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Mai X. Luong
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INVOLVEMENT OF CDP/CUX IN THE REGULATION OF HISTONE H4 GENE EXPRESSION, PROLIFERATION AND DIFFERENTIATION

A Dissertation Presented

By

Mai Xuan Luong

Submitted to the Faculty of the

University of Massachusetts Graduate School

of Biomedical Sciences, Worcester

in partial fulfillment of the requirement for the degree of:

DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES

MAY 7, 2003

CELL BIOLOGY

INVOLVEMENT OF CDP/CUX IN THE REGULATION OF HISTONE H4 GENE EXPRESSION, PROLIFERATION AND DIFFERENTIATION

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By
Mai Xuan Luong

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Department of Cell Biology
May 7, 2003

DEDICATION

This thesis is dedicated to my parents, Xieu Tho Tran and Thuan Luong, who have encouraged and supported me in all my endeavors.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude towards my thesis advisors Drs. Gary and Janet Stein for their support and guidance. Interactions with Gary have provided me with glimpses of the entire forest, whereas conversations with Janet have taught me to begin to examine the intricate details of each leaf. These valuable lessons are much appreciated. I would also like to give thanks to Dr. Andre van Wijnen and Dr. Jane Lian for their help. I am indebted to all those in the Stein laboratory for their friendship: Rosa, Karina, Shirwin, Laura, Ricardo, Kim, Caroline, Rahul, Faiza, Betsy and Judy. My special thanks to Kaleem, Amjad, Soraya, Chris, Ronglin, Hayk and Dan for technical assistance and helpful discussion. I will always cherish the time we've spent together, enjoying fabulous foods and equally satisfying discussions about science and life in general.

I would like to thank my parents for their encouragement and support through the years. They have made tremendous sacrifices for my education, my well-being, and my future. I am very grateful for their unconditional love, patience and understanding. I would also like to thank my brother, sister and Chris for their companionship and support. I am indebted to my uncles (Nghia, Nhu, Nhon, and Hung) and my aunts (Nhi and Truc) who have helped me in different ways. And last, but certainly not least, I would like to thank my husband Jim for his remarkable patience and loving support as I strived to meet the challenges of graduate school.

GENERAL ABSTRACT

INVOLVEMENT OF CDP/CUX IN THE REGULATION OF HISTONE H4 GENE EXPRESSION, PROLIFERATION AND DIFFERENTIATION

By

Mai Xuan Luong

May 7, 2003

Thesis Advisors: Drs. Janet and Gary Stein

Department: Cell Biology

Proliferation and differentiation are essential processes for the growth and development of higher eukaryotic organisms. Regulation of gene expression is essential for control of cell division and differentiation. Normal eukaryotic cells have a limited proliferative capacity, and ultimately undergo cellular senescence and apoptosis. Terminal differentiation of cells is associated with loss of proliferative capacity and acquisition of specialized functions. Proliferation and differentiation are processes required for the creation and maintenance of diverse tissues both during embryonic development and postnatal life. The cell cycle is the process by which cells reproduce, and requires duplication and segregation of hereditary material. Loss of cell cycle control leads to genetic instability and cancer.

Expression of replication-dependent histone genes is tightly coupled to DNA synthesis, thus making histone genes a good model for studying cell cycle regulation. The HiNF-D complex interacts with all five classes (H1, H2A, H2B, H3 and H4) of histone genes in a cell cycle-dependent manner. The CCAAT displacement protein (CDP)/Cux and the tumor suppressor pRB are key components of the HiNF-D complex. However, the molecular interactions that enable CDP/Cux and pRB to form a complex and thus convey cell growth regulatory information onto histone gene promoters are poorly understood. Transient transfection assays show that CDP/Cux represses the histone H4 promoter and that the pRB large pocket domain functions with CDP/Cux as a co-repressor. Direct interaction between CDP/Cux C-terminus and the pRB pocket domain was observed in GST pull-down assays. Furthermore, co-immunoprecipitation assays and immunofluorescence microscopy established that CDP/Cux and pRB form complexes *in vivo* and associate *in situ*. pRB interaction and co-repression with CDP/Cux is independent of pRB phosphosphorylation sites, as revealed by GST pull-down assays and transient transfection assays using a series of pRB mutant proteins. Thus, several converging lines of evidence indicate that complexes between CDP/Cux and pRB repress cell cycle-regulated histone gene promoters.

CDP/Cux is regulated by phosphorylation and acetylation at the C-terminus, which contains two repressor domains and interacts with histone deacetylase HDAC1. *In vivo* function of the CDP/Cux C-terminus in development and gene regulation was assessed in genetically targeted mice (Cutl1^{tm2Ejn}, referred to as

*Cutl1*ΔC). The mice express a mutant CDP/Cux protein with a deletion of the C-terminus including the homeodomain. Indirect immunofluorescence microscopy showed that the mutant protein exhibited significantly reduced nuclear localization in comparison to the wildtype protein. Consistent with these data, DNA binding activity of HiNF-D was lost in nuclear extracts derived from mouse embryonic fibroblasts (MEFs) or adult tissues of homozygous mutant (*Cutl1* ΔC $^{-/-}$) mice, indicating the functional loss of CDP/Cux in the nucleus. No significant difference in growth characteristics or total histone H4 mRNA levels was observed between wildtype and *Cutl1*ΔC $^{-/-}$ MEFs in culture. However, the histone H4.1 (murine FO108) gene containing CDP/Cux binding sites have reduced expression levels in homozygous mutant MEFs. Stringent control of growth and differentiation appears to be compromised *in vivo*. Homozygous mutant mice exhibit stunted growth (20-50% weight reduction), a high postnatal death rate of 60-70%, sparse abnormal coat hair and severely reduced fertility. Hair follicle deformities and severely diminished fertility in *Cutl1*ΔC $^{-/-}$ mice suggest that CDP/Cux is required for normal development of dermal tissues and reproductive functions. Together the data presented in this dissertation provide new insight into the *in vivo* functions of CDP/Cux in the regulation of histone gene expression, growth control and differentiation.

TABLE OF CONTENTS

Page

DEDICATION iii

ACKNOWLEDGEMENTS iv

ABSTRACT v

LIST OF TABLES xiii

LIST OF FIGURES xiv

CHAPTERS

CHAPTER 1: GENERAL INTRODUCTION

Cell Division and the Cell Cycle 1

Histone proteins 5

Histone genes: organization and variants 7

Regulation of replication-dependent histone gene expression 12

Transcriptional Regulation of the Human Histone H4 Gene FO108 14

CDP/Cux 22

CHAPTER 2: MATERIALS AND METHODS

Plasmid construction 28

Bacterial transformations 31

Small-scale DNA preparations 31

Large-scale DNA preparations 32

Preparation of genomic DNA from mice 33

Tissue culture and cell transfection 34

Preparation of mouse embryonic fibroblasts (MEFs) 34

Flow cytometry analysis 35

Cell transfection 36

Luciferase assay 37

Extraction of protein from organs 37

Preparation of nuclear extract 38

Preparation of whole cell lysate 39

Preparation of *in vitro* translated protein 40

GST pull down assays 40

Co-immunoprecipitation assays 43

SDS-PAGE and western blot analysis 43

Antibodies 45

Immunofluorescence microscopy 47

Electrophoretic mobility shift assay (EMSA) 49

Generation of *Cutl1* mutant mice 51

Isolation of total cellular RNA 53

Northern blot analysis 53

Reverse transcriptase PCR (RT-PCR) 54

Southern blot analysis 55

S1 nuclease protection assay 56

Histology 56

Serum testosterone levels 57

Scanning electron microscopy 57

CHAPTER 3: THE TUMOR SUPPRESSOR pRB FUNCTIONS AS A CO-REPRESSOR OF THE CCAAT DISPLACEMENT PROTEIN (CDP)/CUX TO REGULATE CELL CYCLE CONTROLLED HISTONE H4 TRANSCRIPTION

Abstract 58

Introduction 60

Results

CDP/Cux interacts with pRB, Cyclin A, and CDK1 *in vitro* 70

CDP/Cux (CR2-Cterm) interaction with the pRB large pocket is phosphorylation-site independent 72

Direct interaction between CDP/Cux and pRB is mediated by the CDP/Cux C-terminus and the pRB pocket domain 73

CDP/Cux and pRB interact *in vivo* 75

A subset of pRB and CDP/Cux associates *in situ* 77

The N-terminus of CDP/Cux is required for full repression of histone H4 transcription 77

Co-repression of H4 promoter activity by pRB and CDP/Cux 80

The large pocket of pRB functionally interacts with CDP/Cux to repress histone H4 transcription 83

Discussion 86

CHAPTER 4: GENETIC ABLATION OF THE CDP/CUX C-TERMINUS RESULTS IN DECREASED HISTONE H4.1 (FO108) EXPRESSION, HAIR FOLLICLE DEFORMITIES AND REDUCED MALE FERTILITY

Abstract 91

Introduction 93

Results

Loss of CDP/Cux C-terminus results in high neonatal lethality and severe growth retardation 98

Expression of the mutant *Cutl1* allele 101

The CDP/Cux-containing HiNF-D complex is absent in *Cutl1* $\Delta C^{-/-}$ mice 104

Levels of CDP/Cux ΔC protein in the nucleus are significantly reduced in *Cutl1* $\Delta C^{-/-}$ cells 107

Embryonic fibroblasts homozygous for the *Cutl1*ΔC mutation exhibit normal cell growth 110

The *Cutl1* $\Delta C^{-/-}$ mice have reduced fertility 113

The development of the gastrointestinal tract is unperturbed in the *Cutl1* $\Delta C^{-/-}$ mice 115

Cutl1 ΔC mutation results in abnormal dermis and hair loss 117

Discussion 119

CHAPTER 5: GENERAL DISCUSSION

CDP/Cux as a tissue-specific mitogenic factor 125

Implications of CDP/Cux as a tumor suppressor 127

CDP/Cux regulates differentiation 133

REFERENCE LIST 136

LIST OF TABLES

Page

Table 1-1. Replication-dependent histone genes in humans and mice 8

Table 1-2. Variant histone genes 10

Table 1-3. Post-translational modifications of CDP/Cux 24

Table 1-4. Gene targets of CDP/Cut 25

Table 3-1. A subset of pRB-interacting proteins 66

Table 3-2. Knockout phenotypes of genes in the pRB family 68

LIST OF FIGURES

Figure		Page
Fig. 1-1	The restriction point and the cell cycle	2
Fig. 1-2	Cell cycle and restriction point control	4
Fig. 1-3	Cell cycle regulation of histone gene expression. Histone protein biosynthesis is functionally coupled to DNA synthesis	11
Fig. 1-4	Chromatin organization of the histone H4 FO108 gene locus	15
Fig. 1-5	Cell cycle regulated element and cognate binding factors of the histone H4 promoter	18
Fig. 1-6	Diagram of CDP/Cux, its isoforms and evolutionarily conserved domains	20
Fig. 1-7	Diagram of CDP/Cux, its isoforms ans evolutionarily conserved domains	23
Fig. 3-1	CDP/Cux and pRB mutant proteins	62
Fig. 3-2	The CDP/Cux C-terminus interacts with H4 Site II-associated proteins *in vitro*	71
Fig. 3-3	The interaction between CDP/Cux and pRB is direct and phosphorylation-site independent	74
Fig. 3-4	CDP/Cux interacts with pRB *in vivo*	76
Fig. 3-5	Partial colocalization of CDP/Cux and pRB in HeLa cells	78
Fig. 3-6	A subset of CDP/Cux and pRB associates *in situ*	79

Fig. 3-7 The C-terminus of CDP/Cux is required for repression of H4 promoter activity 81

Fig. 3-8 The pRB large pocket is sufficient for CDP/Cux dependent co-repression of H4 transcription 82

Fig. 3-9 Overexpressed mutant pRB proteins retain nuclear localization and moderate expression does not perturb cell cycle distribution 85

Fig. 3-10 Model for the mechanism of HiNF-D repression of histone gene transcription 90

Fig. 4-1 Targeted mutation of the mouse *Cutl1* gene by homologous recombination 99

Fig. 4-2 High post-natal lethality and stunted growth in *Cutl1* ΔC homozygous mutant mice 100

Fig. 4-3 Full-length *Cutl1* mRNA and protein are not expressed in ΔC-/- mice 102

Fig. 4-4 Absence of HiNF-D complex in homozygous mutant mice 105

Fig. 4-5 CDP/Cux ΔC protein does not retain DNA-binding activity 106

Fig. 4-6 Reduced levels of CDP/Cux ΔC mutant protein is detected in the nucleus 108

Fig. 4-7 Significantly reduced levels of CDP/Cux ΔC protein is observed in the nucleus of homozygous mutant MEFs 109

Fig. 4-8 Reduced histone H4.1 mRNA levels in *Cutl1* ΔC mouse embryonic fibroblasts (MEFs) that exhibit normal growth characteristics 112

Fig. 4-9 *Cutl1* ΔC mutant males with normal testicular morphology have reduced serum testosterone levels 114

Fig. 4-10 Normal histologic features of the small intestine from *Cutl1* ΔC-/- mice 116

Fig. 4-11 Hair loss in homozygous mutant *Cutl1* ΔC mice 118

Fig. 5-1 Model for CDP/Cux-pRB interaction in different cell cycle stages ... 130

Fig. 5-2 Model for CDP/Cux as a putative tumor suppressor protein 132

Fig. 5-3 CDP/Cux is involved in the regulation of proliferation, apoptosis and differentiation 134

CHAPTER 1:

GENERAL INTRODUCTION

General Background

Cell division and differentiation are essential processes for the growth and development of higher eukaryotic organisms. Regulation of gene expression is vital for control of cellular growth and differentiation. Normal eukaryotic cells have a limited proliferative capacity, and ultimately undergo cellular senescence and die. In contrast, transformed cells bypass the limit on proliferation and survive in an immortalized state. Terminal differentiation of cells is usually linked with loss of proliferative capacity and acquisition of specialized functions. Proliferation and differentiation are required for the creation and maintenance of diverse tissues both during embryonic development and postnatal life.

Cell Division and the Cell Cycle

Overview. The cell cycle is the process by which cells reproduce, and requires duplication and segregation of hereditary material. The cell cycle is divided into four consecutive phases beginning with Gap 1 (G_1) phase, followed by Synthesis (S) phase then Gap 2 (G_2) phase and ending with Mitosis (M) phase (Fig. 1-1). DNA synthesis and duplication occur in S phase and chromosome segregation occurs during mitosis or M phase. A normal cell in resting state is in Gap 0 (G_0) phase and upon growth stimulation enters G_1 phase. At the restriction point in G_1 phase [Pardee, 1989], the influences of mitogenic and anti-mitogenic signals culminate in an irreversible decision either to commit to the mitotic cell cycle and enter S phase, or to remain in a quiescent state with the option to differentiate or to re-enter the cell cycle

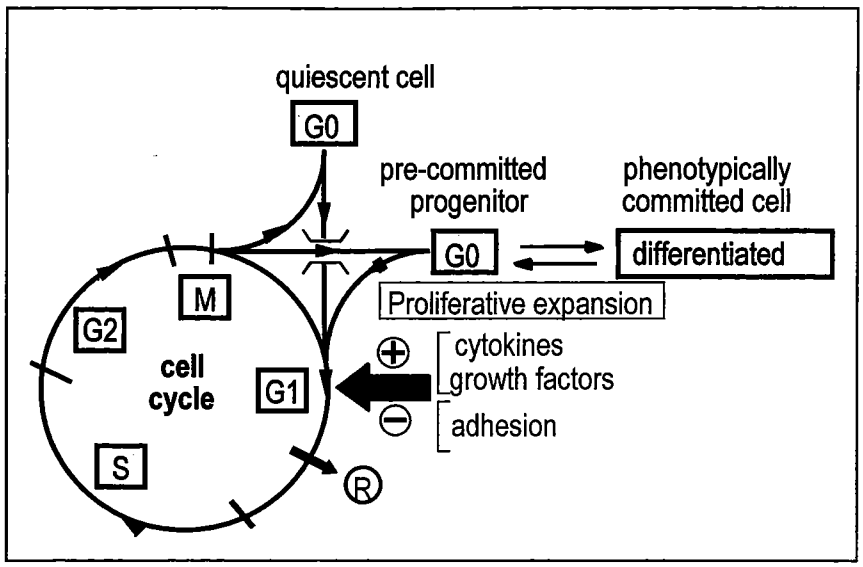


FIG 1-1. The restriction point and the cell cycle. The relative timing of the four phases: Gap 1 (G_1) phase, Synthesis (S) phase, Gap 2 (G_2) phase and mitosis (M) phase are indicated. The relative duration of each phase is depicted. Based on the influences of mitogenic (i.e., cytokines and growth factors) and anti-mitogenic signals (i.e., cell adhesion), the cell makes an irreversible decision at the restriction (R) point in G_1 phase either to commit to the mitotic cell cycle and enter S phase, or to remain in a quiescent state (G_0) with the option to differentiate. Adapted from Stein et al., 1999.

when conditions are more favorable for growth. At checkpoints during the cell cycle, controls operate to regulate the onset of these events and ensure that they occur in the correct sequence, are coordinated with cellular growth, and are corrected for errors in their execution [Nurse, 1994]. Stringent control of how cell cycle events are executed ensures the survival of living organisms, while deregulation of these events increases genomic instability, an important factor in the initiation of cancer and non-malignant disorders.

Cell cycle engines. Cyclin-dependent kinases (CDKs) act as a "cell cycle engine" to drive cells through the cell cycle (Fig. 1-2). Each enzyme complex is composed of a regulatory subunit, the cyclin, and a catalytic subunit, the CDK. With the exception of cyclin D, which is expressed as a delayed early response (early/mid G_1) to mitogen stimulation [Matsushime et al., 1994], most cyclins are expressed in a cell cycle-dependent manner. CDK activity is controlled by the availability of the cyclin subunit [Sherr, 1993], by targeted degradation, by changes in their phosphorylation status [Fisher and Morgan, 1994; Morgan, 1997] and by interaction with CDK inhibitors $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, and INK4 family proteins $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ [Vidal and Koff, 2000].

The CDKs phosphorylate a wide range of structural proteins and transcription factors that control progression through the cell cycle [Stein et al., 2001]. The sequential activity of cyclin D-CDK4/6 complexes [Matsushime et al., 1994; Ohtsubo and Roberts, 1993; Quelle et al., 1993], cyclin E-CDK2 complexes and cyclin A-CDK2 complexes are necessary for progression through G_1 and entry into S phase [Beijersbergen and Bernards, 1996]. Cyclin D and cyclin E-CDK complexes activate

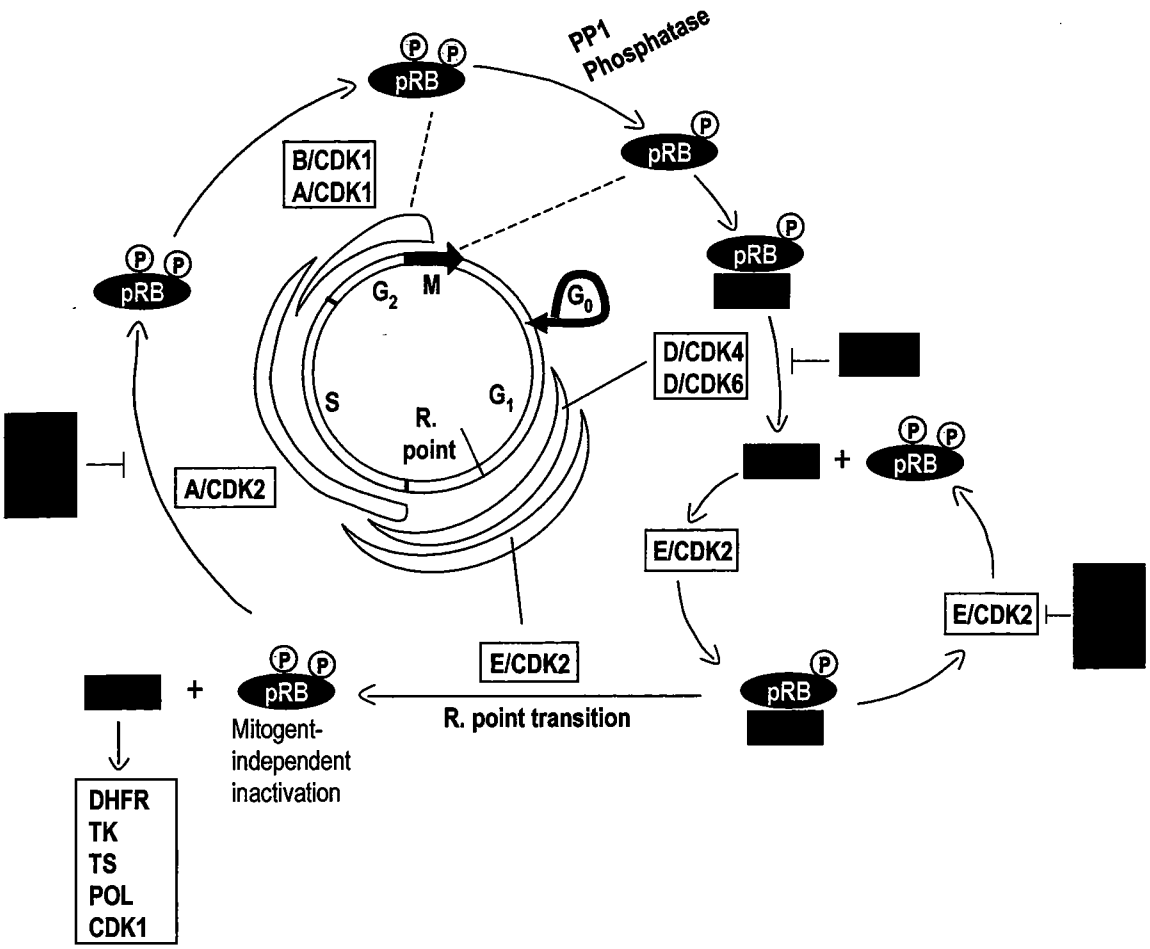


FIG 1-2. Cell cycle and restriction point control. Critical events and key regulators are depicted in the diagram. pRB phosphorylation initiated by Cyclin D-dependent kinases releases pRB-bound E2F. Active E2F transactivates the expression of genes required for S phase progression, including dihydrofolate reductase (DHFR), thymidine kinase (TK), thymidine synthase (TS), DNA polymerase-α (POL), CDK1, cyclin E and E2F itself. This establishes a positive feedback loop promoting pRB phosphorylation by cyclin-E-CDK2, contributing to the irreversibility of the restriction point, and ultimately making pRB phosphorylation by cyclin-dependent kinases (CDKs) mitogen-independent in S and G_2 phase. In mitosis, pRB is dephosphorylated by PP1 phosphatase, which then allows it to bind E2F in G_1.

E2F-dependent transcription of genes required for S phase by phosphorylating pRB retinoblastoma protein and thus blocking its repression of E2F activity. In S phase, phosphorylation of components of the DNA replication machinery by cyclin A/CDK2 complexes mediates the initiation of DNA replication and blocks re-initiation at the same replication foci [Coverley et al., 2002]. In mitosis, cyclin B/CDK1 complexes regulate chromosome condensation by modifying chromatin structure. Cyclin B-CDK1 complexes are also involved in nuclear envelope breakdown through its phosphorylation of nuclear lamins. In addition, both cyclin A/CDK1 and cyclin B/CDK1 complexes promote chromosome segregation by activating topoisomerase II. Thus cyclin/CDK complexes are essential to the proper regulation of cell cycle progression.

Transcriptional control during the cell cycle. Transcriptional modulation of genes that encode cyclins and proteins involved in cell cycle progression is essential throughout interphase. Regulation of gene transcription is mediated by promoter elements and the macromolecular complexes of transcription factors that interact with these regulatory sites in a sequence specific manner. Formation of the macromolecular complexes may be facilitated by specific localization of genes within the nucleus and local concentration of co-regulators at specific subnuclear domains.

Histone proteins

Histone proteins are critical for structural organization of all eukaryotic genomes by facilitating the compaction and condensation of their DNA. Expression of replication-dependent histone genes is temporally and functionally coupled with

DNA synthesis in S phase. Histones are a family of small, basic proteins that bind and package DNA into chromatin within each cell nucleus. There are five major classes of histones: the linker histone H1, and the core histones H2A, H2B, H3 and H4. The core histones form nucleosomes that are the basic packaging unit of eukaryotic DNA. Each nucleosome is composed of two each of the core histones, around which is wrapped 146 base pairs of DNA in approximately two superhelical turns [Luger et al., 1997]. The H1 histones facilitate the organization of linear arrays of nucleosomes into 30 nm chromatin fibers by interacting with the DNA.

Histones are essential for chromatin organization and transcriptional regulation in eukaryotic cells [Jenuwein and Allis, 2001; Stein et al., 1991]. The linker histone H1 has been shown to influence nucleosome positioning, which in turn influences transcription [Archer et al., 1991]. Changes in post-translational modifications of histones, such as acetylation, may affect the ability of histones to influence transcription [Bradbury, 1992; Jenuwein and Allis, 2001; Stein et al., 1991]. Histone acetylation is often associated with DNA replication and transcriptional regulation [Eberharter and Becker, 2002; Magnaghi-Jaulin et al., 2000]. Acetylation of the amino terminal tail domains of core histones destabilizes their interaction with the nucleosomes and thereby facilitates transcription [Pennisi, 1997]. In addition to acetylation, histone tails also undergo adenosine diphosphate- ribosylation, ubiquitination, methylation, and phosphorylation. The C-terminal tails of histones H2A and H2B undergo cell cycle-dependent ubiquitination [Mueller et al., 1985]. Furthermore, ubiquitinated histones are associated with transcriptionally active DNA sequences [Nickel et al., 1989]. Phosphorylation of histones H3 and H1 is also cell

cycle regulated. For example, histone H1 is hyperphosphorylated in late G_2 phase [Mueller et al., 1985]. Because of the essential function of these histone modifications in the tail domains, it appears that nucleosomes not only mediate the structural organization of DNA but also carry epigenetic information that determines both how genes are expressed and how their expression patterns are maintained from one cell generation to the next [Spotswood and Turner, 2002].

Histone genes: organization and variants.

The genomes of various eukaryotic organisms exhibit significant differences in the number and organization of histone genes [Old and Woodland, 1984]. For example, the majority of the histone genes in sea urchins (300 to 600 copies), Drosophila (100 copies) and amphibians (40 to 1600 copies) are arranged as quintets. Each quintet of genes contains one copy of each of the five histone classes. These quintets are frequently arranged in tandemly repeated patterns in the genome. In contrast, human and mouse have only 10-40 copies of the histone genes that are clustered but not tandemly repeated [Heintz et al., 1981; Sittman et al., 1981].

In total there are at least 74 histone genes in the human genome [Heintz et al., 1981; Lichtler et al., 1982; Tripputi et al., 1986; Volz et al., 1997] and a similar number in the mouse genome [Marzluff et al., 2002; Sittman et al., 1981; Wang et al., 1996b; Wang et al., 1996a]. The majority of these genes are present in one large cluster (HIST1) on human chromosome 6 (55 genes) and mouse chromosome 13 (51 genes) (Table 1-1). There are 2 smaller clusters (HIST2 and HIST3) on human chromosome 1 that together contain 10 histone genes. Orthologous *Hist2* and *Hist3*

Table 1-1. Replication-dependent histone genes in humans and mice

Chromosome (histone cluster)	6p21-p22 (HIST1)	*13A2.3 (Hist1)*	1q21 (HIST2)	*3 (Hist2)*	1q42 (HIST3)	*11B2 (Hist3)*	12p13.1 (HIST4)	6G1 *(Hist4)*
H1	6	6						
H2A	12	13	3	3	1	1		
H2B	15	13		1	1	2		
H3	10	9		1	1			
H4	12	10	1	1			1	1
genes in each cluster	55	51	6	10	3	3	1	1

* Adapted from Marzluff et al., 2002

clusters are located on mouse chromosomes 3 and 11, respectively. The remaining histone genes are distributed as single copies throughout the genome. Each of the fourteen histone H4 genes encodes the same protein, and there are only three histone H3 proteins encoded by the twelve histone H3 genes in both humans and mice. In contrast, histones H2a and H2b are composed of at least ten non-allelic variants in each species.

There are three groups of histones: replication-dependent histones are the predominant group, containing 74% of histone genes which are organized into 2 clusters (HIST 1 and HIST2); the two remaining groups are comprised of replacement variants and tissue-specific variants which are solitary genes outside any histone gene cluster [Marzluff et al., 2002]. Replacement variants, such as histone $H1^0$, are mostly synthesized in non-proliferating cells. Their gradual accumulation parallels a decrease of the main type histones of the corresponding class (Table 1-2). Replacement histones are also often synthesized at low levels in proliferating cells [Marzluff et al., 2002]. Expression of tissue-specific histones is restricted to certain tissues. Examples include histone H1oo in mouse oocytes [Tanaka et al., 2001] and testis-specific histone H1t in sperm cells [Wang et al., 1997]. Unlike the mRNAs encoding main type histones, replacement variant mRNAs are polyadenylated and splicing occurs in several variants. Replication-dependent histones represent the majority of histone proteins synthesized in dividing cells. Synthesis of these histones is restricted to S phase of the cell cycle, when DNA is replicated (Fig. 1-3) [Stein and Borun, 1972]. Transcriptional and post-transcriptional mechanisms determine the levels of histone mRNA during the cell cycle. Regulation of histone gene transcription results from the

Table 1-2. Variant histone genes

Histone Variant	Expression Pattern	Introns	Chromosome (Human)	Chromosome (mouse)
$H1^0$	liver, pancreas, brain, testis	no	22	15
H1t	spermatocytes, spermatids	no	6	13
H1oo	oocytes (mouse)	yes	unknown	3
H2A.X	testis, thymus, spleen uterus, ovaries, intestines	no	11	9
H2A.Z	germ cells most somatic tissues	yes	4	1
H2B	spermatid	no	6	13
H3.3A	differentiated adult tissues	yes	1	13
H3.3B	differentiated adult tissues	yes	17	11
H3.4	testicular cells	no	5	1

* Adapted from Marzluff et al., 2002

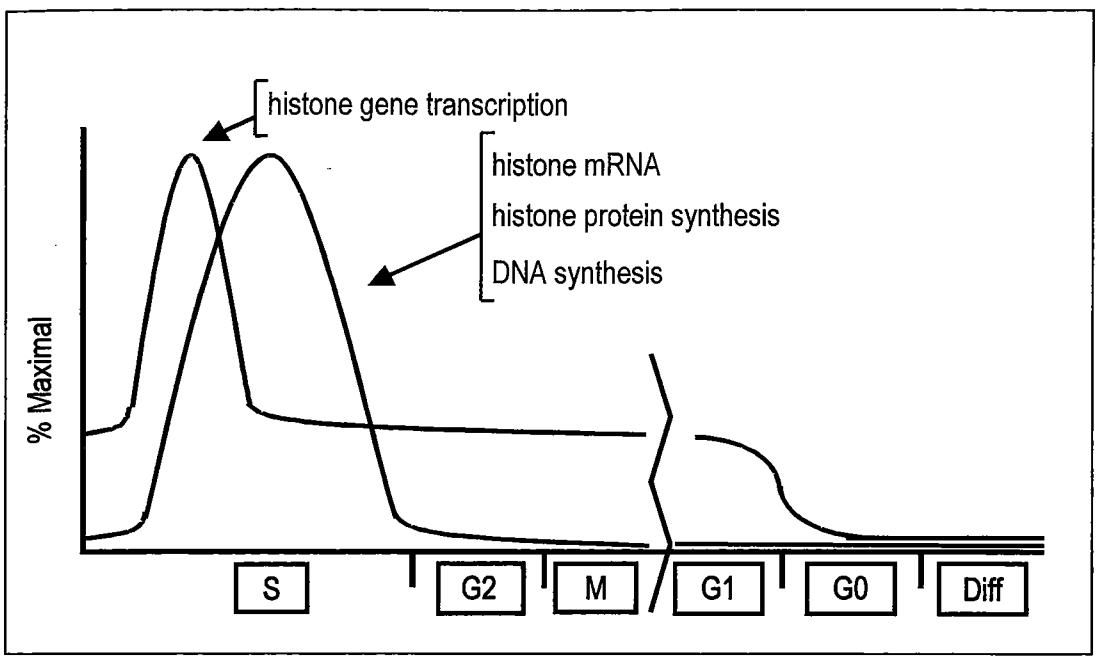


FIG 1-3. Cell cycle regulation of histone gene expression. Histone protein biosynthesis is functionally coupled to DNA synthesis. Cells entering S phase exhibit a concurrent increase in the accumulation of histone mRNA, the level of histone biosynthesis, and the rate of DNA synthesis. A three- to five fold enhancement of histone gene transcription in early S phase precedes the accumulation of histone mRNA. Selective degradation of histone mRNAs occurs in parallel with a decline in DNA synthesis. This figure is adapted from Stein et al., 1996.

collective effects of cell signaling pathways, dynamic chromatin structure and multiple transcription factor interactions. Thus replication-dependent histone genes serve as excellent models for understanding molecular mechanisms involved in cell cycle regulation of gene expression.

Regulation of replication-dependent histone gene expression

Synthesis of replication-dependent histones is tightly coupled to DNA synthesis, occurring exclusively in S phase [Marashi et al., 1982]. Because somatic cells do not have storage pools for histone proteins or histone mRNA, coordination of *de novo* synthesis of all five histone classes in S phase is required for the packaging of newly replicated DNA. Histone biosynthesis occurs at an average rate of several thousand proteins per second throughout S phase. This immense upregulation of histone production is due to a 10-30 fold increase in histone mRNA in S phase, which is a result of altered transcriptional [Baumbach et al., 1987; Heintz et al., 1983] and post-transcriptional regulation [Cleveland and Yen, 1989; Morris et al., 1986].

