrated through serial graded solutions of 100%, 75%, 50%, 30% Ethanol and 1XPBS twice for 5 minutes each. Then sections were incubated in 100% methanol containing 0.4% hydrogen peroxide for 20 minutes to remove endogenous peroxidase activity. 1X PBST washes were repeated and sections were incubated for 1 hour in blocking solution (Vector ABC kit, Vector Inc., CA) and overnight with primary antibody (0.1 μg/ml final concentration for rabbit anti-Cdk1, 2,3,4,5,6,7, or8 antibodies which were either purchased from Santa Cruz or a gift from collaborator's lab). After three washes with 1X PBST for 10 minutes each, sections were incubated with secondary biotinylated anti-rabbit antibodies (ABC kit) overnight, followed by three washes in 1X PBST. ABC reagent was applied for 2 hours and slides were rinsed again three times in 1X PBST for 10 minutes each. Finally, slides were incubated with DAB solution (DAB, research Genetics, Inc.) for 2 minutes to develop a chromophore at the site of antibody binding, dH_2O wash three times, counterstained with methylene blue (1:100 dilution), dehydrated and mounted.

Immunohistochemistry and DNA Fragmentation Double Labeling

Fluorescent detection of DNA fragmentation and Cdk5 expression were as paper by Ahuja et al., 1994. Briefly, sections were twice washed in 1X PBST, post fixed in ethanol: acetic acid (2:1) for 5 min at -20°C, and again washed twice in 1X PBST. Section were placed for 5 min in equilibration buffer (ApopTag kit, Oncor Inc.), and applied with TdT and incubated for 1 hr at 37°C. The slides were immersed in pre-warmed stop/wash buffer for 30 min, rinsed three times with 1X PBST, and anti-

digoxigenin FITC flourescent reagent (Jackson Immunology) was applied and incubated for 30 minutes. After a wash with 1X PBST, slides were incubated for 20 min in 0.3% H_2O_2, followed by two washes with 1X PBST. Blocking solution (ABC kit, Vector Inc.) was applied to the sections for 1hr, and primary antibody [1mg/ml=40mg BSA (Bovine Serum Albumin) + 1uL antibody (100mg/mL, as above) + 1mL 1X PBST] was added and incubated overnight at 4°C. The sections were then washed three times with 1X PBST, incubated with secondary biotinylated antibody (ABC kit, Vector Inc.) overnight at RT, and again rinsed three times in 1X PBST. Fluorescent signal was detected by incubating the slides with cy3-conjugated IgG mouse anti-biotin (Jackson Immunology) for 30 min, followed by washing in 1X PBST for three times at 10 minutes each. The slides were mounted with 90% glycerol and observed under confocal microscope. Using different filters, we were able to capture fluorescent signals from Cy3 and FITC respectively to detect immunohistochemistry and DNA fragmentation signals. We then combined both signals to detect the overlapped signals by computer.

c. DNA characterization technologies:

DNA analysis for fragmentation

We used a nonisotopic DNA end labeling in situ technique, employing digoxigenin-11-dUTP and terminal transferase (ApopTag™ Peroxidase Kit, Oncor, Gaithersburg MD, Zakeri et al., 1994 and Zakeri and Ahuja, 1994). Sections were post-fixed in ethanol: acetic acid (2:1) for 5 minutes at -20°C and washed in 1X PBS twice for 5 minutes each. Endogenous peroxidase was quenched with 0.1% hydrogen peroxide in 1X PBS for 20 minutes and then rinsed in 1X PBS twice for 5 minutes. Sections were equilibrated in equilibration buffer for 20 minutes before the addition of reaction buffer containing TdT (terminal

deoxynucleotidyl transferase) enzyme and digoxigenin-11-dUTP for 90 minutes at 37°C. The reaction was stopped by incubating sections in stop/wash buffer for 30 minutes at 37°C followed by three washes in 1X PBS for 5 minutes each. The digoxigenin-11-dUTP containing oligonucleotide extensions were detected by anti-digoxigenin-peroxidase for 30 minutes followed by four washes in 1X PBS for 5 minutes each. Color development was performed by staining sections in stable diaminobenzidene (DAB, Research Genetics, Inc.) for 4 minutes. Slides were then washed in tap H_2O three times for one minute each, distilled H_2O for 5 minutes. Sections were counterstained with methyl green (0.5% methyl green in 0.1M sodium acetate, pH 4.0) for 3 minutes and washed in dH_2O three times for 1 minute each. Sections were then rinsed in 100% butanol three times for 2 minutes each, followed by three rinses in toluene for 2 minutes each, and mounted with Permount®.

Transformation of plasmid into E.coli.

To prepare competent DH5α (GIBCO BRL) cells, 5 mL of LB (10g bacto-tryptone, 5g bacto-yeast extract (Difco), 10g NaCl in a final volume of 1L, pH 7.0) were inoculated with 200 uL DH5α cells and grown up overnight at 37°C. The next morning, 50 mL of LB broth were inoculated with 0.5 mL of the overnight culture and grown at 37°C for 3 hours (OD_{550} was approximately 0.5 -0.7) and then placed on ice. After 20 minutes the cells were centrifuged at RT, 2500 RPM for 15 min. The pellet was resuspended in 25 mL of ice cold, sterile 0.1 M $CaCl_2$ and left on ice for 20-30 minutes with occasional swirling. The solution was centrifuged again for 10 minutes to pellet cells. The pellet was resuspended in 3 mL 0.1M $CaCl_2$ and placed on ice or stored at 4°C overnight. DNA was diluted to a concentration of 10 ng/μL and 20ng (2 μL) of DNA were added to 300 μL competent cells in a 15 mL conical tube. As a control, 300 μL of cells were placed in a second tube without

DNA. The colonials were gently mixed and left on ice for 0.5 hour with occasional shaking. The cells were then heat shocked at 42°C for 45 seconds to enhance transformation. Then, the cells were cooled down on ice for 2 minutes. 1mL LB broth was added and cells were grown in a 37°C shaking incubator for 45 minutes and then placed on ice. 100 µL and 200 µL of cells were plated out onto separate LB + Amp plates (LB broth, 15 gms agar/L, 50 µg/mL ampicillin (Boehringer Mannheim). On a third plate, 100 µL of cells only were plated out. Plates were incubated overnight at 37°C. The next day a single colony was picked and 5 mL LB + amp broth was inoculated and incubated in shaker at 37°C overnight.

Plasmid preparation

Bacteria from a 250 mL culture of LB broth + amp were pelleted at 5000 RPM for 15 minutes at 4°C. The pellet was resuspended in 10 mL ARS I (alkaline rapid screen, 4.5 gms glucose, 12.5 mL 1M Tris HCl (pH 8.0), and 10 mL 0.5M EDTA (pH 8.0) in a total volume of 500 mL) and one scoop of lysozyme (Jersey Lab and Glove Supply) and incubated on ice for 30 minutes. 20 mL ARS II (2 mL 10N NaOH, 10 mL 10% SDS, and 88 mL H_20) was added and the mixture was swirled and incubated on ice for no more than 5 minutes. 15 mL ARS III (204. 12 gms NaOAc in 500 mL H_20, pH 4.8) were added and the mixture was inverted and incubated on ice for 45 minutes. This mixture was then centrifuged at 12000 RPM for 20 minutes and the supernatant was transferred to a clean tube with equal volume (45 mL) of isopropanol. This solution was mixed well and placed at -70°C. After 20 minutes, it was centrifuged at 12000 RPM for 20 minutes and the pellet was dissolved in 7 mL of 1X TE and 4 mL of 7.5 M NH_4OAc. 2 volumes 100% ethanol was added, mixed well and the tube was placed at -70°C for 20 minutes. This was centrifuged at 12000 RPM for 20 minutes and the pellet was resuspended in 7 mL TE and 10 uL RNase A (10 mg/mL)was

added for a 30 minute incubation at 37°C. 3 mL of 5M NaCl, 2.5 mL 30% PEG/1.5 M NaCl were added, mixed well, and the tube was left on ice for 30 minutes. This was centrifuged at 12000 RPM for 20 minutes and the pellet was dissolved in 560 µL H_20. To this mixture, 70 µL of 10X PK buffer and 70 µL Proteinase K (5 mg/mL) were added for an overnight incubation at 37°C. The next day, 70 µL 3M NaOAc were added and phenol/CH Cl_3 extractions were performed until the interface was clean (~ 4 times). Two and a half volumes of 100% Ethanol were added and DNA was precipitated at -70° for 20 minutes. This solution was centrifuged at 13000 RPM for 15 minutes at 4°C and the pellet was washed with cold 70% ethanol. The pellet was then dried in a Savant speed vacuum, dissolved in 400 µL of dH_20 and the optical density was read at 260nm.

Probe Preparation for In situ hybridization

To prepare radioactive probes, 4 µL 5X transcription buffer (Stratagene), 2 µL 0.1 M DTT (Jersey Lab Supply), 3 µL cold 10 mM CTP/ATP/GTP (combined; Stratagene), 5 µL ^{35}S-UTP (Amersham), 1 µL DNA template (300 ng), 2.5 µL H_2O, 1 µL RNAse inhibitor (Boehringer Mannheim), and 1.5 µL appropriate RNA polymerase were combined in a tube and incubated for 2 hours at 37°C. At this time 1 µL polymerase was added and the mixture was incubated at 37°C. After 30 minutes, 1µL of RNAse free DNAse RQ1 (Promega) was added and the mixture was incubated for 15 minutes at 37°C. 3 µL of total yeast RNA (extracted three times with phenol/chloroform and ethanol precipitated several times to make a stock solution: 10 mg/ml; Boehringer Mannheim) and 25 µL DEP H_2O was added. Alkaline hydrolysis was performed by adding 50 µL of solution A (10 µL 1M DTT, 80 µL 1M $NaHCO_3$, 120 µL 1M Na_2CO_3, and 790 µL H_2O) for 15-30 minutes depending on the length of DNA template. After hydrolysis was completed, the reaction was neutralized with

50 µL solution B (200 µL 1M NaOAc, 10 µL acetic acid, 10 µL 1M DTT, 780 µL H_2O) and run on a 1:1 ratio of fine: medium Sephadex G50 column (Pharmacia Biotech, Sambrook et al., '89) to isolate the labeled probe from unincorporated nucleotides and labeled smaller size fragments. The column was prepared by using a pasteur pipet which was plugged with siliconized glass wool (Supelco) with the tip broken off. The column was equilibrated prior to loading the probe with running buffer (10 mM Tris-HCl, 5 mM EDTA, 0.1% SDS (sodium dodecyl sulfate) and 10 mM DTT added just prior to use), followed by 50 µL total yeast RNA (10 mg/ml), and 500 µL running buffer. Probe containing 1 µL of saturated phenol red to follow free nucleotides was run through and fractions were collected until the phenol red was about 1 inch from the bottom. Tubes containing labeled probe were monitored by geiger counter. Probes were ethanol precipitated with one tenth volume of 3M KAc and 2 volumes of 100% ethanol overnight at -20°C. The next morning, centrifuge to collect pellet, and the probe was dried and resuspended in 10 µL of 1M DTT and 1 µL was counted in CytoScint scintillation fluid (Fisher). A final concentration of 35000 cpm/µL in hybridization buffer was used.

In situ hybridization

In situ hybridization experiments were performed essentially as described by Sassoon and Rosenthal (1993) and developed by Zakeri and Wolgemuth (1997). Sections were washed in 0.85% NaCl and 1X PBS for 5 minutes each, followed by fixation in 4% paraformaldehyde for 20 minutes and two 5 minutes washes in 1X PBS. Sections were then incubated in 2 mg proteinase K in PK buffer (1 M Tris, 0.5 M EDTA) for 7.5 minutes, washed in 1X PBS for 5 minutes, in 0.1 M triethanolamine containing acetic anhydride for 10 minutes, in 1X PBS for 5 minutes, and in 0.85% NaCl for 5 minutes. Slides were dehydrated in graded ethanol

solutions, air dried and hybridized with 9 parts hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM $NaH_2PO_4 \cdot H_20$ (pH 8.0), 10% dextran sulfate, 1X Denhardt's solution, 0.5 mg/ml total yeast RNA) and 1 part [^{35}S]UTP labeled probe in 10 mM DTT for 16-18 hours at 50°C. Posthybridization washes included 5X SSC, 10 mM DTT at 50°C for 30 minutes; followed by 50% formamide, 2X SSC at 65°C for 20 minutes; 1X washing solution (0.4 M NaCl, 10 mM Tris (pH 7.5), and 5 mM EDTA) at 37°C twice for 10 minutes each; 20 mg/ ml RNAse A in 1X washing solution at 37°C for 30 minutes; 1X washing solution at 37°C for 5 minutes; 2X SSC at 37°C for 15 minutes; and 0.1X SSC at 37°C for 15 minutes. The slides were dehydrated, allowed to dry and dipped in photographic NTB-2 emulsion (Kodak) and exposed for two weeks. The slides were developed (1:1; Kodak Dektol developer: H_2O) for 2 minutes, washed for 10 seconds, fixed (Kodak) for 5 minutes, washed with dH_2O for 5 minutes, counterstained with 0.2% toluidine blue for 10 minutes, dehydrated in 50%, 75%, 85%, 95%, 100% (twice) ethanol for two minutes each and xylene (twice) for 5 minutes each. The slides were mounted with Permount® and coverslipped.

Northern blot hybridization

Probe Preparation for Northern blot hybridization: To prepare the random prime probe, 200ng insert fragment of plasmid DNA, 5 µL primer, and 27 µL H_20 were incubated in boiling water for 5 minutes and placed on ice for 2 minutes. To this 10 µL of labeling buffer, 5 µL ^{32}P-dCTP (Amersham), and 2 µL Klenow were added. This was incubated at 37°C. After 1 hour, 13 µL H_20 and 7 µL 10X STE were added. A push column (GIBCO BRL Inc.) was hydrated with 70 µL of 1X STE, after which the probe was pushed through.

Another 70 µL 1X STE was pushed through to wash the column and 1 µL was taken to count. $2X10^7$ counts were used for hybridization.

RNA was separated by using a high-resolution gel (1.5% agarose and 2.2M formaldehyde (Fisher) in 1X MOPS (10X MOPS: 200mM MOPS, 50mM NaOAc, 10mM EDTA, pH 7.0). RNA samples were centrifuged for 30 minutes at 4°C, dried, and resuspended in water to measure optical densities. 20 µg of RNA were placed in separate tubes and ethanol precipitated once again. The dried pellet was resuspended in 10 µL of sample buffer (50% formamide, 1X formaldehyde, 1X MOPS buffer). Samples were heated at 70°C for 5 minutes and quenched on ice to denature DNA. To each sample, 0.5 µL of ethidium bromide (10 mg/ml) and 0.5 µL of bromophenol blue (10 mg/ml in glycerol) were added and mixed. Samples were loaded immediately and the gel was run at 40V for approximately six hours.

To transfer RNA onto membrane, the gel was then pre-soaked in 10X SSC while shaking for 1 hour. The gel was transferred onto Hybond (Amersham) overnight by capillary action, after which it was crosslinked in a stratalinker (Stratagene) at 120,000 microjoules. The blot was prehybridized overnight at 42°C in prehybridization buffer (50% formamide, 5X SSC, 5X Denhardt's, 50 mM $NaPO_4$ pH 7.0, 0.2% SDS, 500 µg/ml salmon sperm DNA). The blot was hybridized in hybridization buffer (50% formamide, 5X SSC, 1X Denhardts, 20 mM $NaPO_4$, 10% Dextran sulfate, and 100 µg/ml salmon sperm DNA (boiled for 5 minutes and quenched on ice just prior to use) containing 2×10^7 cpm probe overnight at 42°C. After hybridization, the blot was washed in 2X SSC, 0.1% SDS for 15 minutes at room temperature, twice in 2X SSC, 0.1% SDS for 20 minutes each at 65°C, in 1X SSC, 0.1% SDS for 20 minutes at 65°C, in 0.1X SSC, 0.1% SDS for 20 minutes at

65°C, and in 0.1X SSC alone for 20 minutes at 65°C. The blot was placed in a plastic bag and exposed to film overnight with intensifying screens at -70°C.

d. Protein characterization technologies:

Cdk5 immunoprecipitation and kinase assay

Equal amounts of CP treated day 10.5 embryos and 1XPBS treated day10.5 embryos were quickly frozen in liquid nitrogen. Tissue extracts were placed in extraction buffer (5 mL of RIP A Buffer per gram of tissue: 50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.05% Na deoxycholate, 0.1% SDS, 0.1mM Na_3VO_4, 1mM DTT, 20 mM glycerophosphate, 2mM EDTA, 1 µg/ml aprotinin, 100 µg/ml methylsulfonylfluoride (in isopropanol), 10 µg/ml leupeptin) and manually homogenized on ice. The lysates were run through a syringe (26.5 gauge needle) until they flowed through easily and were incubated on ice for 15 minutes. Lysates were then centrifuged at 4°C for 15 minutes at 14000 rpm to remove cellular debris. Supernatants were kept on ice. Protein concentration was determined by use of the Biorad protein assay dye reagent (diluted 1 to 4 in H_2O) with the addition of BSA (1 mg/ml) in different quantities for standards or 1-2 µL of each sample. Optical densities of all the standards and samples were read at 595nm. Concentrations were determined by a protein assay standard curve. To 500 µg of tissue extract, 1 µL of anti-Cdk5 was added and tubes were placed on a rocker for 1 hour at 4°C. For the control, 1 µL rabbit IgG (0.1 µg/µL) was added instead. After one hour, 15µL beads (GIBCO BRL ProteinA Agarose) were added and tubes were placed on a rocker at 4° for an hour once again. The mixture was centrifuged briefly to pellet the ProteinA agarose complex and the pellet was then washed three times in RIP buffer. The beads were then incubated in EB buffer (50mM Tris pH 7.5, 10mM $MgCl_2$, 1mM DTT, 20mM EGTA, 80mM B-glycerophosphate, 0.1 mM Na_3VO_4) on

a rocker at 4°C for 5 minutes. Tubes were centrifuged briefly and buffer was removed with a syringe (28.5 gauge needle). The beads were then incubated with kinase buffer (20μL/ reaction: eb buffer + 2.5 Histone HI (Gibco BRL), 5 μM cAMP (Sigma), 10 μm ATP (Boehringer), 2.5 μCi γ-P32 ATP (Amersham) at 30°C for 30 minutes. Twenty μL of 2X Laemmeli buffer (0.1M Tris, 4% SDS, 20% glycerol (Fisher), 10% ß-mercaptoethanol (Sigma), 0.2% bromophenol blue (Fisher)) was added to samples and they were boiled for 2 minutes. Samples were centrifuged for 1 min at 3000 rpm at 4°C and 20μL were loaded into each lane of a 12% acrylamide gel. The gel was run at 13 mA for three hours. The gel was then dried at 80°C for 2hrs and exposed to film (Dupont Cronex) overnight. To prepare the gel, a 12% separating gel (20 μL of 0.5M EDTA, 2.5 ml of 1.5M Tris pH 8.8, 50 μL of 20% SDS; 7.5 μL of 100% TEMED, 4 ml of 30% acrylamide/0.8% bisulfate, 100 μL of 10% ammonium persulfate and 3.3 ml H_2O) and 4.5% stacking gel (10 μL of 0.5 M EDTA, 625 μL of 10mM Tris, pH 6.8, 25μL of 20% SDS, 5 μL of 100% TEMED, 750 μL of 30% Acrylamide/0.8% bisulfate, 50 μL of 10% ammonium persulfate and 3.5 ml H_2O) were made.

Western Blot

Tissue or cell sample were lysed in lysis buffer (50mM Tris, pH7.5, 150mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.15% SDS, 0.1mM sodium vanadate, 1mM DTT, 20mM β–glycerophosphate, 2mM EDTA, 1X protease cocktail tablet (Roche Inc.). The lysis solution was cleared by centrifugation and protein concentrations were determined by assaying with BioRad reagent. Samples were combined with Laemmli loading buffer. Equal amount of protein (approximately 300ng of total protein) were electrophoresed on 10% SDS-polyacrylamide gels at 100 V for 1.5 hours. The gels were electroblotted to

nitrocellulose membranes at 250 mA for 2 h at 4°C. Each membrane was incubated for 1.5 hours at room temperature in 10 mL of PBS pH 7.4 containing 0.1% v/v Tween 20® and 10% w/v skim milk (Difco) to block non-specific reactions, and then washed four times with PBS, 0.1% v/v Tween 20 for 5 minutes. The membranes were incubated with each of the antibody (100µg/mL) in 4 mL PBS, pH 7.4, 0.1% v/v Tween 20 and 10% w/v skim milk for 1.5 h at room temperature, washed 4 times with PBS, 0.1% v/v Tween 20 for 5 minutes, and incubated for 1.5 h at room temperature with 5 mL of a 1:20,000 dilution of horseradish-peroxidase-linked-goat-anti-rabbit IgG (Caltag Laboratories, in PBS 0.1% v/v Tween 20). The membranes were washed 4 times with PBS 0.3% v/v Tween 20 for 5 minutes; 4 times with PBS 0.1% v/v Tween 20 for 5 minutes and once with PBS for 5 minutes. The immunoreactive bands were detected using enhanced chemiluminescence using the protocols described by the supplier (Amersham) except that 1 mL of detection reagent mix was typically used on an 8.5 cm x 5.5 cm membrane sealed by surface tension to Parafilm. The membranes were exposed on Kodak X-OMAT AR film between intensifying screens for various times between 2 and 300 seconds.

3. Results

A. Analysis of the expression of Cdk5 in the developing mouse limb:

In the developing mouse limb, cell death begins at day 10.5, and it increases and peaks at day 14.5 in the interdigital regions. Previous studies from our laboratory reported high expression of Cdk5 by immunohistochemistry in the developing interdigital regions of mouse limb (Ahuja et al. 1994; 1997; Zhang et al.1997). In order to show that Cdk5 expression is restricted to dying cells, we used fluorescent double-labeling for *in situ* DNA fragmentation and Cdk5 protein detection, combined with con-focal microscopy to detect the relationship between Cdk5 expression and apoptotic cell death. As mentioned in Material and Methods, FITC-conjugated label (green fluorescence) was used for detection and analysis of DNA fragmentation (Figure 1C), and Cy3 conjugated label (red fluorescence) was used for detection and analysis of Cdk5 protein expression (Figure 1D). This observation is similar to the detection of DNA fragmentation and Cdk5 protein localization using non-fluorescent methods shown in Figure 1A and 1B respectively. An intensive yellow stain was detected when Cdk5 expression and DNA fragmentation overlaps. As shown in Figure 1 (E-G), there is an overlap between the expression of Cdk5 and cells with fragmented DNA (Figure 1E to1G). These results clearly show that Cdk5 expression is elevated in the cells. The high expression of Cdk5 in apoptotic cells may have a role in the regulation of the apoptotic signaling.

Since we showed that Cdk5 protein expression inceased in cells with fragmented DNA, we went on to investigate when Cdk5 transcriptional activation

is turned on in the developing limb. Using *in situ* hybridization (see Material and Methods), we localized Cdk5 mRNA expression in different parts of the embryo as well as in the developing limbs. Although we detected Cdk5 differential expression at the mRNA level in the spinal cord of the developing embryos (Figure 2B), the up-regulated mRNA did not appear to correlate exclusively with the dying cells indicated by DNA fragmentation and specific up-regulation of the Cdk5 protein (arrows in Figure 2A). The expression of Cdk5 mRNA was uniform in the developing limb (Figure 2D), although there were a number of cells undergoing cell death (arrows in Figure 2F) (Figure 2C and 2E are control experiments). This suggests that Cdk5 mRNA expression is not restricted in apoptotic cells. The accumulation of Cdk5 protein in dying cells shows that Cdk5 may play a role in apoptosis at the protein level.

Finally, we asked if other members of the Cdk family are upregulated in the developing mouse limb. We examined the expression of other Cdks by immunohistochemistry. In contrast to the high level of expression of Cdk5 in apoptotic cells in the interdigital limb, there was no specific signal above background when antibodies against Cdc2 (Cdk1), Cdk2, Cdk4 and Cdk6 protein were used (Figure 3B, 3C, 3E, 3G). Cdk3, Cdk7, and Cdk8 antiserum detected a signal at an overall low level, with no cellular specificity (Figure 3D, 3H, 3I). We concluded that the Cdk5 expressionin the apoptotic cells in the inter-digital region of the limbs is specific to Cdk5.

B. Analysis of the expression of Cdk5 in the developing rat lens:

The unique structure of the developing lens offers several advantages for the study of a different type of cell death as compared to what we see in the developing limb as presented above. In developing rat lens, rudiment lens epithelium cells elongate, invaginate to the hollow cavity of lens vesicle, and become primary fiber cells (Figure 4A, 4B). Then, the nucleus, chromatin, and intracellular organelles of the fiber cells in the center of the lens are degraded from day 17 of the embryo and end at the date of birth (Figure 4C, 4D). At the same time, surrounding secondary fiber cells lie down as concentric shells and also go through the degeneration process (Figure 4E).

As described in Materials and Methods, E18 rat embryos were used for immunohistochemical staining to detect Cdk5 expression. Cdk5 protein was detected at the anterior ends of the lens fiber cells as intense staining signals (Figure 5A, 5C). In certain fiber cells, staining appeared to be both nuclear and cytoplasmic, where there was nuclear apoptosis. Controls slides, stained with Cdk5 antibody in the presence of the blocking antigenic peptide, showed negative signal (Figure 5B). To specifically correlate Cdk5 expression to this special denuclear apoptotic cells, we again used fluorescent double-labeling techniques for *in situ* DNA fragmentation and Cdk5 protein detection to detect the relationship between Cdk5 expression and cell death. FITC-conjugated label (green fluorescence) was used for detection and analysis of DNA fragmentation (Figure 5D), and Cy3 conjugated label (red fluorescence) was used for detection and analysis of Cdk5 protein expression (Figure 5E). As shown in the mouse

limbs, we detected DNA fragmentation and Cdk5 expression in the same cell. Therefore, there is an overlap between the expression of Cdk5 and apoptotic cells.

To investigate how Cdk5 is regulated at the transcriptional level, we performed *in situ* hybridization with radioactively labeled sense and antisense Cdk5 probe (as described in Materials and Methods) and analysed the distribution of Cdk5 mRNA within the lens. Hybridization with the Cdk5 antisense probe showed that Cdk5 is evenly distributed in all of the elongating fiber cells (Figure 6B), as well as fiber cells in the bow region of the lens which cells did not undergo apoptosis (Figure 6B arrow). A control experiment performed with the sense probe showed no hybridization (Figure6A). This suggested that the increased Cdk5 protein expression was not due to the upregulation of its mRNA. This result is in agreement with the finding by our collaborators (Dr. Zelenka, NIH). He showed that there was no increase in the amount of Cdk5 protein as measured by western, while Cdk5 histone H1 kinase activity increased with developmental age (Gao et al.1997). Our results indicate that in the lens fiber cell, increased expression of Cdk5 is not due to an upregulation of messenger RNA, but to post-translational stability of the protein.