Transcriptional regulation. Replication-dependent histone genes are transcribed at basal levels throughout the cell cycle, but the resulting transcripts either are not processed into mature mRNAs or are selectively degraded [Osley, 1991; Stein et al., 1994]. Transcription of histone genes is coordinately upregulated 3-5 fold at the beginning of S phase. Vertebrate histone genes are transcribed by RNA polymerase II, and the sequences that regulate transcription occur 5' to the site of transcription initiation. The histone gene promoters are modular in nature and contain discrete, independently functioning sequence elements that together contribute to the

transcription of each histone gene. Regulatory elements present in the promoters of all 5 classes of histone genes include the histone family-specific hexamer (HEX) element, the TATA box, the CAAT motif and Sp1-binding sites [Osley, 1991; Stein et al., 1994]. In addition, there are elements that are specific to replication-dependent histone genes, and elements that occur only in particular classes of histone genes. Since the HEX element is specific to the histone gene family, it has been proposed that a unique factor binding at this sequence may function to coordinately activate histone gene transcription upon entry into S phase [Osley, 1991]. However, no known cell-cycle function has been attributed to the hexamer element, which acts primarily to maintain maximal levels of transcription. Instead, each kind of vertebrate histone gene contains a different cell cycle regulatory element that binds a distinct regulatory factor with specific activation functions [Artishevsky et al., 1987; La Bella et al., 1988; La Bella et al., 1989; Stein et al., 1994]. Studies in our laboratory have focused on elucidating the mechanisms involved in transcriptional regulation of the replication-dependent histone H4 gene FO108.

Post-transcriptional regulation. All replication-dependent histone mRNAs contain a stem-loop motif at their 3' termini. In the nucleus, histone mRNA transcripts are processed by endonucleolytic cleavage just 3' to the stem-loop structure to produce the mature cytoplasmic mRNA species. Activation of histone transcript processing is the primary post-transcriptional regulatory pathway utilized in eukaryotic organisms upon entry into S phase. Upon completion of S phase, histone mRNA is returned to basal levels. This downregulation is mainly due to rapid mRNA degradation in the cytoplasm, which is regulated by the presence of the stem-loop

motif [Pandey and Marzluff, 1987]. The mRNA half-life is 120 minutes in early S phase and decreases dramatically near the completion of DNA synthesis to 15-20 minutes [Morris et al., 1991].

Transcriptional Regulation of the Human Histone H4 Gene FO108

Modifications of chromatin structure. Control of basal and cell cycle regulated histone H4 transcription is dependent on proper chromatin conformation and coordinated binding of transcription factors to gene regulatory elements. The chromatin structure of the FO108 histone H4 gene was analyzed by examining the levels of nuclease sensitivity throughout the gene [Chrysogelos et al., 1989]. During the cell cycle, the coding region, the 3' segment of the distal promoter (–250 to –600 nt) and the proximal promoter (-70 to –20 nt) exhibit changes in nuclease sensitivity (Fig. 1-4B), which are indicative of changes in chromatin structure at these sites. Sensitivity to DNaseI and S1 nuclease peaks in S phase, then decreases in mitosis and G_1 phase. Micrococcal nuclease analysis of the histone H4 FO108 gene shows that the proximal promoter and the 5' segment of the coding region (-70 to +90 nt) lack normal nucleosomal organization throughout the cell cycle (Fig. 1-4C), which is reflected by a degeneration of the characteristic nucleosomal ladder produced by nuclease digestion [Cho et al., 2002; Moreno et al., 1988]. In addition, there are significant changes in nucleosome structure associated with the 3' segment of the coding region during the cell cycle. In summary, the histone H4 promoter is organized in an open chromatin structure and is accessible to nucleases (and transcription factors) throughout the cell cycle. This is consistent with the

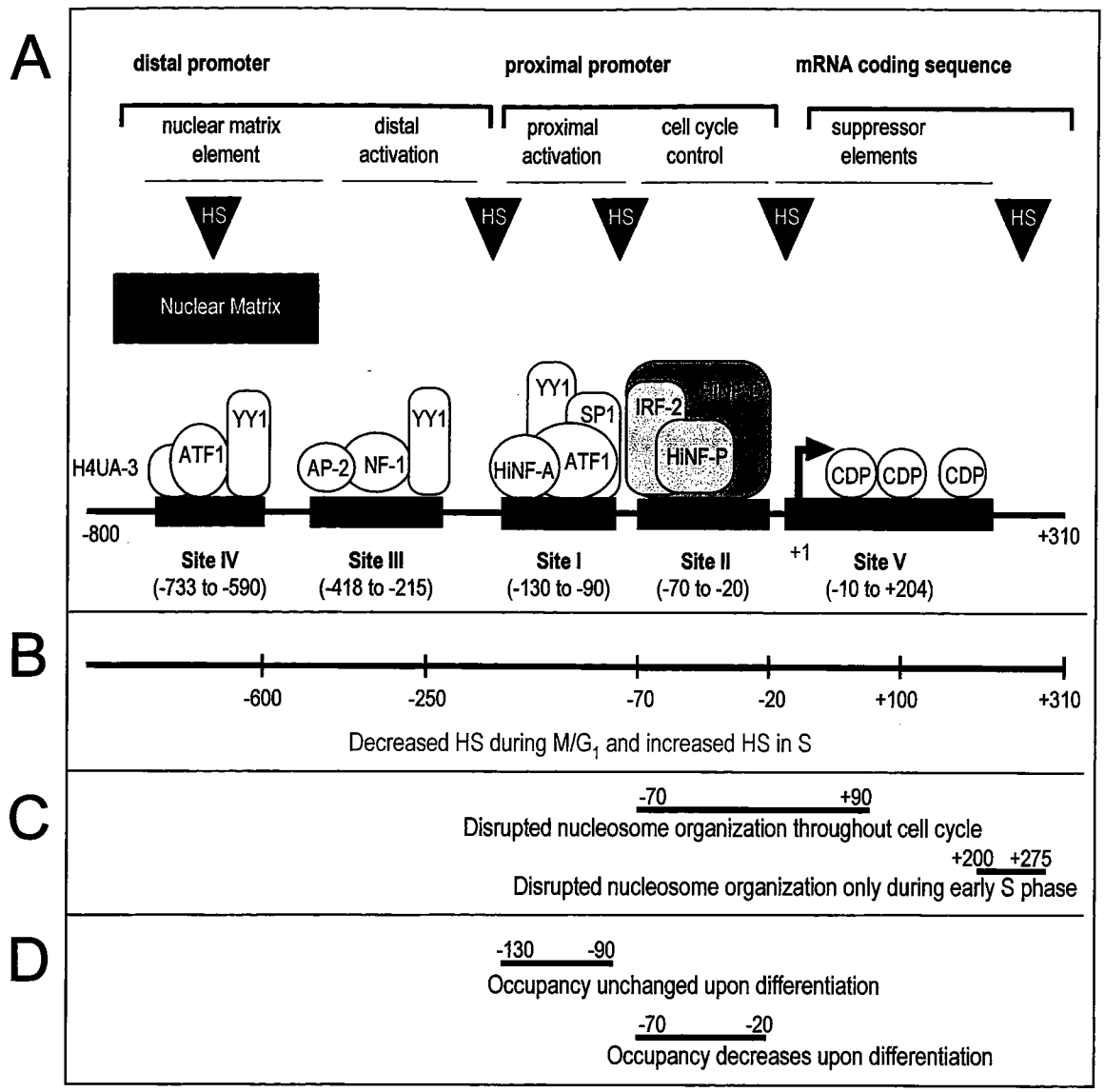


FIG 1-4. Chromatin organization of the histone H4 FO108 gene locus. (A) The diagram depicts the five regulatory elements (Sites I to V), the nucleotides they encompass, and the transcription factors that interact at these sites (Kroeger et al., 1987, Pauli et al., 1989, Dworetzky et al., 1992, Wright et al., 1990, Artyshevsky et al., 1987, Stein et al., 1994, Guo et al., 1995 and 1997, Last et al., 1999). Also indicated are the MNase-sensitive sites and DNaseI-hypersensitive sites (HS, black triangles). **(B)** Regions of nuclease hypersensitivity during the cell cycle (Chrysogelos et al., 1989, Hovhannisyan et al., 2003). **(C)** Nucleosome organization during the cell cycle (Moreno 1988, Hovhannisyan unpublished). **(D)** *In vivo* occupancy of Sites I and II during differentiation, as observed by DNaseI footprinting using LM-PCR (Stein et al., 1989, Hovhannisyan et al., 2003).

observation that basal transcription of the histone gene is constitutive. Upon entry into S phase, transcriptional upregulation is accompanied by modest increases in nuclease sensitivity at several sites, including the coding region. Interestingly, occupancy of the proximal promoter (-70 to –20 nt) is decreased during differentiation, when histone gene transcription is silenced (Fig. 1-4D) [Hovhannisyan et al., 2003; Stein et al., 1989]. Taken together, these observations suggest that discrete regions of the histone H4 gene undergo chromatin modifications that correlate with the observed transcriptional status of the gene.

Histone H4 Gene regulatory elements and cognate binding factors.

Our laboratory has defined and characterized multiple regions involved in the transcriptional regulation of the histone H4 FO108 gene (Fig. 1-4A). The distal promoter region of the histone H4 gene supports maximal transcription. Two elements have been defined *in vitro*, Site III and Site IV. Site IV (-733 to –590) is a transcriptional activator element that binds preferentially to two proteins present in nuclear matrix extracts [Dworetzky et al., 1992; Pauli et al., 1989], YY1 and ATF/CREB [Guo et al., 1995; Guo et al., 1997a]. Site III (-418 to –215) has been shown to increase transcription of reporter genes by two fold [Kroeger et al., 1987]. Binding of YY1, AP-2 and NF1 to Site III was observed by electrophoretic mobility shift assay (EMSA) [Last, 1998; Last et al., 1999a].

In the proximal promoter of the histone H4 FO108 gene, two sites of protein-DNA interaction, Site I (-130 to –90 nt) and Site II (-70 to –20 nt), have been established both *in vitro* and *in vivo* [Pauli et al., 1987; van Wijnen et al., 1987; van

Wijnen et al., 1991d]. Site I of the proximal promoter confers maximal levels of histone H4 gene transcription in proliferating cells, and deletion of Site I reduces transcription rates 4-6 fold *in vitro* [van Wijnen et al., 1989; van Wijnen et al., 1991d]. Several transcription factors that interact with Site I and activate transcription include YY1, Sp1, CREB, ATF-1 and an unidentified complex histone nuclear factor A [Birnbaum et al., 1995; Guo et al., 1997b; Last et al., 1999b; van Wijnen et al., 1989; Wright et al., 1995]. Binding of these regulatory factors to Site I *in vivo* occurs throughout the cell cycle [Pauli et al., 1987].

Site II of the histone H4 proximal promoter is essential for proper initiation of transcription and cell cycle regulation [Ramsey-Ewing et al., 1994]. Site II is occupied by three factors (Fig. 1-5), which are designated histone nuclear factors M, P and D (HiNF-M, HiNF-P, and HiNF-D) [Kroeger et al., 1987; Ramsey-Ewing et al., 1994; van Wijnen et al., 1991c]. HiNF-M was identified as interferon regulatory factor 2 (IRF-2) and interacts at the cell cycle control element on the distal side of Site II [Ramsey-Ewing et al., 1994; Vaughan et al., 1995]. Consistent with its role in cell growth regulation, IRF-2 can activate histone H4 transcription [Vaughan et al., 1995]. In addition, IRF-1, IRF-3, and IRF-7 are also potent activators of the H4 promoter [Xie et al., 2001] Binding of IRF-2 to Site II is cell cycle regulated, with maximal levels in S phase [Shakoori et al., 1995; Vaughan et al., 1998]. Recently identified, HiNF-P is a 65-kDa zinc-finger protein that interacts with the histone H4 promoter *in vivo* [Mitra et al., 2002b]. Like IRF-2, HiNF-P can activate histone H4 transcription and binds Site II in a cell cycle-dependent manner [Mitra et al., 2002b].

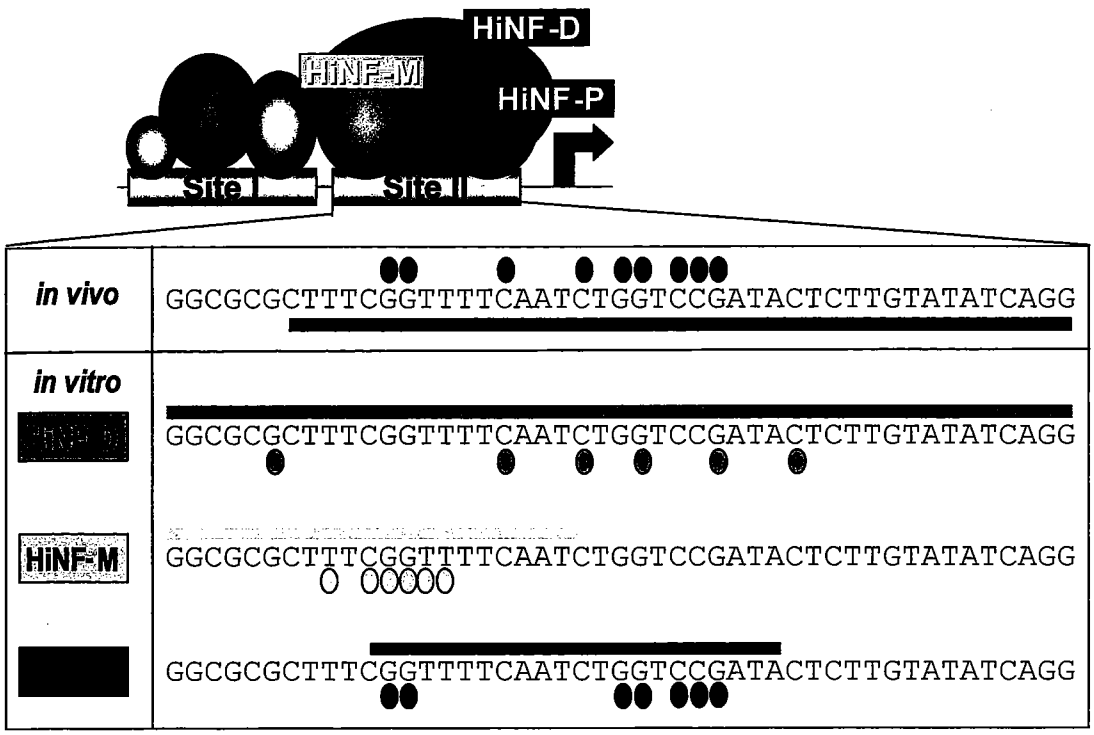
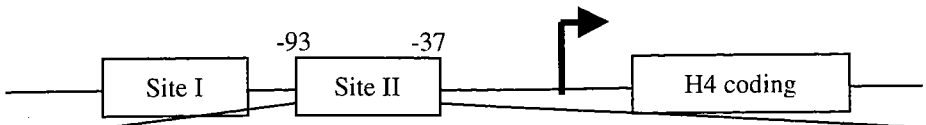


FIG 1-5. Cell cycle regulated element and cognate binding factors of the histone H4 promoter. Site II is occupied by HiNF-D, HiNF-M, and HiNF-P. *In vivo* occupancy of Site II was detected by DNase I footprinting (solid dark blue bar) and DMS finger-printing (dark blue ovals)(Pauli et al., 1987, Ramsey-Ewing et al., 1994). *In vitro* protein/DNA interactions observed by EMSA (light blue, yellow and red bars) and methylation interference assays (light blue, yellow and red ovals) using gel-purified HiNF-D complexes or fractionated extracts enriched for HiNF-M or HiNF-P are depicted (van Wijnen et al., 1987).

The third factor that interacts with Site II is HiNF-D, a protein complex that consists of growth regulatory factors cyclin A, CDK1, pRB, and the homeobox protein CDP/Cux, which is the DNA binding subunit [van Wijnen et al., 1994; van Wijnen et al., 1996]. HiNF-D binding activity is proliferation-specific and is not observed in differentiated cells [Holthuis et al., 1990; van Wijnen et al., 1989; van Wijnen et al., 1994]. HiNF-D interaction with Site II (-70 to –20) is restricted to S phase in normal cells but is constitutive in cancer cells [Holthuis et al., 1990; Owen et al., 1990; van Wijnen et al., 1989]. Based on the observed binding of HiNF-D to wildtype (FO108) and mutant Site II sequences [van Wijnen et al., 1992], approximately half of the human histone H4 genes are predicted to bind HiNF-D (Fig.1-6).

In addition to histone H4, HiNF-D also interacts with the promoters of histones H3 (-139 to –67) and H1 (-213 to –92) [Owen et al., 1990; van den Ent et al., 1994; van Wijnen et al., 1991c; van Wijnen et al., 1996]. In contrast to histone H4, HiNF-D binding to the H3 and H1 promoters requires sequences of both the analogous Site I and Site II. Occupation of the H1, H3 and H4 promoters by HiNF-D was observed *in vitro* by DNaseI footprinting and dimethyl sulfate (DMS) fingerprinting using electro-eluted HiNF-D/DNA complexes [van den Ent et al., 1994; van Wijnen et al., 1991c; van Wijnen et al., 1991d]. HiNF-D binding to the H3 and H1 genes occurs in proliferating but not differentiated cells, and is cell cycle-regulated in normal diploid cells but not in tumor cells. Thus through its interaction with the promoters of multiple histone gene classes, HiNF-D may be involved in the coordinate regulation of these genes during the cell cycle.



Gene	Accession#		** * * ** ***			binding
H4/n	M16707	cgctttc	ggttttcaatctggtccg	atactcttg	tatatca	+++
H4/a	X60481	gcttttc	agtctctgt----gtccg	ctggaggtggggcagg		
H4/b	X60482	aactgat	gg-ctcagttaatggctg	caagtatag	tgtgtgt	
H4/c	M60749	ccaattc	agtctccaa-aaggtccg	cataattga	tatataa	yes
H4/d	X60483	tctttca	ggttctcagttcggtccg	ccaactgtc	-gtataa	no
H4/e	X60484	tctttca	ggttctcagttcggtccg	ccaactgtc	-gtataa	no
H4/fψ	X60485	tgttttt	ggtttttacctcgggtct	tttgtcgtg	ccttaaa	
H4/g	X60486	ctcatgt	tgttttcaatcaggtccg	ccaagtttg	tatttaa	yes
H4/h	X60487	cgctttc	agtcttcaattaggtccg	aattcccgg	catataa	
H4/i	X67081	cgctttc	agttctcaataaggtccg	atgttcgtg	tataaat	yes
H4/j	Z80787	cgttttc	agtcttcatttaggtccg	aattcccgg	catataa	
H4/k	X83548	tgtggag	agttcccaatcaggtccg	atttattac	tatataa	yes
H4/l	Z80788	gtctttc	agttttcaaaaaggtcta	ctctaccgt	tatataa	yes
H4/m	AB000905	ctcttcc	ggttttcagtctggtccg	cagaggtta	cccataa	
mH4/n	V00753	cgctttc	agttttcaatctggtccg	atcctctca	tatatta	+++
mH4/g	X13235	acgctct	agttttcaaccaggtccg	cagaaggcc	tatttaa	++
mH4.3	X13236	cacttga	agttctcaaccaggtccg	attagagcc	tataata	+
mH4-D	U62672	ggttttc	-gtttgtgt--gattctg	ttgggggtgggagatca		
H4mut	ST-8	cgctttc	ggttttcaatctggtccg	atctgtcac	gcgctca	+
H4mut	GT-9	cgctttc	ggttttcaatcgtcgaat	gcactcttg	tatatca	+++
H4mut	SUB-11	cgctttc	ggttctcagttcggtccg	ccactcttg	tatatca	no
H4mut	MC-7	cgctgtc	tatgttacatatggtccg	atactcttg	tatatca	+

FIG 1-6. Observed or predicted HiNF-D binding to different histone H4 genes.
Site II of the human histone H4/n (FO108) gene has high sequence similarity to other histone H4 genes in humans and mice. The asterisks above the sequence indicate methylation-protected protein/DNA contacts for the FO108 gene {173}. Based on mutagenesis studies of Site II sequences [van Wijnen et al., 1992], HiNF-D is predicted to bind to nearly half of the histone H4 genes in humans. Observed (bold font) and predicted (regular font) HiNF-D binding activities are indicated in the right column. The relative binding strength of HiNF-D is reflected by the number of plus signs. mH4 sequences represent murine histone H4 genes [vander Meijden et al., 1998].

Three observations implicate HiNF-D as a transcriptional activator. First, HiNF-D contributes to transcriptional activation of histone H4 when the HiNF-M binding site has been mutated or when HiNF-M is absent in the cell [Aziz et al., 1998b]. Second, HiNF-D activity is correlated with histone H4 mRNA levels in several mouse tissues: high levels are present in adult spleen and thymus, and fetal liver; and low levels are observed in adult liver [van Wijnen et al., 1991a]. Lastly, in HeLa cell lines containing stably integrated histone H4 promoter fused to a chloramphenicol acetyl transferase (CAT) reporter gene, mutations that abrogate HiNF-D interaction with Site II cause a delay both in transcriptional upregulation and downregulation of the histone H4 gene by two hours [Aziz et al., 1998a]. Therefore, HiNF-D may also act as a suppressor of histone H4 gene transcription. Forced expression of CDP/Cux, the DNA-binding subunit of HiNF-D, results in transcriptional repression [van Wijnen et al., 1996]. Using EMSA, as well as methylation interference and DNaseI footprinting analyses, CDP/Cux was identified as the factor that interacts with three transcriptional suppressor elements at Site V (-10 to +210) in the coding region of the histone H4 FO108 gene [Last et al., 1998]. In addition, maximal HiNF-D interaction with Site II and Site V occurs in mid to late S phase, when histone gene transcription is downregulated [Last et al., 1998; Shakoori et al., 1995; van Wijnen et al., 1997]. Although many observations support a role for CDP/Cux in the regulation of histone gene expression, the molecular mechanisms that mediate this regulatory function is poorly understood.

CDP/Cux

Human CCAAT Displacement Protein (CDP)/Cux is encoded by the *Cutl1* (Cut-like 1) gene, a candidate tumor suppressor gene located on chromosome 7 at band 22, a region that is often deleted in several cancers [Ishwad et al., 1997; Zeng et al., 1997; Zeng et al., 1999]. *Cutl1* spans at least 340 kb and consists of 33 exons. At least six isoforms are generated as a result of alternative transcription initiation, splicing and polyadenylation (Fig. 1-7). Human CDP is a homolog of the Drosophila homeodomain protein Cut, canine Clox, murine Cux-1 and rat CDP2. CDP/Cux is expressed in many cell lines and tissues [Nepveu, 2001; Neufeld et al., 1992]. CDP/Cux contains five evolutionarily conserved domains: the coiled-coil domain, which is a motif that mediates protein-protein interaction, and four DNA binding domains [Cut Repeat 1 (CR1), CR2, CR3 and the homeodomain (HD)] (Fig. 1-7). Individual Cut Repeats cannot bind to DNA as a monomer; however several combinations of domains (CR1+CR2, CR3+HD, CR1+HD, and CR2+HD) are able to bind DNA with a high affinity [Moon et al., 2000]. Acetylation by PCAF or phosphorylation by protein kinase C, casein kinase II or cyclin A/CDK complexes results in decreased DNA binding activity of CDP/Cux (Table 1-3) [Coqueret et al., 1996; Coqueret et al., 1998c; Coqueret et al., 1998a; Li et al., 2000; Santaguida et al., 2001].

Forced expression of CDP/Cux results in the repression of multiple genes (Table 1-4) [Nepveu, 2001], including histone H4 FO108 [van Wijnen et al., 1996]. In addition to histone genes, CDP/Cux has been shown to regulate the expression of

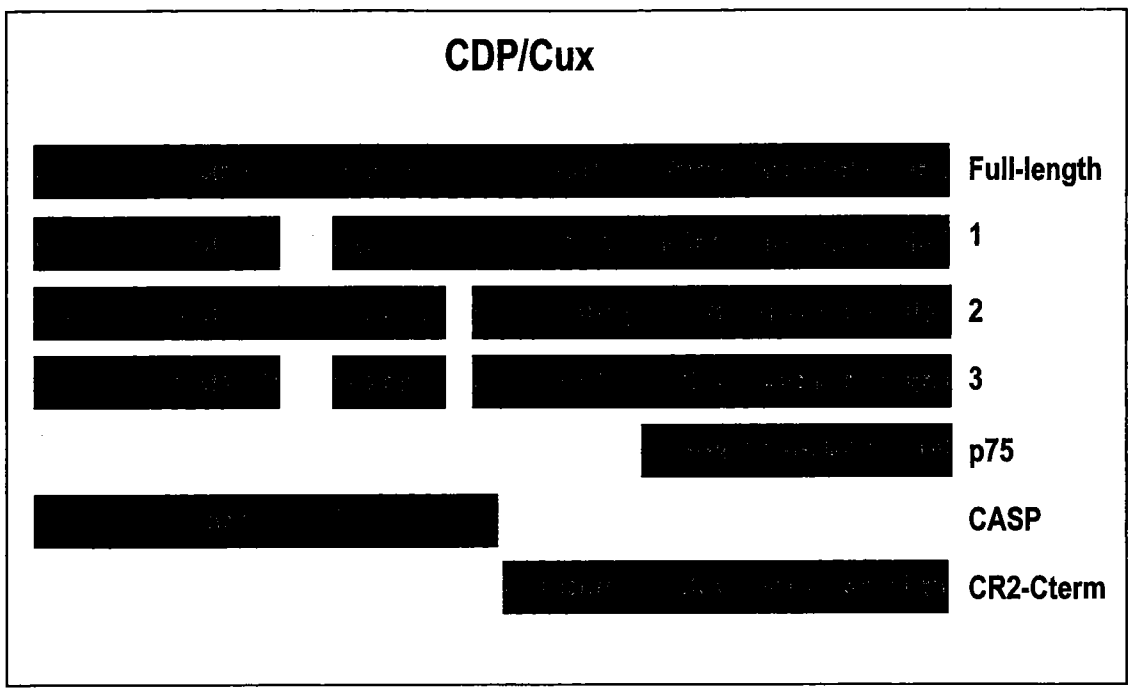


FIG 1-7. Diagram of CDP, its isoforms and evolutionarily conserved domains. Full-length CDP/Cux contains a coiled-coil domain (CC), three Cut repeats (CR1, CR2, CR3), a homeodomain (HD), and two repressor (R) domains. Isoforms 1, 2, 3, CDP p75, and CDP alternatively spliced product (CASP) result from alternative transcription, splicing and polyadenylation. CDP/Cux (CR2-Cterm) is a product of S phase-specific protease cleavage and CDP p75 results from transcription initiation within intron 20. CASP contains the CC and a C-terminus unique to this isoform. This figure was adapted from Zeng et al., 2000.

Table 1-3: Post-translational modifications of CDP/Cux

Interactor (Region)	Interacting CDP Region	*Interaction	Enzyme	CDP/Cux substrate	DNA binding
HDAC-1	CR3-Cterminus	*in vitro*	ND	ND	ND
CBP N-terminus (1-786)	ND	*in vivo* *in vitro*	ND	ND	ND
PCAF	ND	*in vitro*	ND	Region between CR3 and HD	decreased
ND	ND	ND	CK II	Ser^{100} (CR1) Ser^{789} (CR2) Ser^{972} (CR3)	decreased
ND	ND	ND	PKC	Thr^{415} (CR1) Thr^{804} (CR2) Ser^{987} (CR3)	decreased
CyclinA/CDK1 CyclinA/CDK1 CyclinA/CDK2	ND HD plus cy motif	*in vivo* *in vitro* *in vitro*	A/CDK1	Ser^{1237} Ser^{1270} (CR3-HD)	decreased
Cdc25A	ND	*in vitro* *in vivo*	Cdc25A phosphatase	CR3HD	increased
Large T antigen	ND	*in vivo*	ND	ND	ND

* Interactions described as "*in vivo*" were detected by co-immunoprecipitation assays.
Casein kinase II (CKII); Protein kinase C (PKC); not determined (ND)

Table 1-4. Target Genes of CDP/Cux

Genes repressed in proliferating precursor cells	Function(s)
Human γ globin	Cellular oxygenation
Xenopus β globin	Cellular oxygenation
Murine NCAM	Neural cell adhesion molecule
Human tryptophan hydroxylase	Serotonin neurotransmitter biosynthesis
Rat tyrosine hydroxylase	Catecholamine neurotransmitter biosynthesis
Human hepatic-specific cholesterol-7α Hydroxylase	Bile acid biosynthesis
Rat osteocalcin	Bone development
Human cystic fibrosis transmembrane conductance regulator	Ion transport (epithelial cells)
Human phagocyte-specific gp91-phox	Immune function
Immunoglobulin heavy chain	Immune function
T cell receptor β, matrix attachment region (MAR)	Immune function
β-MHC (major histocompatibility gene)	Immune function
CD8α gene, (MAR)	Immune function
Neutrophil-specific lactoferrin	Immune function
Human C/EBPε	Immune function
Neutrophil collagenase	Immune function
p21	Growth inhibition
Human Papillomavirus Type 6 Long Control Region	Epithelial lesions
Mouse mammary tumor virus	Mammary carcinomas
Genes expressed in proliferating cells that are repressed by CDP/Cut	
Histone H1 (human and *xenopus*)	Proliferation
Histone H2B-1 (*xenopus*)	Proliferation
Histone H2A and H2B (*xenopus*)	Proliferation
Histone H3 (human)	Proliferation
Histone H4 (human and *xenopus*)	Proliferation
Murine c-myc	Proliferation
Thymidine kinase	Proliferation
Rat c-mos	Proliferation

* Adapted from Nepveu, 2001.

genes involved in proliferation including *p21*^{Cip1} and *c-myc* [Coqueret et al., 1998a; Dufort and Nepveu, 1994]. Furthermore, promoter activity of genes involved in differentiation such as *osteocalcin* and the phagocyte-specific gene *gp91-phox* have also been shown to be regulated by CDP/Cux, in complex with cyclin A and pRB-related p107 [van Gurp et al., 1999; van Wijnen et al., 1996]. CDP/Cux represses the promoter activity of many genes that have immune functions, and has been shown to interact with the matrix attachment regions of several genes, including the *CD8*α [Banan et al., 1997] and *T cell receptor* β genes [Chattopadhyay et al., 1998]. Many of the identified target genes of CDP/Cux are repressed in proliferating precursor cells and are turned on as cells undergo terminal differentiation. Consistent with its proposed role as a transcriptional repressor, CDP/Cux binding to these genes is maximal in proliferating cells and is significantly decreased in differentiated cells. Transcriptional repression by CDP/Cux is mediated by two mechanisms: displacement of activators through competition for occupancy of a binding site [Barberis et al., 1987; Skalnik et al., 1991]; and active repression by two repressor domains in the C-terminus, perhaps through direct recruitment of HDAC-1 [Li et al., 2000, Mailly et al., 1996].

In addition to gene repression, CDP/Cux has also been observed to activate several genes: *rat tyrosine hydroxylase* gene [Yoon and Chikaraishi, 1994], and S phase-specific genes *DNA polymerase-*α (*pol-*α), *dihydrofolate reductase* (*DHFR*), *carbamoyl-phosphate synthase-aspartate carbamoyltransferase-dihydroorotase* (*CAD*), and *cyclin A* gene [Goulet et al., 2002; Moon et al., 2001; Truscott et al., 2003]. CDP/Cux activates the *tyrosine hydroxylase* gene by enhancing the binding of

ITF2 to the gene promoter and both transcription factors are required for gene activation. CDP/Cux interacts with the *pol-α* gene *in vivo* and forced expression of CDP/Cux activates endogenous *pol-α* gene expression. Taken together these observations suggest that CDP/Cux may be a bifunctional regulator of cell growth and tissue-specific gene expression.

CDP/Cux has been implicated in the regulation of histone gene expression. Interaction between histone promoters and CDP/Cux was originally demonstrated with histone H2B from sea urchin [Barberis et al., 1987]. CDP/Cux has since been found to interact with the promoters of all five classes of histones genes (Table 1-4): human histones H1, H3 and H4 [van den Ent et al., 1994; van Wijnen et al., 1996] and H1, H2A, H2B, H3 and H4 genes in *Xenopus* [El-Hodiri and Perry, 1995]. Purified CDP/Cux can interact with chromatin, as reflected by its ability to bind the histone H4 promoter reconstituted into nucleosome cores [Last et al., 1999b].

The research described in this thesis was undertaken to determine how the proliferation-specific factor, HiNF-D/CDP/Cux, may regulate growth control and transcription of the replication-dependent histone H4 gene FO108. One objective was to establish the molecular interactions that can account for the assembly of the HiNF-D complex and the post-translational modifications that may govern these interactions. The second goal was to investigate the mechanism(s) involved in CPP/Cux-mediated repression of histone H4 transcription. The third objective was to assess the *in vivo* function of CDP/Cux by characterizing a *Cutl1* mutant mouse.