Finally, we asked if Cdk5 had specific expression in the dying lens fiber cells, as compared to other Cdks. Using immunohistochemical staining, we found that there was no specific signal when antibodies against Cdk2 and Cdk4 proteins were used (Figure 7A, 7B). Cdk7 and Cdk8 antiserum detected a low expression of those proteins, but the expression was not localized in the dying fiber cells

(Figure 7C, 7D). These results, as in the mouse limb, indicated specific Cdk5 expression in the dying fiber cells in the rat lens.

Our results showed that, in normal developing limb and lens, only Cdk5 expression increased specifically in dying cells, but not other Cdks. Furthermore, this upregulation is not at the level of transcription, and may not be at the level of translation. It may be regulated after translation.

C. Analysis of the association of Cdk5 with cell death in induced models of cell death:

To examine if the expression of Cdk5 and its kinase activity are increased with induction of cell death, we investigated two different apoptotic model systems. One of them was cyclophosphamide (CP). CP is an alkylating agent that disrupts normal embryonic development by inducing excessive cell death. By using different apoptotic markers—such as phagocytic cell detection, lysosomal activity assay, and DNA fragmentation assay—cell death in day 10.5 Our lab has confirmed that cell death in CP-treated mouse embryo is apoptosis (Zhu et al. 2002).

Having established the CP-induced excessive cell death in the treated embryos, we examined if Cdk5 expression was altered in this apoptotic model. Near serial sections of CP-treated embryos were stained for the presence of Cdk5 protein (Figure 8D, 8E, 8F), as well as for DNA fragmentation (Figure 8A, 8B, 8C). As shown in Figure 8 (D, E, F), a high level of the expression of Cdk5 protein was detected in treated embryos. Interestingly, the level of cell death by detection of DNA fragmentation was less than the level of detectable Cdk5

protein (Figure 8B, E). We attribute this to the fact that either Cdk5 protein localized in the cytoplasm is a stable protein that can easily be detected in the fragmented cells with or without nuclei, and /or that the appearance of Cdk5 protein is before the fragmentation of DNA, and stored after cell fragmentation.

To better correlate the expression of Cdk5 with DNA fragmentation and cell death, we used fluorescent double-labeling for *in situ* DNA fragmentation and Cdk5 protein detection. The results were analyzed by con-focal microscopy. As mentioned in Material and Methods, FITC-conjugated label (green fluorescence) was used for detection and analysis of DNA fragmentation (Figure 9A), and Cy3 conjugated label (red fluorescence) was used for detection and analysis of Cdk5 protein expression (Figure 9B). Figure 9 (C, D) shows that there is an overlap between the expression of Cdk5 and cells with fragmented DNA, as indicated by the presence of the intense yellow staining (Figure 9C to 9D). As we showed previously, in normal embryonic development, Cdk5 is also upregulated artificially induced cell death, and there is a correlation between the Cdk5 expression and cell killing.

Similar to the limb and the lens, Cdk5 expression in CP-treated embryos was not regulated at the level of transcription in *in situ* hybridization (Figure 10A). In addition, we detected the same Cdk5 mRNA expression in control and CP-treated embryos by Northern Blot (Figure 10B).

Furthermore, we used Histone H1 kinase assay to detect its kinase activity. A slight increase of Cdk5 protein expression was detected in CP-treated embryos by western blot (Figure 11A). However, the kinase activity of CP treated embryo

was about 300 percent higher than that of the control (Figure 11B). Taken together, these results suggest that the regulation of Cdk5 during cell death was not due to an upregulation of mRNA, or protein, but post-translation regulation of Cdk5 kinase activity.

D. Regulation of Cdk5 in induced cell death using COS-7 cells as a model

In vivo models, such as CP-treated embryos, developing limb and lens, are difficult to manipulate. It is therefore useful to develop an *in vitro* model. We developed a cell culture model by using COS-7 cells. COS-7 cells are a commonly used monkey kidney fibroblast-like cell line. We found that COS-7 cells can undergo apoptosis after being treated with several cell death inducing agents, such as camptothecin (CPT) and cycloheximide (CHX). CPT interacts with DNA and interrupts DNA synthesis at transcriptional level (Tinbrell et al. 1991), and cycloheximide (CHX) interruptes protein synthesis at the translation level by blocking the peptide translocation reaction on ribosomes (Tinbrell et al. 1991).

First, we tested that CHX and CPT can kill COS-7 cells by apoptosis. COS-7 cells were treated with CHX or CPT for 18hrs, and the level of cell death was examined by trypan blue staining (see Materials and Methods). Cell death ratio was calculated by counting the percentage of blue cells, since dead cells lose their membrane integrity, and this allows the preferential uptake of Typan Blue. While living cells will remain undyed. The percentage of blue cells to total cells

indicated the number of dead cells. We found that 45% of the cells treated with CHX and 55% of the cells treated with CPT died by apoptosis after 12 hours treatment (Figure 12A). DNA fragmentation confirmed the apoptotic nature of cell death by these cell death-inducing compounds as shown in Figure 12B.

Having established that these compounds can induce cell death in COS-7 cells, we examined if Cdk5 protein expression and kinase activity was modulated before and after treatment with CHX and CPT.Western blot (Figure 13A) analysis did not reveal an increase of Cdk5 protein expression after the treatment and cell death. However, Cdk5 Histone H1 kinase activity did show a significant increase upon induction of cell death with both compounds. These results confirmed the relationship between Cdk5 kinase activities to induction of cell death using in vitro cell death model (Figure 13B). This model provides us with an in vitro system that can be manipulated to study the underlying role of Cdk5 activation in cell death

4. Conclusion

In this study, we mapped the expression pattern between cyclin-dependent kinases and cell death in normal developing limb, normal developing lens, abnormal CP-treated mouse embryo, and induced apoptosis in COS-7 cells. The results showed that only one cyclin-dependent kinase—cyclin-dependent kinase 5 (Cdk5) is associated with apoptosis. In addition, in all these systems, the kinase activity of Cdk5 was increased in apoptosis. This increase was regulated at neither transcriptional level, nor translation level, but regulated after translation. Although Cdk5 belongs to a family of kinases, other members of the family were not involved in the regulation of cell death (Luo et al. 1994).

Cdk5 is associated with cell death:

Previous Cdk5 studies were focused primarily on its function in neuronal differentiation. Cdk5 is highly expressed in brain tissues (Tsai et al.1994, Patrick et al.1998). By using *in vivo*, as well as *in vitro* systems, we first demonstrated that Cdk5 is the only member of the Cdk family that is likely to be involved in apoptitic cell death in the developing mouse limb, developing rat lens, cyclophosphamide-treated developing mouse embryo, and apoptotic COS-7 cell. Recently, Patrick et al. (1999), Lee et al. (2000), and Ahlijanian et al. (2000) have shown that active Cdk5 can induce cell death in COS-7 cell line, their data confirmed our finds.

Immunohistochemistry and DNA fragmentation double-labeling experiments showed that there was always more Cdk5 expression signal than DNA fragmentation signal. This maybe the case because the expression of Cdk5 happens at

an early stage of apoptosis, and is constantly activated for a certain period during the process.

Cdk5 is not regulated at transcriptional level during cell death:

The expression pattern of Cdk5 mRNA also provided some insight into Cdk5's possible regulatory mechanism during apoptosis. By using *in situ* hybridization, Cdk5 mRNA was found ubiquitously expressed in all tissues; there was no difference between apoptotic cells and living cells. This strongly suggests that the transcription level of Cdk5 is not changed in the dying cells. *In situ* hybridization did not indicate any obvious differences in the level of Cdk5 mRNA between the nucleus and the cytoplasm. In addition, Cdk5 protein expression did not increase in apoptotic cells.

The Cdk5 kinase activity increases during cell death:

Western blot analysis showed that there was no increase in the expression of Cdk5 in dying cells, although there was a strong signal when tissues were stained by immunohistochemistry. This indicates that the total amount of Cdk5 protein did not increase dramatically during cell death.

In addition, Cdk5 kinase activity could be elevated when there is more cell death. For example, after embryos are treated with CP, there is a significant increase in cell death. Parallel to this, we detected an increase of Cdk5 kinase activity. In the induced apoptotic COS-7 *in vitro* model, Cdk5 activity increased under treatments with all the cell death-inducing compounds. Data from retinoid acid-treated mouse

embryo (which increases cell death in the interdigital area) (Ahuja et al. 1997) also showed that Cdk5 expression and its activity increased along with more cell death. These results suggest that Cdk5 might be activated by its regulators.

All these data suggest that Cdk5 is activated during cell death. However, it is not upregulated at the transcriptional level, or the translational level but at the post-translational level. Therefore, it is necessary to investigate how Cdk5 is regulated during apoptosis. Yet the exact mechanism of Cdk5 regulation during cell death is still under study. There are several possibilities. One is that Cdk5 is activated by one or more of its activators during apoptosis. Until now, many proteins have been reported which can form hetero-dimer with Cdk5, such as p25, p35, p39, and p67 (Munc-18). None of them, however, has been reported directly related to apoptosis. Therefore, it is essential to determine whether or not there are unique factors that can regulate Cdk5 activity during apoptosis.

Chapter II: Identification of Cdk5 interactive proteins during cell death

1. **Objective**

2. **Material and Methods**

3. **Results**
   - A. **Identifying Cdk5 interactive protein(s) by the yeast two-hybrid system**
   - B. **Identifying Cdk5 interactive protein(s) by ^{35}S-Methionine incorporation and immunoprecipitation**

4. **Discussion: p35/p25 were identified as Cdk5 interactive proteins during apoptosis.**

Results and discussion:

Section II: Identification of Cdk5 regulators in apoptotic cell death

1. Objective

Like other Cdks, Cdk5 is associated with several regulatory factors. Cdk5 activation requires its interaction with other proteins, including p23 (Uchida et al.1994; Ishiguro et al.1994); p25 (Ishiguro et al, 1994; Uchida et al, 1994; Patrick et al, 1999; Lee et al, 2000; Agarwal et al. 2001; Tarricone et al. 2001, Lim et al. 2001), p35 (Tsai et al, 1994; Alvarez et al. 2001; Van et al. 2001), p39 (Tang et al, 1995), cyclin E (Miyajima et al. 1995; Donnellan et al. 1999) and Munc-18 (also called p67) (Shuang et al, 1998; Fletcher et al, 1999). In Hela cells (Moorthamer et al.'1999a, b), using the yeast two-hybrid system, Cdk5 was shown to associate with several proteins that inhibited its function. These include the ribosomal protein L34 and the DNA binding protein (dbpA) (Moorthamer et al.1999). However, the precious mechanism of these interactions and their functions are not clear.

Our studies have illustrated the correlation between Cdk5 and apoptosis. Cdk5 kinase activity was activated during cell death, and this increased activation was regulated after the translation, not at the level of transcription or the level of translation. Hence, we investigated whether or not there are any proteins that could be involved in the regulation of Cdk5 (i.e., may activate or inhibit its kinase activity) in dying cells. To identify which protein(s) may regulate Cdk5 kinase activity during cell death, we used the yeast two hybrid system and ^{35}S-Methionine in immunoprecipitation.

First, we constructed a cDNA library from messenger RNA of CP-treated mouse embryonic cells where there is massive cell death (as described in previous section). We then used Cdk5 as bait to screen this library for Cdk5-associated proteins by using a yeast two-hybrid system.

Secondly, we labeled COS-7 cells with ^{35}S-Methionine and treated the cells with CPT or CHX as shown previously. After treatment, the cells were lysed and the total cell proteins were immunoprecipitated using anti-Cdk5 antibody. We anticipated that proteins interacting with Cdk5 would be also immunoprecipitated and identified.

2. Materials and Methods

1) Introduction of methods:

a. Yeast Two-Hybrid System:

The yeast two-hybrid assay is based on the fact that many eukaryotic trans-acting transcription factors are composed of physically separable, functional independent domains. Such regulators often contain a DNA-binding domain that bind to a specific enhancer-like sequence, which in yeast is referred to as an upstream activation site UAS). One or more activation domains direct the RNA polymerase II complex to transcribe the gene downstream of the UAS. Both the DNA-BD and the AD are required to activate a gene and normally, as in the case of the native yeast GAL4 protein, the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD peptides do not directly interact with each other and thus cannot activate the responsive genes. However, if the DNA-BD and AD can be brought into close physical proximity in the promoter region, the transcriptional activation function will be restored. In principle, any AD can be paired with any DNA-BD to activate transcription, with the DNA-BD providing the promoter specificity. The yeast two-hybrid system provides a sensitive method for detecting relatively weak and transient protein-protein interactions (Fig.14).

b. ^{35}S-Methionine incorporation and Immunoprecipitation Assay:

Immunoprecipitation combined with ^{35}S-Methionine incorporation is a widely used method for the analysis of association of target protein and its regulators in a complex mixture. At the beginning of this method, cells are cultured in medium

containing ^{35}S labeled-Methionine. Therefore, all newly synthesized proteins will be labeled by ^{35}S labeled-Methionine. The protein complex of interest can be concentrated and immunoaffinity-purified via a specific antibody followed by precipitating with a special agarose (Protein A/G). Often immunoprecipitated complexes are functionally fully active and each factor in the complex can be further analyzed by activity analysis or by running the concentrated complex on SDS-polyacrylamide gels to analyze each factor in the complex separately.

2) Antibody and Genes:

Antibodies: Anti-Cdk5 (C-8), and Anti-p35 (C-19) are from Santa Cruz Inc.

Gene: human Cdk5 cDNA gene is gift from Dr. Tsai in Harvard University.

pAS1-CYH2-Grb2, and pAS1-CYH2-Snf1 construct is gift from Shaosong Yang in SUNY/Stony Brook.

Vectors: pCITE-a is from Novagen Inc. All other vectors used in yeast two-hybrid system are purchased from Clontech Inc.

3) Detail methods:

a. Yeast Two-Hybrid System related methods:

1. Embryo and CP (Cyclophosphamide) treatment

As described in Section I. Embryos were saved in liquid nitrogen.

2. Total RNA isolation from embryos:

Embryos were taken from liquid nitrogen (weight about 50mg), homogenized in liquid nitrogen, and the tissue was lysed in 5ml Ultraspec RNA (Biotecx Laboratory, Inc. Cat# BL-10050, Tel. 1-800-535-6286). This mixture was transferred to 5 tubes, vortexed, and incubated on ice for 10 min. 200ul of phenol-chloroform was added,

vortexed, centrifuged, and the top layer was removed to another tube. Then 100ul chloroform was added, vortexed, centrifuged, and the top layer was again removed to a clean tube. Two volumes of ethanol were added, and the tubes were incubated on dry ice for 30min. Samples were spun for 30min, and the pellets were dissolved in water. The $O.D._{260}$ was read to check the quality of RNA.

3.Poly A+ RNA isolation:

We used a Clontech mRNA separator Kit (Cat#PT1353-1) to separate mRNA from total RNA. Total RNA was heated at 70^0C for 6min. Remove the RNA sample from 70^0C, sit at RT for 10min, add 1/5 volume of sample buffer and mix well. Quickly transfer the sample to fresh column, let sit 10min at RT for sufficient binding. Centrifuge the column at 350g for 2min at 4^0C. Wash the column with 0.3ml of high salt buffer, centrifuge at 350g for 2min at 4^0C, repeat twice. Wash the column three times with 0.6ml aliquots of low salt buffer by centrifuge at 350g for 2min at 4^0C. Transfer the column to a 1.5ml tube, add 0.4ml of elution buffer (pre-warmed in 65^0C), spin at 350g for 2min, repeat twice. Add 1/10 volume of 2M KOAc (pH5.0), and 3 volume of EtOH, store at -20^0C. Read $O.D._{260}$ to check the quality of mRNA.

4. Large Scale Yeast RNA preparation for yeast transformation:

Culture 200mL yeast in YPD complete medium. The next day, yeast was centrifuged and the pellet was resuspended in 22ml of buffet A (50mM Tris, 10mM EDTA pH8) in a 250mL capped flask. Two ml of 10% SDS and 25ml of phenol preheated to $65^0C$was added and the culture was shaken at 65^0C for 6 minutes. Chill in a liquid N_2 by swirling until phenol crystals form on the side of the flask. Spin in 30ml tubes for 5min at 5000rpm. Remove supernatant and add to another 250ml flask

containing 25ml of preheated phenol and shake at 64^{0}C for 6min, chill in liquid N_2 again. Spin at 5000rpm for 5min again to separate the phases. Remove the supernatant, add 25mls of chloroform, shake at room temperature for three minutes and spin in a table top centrifuge for 5min at maximum velocity. Remove the supernatant and EtOH precipitate by the addition of 2 volumes of EtOH.

5. CP treated Day 10.5 mouse embryonic cDNA library construction:

CP treated day10.5 mouse embryo PolyA$^+$RNA was used in a reverse transcription reaction following the Clontech protocol for the Two-hybrid cDNA Library Construction Kit (PT1113-1). To one tube, add 5ug PolyA$^+$RNA sample and 5ul Random p(dN)6 primers. To another tube add 5ug Poly A$^+$RNA sample and 5ul Oligo(dT)25(dN) primers. Add DEPC-H_2O to a final volume of 12.5ul. Heat the tube at 70^{0}C for 3min. Cool the tubes in an ice bath for 2min. Add 2.2ul H_2O, 5ul 5Xfirst-strain buffer, 0.5ul DTT, 1.3ul dNTP and 1ul alpha-32P dCTP (800Ci/mmol). Add 2.5ul (500unit) of MMLV reverse transcriptase to each reaction tube. Mix and incubate at 42^{0}C for 1.5hr. Add 123.5ul H_20, 40ul 5X second-strain buffer, 1.5ul dNTP mix, and 10ul second-strand enzyme cocktail. Incubate at 16^{0}C for 2hr. Add 3ul (15unit) of T4 DNA polymerase, incubate at 16^{0}C for 30min. Add 10ul 0.2M EDTA to stop the reaction. Add 200ul Phenol:chloroform:isoamyl alcohol and extract. Spin down. Combine top layers of both tubes, chloroform extract. Spin down. Remove top layer to another tube. Add 200ul 4M Ammonium acetate and 1ml ethanol to precipitate, spin down pellet. Dissolve the pellet cDNA in 15ul H_2O. Add 3ul 10X Adaptor buffer, 3ul 10mM ATP, 7ul EcoRI adaptors and 2ul T4 DNA ligase (100unit). 16^{0}C incubate overnight. Add 3ul 0.2M EDTA to stop the

reaction. Add 70ul H_2O. Phenol extract, chloroform extract this mixture, and take the top layer to a clean tube. Add 3M sodium acetate (20ul) and 500ul ethanol to precipitate. Resuspend the pellet in 14ul H_20. Add 2ul 10X Adaptor buffer, 2ul 10mM ATP, 2ul T4 polynucleotide kinase. Incubate at 37^{0}C for 30min. Add 2ul of 0.2M EDTA to stop the reaction. Incubate reaction at 70^{0}C for 15min. Cool on ice for 2min. Run cDNA fractionation column, collect the first high counts fraction. Ethanol precipitate. Dissolve the pellet in 7ul H_20, take 1ul run agarose gel. Dry down the gel and expose to x-ray film. Good quality cDNA should have a smear at 0.4kb to 4kb.

Ligation of Adaptor-ligated dsDNA to pGAD10:

Mix 1ul of vector (0.15ug/ul), 10ul plasmid ligation buffer, 1ul 10mM ATP, 1ul T4 DNA ligase (100u/ul) in each of three tubes. Then add 1ul, 1.5ul, 2ul of cDNA in each tube respectively, incubate at 16^{0}C for exactly 30min. Add 80ul H_20 and 1.5ul glycogen, mix, then add ethanol to precipitate in -70^{0}C.

Electroporate transformation of Recombinant Library Plasmid

For each transformation, thaw 50ul electrocompetent (Clontech) DH5α cells on ice, add 15ul ligated DNA, mix throughly with a pipette tip. Transfer the mixture to a chilled 0.1-cm electroporate cuvette. Set up the electroporator using the E.coli Pulsar (BioRad). Transform the DNA by pulsing the mixture at 25kV for 0.4 msec. Immediately add 150ul LB broth to the cuvette and transfer the entire volume to the pre-labeled polypropylene tubes containing 800ul LB broth. Incubate the tube in 37^{0}C for one hour with shaking. At the end of the one hour incubation, remove 10ul of transformation mixture and add it to an eppendorff tube with 40ul LB broth, mix

gently by swirling, and then spread the 50ul aliquot on a pre-warmed 90-mm LB plate containing 50ug/ml of ampicillin. Incubate the plate at 37^{0}C over night. Next day, examine the plate. Then divide the total volume of the left unamplified library by 40, spread the calculated volume of the transformation culture on a 150-mm, pre-warmed LB agar plate containing 50ug/ml of ampicillin. Incubate at 37^{0}C overnight.

Plasmid Library Total DNA Extraction

Take out the plasmid library, and 8ml of LB broth to each plate, scrape the colonies off the agar and pour the suspension into a large, sterile, pre-chilled polypropylene vessel. Remove the desired portion of the library for frozen stock. Determine the titer of the library according to the plasmid library titer protocol from "Molecular Cloning" (Second Edition). Collect the cells by centrifuging 10min at 6000g in 4^{0}C. Resuspend pellet in 4ml Solution I (50mM Glucose, 10mM EDTA, pH8.0, 25mM Tris., pH8.0). Then add 1ml of 25mg/ml hen egg white lysozyme in solution I, resuspend the pellet completely in the solution and allow it to stand 10min at room temperature. Add 10ml freshly prepared solution II (0.2mM NaOH, 1% SDS), mix by stirring gently until solution becomes homogeneous and clear, let stand on ice for 10min. Add 7.5ml solution III (3M potassium acetate) and again stir gently until viscosity is reduced and a large precipitate forms, stand on ice for 10min. Centrifuge 10min at 20,000g in 4^{0}C. Transfer the supernatant into a clean centrifuge tube, add 0.6 volume of isopropanol, mix by inversion, let stand 5-10min at room temperature. Centrifuge 10min at 15,000g in 4^{0}C, wash the pellet with 2ml of 70% ethanol, centrifuge briefly, collect the pellet, dry under vacuum. Resuspend pellet in 4ml TE buffer. Add 4.4g CsCl, dissolve, and add 0.4ml of 10mg/ml ethidium bromide.

Transfer the solution to a 5ml ultracentrifuge tube. Top up the tube. Band plasmid by centrifuging overnight at 350,000g in 20^{0}C. Carefully remove the tube from the centrifuge. Insert a 20-G needle gently into the top of the tube. Recover the plasmid band (the lower of the two bands) by inserting a 3ml syringe with a 20-G needle attached into the side of the tube about 1cm below the plasmid band. Insert the needle with the beveled side up. Extract the EtBr with H_2O-saturated N-butanol until the top butanol is colorless (no longer pink). Add 2X volume of TE, 6X volume of ethanol, leave in -70^{0}C for 20min. Centrifuge the mixture at 20,000g in 4^{0}C for 20min, collect the pellet. Wash the pellet with 70% ethanol, dry DNA by vacuum. Resuspend the pellet in 1ml TE, measure the O.D. to qualify the concentration of DNA.

***6.* Library Scale Yeast Transformation:**

Using a colony from the plate, inoculate 50ml SC-Trp (Clontech) medium and grow overnight at 30^{0}C. The next day, take approximate amount in 500ml YPD complete medium (Clontech) in order to have $O.D._{600}$ at about 0.2-0.3. Incubate 3-4 hours, to let the $O.D._{600}$=0.5-0.8. Harvest cells at 5000rpm for 10min. Wash the cells once with about 100ml LiSORB (10mM LiAC, 10mM Tris pH8.0, 1mM EDTA, 1M Sorbitol), and resuspend in 50ml LiSORB, incubate at 30^{0}C for 30min. Harvest the cells again at 5000rpm for 10min, resuspend in 625ul LiSORB, incubate on ice.

Prepare carrier DNA mix by adding 200ul of yeast total RNA (20mg/ml), 800ul LiSORB, and 40ug library DNA together. Mix 100ul of competent yeast cells and 100ul DNA mixture, incubate at 30^{0}C for 30min. Add 900ul 40% PEG3350 in LiAc/TE (100mM LiAC, 10mM Tris pH8, 1mM EDTA) and incubate at 30^{0}C for

30min. Pool the cells and add 100ml Sc-Trp, Leu liquid media, shake at 30^{0}C for 3 hours, harvest the cells and resuspend on 6ml of SC-His, Trp, Leu liquid media, plate 300ul on each SC-His, Leu, with 25mM 3-aminotriazole). Colonies will grow visible after 3-5 days.

7. Small Scale Yeast Transformation:

Inoculate one colony in 10ml SC-Trp liquid media, vortex vigorously and incubate at 30^{0}C overnight. Transfer the culture to 300ml YPD media to O.D.$_{600}$=0.2-0.3. Incubate for 3 hours at 30^{0}C with shaking to get O.D.$_{600}$=0.5-0.8. Centrifuge the cells at 1000g for 5min at room temperature. Resuspend the pellet in 50ml LiAC/TE (100mMLiAC, 10mM Tris ph8, 1mMEDTA), centrifuge the cells again at 1000g for 5min, resuspend the pellet in 1.5ml LiAC/TE. Mix 0.1ug of Active binding construct and 0.1mg of total yeast RNA together, add to 100ul of competent yeast cells, add 0.6ml PEG/LiAC (40% PEG3350, 100mMLiAC), vortex to mix. Incubate at 30^{0}C for 30min with shaking. Add 70ul DMSO, gently mix by inversion. Heat shock at 42^{0}C for 15min, chill cells on ice for 2min. Pellet the cell, resuspend in 0.5ml TE, plate on selective plates.

8. Preparation of Plasmid DNA from Yeast:

Inoculate one colony in 5ml selective liquid media overnight. Pellet the cell and resuspend the pellet in 200ul of solution A (100mM NaCl, 10mM Tris-HCl pH8, 1mM EDTA, 0.1%SDS). Add glass beads (0.45mm diameter) until just below the level of the liquid. Mix vigorously on a vortex mixer for 1min. Phenol extract the sample containing the glass beads with an equal volume of Tris-buffered phenol pH8. Repeat the phenol extraction, then extract once with an equal volume of

chloroform/isoamyl alcohol (24:1). Ethanol precipitate with 300mM NaOAc. Wash the pellet with 80% ethanol (-20^{0}C). Resuspend the dry pellet in 50ul of TE (10mM TrispH8, 1mM EDTA). Take 10ul of DNA transform to *E.coli*.

9. Yeast Large Scale RNA Preparation:

Grow 500ml of yeast in YPD complete media overnight. Pellet cells and resuspend in 22ml of 50mM Tris, 10mM EDTA pH8.0, place in a 250ml capped flask. Add 2ml of 10%SDS and 25ml of phenol preheated in 65^{0}C, and shake at 65^{0}C for 6min. Chill the mixture in liquid nitrogen by swirling until phenol crystals form on the side of the flask. Centrifuge in 30ml Sarstadt tube for 5min at 5000rpm. Remove supernatant and add to another 250ml flask containing 25ml preheated phenol and shake at 65^{0}C for 6min, chill in liquid nitrogen again. Centrifuge at 5000rpm again to separate the phases, remove the supernatant with RNAse free pasteur pipets and add 25ml chloroform, shake at room temperature for three minutes and spin in a table-top centrifuge for 5min at maximum velocity. Remove the supernatant and precipitate the RNA by the addition of 2 volume of ethanol. Resuspend RNA in RNAse free water.