CHAPTER 2

MATERIALS AND METHODS

Plasmid construction

All oligonucleotides used in this study were synthesized using a Beckman 1000M synthesizer and all constructs were subjected to automated sequencing (Applied Biosytems ABI Model 377, Foster City, CA) to verify correct orientation of the inserted DNA fragments and absence of chemical synthesis-related mutations. Mammalian expression plasmids for myc-epitope tagged CDP proteins were prepared as follows: DNA fragment (*Not*I/*Xho*I) encompassing full-length CDP was isolated from MT2-CDP [Neufeld et al., 1992] and inserted into the *Not*I/ *Xho*I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA) containing a myc epitope tag (pcMyc). To place the CDP coding sequence in frame with the myc tag, a 14-basepair oligonucleotide (5' CGA GCA AGC TTG CT; eliminates the *Cla*I site upon insertion) was inserted at a *Cla*I site near the 5' end of the CDP insert. To clone the myc-tagged deletion mutant protein CDP (CR2-Cterm) [a 110 kD deletion mutant that encompasses Cut repeat 2 (CR2) to the C-terminus of CDP], the CDP coding sequences were isolated as an *Eco*RI/*Xho*I fragment from the GST/CDP (CR2-Cterm) construct (kindly provided by Ellis Neufeld, Children's Hospital, Boston), and inserted into similarly digested pcMyc plasmid. Expression vectors for Xpress-tagged wildtype and deletion mutant Cux proteins were cloned from pBSTR1/Cux plasmid (kindly provided by Dr. Alain Nepveu, McGill University, Montreal, Canada) as follows: full-length Cux cDNA was excised as a *Eco*RI/*Xba*I fragment and inserted into pcDNA3.1; ΔC Cux (aa 1-1058) was generated by inserting an *Eco*RV/*Xho*I fragment into pcDNA3.1. Reporter gene assays were performed with the H4/Luc construct which has been described previously [Xie et al., 2001].

- **Restriction digestion of DNA**

In general, restriction digestions were performed as described below with enzymes and buffers obtained from New England Biotechnology (NEB, Beverly, MA). In a total volume of 50 µl, DNA (10 µg), 1x digestion buffer, and 10-20 units of restriction enzyme were incubated at 37°C for 3 hours. The enzyme was then heat inactivated at 65°C for 20 minutes. Digested DNA was precipitated at –70°C for ten minutes with 5 volumes of 100% ethanol (250 µl) and 1/10 volume of 3 M sodium acetate pH 5.2 (5 µl). The DNA pellet was obtained by centrifugation at 14,000 rpm for 30 minutes at 4°C, washed with 70% ethanol, air dried and resuspended in 20 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA).

Digestion Buffers	**Composition**
Buffer 1	10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.0 at 25°C)
Buffer 2	10 mM Tris-HCL, 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl (pH 7.9 at 25°C)
Buffer 3	50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl (pH 7.9 at 25°C)
Buffer 4	20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT (pH 7.9 at 25°C)

- **Dephosphorylation of DNA**

The digested pcMyc vector was treated with 20 units of calf intestine alkaline phosphatase (NEB) for 30 minutes 37°C to prevent vector self-ligation. The dephosphorylation reaction was performed in the NEB buffer used for digestion as this enzymes is active all 4 buffer conditions. The enzyme was heat inactivated in the presence of 3 mM EDTA at 65°C for 15 minutes.

- **Gel purification of digested DNA fragments**

Digested and dephosphorylated plasmid DNA fragments were separated by agarose gel electrophoresis. The appropriate bands were excised and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. In brief, the weight of the gel slice was determined and three volumes of Buffer QG were added to 1 volume of the gel slice (100 mg is approximately 100 μl). The gel slice was dissolved at 50°C for 10 minutes and then mixed with one volume of isopropanol. To bind the DNA to the matrix, the solubilized gel was applied to the QIAquick gel extraction column and centrifuged for 1 minute at 4,000 rpm. The column was washed with 0.5 ml of Buffer QG, followed by 0.75 ml of Wash Buffer PE. Residual ethanol from the Buffer PE was removed by additional centrifugation for 1 minute. The column was then placed in a clean microfuge tube and DNA was eluted with 30-50 μl of Buffer EB (10 mM Tris-HCl, pH 8.5).

- **DNA ligation reactions**

The concentration of purified vector and insert DNA (1 μl each) was visually estimated by agarose gel electrophoresis. DNA ligation was performed by incubating a mixture containing 50 ng of vector, 50 or 150 ng of insert, 2 μl T4 DNA ligase buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 mg/ml BSA (NEB)] and 400 Units T4 DNA ligase (NEB) at 22°C overnight.

Bacterial transformations

Ligated DNA (2 μl) was mixed with competent HB101 bacterial cells (50 μl) and incubated on ice for 10 minutes. Cells were heat shocked at 42°C for 90 seconds and placed on ice for 5 minutes. Luria-Bertani (LB) (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl) medium (500 μl) was added to the cells and incubated at 37°C for 1 hour with shaking. Cells were then collected by centrifugation at 4000 rpm for 3 minutes, resuspended in LB (50 μl), spread on LB Ampicillin (100 μg/ml) (LB-Amp) plates and incubated overnight at 37°C.

Small-scale DNA preparations

Using QIAprep spin miniprep kit (Qiagen), plasmid DNA was isolated from an overnight LB-Amp culture (5 ml) inoculated with cells from a single colony. Cells were collected by centrifugation at 2,000 rpm using a low-speed swing-rotor centrifuge (IEC CRU-5000) (International Equipment Co., Needham, MA) and resuspended in 250 μl of Buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA;

10 μg/ml RNase A). Cells were lysed for 5 minutes at room temperature upon addition of Buffer P2 (250 μl; 200 mM NaOH; 1% SDS). Buffer N3 (350 μl; contains guanidine hydrochloride to bind the DNA to the silica in the column) was then added to the cell lysate and the sample was centrifuged for 10 minutes. Using a vacuum manifold, the plasmid DNA in the supernatant was bound to a QIAquick spin column, washed with 0.5 ml of buffer PB (a wash buffer that contains guanidine hydrochloride and isopropanol) and 0.75 ml of Buffer PE (a low salt buffer containing 80% ethanol). The column was centrifuged for 1 minute at 14,000 rpm to remove the residual ethanol. The DNA was then eluted in a new tube with 50 μl of Buffer EB (10 mM Tris-HCl, pH 8.5).

Large-scale DNA preparations

A small culture (5 ml) inoculated with cells from a single colony was grown in LB-Amp for 5-8 hours at 37°C in a shaking incubator. This culture was then used to inoculate 300 ml of LB-Amp and incubated overnight at 37°C in a shaking incubator. The bacterial cells were centrifuged in a Beckman JA-10 rotor (Beckman Instruments Inc., Fullerton, CA) at 5,000 rpm for 10 minutes. Plasmid DNA was extracted using the Qiagen QIAfilter Maxiprep kit. Cells were resuspended in 10 ml of Buffer P1, which contains RNase A, and lysed with Buffer P2 (10 ml) for 5 minutes at room temperature. The cell debris was precipitated with 10 ml of Buffer P3 (3.0 M potassium acetate, pH 5.5) at room temperature for 30 minutes. Using the QIAfilter, the supernatant was separated from the precipitate and loaded onto the tip-500 column, which was equilibrated with 10 ml of Buffer QBT (750 mM NaCl;

50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100). The column was washed 3 times with Buffer QC (1.0 NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol) and DNA was eluted with 15 ml of Buffer QF (1.25 M NaCl; 50 mM Tris-Cl pH 8.5; 15% isopropanol) in a 50 ml corex tube. The eluted DNA was precipitated with isopropanol (10.5 ml) and centrifuged at 10,000 rpm for 45 minutes at 4°C. The DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. The DNA pellet was air dried and resuspended in 500 µl of TE, pH 8.0.

Preparation of genomic DNA from mice

Genomic DNA was isolated from tail biopsies (0.4 cm) using the Qiagen DNeasy Tissue Kit, according to the manufacturer's instructions. Each tail was digested in a mixture containing Buffer ATL (180 µl) and proteinase K (20 µl) overnight at 55°C in a hybridization oven. A Buffer AL-ethanol (1:1) mixture was added and vortexed for 5 seconds. The mixture was then pipetted into the DNeasy mini column and centrifuged at 8,000 rpm for 1 minute. The column was placed in a new 2-ml collection tube and washed with Buffer AW1 (500 µl) followed by wash Buffer AW2 (500 µl). Residual ethanol present in the wash buffers was removed by an additional 3-minute centrifugation at 12,000 rpm. DNA was then eluted with Buffer AE (200 µl) into a 1.5-ml microfuge tube and the DNA concentration was calculated from the absorbance of the eluted DNA at 260 nm, which was measured using the Beckman spectrophotometer.

Cell culture

All cell lines used in this study were propagated according to culture conditions suggested by the American Type Culture Collection (Manassas, VA; http://www.atcc.org). In brief, actively proliferating cultures of mouse embryonic fibroblasts (MEFs), NIH/3T3, HeLa, and COS-7 cells were maintained at subconfluence in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 μM L-glutamine, at 37°C in humidified air containing 5% CO_2.

HeLa cells are HPV-18 transformed human cervical carcinoma epithelial cells that express wild type pRB [Scheffner et al., 1991], but expression of the HPV-18 derived E7 protein alters the cell growth regulatory functions of the pRB protein [Chellappan et al., 1992]. COS-7 cells are SV40 transformed monkey kidney fibroblasts that express wild-type SV40 T antigen, which is known to interact with endogenous pRB and alter its function [Hamel et al., 1990]. NIH/3T3 cells are derived from mouse embryo cultures, have relatively normal cell growth characteristics and express endogenous pRB [Banks et al., 1990]. Apart from differences in pRB status, HeLa, COS-7 and NIH/3T3 cells all contain CDP/Cux binding activity as reflected by detection of the CDP/Cux-containing HiNF-D complex [van der Meijden et al., 1998; van Wijnen et al., 1996].

Preparation of mouse embryonic fibroblasts (MEFs)

Embryos were harvested from pregnant mothers that were at day 12.5 of gestation. A part of the embryo was removed for genotyping. The remainder of the

embryo was passed through an 18-gauge needle with 1 ml of Trypsin-EDTA (Life Technologies, Rockville, MD) to break up the tissue and incubated for 5 to 10 minutes at 37°C in a CO_2 incubator. Primary fibroblasts were then plated with 20 ml complete DMEM (10% FBS). The growth rates were determined by plating MEFs derived from the same litter at a density of $4x10^6$ cells per plate (100 mm). On the indicated days, MEFs were trypsinized and the number of cells per plate was determined by sampling each plate 4 times. Cells were loaded onto a hemocytometer and cells in each of the two center squares were counted.

Flow cytometry analysis

Proliferating MEFs were harvested for flow cytometry analysis with Trypsin-EDTA, washed 3 times with PBS, and stained with a propidium iodide solution (2 mM $MgCl_2$, 20 μg/ml propidium iodide, 50 μg/ml RNase). Cell cycle phase estimates were obtained with a FACS Scan Cytometer equipped with pulse processing electronics (Becton Dickinson, San Diego, CA). For each sample, 15,000 doublet discrimination events were collected and analyzed using MODFIT software (Verity House Software, Topsham, Maine). Flow cytometry analysis was performed by the Flow Cytometry Facility at the University of Massachusetts Medical Center. The data were subsequently analyzed by Dr. Stephen Baker (Information Services at UMass) using the Tukey HSD statistical test.

Cell transfection

To determine histone H4 promoter activity, NIH/3T3 cells were plated in 6-well culture plates at a density of 1.5 x 10^5 cells per well and were transiently transfected 22 hr after plating. To measure H4 gene promoter activity, H4 promoter/Luciferase (Luc) reporter gene construct (1 μg) and a promoterless luciferase reporter construct (pGL2)(1 μg) were cotransfected with different amounts of expression vectors as indicated in the figure legends.

Transfection of COS-7, NIH/3T3 and HeLa cells was performed as follows: cells were washed twice with phosphate buffered saline (PBS) and transfected with DNA using the lipid-based compound Superfect (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Briefly, DNA was diluted in 1 ml of serum free medium. Superfect (7 μl) was then added to this mixture. The mixture was incubated at room temperature for 15 minutes to allow the formation of lipid-DNA complexes. The lipid-DNA mixture was then diluted with 5 ml of complete medium and 1 ml was added to each of the wells of a six well plate, which were washed twice with PBS. Cells were incubated with transfection mixture at 37°C in 5% CO_2 for 2 to 3 hours. The transfection mixture was aspirated and cells were washed once with PBS and then incubated at 37°C in DMEM medium supplemented with 10% fetal calf serum for 22-24 hours. For immunoprecipitation and GST pull-down assays, cells were plated in 100 mm plates at a density of $0.5X10^6$ cells per plate. Cells were transfected with 10 μg of different expression constructs essentially as described above.

Luciferase assays

Reporter gene activity was measured by luciferase assays. Cells were washed twice with 1 x PBS buffer and lysed with 250 μl of Reporter Lysis Buffer (Promega Corp., Madison, WI) at room temperature for 10 minutes. Cell lysates were collected and used immediately for reporter gene assay or stored at -70°C. Luciferase activity was determined using the luciferase assay system (Promega Corp., Madison, WI). Cell lysate (10 μl) was mixed with luciferase reagent (100 μl) containing substrate for firefly luciferase and luminescence was determined for 12 seconds using the Monolite TM 2010 luminometer (Analytical Laboratory, San Diego, CA).

Extraction of protein from organs

Mice were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and euthanized by cervical dislocation. Lung and brain tissues were harvested from adult mice, rapidly frozen and reduced to powder in liquid nitrogen by a Bessman tissue pulverizer (Fischer Scientific, Pittsburgh, PA). The tissue powder was transferred to a 50 ml conical tube and thawed on ice with 30 ml ice-cold buffer R (10 mM KCl, 10 mM HEPES/pH7.5, 0.5% Triton, 300 mM sucrose, 3 mM $MgCl_2$). The suspension was then passed through a Sweeny filter (Millipore, Bedford, MA) to separate released cells from the extracellular matrix. Cells were centrifuged at 1,500 rpm using a low-speed swing-rotor centrifuge. To prepare whole cell extract, the cell pellet was resuspended in 200 μl RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and centrifuged at 15,000 rpm for 20 minutes at 4°C. The supernatant was rapidly frozen in liquid N_2 in 25 μl aliquots and

stored at –70°C as whole cell lysate. To prepare nuclear extract, the cell pellet was resuspended in 1 ml Buffer A (10 mM HEPES/ pH 7.5, 10 mM KCl), transferred to a 1.5 ml tube and centrifuged at 6,000 rpm for 1 minute. The nuclear pellets were extracted with 300-600 µl Buffer C (400 mM KCl, 25 mM HEPES/pH 7.5, 25 % glycerol) for 30 minutes on ice and rapidly frozen in 50 µl aliquots. All buffers used were supplemented with Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN).

Preparation of nuclear extract.

Nuclear extracts were prepared by a modified Dignam method [Dignam et al., 1983]. HeLa cells and mouse embryonic fibroblasts were plated in 100 mm plates at a density of 0.5×10^6. Cells were collected at 70% confluence by washing twice with ice-cold PBS. The whole isolation procedure was carried out on ice. Cells were scraped, transferred to an Eppendorf tube and microfuged at full speed for 30 seconds at 4°C. Cells were gently resuspended in 400 µl of NP-40 lysis buffer [10 mM Tris (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl, 0.5% Nonidet P-40 (NP-40)] supplemented with Complete protease inhibitor cocktail and incubated on ice for 20 minutes. Cell lysate was microfuged at 7000 rpm for 5 minutes at 4°C and the pellet was resuspended in 400 µl of hypotonic buffer [10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl]. Nuclei were collected by centrifugation at 10,000 rpm for 2 minutes. Supernatant containing the cytosolic portion was discarded and the pellet was extracted in 100 µl of extraction buffer [20 mM HEPES (pH 7.9), 1.5 mM

$MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 20% glycerol] for 1 hour with end-to-end rotation. The extracted nuclei were microfuged at full speed for 5 minutes at 4°C. Aliquots (20 μl) of the supernatant (nuclear extracts) were immediately frozen in liquid nitrogen and stored at –80°C until further use. Protein concentration of nuclear extracts was determined by Bradford assay.

Nuclear extract (420 mM KCl) from proliferating HeLa cells was loaded onto a phospho-cellulose column and eluted with a KCl 200-400 mM buffer as described previously [van Wijnen et al., 1992; Vaughan et al., 1995]. This KCl fraction (3 μg/μl), enriched for HiNF-D activity, was diluted 1 fold with Buffer X (18.2 mM dibasic sodium phosphate, 3.4 mM monobasic sodium phosphate, 2% Nonidet P-40, 1% sodium deoxycholate and 0.2% SDS) and used in GST pull-down assays to examine the interactions between HiNF-D components.

Preparation of whole cell lysate

Transiently transfected HeLa cells expressing wild type pRB, large pocket (LP) pRB and mutant large pocket (mLP) pRB were harvested 22 h post transfection. Cells were lysed with RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with Complete protease inhibitor cocktail, quickly frozen in liquid nitrogen and rapidly thawed at 37°C. The lysate was microfuged at 12,000 rpm for 15 minutes at 4°C and the supernatant was used for GST pull-down assays.

Preparation of *in vitro* translated protein

In vitro translated proteins were prepared as follows: the expression constructs pcDNA pRB A/B and CMV cyclin A were subjected to coupled *in vitro* transcription and translation with [^{35}S] methionine in rabbit reticulocyte lysate according to the manufacturer's instructions (Promega, Madison, WI). The reaction mixture contained RNasin ribonuclease inhibitor (30 units) (Promega, Madison, WI), TnT reaction buffer (2 μl), TnT amino acid mixture minus methionine (1 μl), expression plasmid (1.2 μg), TnT rabbit reticulocyte lysate (25 μl), TnT T7 RNA polymerase (1 μl) and ^{35}S-labeled L-methionine (1 mM or 15 μCi) (Sigma, St. Louis, MO). The appropriate amount of nuclease-free double-distilled water (dd water) was added to bring the total reaction volume to 60 μl. The reaction mixture was briefly vortexed then microfuged for 5 seconds at 12,000 rpm. Following a 2-hour incubation at 30°C, glycerol (20 μl) and buffer X (80 μl) were added to the lysate. *In vitro* translated cyclin A (60 μl/reaction) or pRB A/B pocket (37 μl /reaction) were then used in GST pull-down assays.

GST pull down assays

- **Preparation and transformation of competent BL21 bacterial cells**

An overnight culture (500 μl) inoculated with cells from a single colony was diluted in LB-Amp (10 ml) medium. The optical density of the culture at 600 nm was measured intermittently using a spectrophotometer (Beckman Instruments, Fullerton, CA). When the optical density of the culture reached 0.5, the exponentially growing cells were centrifuged for 5 minutes at 2000 rpm using a low-speed swing-rotor

centrifuge. The supernatant was poured off and the cell pellet was resuspended with 0.1 M ice cold $CaCl_2$ (5 ml). The cells were incubated on ice for one hour, centrifuged 5 minutes at 2000 rpm, resuspended in 0.1 M $CaCl_2$ (1 ml) and again incubated on ice for another 2 hours. Cells (200 ml) were then transformed with plasmids (100 ng) that express the ampicillin resistance gene and the glutathione-*S*-transferase (GST) gene or GST fused to CDP/Cux (CR2-Cterm). Cells were incubated on ice for 30 minutes, heat shocked at 42°C for 90 seconds and plated on LB-Amp plates.

- **Purification of GST proteins**

An overnight culture (1 ml) inoculated with a single transformed colony was diluted with LB-Amp (100 ml) and incubated with vigorous shaking at 37°C until the optical density (at 595 nm) of the culture reached 0.7. Cells were induced to express GST proteins by incubation with isopropyl-beta-D-thiogalactoside (IPTG; 0.1 mM) for 5 hours, and centrifuged for 5 minutes at 2000 rpm. The supernatant was poured off and the cell pellet was resuspended with ice cold Buffer A (5 ml) supplemented with the Complete protease inhibitor cocktail and dithiothreitol (DTT; 1 mM). Using the sonic dismembrator (Fisher Scientific, Pittsburgh, PA), cell lysis was achieved by sonicating cells three times for 15 seconds. The sample was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was incubated with glutathione-Sepharose beads (500 µl; Amersham Pharmacia Biotech; Uppsala, Sweden) pre-washed with ice cold Buffer A (5 ml) on a stirring wheel at 4°C for 3 hours. Beads were washed 3 times with Buffer A (5 ml) and 2 times with Buffer A' (10 mM

HEPES/ pH 7.5, 0.1 M NaCl). After the last wash, beads were resuspended in Buffer A' (500 μl). To determine the concentration of the GST proteins bound to the beads, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below) along with bovine serum albumin (BSA), which served as a pre-calibrated protein concentration standard. Proteins were visualized with GelCode Blue Stain Reagent (Pierce, Rockford, IL) and a gel documentation system (Alpha Imager; Alpha Innotech Corp., San Gabriel, CA) was used to measure the intensity of the protein bands.

- **GST pull-down assay**

GST pull-down assays were performed using a GST moiety fused to CDP/Cux (CR2-Cterm) protein. The resins containing the bound GST or GST-CDP/Cux (CR2-Cterm) fusion proteins were each incubated with endogenous, over-expressed or *in vitro* translated ^{35}S-labeled proteins. Protein preparations were precleared with glutathione- Sepharose beads prior to incubation with 2 μg of GST or GST-CDP (CR2-Cterm) at 4°C for 16 hours. Beads were then centrifuged and washed three times with wash buffer (10 mM Tris-HCl/ pH8, 50 mM NaCl, 2 mM EDTA/ pH8 and 0.2% NP-40). Bound proteins were resuspended in 2X Laemmli gel loading buffer (62.5 mM Tris-HCl/ pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, and bromophenol blue), separated by SDS-PAGE and analyzed by western blot. Gels containing ^{35}S-labeled samples were dried under vacuum at 80°C for 1 hour and subjected to autoradiography.

Co-immunoprecipitation assays

COS-7 and HeLa cells were transfected with expression constructs for pRB and/or Myc-tagged CDP proteins. Thirty hours after transfection, cells were washed, scraped and solubilized in RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). After 20 minutes on ice, the cell lysates were centrifuged and supernatants were pre-cleared for 30 minutes at 4°C. The supernatant was then incubated overnight with anti-pRB antibody or anti-Myc antibody (to immunoprecipitate CDP) at 4°C, followed by incubation with protein A/G Plus-Sepharose beads (40 μl) for 1 hour at 4°C. The beads were washed four times with the wash buffer. The immune complexes were then eluted by boiling for 5 minutes in Laemmli gel loading buffer, subjected to SDS-PAGE and analyzed by western blot.

SDS-PAGE and western blot analysis

Cell lysates or immunoprecipitated complexes were subjected to SDS-PAGE and western blotting as follows: to prepare two mini-gels for Mini-PROTEAN II electrophoresis system (BIORAD Laboratories, Hercules, CA) the appropriate amount of ProtoGel (29.5% acrylamide: 0.5% *bis*-acrylamide), ProtoGel buffer (1.5M Tris-HCl pH 8.8, 10% SDS) and deionized water were mixed and polymerized in the presence of 0.1% ammonium persulphate (APS) and N,N,*N′*,*N′*-tetramethylethylenediamine (TEMED). This mixture of resolving gel was poured into the pre-assembled Mini-PROTEAN II apparatus. After the resolving gel was polymerized, stacking gel (for 5ml: 0.65 ml ProtoGel, 1.25 ml ProtoGel stacking buffer (1M Tris-HCl pH 6.8, 10% SDS), 3 ml of deionized water, 0.1% APS and

TEMED) was prepared and a comb with desired number of wells was inserted avoiding any bubbles. After the stacking gel was polymerized, the comb was removed, wells were thoroughly washed with deionized water and the assembly was placed in a Mini-PROTEAN II gel running chamber. The samples were loaded onto gels and resolved in 1X SDS-PAGE running buffer (200 mM glycine, 25 mM Tris-HCl pH 7.4, 1% SDS) under an electric field of 100 V for 1-2 hours. Gels were then subjected to protein transfer onto an immobilized nylon membrane [Immobilin-P (Millipore Corp., Bedford, MA)] using a semi-dry transfer apparatus (Owl Separation Systems, Portsmouth, NH). For transfer, gels were rinsed in western blotting transfer buffer 1 (48 mM Tris-HCl pH 7.4, 40 mM glycine, 3.75% SDS, 20% methanol) and placed onto Whatmann 3MM filter paper pre-soaked in transfer buffer. Then the membrane was placed onto the gel avoiding any bubble. Another 3MM Whatmann filter paper, pre-soaked in the transfer buffer was placed onto the membrane and proteins were transferred from gel to membrane under constant current of 10V for 30 minutes. For analysis of full-length CDP/Cux protein, blotting transfer buffer 2 (48 mM Tris-HCl pH 7.4, 40 mM glycine, 0.1% SDS) was used.

After transfer, membrane was blocked with 5% skim milk in PBS at room temperature for 30 minutes. The membrane was incubated overnight with appropriate dilution of primary antibody in PBS containing 1% skim milk (Bio-Rad, Hercules, CA) and 0.05% Triton-X 100 (Sigma-Aldrich, St. Louis, MO) at 4°C with shaking. Membrane was extensively washed with PBST (PBS containing 0.05% Triton-X 100) and incubated with 1:5000 dilution of appropriate secondary antibody conjugated with horseradish peroxidase enzyme at room temperature for one hour with shaking.

After extensively washing the membrane with PBST, proteins were detected by incubating membranes for 1 minute with substrate for peroxidase enzyme (ECL western blotting detection reagent; Amersham-Pharmacia Biotech Inc., Piscataway, NJ). Membrane was then exposed to an autoradiographic film for varying time points and film was developed in an X-ray developer.

Antibodies

Antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA) unless otherwise mentioned. Different antibodies and their dilutions used for various applications throughout this study are as follows (IF: immunofluorescence; WB: western blot analysis; IP: immunoprecipitation; EMSA):

Primary antibody	**Application (dilution)**	**Company**
mouse monoclonal anti-HA	IF (1:3000)	Santa Cruz
mouse monoclonal anti- Xpress	IF (1:500)	Invitrogen Corp., Carlsbad, CA
mouse monoclonal anti-Myc (clone 9E10)	IF (1:2000) WB (1:5000) IP (6 μg)	Zymed Laboratories, San Francisco, CA
rat monoclonal anti-BrUTP	IF (1:20)	Harlan Sera Laboratories, Leicestershire, England
mouse monoclonal anti-coilin	IF (1:100)	Smith *et al.*, 1995
mouse monoclonal anti-PML	IF (1:1000)	Santa Cruz

anti-HiNF-P	IF (1:100)	Santa Cruz
rabbit polyclonal anti-Sp1	IF (1:100) WB (1:1000)	Santa Cruz
guinea pig polyclonal anti-CDP (epitope = full-length CDP)	IF (1:100) WB (1:1000) EMSA (0.5-2 μg)	Dr. Ellis Neufeld Children's Hospital Boston, MA
goat polyclonal anti-CDP (epitope = C-terminus)	WB (1:1000)	Santa Cruz
rabbit polyclonal anti-CASP	IF (1:100)	Dr. Ellis Neufeld
mouse monclonal anti-pRB (epitope = full-length pRB) "pRB IF8"	IF (1:100) WB (1:200) IP (6 μg)	Santa Cruz
mouse monclonal anti-pRB (epitope = aa 300-380) "pRB C36"	IF (1:100)	BD Biosciences Pharmingen San Diego, CA
mouse monclonal anti-pRB (epitope = aa 393-572) "pRB XZ104"	WB (1:100)	BD Biosciences Pharmingen San Diego, CA
rabbit polyclonal anti-p107	WB (1:1000)	Santa Cruz
rabbit polyclonal anti-Cdk2	WB (1:5000)	Santa Cruz
mouse monoclonal anti-CDK1	WB (1:1000) IF (1:100)	Santa Cruz

mouse monoclonal anti-cyclinA	WB (1:1000) IF (1:100)	Santa Cruz
rabbit polyclonal anti-cyclin E	EMSA (0.5-2 μg)	Santa Cruz

Horseradish peroxidase conjugated secondary antibodies for western blot analysis were used at a dilution of 1:5000. For immunofluorescence, either Alexa 488 or Alexa 568 secondary antibodies (Molecular Probes, Eugene, OR) were used at a dilution of 1:200 or 1:400, respectively To detect rat monoclonal antibody raised against BrUTP, a donkey anti rat secondary antibody conjugated with Texas-Red fluorochrome was used at a dilution of 1:100 (Molecular Probes, Eugene, OR).

Immunofluorescence microscopy

Cells were grown on gelatin-coated cover slips (Fisher Scientific, Springfield, NJ) at a density of $0.08X10^6$ cells per well (35 mm). For whole cell preparations, cells were rinsed twice with ice-cold PBS and fixed in 3.7% formaldehyde in PBS for 10 minutes on ice. After rinsing twice with PBS, the cells were permeabilized in 0.1% Triton X-100 in PBS, and rinsed twice with PBSA [0.5% bovine serum albumin (BSA) in PBS] followed by antibody staining. Antibody staining was performed by incubating whole cell preparations with appropriate dilutions of antibodies against the proteins of interest for 1 hr at 37°C. Coverslips were rinsed 4 times with PBSA before incubation with secondary antibodies for 1 hr at 37°C. Cells were rinsed 4 times with PBSA and then stained with 4', 6-diamidino-2-phenylindole (DAPI) in PBSA

containing 0.1% Triton X-100 (PBSAT) for 5 minutes. Coverslips were washed once with PBSAT and twice with PBS. Coverslips were then mounted onto glass slides with Vecta Shield (Vector Laboratories, Burlingame, CA) or ProLong Antifade Kit (Molecular Probes Inc., Eugene, OR). Immunofluorescent signals were detected using an epifluorescence microscope attached to a charged-coupled device (CCD)-camera or the Leica True Confocal Scanning Spectrophotometer. The images were captured and analyzed with the Metamorph and Adobe Photoshop software programs. Statistical image analysis was performed using the MATLAB image processing toolbox (Mathworks Inc., Natick, MA). Cross-correlation, which measures the degree of signal overlap between two images, was calculated essentially as described [van Steensel et al., 1996]. Here the correlation is expressed as the average percent colocalization of several image pairs. To determine that the observed correlation is not due to random signal overlap, one image from each image pair was rotated ten degrees and the cross-correlation was calculated. If the observed colocalization were random, rotation of the image would not change the degree of signal overlap. Differences between rotated and unrotated cross-correlation values were assessed by two-tailed paired student's t-test.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed to test for HiNF-D DNA binding activity [van Wijnen et al., 1991b]. The histone H4/Site II and gp91-phox probes were EcoRI-HindIII inserts from plasmids pFP202 and pFp-Puc, respectively. The Sp1 probe synthesized using a Beckman 1000M synthesizer is the consensus binding sequence [5'-ATTCGATCGGGGCGGGGCGAGC-3'].

- **End-labeling of the H4 and gp91-phox probes**

Plasmids (100 μg) pFP202 and pFp-Puc were digested with EcoRI (200 units) for 2 hours at 37°C, and dephosphorylated by calf intestinal phosphatase as described above. Phenol/chloroform extraction was performed by combining the DNA mixture with 200 μl of a solution containing phenol, chloroform and isoamyl alcohol (25:24:1). Following a 3-minute centrifugation at 12,000 rpm, DNA in the supernatant fraction was precipitated with ethanol and sodium acetate as described above and resuspended in TE (50 μl). For labeling reaction, linearized DNA (20 μg), T4 polynucleotide kinase buffer [70 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT], [γ-^{32}P] dATP (100 μCi), and T4 polynucleotide kinase (10 units) were mixed together in an Eppendorf tube and the final volume was adjusted to 30 μl with distilled water. The labeling was performed at 37°C for 1 hour and the enzyme was heat inactivated at 65°C for 1 hour. The labeled DNA was precipitated and digested with Hind III restriction enzyme for 4 hours at 37°C. To isolate the labeled probe insert, the DNA fragments mixed with bromophenol blue (BPB) dye

were separated by electrophoresis in a polyacrylamide gel (4%) in 0.5X TBE (Tris borate/EDTA) at 200V for 1.5 hour. The probe insert, which co-migrates with the BPB dye, was excised and purified using the QIAquick gel extraction kit as described. The labeled probe (2 μl) was mixed with 4 ml of Ecoscint™ H solution (National Diagnostic, Atlanta, GA) and counts per minute were measured in a scintillation counter.

- **End-labeling of the Sp1 oligonucleotide**

The top strand of the Sp1 (100 ng) oligonucleotide was labeled at the 5' end as described above. To generate a double-stranded DNA fragment containing the binding site, complementary bottom oligo (400 ng) was added to tubes together with labeled top strand and boiled for 5 minutes. Tubes were slow cooled to room temperature in the same beaker to facilitate annealing. The unincorporated nucleotides were removed by QIAquick Nucleotide Removal Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. Labeling efficiency was determined using a scintillation counter.

- **Protein/DNA binding reactions**

Protein/DNA binding reactions with H4/SiteII and gp91-phox probes were performed by combining ^{32}P-labeled probe DNA (10 fmole), non-specific competitor DNA (1 μg poly(dG·dC) and 100 ng poly(dI·dC), and nuclear extract (1-10 μg). Protein/DNA binding reactions with the Sp1 probe were performed with 1 μg poly(dG·dC) as non-specific competitor DNA. Oligo competition assays were

performed in the presence of 100-fold molar excess (1 pmole) of the unlabeled TM-3 oligo (self-competition with wildtype H4/SiteII) [Aziz et al., 1998a], the gp91phox oligo [Skalnik et al., 1991] or the Sp1 oligo. Non-specific competition was performed with the SUB-11 oligonucleotide containing a mutated H4/Site II [Aziz et al., 1998a]. Immuno-reactivity EMSAs were performed by pre-incubating antibodies (0.5-2 μg) with proteins on ice for 15 minutes prior to the addition of probe DNA. Antibody against cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a nonspecific antibody control. Electrophoresis of protein/DNA complexes was performed in a 4% non-denaturing acrylamide:bisacrylamide (80:1) gel with 0.5X TBE buffer. Electrophoresis was performed for 2 to 3 hours at 200 V. Gels were dried under vacuum at 80°C for 2 hours and subjected to autoradiography.

Generation of *Cutl1* mutant mice

Cutl1 mutant mice were generated by Dr. DongXia Xing in the laboratory of Dr. Ellis Neufeld (Children's Hospital, Division of Hematology, Boston, MA).

- **Targeting construct for *Cutl1* mutant mice**

A targeting vector was constructed which contains an SV40 early promoter-neomycin (neo) resistance cassette (Invitrogen, Carlsbad, CA) for positive selection, and a phosphoglycerate kinase (HSV-TK) cassette [Tybulewicz 1991] for negative selection. From the CDP/Cux locus, a 696 bp *Hind*III (intron 19) and *Xho*I (exon 20-12 bp into the homeodomain) fragment was used as 5' homology and a 6kb *Bam*HI fragment was used as 3' homology.

- **Homologous recombination into ES cells**

CJ7 ES cells were electroporated with 50 μg of linearized targeting vector followed by a week of selection with G418 (200 μg/ml) and gancyclovir (2 μM). G418/gancyclovir resistant clones were screened by Southern blot analysis of *Bam*HI digested genomic DNA, probed with a radiolabeled fragment located 5' to the targeted sequence. One correctly targeted heterozygous ES clone had a normal diploid karyotype and was subsequently used to inject blastocysts.

- **Generation of chimeric mice carrying the mutant allele**

C57Bl/6J blastocysts were injected with the embryonic stem (ES) clone and transferred to the uteri of 2.5-day pseudopregnant female mice. Chimeras identified by agouti coat color were mated with C57Bl/6J females to generate F1 progeny. The genotypes of F1 mice were analyzed by Southern blot analysis with *Bam* HI digested genomic DNA from tail biopsies, as described above. PCR analysis was also performed to determine mice genotypes, using CDP/Cux intron 19 forward primer HD-F (5'-CAGGGTTTTAT TTGGGGGC TTT TT-3') with exon 20 reverse primer HD-R1 (5'-AAGTTCCTCGATGGTTT TT-3') from the wildtype allele which produced a 596 bp product, or a 1.06 kb product with neo reverse primer HD-R2 (5'-GCATCGCCTTCTATCCGCCTTCTTG-3') from the mutant allele. PCR reactions were performed by adding genomic DNA to a mixture containing 10 mM Tris-HCl, 150 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 25 pmol of each primer and 5 U of Taq polymerase (Promega Corp., Madison, WI). Following a 2-

minute denaturation at 95°C, PCR reactions were amplified for 30 cycles (94°C, 30 s; 62°C, 30 s; 72°C, 1 minute). F1 mice were crossed to generate F2 litters, and the genomic PCR analysis described above was performed to detect the wildtype (0.6 kb) or mutant allele (1.0 kb).

Isolation of total cellular RNA

Total cellular RNA was isolated from proliferating MEFs with the TRIzol Reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions. In brief, cells from one 100mm plate were washed twice with ice cold 1X PBS, scraped in 1ml PBS and transferred to an Eppendorf tube. Cell pellets were obtained by centrifugation at 14000 rpm for 30 sec. Cell pellets were then thoroughly re-suspended in 1 ml TRIzol reagent and incubated at room temperature for 5 minutes. Then 0.2 ml chloroform was mixed with the cell lysate. The two phases were allowed to separate at room temperature for 10 minutes, followed by centrifugation at 14000 rpm for 15 minutes. The upper clear phase containing total cellular RNA was transferred to a new Eppendorf tube and 0.5 ml isoproponal was added to precipitate RNA. Following a 10-minute incubation at room temperature, RNA was pelleted by centrifugation at 14000 rpm for 15 minutes. The RNA pellet was washed with 75% ethanol and re-suspended in 50μl RNase free deionized water.

Northern blot analysis

Total cellular RNA (20 μg) was resolved in a 1% denaturing agarose gel (3.7% formaldehyde, 1X MOPS buffer (40 mM 3-[N-Morpholino]propanesulfonic

acid (MOPS) pH 7.0, 10 mM sodium acetate, 1 mM EDTA), 1% agarose) for 2-3 hours and transferred overnight onto Hybond N^+ membrane in 10X SSC buffer (1.5 M sodium chloride, 0.15 M sodium citrate pH 7.4). The RNA was cross-linked to the membrane after transfer and pre-hybridized at 65°C with Hybridization Buffer [0.7% SDS, 5X Denhardt's Solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 5X SSPE (0.9 M NaCl, 50 mM $NaPO_4$ pH 7.4, 5 mM EDTA)] for 1 hour. To label the probe for northern blotting, 25 ng of the histone H4 cDNA (1kb fragment including the entire coding region) was boiled in 45 μl TE buffer for 5 minutes and transferred to a RediPrime labeling tube (Amersham-Pharmacia Biotech Inc., Piscataway, NJ). Upon addition of 50 μCi of [^{32}P] α dCTP, the reaction mixture was incubated at 37°C for 10 minutes and the reaction was stopped by adding 10 mM EDTA. Unincorporated radioactive nucleotides were removed by passing the reaction mixture through a ProbeQuant™ G-50 Micro columns (Amersham-Pharmacia Biotech Inc., Piscataway, NJ). Counts per minutes (cpm) in the flow through were measured using a scintillation counter. The blot was incubated with $1x10^6$ cpm of labeled probe per ml of hybridization buffer at 65°C overnight. The blot was extensively washed with low (0.1X SSC, 0.1% SDS) and high (2X SSC, 0.1% SDS) stringency buffers and the signal was visualized by autoradiography.

Reverse transcriptase PCR (RT-PCR)

To perform reverse transcription polymerase chain reaction (RT-PCR), a mixture of 2 μg total cellular RNA, 2.5 μM oligo dT, 1 mM dNTP and 10 U Moloney Murine Leukemia Virus Reverse Transcriptase (Promega Corp., Madison, WI) was

incubated for 1 hr at 37°C followed by a 15-minute heat inactivation at 95°C. The resulting cDNA mixture (2 μl) was used in PCR reaction containing 1 mM dNTP, 1 mM $MgCl_2$, 10 U Taq polymerase (Promega Corp.) and primers (20 μM) that spanned the C-terminus of CDP. Following a 5-minute denaturation at 95°C, PCR reactions were amplified for 30 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s) and terminated with a final elongation step at 72°C for 10 minutes. Primers for murine CDP/*Cux* included: primer 22F (5'- TCTCCGACCTCCTTGCCCG-3'); primer 23F (5'- GCCCCCAGCCACAACACCA-3'); primer 23R (5'TGGTGTTGTGGCTGGG GGC-3'); primer 24F (5'-GGAGAGGACGCCGCTACC-3'); primer 24R (5'- GGCT TCCAGCTTGAATCTCC-3'); primer NeoR1 (5'- CCATCAGAAGCTGACTC-3') and primer NeoR2 (5'- GAAGAACGAGATCAGCAGCC-3'). RT-PCR products were visualized in a 1% agarose/ethidium bromide gel and transferred to Hybond-N^+ membrane (Amersham Pharmacia Biotech, Arlington Heights, IL) for the subsequent Southern blot analysis.

Southern blot analysis

Blots were hybridized with a random-primed (Prime-It kit; Stratagene, La Jolla, CA), ^{32}P-labeled cDNA probe for CDP/*Cux* (1.7 kb EcoRI/DraII fragment spanning Cut Repeat 3 to C-terminus) at 65°C overnight. The blot was washed and subjected to autoradiography as described for northern blot analysis.

S1 nuclease protection assay

S1 nuclease protection (S1) assays were performed according to the manufacturer's protocol (Ambion, Austin, TX) with minor modifications. Oligonucleotides complementary to the mRNA cap site of the mouse genes encoding histone H4.1 (mouse homologue of the human histone H4 FO108 gene) were used as probes [van der Meijden et al., 1998]. A cytoplasmic β-actin probe served as an internal control. The probes were labeled with ^{32}P-γ-ATP and T4 Polynucleotide kinase and approximately 100 fmol of each probe was used per reaction. The probes were first denatured at 94°C, then hybridized to total RNA (10 μg) overnight at 16°C. The samples were subsequently digested for 40 minutes at 37°C by S1 nuclease (Ambion, Austin, TX). The reaction was stopped by addition of Stop Solution and digested fragments were purified by ethanol precipitation. The pellets were dissolved in loading buffer and separated in a 6% denaturing polyacrylamide gel (SequaGel, Hessle Hull, England) alongside undigested probes. Gels were exposed and analyzed by Phosphor imaging (PhosphorImager Storm 840, Molecular Dynamics, Inc., Sunnyvale CA).

Histology

Tissues were fixed for 24 hours at 4°C on a shaking platform in Bouin's fixative (5% acetic acid, 24% formaldehyde, 71% picric acid) or in 10% buffered neutral formalin (VWR Scientific Products, West Chester, PA). Samples were washed 5 times with PBS, passed through a series of graded ethanol solutions and then embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin,

examined, and photographed. Histology slides were prepared by the diabetes endocrinology research center morphology core at UMass.

Serum testosterone levels

Blood was collected from wildtype, heterozygous and homozygous mutant *Cutl1* ΔC mice by cardiac puncture immediately after the mice were sacrificed by cervical dislocation. Blood samples were kept on ice for 10 minutes and subsequently subjected to centrifugation at 4°C for 10 minutes at 6,000 rpm. The supernatant was aliquoted and stored at –70°C until assayed. Serum samples were measured using a Coat-A-Count total testosterone kit as directed by the manufacturer (Diagnostic Products Corp., Los Angeles, CA). Control samples were obtained from littermates or age-matched males.

Scanning electron microscopy

Multiple dorsal hairs were manually removed from a homozygous mutant and a control mouse (both 5-month old females). These hairs, representing the four major hair types (zigzag, guard, auchene, and awl), were prepared for scanning electron microscopy (SEM) as previously described [Sundberg et al., 1994; Yamanaka et al., 1997] and screened for abnormalities.

CHAPTER 3:

THE TUMOR SUPPRESSOR pRB FUNCTIONS AS A CO-REPRESSOR OF THE CCAAT DISPLACEMENT PROTEIN (CDP)/Cux TO REGULATE CELL CYCLE CONTROLLED HISTONE H4 TRANSCRIPTION

GUPTA, LUONG ET AL. 2003, JOURNAL OF CELLULAR PHYSIOLOGY. ACCEPTED.

Co-immunoprecipitation assays in COS-7 cells and gene reporter assays with CDP/Cux CR2-Cterm were performed by Dr. Sunita Gupta. Immunofluorescence using expression vectors for pRB proteins was performed by Michael Luong. Colocalization of pRB and CDP/Cux was calculated with the assistance of Daniel Young.

ABSTRACT:

The CCAAT displacement protein (CDP)/Cux performs a key proliferation-related function as the DNA binding subunit of the cell cycle controlled HiNF-D complex. HiNF-D interacts with all five classes (H1, H2A, H2B, H3 and H4) of cell-cycle dependent histone genes, which are transcriptionally and coordinately activated at the G_1/S phase transition independent of E2F transcription factor. In addition to CDP/Cux, cell cycle regulators pRB, CDK1 and cyclin A are also present in the HiNF-D complex. However, the molecular interactions that enable these components to form a complex and thus convey cell growth regulatory information onto histone gene promoters are not fully understood. Results from GST pull-down assays showed that the CDP/Cux C-terminus (CR2-Cterm) was sufficient for interaction with pRB, CDK1 and cyclin A.

HiNF-D complex formation on histone gene promoters *in vitro* is upregulated in S phase, which is subsequent to hyperphosphorylation of pRB [van Wijnen et al., 1997]. Thus it was postulated that CDP/Cux interacts with the hyperphosphorylated form of pRB. To test this hypothesis, GST pull-down assays were performed with pRB mutant proteins. CDP/Cux (CR2-Cterm) interaction was observed with full-length pRB, wildtype large pocket domain (LP) and mutant LP which is hypophosphorylated. Thus pRB-CDP/Cux interaction *in vitro* does not require hyperphosphorylation of pRB. Direct interaction between CDP/Cux (CR2-Cterm) and the pRB pocket domain was observed in GST pull-down assays using *in vitro* translated pRB protein. Furthermore, co-immunoprecipitation assays and *in situ*

immunofluorescence microscopy established that CDP/Cux and pRB form complexes *in vivo* and associate *in situ*. To assess the functional consequences of CDP/Cux-pRB interactions, gene reporter assays were performed. Both CDP/Cux and pRB repressed the H4 gene promoter and when co-expressed, these proteins cooperate as repressors. The pRB-pocket domain was sufficient for co-repression with CDP/Cux. Thus, several converging lines of evidence indicate that a CDP/Cux- pRB complex represses cell cycle-regulated histone gene transcription.

INTRODUCTION:

Cell proliferation is regulated by a complex and interdependent series of biochemical events involving cell cycle specific modifications in gene expression. The S-phase specific expression of histone genes represents one of the earliest characterized examples of cell cycle dependent gene regulation and provides a paradigm for understanding gene regulatory signaling mechanisms operative at the G_1/S transition [Prescott, 1966; Stein et al., 1996]. Histone gene expression in mammalian cells is both temporally and functionally coupled with DNA replication [Dominski and Marzluff, 1999; Osley, 1991; Stein et al., 1984; Stein et al., 1996]. Cell cycle dependent modulations of histone gene transcription provide the initial rate-limiting step in the induction of histone gene expression at the G_1/S phase transition.

Cell cycle control of histone H4 gene transcription requires a critical multipartite promoter element, Site II, that interacts with three distinct histone nuclear factors (HiNFs) [Aziz et al., 1998b; Ramsey-Ewing et al., 1994; van der Meijden et al., 1998; van Wijnen et al., 1989; van Wijnen et al., 1992; Vaughan et al., 1998; Xie et al., 2001]. Several other promoter elements, transcription factors and/or co-factors also contribute to the regulation of histone H4 gene transcription in the context of a dynamic and transcriptionally active chromatin organization [Hovhannisyan et al., 2002; Last et al., 1998; Last et al., 1999a; Mitra et al., 2001; Staal et al., 2000; Stein et al., 1996]. Overlapping recognition sequences within Site II for HiNF-M, HiNF-P and HiNF-D together modulate H4 gene transcription levels by at least an order of

magnitude. This composite organization of Site II supports responsiveness to multiple signaling pathways that modulate activities of the H4 gene transcription factors during the cell cycle. HiNF-M has been identified as the oncoprotein IRF-2 [Vaughan et al., 1995] and HiNF-P is a 65 kDa Zinc finger protein that links the growth factor dependent NPAT/Cyclin E/CDK2 pathway to cell cycle control of H4 gene transcription [Mitra et al., 2002b]. The HiNF-D complex is composed of the homeodomain protein CDP/Cux and the cell cycle regulators pRB, CDK1 and cyclin A [Shakoori et al., 1995; van Wijnen et al., 1989; van Wijnen et al., 1994; van Wijnen et al., 1996; van Wijnen et al., 1997]. Although components of the HiNF-D complex have been identified, it is not clear how they interact with one another. Studies using gene replacement strategies and forced expression of CDP/Cux have revealed a role for CDP/Cux in the regulation of cell growth and differentiation [Ellis et al., 2001; Ledford et al., 2002; Luong et al., 2002; Mitra et al., 2002b; Quaggin et al., 1997; Vaughan et al., 1998; Xie et al., 2002]

The functional interactions of CDP/Cux (and/or HiNF-D) with the promoters of histone [Barberis et al., 1987; van den Ent et al., 1994; van Wijnen et al., 1996; Wu and Lee, 2002], c-Myc [Dufort and Nepveu, 1994], p21 [Coqueret et al., 1998a], c-Mos [Higgy et al., 1997], transforming growth factor β type II receptor [Jackson et al., 1999], and thymidine kinase [Kim et al., 1997] genes indicate that this factor is a major component of a gene regulatory mechanism that controls cell growth [Nepveu, 2001]. CDP/Cux contains four independent DNA binding domains [Aufiero et al., 1994; Harada et al., 1995; Mailly et al., 1996; Moon et al., 2000] (Fig. 3-1A) and as a result may exhibit multiple conformational modes to support promoter recognition.

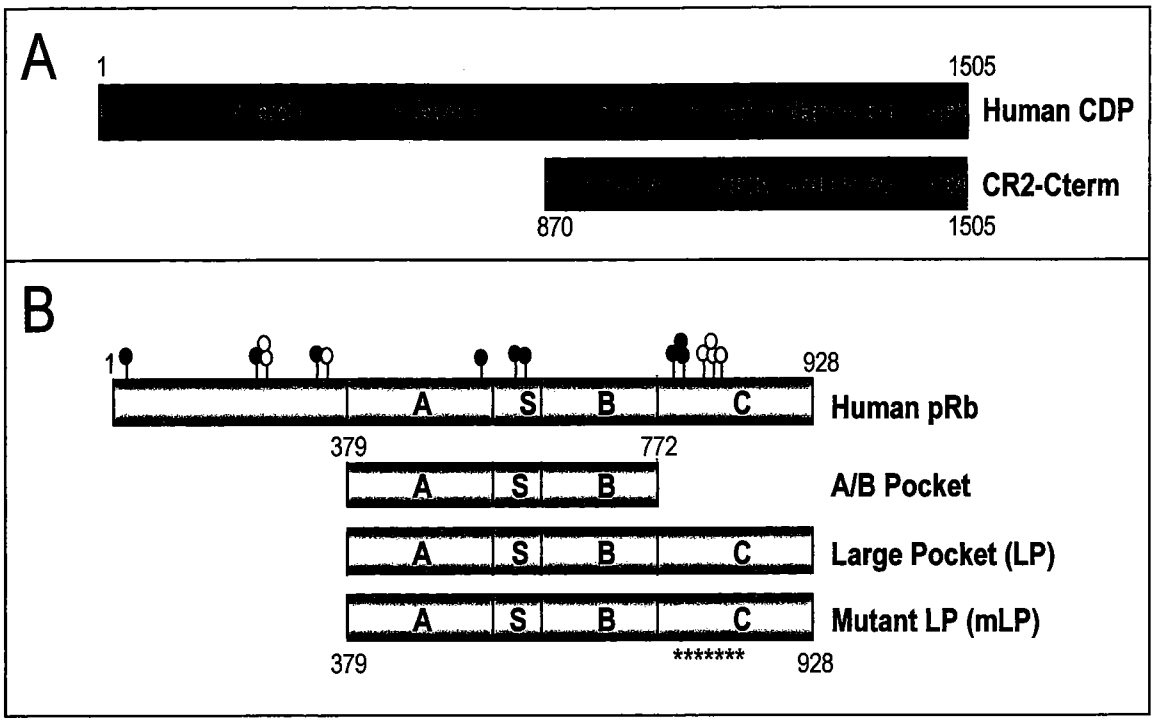


FIG 3-1. CDP/Cux and pRB mutant proteins. (A) Structure of the two CDP/Cux proteins tested in our studies. The full-length protein spans a coiled-coil (CC) domain, three Cut Repeats (CR) and a homeodomain (HD). **(B)** Structure of pRB proteins. In contrast to the spacer (S) region, domains A and B within the pRB protein are conserved across species. pRB contains 16 potential Ser/Thr-Pro motifs (ovals) and seven of these sites have been shown to be phosphorylated *in vivo* (yellow ovals). All seven potential phosphorylation sites (ovals) within domain C are mutated (red stars) in phosphorylation-deficient mutant large pocket (mLP) protein.

In addition, the DNA binding and/or transcriptional activities of CDP/Cux may be regulated by interactions with different members of the pRB family (i.e., p105 and p107)[van Gurp et al., 1999; van Wijnen et al., 1996; van Wijnen et al., 1997] and cyclin/CDK proteins [Shakoori et al., 1995; van Wijnen et al., 1994] [Santaguida et al., 2001]. Post-translational modifications that regulate CDP/Cux activity include phosphorylation [Coqueret et al., 1996; Coqueret et al., 1998c; Santaguida et al., 2001], dephosphorylation [Santaguida et al., 2001; van Wijnen et al., 1991d], and proteolytic processing [Moon et al., 2001; Moon et al., 2002]. Furthermore, the ability of CDP/Cux to bind nucleosomal DNA [Last et al., 1999b] and to recruit histone modifying proteins (e.g., the histone H1 kinase cyclinA/CDK1, histone deacetylase HDAC-1, and the histone acetyl transferases PCAF and CBP) [Li et al., 2000; van Wijnen et al., 1994] [Li et al., 2000] suggests that the protein may contribute to modifications in the chromatin architecture of its target genes.

CDP/Cux interacts with the promoters of all five classes (i.e., H1, H2A, H2B, H3 and H4) of histone genes and consequently may coordinately regulate their transcriptional activation at the G_1/S phase transition or their repression in mid- to late S phase [Barberis et al., 1987; van den Ent et al., 1994; van Wijnen et al., 1996; Wu and Lee, 2002]. It has been well documented that the interaction of the CDP/Cux containing HiNF-D complex with histone gene regulatory elements is proliferation-specific and cell cycle regulated with respect to S phase [Holthuis et al., 1990; Last et al., 1998; Shakoori et al., 1995; van Wijnen et al., 1992; van Wijnen et al., 1997; Wright et al., 1992]. Our laboratory has shown that this interaction is important for the timing of maximal histone H4 gene transcription during the cell cycle [Aziz et al.,

1998a]. When cell growth is stimulated by growth factors, HiNF-D complex formation on histone gene promoters *in vitro* is upregulated and occurs subsequent to hyperphosphorylation of pRB [van Wijnen et al., 1997]. Thus it was postulated that CDP/Cux interacts with the hyperphosphorylated form of pRB. To test this hypothesis, a detailed analysis of pRB-CDP/Cux interaction was performed in this chapter.

pRB protein is encoded by the retinoblastoma gene which was the first tumor suppressor gene to be identified [Friend et al., 1986]. Mutations in the retinoblastoma gene have been detected in retinoblastoma tumors and other cancers such as osteosarcoma, small cell lung cancer, prostate cancer, and breast cancer [Harbour and Dean, 2000]. The tumor suppressor activity of pRB was demonstrated by its ability to inhibit the malignant phenotype when reintroduced into pRB-deficient tumor cells [Huang et al., 1988]. The retinoblastoma gene encodes a 928-amino acid phosphoprotein, pRB, which is synthesized throughout the cell cycle and contains several functional domains (Fig. 3-1A). Interaction of domain A with domain B forms the pRB minimal pocket, and the pRB large pocket consists of domains A, B and C. pRB arrests cells in the G_1 phase of the cell cycle [Weinberg, 1995] by repressing genes required for the G_1-to-S-phase transition. A major target of pRB is the E2F family of transcription factors. The E2F transcription factors regulate the transcription of many genes that are important in cell-cycle progression and that are repressed by pRB. Tumor suppression by pRB requires an intact pocket domain (Fig. 3-1A), which is disrupted by most naturally occurring tumor-promoting mutations. Viral oncoproteins that disrupt pRB function also target the pocket domain [Kaelin, Jr. et

al., 1990; Larose et al., 1991]. Sequences in the pRB pocket domain are required for interaction with E2F and several other cellular proteins. pRB can repress transcription by at least two mechanisms. pRB large pocket binds the transactivation domain of E2F, thereby blocking the ability of E2F to activate transcription (Table 3-1). In addition, the pRB pocket can actively repress transcription when it is tethered to promoters. Active repression by pRB is mediated by its recruitment of histone deacetylases (HDACs), BRG and BRM (the two ATPase components of the human SWI/SNF chromatin remodeling complex), and DNA methylase DNMT1 (Table 3-1).

Progression of the cell cycle normally occurs when pRB is inactivated by phosphorylation catalyzed by cyclin-CDK complexes. pRB has 16 potential sites for CDK phosphorylation, and it oscillates between hypophosphorylated and hyperphosphorylated forms during the cell cycle. pRB is in an unphosphorylated state in G_0, hypophosphorylated in early G_1 and hyperphosphorylated in late G_1 until M phase, when it is dephosphorylated [Ezhevsky et al., 1997]. It is thought that phosphorylation of pRB is mediated by cyclin D-CDK4/6 complexes in early G_1, cyclinE-CDK2 complexes in late G_1, and cyclin A-CDK2 may maintain pRB in the hyperphosphorylated state during S phase [Sherr, 1996]. Phosphorylation of the C-terminal region of pRB by CDK4/6 displaces HDAC from the pocket domain, thereby relieving transcriptional repression by pRB. This facilitates an intramolecular interaction that leads to phosphorylation of the pocket by CDK2 and disruption of pocket structure.

Studies of pRB-deficient mice and embryonic fibroblasts have yielded additional insight into the function(s) of this protein. pRB is required for normal

Table 3-1: A subset of pRB-interacting proteins

pRB-binding proteins that are cell-cycle regulators		
Interactor	**Region of pRB**	***In vivo**
Cyclin A	not mapped	yes
Cyclin A1	large pocket	yes
Cyclin D1	A/B pocket	yes
Cyclin D2	A/B pocket	-
Cyclin D3	A/B pocket	-
Cyclin E	not mapped	-
CDK1	not mapped	yes
CDK2	C domain	-
p21	A/B pocket	yes
$p57^{KIP2}$	not mapped	-
pRB-binding proteins which function to regulate transcription		
E2F-1	large pocket	yes
E2F-2	large pocket	yes
E2F-3a	large pocket	yes
E2F-3b	not mapped	yes
E2F-4	large pocket	yes
DP-1	not mapped	yes
DP-2	not mapped	yes
HDAC-1	A/B pocket	yes
HDAC-2	A/B pocket	yes
HDAC-3	A/B pocket	yes
BRG1	A/B pocket	yes
hBrm1	A/B pocket	yes
DNMT1	A/B pocket	yes
AP-2	large pocket	yes
Pax-3]	A/B pocket	-
Pax-5]	large pocket	-
Pax-6] homeodomain proteins	not mapped	yes
Phox]	not mapped	-
Chx10]	A/B pocket	-

**In vivo* interaction that has been detected between endogenous pRB and its respective binding protein in mammalian cell extracts. This table was adapted from Morris and Dyson 2001.

mouse development as nullizygous mice die in utero at embryonic day 16.5 (Table 3-2) [Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992]. The most prominent abnormalities in these embryos are defective hematopoiesis, ectopic mitosis and apoptosis in the central nervous system (CNS). pRB heterozygous mice are normal but have a high predisposition to pituitary tumors. Primary fibroblasts derived from pRB-deficient embryos exhibit a shortened G_1 phase, elevated and accelerated expression of cyclins, activation of E2F-responsive genes and apoptosis [Almasan et al., 1995; Herrera et al., 1996]. Loss of pRB function activates the p53 apoptotic pathway, which may serve as an intrinsic protective mechanism for eliminating cells in which the pRB pathway is deregulated [Morgenbesser et al., 1994]. A possible link between pRB and p53 is the free E2F, which is released when pRB function is lost. Transgenic mice in which pRB was inactivated by SV40- T antigen developed slowly growing tumors with high apoptotic rates, whereas an additional inactivation of p53 or E2F resulted in rapidly growing tumors due to decreased apoptosis [Pan et al., 1998; Symonds et al., 1994]. Taken together, these observations suggest that release of the free E2F1 resulting from loss of pRB function is responsible for triggering much of the p53-dependent apoptosis.

Two other proteins, p107 and p130, show high homology with pRB within domains A and B, and they also bind viral oncoproteins and E2F [Ewen et al., 1991; Hannon et al., 1993]. All three proteins can inhibit E2F-responsive promoters [Zamanian and La Thangue, 1993], recruit HDAC via the pocket domain [Ferreira et al., 1998], actively repress transcription [Bremner et al., 1995; Starostik et al., 1996], and arrest cellular growth when overexpressed [Claudio et al., 1994; Harbour and

Table 3-2: Knockout phenotypes of genes in the pRB family

Genotype	Phenotype	Major abnormalities
$RB^{-/-}$	Embryonic lethality (E13.5-16.5)	Defective neurogenesis & hematopoiesis
$RB^{+/-}$	Tumor predisposition	Complete penetrance of pituitary tumors
$p107^{-/-}$	Normal	None
$p130^{-/-}$	Normal	None
$RB^{+/-}$;$p107^{-/-}$	Growth retardation increased mortality	Pituitary tumors retinal dysplasia
$RB^{-/-}$;$p107^{-/-}$	Embryonic lethality (E11.5)	Accelerated apoptosis in liver & CNS
$p107^{-/-}$;$p130^{-/-}$	Neonatal lethality	Defective endochondral bone & limb development

*Adapted from Lin et al., 1996.

Dean, 2000; Starostik et al., 1996]. Although pRB, p107 and p130 are closely related, they have several distinct biochemical properties. For example, in p107 and p130, the spacer region between domains A and B mediates inhibition of cyclin A-CDK2 complexes, which results in growth suppression by p107 [Zhu et al., 1995]. In contrast, the spacer region in pRB has no known function. In addition, pRB and p107 interact with E2F proteins in G_1 and S phase of cycling cells but p130 binds E2F4-5 in quiescent cells and differentiating muscle cells [Corbeil et al., 1995]. Analysis of mice in which these pRB-related genes have been inactivated suggests that these genes have a partial overlap in function (Table 3-2). Deletion of pRB leads to embryonic lethality in midgestation, whereas the additional loss of p107 results in an earlier lethality [Lee et al., 1996]. p107 and p130 knockout mice develop normally but the double knockout mice have defective bone development, shortened limbs and neonatal lethality [Cobrinik et al., 1996]. Heterozygous mice ($pRB^{+/-}$) develop normally but the additional loss of p107 function ($pRB^{+/-}$, $p107^{-/-}$) results in growth retardation and early mortality [Lee et al., 1996].

To understand the molecular basis of the interactions between pRB and CDP/Cux as components of the multi-subunit HiNF-D complex that controls histone gene expression, GST pull down assays, co-immunoprecipitations, and transient transcriptional assays were performed. Studies presented in this chapter indicate that CDP/Cux forms protein/protein complexes with pRB in the absence of DNA and that pRB cooperates with CDP/Cux in the repression of H4 gene transcription. These data support the concept that complexes between CDP/Cux and pRB are important cell cycle regulators of transcription during S phase.

RESULTS:

CDP/Cux interacts with pRB, Cyclin A, and CDK1 *in vitro*

The HiNF-D complex represents an electrophoretically stable protein/DNA complex that is immuno-reactive with antibodies against CDP/Cux, as well as the cell cycle regulatory factors pRB, cyclin A and CDK1 [van Wijnen et al., 1994]. HiNF-D can be chromatographically fractionated over multiple ion exchange resins indicating that this factor may form a distinct biochemical entity in the absence of DNA [van Wijnen et al., 1992]. However, it has not been possible yet to purify the HiNF-D complex to homogeneity, and the size of the complex is not known. To determine the molecular weight of HiNF-D, electromobility shift assays (EMSAs) were performed using HeLa nuclear extract and a ^{32}P-labeled probe spanning histone H4 Site II. Protein markers were electrophoresed in the same gel and detected by Coomassie dye (Fig. 3-2A). Specific HiNF-D activity was verified by oligonucleotide competition assays. Based on the migration of HiNF-D and the marker proteins, the observed molecular weight of HiNF-D is greater than 340 kDa (Fig. 3-2A), consistent with the estimated sum (400 kDa) of the molecular weights of the HiNF-D components [i.e., CDP/Cux (190 kDa), pRB (110 kDa), cyclin A (60 kDa) and CDK1 (34 kDa)]. Thus the previously identified components of HiNF-D can account for the observed molecular weight.