10. X-Gal Colony Filter Assay:

Label Schleicher and Schuell circular nitrocellulose filters with a ballpoint pen. Lay the filter onto the plate of yeast colonies and allow it to wet completely and place orientation markers on filters and plates with ink and a needle. Lift the filter off the plate carefully to avoid smearing the colonies and place the filter in liquid nitrogen for 1min to permeabilize the cell. Carefully remove the filter from the liquid nitrogen and place cell side up in a petri dish that contains 3MM chromatography paper

soaked with 0.3ml/square inch of Z buffer (16.1g $Na_2HPO4.7H_2O$, 5.5g $NaH_2PO_4.H_2O$, 0.75g KCl, 0.25g $MgSO_4.7H_2O$, and 2.7ml 2-mercaptoethanol) with 1mg/ml X-Gal. Incubate at 30^0C overnight for development of color.

11. Plasmid Library Titer Measurement:

Take 1ul of the library, dilute it to 10^3 and 10^6. Take 1ul of 10^3 dilution, dilute it in 50ul of LB broth, plate it in LB agar plate with 50ug/ml ampicillin. Remove 50ul and 100ul of 10^6 dilution, plate in LB agar plate with 50ug/ml ampicillin. Invert the plates and incubate at 37^0C overnight. The next day, count the number of colonies to determine the titer (cfu/ml). Calculate the titer according to the following formulas: Colony numbers in 10^3 dilution plate $X10^3X10^3$=cfu/ml. Or: colony number in 10^6 dilution plates $X10^3X10^3X10^3$=cfu/ml

b. ^{35}S-Methionine incorporation and Immunoprecipitation Assay related methods:

1. ^{35}S-Methionine incorporation:

COS-7 cells are cultured in optimized medium (DMEM medium with 10% fetal bovine serum, 1X L-glutamine, and 1X antibiotic) until 80% coverage. Change to minimum medium (DMEM medium with no amino acid) and culture for 4 hours. Add ^{35}S-Methionine from Amersham (final concentration 1mCi/ml) with other 1X amino acid into cultured minimum medium. Continue culture for 8 hours. Wash cells twice with ice-cold 1XPBS. Scrape the cells off the plate, spin and collect the cells. Cells were quickly frozen in liquid nitrogen, store in -70^0C.

2. Immunoprecipitation Assay:

Suspend cell pellet from previous step in lysis buffer (50mM Tris-HCL at pH7.5, 150mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 1X complete

protease inhibitor from Roche Pharmaceutical Co.) at a final concentration of 10^7 cells/ml. Quickly freeze and thaw cells once. Homogenize the cells. Centrifuge homogenized suspension at 12,000Xg for 10 min at 4^0C, collect the supernatant. Add 50ul pre-cleaned protein A agarose (Roche Pharmaceutical Co.) for every 1ml supernatant. Incubate over night at 4^0C on a rocking platform. Collect complexes by gravity sedimentation at 12,000g for 20 seconds. Wash the pellet carefully with lysis buffer again. Wash the pellet twice with high salt wash buffer (50mM Tris-HCL at pH7.5, 500mM NaCl, 0.1% Nonidet p40, and 0.05% sodium deoxycholate). Wash the pellet twice with low salt wash buffer(50mM Tris-HCl at pH7.5, 0.1% Nonidet P40, and 0.05% sodium deoxycholate). Add 30ul gel loading buffer to the agarose pellet. Heat to 100^0C for 5 min. Centrifuge at 12,000g for 20 seconds. Load 15ul on (12%) SDS-polyacrylamide gel. Dry gel for 2 hours at 80^0C. Expose over night.

3. Western Blot:

As described in Section I.

3. Results

A. Identification of Cdk5 interactive proteins during apoptosis by a yeast two-hybrid system:

The principle of the yeast two-hybrid system and related methods is described in Materials and Methods. To screen for Cdk5 interactive proteins during apoptosis as illustrated in Figure 15, one AD fusion apoptotic gene enriched cDNA library was first constructed (Figure 15). Simultaneously, a DNA-BD/Cdk5 target plasmid (called "bait") was constructed, and expressed in the yeast strain (Y190-Cdk5).

Construction of an AD fusion apoptotic gene enriched cDNA library:

To prepare an AD fusion apoptotic gene enriched cDNA library, we selected the CP-treated day 10.5 mouse embryo. As discussed previously, extensive cell death is detected in day 10.5 mouse embryos which treated with CP. This massive cell death could provide enough RNA to make one yeast two-hybrid cDNA library.

A, total RNA was isolated from CP-treated mouse embryos, as described in Materials and Methods (Figure 16A). There are two bands shown 18S and 28S rRNA. The ratio between two bands is about 1:3, which guarantees good quality of total RNA.

B, messenger RNA was isolated from total RNA. The mRNA was used in a reverse transcription reaction, in order to get double-stranded cDNA. Figure 16B

shows a smear from 0.5Kb to about 4Kb, which indicates good quality and wide coverage of the cDNA fragments.

C, the double-stranded cDNA was cloned into a yeast two-hybrid system DNA-AD vector pGAD10 EcoRI site (Figure 16C).

D, the quality of the cDNA library was determined by measuring the titer of the plasmid library ($1.3X10^9$cfu/ml), and the library complexity (10 out of 15 randomly picked clones from the library had insert at EcoRI site) (Figure 16D), as described in Materials and Methods.

Construction of a DNA-BD/Cdk5 plasmid and the set up of a Y190-Cdk5 yeast strain:

The "Bait", DNA-BD/Cdk5 target construct, was made from DNA-BD vector pAS1-CYH2 (Figure 17A) and human Cdk5 cDNA, a kind gift from Dr. Tsai (Figure 17B). In order to clone Cdk5 into the vector with correct reading frame, the Cdk5 gene was first cloned into a "switch" vector, pCITE-a, to make pCITE-a-Cdk5 (Figure 17C). Then, this Cdk5 gene with one shifted reading-frame was cloned into pAS1-CYH2 vector at NcoI and SalI site (Figure 17D). After sequencing to confirm the correct clone, pAS1-CDK5 (Fig. 17D) was transformed into the host yeast strain Y190 (Figure 15). The selected bait (Cdk5) expression yeast strain (Y190-Cdk5) was grown on SD/His^- minimum medium to maintain the foreign plasmid. High Cdk5 expression in Y190-Cdk5 was detected by Western blot by using Cdk5 antibody (Figure 17E).

Screening the Cdk5 interactive protein:

As described in Figure 15, CP-treated cDNA library plasmid was transformed into the Y190-Cdk5 bait expression strain (Figure 15). Transferred yeast was growing on SD/His^-/Trp^-/Leu^- yeast minimum solid media for 5 days. After performing colony-lift filter assay for β–galactosidase as described in Materials and Methods, nine colonies were shown to be positive. Because there were more than 10^8 colonies on each plate, in order to purify one single clone, all positive clones had to be replated at a low titer (less than 200) to obtain a single colony. In addition, a colony-lift filter assay for β–galactosidase was performed again, and Figure 18 shows one of these plates. Then, all nine colonies were streaked on SD/Trp^-/Leu^- yeast minimum medium to eliminate DNA-BD/Cdk5 plasmid. At the same time AD/library plasmid could be segregated within a single colony (Figure 15). After the plasmid was extracted from these nine colonies, the inserted library gene was verified by digesting the AD/library plasmid with EcorR1 (Figure 19A). Next, we transformed each extracted AD/library plasmid back to Y190-Cdk5 strain (Figure 15), and performed colony-lift filter assay for β–galactosidase again. Figure 19B shows that 4 out of 9 colonies (colony 1-3, 21-1, 71-2, 91-1) gave blue positive signals. All other colonies did not turn blue, which indicates that they are false positives. Similarly, we repeated the yeast two-hybrid in order to find more Cdk5 interactive proteins. Figure 19C shows that 5 out of 13 colonies (colony 107, 108, 109, 111,114) gave blue positive signals, while the others did not.

Further verification of positive clones:

To eliminate false positive colonies, which can non-specifically activate LacZ, two widely used control baits, DNA-BD/Grb2 and DNA-BD/Snf4 bait plasmid, were used. Grb2 gene (growth factor binding protein) contained the SH2 and SH3 domains, which specifically interacted with the phosphotyrosine motif and proline rich motif (Pierre et al.1995). Snf4 served as adaptors that specifically bind with Snf1, which is a protein kinase (Rong et al.1996). Each control bait plasmid was transformed into Y190 yeast strain as Cdk5 bait into Y190 as described in Material and Methods, but we then used the Grb2 or Snf4 gene instead of DNA-BD/Cdk5 bait plasmid. The Y190-Grb2 and Y190-Snf4 strains, which express Grb2 or Snf4 proteins were selected by western blot analysis.

All of the 9 plasmids from positive clones (4 from the first run yeast two-hybrid screening, and 5 from the second run) were transformed into both strains respectively. Figure 20 shows that 8 out of 9 plasmids turned blue in the colony-lift filter assay for β–galactosidase and shows interaction with both Grb2 and Snf4. This assay result indicated that these clones could non-specifically interact with DNA-BD/bait. Only one clone (No.1-3) remains white, which shows that this clone interacts specifically with Cdk5 and not with Grb2 or Snf4. Sequencing analysis of this plasmid (Figure 21) showed that clone 1-3 had 100% homology to the mouse p35 gene (Figure 22A).

B. Cdk5 and p35 interaction during apoptosis as shown by ^{35}S-Methionine incorporation and immunoprecipitation:

To get more information about Cdk5 interactive proteins during apoptosis, and to confirm the results from the yeast two-hybrid system, ^{35}S-Methionine incorporation and immunoprecipitation assays were performed. Because it was very difficult to incorporate ^{35}S-Met into *in vivo* embryo cells, we used an *in vitro* model instead. From previous data, when COS-7 was treated with camptothecin (CPT) or cycloheximide (CHX), CDK5 Histone H1 kinase activity increased along with apoptosis (Figure 13). Therefore, COS-7 cells were cultured in cell culture medium with ^{35}S- labeled Methionine when treated with CPT or CHX (as described in Materials and Methods). Figure 23B shows extensive proteins were labeled in the control, CHX- and CPT-treated COS-7 cells.

We used Cdk5 antibody, as described in Materials and Methods, to immunoprecipitate Cdk5 and interacted proteins. Proteins were detected on X-ray film after electrophoresis (Figure 23A). Two major bands were detected. One band was about 65KD, and was present in control, CHX-, and CPT-treated cells. The other band was about 25KD, and was unique to CHX-, and CPT-treated cells. According to the published data from other labs, this is a cleavage of p35 to p25 during cell death (Tsai et al. 1994; and Lee et al. 2000), our protein (the 25KD band) may correspond to the p25 protein described by Tsai et al. The 65KD band was also present in the control healthy cells and therefore is not related to apoptosis.

To confirm that the 25KD band was p25 protein, we used the p35 antibody to do Western Blots on CHX and CPT treated COS-7 cells. The results showed that p25 protein is expressed in both total protein (Figure 24B), and CDK5 immunoprecipitated protein during cell death (Figure 24A). At the same time, the

expression of Cdk5 remained the same, which suggested that Cdk5 expression did not increase (Figure 24C and D). These data suggests that p25 is one of the proteins that can form a complex with Cdk5 during the cell death.

4. Discussion

Through two different approaches, our study first demonstrates that p35/p25 is associated with Cdk5 during apoptosis. Early studies showed that Cdk5 associated with p35/p25 only in bovine brain, suggesting a role during neuronal cell differentiation (Lew et al. 1994; Tsai et al. 1994). However, our findings showed that Cdk5 interacts with p25/p35 and might play a role during cell death or act in a common path during the progression of cell death regardless of how the cells are signaled to die. Recent reports from other groups also showed that the Cdk5/p25 complex might play a role during apoptosis in Alzheimer's disease (Patrick et al. 1999), which confirmed our findings. In addition, our findings extended the earlier studies and indicate that Cdk5-p25/p35 interaction is not merely neuron-specific, but may occur to other tissues and cells.

We used a CP-treated day 10.5 mouse whole embryo cDNA library to screen for Cdk5-associated proteins during cell death through yeast two-hybrid system. The finding of p35 as a Cdk5 associated protein in dying cells raised a question: Did p35 express because of cell death which was induced by CP, or it is just a domestic protein which normally expressed at day 10.5? Previous data of p35 expression pattern from other groups showed that low expression of p35 was first seen in migrating young postmitotic neurons and rapidly increases as they mature (Nikolic et al.'1996). During mice embryonic development, p35 expression was detected between developmental embryonic day 15 (E15) and postnatal day 0 (P0) (Delalle and Tsai'1997). Therefore, during embryonic development, the temporal pattern of cdk5 and p35 expression and thus of the cdk5/p35 kinase activity, closely

paralleled the extent of corticogenesis after embryonic development day 10.5 (Nikolic et al.'1996; Delalle and Tsai'1997). Therefore, we confirmed that the increase of Cdk5/p35 kinase activity is related cell death, and eliminated the possibility that p35 is from endogenous expression.

From different approaches, we got p35 from yeast two-hybrid system and its truncated form p25 from immunoprecipitation. This suggested to us the desirability of investigating what caused the cleavage. Thus, we investigated the sequence of the approximate cleavage site to yield a 25KD C-terminal protein (Figure 31A). The surrounding sequence of p35 (SwissProt) is listed below:

2 mgtvlslsps yrka**tlfedg** aatvghytav qnsk**nakdkn** ikrhsiisvl pwkrivavsa
61 kknskkvqp nssuqnnith lnnenlkksl scanlst**fa**q pppaqppapp asqlsgsqtg
121 vsssvkkaph pavssagtpk rvivqastse llrclgeflc rrcyrlkhls **qtdpvixlrs**
181 **vdrslllqgw qdqgfitpan** vvflym**lcrd** viss**evgsdh** elqavlltcl ylsysymgne
241 isyplkpflv **esckeafwdr** clsvinlmss **kmlqinadph** yftq**vfsdlk** nes**gqedkkr**
301 lllgidr

The cleavage site to yield p25 (FAQPPPAQPPAPP) is in indicated in large font and underlined. All "xxxd" sequences, which are caspase-specific recognition sites, are indicated in bold. There are no particularly good caspase cleavage sites in p35. Thus, it is unlikely that p25 is generated by primary activation of caspases. An alternative mechanism should exist for the cleavage of p35 to p25. Tsai et al. has shown that calpain, which is a calcium-dependent cystein protease, could convert p35 to p25, and then involved in apoptosis (Lee et al.2000).

Utilizing two different assays (yeast two hybrid and immuno-precipitation), we got two proteins interacting with Cdk5. Using the yeast two-

hybrid system, we got p35 as a Cdk5-associated protein, while in immunoprecipitation, we could only get p25. This discrepancy could be due to the short half life of p35 (about 20-30 minutes), compared to a much more stable p25 (about 5 hours) (Patrick et al.1999). In CP-treated embryos, apoptotic cells are not synchronous, and their percentage is not as high as in cell lines. In addition, the yeast two-hybrid system was dependent on cloned cDNA expression, and the final clone was verified by DNA sequence. However, p25 could not be detected through this system, because p25 is generated by cleavage of p35. Data from Dr. Tsai's lab, as well as our lab, has shown that in the initial stage of cell death, p35 is quickly converted to p25, which is more stable and prolonging Cdk5 activation. The activated Cdk5 could hyperphosphorylate and disrupt the cytoskeleton (Patrick et al.1999; Lee et al.2000).

Western blot on ^{35}S-Methionine incorporated COS-7 total cell lysate shows that p35 could be detected in dying cells. The same cell lysates were used for immunoprecipitation, but p35 was not detected. This suggests that p35 is rapidly converted to p25. During apotosis, after p35 is quickly converted to p25, p25 could be pulled down by immunoprecipitation. In total cell lysates, the concentration of p35 was constant because it had to be constantly produced, in order to be cleaved to p25. In addition, Cdk5 protein expression was not changed in both total cell lysates and immunoprecipitation (Figure 24C, 24D). This was consistent with our previous results.

Chapter III: Functional Analysis of p35/p25 during Cell Death

1. Objective

2. Material and Methods

C. Results

- **A. Expression analysis of p35/p25 during cell death in developing mouse limbs.**
- **B. Expression analysis of p35/p25 during cell death in developing lens.**
- **C. Expression analysis of p35/p25 during cell death in CP treated mouse embryos.**
- **D. Expression and characterization of p35/p25 in induced COS-7 cell death.**

D. Discussion: p35/p25 is expressed during cell death. DNA damage, protein synthesis interruption can cause the conversion of p35 to p25. This conversion may process through cycloheximide mediated pathway.

Results and discussion:

Section III: Functional Analysis of p35/p25 during Cell Death

1. Objective

Using the yeast two-hybrid system and immunoprecipitation, we showed that p35/p25 associated with Cdk5 during cell death. To further characterize the role of p25/p35 during cell death, we investigated the roles of p35/p25 during cell death. By using several models, including: two models of normal development (developing mouse limb and rat lens), one model of abnormal development (cyclophosphamide-treated mouse embryo), and one model of *in vivo* induced cell death (COS-7 cell line) to investigate:

1. The expression pattern of p35 in these models.
2. Whether p35 was regulated at the transcriptional level, or the translational level.
3. Using an *in vitro* model, COS-7 cell line, we investigated the role of p25 in the activation of Cdk5 during apoptosis.

2. Material and Methods

a. Materials used in the experiences:

Animals and Treatments
As described in Chapter I.

Slide preparation
As described in Chapter I

Tissue fixation and preparation for frozen sections
As described in Chapter I

Probes and Antibodies
In situ probe: The p35 construct was a gift from Dr. Zelenka, National Institute of Health. It is a 250bp insert digested at NotI (for antisense) and BamHI (for sense).

Antibodies: Anti-Cdk5 (C-8), and Anti-p35 (C-19) are from Santa Cruz Inc.

Vector: pCMS-EGFP was from Clontech Inc.

b. Cell biology technologies:

Trypan blue staining
As described in Chapter I

Immunohistochemistry
As described in Chapter I

Immunohistochemistry and DNA fragmentation double labeling
As described in Chapter I

Cell transfection
As described in Chapter I

c. DNA characterization technologies:

Transformation
As described in Chapter I

Plasmid preparation
As described in Chapter I

In situ hybridization
As described in Chapter I

DNA analysis for fragmentation
As described in Chapter I

Northern blot hybridization
As described in Chapter I

d. Protein characterization technologies:

immunoprecipitation and kinase assay
As described in Chapter I

Western blot
As described in Chapter I

3. Results

After identifying the interaction of p35/p25 with Cdk5 during cell death, we investigated its expression pattern and regulation in various cell death models. We detected p35/p25 expression in the developing limbs, developing lens, CP-treated mouse embryos and apoptotic COS-7 cells.

A. Expression of p35/p25 during cell death in the developing mouse limb:

To investigate the p35/p25 expression pattern in the developing limb, two p35 antibodies were used. One was specific to the C-terminal region of p35, which recognized both the p35 and p25 shared sequence, while another was specific to the N-terminal region of p35 protein, which recognizes only p35. Serial limb sections of day 14.5 embryos were used for immunohistochemistry (see Materials and Methods). Antibodies to p35 proteins showed a specific expression pattern which was similar to that of Cdk5 expression (Figure 25A). There was a specific signal with the antibody against the C-terminal end of p35. This signal could be completely blocked by p35 peptide. The antibody specific to the p35 N-terminal end did not give a signal above background (data not shown), which suggested that the expression was specific to p25, but not p35. To localize p35/p25 expression in apoptotic cells in the developing mouse limb, we used fluorescent double-labeling for *in situ* DNA fragmentation and p35 protein detection. The sections were analyzed by confocal microscopy (see Materials and Methods). The result showed that the expression of p35 was localized to the cells

with fragmented DNA (Figure 25B-E). Thus, our results showed that p35/p25 was upregulated in the dying cells.

Since the expression of p35 was co-localized in the interdigital region of the limb, we asked if the expression of p35 mRNA was upregulated as well. *In situ* mRNA hybridization analysis (see Materials and Methods) indicated that there was no differential expression of the protein in healthy and dying cells (Figure 26). Furthermore, western blot analysis (see Materials and Methods) of day12.5 and day13.5 mouse limb (Ahuja et al. 1997) showed that there was no significant increase in p35 expression. Western blot analysis using mouse limbs and p25 antibody did not show an upregulation of the protein. We believe that this is due to the low percentage of apoptotic cells in the limb tissue, and the expression of p25 is too low to be detected. Or, apoptosis is not synchronous in this *in vivo* system.

Overall, these results suggested that the regulation of Cdk5 by p35/p25 was not at the transcriptional or translational level.

B. Expression of p35/p25 during cell death in the developing rat lens:

Serial rat lens sections of day 18 embryos were used for immuno-histochemistry using p35 antibodies (C-terminal). The expression pattern of p35/p25 was similar to that of Cdk5 (Figure 27). In certain fiber cells, staining is shown to be both nuclear and cytoplasmic (Figure 27B, 27C arrows). Controls performed with normal rabbit serum were negative (Figure 27A). This result showed that the expression of p35/p25 might increase during apoptosis.

Then, the mRNA expression pattern was investigated to illustrate whether p35 was regulated at transcriptional level. *In situ* hybridization (see Materials and Methods) showed that a p35 was evenly distributed in all of elongating fiber cells (Figure 28B), and fiber cells in the bow region of the lens, the region which did not undergo apoptosis (Figure 28B arrow). In addition, strong hybridization was found in the neural retina and cornea (Figure 28B). In control experiments, sense probe did not show any hybridization (Figure 28A). This suggested that the increased p35/p25 protein was not due to upregulation of its mRNA. These results suggested that p35/p25 might not be upregulated at the transcriptional level during cell death in the lens, similar to that we found in the limbs.

C. Expression of p35/p25 during cell death in CP-treated mouse embryo

To determine the expression pattern of p35/p25 in CP-treated day 10.5 mouse embryos, we used immunohistochemistry by p35 antibodies (C-terminal). A specific expression pattern of p35 was shown and similar to that of Cdk5 (Figure 29A). Western blot analysis showed that there was a slight increase of p35 in CP-treated embryo (Figure 29B). We also detected an increase in p25 expression (Figure 29B). This indicated that p25 was generated during apoptosis. This result confirmed our earlier findings that p25 was generated during apoptosis in COS-7 cells.

We then asked whether the elevation of the p35/p25 protein reflected an up-regulation of its messenger RNA. Using *in situ* hybridization, we localized the

p35 mRNA to the different areas of the embryo sections. No differential expression of p35 mRNA in the developing embryo (Figure 30A). Northern blot analysis also showed that there was no increase of p35 mRNA(Figure 30B). These results suggested that the regulation of p35 during cell death was not at the level of transcription.

In summary, all these results showed that p35/p25 was specifically related with apoptotic cells. The increase of p35/p25 is related with post-translational modifications.

D. Expression and characterization of p35/p25 in induced COS-7 cell death.

Using different *in vivo* models, we found that p35/p25 was expressed in apoptotic cells, and the expression was associated with the activation of Cdk5 protein. To investigate how Cdk5/p35/p25 complex functions during apoptosis, it was essential to find a cell death model in which Cdk5 activity could be manipulated. Among several induced cell death models that have been studied, we selected COS-7 cells induced to die by cell death-inducing reagents. As presented before Cdk5 activity is increased in apoptotic COS-7 cell. We examined the expression of p35/p25 in COS-7 cells treated with various cell death-inducing reagents.

Western blot analysis clearly showed an increase of p25 in cells treated with cell death-inducing reagents: cycloheximide (CHX) and camptothecin (CPT) (Figure 30C). We did not detect an increase of p35 expression in same samples. As

we showed previously, Cdk5 kinase activity increased in CHX and CPT treated COS-7 cells (Figure 13B). These results suggest that the increase of Cdk5 activity in apoptotic COS-7 cells is regulated by the association of Cdk5/p25 complex.

To further investigate how Cdk5/p25 regulates during apoptosis, p35 anti-sense gene (from pcDNA3-hp35) was cloned into expression vector pCMS-EGFP (Figure 31), which could generate fluorescent signal after being transfected into cells.

First, we examined the transfection efficiency. Since the expression of EGFP gene generates a fluorescent signal, it was easy to verify the transfection and expression efficiency. As shown in Figure 32, no fluorescent signal was detected in the untransfected cells (Figure 32A, 32B), while there were many fluorescent signals in pCMS-anti-p35 transfected cells (Figure32C, 32D). The number of fluorescent signals showed that more than 35% of cells were transfected. Since anti-p35 gene is in the same construct with EGFP gene, these cells should also express the anti-p35 gene. We expected that antisense gene should also be transcripted and block the p35 mRNA and thus p25 generation. Figure 33A showed that there was no p25 expression in p35 antisense transfected cells.

These antisense transfected cells were then treated with cell death-inducing reagents, and the expression of p25, Cdk5, as well as Cdk5 histone H1 kinase activity was investigated. Western blot analysis shows that the expression of p25 was blocked in pCMS-anti-p35 transfected COS-7 cells (Figure 33A). Under these experimental conditions, Cdk5 expression remained constant (Figure 33B). However, induced Cdk5 histone H1 kinase activity was blocked (Figure 33C).

Interestingly, when we looked for cell visibility in p35 antisense transfected cells, we found that CHX and CPT had different effects on cell survival. When p25 expression was blocked, in CPT treated COS-7 cells, cell counts shows that apoptosis was not prevented compare with CPT treated normal COS-7 cells (Figure 34A), On the other hand, when these cells are treated with CHX, cell counts shows that cell apoptosis was blocked compare with CHX treated normal COS-7 cells (Figure 34A). In CHX treated cells, the cell death rate dropped from about 43% to about 30% (Figure 34A), and the DNA fragmentation signals could hardly be detected (Figure 34B).

These results suggest that apoptosis induced by CHX and CPT might follow different apoptotic pathways. Active Cdk5 could be involved in CHX-induced cell killing, but CPT may not require active Cdk5 to trigger apoptosis.

4. Discussion

p25 is the C-terminal fragment of p35 generated by calpain (Lee et al.2000) (Figure 35A). Protein-protein interaction studies have shown that both p25 and p35 can bind to Cdk5 (Lew et. al.1994; Tsai et. al.1994). Recent studies show that dimerization of Cdk5/p25, as well as that of p35/Cdk5, can form tau protein kinase II. The kinase phosphorylates tau protein, destabilizes the cytoskeleton, and then leads to cell death (Patrick et al.1999; Kusakawa et al.2000; Lee et al.2000; Ahlijanian et al.2000; Smith et al. 2002).

Our findings confirm that p25 regulates Cdk5 kinase activity during apoptosis. In mouse embryonic limb and rat developing embryonic lens, immunohistochemistry shows a slight increase of p35 expression during cell death; this up-regulation is correlated with induced Cdk5 activity. In CP-treated embryo, however, we found that it was p25, but not p35, that increased during cell death. Since p25 is the truncated form of p35 (Figure 35A), and both of them share the same C-terminal sequence, it is not possible to recognize which of the two proteins is present in immunohistochemistry studies by using an antibody that can recognize the common C-terminal region. By using an antibody that can recognize the N-terminal region of p35, which is not a fragment of p25, we showed that p25 is the likely partner of Cdk5 involved in the activation of apoptosis.