To test whether CDP/Cux can form stable protein/protein complexes with non-DNA binding partner proteins in the HiNF-D complex, pull-down assays were performed using glutathione-S-transferase (GST)-CDP/Cux fusion protein. To date

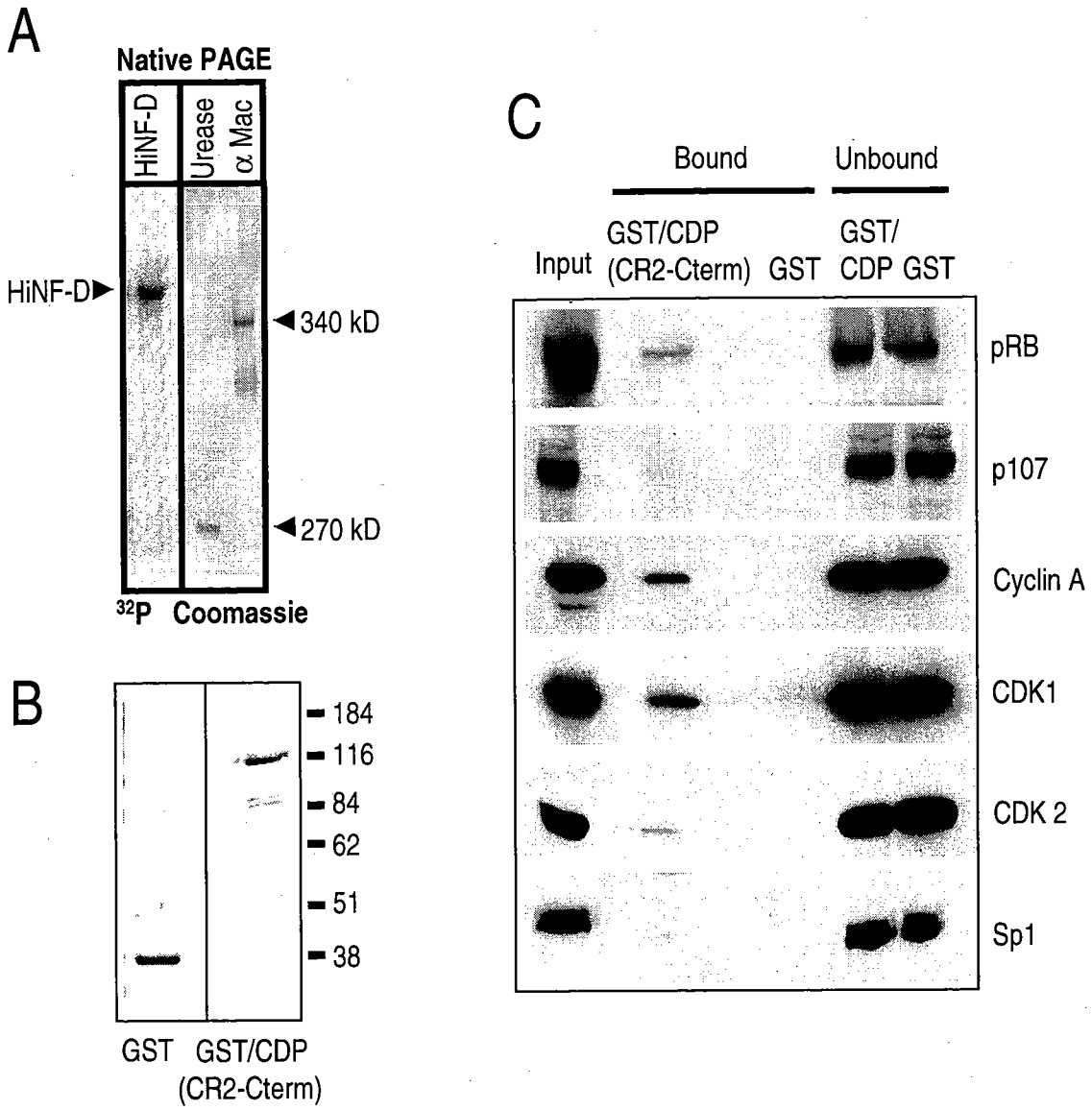


FIG 3-2. The CDP/Cux C-terminus interacts with H4 Site II-associated proteins *in vitro*. (A) HiNF-D is greater than 340 kDa. (left) Electromobility Shift Assay (EMSA) was performed with ^{32}P-labeled oligos spanning Site II of the histone H4.1 promoter and nuclear extract (1.5 μg) prepared from proliferating HeLa cells. (Right). In the same polyacrylamide gel (4.4%) used in EMSA, urease (4μg) and α-macroglobulin (1μg) protein markers were subjected to electrophoresis and were detected with Coomassie dye. **(B)** GST and GST-CDP/Cux (CR2-Cterm, amino acid 853-1505) proteins conjugated to glutathione Sepharose beads were subjected to SDS-PAGE and visualized by Coomassie dye to confirm protein integrity. **(C)** CDP/Cux specifically and selectively binds pRB, cyclin A and CDK1. HeLa nuclear proteins (690 μg) were used in pull-down assays with 2 μg of either GST or the GST-CDP/Cux (CR2-Cterm) fusion protein. Bound proteins were separated by SDS-PAGE and subjected to western blot analysis with antibodies to pRB, p107, cyclin A, CDK1, CDK2 and Sp1.

expression of full-length CDP/Cux as a recombinant GST fusion protein has not been possible. Therefore a truncated CDP/Cux fusion protein was generated using a C-terminus region, CR2-Cterm, which is an S phase-specific isoform of CDP/Cux (Fig. 3-1A). *In vitro* interactions of endogenous pRB, cyclinA, and CDK1 present in HeLa nuclear extract with recombinant GST-CDP/Cux (CR2-Cterm) were investigated. Interacting proteins were eluted and subjected to western blot analysis using a panel of antibodies against the known HiNF-D subunits. Results show that pRB, cyclin A and CDK1 are capable of binding to GST-CDP/Cux (CR2-Cterm), but these proteins do not interact with GST alone (Fig. 3-2C). For comparison, Sp1 does not bind to GST-CDP/Cux (CR2-Cterm), thus demonstrating that the interactions detected in these experiments are selective and specific. Furthermore p107 and CDK2 show limited binding to GST-CDP/Cux (CR2-Cterm) as compared to pRB and CDK1, respectively. Hence, the GST pull-down assays establish that the S phase-specific cleavage product of CDP/Cux, CR2-Cterm, supports interactions with pRB, cyclin A, and CDK1.

CDP/Cux (CR2-Cterm) interaction with the pRB large pocket is phosphorylation-site independent

The large pocket (referred to as LP in this dissertation, amino acids 379-928) is the minimal growth-suppressing domain of pRB, and nearly all of the germline tumor-derived mutations of pRB occur within this region. In addition, most of the known pRB interactions with other proteins require an intact pocket domain [Morris and Dyson, 2001] (Table 3-1). To test whether CDP/Cux interacts with the large

pocket of pRB, pull-down assays were performed with GST-CDP/Cux (CR2-Cterm) and HeLa lysates containing exogenously expressed full-length or mutant pRB proteins (Fig. 3-1B). Results indicate that the CDP/Cux C-terminus interacts with full-length pRB and large pocket to a similar extent (Fig. 3-3A). Thus the pRB large pocket alone is sufficient to mediate interaction between CDP/Cux and pRB.

When hyperphosphorylated by cyclin/Cdk complexes during G_1 phase, pRB can no longer sequester E2F or exert its growth suppressing activity through E2F. To determine whether phosphorylation of the large pocket is required for interaction with CDP/Cux, GST-CDP/Cux pull-down assays were performed using overexpressed mutant large pocket (mLP). This mutant pRB protein has mutations in seven of the nine phosphorylation sites. Results show CDP/Cux (CR2-Cterm) interacts with mLP (Fig. 3-3A), suggesting that the CDP/Cux-pRB interaction is independent of phosphorylation sites in the large pocket domain.

Direct interaction between CDP/Cux and pRB is mediated by the CDP/Cux C-terminus and the pRB pocket domain

The data described above show that CDP/Cux (CR2-Cterm) can interact with pRB large pocket in the absence of cell signaling-dependent post-translational modifications. However, it is unclear whether the observed GST-CDP/Cux interaction with pRB large pocket is direct or mediated by bridging proteins present in the HeLa nuclear extract. To test these possibilities, pull-down assays were performed with GST-CDP/Cux (CR2-Cterm) and *in vitro* translated ^{35}S-labeled A/B pocket or ^{35}S-labeled cyclin A. Results show that GST-CDP/Cux (CR2-Cterm)

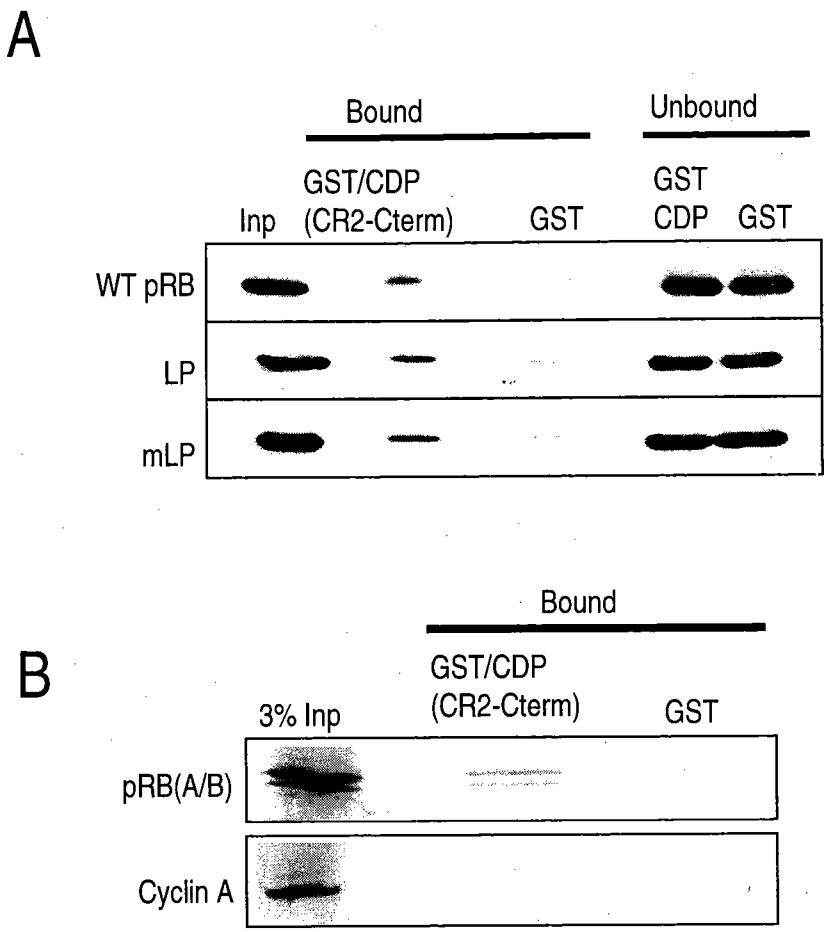


FIG 3-3. The interaction between CDP/Cux and pRB is direct and phosphorylation-site independent. (A) Wild type pRB, a truncated pRB protein spanning the large pocket (LP, amino acids 379-928) and a phosphorylation deficient large pocket protein (mLP) interacts specifically with GST-CDP/Cux (CR2-Cterm). HeLa cell lysates (1 mg) containing over-expressed pRB proteins were used in pull-down assays with 2 μg of GST or GST-CDP/Cux (CR2-Cterm) proteins. **(B)** CDP/Cux (CR2-Cterm) interacts directly with the minimal pRB A/B pocket (amino acids 379-772). GST pull down assays were performed by mixing GST or GST-fusion proteins with *in vitro* translated ^{35}S-labeled proteins. Proteins from the input (Inp), bound and unbound fractions were separated by SDS-PAGE and detected by autoradiography.

specifically binds the pRB minimal A/B pocket domain but not cyclin A (Fig. 3-3B). Thus the CDP/Cux (CR2-Cterm)/ cyclin A interaction observed with HeLa nuclear proteins (Fig. 3-2C) may be mediated through an accessory factor (e.g., pRB). More importantly, CDP/Cux (CR2-Cterm) and the pRB A/B pocket are sufficient for direct interaction between CDP/Cux and pRB

CDP/Cux and pRB interact *in vivo*

To assess whether CDP/Cux and pRB formed stable complexes within intact cells, co-immunoprecipitation assays were performed. Interactions of both endogenous and co-expressed pRB with CDP/Cux were assessed in COS-7 cells transiently expressing myc-tagged CDP/Cux protein. Immunoprecipitated proteins were detected by western blot using antibodies against the myc tag or the pRB protein. The results show that pRB co-immunoprecipitates with full length CDP/Cux (Fig. 3-4, panels A and B) and to a lesser extent with CDP/Cux (CR2-Cterm) (Fig. 3-4, panels C and D). Specific immunoprecipitation was not observed with normal mouse IgG that was used as a negative control. In addition, CDP/Cux also forms complexes with pRB in HeLa cells (Fig. 3-4E). Thus optimal CDP/Cux-pRB interaction requires the CDP/Cux N-terminus, which includes a coiled-coil domain, a protein-protein interaction motif. Taken together, these data establish that CDP/Cux and pRB form specific protein/protein complexes in mammalian cells.

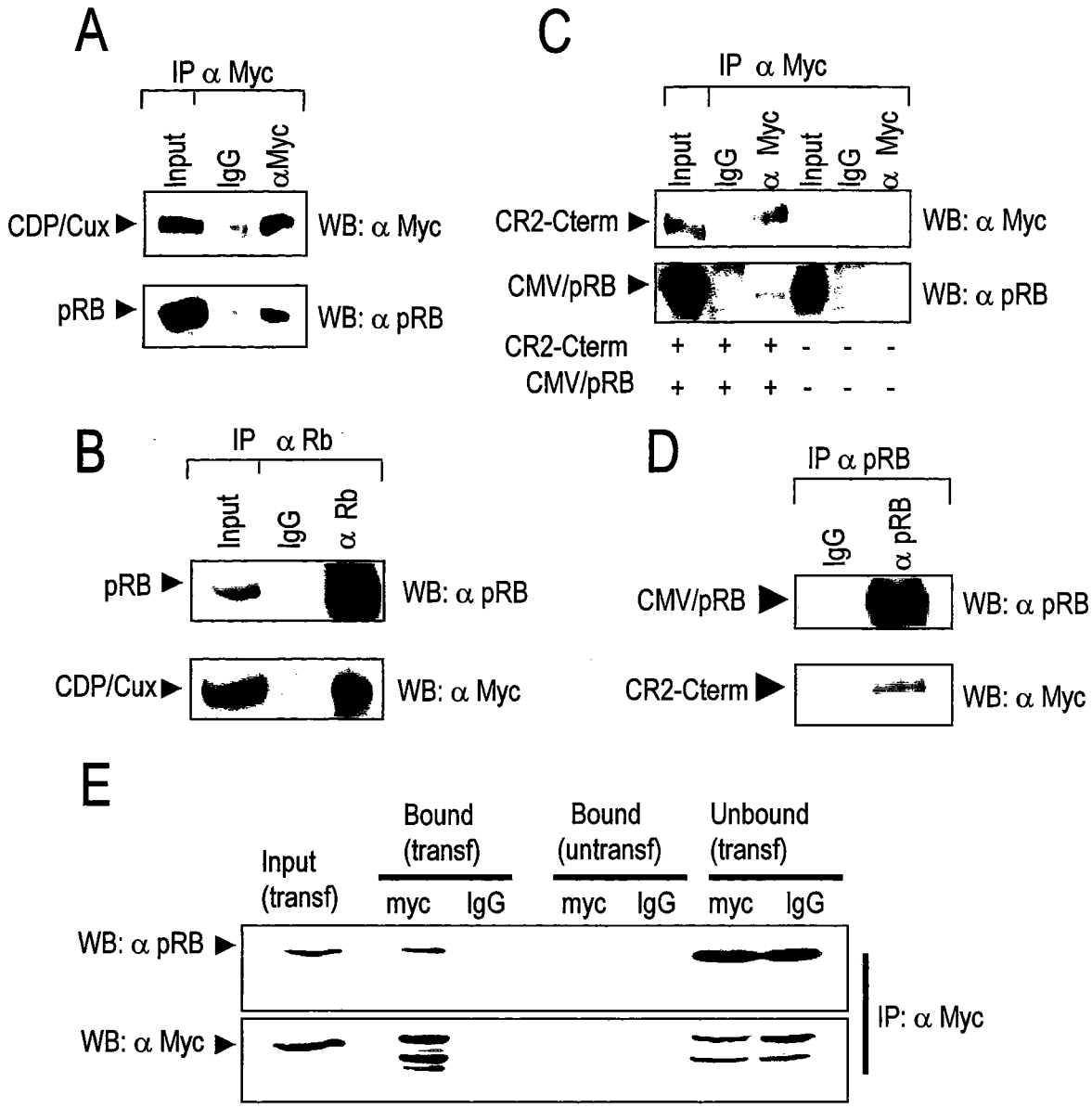


FIG 3-4. CDP/Cux interacts with pRB *in vivo*. Cos-7 cells were cotransfected with myc-tagged full length CDP/Cux or CR2-Cterm to detect interaction with endogenous (panels A and B) or co-expressed (panel C and D) pRB by co-immunoprecipitation assays. Cells were harvested 32 h after transfection and co-immunoprecipitation was performed using myc antibody (panels A and C) or the pRB antibody (panel B and D). Bound proteins were detected by western blot with the respective antibodies. Input represents 3% of bound sample (panel A and B), 10% of the bound sample (panel C). Exogenously expressed pRB and Myc-CDP/Cux form complexes in HeLa cells (panel E). Untransfected lysate (untransf) was used as a negative control. Western blot analysis of proteins that were not immunoprecipitated (unbound fraction) was performed to assess protein integrity following overnight incubation at 4°C.

A subset of pRB and CDP/Cux co-localize *in situ*.

Because pRB and CDP/Cux exist in a complex *in vivo,* their association *in situ* within the nucleus was assessed. Expression of endogenous CDP/Cux and pRB proteins were detected by *in situ* immunofluorescence and analyzed by confocal microscopy. Colocalization of two proteins was assessed by using a cross-correlation function that establishes the degree of signal overlap between two images (see Materials and Methods). On average, colocalization of pRB with CDP/Cux was 35% in HeLa cells (Fig. 3-5) and 47% in T98G glioblastoma cells (Fig. 3-6A). Specificity of colocalization was demonstrated by determining that the observed correlation is not due to random signal overlap. One image from each image pair was rotated ten degrees and the cross-correlation was measured (Fig. 3-6B). If the observed colocalization were random, rotation of the image would not change the degree of signal overlap. Differences between rotated and unrotated cross-correlation values were assessed by paired student's t-test. Our analysis shows that the majority of the observed colocalization of CDP/Cux and pRB is not due to random signal overlap ($p<0.001$) in both T98G glioblastoma and HeLa cells.

The N-terminus of CDP/Cux is required for full repression of histone H4 transcription

CDP/Cux has been shown to repress H4 promoter activity [van Wijnen et al., 1996], and the C-terminal region of CDP/Cux that encompasses its DNA binding domains is involved in transcriptional inhibition of CDP/Cux responsive promoters [Mailly et al., 1996; Moon et al., 2000]. CDP/Cux has been shown to undergo

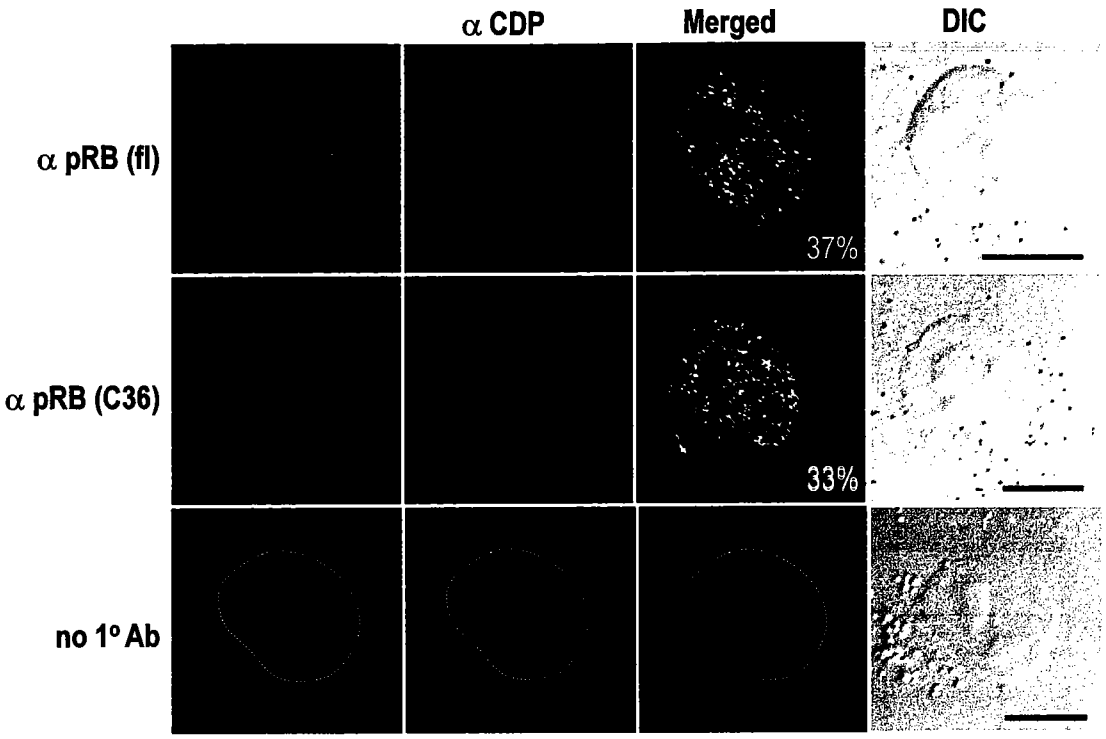


Figure 3-5. Partial colocalization of CDP/Cux and pRB in HeLa cells. Cells grown on gelatin-coated coverslips were fixed, permeabilized and incubated with antibodies against full-length CDP/Cux and pRB, then analyzed by confocal microscopy. The bar represents 10μm. (α pRB fl) mouse monoclonal antibody raised against full-length pRB (Santa Cruz); (α pRB C36) mouse monoclonal antibody raised against amino acids 300-380 of pRB; (no 1° ab) no primary antibodies were added to these coverslips; (DIC) differential interference contrast image. Merged inset is percent colocalization calculated as described in Materials and Methods. Four cells were analyzed with each pRB antibody.

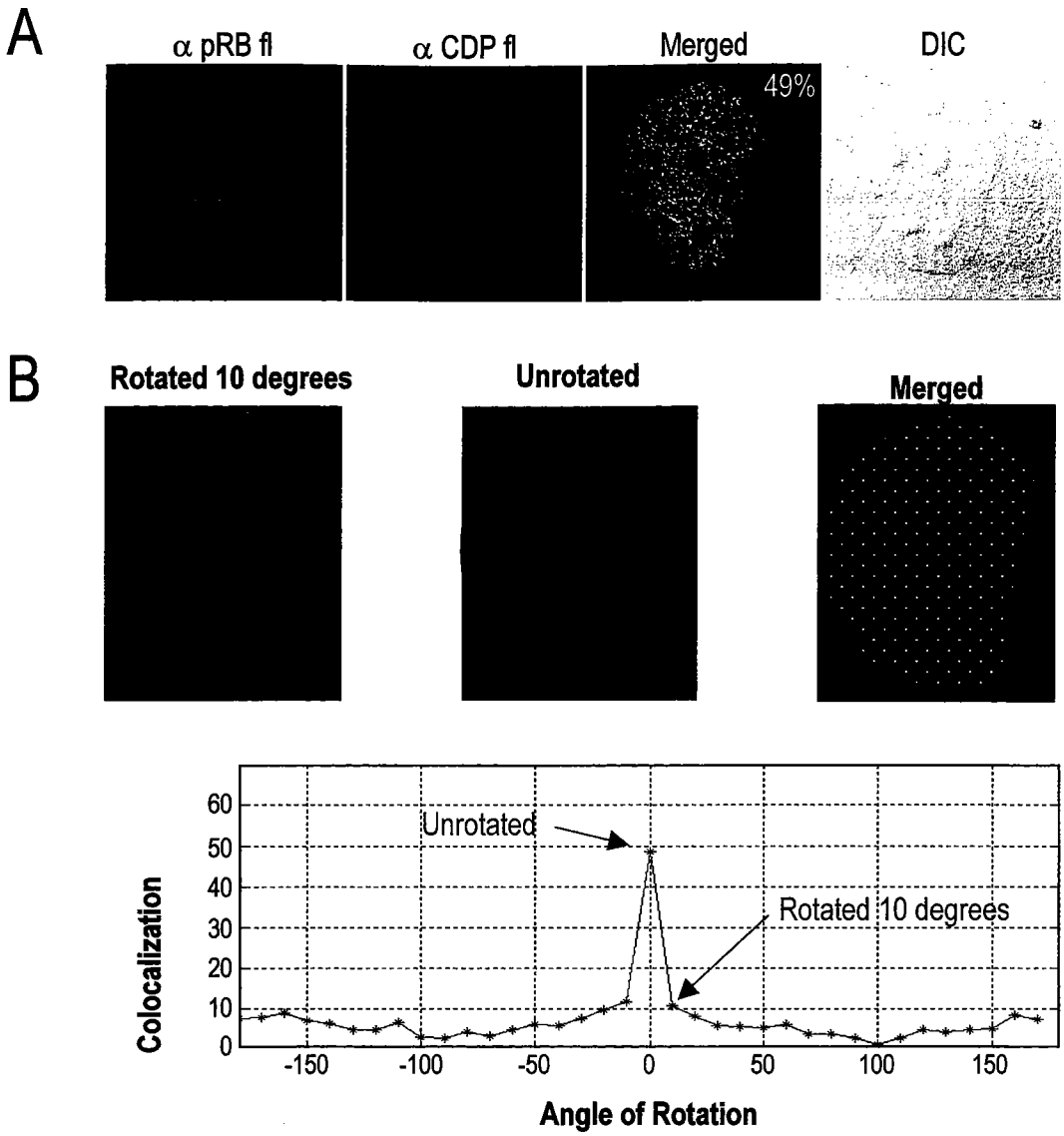


FIG 3-6. A subset of CDP/Cux and pRB associates *in situ*. T98G glioblastoma cells grown on gelatin-coated coverslips were fixed, permeabilized and incubated with antibodies against full-length CDP/Cux or pRB as indicated, then were analyzed by confocal microscopy. **(A)** Merged inset is percent colocalization calculated as described in Materials and Methods. Seven cells were analyzed with α pRB (fl) and four cells were analyzed with α pRB C36, a mouse monoclonal antibody raised against amino acids 300-380 of pRB. Bar represents 10μm. (DIC) differential interference contrast image. **(B)** Colocalization of CDP/Cux and pRB is not due to random signal overlap. One image is rotated 10 degrees and the colocalization of signal in the remaining area of overlap (white dots) is calculated. Rotation by 10 degrees results in a significant change in colocalization (from 49% to 10%).

proteolytic cleavage in S phase [Moon et al., 2001]. The cleavage product, CDP/Cux (CR2-Cterm) protein, lacks the N-terminus of CDP/Cux and spans Cut repeat 2 (CR2) to the C-terminus (Cterm) (Fig. 3-1A). To assess whether CDP/Cux (CR2-Cterm) represses histone H4 gene transcription, reporter gene assays were performed with constructs expressing myc-tagged full-length or CR2-Cterm CDP/Cux proteins in NIH/3T3 cells (Fig. 3-7A). The data show that CDP/Cux (CR2-Cterm) mediates repression but to a lesser extent than the full-length protein (Fig. 3-7A). Both CDP/Cux proteins are expressed in a dose-dependent manner and are localized to the nucleus (Fig. 3-7, panels B and C). Thus, the N-terminal sequences contribute to the repressive potential of CDP/Cux on histone H4 gene transcription. Although CR2-Cterm lacks one of the DNA-binding Cut repeats, this mutant protein retains the ability to bind to the histone H4 promoter and CDP/Cux consensus binding sites [Last et al., 1999b; Moon et al., 2001; van der Meijden et al., 1998]. Decreased repression of the H4 promoter by CR2-Cterm may be due to reduced protein-protein interactions, since absence of the N-terminus resulted in decreased CDP/Cux-pRB interactions (Fig. 3-4).

Co-repression of H4 promoter activity by pRB and CDP/Cux

CDP/Cux and pRB form complexes *in vivo* and associate *in situ*. To assess functional consequences of these interactions on transcriptional regulation of histone genes, pRB was co-expressed with CDP/Cux and H4 promoter activity was measured in NIH/3T3 cells. The data show that expression of pRB or CDP/Cux alone reduces H4 promoter activity by 4 and 18 fold, respectively (Figure 3-8). When pRB is co-

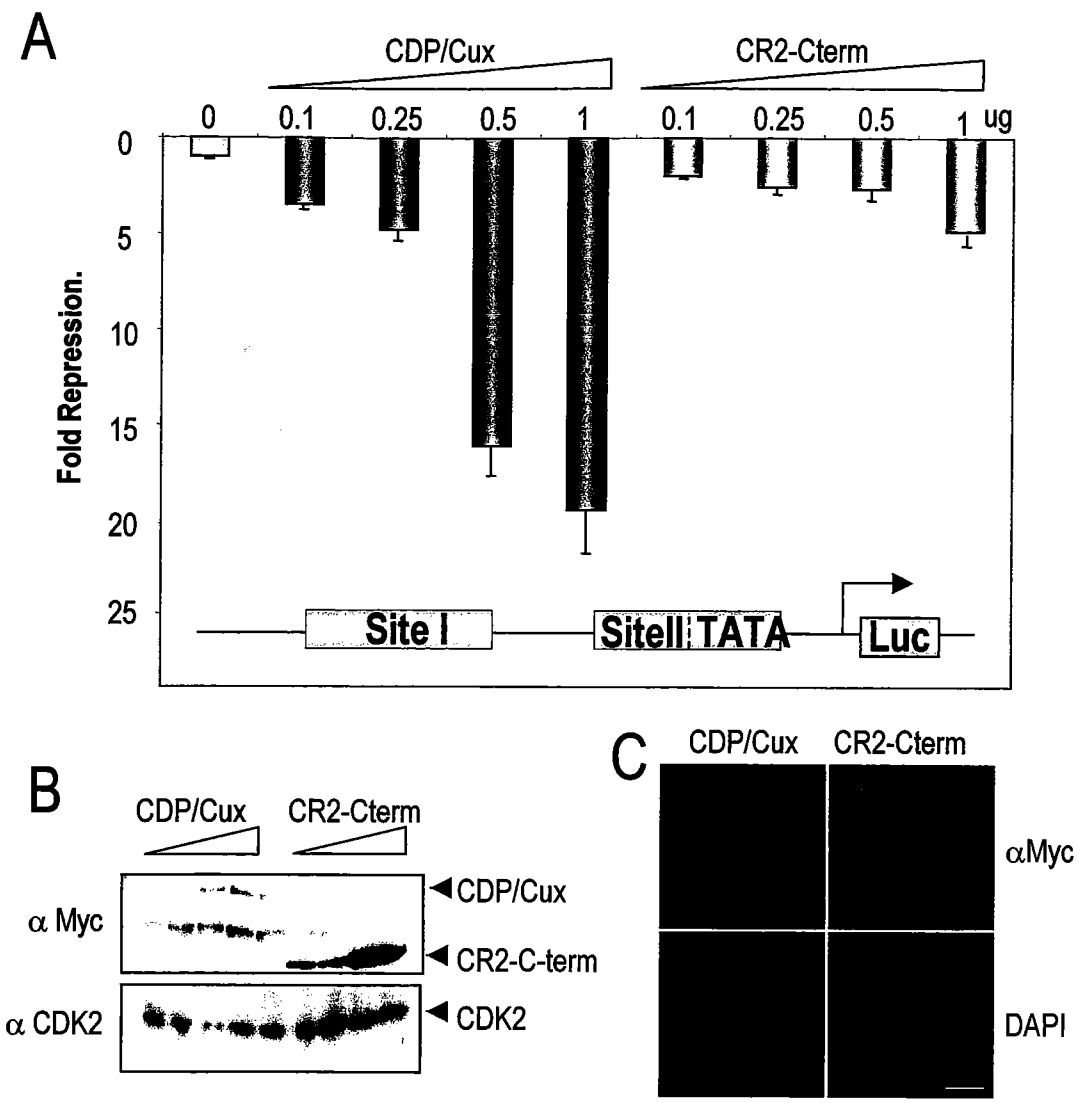


FIG 3-7. The C-terminus of CDP/Cux is required for repression of H4 promoter activity. (A) NIH/3T3 cells were cotransfected with the H4/Luc reporter construct (1 μg) and increasing amounts (0.1 to 1 μg) of the CMV driven myc-tagged full length CDP/Cux or mutant CDP/Cux (CR2-Cterm) expression plasmid. Luciferase assays were performed 24 h after transfection. Inset: Schematic diagram of H4 promoter–luciferase reporter construct encompassing Sites I and II used in this study. **(B)** The same samples used in the luciferase assays were analyzed by western blotting of a 6% SDS polyacrylamide gel. CDP/Cux was detected with the c-myc antibody and CDK2 was detected as an internal control for protein loading. **(C)** Nuclear localization of Myc-tagged wild type CDP/Cux and CDP/Cux (CR2-Cterm) proteins was assessed by immunofluorescence microscopy in HeLa cells using a monoclonal antibody against c-myc. Chromatin is visualized by DAPI staining. The bar represents 10 μm.

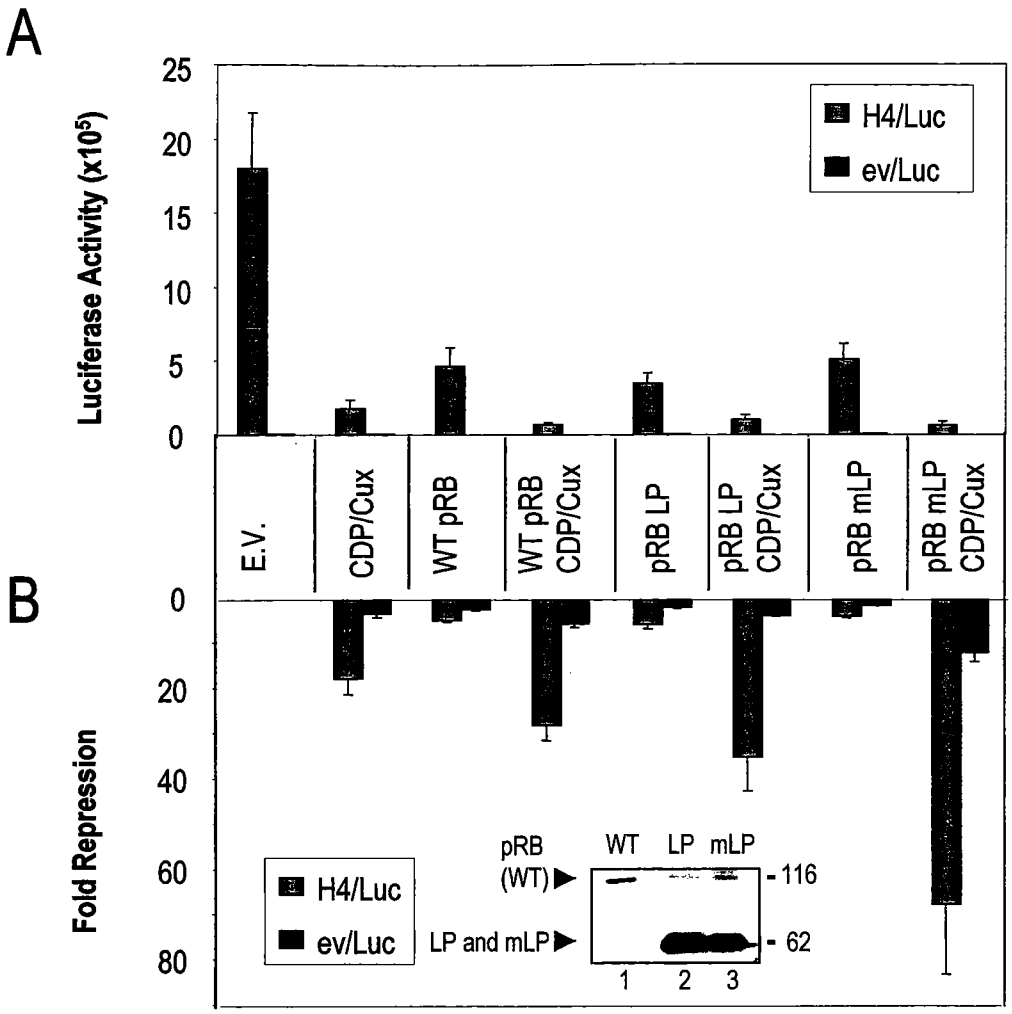


FIG 3-8. The pRB large pocket is sufficient for CDP/Cux dependent co-repression of H4 transcription. NIH/3T3 cells were transfected with an H4 promoter-driven luciferase reporter construct (H4/Luc) or a promoterless luciferase construct (ev/Luc) or and cotransfected with expression vectors for CDP/Cux and/or pRB proteins. Cells were harvested 22 h after transfection and total cell lysate was assayed for luciferase activity or subjected to SDS-PAGE and western blot analysis. Luciferase values **(A)** and fold repression **(B)** are shown. Inset: Overexpressed wildtype pRB (lane 1), wildtype large pocket domain (LP, lane 2), and mutant large pocket domain (mLP, lane 3) proteins were detected using the pRB (XZ104) antibody which is directed against the pRB pocket. Endogenous pRB was also detected in lanes 2 and 3. The amount of DNA in each well was maintained at a constant level by supplementing the transfection mixture with the empty expression vector. Co-transfection experiments with CDP/Cux and pRB proteins were performed with 1 μg of each vector with the exception of vectors for pRB large pocket (500 ng). Error bars represent standard error of the mean where n=12. (e.v.) empty vector backbone for CDP/Cux and pRB expression constructs.

expressed with CDP/Cux, additive repression (25 fold) of reporter gene expression was observed. Thus pRB and CDP/Cux interact and independently repress histone H4 transcription in NIH 3T3 cells. Co-repression by pRB with CDP/Cux was also observed in HeLa, Cos-7 and Panc-1 cells [Gupta et al., 2003].

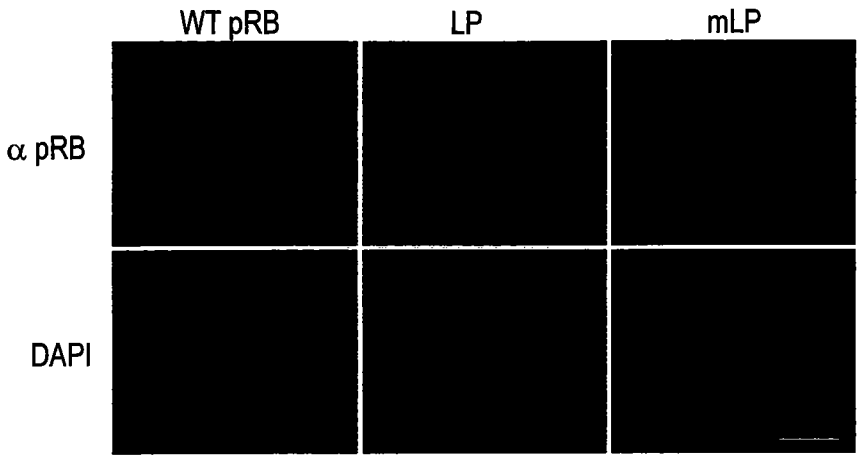
The large pocket of pRB represses histone H4 transcription with CDP/Cux

CDP/Cux C-terminus interacts with the wild type (LP) and phosphorylation mutant large pocket (mLP) of pRB *in vitro* (Fig. 3-3B). To test whether the large pocket of pRB is sufficient for co-repression of histone gene transcription, H4/Luciferase reporter construct, CDP/Cux and pRB constructs were cotransfected into NIH/3T3 cells (Fig. 3-8). Results show that H4 promoter activity is strongly repressed by forced expression of CDP/Cux (~17 fold), whereas forced expression of the large pocket (LP) or mutant large pocket (mLP) alone causes a moderate repression of H4 gene transcription (~6 and ~4 fold, respectively) similar to wildtype pRB (~4 fold) (Fig. 3-8B). Co-expression of CDP/Cux with pRB or wild type pRB large pocket results in increased repression of the H4-driven luciferase reporter (~26 and ~35 fold, respectively). Taken together, these data suggest that pRB large pocket is sufficient for co-repression with CDP/Cux.

To determine whether CDK phosphorylation sites in the large pocket are required for this interaction, CDP/Cux was co-expressed with the phosphorylation site-deficient mutant PSM7 large pocket. We find that the phosphorylation mutant large pocket and CDP/Cux function together as strong repressors of H4 gene transcription (~67 fold) (Fig. 3-8B). Increased co-repression by large pocket proteins,

as compared with wildtype pRB, may be due to the difference in expression levels of large pocket proteins and wildtype full-length pRB (Fig. 3-8B; inset). Increased co-repression by mutant large pocket (mLP) may be due to the recruitment of additional proteins, because many pRB co-repressors including mSin3A, NcoR and HDAC proteins have been shown to bind the hypophosphorylated, but not the hyperphosphorylated, form of pRB [Morris and Dyson, 2001]. Thus the CDP/Cux functional interaction with pRB large pocket does not require large pocket phosphorylation sites. Immunofluorescence microscopy was used to confirm that the overexpressed pRB mutant proteins were localized to the nucleus (Fig. 3-9A). Because prolonged overexpression of pRB proteins may result in a G_1 cell cycle block [Angus et al., 2002], flow cytometric analysis was performed in parallel with these experiments. No significant effect on the cell cycle distribution resulted from expression of pRB proteins for 22 hours (Fig. 3-9B). Altogether, results from gene reporter assays using pRB mutant proteins indicate that pRB large pocket is sufficient for co-repression with CDP/Cux and that this co-repression is independent of pRB large pocket phosphorylation sites.

A



B

Phase	E.V.	WT pRB CDP/Cux	LP CDP/Cux	mLP CDP/Cux
G_0-G_1	55	53	58	56
G_2-M	10	8	8	8
S	35	39	34	36

FIG 3-9. Overexpressed mutant pRB proteins retain nuclear localization and moderate expression does not perturb cell cycle distribution. (A) Mutant pRB proteins retain nuclear localization. HeLa cells transfected for 22h with 500ng of expression vectors were subjected to immunofluorescence microscopy using mouse monoclonal antibody against full-length pRB. The bar represents 10μm. **(B)** NIH/3T3 cells overexpressing CDP/Cux and pRB proteins were harvested 22 h after transfection and cell cycle distribution was determined by flow cytometric analysis. (WT pRB) wild type pRB; (LP) pRB large pocket; (mLP) phosphorylation mutant pRB LP.

DISCUSSION:

The experiments in this chapter present multiple lines of evidence indicating that CDP/Cux and pRB independently repress histone H4 transcription and directly interact as a protein/protein complex. These findings provide a molecular mechanism for the integration of the activities of CDP/Cux and pRB in the HiNF-D protein/DNA complex that interacts with the Site II element of histone H4 genes. Converging data obtained by the combined application of GST pull-down assays, immunoprecipitations, yeast and mammalian two-hybrid analyses [Gupta et al., 2003], as well as immunofluorescence microscopy, all indicate that CDP/Cux and pRB form a complex both *in vitro* and in intact cells. Previous data from our laboratory have indicated that CDP/Cux forms promoter-selective protein/DNA complexes *in vitro* with pRB on the cell cycle controlled histone H4, H3 and H1 promoters or with the pRB-related protein p107 on the tissue-specific osteocalcin and gp91phox promoters [van Gurp et al., 1999; van Wijnen et al., 1996]. Based on these results, we propose that complexes between CDP/Cux and pRB are biologically significant and perform important cell cycle related gene-regulatory functions.

The pRB tumor suppressor modulates cell cycle progression through transcriptional regulation of genes required for the G_1 to S transition [Morris and Dyson, 2001]. It can directly bind to and inactivate certain promoter bound transcription factors, the most notable of which is E2F. Data presented in this study indicate that CDP/Cux provides an E2F-independent mechanism for a cell growth regulatory function of pRB in mammalian cells. Although it is well documented that

E2F is deregulated as a result of viral or cellular modifications in pRB, previous findings from our laboratory show that the level of the HiNF-D complex (i.e., the protein/DNA complex that is observed with probes spanning the Site II cell cycle element) is elevated in HeLa and COS-7 cells, as well as in other transformed (or tumor-derived) cell lines [Holthuis et al., 1990; van Wijnen et al., 1992]. Thus it appears that formation of CDP/Cux-pRB complexes is not adversely affected by viral modifications of pRB. Consistent with this concept, pRB repression of histone H4 promoter activity was observed in tumor-derived and/or virally transformed cell types. It has previously been shown that paired-like homeodomain proteins (e.g., Pax-3, MHox, Chx10) can interact with pRB through their conserved homeodomains [Wiggan et al., 1998] [Cvekl et al., 1999; Eberhard and Busslinger, 1999]. Consistent with these observations, results from immunoprecipitation experiments indicate that CDP (CR2-Cterm) encompassing the homeodomain was sufficient for CDP/Cux interaction with pRB.

The temporal correlation in S phase between maximal HiNF-D interaction with the H4 Site II element [Last et al., 1998; Shakoori et al., 1995; Stein et al., 1998] and the presence of the CR2-Cterm cleavage product of CDP/Cux [Santaguida et al., 2001] suggests that CR2-Cterm may be a component of HiNF-D. Data from GST pull-down experiments show that the CDP/Cux (CR2-Cterm) moiety is sufficient for interaction with HiNF-D subunits cyclin A, CDK1 and pRB, and for direct interaction with the pRB minimal pocket. However, reporter transcriptional assays and co-immunoprecipitation experiments indicate that the N-terminus of CDP/Cux, which contains a coiled-coil protein interaction motif and the DNA-binding domain Cut

repeat 1, is required for full repression of histone H4 transcription and optimal levels of complex formation with pRB *in vivo*. Thus it is likely that full-length CDP/Cux, rather than the CR2-Cterm isoform, is involved in the regulation of histone gene transcription.

The CDP/Cux-dependent transcriptional repressor function of pRB on histone genes operates independently of E2F, because H4 gene promoters do not contain E2F binding sites [van Wijnen et al., 1996]. Results from GST pull-down studies show that the structural determinants of pRB that support the CDP-pRB interaction are similar to those required for E2F. Specifically, both CDP/Cux and E2F interact with the pRB large pocket, and neither protein depends strictly on contacts with CDK phosphorylation sites in the pRB large pocket [Knudsen and Wang, 1997]. Because of similarities in the binding characteristics of E2F and CDP/Cux with pRB, these proteins may compete for binding to pRB under biological conditions where they are expressed simultaneously. However, during cell growth stimulation (e.g., IL-3 dependent cell cycle entry of FDC-P1 hematopoietic progenitor cells) when cells progress into S phase, HiNF-D protein/DNA complex are dramatically elevated following hyperphosphorylation of pRB in late G_1 [Shakoori et al., 1995; van Wijnen et al., 1997]. Since maximal HiNF-D complex formation occurs when pRB is in the hyperphosphorylated state, this finding suggests that the disruption of E2F/pRB complexes by pRB phosphorylation may increase the availability of pRB for interaction with CDP/Cux.

The CDP/Cux-pRB interaction may be of broader significance in regulating transcription during the cell cycle. Apart from regulating human histone H4 gene

transcription, CDP/Cux represses the hamster thymidine kinase (TK) promoter [Wiggan et al., 1998] and complexes containing pRB-related proteins interact with the TK promoter [Kim et al., 1996]. Recent data by Lee and colleagues have provided evidence suggesting that the combined actions of CDP/Cux and pRB at the histone H3.2 and TK promoters may contribute to cell cycle control of transcription [Kim et al., 1996; Kim et al., 1997; Wu and Lee, 1998; Wu and Lee, 2002]. These investigators showed that AP-2 interacted with both pRB and CDP/Cux, and postulated that CDP/Cux repression of the histone H3.2 promoter was mediated by recruitment of the AP-2/pRB complex [Wu and Lee, 2002]. The H4, H3.2 and TK genes are transiently activated at the G_1/S phase transition, while the levels of the DNA binding complex between CDP/Cux and pRB (i.e., HiNF-D complex) are maximal in mid to late S phase [Last et al., 1998; van Wijnen et al., 1997]. Therefore, it is possible that CDP/Cux-pRB co-repression attenuates transcription of the H4, H3.2 and TK genes during later stages of S phase when DNA synthesis rates are decreased and the demand for histones and nucleotides is diminished (Fig. 3-10).

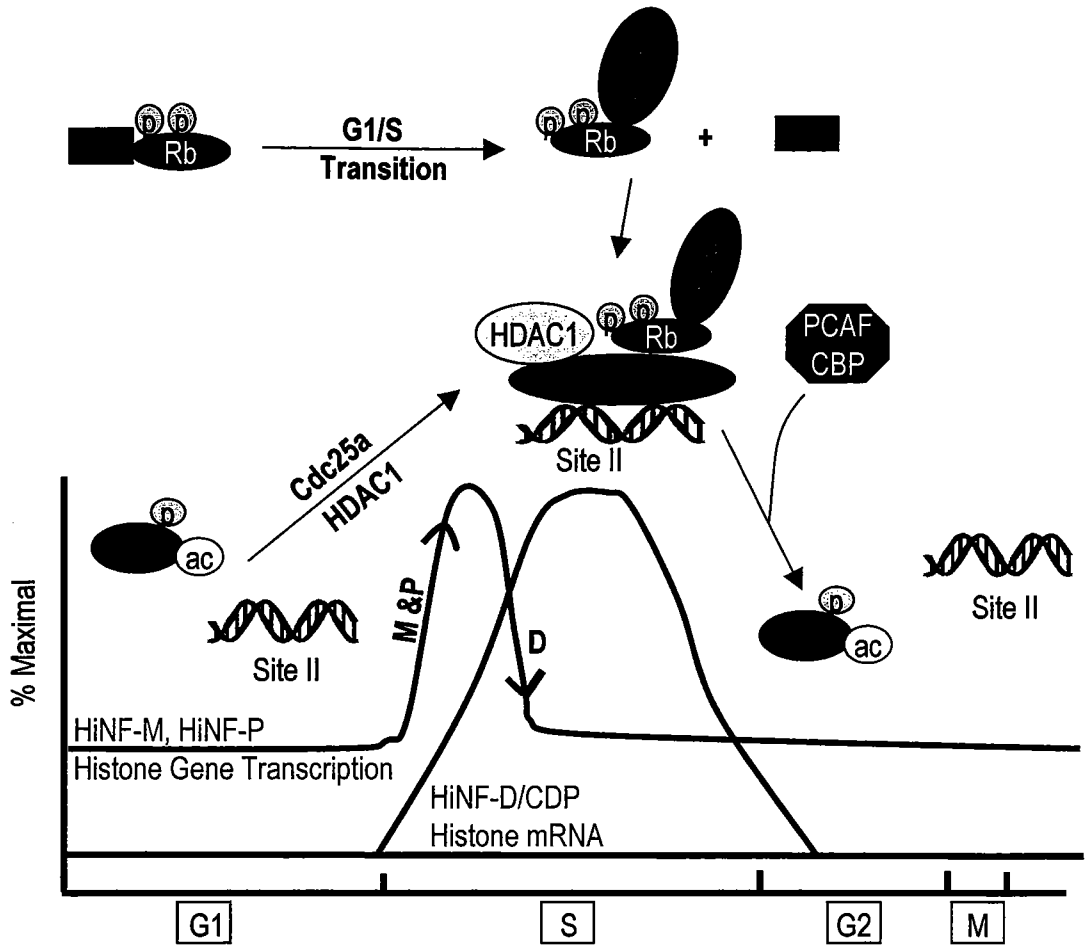


FIG 3-10. Model for the mechanism of HiNF-D repression of histone gene transcription. The red curve represents levels of HiNF-M, HiNF-P and histone gene transcription during the cell cycle. HiNF-D is observed only in S phase of normal cells (blue curve). Consistent with a role in gene repression, maximal levels of HiNF-D occur after the peak of histone gene transcription. CDP/Cux has minimal DNA binding activity outside of S phase. Phosphorylation and acetylation of CDP reduces its DNA binding activity. Thus CDP/Cux interaction with cdc25A, which is activated in early G_1, may lead to occupation of histone H4 Site II by CDP/Cux. Direct interaction with HDAC1 enhances binding of CDP/Cux to DNA, which may result in the displacement of activators HiNF-M and HiNF-P from Site II. Recruitment of pRB-cyclin A-CDK1 complexes, results in the phosphorylation of CDP/Cux which would lead to loss of Site II binding at the end of S phase. Acetylation by PCAF may further disrupt the HiNF-D complex.

CHAPTER 4:

GENETIC ABLATION OF THE CDP/CUX C-TERMINUS RESULTS IN DECREASED HISTONE H4.1 (FO108) EXPRESSION, HAIR FOLLICLE DEFORMITIES AND REDUCED MALE FERTILITY

LUONG ET AL. 2002, MOLECULAR AND CELLULAR BIOLOGY **22:**1424-1437

The *Cutl1* ΔC mutant mice were generated and genotyped by Southern blot analysis by Dr. DongXia Xing in the laboratory of Dr. Ellis Neufeld (Children's Hospital, Division of Hematology, Boston, MA). Electron micrographs were obtained from Dr. J. P. Sundberg (Millennium Pharmaceuticals, Inc, Cambridge, MA). S1 nuclease protection assays were performed by Caroline van der Meijden (University of Massachusetts Medical Center, Department of Cell Biology, Worcester, MA).

ABSTRACT

Murine CDP/Cux, a homologue of the Drosophila cut homeodomain protein, modulates the promoter activity of cell cycle-related and cell type-specific genes. CDP/Cux interacts with histone gene promoters as the DNA-binding subunit of a large nuclear complex (HiNF-D). CDP/Cux is a ubiquitous protein containing four conserved DNA binding domains: three cut repeats (CR) and a homeodomain. In this study, we analyzed genetically targeted mice (Cutl1^{tm2Ejn}, referred to as *Cutl1* ΔC) that express a mutant CDP/Cux protein with a deletion of the C-terminus including the homeodomain. In comparison to the wildtype protein, indirect immunofluorescence showed that the mutant protein exhibited significantly reduced nuclear localization. Consistent with these data, DNA binding activity of HiNF-D was lost in nuclear extracts derived from mouse embryonic fibroblasts (MEFs) or adult tissues of homozygous mutant (*Cutl1* ΔC $^{-/-}$) mice, indicating the functional loss of CDP/Cux in the nucleus. No significant difference in growth characteristics or total histone H4 mRNA levels was observed between wildtype and *Cutl1* ΔC $^{-/-}$ MEFs in culture. However, the histone H4.1 (murine homologue of human FO108) gene containing CDP/Cux binding sites have reduced expression levels in homozygous mutant MEFs. Stringent control of growth and differentiation appears to be compromised *in vivo*. Homozygous mutant mice exhibit stunted growth (20-50% weight reduction), a high postnatal death rate of 60-70%, sparse abnormal coat hair and severely reduced fertility. The deregulated hair cycle and severely diminished

fertility in *Cutl1* $\Delta C^{-/-}$ mice suggest that CDP/Cux is required for the developmental control of dermal and reproductive functions.

INTRODUCTION

CDP (CCAAT Displacement Protein)/Cux is a transcription factor involved in the regulation of cell growth and differentiation related genes (Table 1-4) [Nepveu, 2001]. Human CDP, its canine (Clox), murine (Cux-1) and rat (CDP2) homologs, and the Drosophila Cut protein have four DNA binding domains in common (Fig. 1-7) [Andres et al., 1992; Nepveu, 2001; Neufeld et al., 1992; Valarche et al., 1993; Yoon and Chikaraishi, 1994]: a unique homeodomain and three Cut repeats, which are similar regions of 70 amino acids (Fig. 1-7). The role of *Drosophila Cut* in cell-fate determination has been well defined by the phenotypes of various mutant flies. Mutations within the *cut* locus that disrupt the coding region cause embryonic lethality, whereas some mutations in the enhancer regions result in viable mutant flies with malformations in the leg and wing where *cut* fails to express [Jack et al., 1991; Jack, 1985; Johnson and Judd, 1979]. CDP/Cux is expressed in most adult and fetal tissues [Vanden Heuvel et al., 1996b] and its DNA binding activity is ubiquitous among various mammalian cell lines [Lievens et al., 1995]. Phosphorylation by protein kinase C and casein kinase II or PCAF-mediated acetylation of the CDP/Cux homeodomain inhibits DNA binding activity (Table 1-3) [Coqueret et al., 1996; Coqueret et al., 1998b; Li et al., 2000]. Stable CDP/Cux-DNA complexes are detected in proliferating cells but are undetectable upon cellular differentiation of HL60 promyelocytic leukemia cells and fetal rat calvarial cells [Lievens et al., 1995; Owen et al., 1990; Skalnik et al., 1991]. CDP/Cux has been shown to bind to a variety of promoters or enhancer sequences of genes involved in differentiation, including

myeloid cytochrome gp91-phox [Skalnik et al., 1991], dog heart myosin heavy chain [Andres et al., 1992], rat tyrosine hydroxylase [Yoon and Chikaraishi, 1994], human γ-globin [Superti-Furga et al., 1988], *Xenopus* β-globin, and mouse N-CAM [Valarche et al., 1993]. Transfection experiments suggest that CDP/Cux functions as a repressor of these target genes in proliferating precursor cells. Upon terminal differentiation, these target genes are induced when CDP/Cux DNA binding activity is downregulated. Two mechanisms of repression by CDP/Cux have been proposed: "passive repression" by competition with activators for occupancy of binding sites [Barberis et al., 1987; Neufeld et al., 1992; Skalnik et al., 1991], and "active repression" possibly through the interaction of CDP/Cux with HDAC1 [Mailly et al., 1996].

The role of CDP/Cux in mammalian cell growth control is reflected by its functional interaction with the promoters of the five major classes of histone genes (H1, H2A, H2B, H3 and H4), as well as genes encoding modulators of proliferation including c-myc and p21 [Coqueret et al., 1998a; Dufort and Nepveu, 1994; van Wijnen et al., 1991c; van Wijnen et al., 1996]. CDP/Cux DNA binding activity is highest during S-phase [Holthuis et al., 1990; Last et al., 1998; van Wijnen et al., 1997; Wright et al., 1992], when p21 expression is downregulated [Wright et al., 1992] and histone H4 expression is maximal [Coqueret et al., 1998a; van Wijnen et al., 1996]. The interaction of CDP/Cux with the promoters of all major histone classes during *Xenopus* development implicates CDP/Cux in the coordinate control of histone gene expression during the cell cycle and early development [El-Hodiri and Perry, 1995]. Moreover, eliminating CDP/Cux binding to the human histone H4

promoter alters the timing of maximal transcription during S phase [Aziz et al., 1998a]. Although CDP/Cux may have a bifunctional role, it is generally considered as a repressor, possibly exerting its regulatory activity in conjunction with other nuclear proteins [Coqueret et al., 1998a; Li et al., 2000; Mailly et al., 1996; van Wijnen et al., 1994]. For example, transcriptional regulation of human histone H4 is mediated in part by the promoter complex HiNF-D, which is composed of CDP/Cux, pRb, cyclin A, and CDK1 [van Wijnen et al., 1994].

The developmental expression pattern of CDP/Cux has been monitored in mice that have a *lacZ* gene expressed from the *Cutl1* gene locus [Ellis et al., 2001]. *Cutl1* expression is detected in most tissues at embryonic day 8.5 (E8.5) and, starting with E12.5, becomes gradually limited to a subset of organs. *Cutl1* is expressed in the epithelial compartment of the developing whisker, tooth, choroids plexus, pituitary, thyroid, salivary gland, pancreas, kidney and lung. In addition, expression of *Cutl1* is also observed in striated and smooth muscles of various organs and in hypertrophic chondrocytes of developing bones [Ellis et al., 2001]. *Cutl1* is most highly expressed in the testis [Vanden Heuvel et al., 1996b], specifically in postmeiotic round and elongating spermatids, but not in mature sperm [Ellis et al., 2001].

There is limited insight into the functions of mammalian *Cutl1 in vivo*. The human *Cutl1* gene located at 7q22 has been implicated as a tumor suppressor gene in several studies [Ishwad et al., 1997; Zeng et al., 1997; Zeng et al., 1999]. Loss of heterozygosity (LOH) was detected in a subset of uterine leiomyomas and breast tumors. In all instances, the smallest deleted region included polymorphic markers that are located within or close to the *Cutl1* gene. *Cutl1* mRNA levels were reduced in

eight out of 13 mammary tumors [Zeng et al., 1999]. Furthermore, CDP/Cux has been shown to repress transcription from the promoters of the mouse mammary tumor virus and the human papillomavirus [Ai et al., 1999; O'Connor et al., 2000; Zhu et al., 2000]. Interestingly, immunocomplexes of CDP/Cux and Polyomavirus Large T (PyV LT) antigen were detected in leiomyoma and breast tumors induced in transgenic mice expressing the PyV LT antigen [Webster et al., 1998]. The existence of such complexes suggests that induction of these tumors may involve the sequestration of CDP/Cux by PyV LT antigen. However, it is also possible that CDP/Cux may be associated with the process of tumorigenesis since CDP/Cux levels were elevated during tumor progression in the PyV LT mice as well as in several breast tumor cell lines [Zhu et al., 2000].

Studies involving *Cutl1* mutant mice have provided further insight into the *in vivo* function of CDP/Cux. Forced expression of CDP/Cux in mice resulted in multiorgan hyperplasia and organomegaly [Ledford et al., 2002]. Cutl1^{tm1Ejn} mice with a deletion of Cut Repeat 1 exhibit a mild phenotype including curly vibrissae, wavy hair and high postnatal lethality in litters born to homozygous mutant mothers due to the mothers' impaired lactation [Tufarelli et al., 1998]. In this study, insertional mutagenesis to inactivate CDP/Cux was used by targeting its homeodomain DNA binding region. The results presented in this chapter show that this mutation (Cutl1^{tm2Ejn}) prevents CDP/Cux from accumulating in the nucleus as a functional DNA binding complex (i.e., HiNF-D). Homozygous mutant mice (Cutl1$^{tm2Ejn/tm2Ejn}$), hereafter referred to as *Cutl1* $\Delta C^{-/-}$ mice, display high postnatal lethality, growth retardation, hair loss and severely reduced male fertility. Despite the

ubiquitous expression of CDP/Cux, these data indicate that its absence from the nucleus results in limited disturbance of normal tissue development. In addition, overall expression of histone H4 genes was unaltered in homozygous mutant embryonic fibroblasts.

RESULTS

Loss of CDP/Cux C-terminus results in high neonatal lethality and severe growth retardation

A targeting construct was generated to functionally inactivate CDP/Cux by introducing a premature translational termination codon in exon 20 that encodes the beginning of the homeodomain, a DNA binding region of CDP/Cux (ΔC; Fig. 4-1A). The genotypes of heterozygous mice generated from ES clones with the correctly targeted allele were determined by Southern blot analysis (Fig. 4-1B). Heterozygous mice were interbred and PCR analysis
(Fig. 4-1C) demonstrates that the mutation is transmitted to heterozygous and homozygous offspring at the expected frequency (Fig. 4-2A).

Mice heterozygous for the *Cutl1* ΔC mutation ($\Delta C^{+/-}$) are normal in appearance and are fertile. Homozygous mutant ($\Delta C^{-/-}$) pups are indistinguishable from their littermates at birth. The *Cutl1* $\Delta C^{-/-}$ pups failed to thrive but they nursed normally evident by the milk evident present in their stomachs during the first 2 to 3 days after birth. By the end of the first week after birth, many *Cutl1* $\Delta C^{-/-}$ pups appear considerably smaller than their littermates and most die at this stage (Fig. 4-2B). More than 70% of *Cutl1* $\Delta C^{-/-}$ pups failed to survive to weaning age (Fig. 4-2C), which may be related to the fact that these pups suffer from purulent rhinitis characterized by mucosal and submucosal purulent infiltrates within the nasal turbinates (Dr. E.J. Neufeld, unpublished data). The homozygous mutant mice that do survive to adulthood have a normal life span but are severely growth retarded and

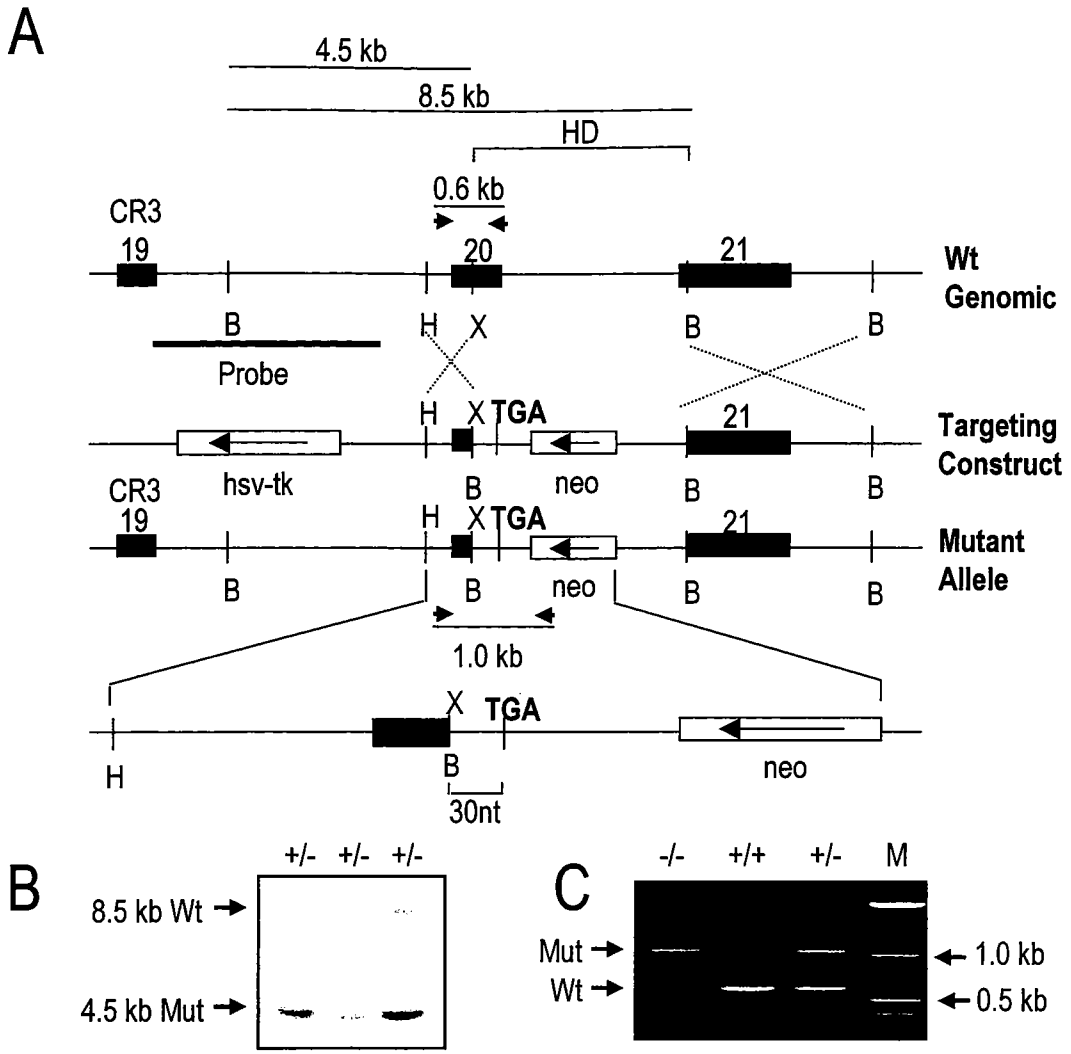
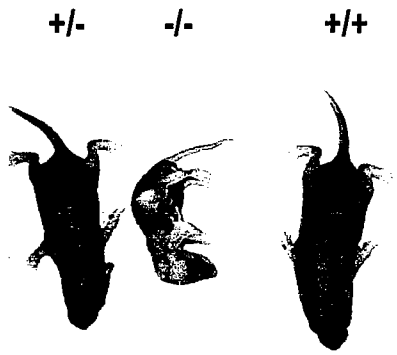


FIG 4-1. Targeted mutation of the mouse *Cutl1* gene by homologous recombination. **(A)** Top: A schematic diagram of the wildtype *Cutl1* allele with the positions of exons 19-21 indicated. Arrows denote the positions of the primers used in PCR-based genotyping. Middle: Targeting construct with the neo gene in an antisense orientation. Bottom: Predicted mutant allele resulting from homologous recombination between the wildtype allele and the targeting construct. Ten amino acids encoded by 30 nucleotides of the poly-linker sequence in the targeting construct are added to the open reading frame of the mutant Cux transcript before reaching the stop codon. A *Bam*HI site introduced in the construct gives a diagnostic fragment of 4.5 kb. **(B)** Southern blot analysis of 3 heterozygous embryonic stem cell clones displaying both the 8.5- and the 4.5 kb *Bam*HI fragments corresponding to the wildtype and mutant allele, respectively. **(C)** Genotyping of pups from F2 generation with primers in intron 19 (HD-F), exon 20 (HD-R1), and the neomycin cassette of the targeting vector (HD-R2). HD-R1 sequence is in the region of exon 20 that is deleted from the mutant allele.