In CP-treated mouse embryo, we could detect the expression of p25 by western blot, but not in the limb and lens. This might be due to the small percentage of apoptotic cell in the developing limb and lens. In CP-treated mouse embryo, the number of dying cells increased and therefore the amount of p25 protein was enough to be detected.

Western blot analysis showed that p25, not p35, increased during cell death. The mechanism of p35 cleavage to provide p25 could be probably explained as follows. The half-life of p35 is only about 20-30 minutes, which could not constantly activate Cdk5. During apoptosis, p35 could be cleaved into p25, which has a much longer half-life. The accumulated p25 in apoptotic cells could cause prolonged activation of Cdk5. p25/Cdk5 is more potent than p35/Cdk5 in phosphorylating protein substrates such as tau and neurofilaments (Patric et al.1999). Thus, activated p25/Cdk5 might trigger the hyperphosphorylation of the microtubule-associated protein tau. Tau hyperphosphorylation could cause microtubule destabilization. This might be associated with morphological degeneration and cytoskeletal disruption, and in turn, cause apoptosis (Tsai et al. 2001).

In the functional study of Cdk5/p25 during cell death, we found that the increased p25 expression was common in CHX and CPT treated cells (Figure 33A). It was reported that some cell death signals, including oxidative stress, excitotoxin, and amyloid β–peptide (Aβ), can increase Ca^{2+} concentration, and consequently activate calpain to cleave p35 to p25. p25 could bind Cdk5 with high affinity, and keep Cdk5 activated. Activated Cdk5 could disrupt the cytoskeleton and cause cell death (Lee et al.2000, Ahlijanian et al.2000). In our study, we interrupted DNA synthesis by treating embryos with Cyclophosphamide (CP) or cells with Camptothecin (CPT); as well as interrupted protein synthesis by treating cells with Cycloheximide (CHX). We found that all these compounds increase the generation of p25. This indicated that, besides oxidative stress, amyloid β–peptide, and excitotoxin signals from outside, cell death signals from inside, such as DNA damage and protein synthesis interruption, could also cause the p25

expression by cleavage of p35 (Figure 35B). Thus, p25 may play a central role in the activation of apoptosis.

We found that Cdk5/p25 might be involved in CHX-induced apoptosis pathways. When p35/p25 expression was blocked by p35 antisense, we were able to partially block cell death induced by CHX. However, the cell death induced by CPT could not be blocked in the same way. This suggested that p25/Cdk5 are required in the triggering of apoptosis by CHX. A possible mechanism by which CHX kills cells is as follows: CHX blocks the translocation reaction on ribosome, leading to the inhibition of protein synthesis. As a result of the combination of the protein synthesis interruption and the short half-life of p35 (20-30 minutes), the level of p35, as well as p25, might drop quickly. Without the presence of p35/p25, Cdk5 could not be activated, and then the cytoskeleton could not be disrupted, and the process of apoptosis is blocked. On the other hand, because p35/p25 is not regulated at the transcriptional level, blocking DNA synthesis by CPT could not affect the Cdk5 activity. Thus, cell death could not be affected by CPT.

Conclusion:

We have used four models to study the role of Cdk5 during cell death. These four models include: the normal developing embryonic mouse limb, the normal developing embryonic rat lens, the CP-treated mouse embryo, which cell death is enhanced, and the CHX, or CPT-treated COS-7 cells, in which cell death is induced. The predominant themes of this work have been the importance and regulation of Cdk5 during cell death. Our studies by using these four models illustrated that Cdk5 expression is correlated with cell death process, it is regulated at the post-translation level by increase of it's kinase activity. By using a yeast two-hybrid system and ^{35}S-Met incorporation pull down experiment, we demonstrated that the Cdk5's activity is regulated by p25, which is the truncated form of p35—one of Cdk5 activator. The relationships between Cdk5 and p25 were further studied by several experiments. We showed that the appearance and induction of p25 in our different cell death systems correlates with the induction of kinase activity of the Cdk5 protein, as well as cell death. Additionally, induced apoptosis by CHX can be partially blocked when we inhibited p35/p25 expression, which indicates that Cdk5/p25 complex maybe involved in CHX induced cell death pathway at that p25 is needed for Cdk5 kinase activity.

FIGURES

Figure 1.Localization of apoptotic cell death and Cdk5 expression in mouse embryonic limbs.

A. DNA fragmentation assay on frozen section from Day 14.5 embryonic hindlimbs (10X). Black arrows indicate apoptotic cells in the interdigital region of the developing limb.
B. Frozen section from Day 14.5 embryonic hindlimbs (10X) were stained with Cdk5 antibody (c-8). Black arrows indicated Cdk5 expression cells.
C. DNA fragmentation with FITC shows a dim background with a bright punctuation in cells with the fragmented DNA in the interdigital regions (4X).
D. The detection of Cdk5 by cy3 shows a dim background with the bright punctuation of the Cdk5 expression in the cells of the interdigital region (4X).
E. The confocal overlaid image shows intense yellow staining where the two signals of green and red overlap in the same area (4X).
F. An enlargement of the region indicated in panel E by the box (10X).
G. An enlarged of panel F as indicated in the box (40X).

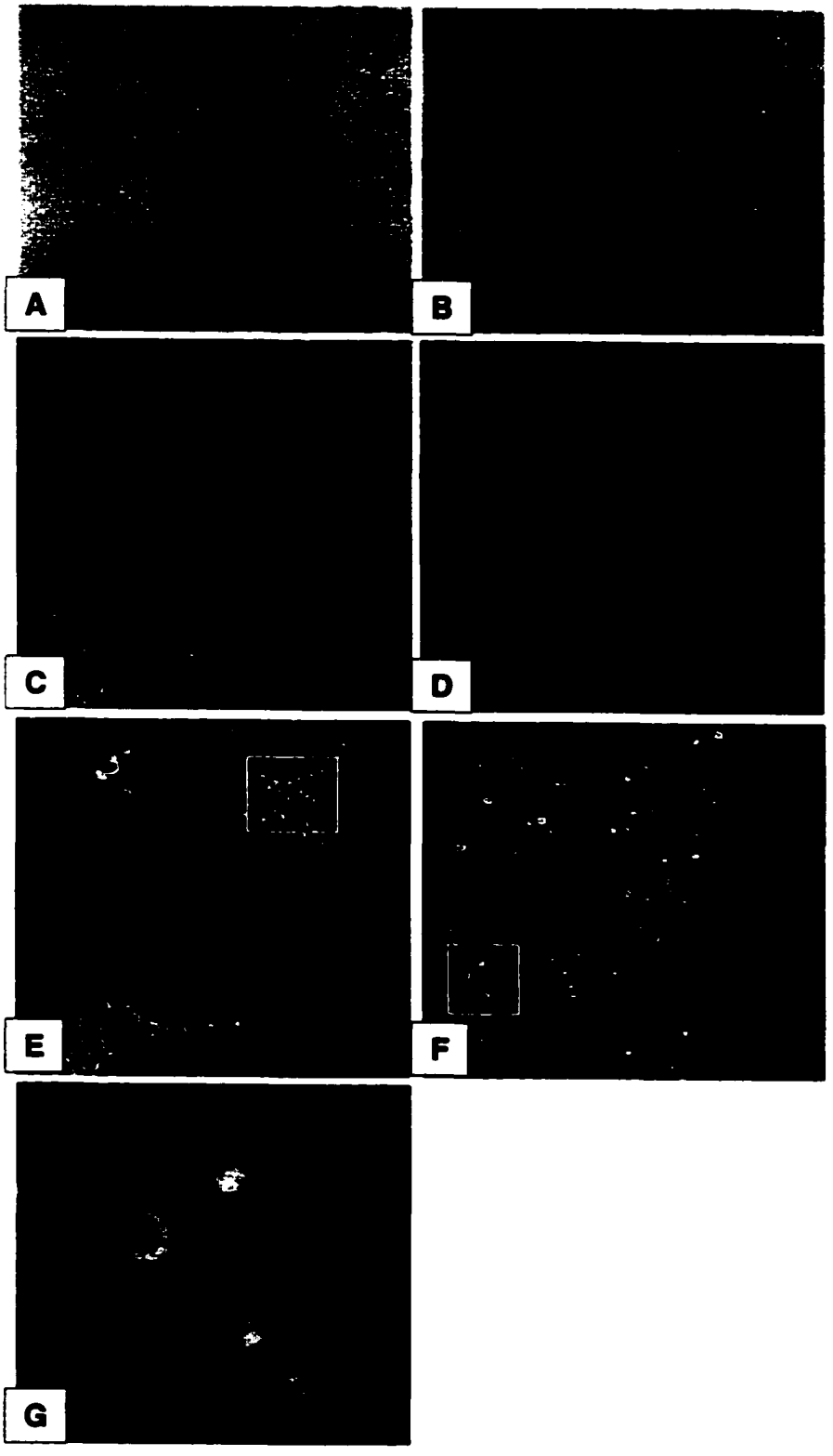


Figure 1

Figure 2. Expression of Cdk5 mRNA in the dying cells by *in situ* hybridization.

A. Immunohistochemical analysis for the localization of Cdk5 protein in the dorsal root ganglia (DRG). The arrows indicated the DRG with a few punctuated darkly stained cells representing the dying cells that are expressing Cdk5 protein.

B. A darkfield image of the mRNA expression of the Cdk5 using the antisense probe. Expression is relatively high throughout the DRG and is not limited to the dying cells.

C-F. The interdigital region of the developing hand plate in which we can find dying cells (as indicated by arrow in panel E and F). In panel C (darkfield) and E (lightfield), Cdk5 sense probe was used to detect control background levels. In panel D (darkfield) and F (lightfield), antisense Cdk5 probe was used. The reddish cells in the dark field represent the dying cells with condensed nuclei or the neighboring phagocytic macrophages that usually refract light in dark field.

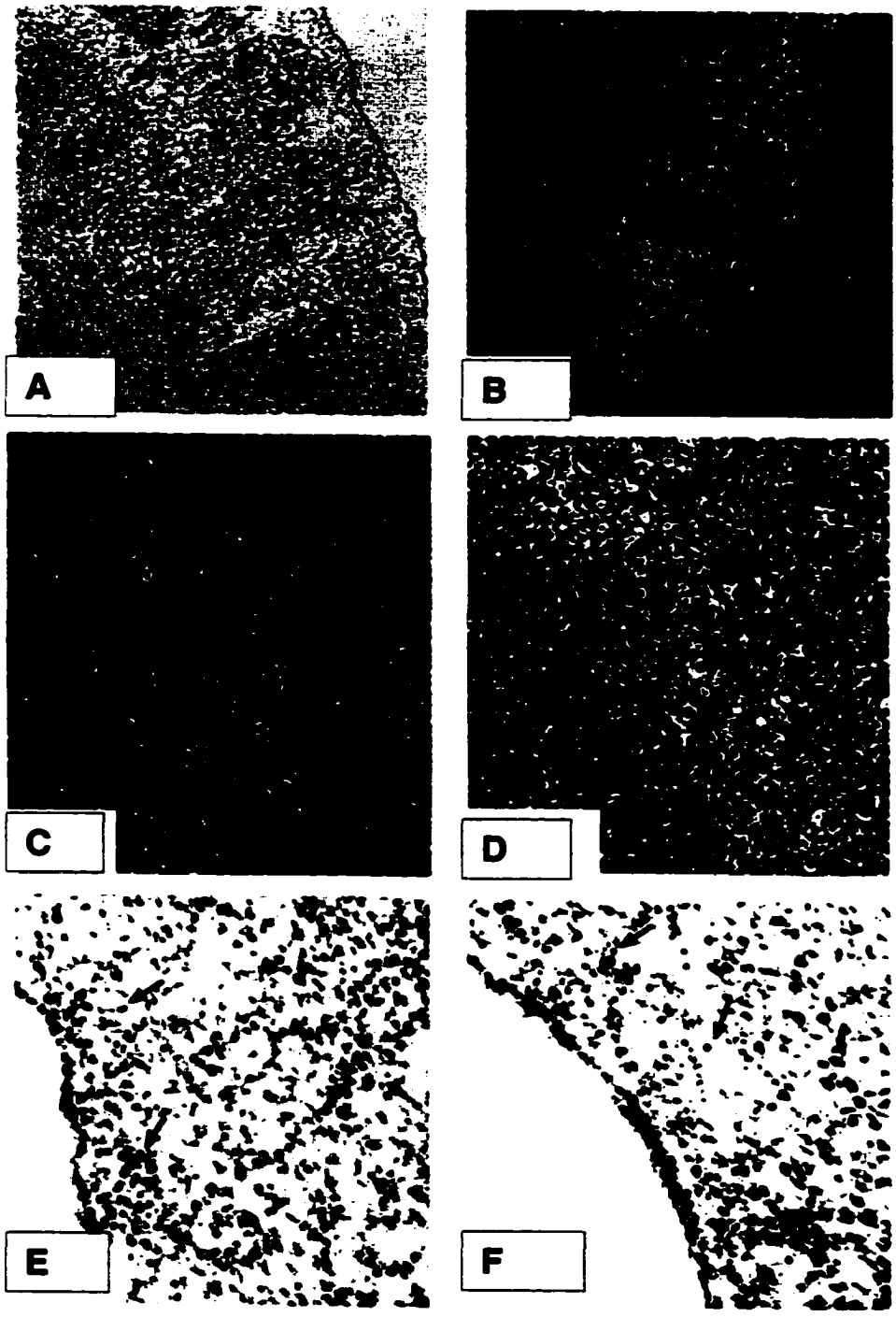


Figure 2

Figure 3. The expression of Cdks in cell death of the developing limb is unique to Cdk5. Serial sections of day14.5 mouse hand plate were used for the detection of different Cdk proteins by immunohistochemistry. A=control Cdk5 protein; B=Cdc2 (Cdk1); C=Cdk2; D=Cdk3; E=Cdk4; F=Cdk5; G=Cdk6; H=Cdk7; I=Cdk8. Arrows indicated that there is Cdk5 expression (F) in the interdigital region of limbs. Other activities seen are nonspecific.

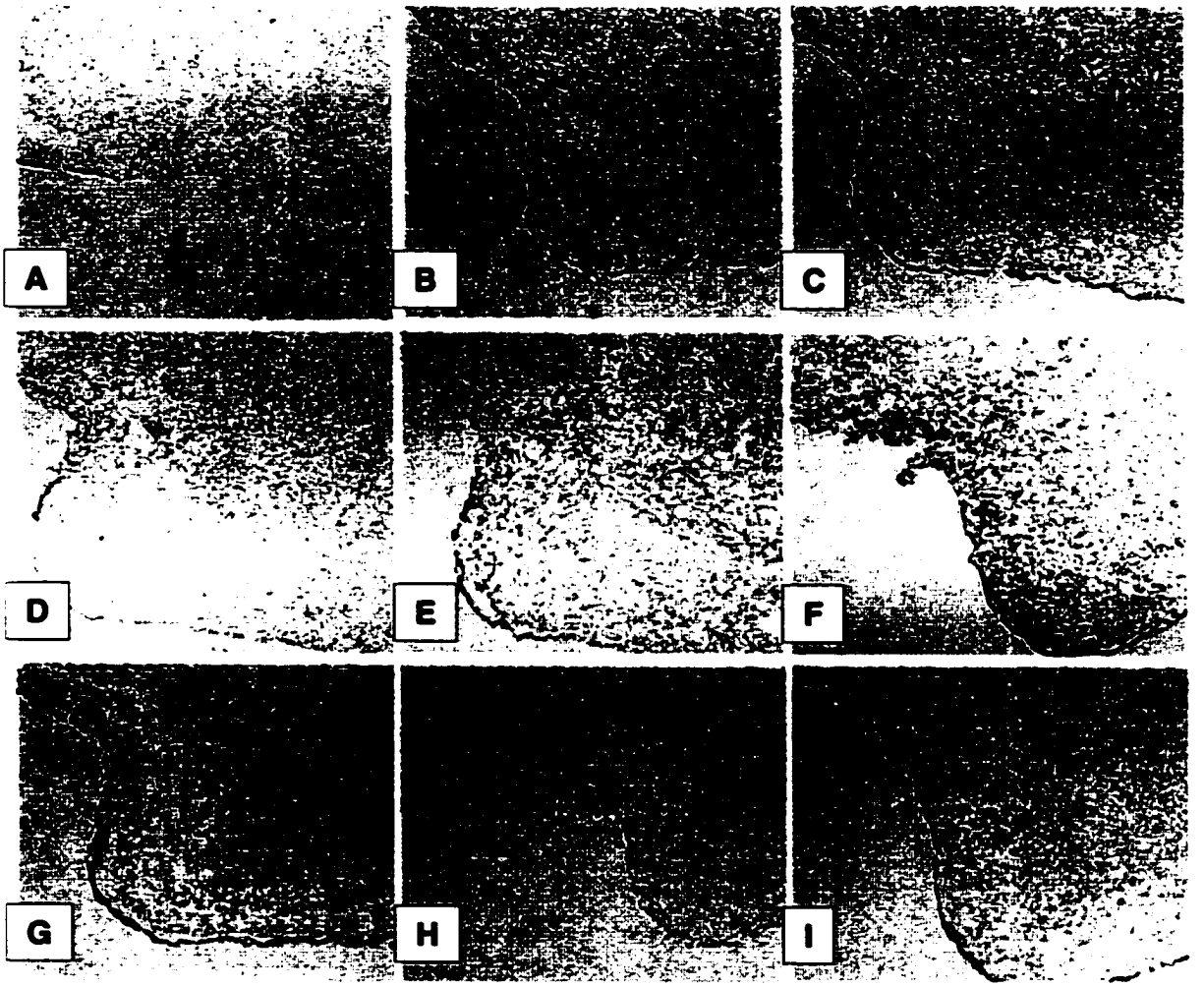


Figure 3

Figure 4. Development process of the lens fiber cells.
Panel A shows lens vesicle.
Panel B shows elongation of the interior cells, producing lens fibers.
Panel C shows lens filled with crystalline-synthesizing cells.
Panel D shows new lens cells derived from anterior lens epithelium. Center lens fiber cells nuclei disappear.
Panel E shows as the lens grows, new fibers differentiation, and nuclei degenerate.

From Developmental Biology (2nd edition)
Author: Scott F. Gilbert

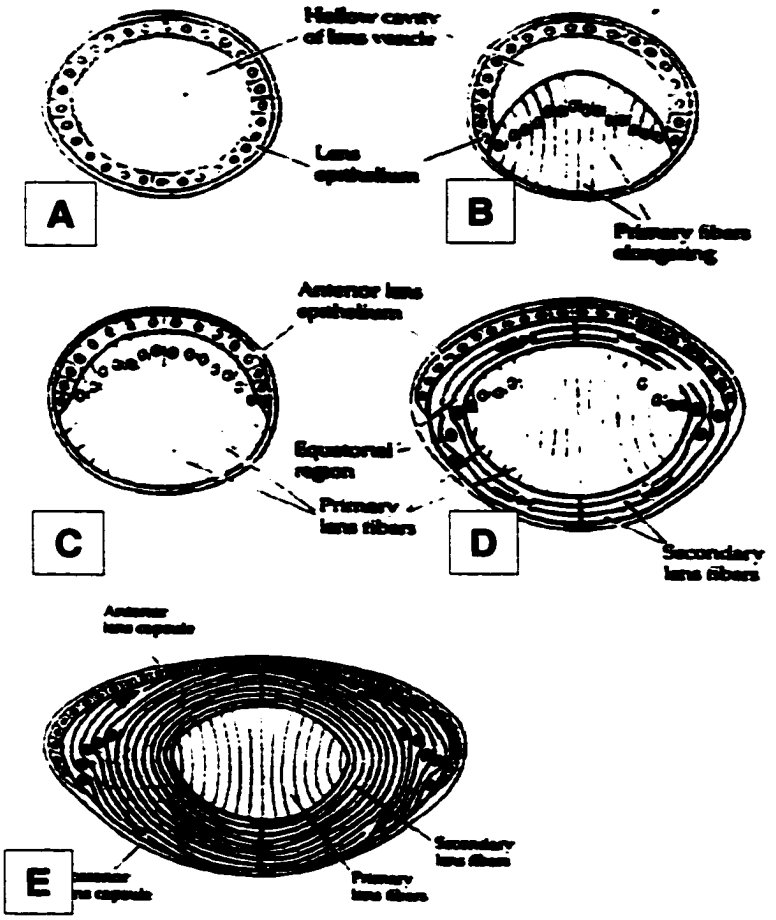


Figure 4

Figure 5. Localization of Cdk5 expression and DNA fragmentation in developing rat lens at E18. Near serial section of paraffin-embedded rat lens were exposed to antibodies for immunohistochemistry staining, or DNA fragmentation staining as described under Materials and Methods.

Panel A shows section incubated with antihuman Cdk5 (c-8), with cornea (C), lens (L), and retina (R) as indicated.

Panel B shows control section incubated with normal rabbit serum.

Panel C is higher magnification of the anterior region of the lens from panel A. Positive staining in the anterior tips of the fiber cells (L), and in some primary lens fiber cell nuclei (black arrow) were shown.

Panel D shows DNA fragmentation with FITC. A dim background with a bright green punctuation in cells indicated the fragmented DNA (white arrows).

Panel E shows the detection of Cdk5 by cy3. A dim background with the bright red punctuation indicated the Cdk5 expression in the fiber cells (white arrows). (C=cornea, L=lens, R=retina)

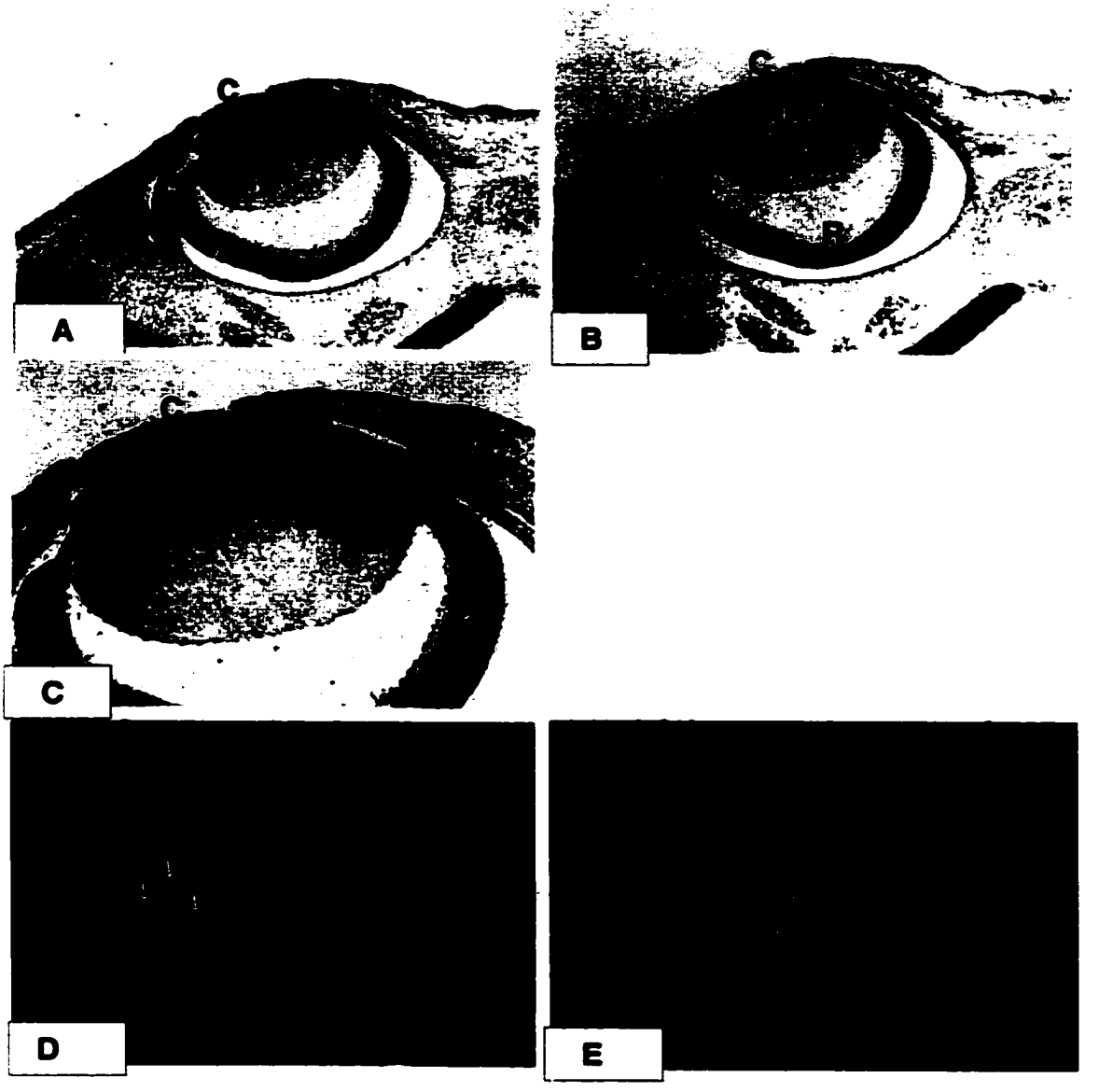


Figure 5

Figure 6. Expression of Cdk5 mRNA in the dying cells by *in situ* hybridization.
Panel B is the hybridization with radioactively labeled Cdk5 sense riboprobe photographed with darkfield illumination.
Panel C is the hybridization with radioactively labeled Cdk5 antisense riboprobe photographed with darkfield illumination. Arrow indicates elongating fiber cells near the bow region of the lens.
(C=cornea, L=lens, R=retina)

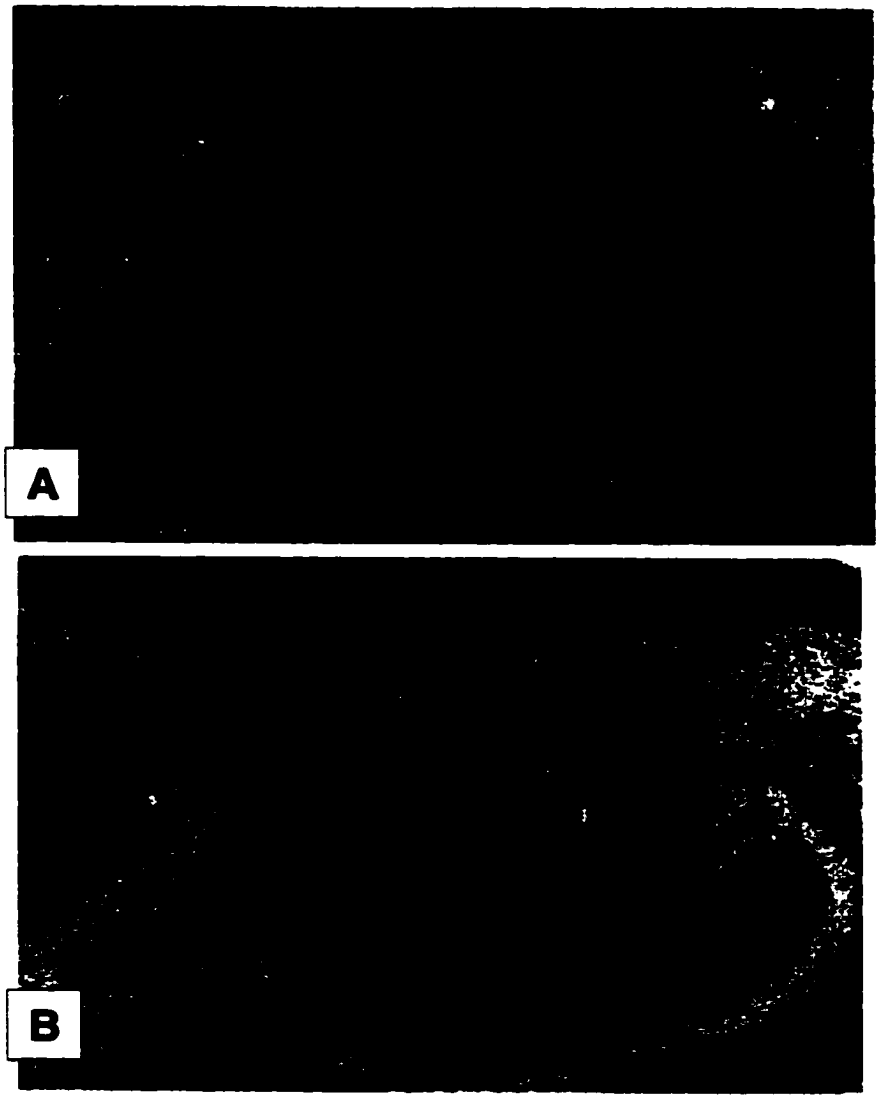


Figure 6

Figure 7. The expression of Cdks in cell death of the developing lens is unique to Cdk5. Serial sections of E18 rat lens were used for the detection of different Cdk proteins by immunohistochemistry. A=Cdk2, B=Cdk4, C=Cdk7, D=Cdk8, E=control of normal rabbit serum. No signal above background of Cdk2 and Cdk4, none specific activities in nuclei only of Cdk7 and Cdk8 which indicated by arrows.

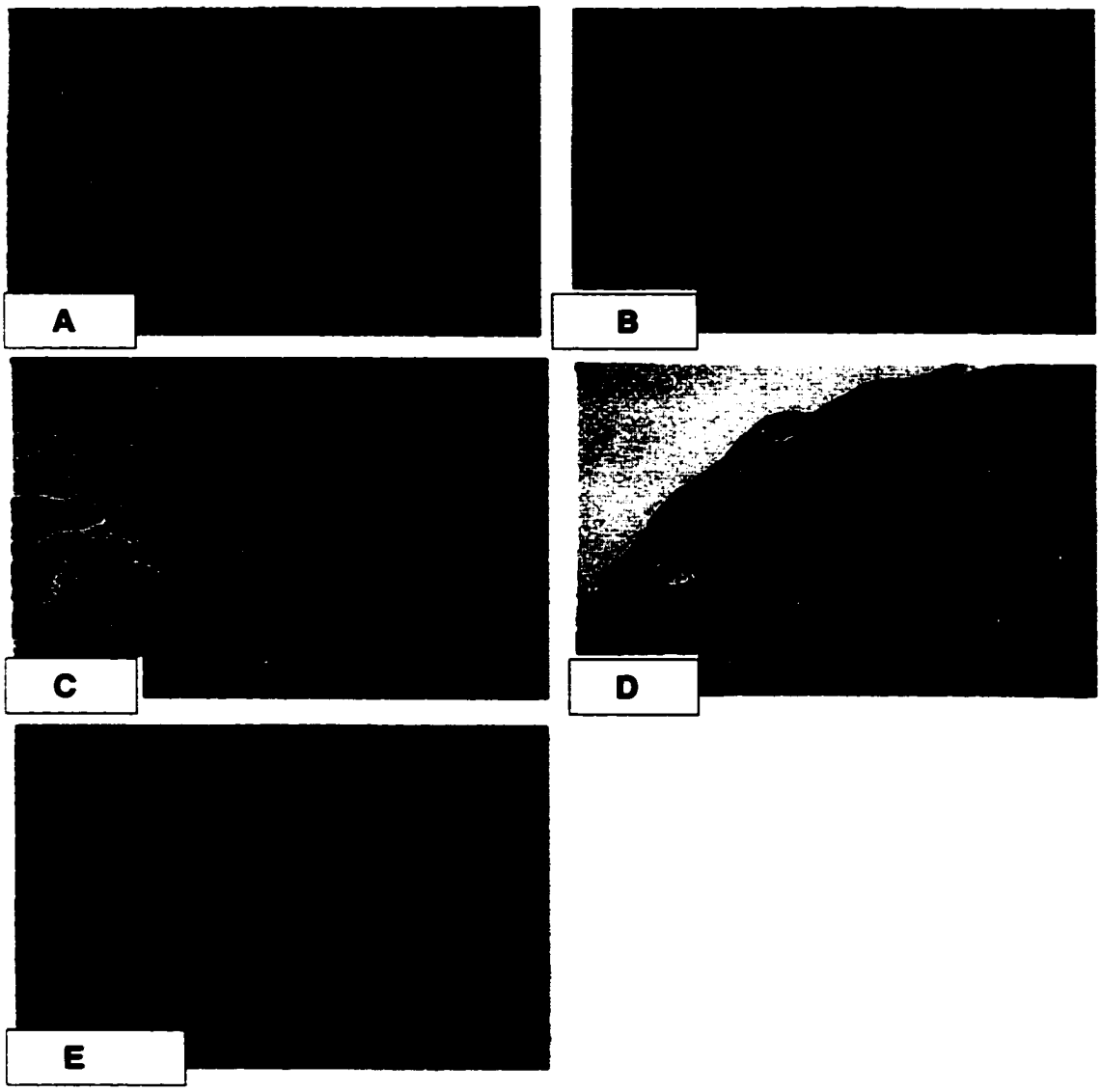


Figure 7

Figure 8. Localization of Cdk5 expression with apoptotic cell death in CP-treated day10.5 mouse embryos. Pregnant female mice were injected intra-peritoneally with 10mg CP/kg body weight as described in Materials and Methods. Near serial frozen sections were processed for different detections.

A. DNA fragmentation assay on frozen section from CP-treated mouse embryo.
B. An enlargement section of the region indicated in panel A by the box. Black arrows show apoptotic cells.
C. An enlarged section of panel A as indicated in the box. Black arrows show apoptotic cells.
D. Frozen section from CP-treated mouse embryos were stained with Cdk5 antibody (c-8).
E. An enlargement section of the region indicated in panel D by the box. Black arrows show Cdk5 expressed cells.
F. An enlarged section of panel D as indicated in the box. Black arrows show Cdk5 expressed cells.

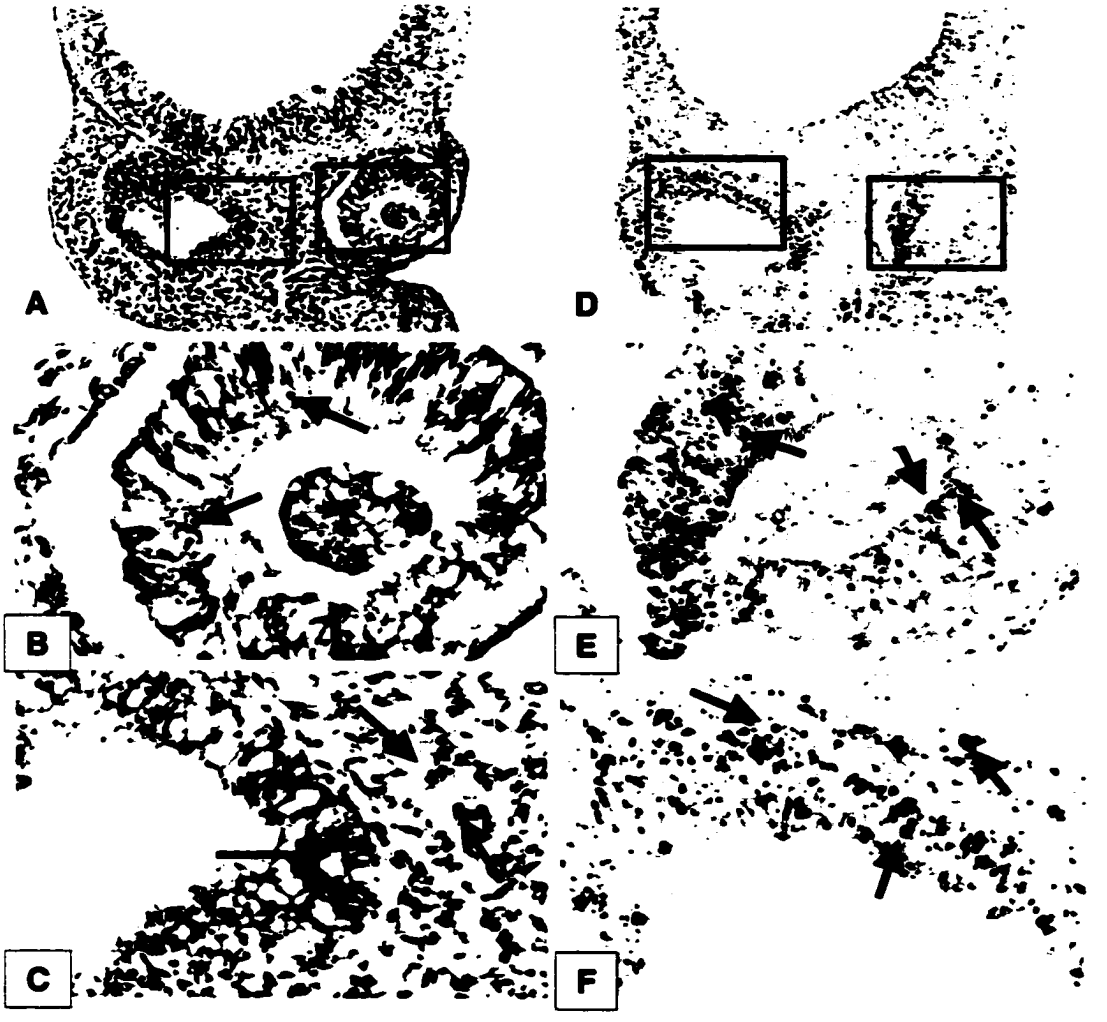


Figure 8

Figure 9. Cdk5 expression is associated with cell death induced in CP-treated embryos. Pregnant female mice were injected intra-peritoneally with 10mg CP/kg body weight as described in Material and Methods. Near serial frozen sections were processed for different detections.

A. DNA fragmentation with FITC shows a dim background with a bright punctuation in cells with the fragmented DNA.
B. The detection of Cdk5 by cy3 shows a dim background with the bright punctuation of the Cdk5 expression in the cells.
C. The confocal overlaid image shows intense yellow staining where the two signals of green and red overlap in the same area.
D. An enlargement of panel E.

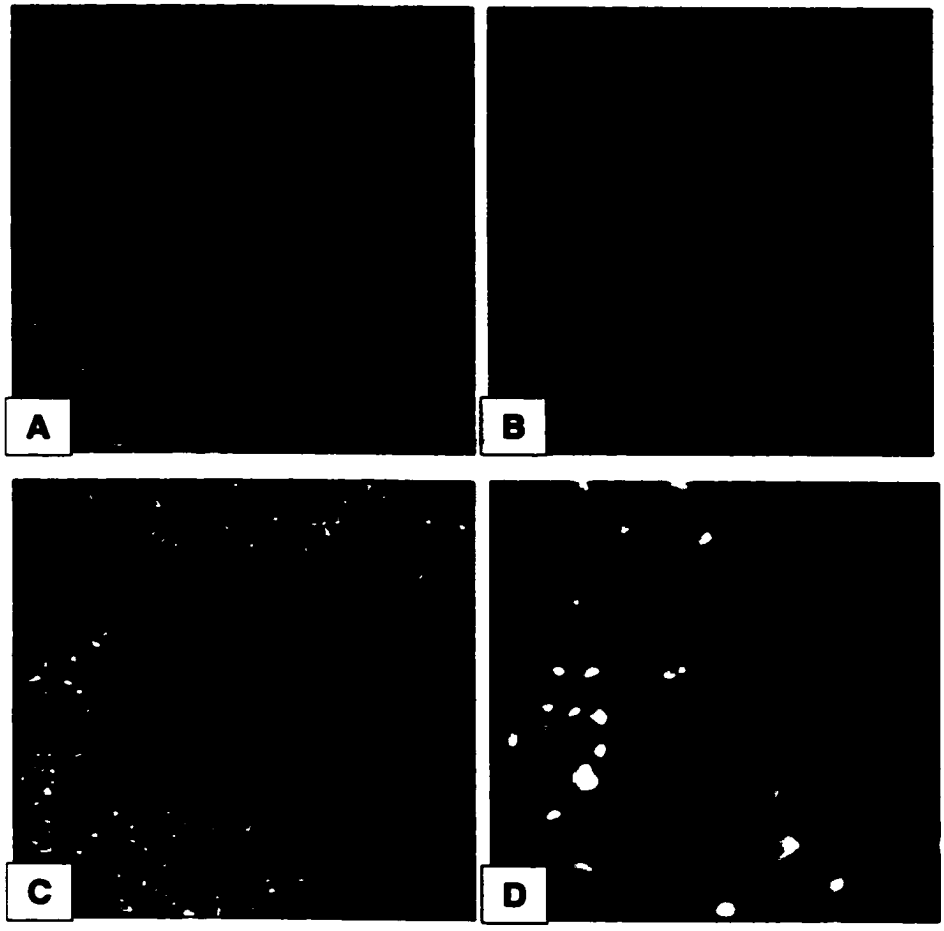


Figure 9

Figure 10. Expression of Cdk5 mRNA in the dying cells by in situ hybridization and northern blot. Pregnant female mice were injected intra-peritoneally with 10mg CP/kg body weight as described in Material and Methods.

A. Panel a represents the Cdk5 sense probe to detect control background levels. Panel b represents signals from antisense Cdk5 probe.

B. The northern blot of control embryo and CP treated embryo. There is no different Cdk5 mRNA expression.

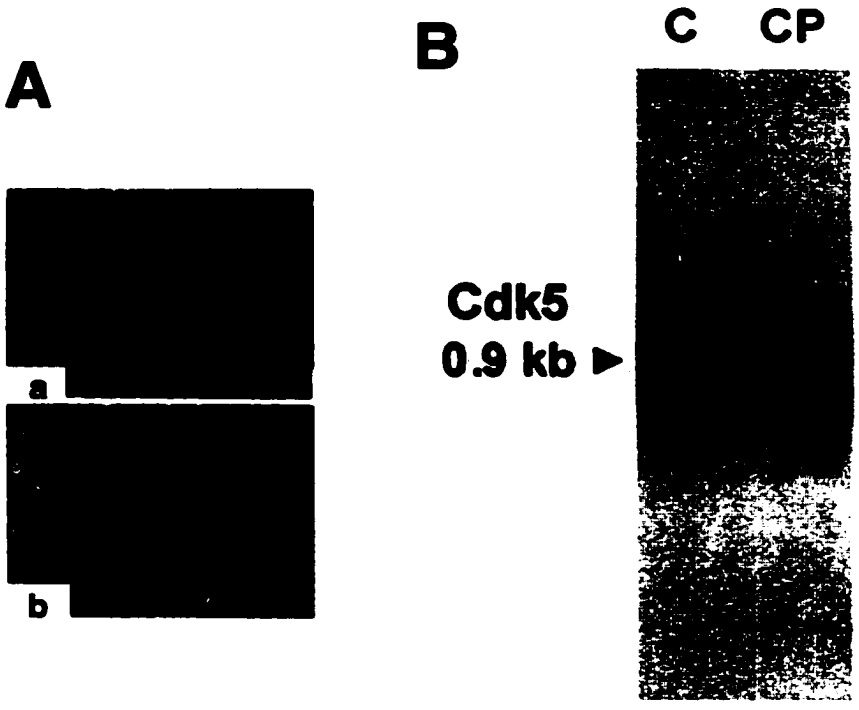


Figure 10

Figure 11. Cdk5 is not up-regulated in dying cells at translation level but at post-translation level. Pregnant female mice were injected intra-peritoneally with 10mg CP/kg body weight as described in Material and Methods.

Panel A shows the western blot to compare the expression of Cdk5 protein of control and CP treated embryo. Left: coomassie blue staining of both control and CP treated embryo. Middle: western blot of both tissues. Right: Quantity of the bands in western blot. There is no significant increase of Cdk5 expression in CP treated embryo.

Panel B shows the Histon H1 kinase activity assay of control and CP treated embryo. A significant increase of Cdk5 Histon H1 kinase activity in CP treated mouse embryo (3X higher than that of control) was detected.

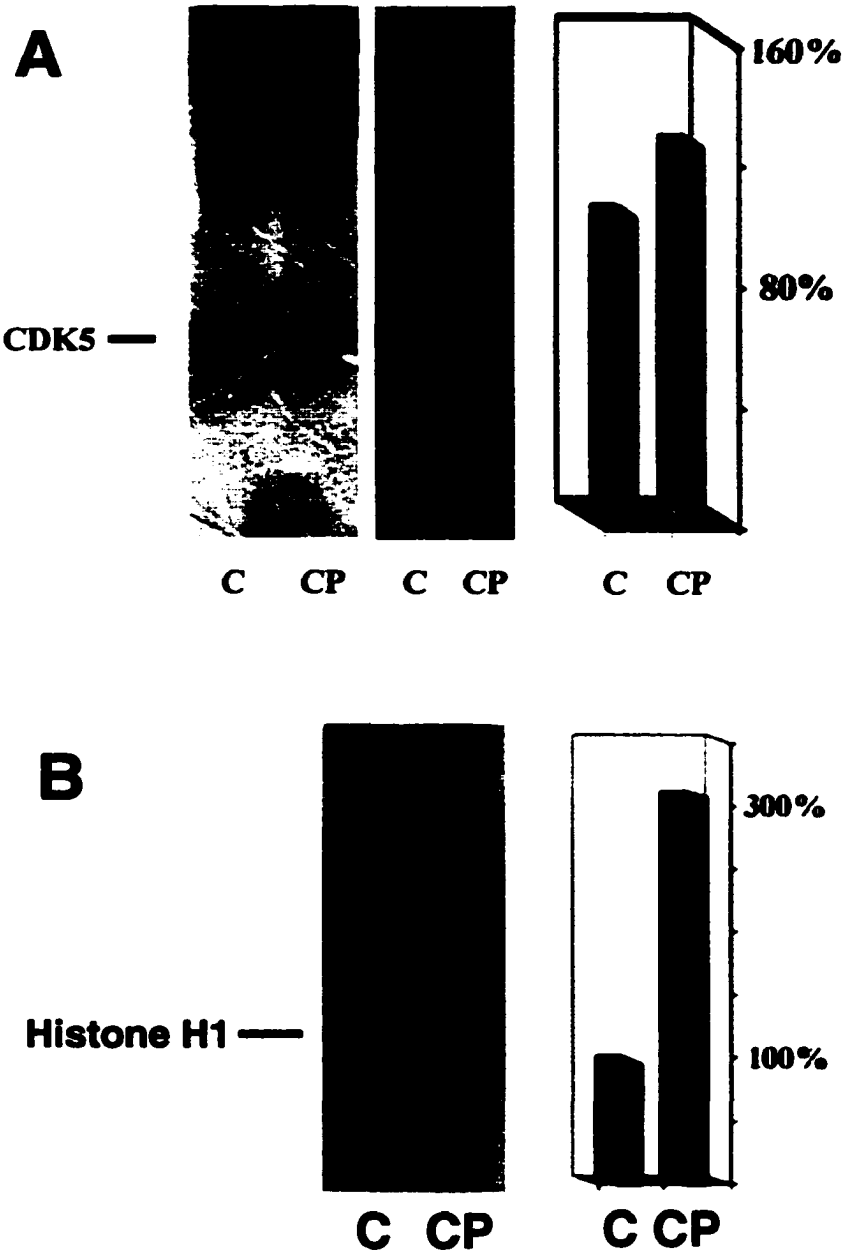
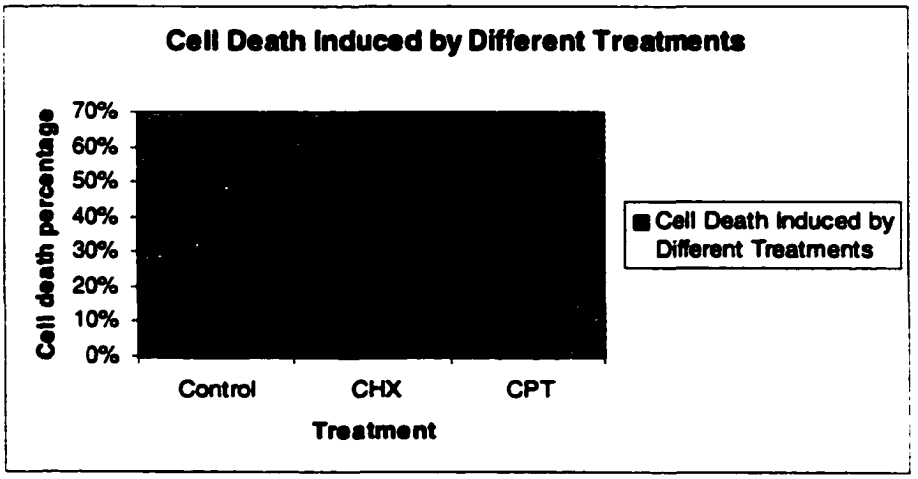


Figure 11

Figure 12. Identification of Cycloheximide (CHX) or Camptothecin (CPT) induced COS-7 cell death is apoptosis. COS-7 cells were cultured in DMEM cell culture medium with 10% FBS. Before each treatment, medium was changed to DMEM with 0% FBS, with CHX (100ug/ml final concentration), or CPT (15uM final concentration). Control cells did not receive CHX or CPT treatment. After 18 hours, cells were collected, washed, and used for various assays for different detections.

A. Trypan Blue staining showed that there was about 45% cell death in CHX treatment, and 55% cell death in CPT treatment.
B. DNA fragmentation assay showed that both CHX and CPT treatment leads to fragmentation.

A



B

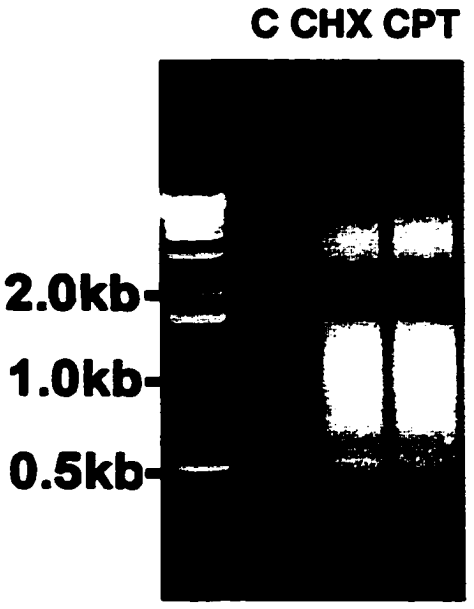


Figure 12

Figure 13. Cdk5 expression and its kinase activity assay in apoptotic COS-7 cells. CHX or CPT treated COS-7 cells were collected and washed for Western blot and Histon H1 kinase assay.

A. Western blot analysis shows that there is no significant change in Cdk5 protein expression.
B. Kinase activity shows that, in both CHX and CPT induced COS-7 cell death, Cdk5 histon H1 kinase activity increased.

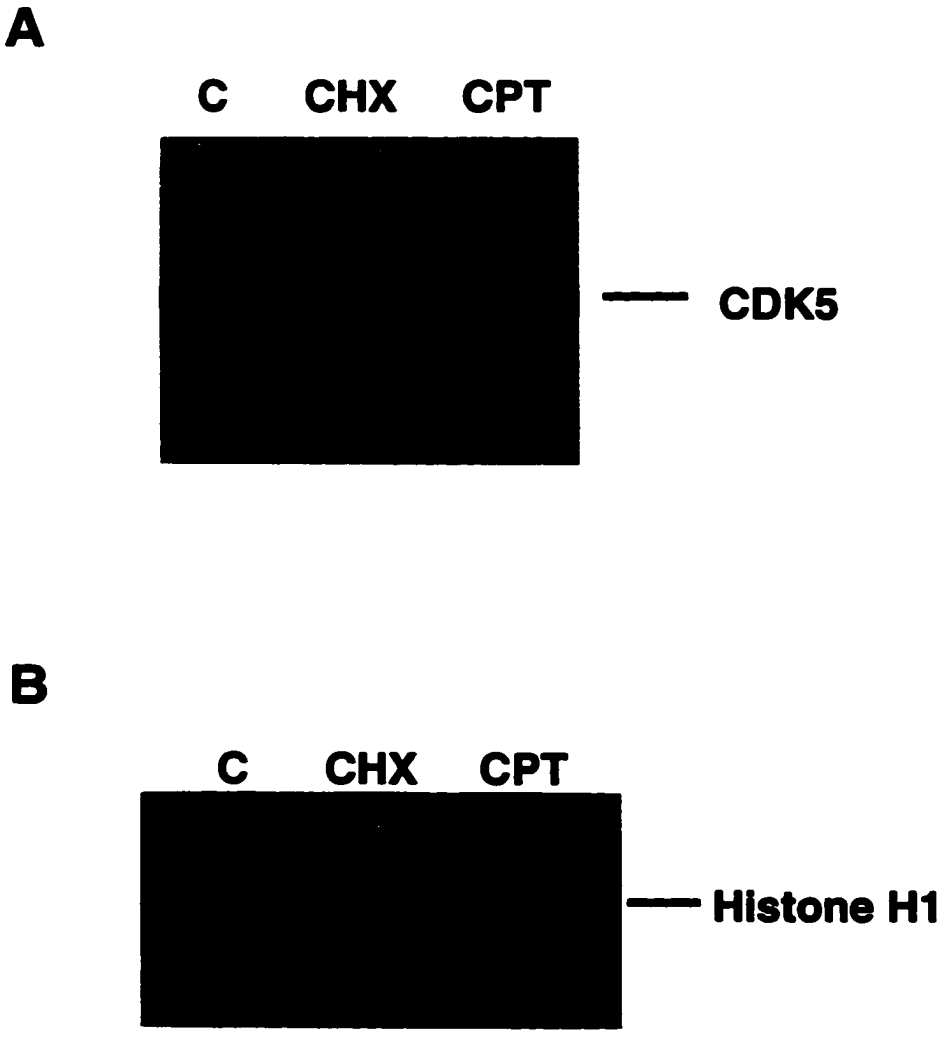
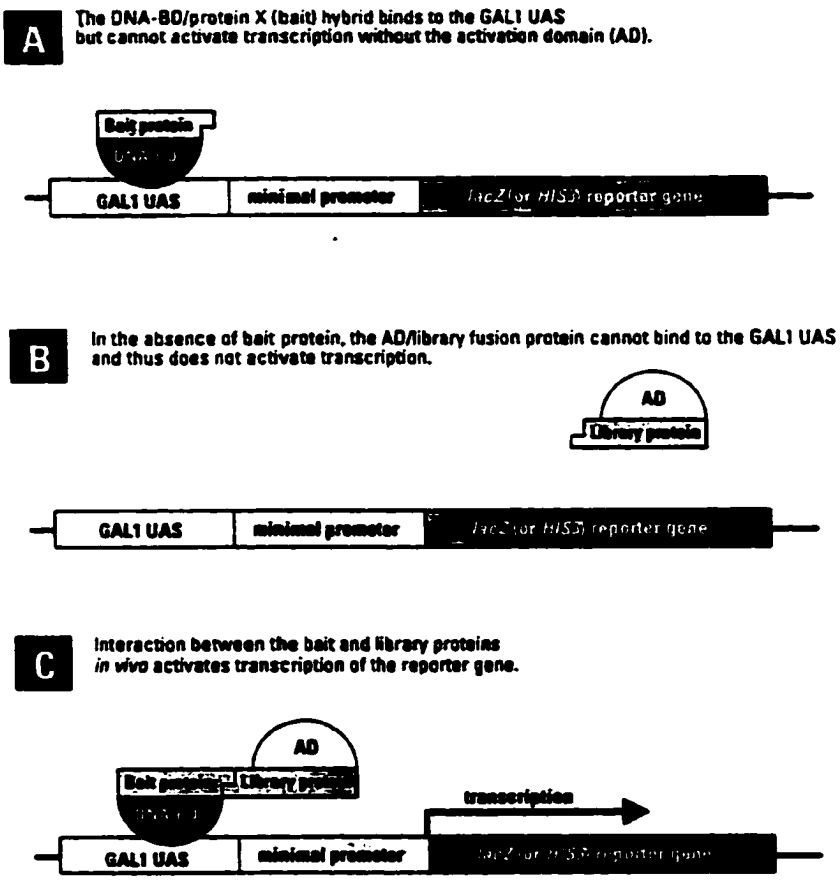


Figure 13

Figure 14. Schematic diagram of the MACHMAKER GAL-4based TWO-Hybrid System from Clontech Inc.