A

Stage	Total	Genotype +/+	+/-	-/-
Prenatal (E 12.5-14.5)	82	20 25%	43 52%	19 23%
Postnatal (day 1)	125	22 18%	73 58%	30 24%
Postnatal (day 14)	181	56 31%	114 63%	11 6%

B



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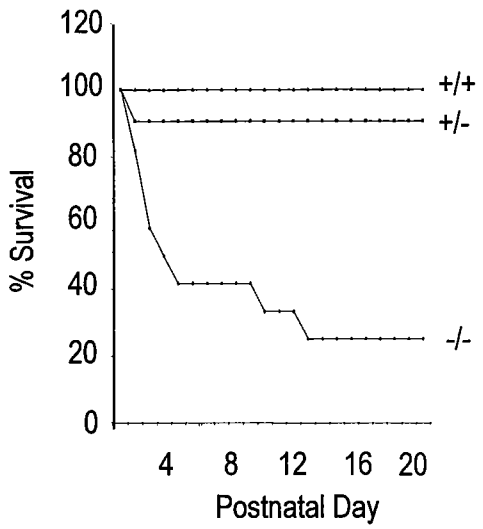

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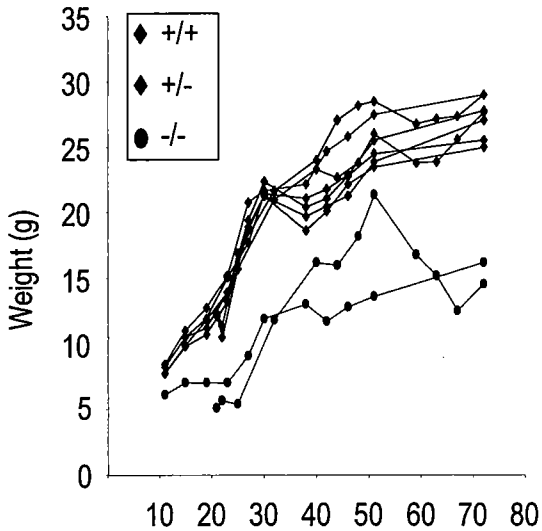

FIG 4-2. High post-natal lethality and stunted growth in *Cutl1* ΔC homozygous mutant mice. (A) Genotype distribution of progeny from heterozygous matings. Data for postnatal day 1 litters are based on those litters in which the genotype of all born pups were determined. **(B)** A litter with a moribund *Cutl1* $\Delta C^{-/-}$ pup was photographed within the first week after birth. **(C)** Of 60 mice screened, more than 70% of homozygous mutant mice die by postnatal day 10. Wildtype and nullizygous mice (n=13), heterozygous mice (n=34). **(D)** *Cutl1* $\Delta C^{-/-}$ mice exhibit stunted growth and weigh significantly less than their littermates. Graph represents the growth curve of 7 males from two litters.

weigh 30-50% less than their normal littermates (Fig. 4-2D). Thus the *Cutl1* ΔC mutation has an effect on viability, general growth, and susceptibility to bacterial infections.

Expression of the mutant *Cutl1* allele

To assess the effect of the mutation on *Cutl1* expression, *Cutl1* transcripts were analyzed by using RT-PCR with primers spanning exon 19-21 (Fig. 4-3A). Total RNA was isolated from proliferating mouse embryonic fibroblasts (MEFs) derived from *Cutl1* homozygous ($\Delta C^{-/-}$), heterozygous ($\Delta C^{+/-}$) and wildtype ($\Delta C^{+/+}$) embryos. The expected RT-PCR product (258 bp) with primers 19F and 20R in exons 19 and 20 was detected in all RNA samples, indicating that *Cutl1* transcripts were made in all three types of MEFs. A 441-bp product was detected using primer pair 19F and 21R spanning exons 19 to 21 in *Cutl1* $\Delta C^{+/+}$ and $\Delta C^{+/-}$ samples. However, this product was absent in *Cutl1* $\Delta C^{-/-}$ samples, establishing that insertional mutagenesis created the designed truncation in the 3' end of the mutant *Cutl1* transcripts (Fig. 4-3B, top panel). Southern blot analysis showed that a probe spanning exons 19 to 21 hybridized with the RT-PCR products from *Cutl1* primers but not with products from GAPDH primers (Fig. 4-3B, bottom panel). Furthermore, two chimeric RT-PCR products (307 bp with primers 19F and NeoR1, and 377 bp with primers 20F and NeoR2) were detected with primers spanning exon 19 and the neomycin cassette in heterozygous and homozygous mutant but not in wildtype embryos as expected (Fig. 4-3C). Taken together the RT-PCR data indicate that the

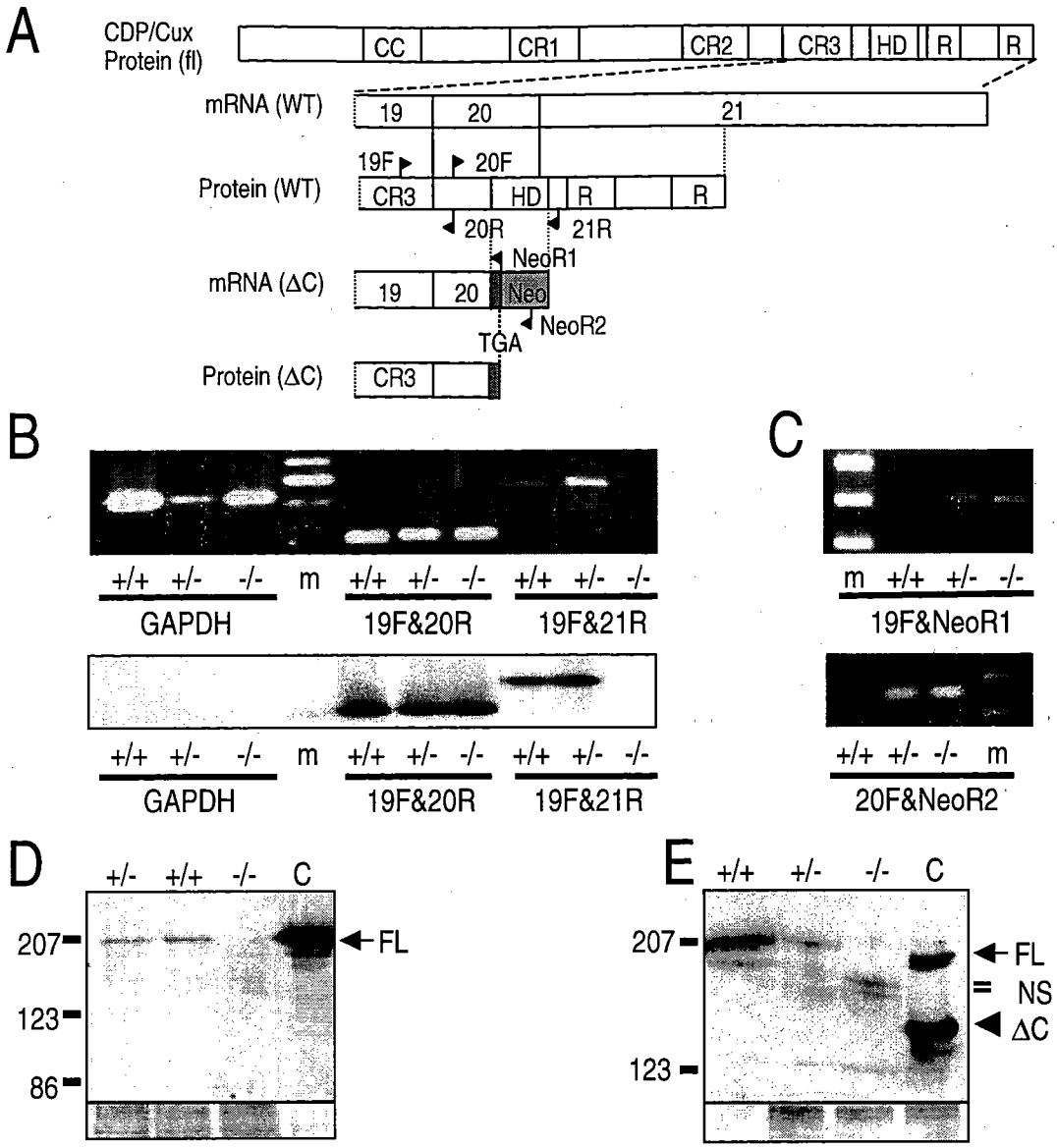

FIG 4-3. Full-length *Cutl1* mRNA and protein are not expressed in *Cutl1* ΔC$^{-/-}$ mice. (A) A schematic diagram of wildtype and mutant *Cutl1* mRNA and protein. Arrows indicate the positions of the primers used in RT-PCR analysis. Dark gray rectangle represents 30 nucleotides that are added to the mutant transcript from the poly-linker sequence of the targeting construct. **(B,C)** Total lung RNA (2 μg) was used in RT-PCR assays with various primers spanning exons 19 to 21 of the *Cutl1* gene. **(B)** Bottom: Southern blot of RT-PCR products using a radiolabeled probe spanning exons 20 to 21. **(D)** Top: Western blot analysis of lung extracts (30 μg) with a polyclonal antibody against CDP/Cux C-terminus. Bottom: Coomassie stain of the SDS-PAGE gel after transfer to the PVDF membrane used in the above Western blot. Control lane (C): whole cell extract of HeLa cells transfected with pcDNA-CDP/Cux (fl). **(E)** Top: Western blot analysis of lung extracts with a polyclonal antibody against full-length CDP/Cux. Bottom: Coomassie stain of the SDS-PAGE gel. Control lane (C): whole cell extract of HeLa cells transfected with pcDNA-CDP/Cux(fl) and pcDNA-CDP/Cux(ΔC). Full-length CDP/Cux (Fl), nonspecific bands (NS), C-terminal truncated CDP/Cux (ΔC).

targeted allele expresses a chimeric truncated transcript in which the homeodomain encoding sequences were replaced with sequences from the neo cassette.

To characterize the expression from the mutant *Cutl1* transcript, whole cell lysates derived from lungs of wildtype, heterozygous and homozygous mice were analyzed using antibodies against the C-terminus of CDP/Cux (Fig. 4-3D) or the full-length protein (Fig. 4-3E). A major band at ~200 kDa, which co-migrates with the overexpressed full-length CDP/Cux protein in the control lanes, is detected with both antibodies in extracts from wildtype and heterozygous but not homozygous mutant mice. Coomassie-staining of the SDS-PAGE gels shows that commparable amounts of protein were blotted (bottom panels of Fig. 4-3D and 4-3E). These data indicate that *Cutl1* $\Delta C^{-/-}$ mice express a truncated CDP/Cux protein that lacks the C-terminus. The CDP/Cux ΔC protein that is expected to be produced in homozygous mutant mice is approximately 150 kDa. However in lung extracts derived from *Cutl1* $\Delta C^{-/-}$ mice, we note that the antibody against full-length CDP/Cux detects diffuse and faint bands at ~170 kDa (Fig. 4-3E). These bands do not co-migrate with the overexpressed CDP/Cux protein carrying the identical ΔC mutation. Similar bands are observed when using an antibody against a C-terminal epitope that is not present in the CDP/Cux ΔC protein (Fig. 4-3D), indicating that these faint bands are not specific for the CDP/Cux protein. Although the truncated protein is produced at a level below detection by our Western blot analysis in lung extracts, immunofluorescence microscopy data indicate that it is expressed at low levels in mouse embryonic fibroblasts (MEFs) and in other cell types [Sinclair et al., 2001].

The CDP/Cux-containing HiNF-D complex is absent in *Cutl1* ΔC $^{-/-}$ mice

To investigate whether the CDP/CuxΔC protein retains the ability to form protein-DNA complexes, electrophoretic mobility shift assays (EMSAs) were performed with nuclear extracts from MEFs, as well as from lung and brain of wildtype, heterozygous and homozygous mutant mice (Fig. 4-4). CDP/Cux is highly expressed in lung and brain tissues. Wildtype CDP/Cux protein has been shown previously to interact with Site II of the cell-cycle regulated histone H4 gene as a multi-component protein-DNA complex (HiNF-D) containing cyclin A, CDK1 and pRB [van Wijnen et al., 1994; van Wijnen et al., 1996]. EMSAs show a low mobility complex (HiNF-D) in wildtype and heterozygous extracts but not in homozygous mutant extracts (Fig. 4-4A), suggesting that the CDP/CuxΔC protein has lost the ability to form protein-DNA complexes. The identity of the HiNF-D complex was established by competition with wildtype and mutant oligonucleotides (Fig. 4-4A) and by immuno-reactivity in EMSAs with a CDP/Cux antibody (Fig. 4-4B). Cyclin E antibody was used as a negative control (Fig. 4-4B).

Since CDP/Cux has previously been shown to bind the myeloid-specific gp91-phox promoter in complex with the pRb-related p107, the binding of the CDP/Cux ΔC protein to this promoter was also tested. The data show that the probe forms a CDP/Cux-containing complex with nuclear proteins from *Cutl1* $\Delta C^{+/+}$ and $\Delta C^{+/-}$, but not $\Delta C^{-/-}$ mice (Fig. 4-5, panels A and B). EMSAs were also performed with an Sp1 probe using nuclear extract from MEFs, as well as lung and brain tissues from mice of all genotypes. The results show a similar Sp1 binding activity in all protein preparations (Fig. 4-5C), suggesting that the quality of the nuclear extract is

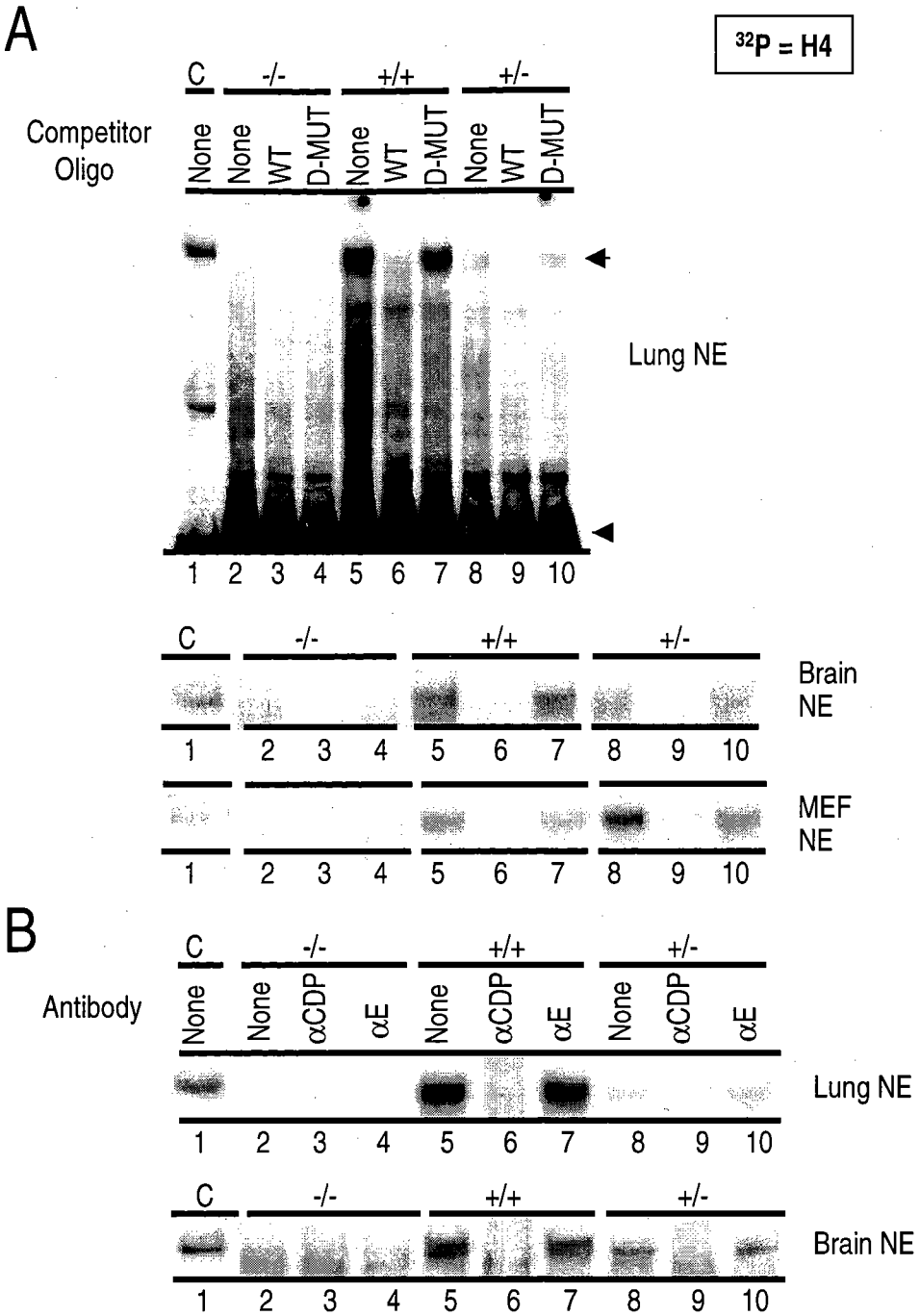

FIG 4-4. Absence of HiNF-D complex in homozygous mutant mice. Electro Mobility Shift Assay (EMSA) was performed with ^{32}P-labeled ds-oligos spanning Site II of histone H4.1 promoter, and nuclear extract (3 μg) prepared from adult lung, adult brain and proliferating embryonic fibroblasts (as indicated). **(A)** Competition with unlabeled wildtype oligonucleotides and mutant oligonucleotides (SUB 11) which has no significant HiNF-D binding activity. **(B)** Immuno mobility shift assay with antibodies against full-length CDP/Cux and cyclin E (αE) as a control. Arrows indicate the HiNF-D complex. Arrowhead indicate unbound labeled probe. Control lane (C): HeLa nuclear extract was used as a positive control for the presence of HiNF-D complex.

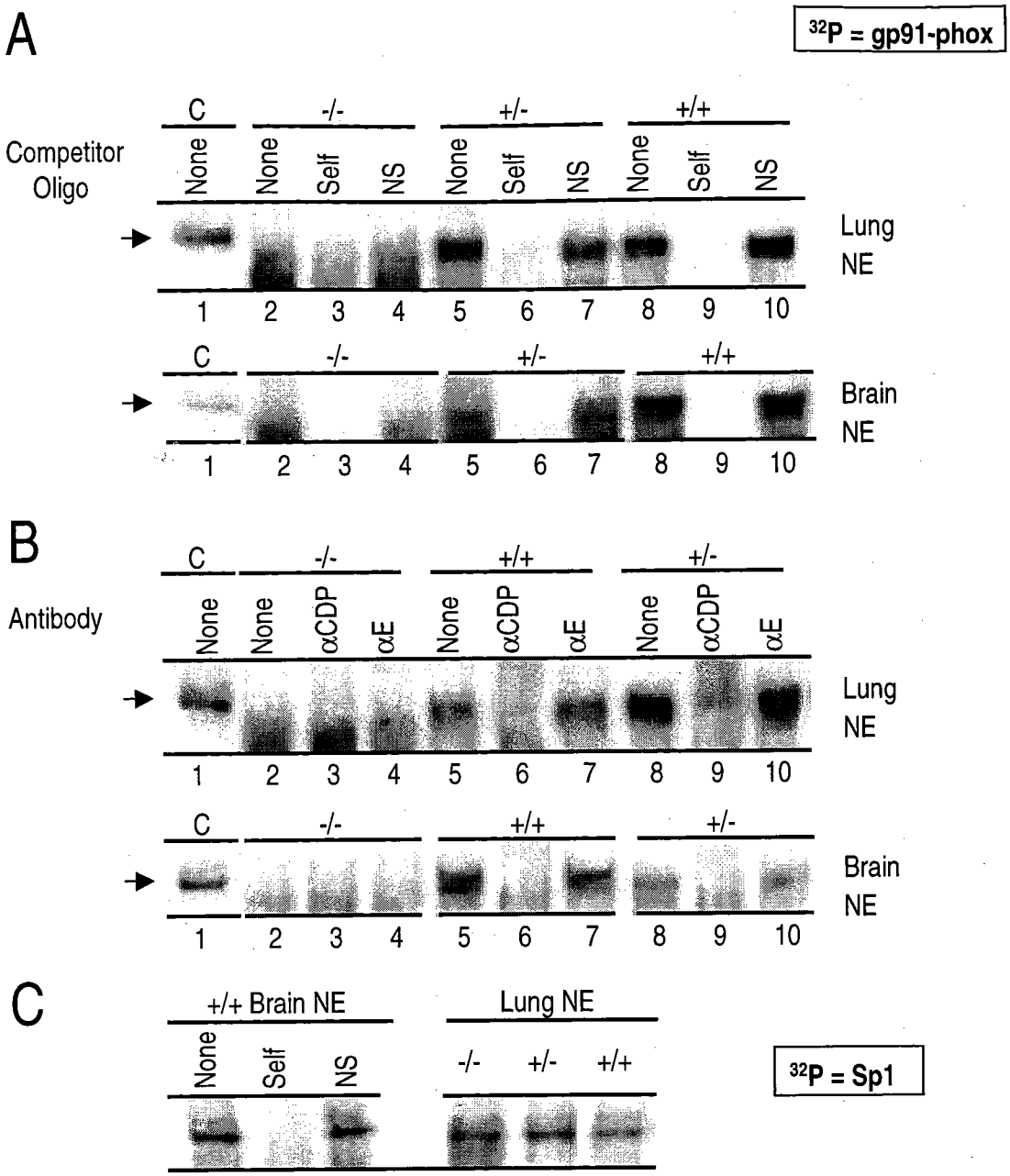

FIG 4-5. CDP/Cux ΔC mutant protein does not retain DNA-binding activity. EMSA with lung and brain nuclear extracts (3 μg) was performed with ^{32}P-labeled double-stranded oligonucleotides containing gp91-phox promoter sequences (A, B) or Sp1 consensus binding site (C). **(A)** Competition with unlabeled wildtype and nonspecific (SUB 11) oligonucleotides. **(B)** Immuno mobility shift assay with antibody against full-length CDP/Cux and cyclin E (αE, 2 μg) as control. Arrows indicate CDP/Cux-containing complexes. **(C)** Left panel: Competition with unlabeled wildtype Sp1 and nonspecific competitor (SUB 11) oligonucleotides. Right panel: EMSA with lung extracts (3 μg). Lane (C): HeLa nuclear extract was used as a positive control for the presence of HiNF-D complex (38). Arrows indicate protein/DNA complexes containing CDP.

comparable for all genotypes. Deletion of the CDP/Cux C-terminus results in the absence of CDP/Cux-containing protein/DNA complexes, indicating that the C-terminus is required for complex formation.

Levels of CDP/Cux ΔC protein in the nucleus are significantly reduced in *Cutl1* $\Delta C^{-/-}$ cells

To address the possibility that the absence of CDP/Cux-containing protein/DNA complexes is due to its exclusion from the nucleus, immunofluorescence microscopy was performed with a polyclonal antibody against full-length CDP/Cux. Strong nuclear immunofluorescence was observed for CDP/Cux in wildtype and heterozygous MEFs but the nuclear signal is significantly reduced in homozygous mutant MEFs (Fig. 4-6). To ensure that overall protein antigenicity is preserved during cellular extraction, MEFs were also incubated with antibody against Sp1. Strong Sp1 nuclear staining is observed in *Cutl1* $\Delta C^{-/-}$ MEFs, as well as $\Delta C^{+/+}$ and $\Delta C^{+/-}$ MEFs (Fig. 4-6). To verify that the CDP/Cux ΔC protein is localized inside the nucleus, rather than on its surface, confocal microscopy was performed with antibody against full-length CDP/Cux. A nuclear signal is detected in *Cutl1* $\Delta C^{-/-}$ MEFs, albeit at a greatly reduced intensity as compared with the wildtype signal, indicating that the CDP/Cux ΔC protein produced in mutant mice retains the ability to localize to the nucleus (Fig. 4-7A). A faint and diffuse cytoplasmic signal is present in *Cutl1* $\Delta C^{+/+}$ MEFs, but a pronounced reticular pattern is observed in *Cutl1* $\Delta C^{-/-}$ MEFs with antibody against full length CDP/Cux (Fig. 4-6 and 4-7). Consistent with the reticular staining pattern, the N-terminus coding sequences remaining in the

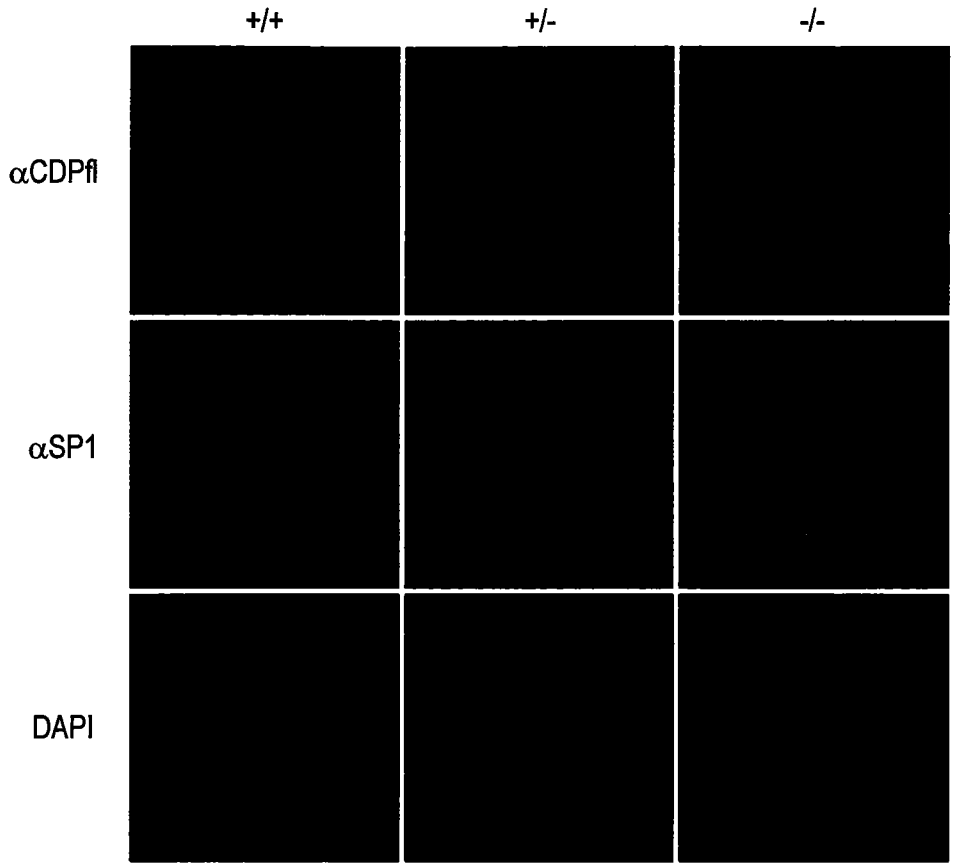

FIG 4-6. Reduced levels of CDP/Cux ΔC mutant protein is detected in the nucleus. Embryonic fibroblasts grown on gelatin-coated coverslips were fixed as whole cells, permeabilized and were incubated with an antibody against full-length CDP/Cux (αCDPfl) or an antibody against Sp1 (αSp1) at a 1:100 dilution. Cell nuclei were visualized chromatin staining with DAPI.

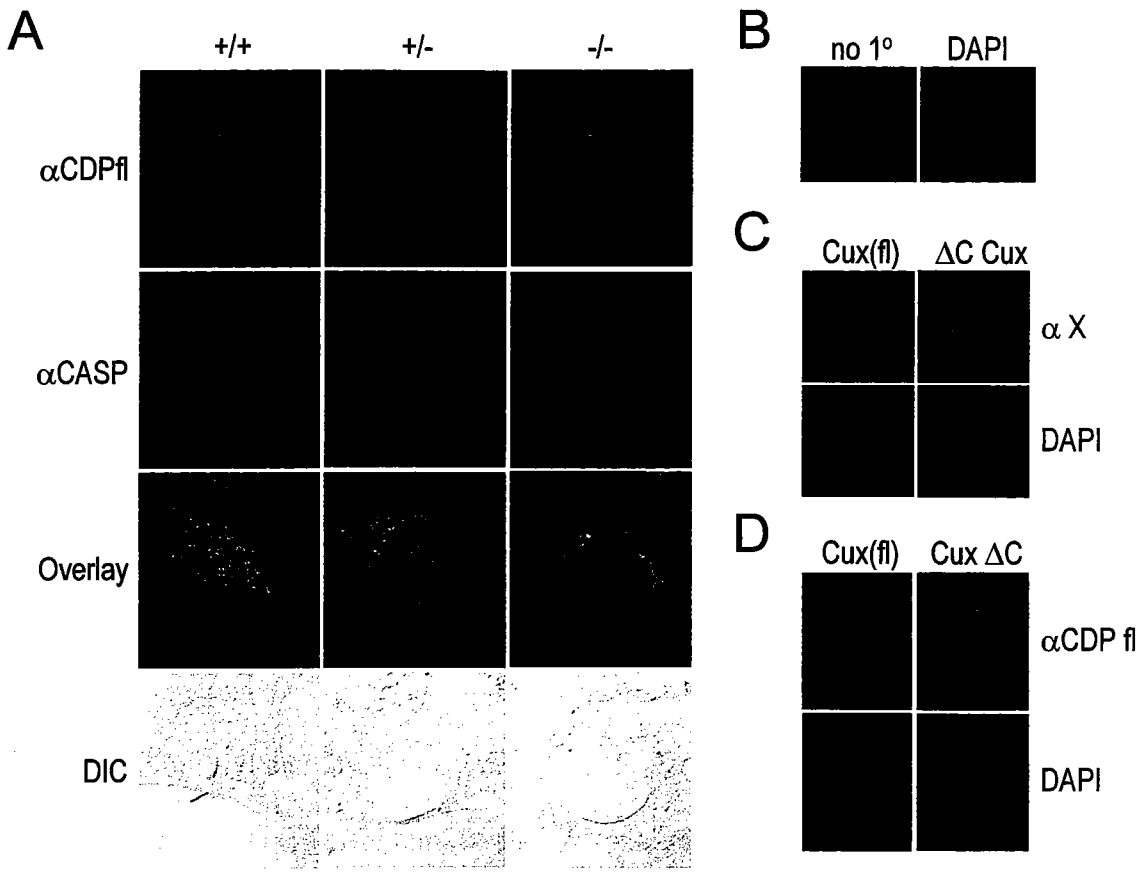

FIG 4-7. Significantly reduced levels of CDP/Cux ΔC protein is observed in the nucleus of homozygous mutant MEFs. (A) Cultured embryonic fibroblasts grown on gelatin-coated coverslips were fixed, permeabilized, incubated with guinea pig antibody against full-length CDP/Cux (αCDP fl) or rabbit anti-CASP (αCASP) antibody, and analyzed by confocal microscopy. (DIC) differential interference contrast **(B)** MEFs (+/-) were subjected to digital microscopy in the absence of primary antibody. Cells were incubated with fluorescein-tagged anti-guinea pig antibody. **(C and D)** HeLa cells were transfected with vectors expressing Xpress-tagged CDP/Cux full-length (Cux fl) and C-terminally truncated (ΔC Cux) proteins. CDP/Cux proteins were analyzed by immunofluore microscopy using an antibody against full-length CDP/Cux (α CDP) and an anti-Xpress antibody (αX).

mutant allele encode an extensive coiled-coil domain that has the potential to form filamentous structures. This coiled-coil domain is also present in an alternatively spliced CDP/Cux variant called CASP. It is likely that the polyclonal antibody against full-length CDP/Cux recognizes CASP as well. To determine which protein is responsible for the perinuclear signal, we performed confocal microscopy with a CASP-specific antibody. A perinuclear signal is detected by the CASP-specific antibody and colocalizes with the cytoplasmic staining observed with the antibody against full-length CDP/Cux (Fig. 4-7A). No specific signal is detected in MEFs when the primary antibody against CDP/Cux was omitted (Fig. 4-7B). In addition, HeLa cells were transfected with vectors expressing an epitope (Xpress)-tagged full-length or CDP/Cux ΔC protein. The expressed full-length and truncated proteins are localized to the nucleus, as determined by immunofluorescence microscopy using antibodies against the Xpress epitope (Fig. 4-7C) and CDP/Cux (Fig. 4-7D). The difference in localization of endogenous and exogenously expressed CDP/Cux ΔC proteins may be due to differential transcriptional regulation. Taken together, these findings establish that deletion of the 3' end of the *Cutl1* gene results in a significant loss of nuclear functions of the gene product.

Embryonic fibroblasts homozygous for the *Cutl1*ΔC mutation exhibit normal cell growth

CDP/Cux has been shown to regulate the promoters of genes involved in cell growth regulation including histone H4, c-myc and p21 [Coqueret et al., 1998a; Dufort and Nepveu, 1994; van Wijnen et al., 1991c; van Wijnen et al., 1996]. We

examined whether the functional loss of CDP/Cux in the nucleus affects cell growth control using flow cytometric analysis to assess the DNA content of the cells and the relative number of cells present in specific cell-cycle stages. The results did not reveal a significant difference in growth characteristics between non-synchronized proliferating homozygous and wildtype MEFs ($p>0.05$) (Fig. 4-8A). Furthermore, the growth rates of *Cutl1* $\Delta C^{-/-}$, $\Delta C^{+/-}$ and $\Delta C^{+/+}$ MEF were determined and found to be comparable (Fig. 4-8B). Expression of histone H4 genes, which is tightly coupled to DNA replication and is a specific marker for cells in S-phase, was assessed by northern blot analysis. As a probe, a DNA segment was used that encompasses the entire coding sequence, which cross-hybridizes with mRNA transcribed from the 12 known histone H4 gene copies in mice. Consistent with the flow cytometry results, Northern blot analysis reveals overall histone H4 mRNA levels from wildtype, heterozygous and homozygous mutant MEFs to be comparable (Fig. 4-8C), indicating that a similar proportion of the cells in each population is in S-phase.