Schematic diagram of the MATCHMAKER GAL4-based Two-Hybrid Systems. The DNA-BD is a.a. 1–147 of the yeast GAL4 protein, which binds to the GAL1 UAS upstream of the reporter gene. The *lacZ* and *HIS3* reporter genes are separate constructs integrated in the yeast genome. The AD is a.a. 768–881 of the GAL4 protein and has transcriptional activation function.

Figure 14

Figure 15. Flow chart for performing a yeast two-hybrid screening of an AD fusion library.

Flow chart for performing a two-hybrid screening of an AD fusion library

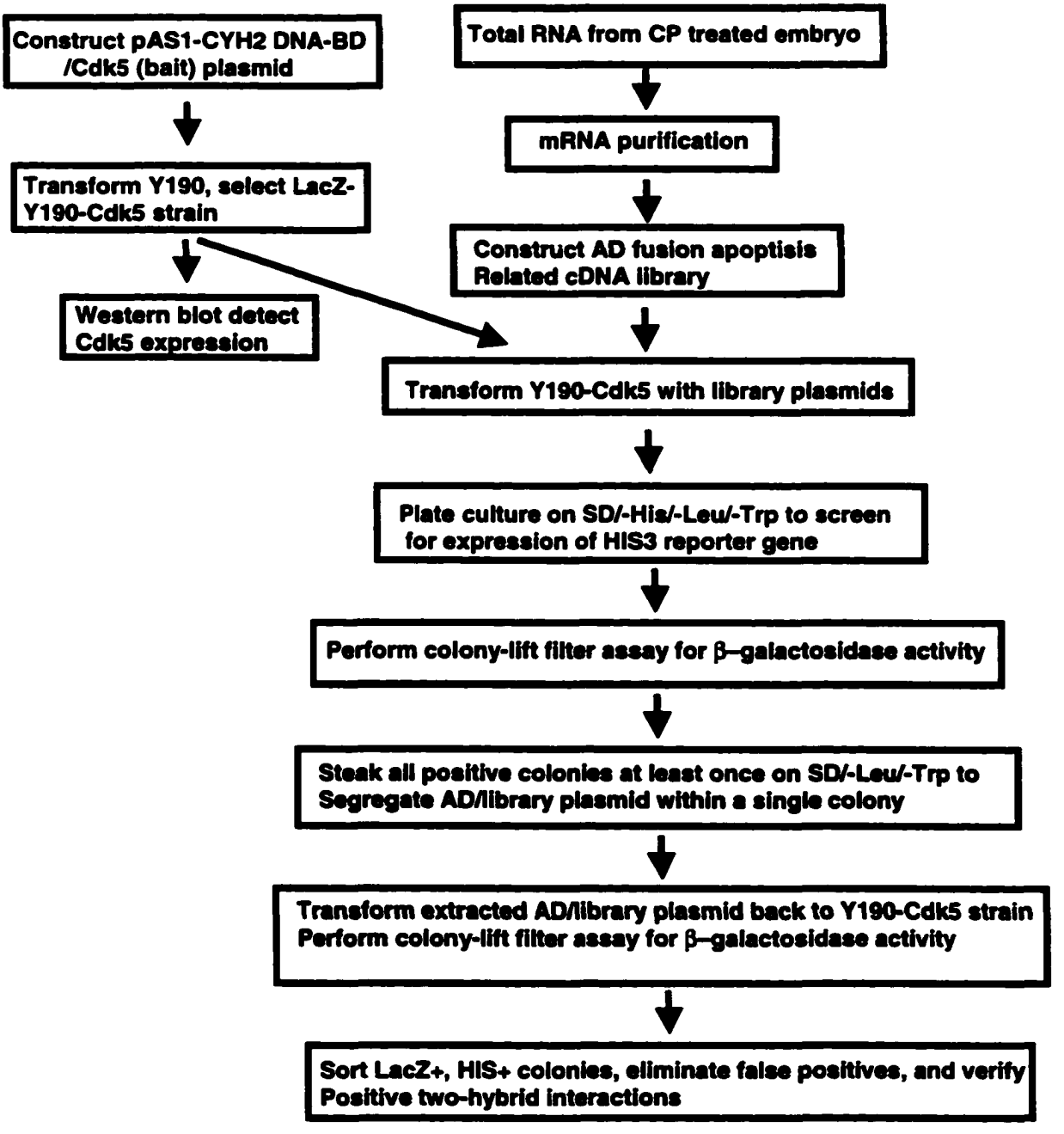


Figure 15

Figure 16. Construction and qualification of AD fusion CP-treated embryo cDNA library.

A. Total RNA from CP-treated mouse embryo. Two bright bands show 18S and 28S ribosomal RNA. The brighten ration between 18S and 28S is about 1:3, which shows high quality of total RNA.
B. Purified CP-treated embryo mRNA was used for reverse transcription to make cDNA. 32P-ATP incorporated cDNA shows a bright smear between 500bp to 4kb on electrophoresis, which indicated high quality of cDNA.
C. Map of AD fusion library vector. The cloning site is at EcoRI.
D. Quality control of AD fusion cDNA library. 15 random colonies were picked, plasmids were extracted, and digested with EcoRI. 10 out of 15 has insert gene, which indicates the complexity of the library is good.

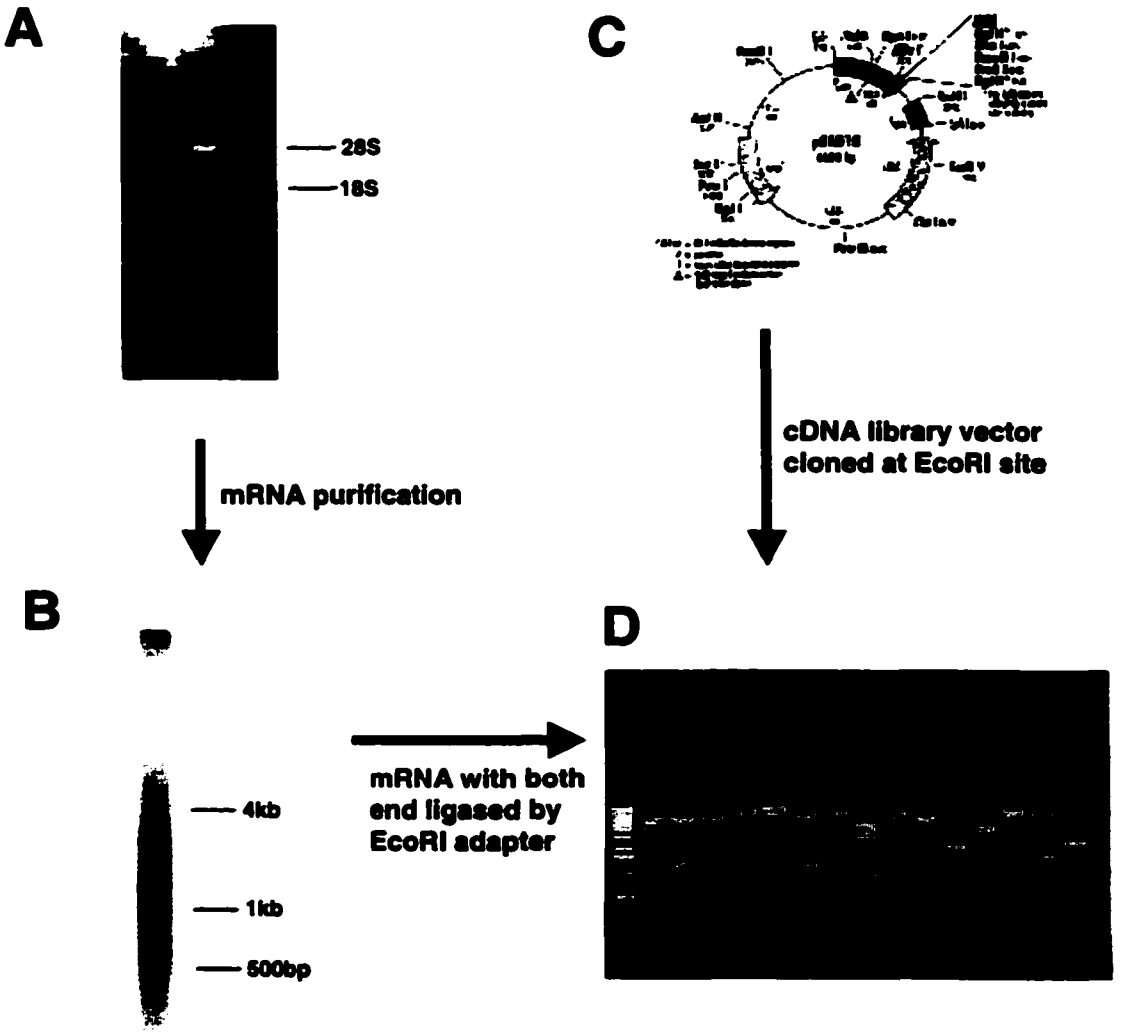


Figure 16

Figure 17. Construction and characterization of Cdk5 expressed host yeast Y190-Cdk5 strain.

A. Map of DNA-BD vector.
B. Map of Cdk5 contained plasmid (gift from Dr. Tsai).
C. Cdk5 was cloned into pCITE-a vector at BamH1 site to shift one base pair in order to clone into DNA-BD vector in frame.
D. Cdk5 gene was cloned into DNA-BD vector (pAS1-CYH2) at Nco1 and Sal1 site to make Cdk5 expression construct (pAS-Cdk5)
E. Western blot shows Cdk5 expressed in host yeast strain (Y190).

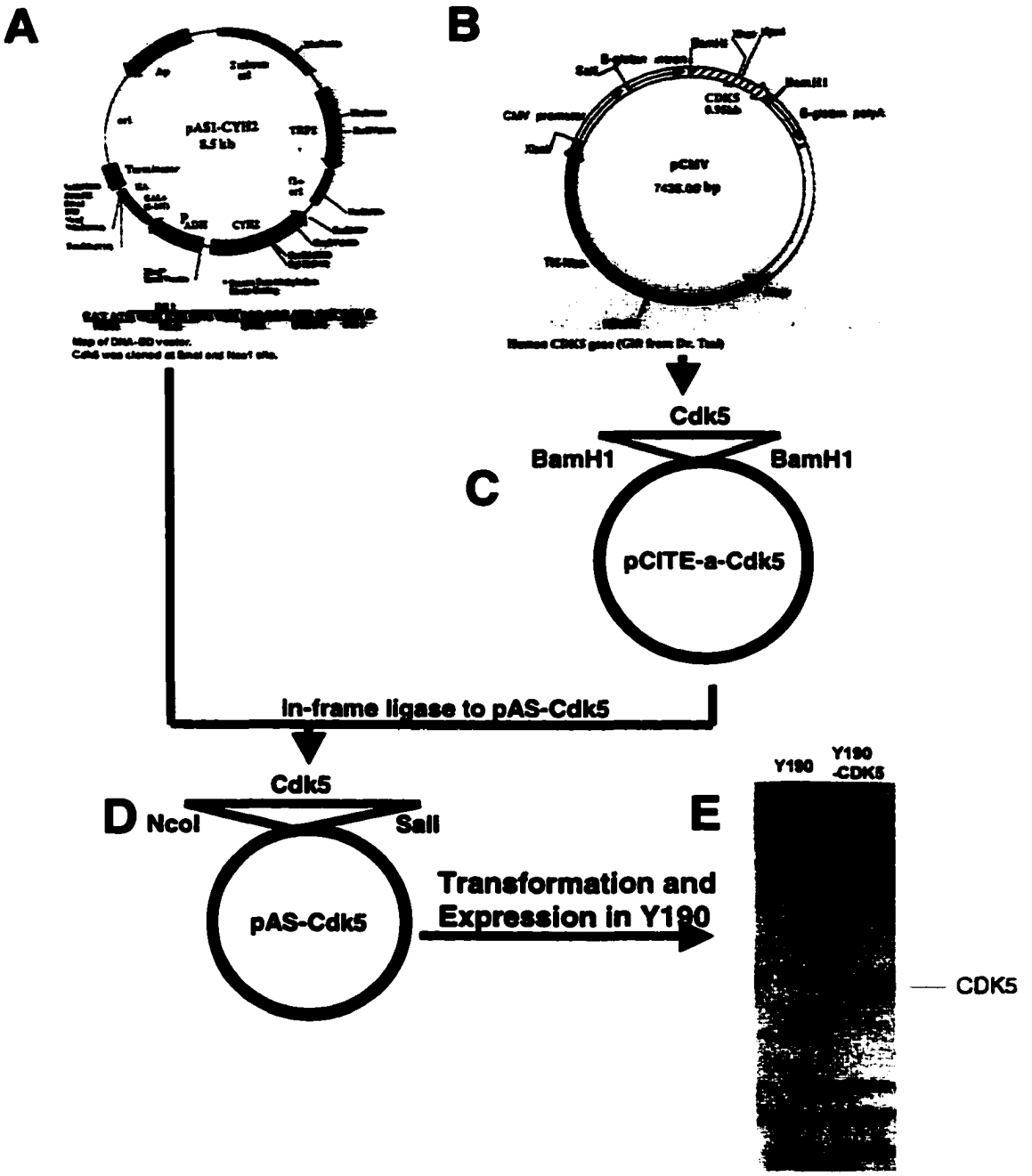


Figure 17

Figure 18. Colony–lift filter assay for β–falactosidase activity on one of the positive colonies in lower titer to purify single pure colony.

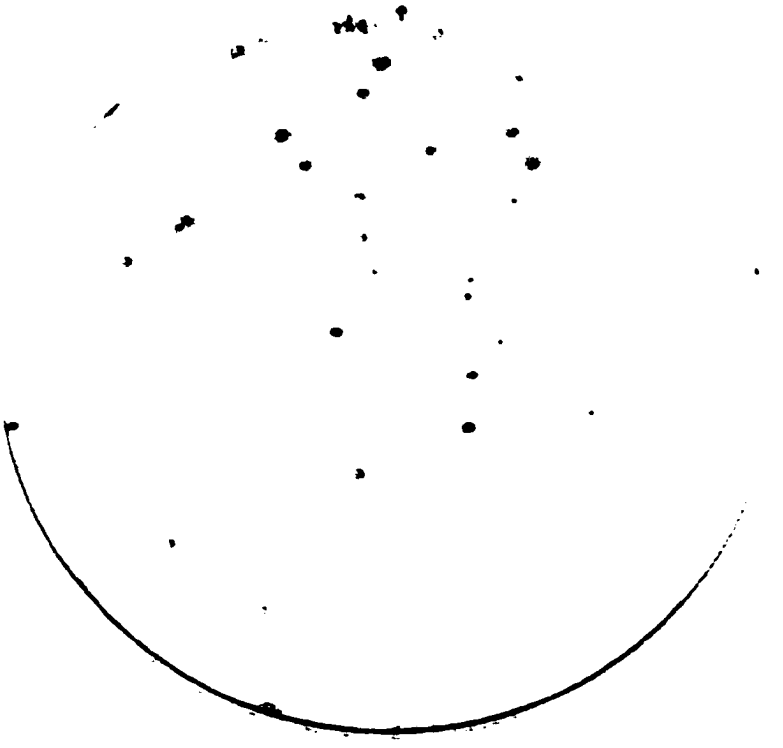


Figure 18

Figure 19. Characterization of positive colonies.

A. EcoR1 digestion of purified plasmids from positive AD fusion colonies after steak the DNA-BD plasmid. All colonies had AD plasmid, except plasmid from colony 61-1 which could not be recovered.

B. All AD plasmids were transformed back to Cdk5 expressed yeast strain (Y190-Cdk5), Colony–lift filter assay for β–falactosidase activity was performed again to eliminate the false positive. Colony 1-3, 21-1, 71-1, and 91-1 turned to blue, which showed positive colonies. Other colonies turned to white that indicated false positive.

C. To repeat the screen. We made another AD fusion CP-treated embryo cDNA library. Follow the same procedure. 13 colonies were selected. After AD fusion plasmids of these colonies were purified, we performed colony–lift filter assay for β–falactosidase activity. Colony 107, 108, 109, 111,114 turned to blue, which showed positive colonies. Other colonies turned to white, which indicated false positive.

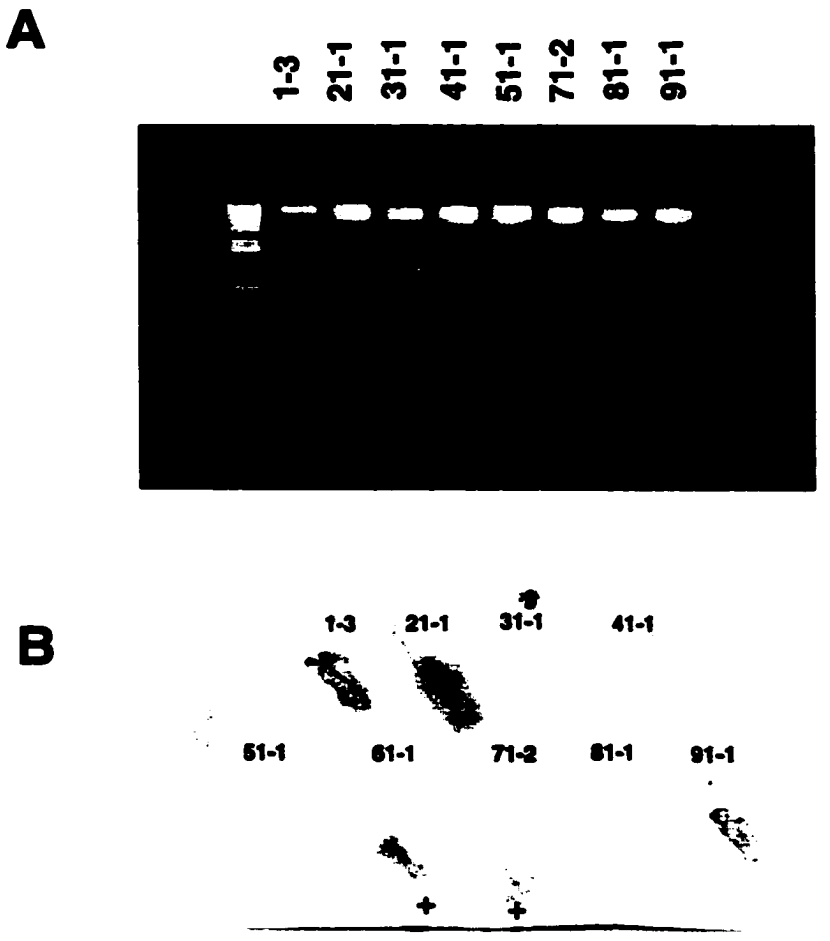


Figure 19

Figure 20. Eliminate none specific false positive.

All positive colonies were transformed into host yeast strain which Cdk5 was replaced by GRB2 or Snf4 genes to identify if the positive AD colony could specific interact with Cdk5 or not.

A. Using GRB2 to replace Cdk5 as DNA-BD plasmid, colony–lift filter assay for β–galactosidase activity showed that only colony 1-3 remained white, which indicated no interaction with GRB2 protein. All others turned blue, which indicated they could interacte with GRB2 protein.
B. Using Snf4 to replace Cdk5 as DNA-BD plasmid, colony–lift filter assay for β–falactosidase activity showed that only colony 1-3 remained white, which indicated no interaction with Snf4 protein. All others turned blue, which indicated they could interacte with Snf4 protein and therefore are false positive clones.

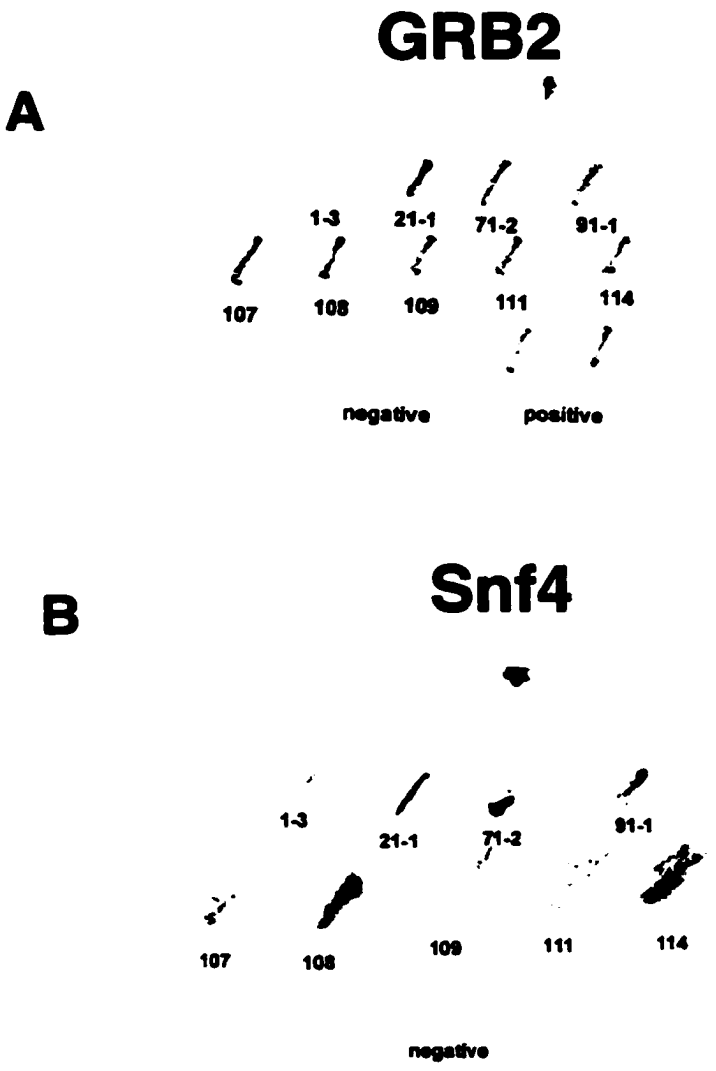


Figure 20

Figure 21. DNA Sequence of 5-end of clone 1-3.

1-3_G4A

```
GNNNNGGGAG ATTANNTTGA GTTACATCTT CTATTCGATG ATGAAGATAC 50
CCCACCAAAC CCAAAAAAAG AGATCTCTAT GGCTTACCCA TACGATGTTC 100
CAGATTACGC TAGCTTGGGT GGTCATATGG CCATGGAGGC CCCCATGGGC 150
ACGGTGCTGT CCCTATCCCC CAGCTATCGG AAGGCCACAC TGTTTGAGGA 200
TGGCGCGGCC ACCGTGGGCC ACTACACGGC CGTGCAGAAC AGCAAGAACG 250
CCAAGGACAA GAACCTGAAG CGGCACTCCA TCATCTCCGT GCTGCCTTGG 300
AAGAGGATCG TGGCGGTGTC AGCGAAGAAG AAGAACTCCA AGAAAGCGCA 350
GCCCAACAGC AGCTACCAGA GCAACATCGC GCATCTCAAC AATGAGAACC 400
TGAAGAAGTC GCTGTCCTGT GCCAACCTGT CCACATTCGC CCAGCCCCCG 450
CCGGCTCAGC CGCCCGCACC CCCAGCCAGC CAGCTTTCTG GCTCCCAGAC 500
TGGGGTCTCC TCTTCTGTCA AGAAGGCCCC GCACCCTGCC ATCACCTCTG 550
CAGGGACACC CAAACGGGTC ATCGTCCAGG CGTCCACTAG TGAGCTGCTG 600
CGCTGCCTGG GTGAGTTTCT CTGCCGCCGG TGCTACCGCC TGAAGCACTT 650
GTCCCCAACG GACCCCGTGC TCTGGCTGCG CAGCGTGGAC CGCTCCCTGC 700
TTCTGCAGGG CTGGCAGGAC CAGGGTTTCA TCACACCGGC CAATCGTGGT 750
CTTCCTCTAC ATGCTCTGCA GGGATGTCAT CTCCTCTGAG GTGGGCTCCG 800
GATTCACGAG CTCCAGGCCC GTCCTGCTTG ACATTGCCTT GTACCTTCTT 850
CTTACTTCCT TACATTGGGG CCAAACGNAG AATCGGGGGG ATTCCCGAAA 900
TTTCCGAAGC TTCCGANAAN GAATCTTATT GAAATTCGGT AANANTACTT 950
GGAAAAAAAA CCCCCGCAAA GTTTTCAACT TTTNAAACTT GNGGCAATTC 1000
GGGGCCACCC AATNNTTAAA ATTTTCNTTT TCANTTTTAA TAACCATTCG 1050
GNTTTTTGGC CCTTTNTTTT TTTTGGTAAA CTTTTANCTT CCTTTTAAAG 1100
TTTTCNAATT NTTTGGGCCC TTTTAAAACC TNTNGANTCT TTTANNAATT 1150
TTTTTAAATG GAACTTAAAA NTTAATGGCC CATNNTTTTT TTTTGGGANC 1200
CTTAAAATTT TTNN                                        1250
```

Figure 21

Figure 22. Sequence homology analysis of clone 1-3 to p35 sequence of mouse and human.

A. DNA sequence analysis shows that the sequence of clone 1-3 has 100% homology to that of mouse p35, and 92% to that of human p35 gene.

B. Protein sequence analysis shows that protein sequence of clone 1-3 has 100% homology to that of mouse p35, and 97% to that of human p35 protein.

A

DNA sequence Analysis:

```
Clone1-3: ccatgggcacggtgctgtccctatcccccagctatcggaaggccacactgtttgaggatg
Mouse:    ccatgggcacggtgctgtccctatcccccagctatcggaaggccacactgtttgaggatg
Human:    ccatgggcacggtgctgtccctgtctcccagctaccggaaggccacgctgtttgaggatg

Clone1-3: gcgcggccaccgtgggccactacacggccgtgcagaacagcaagaacgccaaggacaaga
Mouse:    gcgcggccaccgtgggccactacacggccgtgcagaacagcaagaacgccaaggacaaga
Human:    gcgcggccaccgtgggccactatacggccgtacagaacagcaagaacgccaaggacaaga

Clone1-3: acctgaagcggcactccatcatctccgtgctgccttggaagaggatcgtggcggtgtcag
Mouse:    acctgaagcggcactccatcatctcggtgctgccttggaagaggatcgtggcggtgtcag
Human:    acctgaagcgccactccatcatctccgtgctgccttggaagagaatcgtggccgtgtcgg

Clone1-3: cgaagaagaagaactccaagaaagcgcagcccaacagcagctaccagagcaacatcgcgc
Mouse:    cgaagaagaagaactccaagaaggcgcagcccaacagcagctaccagagcaacatcgcgc
Human:    ccaagaagaagaactccaagaaggtgcagcccaacagcagctaccagaacaacatcacgc

Clone1-3: atctcaacaatgagaacctgaagaagtcgctgtcctgtgccaacctgtccacattcgccc
Mouse:    atctcaacaatgagaacctgaagaagtcgctgtcctgtgccaacctgtccacattcgccc
Human:    acctcaacaatgagaacctgaagaagtcgctgtcgtgcgccaacctgtccacattcgccc

Clone1-3: agccccgccggctcagccgcccgcaccccagccagccagctttctggctcccagactg
Mouse:    agccccgccggctcagccgcccgcaccccagccagccagctttctggctcccagactg
Human:    agccccaccggcccagccgcctgcaccccggccagccagctctcgggttcccagaccg

Clone1-3: gggtctcctcttctgtcaagaaggccccgcaccctgccatcacctc
Mouse:    gggtctcctcttctgtcaagaaggccccgcaccctgccatcacctc 100% Homology
Human:    ggggctcctcctcagtcaagaaagcccctcaccctgccgtcacctc  92% Homology
```

B

Protein Sequence Analysis:

```
Clone1-3: MGTVLSLSPSYRKATLFEDGAATVGHYTAVQNSKNAKDKNLKRHSIISVLPWKRIVAVSA
Mouse:    MGTVLSLSPSYRKATLFEDGAATVGHYTAVQNSKNAKDKNLKRHSIISVLPWKRIVAVSA
Human:    MGTVLSLSPSYRKATLFEDGAATVGHYTAVQNSKNAKDKNLKRHSIISVLPWKRIVAVSA

Clone1-3: KKKNSKKAQPNSSYQSNIAHLNNENLKKSLSCANLSTFAQPPPAQPPAPPASQLSGSQ
Mouse:    KKKNSKKAQPNSSYQSNIAHLNNENLKKSLSCANLSTFAQPPPAQPPAPPASQLSGSQ — 100%
Human:    KKKNSKK QPNSSYQ NI HLNNENLKKSLSCANLSTFAQPPPAQPPAPPASQLSGSQ — 97%
```

Figure 22

Figure 23. ^{35}S-Methionine incorporation and immunoprecipitation analysis to identify Cdk5 associated proteins in CP-treated mouse embryo.

A. Immunoprecipitation was done with anti-Cdk5. After immunoprecipitation, beads were washed and analyzed in SDS-polyacrylamide gel and autography. There are two band appeared. One band is about 25KD only expressed in CHX or CPT treated COS-7 cells, which may be p25 protein. Another band is about 65KD expressed in CHX or CPT treated cells, as well as control, which is a none specific binding protein.

B. Same samples as in panel A but without immunoprecipitation, which is cell total lysis was analyzed by SDS-polyacrylamide gel and autograph. It shows many bands have been labeled.

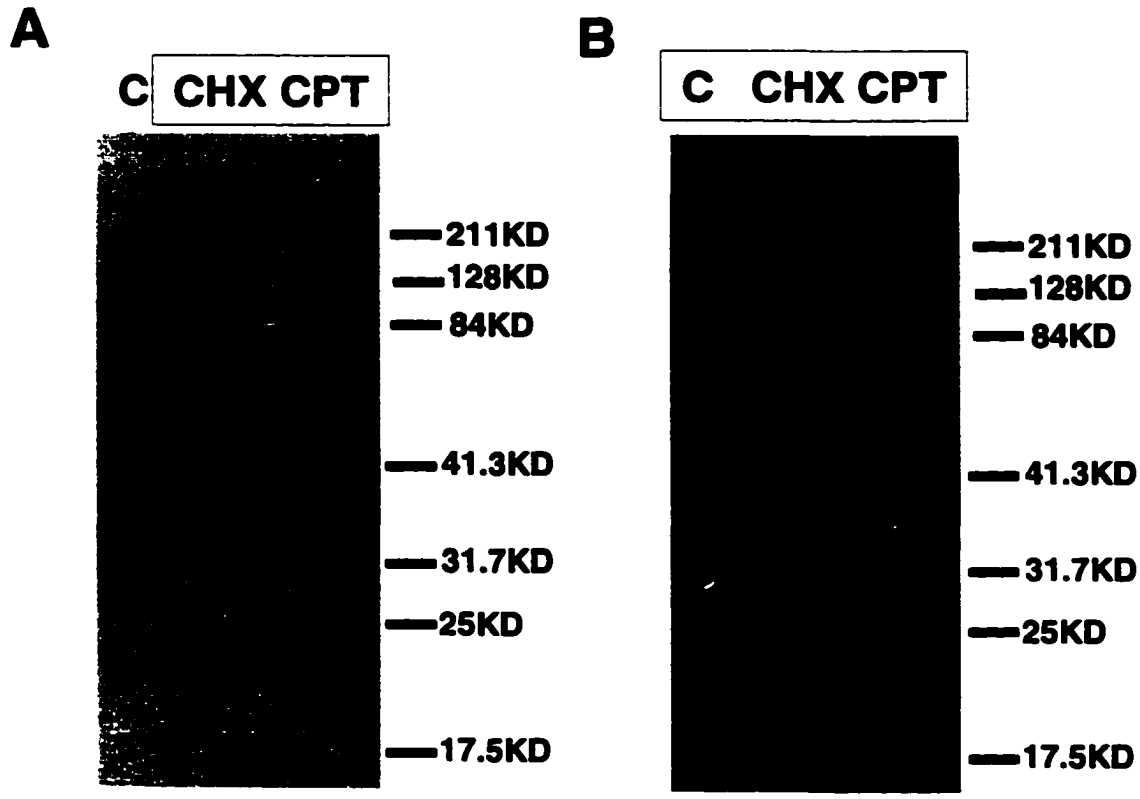


Figure 23

Figure 24. The 25KD protein from immunoprecipitations is the p25 protein.
Same samples, as Figure 23, were run on SDS-polyacrylamide gel, and transferred to PVDF membrane for Western blot analysis.

A. Western blot using anti-p35 (C-19) on immunoprecipited samples show a specific p25 band in CHX or CPT treated COS-7 cells. This confirmed that the 25KD band we detected in Figure 23 in immunoprecipitation is p25.
B. Western blot using anti-p35 (C-19) on total lysis shows that p25 appears in CHX or CPT treated, while p35 expression had no change in all three samples.
C-D. Western blot by using anti-Cdk5 (C-8) on both immunoprecipitation and total lysis shows that there is no change of Cdk5 expression.

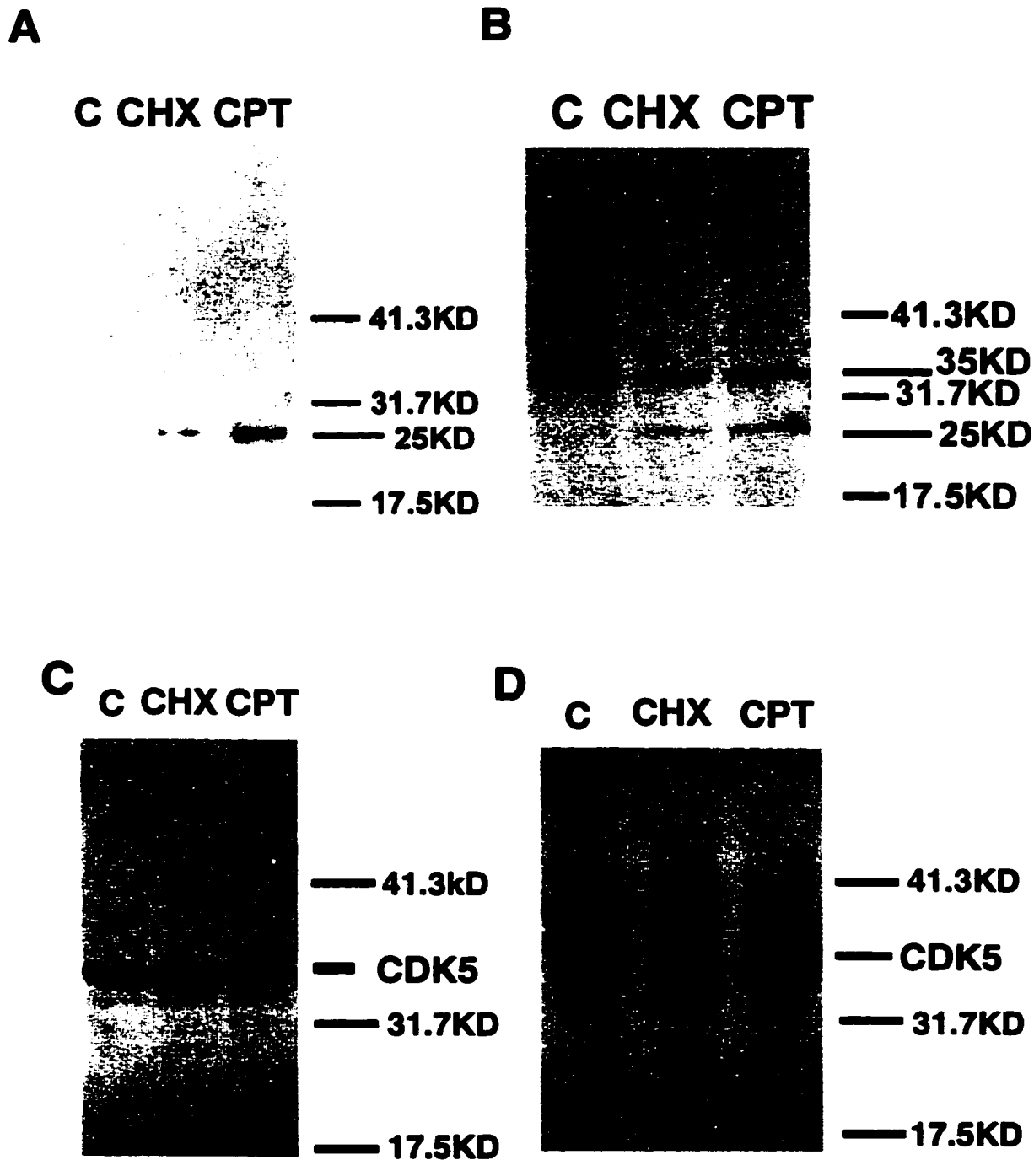


Figure 24

Figure 25. Localization of apoptotic cell death and p35/p25 expression in mouse embryonic limbs.

A. Frozen section from day 14.5. Embryonic hindlimbs were stained with p35 antibody (c-19). Black arrows indicated p35 expression.
B. DNA fragmentation with FITC shows a dim background with a bright punctuation in cells with the fragmented DNA in the interdigital regions.
C. The detection of p35 by cy3 shows a dim background with the bright punctuation of the Cdk5 expression in the cells of the interdigital region.
D. The confocal overlaid image shows intense yellow staining where the two signals of green and red overlap in the same area.
E. An enlargement of the region of panel D.

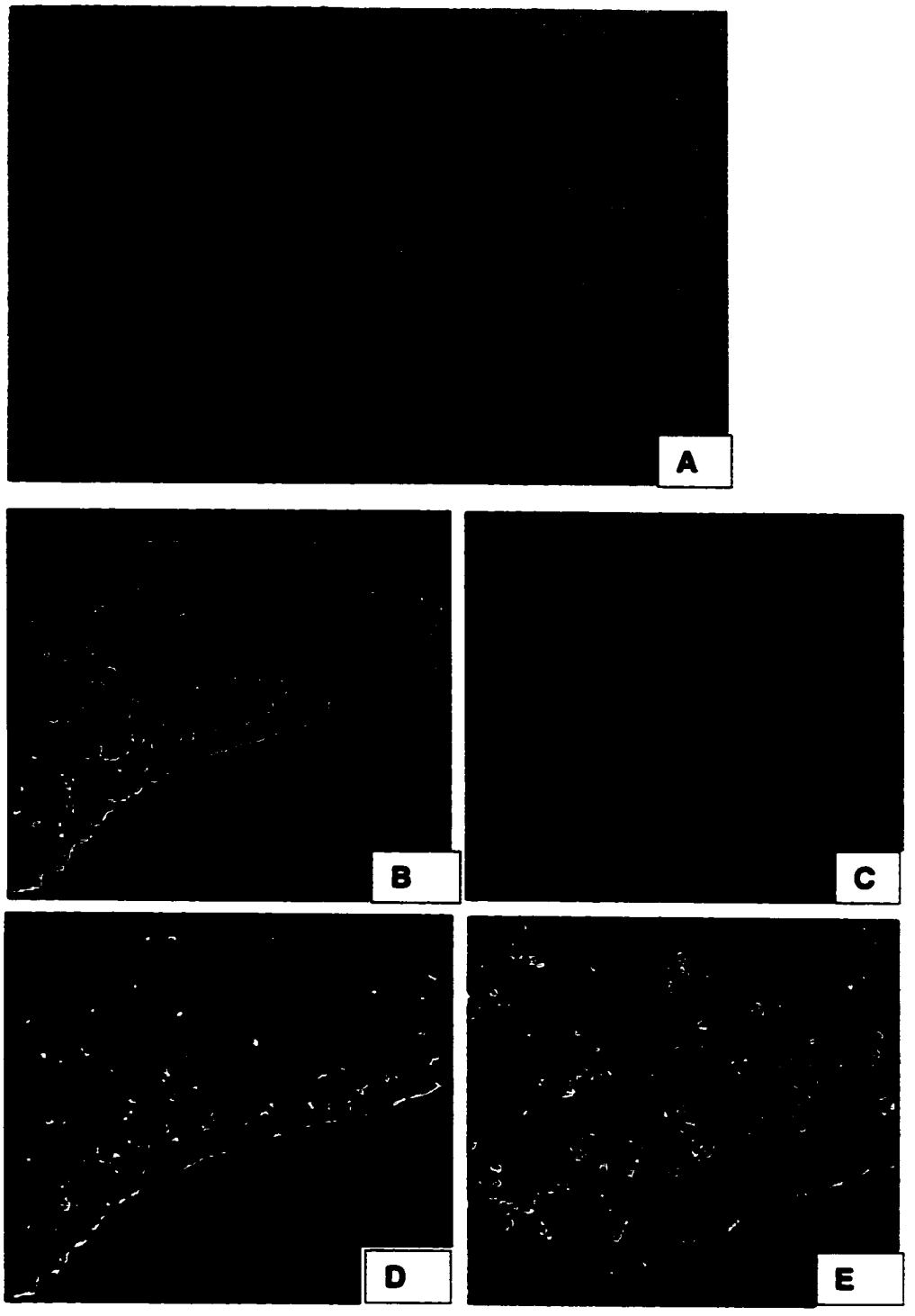


Figure 25

Figure 26. Expression of p35 mRNA in dying cells by *in situ* hybridization.
Panel A (lightfield) and C (darkfield) p35 sense probe was used to detect control background levels.
Panel B (lightfield) and D (darkfield), antisense p35 probe was used.
The reddish cells in the dark field represent the dying cells with condensed nuclei or the neighboring phagocytic macrophages that usually refract light in dark field. Although there was expression of p35 in the mouse limb cells, there was no differential expression in the interdigital region or within the dying cells.

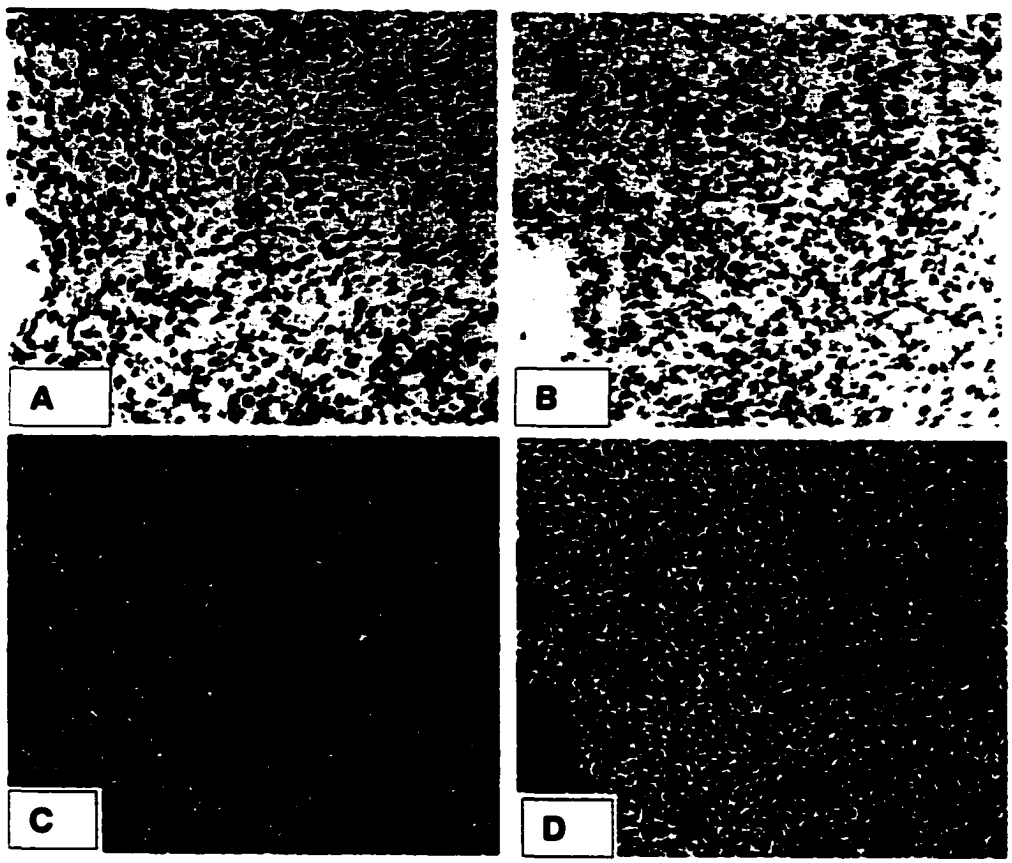


Figure 26

Figure 27. Localization of p35/p25 expression in developing rat lens at E18. Near serial section of paraffin-embedded rat lens were exposed to antibodies for immunohistochemistry staining as described under Materials and Methods.
Panel A shows control section incubated with normal rabbit serum.
Panel B shows section incubated with antihuman p35 (c-19), with cornea (C), lens (L), and retina (R) as indicated.
Panel C is higher magnification of the anterior region of the lens from panel B. Positive staining in the anterior tips of the fiber cells, and in some primary lens fiber cell nuclei (black arrow) are shown.

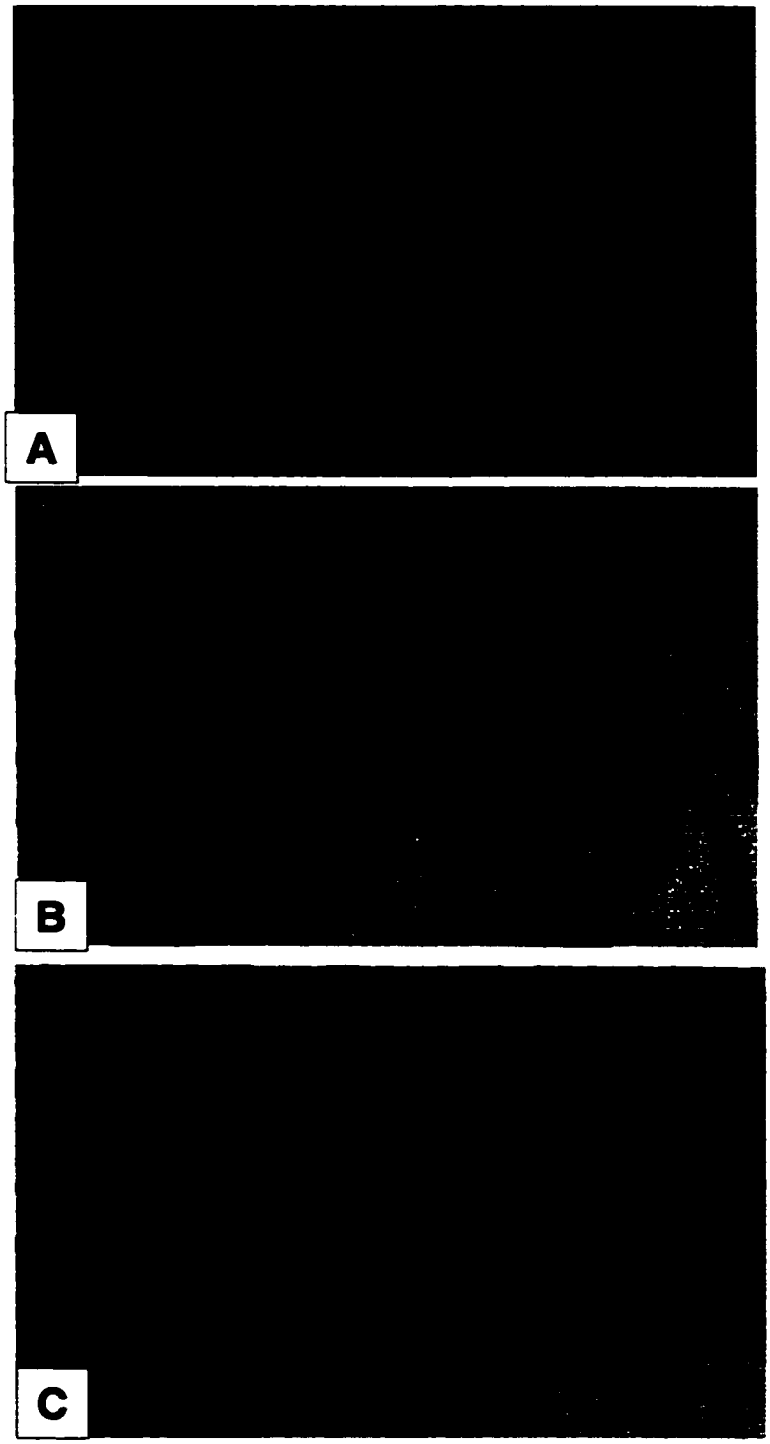


Figure 27

Figure 28. Expression of p35 mRNA in the dying cells by *in situ* hybridization.
Panel A: hybridization with radioactively labeled p35 sense riboprobe photographed with darkfield illumination.
Panel B: hybridization with radioactively labeled p35 antisense riboprobe photographed with darkfield illumination. Arrow indicates elongating fiber cells near the bow region of the lens.
(C=cornea, L=lens, R=retina)

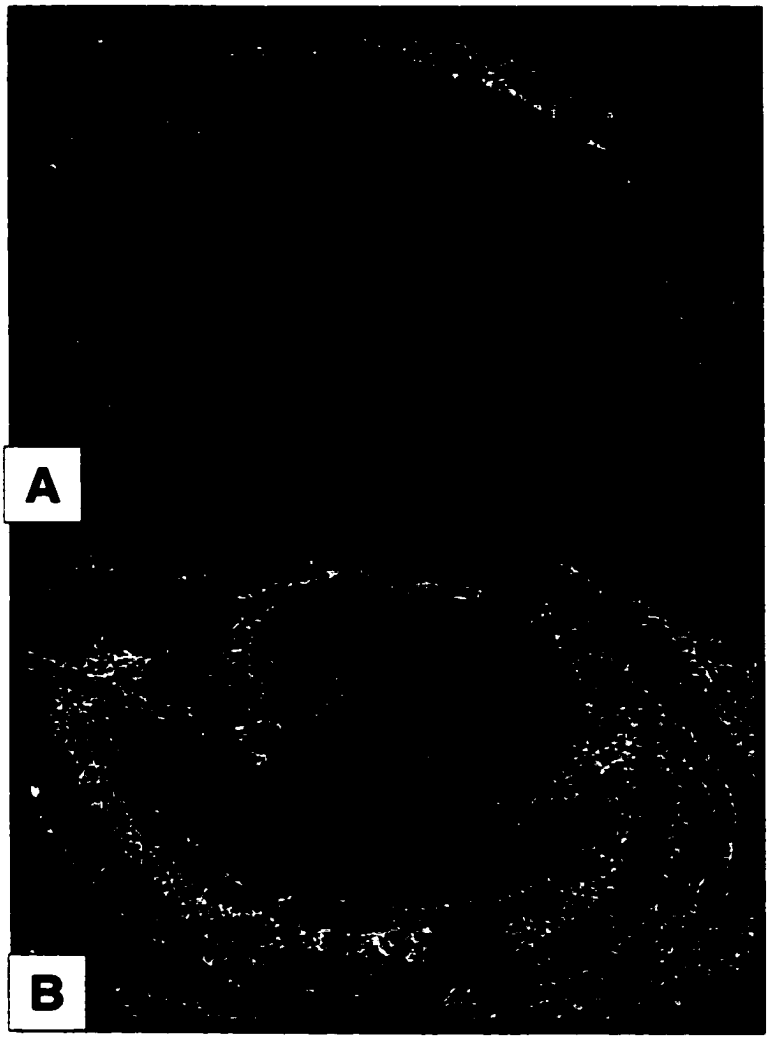


Figure 28

Figure 29. Localization of p35/p25 expression with apoptotic cell death in CP-treated day10.5 mouse embryos. Pregnant female mice were injected intra-peritoneally with 10mg CP/kg body weight as described in Materials and Methods. Embryos were processed for different assays.

A. Frozen sections from CP-treated mouse embryos were stained with p35 antibody (c-19). Many cells showed p35 expression (arrows).
B. Western blot showed that p25 is expressed in CP treated mouse embryo, while there is no significant change in p35 expression.

A

B

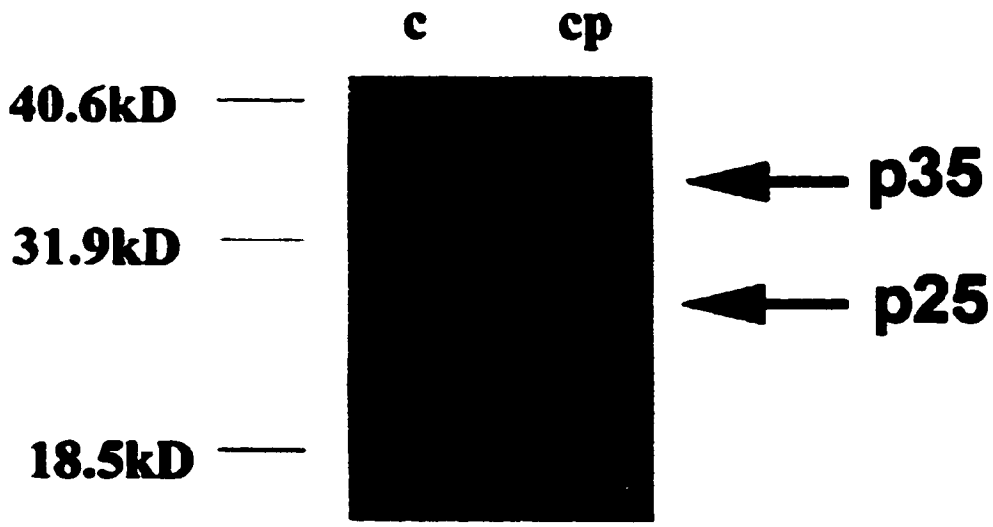


Figure 29

Figure 30.

A shows expression of p35 mRNA in dying cells by in situ hybridization. Pregnant female mice were injected intra-peritoneally with 10mg CP/kg body weight as described in Material and Methods. Panel A represents the p35 sense probe to detect normal developing mouse embryos to determine control background levels. Panel b shows p35 sense probe to detect CP-treated developing mouse embryo to determine control background levels in abnormal embryos Panel c represents signals from antisense p35 on normal developing mouse embryos. Panel d shows signals from antisense p35 probe on CP-treated mouse embryo. There is no different expression of p35 between p35 sense and antisense probe on both normal and CP-treated mouse embryos.

B shows the northern blot of control embryo and CP treated embryo. There is no different Cdk5 mRNA expression.

C shows p35/p25 expression in CHX, or CPT treated COS-7 cells. p25 protein appeared in CHX, or CPT treated cells. There is no p35 expression change in CHX or CPT treated COS-7 cells, as well as control.

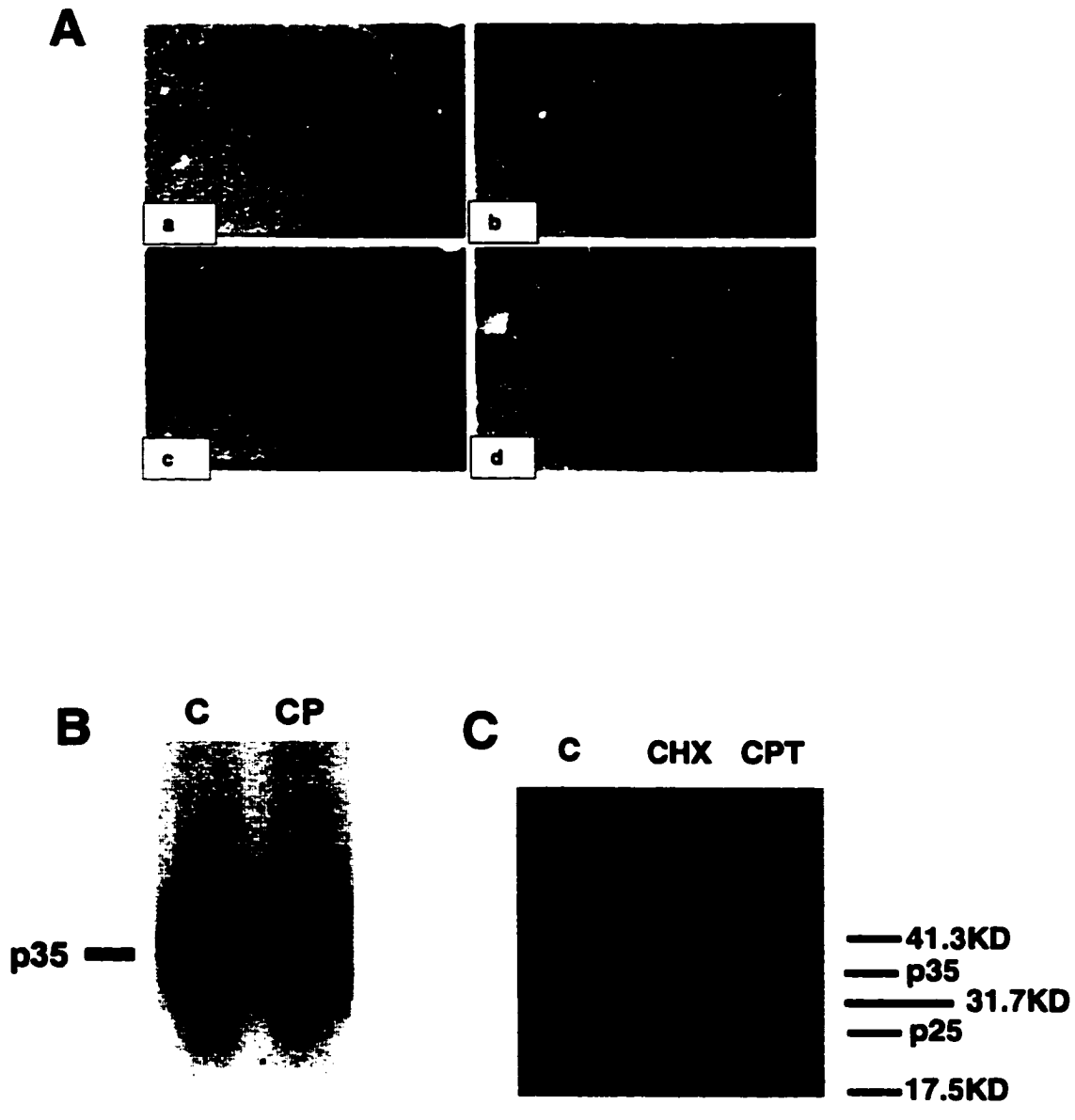


Figure 30

Figure 31. Strategy of making p35 anti-sense expression plasmid (pCMS-anti-p35).
A. Map of p35 gene plasmid. Hp35 gene was released at 5'-KpnI and 3'-EcoRI site.
B. Map of expression vector (pCMS-EGFP). It can express foreign gene, as well we EGFP gene, which can produce fluorescent signal.
C. Map of p35 anti-sense expression plasmid. hp35 gene was cloned at pCMS-EGFP vector EcoR1 and KpnI site (pCMS-anti-p35).

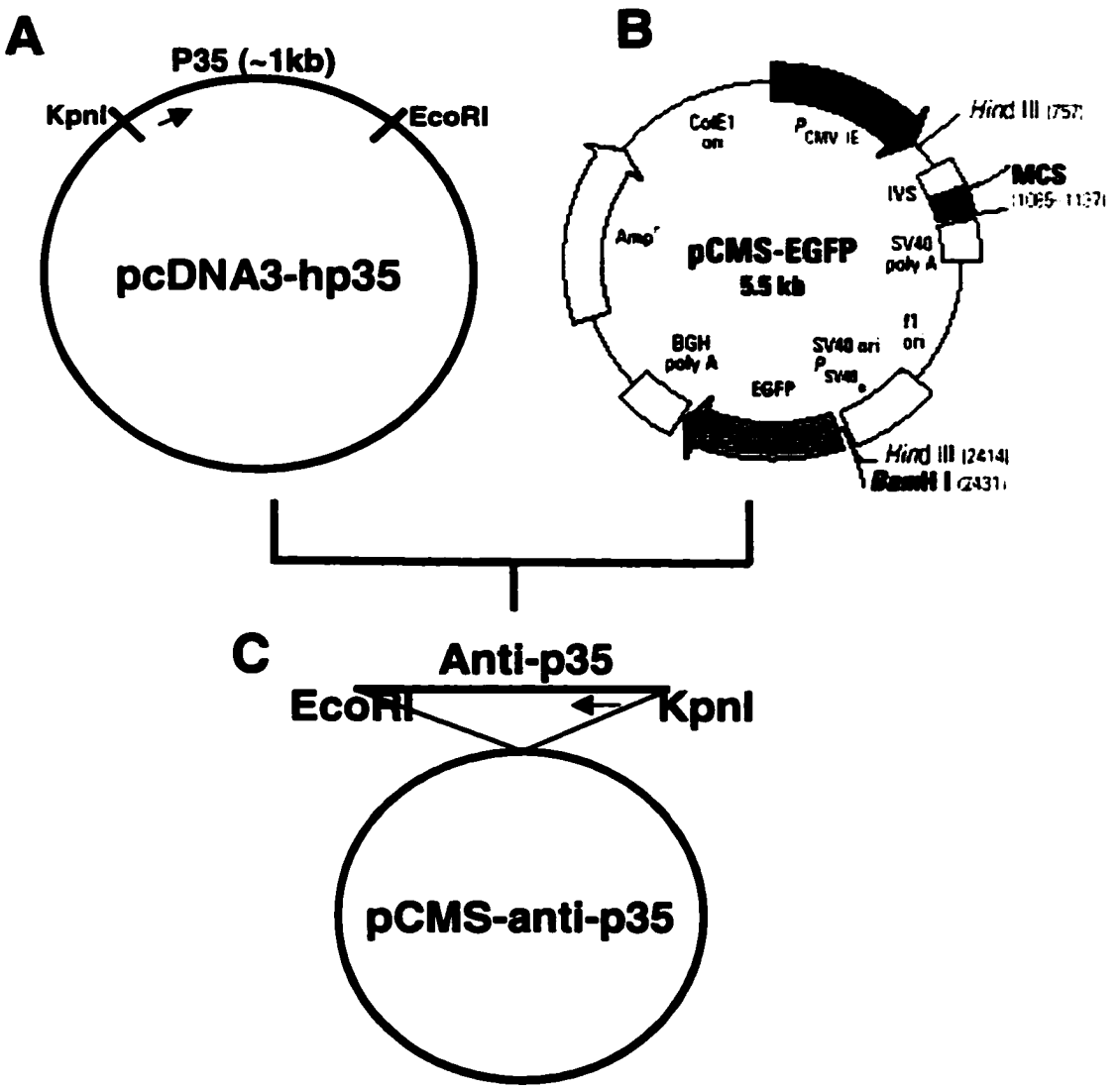


Figure 31

Figure 32. Detection of transfection efficiency of pCMS-anti-p35.

A-B. control cells without transfection. Panel A (dark field) shows no fluorescent signal. Panel B (light field) shows cells are alive.

C-D. pCMS-anti-p35 transfected cells. Panel C (dark field) shows transfected cells expressing fluorescent signals. Panel D (light field) shows cell morphology.

E-F. An enlargement of the region of panel C and D.

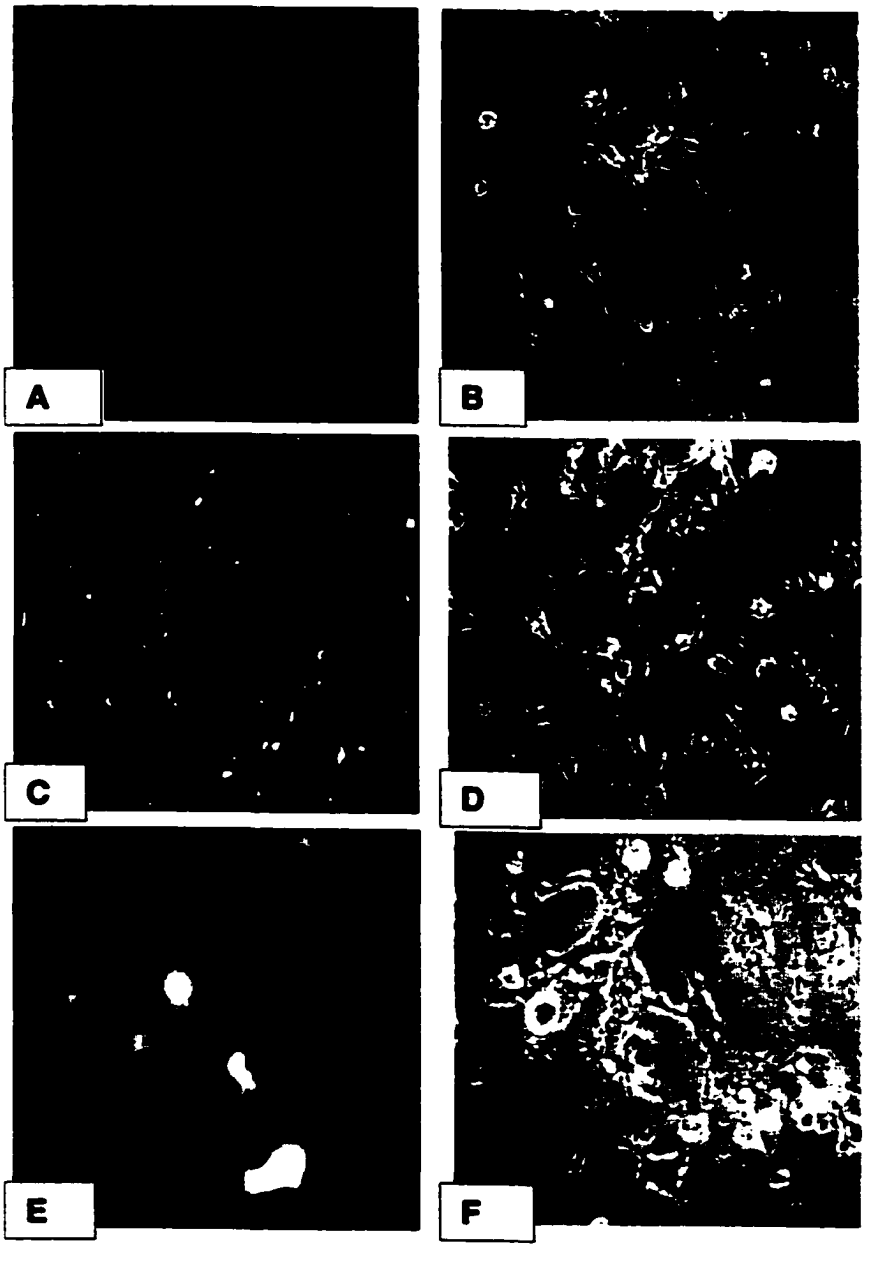


Figure 32

Figure 33. Detection of p25, Cdk5 and Cdk5 kinase activity in anti-p35 transfected and untransfected COS-7 cells treated with CHX or CPT.

A. Western blot shows that p25 expressed in normal COS-7 cells when treated with CHX or CPT. But not expressed in pCMS-anti-p35 transfected cells.
B. Western blot shows that there is no changes of Cdk5 expression.
C. Histon H1 kinase activity assay shows that in untransfected COS-7 cells, Cdk5 kinase activity was elevated under the treatment of CHX or CPT. While, in pCMS-anti-p35 transfected cells, Cdk5 kinase activity does not differ from controls.

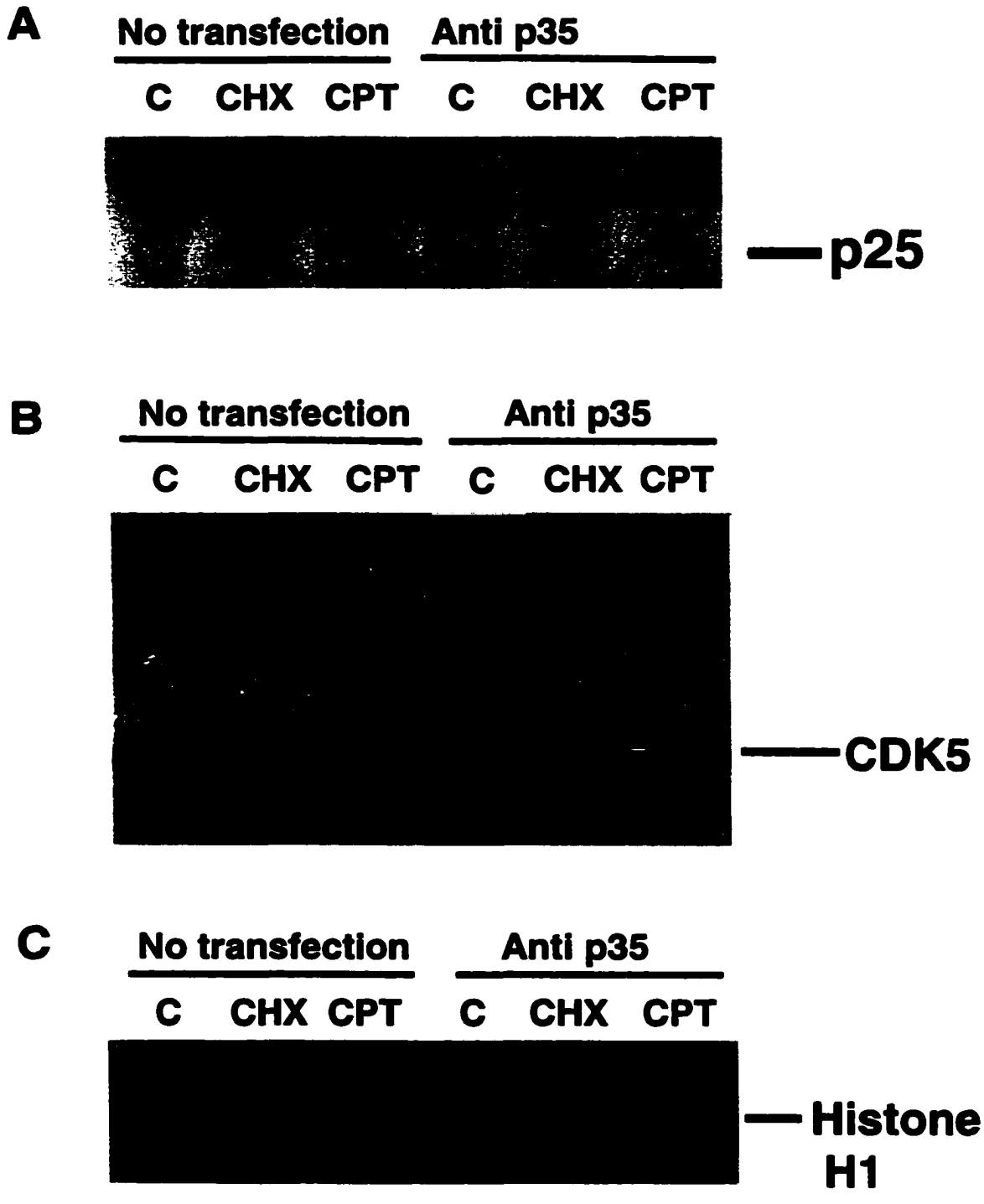


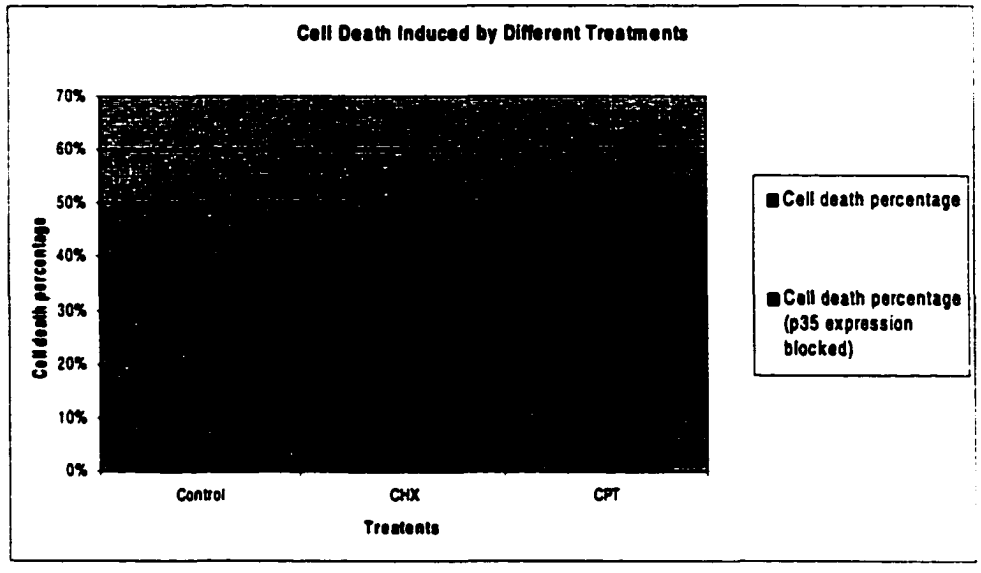
Figure 33

Figure 34. Identification of Cycloheximide (CHX) and Camptothecin (CPT) induced COS-7 cell death without p25 expression. p35 anti-sense transfected COS-7 cells were cultured in DMEM with 10% FBS. Before each treatment, COS-7 cells were placed in DMEM without FBS and exposed to CHX (100ug/ml final concentration), or CPT (15uM final concentration). Control cells received only solvent. After 18 hours, cells were collected and washed for different analysis.

A. Trypan Blue staining showed that there was about 15% decrease of cell death in CHX treated cells. There was no change of cell death in CPT treated cells.

B. Agarose gel electrophoresis of DNA collected from CHX and CPT treated cells showed a decrease in DNA fragmentation in cells treated with CHX and p35 antisense. There was no difference in cells treated with CPT.

A



B

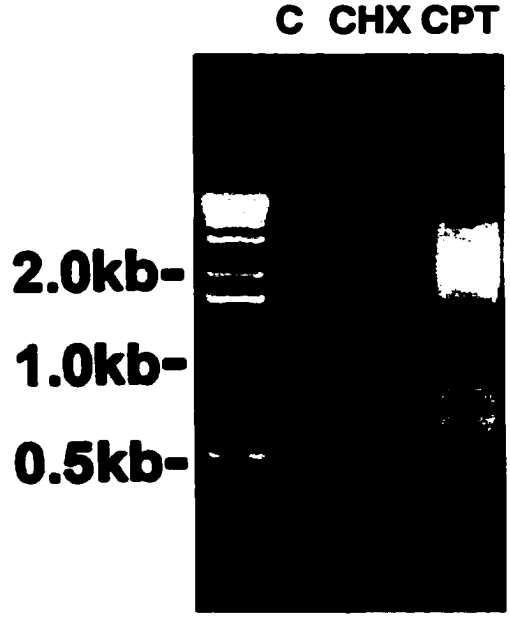
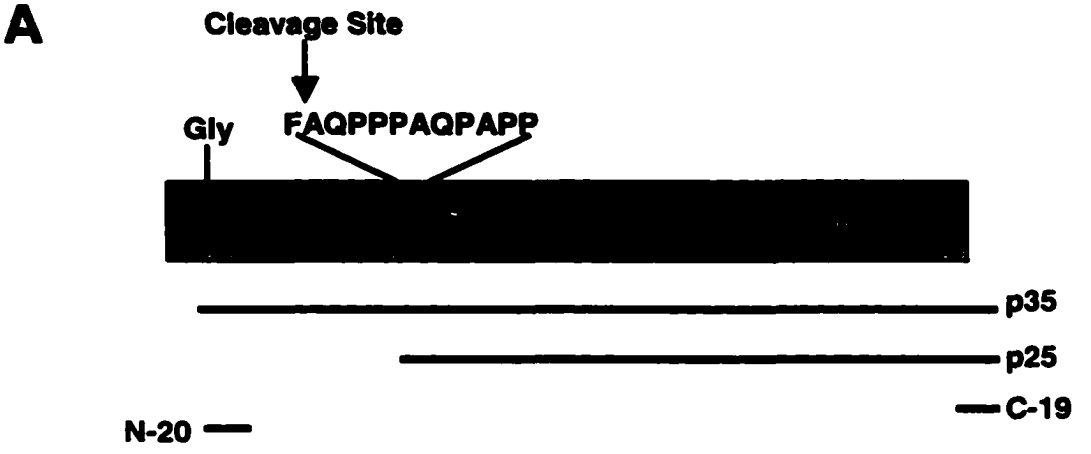


Figure 34

Figure 35.

A. Shows the cleavage site on p35 to convert p35 to p25. C-19 is the anti-p35 antibody (C-19) recognition domain, which can recognize both p35 and p25.
B. Shows the toxic processes activate the protease calpain, which results in p25 production and activation of Cdk5. Factors include Aβ, oxidative stress, excitotoxins, as well as DNA damage and Protein synthesis interruption.



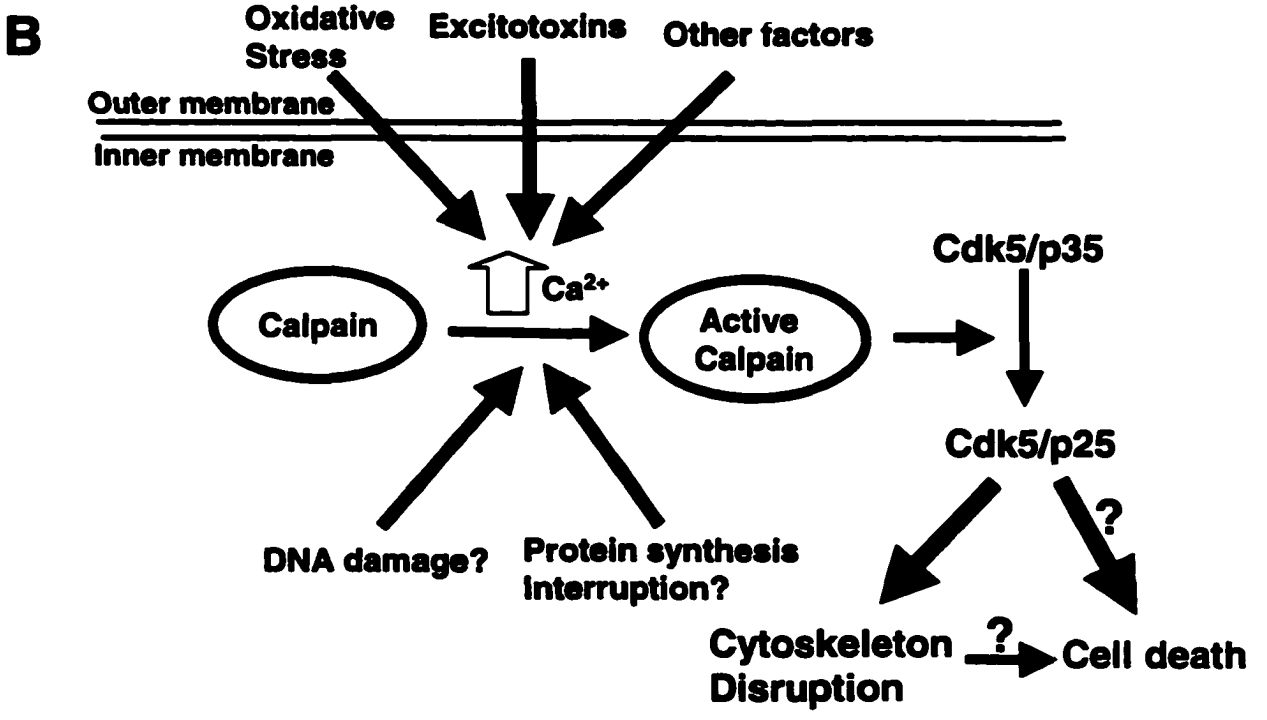


Figure 35

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