Previous studies from our group have focused on one specific histone H4 gene in human and mouse (referred to as FO108 and H4.1, respectively) to understand the regulatory role of CDP/Cux in cell cycle-controlled transcription [van der Meijden et al., 1998; van Wijnen et al., 1996]. To selectively detect expression of the mouse H4.1 gene, S1 nuclease protection assays were performed. Similar to its human counterpart, the murine H4.1 gene is cell cycle regulated and interacts with CDP/Cux [van der Meijden et al., 1998]. S1 nuclease protection analysis was performed with the RNA described above using a 50-bp probe encompassing the divergent 5' flanking region of H4.1. The results suggest that expression of the H4.1 gene is

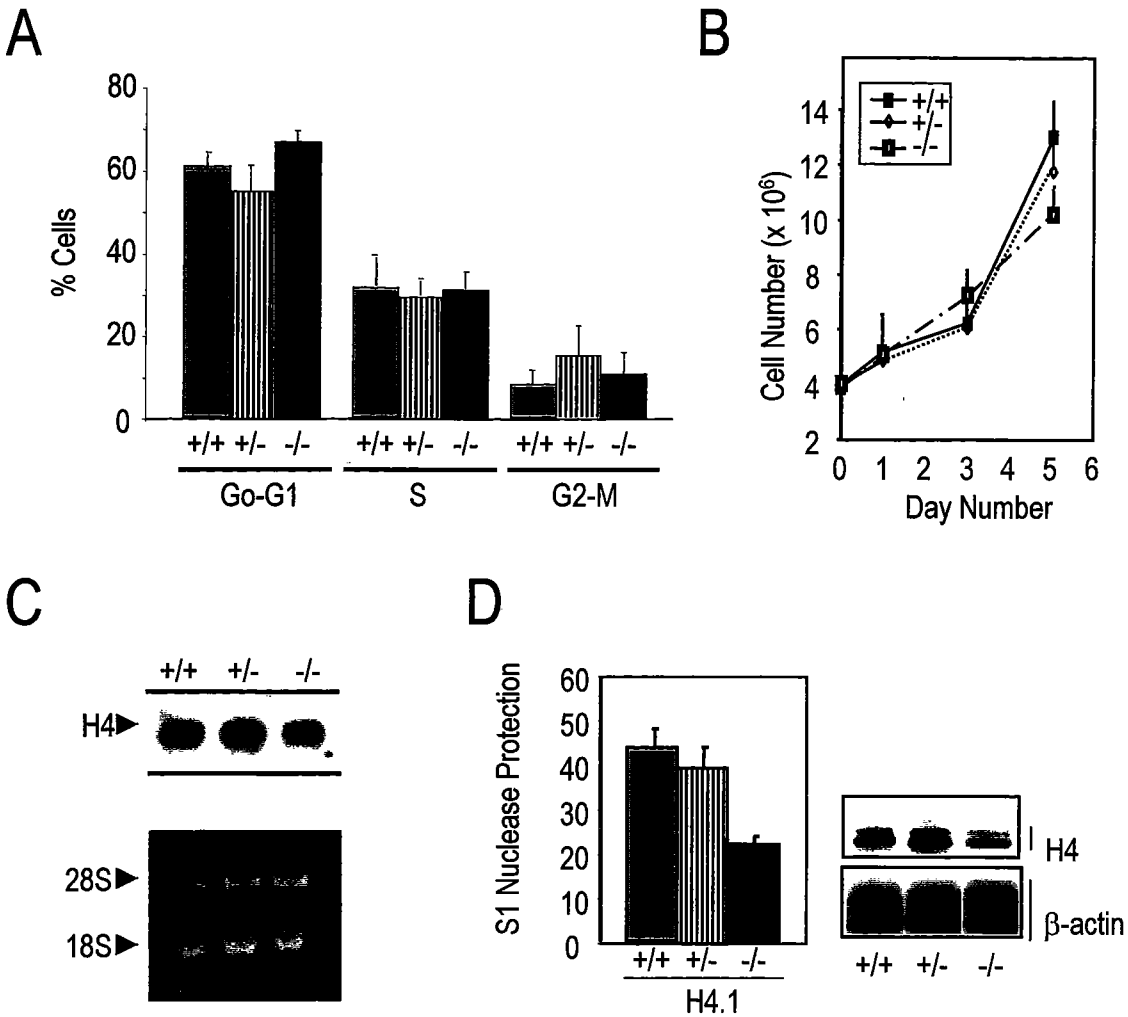

FIG 4-8. Reduced histone H4.1 mRNA levels in *Cutl1* ΔC mouse embryonic fibroblasts (MEFs) that exhibit normal growth characteristics. (A) Cell-cycle distribution of proliferating homozygous mutant MEFs and wild-type MEFs was comparable. Cell-cycle estimates were obtained by FACS analysis for proliferating MEFs stained with a propidium iodide solution. **(B)** *Cutl1* $\Delta C^{-/-}$ MEFs exhibit normal growth rates. MEFs were plated in triplicate at $4x10^6$ cells per plate on day zero and the number of cells per plate was determined on days 1, 3 and 5. **(C)** Total histone H4 mRNA is expressed at comparable levels in proliferating wild-type and *Cutl1* $\Delta C^{-/-}$ MEFs. Top: Northern blot of total RNA (20 μg) was performed using a probe spanning the entire coding region of the human histone H4 gene. Bottom: ribosomal RNA (28S and 18S indicated) visualized by ethidium bromide. **(D)** S1 nuclease protection assays show a two-fold decrease in histone H4.1 mRNA expression in homozygous mutant MEFs. Labeled oligonucleotides complementary to the cap site of H4.1 were hybridized to total RNA (10 μg) from proliferating MEFs, and digested with the S1 nuclease. A β-actin probe served as an internal control. **(Left)** The histogram represents a composite of the ratios of the histone levels in 5 experiments. **(Right)** Autoradiograph of a representative S1 nuclease protection experiment.

moderately reduced in $\Delta C^{-/-}$ MEFs as compared to wildtype MEFs (Fig. 4-8D). The reduction in the expression of H4.1 gene is consistent with the role of CDP/Cux in regulating histone gene transcription, but the moderate nature of the decrease suggests that there are compensatory mechanisms. It has been well established that histone gene expression is regulated by both transcriptional and post-transcriptional mechanisms, and that mRNA stabilization may offset transcriptional changes [Stein et al., 1996].

The *Cutl1* $\Delta C^{-/-}$ mice have reduced fertility

One of the most striking features of the *Cutl1* ΔC mouse is that the reproductive fitness is greatly compromised in the homozygous mutant males. In general, *Cutl1* $\Delta C^{-/-}$ males rarely produce offspring, even when mated with wildtype females. For example, only one of 25 *Cutl1* $\Delta C^{-/-}$ males sired two litters of pups. To assess whether *Cutl1* $\Delta C^{-/-}$ mice have defects in testicular development or function, gross anatomical and histological studies were performed. The weights of the testes dissected from *Cutl1* $\Delta C^{+/+}$, $\Delta C^{+/-}$ and $\Delta C^{-/-}$ adult mice were comparable (Fig. 4-9A). Serum testosterone levels in *Cutl1* $\Delta C^{+/-}$ and $\Delta C^{-/-}$ males were significantly lower than those in *Cutl1* $\Delta C^{+/+}$ male littermates (Fig. 4-9B). Heterozygous males are fertile and yet display the same reduced levels of testosterone as the homozygous mutant males. Abundant maturing germ cells were observed in the seminiferous tubules and epidydimis of wildtype, heterozygous and homozygous mutant male littermates at seven months (Fig. 4-9, panels C and D). Although germ cell numbers were unaffected by the *Cutl1* ΔC mutation, it is possible that reduced fertility in *Cutl1* $\Delta C^{-/-}$

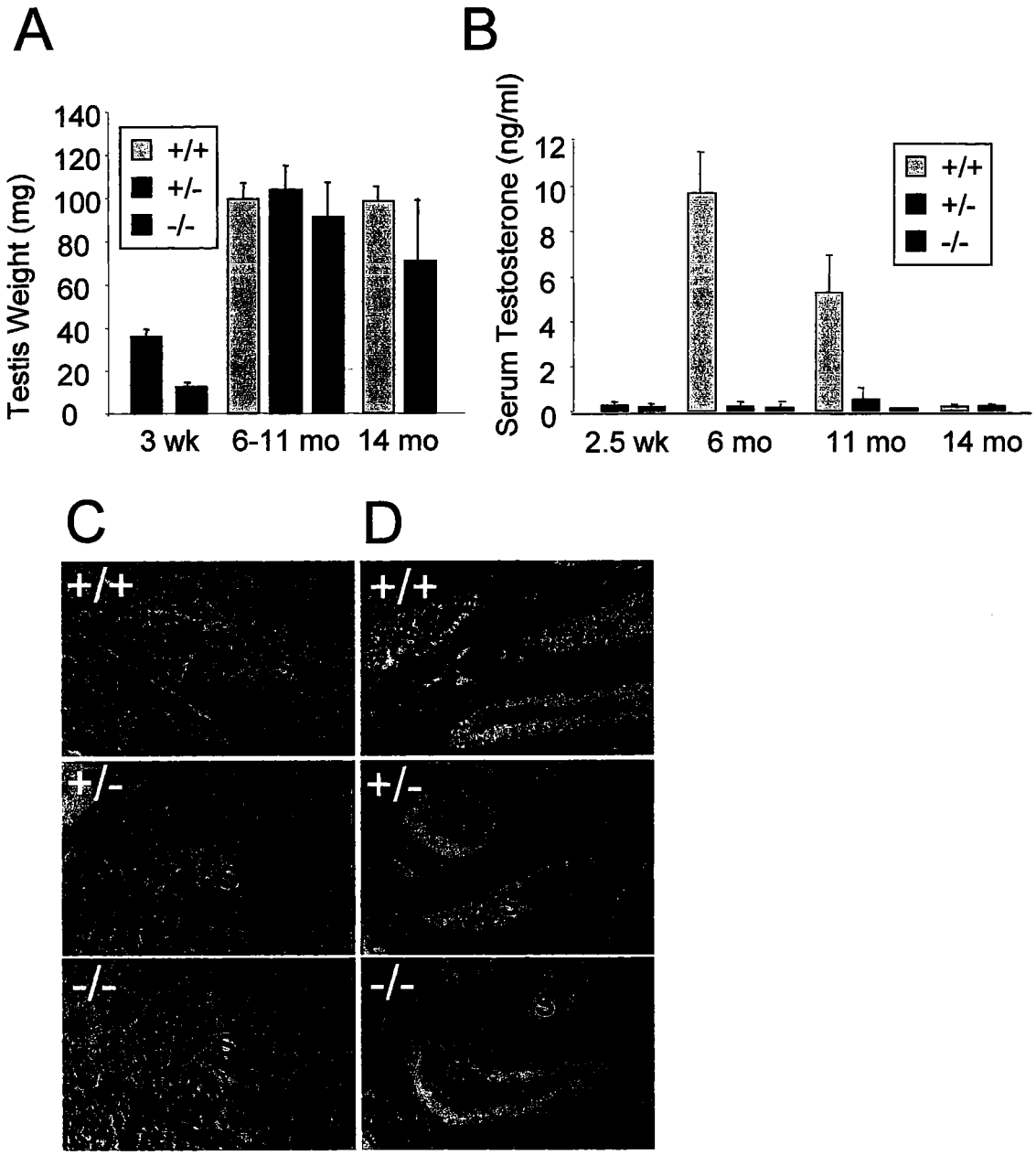

FIG 4-9. *Cutl1* ΔC mutant males with normal testicular morphology have reduced serum testosterone levels. (A) Testes were weighed from mice at age 3 week (4 mice), 10-11 months (9 mice) and 14 months (4 mice). **(B)** Total serum testosterone levels from mice at age 2-3 weeks (6 mice), 6 months (3 mice), 10-11 months (11 mice) and 14 months (4 mice) were measured by radioimmunoassay. **(C)** Micrograph (25x) of hematoxylin and eosin stained sections of testis and **(D)** epididymis from male littermates at 7 months. (S) Sperm, (I) Interstitial Cells.

males is due to defects in sperm motility or other functions that remain to be investigated.

The development of the gastrointestinal tract is unperturbed in the *Cutl1* ΔC $^{-/-}$ mice

Although observed to feed normally, *Cutl1* ΔC$^{-/-}$ mice are severely growth retarded. This runted phenotype may be a result of defective nutrient absorption. Therefore a thorough histological examination of the gastrointestinal (GI) tract in these mice was performed. One potential cause of malabsorption is structural defects in the small intestine, where the majority of nutrient absorption occurs. Careful examination of histological sections stained with H&E revealed no structural abnormalities in the small intestine of mutant mice (Fig. 4-10). The height of the villi (Fig. 4-10A) in *Cutl1* ΔC$^{-/-}$ mice appears normal. In addition, a comparable number of narrow stem cells (Fig. 4-10B), undergoing mitotic activity to reconstitute the cell population of the crypt and villus, are observed in the crypts of Lieberkuhn. Also present are the major cell types with protective and absorptive functions in the small intestine, namely the mucus-secreting goblet cells (Fig. 4-10B) and the surface absorptive cells (Fig. 4-10C). The structure of the brush border (Fig. 4-10C), where many of the enzymatic activities of digestion occur, appears intact in the *Cutl1* ΔC $^{-/-}$ mutant mice. Thus the *Cutl1* ΔC mutation does not appear to disturb the development of the small intestines.

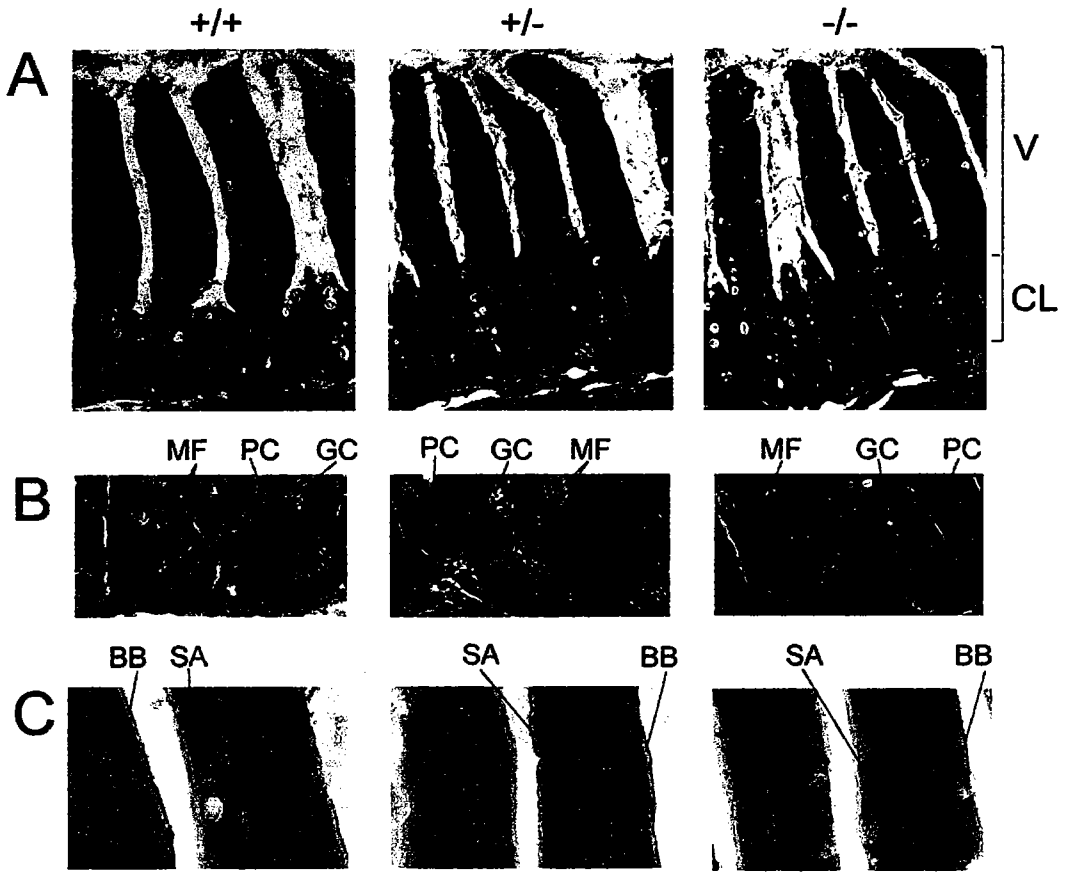

FIG 4-10. Normal histologic features of the small intestine from *Cutl1* ΔC$^{-/-}$ mice. Photomicrographs of H&E Sections (7 μm) of the small intestine. **(A)** Villi (V) and crypts of Lieberkuhn (CL). **(B)** Enlarged view of the boxed area in (A) depicting stem cell mitotic figures (MF), Paneth cells (PC) and goblet cells (GC). **(C)** High power micrograph of the villi. Surface absorptive cells (SA), a columnar type of epithelium with a brush border (BB).

Cutl1 ΔC mutation results in abnormal dermis and hair loss

Homozygous mutant pups begin to shed hair two to three weeks after birth. By one month, the mice are completely bald except for very thin hair covering parts of the ventral region and head (Fig. 4-11A). Re-growth of the coat hair occurs gradually over a period of several months and is more evident in female than in male $\Delta C^{-/-}$ mice. The re-grown coat hair on *Cutl1* $\Delta C^{-/-}$ mice has a distinctive light gray color and appears longer than hair of wildtype animals. In contrast to wildtype control in which there was a thick mat of normal hair, the mutant skin is covered by hairs but they are scant and distorted, appearing to be very thin in diameter. Wildtype mice had normal awl and guard hairs, which are straight and are of uniform diameter along their length with distinct cuticular scales (Fig. 4-11B, panels 1, 2 and 3). Regardless of where they are found on the body, the hair fibers from *Cutl1* $\Delta C^{-/-}$ mice are essentially uniform in size, with a variety of deformities including kinky, twisted, and flattened characteristics (Fig. 4-11B). No vibrissae are evident in the muzzle skin of nullizygous mice, only irregularly wavy hairs that are short and similar to the coat hair (Fig. 4-11A). In summary, these findings suggest that hair fiber types produced in *Cutl1* $\Delta C^{-/-}$ mice are all of one type with loss of other types or that all hairs produced are so deformed they cannot be separated into the various anatomical fiber types. Thus, the genetic mutation we introduced into the *Cutl1* gene locus apparently causes abnormal formation of the coat and vibrissae hair fibers.

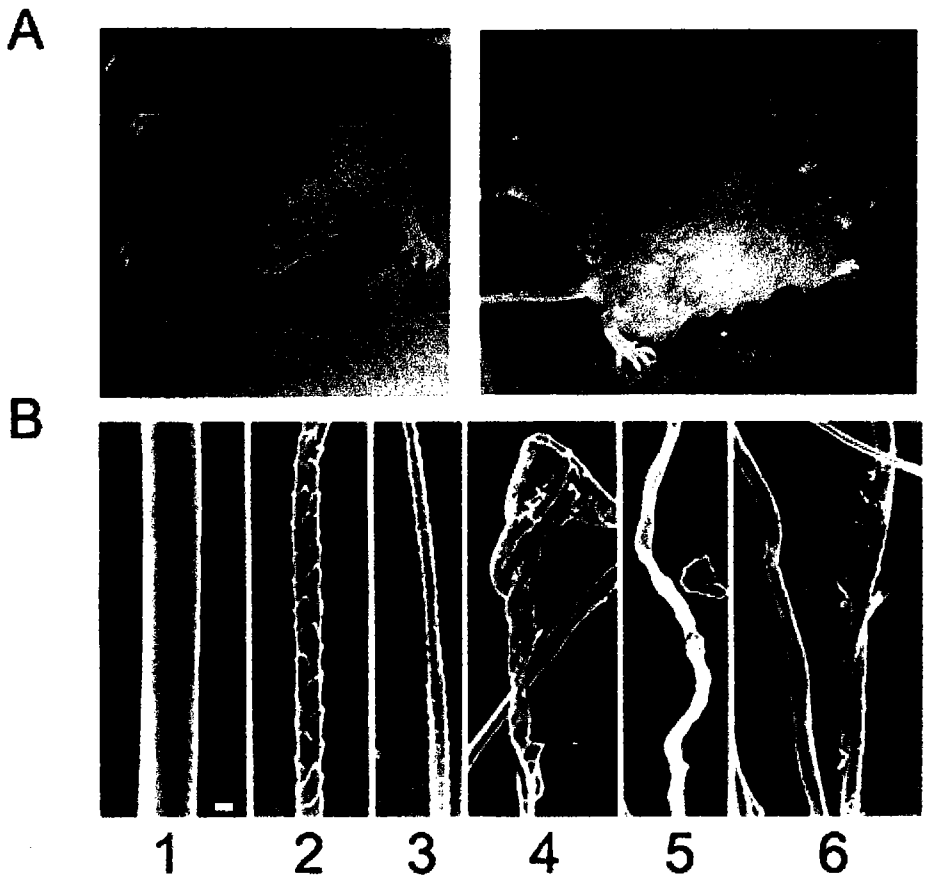

FIG 4-11. Hair Loss in homozygous mutant *Cutl1* ΔC mice. (A) *Cutl1* $\Delta C^{-/-}$ mice with extensive hair loss as compared to its wildtype sibling at 1 month (left) and partial re-growth of abnormal hair at 4 months (right). **(B)** Scanning electron micrographs of plucked hair from five month old mice. The wildtype mouse had normal awl (panel 1) and guard hairs (panels 2 and 3). In contrast, its homozygous mutant littermate had severely deformed hair fibers that could not be typed (panels 4, 5 and 6). Deformed hairs were corkscrew-like in appearance with no evidence of cuticular scales. Bar = 10 μm.

DISCUSSION

To investigate the *in vivo* role of the CDP/Cux C-terminus, mice carrying a genetic deletion of the homeodomain and C-terminus of the protein (*Cutl1* ΔC mice) were characterized. Nuclear signal originating from the truncated CDP/Cux protein is dramatically decreased in homozygous mutant mice, suggesting a significant reduction of its nuclear activity. Consistent with the proposed role of CDP/Cux in the regulation of histone genes [El-Hodiri and Perry, 1995; van Wijnen et al., 1996], altered expression of the histone H4.1 (murine FO108) gene was observed in embryonic fibroblasts homozygous for the ΔC mutation. The genetic mutation in the *Cutl1* gene resulted in abnormal formation of the hair fibers and reduced fertility.

In addition, homozygous mutant mice have increased susceptibility to bacterial infections and suffer from purulent rhinitis characterized by mucosal and submucosal purulent infiltrates within the nasal turbinates (Dr. E.J. Neufeld, unpublished data). Due to enhanced apoptosis, *Cutl1* $\Delta C^{-/-}$ mice have fewer T cells in the thymus and fewer B cells in the bone marrow [Sinclair et al., 2001]. These lymphoid defects are not due to defective antigen receptor rearrangement. Histopathological examination of bone marrow and sternebrae reveals a relative hyperplasia of myeloid cell types in *Cutl1* $\Delta C^{-/-}$ mice [Sinclair et al., 2001]. These data suggest that CDP/Cux is required for normal dermal tissue development, reproduction and the ability to resist microbial infections. A previous study characterizing genetically targeted mice that lack only Cut repeat 1 (due to exon skipping) described a mild phenotype consisting of curly vibrissae, wavy hair and

high pup loss due to impaired lactation in homozygous mutant mothers [Tufarelli et al., 1998]. The mice characterized in the present study lack the entire C-terminus of CDP/Cux and have a more severe phenotype.

Heterozygous mice are fertile and appear indistinguishable from wildtype animals. This finding suggests that haploinsufficiency of the wildtype allele has no phenotypic effect and that the mutant allele does not act in a dominant negative manner. Homozygous mutant (*Cutl1* $\Delta C^{-/-}$) mice are viable at birth and are born at the expected frequency but have a high level of neonatal lethality. Although the cause of the neonatal lethality remains unclear, it does not appear to be a result of impaired feeding because milk is clearly visible in the abdominal cavity of moribund pups. The small proportion of surviving *Cutl1* $\Delta C^{-/-}$ mice has a normal life span but these animals are severely growth retarded. The cause of this failure to thrive is currently unknown. However, growth retardation is not due to general defects in cell proliferation because the cell growth characteristics of homozygous mutant embryonic fibroblasts appear to be normal. Furthermore, there do not appear to be any anatomical or histological abnormalities in the gastrointestinal tract of the *Cutl1* $\Delta C^{-/-}$ mice that may lead to defective absorption of nutrients. Interestingly, increased expression of the brush-border enzyme sucrase-isomaltase (IS), which acts in the final step of small intestinal digestion of dietary starch to glucose, was observed in *Cutl1* $\Delta C^{-/-}$ mice [Boudreau et al., 2002]. It is unclear how increased IS expression may affect nutrient absorption. However, it is possible that the expression or activity of other brush border enzymes is deregulated, which may lead to malabsorption in *Cutl1* $\Delta C^{-/-}$ mice.

Aberrant coat hair, hair loss and a high level of male infertility were the most obvious tissue-specific defects in *Cutl1* $\Delta C^{-/-}$ mice. Consistent with these data, mice lacking CR3 to the C-terminus (ΔCR3-Cterm) exhibit an abnormal pelage due to disrupted hair follicle development [Ellis et al., 2001]. The *Cutl1* ΔCR3-Cterm mutation results in impaired differentiation of the inner root sheath (IRS) that surrounds the hair shaft, and deregulation of *Sonic hedgehog* and IRS-specific gene expression. Cell proliferation, however, was unaffected in the *Cutl1* ΔCR3-Cterm hair bulb. These data suggest that CDP/Cux functions in cell-lineage specification during hair follicle morphogenesis. Interestingly, homozygous deletion of the mouse *ovo1* gene, which encodes a zinc finger transcription factor, has a similar phenotype including growth retardation, aberrant coat hair and a reduced ability to reproduce [Dai et al., 1998]. In addition, *Cutl1* $\Delta C^{-/-}$ mice have similarities to the lanceolate hair (*lah*) mutant mice that are runted, alopecic and lacking vibrissae. The *lah* mutant mice also have follicular dystrophy and lance-shaped broken ends of hair fibers in the muzzle skin [Sundberg et al., 2000]. Several homeobox-containing genes have been shown to be differentially expressed in the dermis and epidermis of fetal and adult skin [Detmer et al., 1993; Stelnicki et al., 1997]. For example, when the homeodomain protein Msx-2 is overexpressed under the control of a keratin promoter in transgenic mice, the animals have a thickened epidermis, shorter coat hair and a reduced matrix region [Wang et al., 1999]. Thus our data contributes to the list of regulatory factors involved in general growth control and dermal tissue development.

A considerable reduction in the fertility was observed in *Cutl1* $\Delta C^{-/-}$ mice and many factors may potentially contribute to this reduced fertility. However, it is

unlikely serum testosterone is directly related to the reduced fertility of *Cutl1* $\Delta C^{-/-}$ males because fertile heterozygous males display the same low testosterone levels as observed in the homozygous males. Adult homozygous mutant males are smaller than wildtype and heterozygous females; this difference in size may affect the mating interactions and thus lower the mating frequency. Furthermore, *Cutl1* $\Delta C^{-/-}$ mice have lymphoid abnormalities and suffer from purulent rhinitis [Sinclair et al., 2001] which could disrupt their sense of smell. Interestingly, the *Cutl1* $\Delta C^{-/-}$ mice have many phenotypes in common with the p73-deficient mice, such as runted appearance, high rates of mortality, severe rhinitis and reduced fertility. As has been shown for the p73-deficient mice, it is possible that lack of interest of *Cutl1* $\Delta C^{-/-}$ male mice in sexually mature females is due to defects in the sensory pathways, such as the absence of expression of pheromone receptors V1R and V2R [Yang et al., 2000]. A previous study suggested that a truncated CDP/Cux protein uniquely expressed in the testis may be involved in testis development and the regulation of gene expression during spermatogenesis [Vanden Heuvel et al., 1996b]. However, the histological sections of the adult testis and epididymis of nullizygous *Cutl1* ΔC mice reveal maturing sperm cells in both organs. Hence, perturbation of the sensory pathways, rather than the absence of germ cells, may contribute to the reduced fertility in the *Cutl1* $\Delta C^{-/-}$ male mice.

In the absence of HiNF-D complex formation, homozygous mutant MEFs exhibit a moderate reduction of H4.1 gene expression, which may be due to decreased transcription of the H4.1 gene. These results are consistent with the postulated role of HiNF-D as a transcriptional regulator of histone genes and suggest possible

mechanisms for HiNF-D function *in vivo*. HiNF-D may suppress the activity of a transcriptional repressor, so that in its absence repression of H4 transcription is increased. The DNA-binding subunit of HiNF-D, CDP/Cux, has been shown to repress transcription of the p21 gene, which can block cell-cycle progression in G_1 phase by inhibiting the activities of cyclin-dependent kinases and the proliferating cell nuclear antigen whose role is to confer processivity to DNA polymerase δ □[Coqueret et al., 1998a]. Thus the absence of CDP/Cux function would predict an increase in p21 activity, which may lead to a cell-cycle block and decreased histone gene expression. Although decreased histone H4.1 mRNA levels were observed in *Cutl1* $\Delta C^{-/-}$ MEFs, no changes in growth characteristics were detected in these cells. Hence, the observed decrease in histone expression was not due to a cell-cycle block mediated by increased p21 activity. HiNF-D may act as a transcriptional activator of the histone H4.1 gene, since the expression of this gene is downregulated in its absence. Several observations implicate HiNF-D as a transcriptional activator of histone gene transcription: HiNF-D contributes to transcriptional activation of histone H4 when the HiNF-M binding site has been mutated or when HiNF-M is absent in the cell [Aziz et al., 1998a]; HiNF-D activity is correlated with histone H4 mRNA levels in several mouse tissues [van Wijnen et al., 1991a]; and mutations that abrogate HiNF-D interaction with Site II cause a delay in H4 transcriptional upregulation. In addition, CDP/Cux has been shown to transactivate the rat tyrosine hydroxylase gene [Yoon and Chikaraishi, 1994] and the DNA polymerase α gene [Moon et al., 2001; Truscott et al., 2003]. Downregulation of histone H4.1 expression did not change the overall histone H4 mRNA levels, which is consistent with studies in which genetic

deletion of two somatic histone H1 genes results in unaltered H1 expression due to compensation by the remaining H1 genes [Fan et al., 2001]. Furthermore, the DT40 chicken B cell line in which half the histone genes were deleted exhibited unaltered growth rates, and steady-state levels of histone mRNA were maintained by increased expression of the remaining histone genes [Takami et al., 1997].

Additional lines of evidence support the role of CDP/Cux in the regulation of growth and differentiation. Deletion of Cut Repeat 3 and the C-terminus of CDP/Cux results in a truncated protein that is retained in the cytoplasm and fails to repress gene transcription [Ellis et al., 2001]. Mice homozygous for this null mutation (ΔCR3-Cterm) die shortly after birth due to retarded differentiation of the lung epithelia. A less severe delay in lung development allows *Cutl1* ΔCR3-Cterm$^{-/-}$ mice on an outbred background to survive beyond birth. Like *Cutl1* ΔC$^{-/-}$ mice, ΔCR3-Cterm$^{-/-}$ mice also exhibit growth retardation. Growth retardation was not due to dysfunction of the thyroid or pituitary glands since levels of growth hormone and thyroid-stimulating hormone are comparable in wildtype and homozygous mutant mice. Interestingly, transgenic mice overexpressing CDP/Cux exhibit multiorgan hyperplasia and downregulation of p27 during nephrogenesis [Ledford et al., 2002]. Transient transfection experiments showed that CDP/Cux is a repressor of p27 promoter activity. Thus these data suggest that CDP/Cux regulates cell proliferation during nephrogenesis by inhibiting expression of p27. This is consistent with the finding that CDP/Cux, as a component of the HiNF-D complex, is a marker for proliferation that is restricted to S phase in normal cells but is constitutive in cancer cells [Holthuis et al., 1990; Owen et al., 1990; van Wijnen et al., 1989].

CHAPTER 5

GENERAL DISCUSSION

The experiments described in this dissertation were initiated: 1) to establish the molecular interactions that enable components of HiNF-D to form a complex and the post-translational modifications that may govern these interactions; 2) to investigate the mechanism(s) involved in CDP/Cux-mediated repression of histone H4 transcription; and 3) to assess the biological role of CDP/Cux in the regulation of proliferation and histone gene expression using a mouse model. Molecular biology and histological techniques were used to characterize a *Cutl1* mutant mouse and to elucidate regulatory mechanisms that govern expression of replication-dependent histone genes during proliferation. Together the data presented here strongly implicate the involvement of CDP/Cux in the regulation of growth control, histone gene expression and differentiation.

CDP/Cux as a tissue-specific mitogenic factor

Several observations suggest that CDP/Cux functions as a mitogenic factor. Cellular division involves DNA replication, a process that requires several enzymes including DNA polymerases. The S phase-specific isoform (CR2-Cterm) of CDP/Cux binds the DNA polymerase α (pol α) gene *in vivo* during S phase and forced expression of CDP/Cux stimulates endogenous pol α gene expression [Truscott et al., 2003]. CDP/Cux also activates transcription of other S phase-specific genes, including dihydrofolate reductase (DHFR) and cyclin A [Truscott et al., 2003]. Furthermore, forced expression of CDP/Cux represses expression of the CDK inhibitor $p21^{Cip1}$ [Coqueret et al., 1998a]. Thus CDP/Cux may facilitate cell cycle

progression by activating genes required for S phase and repressing genes that inhibit cell cycle progression.

In this study, mice with minimal nuclear CDP/Cux function (*Cutl1* ΔC mice) have decreased body weight, which may be due to a proliferation defect. However, fibroblasts derived from homozygous mutant embryos exhibit normal growth rates (Fig. 4-8B, and Luong et al., 2002), indicating that CDP/Cux is not involved in growth regulation in these cells. Proliferation defects in other tissues remain to be investigated. Several studies have implicated CDP/Cux as an oncogene. Increased expression of a specific CDP/Cux isoform, consisting of Cut repeat 3 to the C-terminus (CR3-Cterm), was observed in breast tumors [Goulet et al., 2002]. In invasive tumors, a significant association was established between higher expression levels of CDP/Cux CR3-Cterm and a diffuse infiltrative growth pattern [Goulet et al., 2002]. In polycystic kidneys, CDP/Cux is expressed in cyst epithelium, but is minimally expressed in normal kidney tissue [Vanden Heuvel et al., 1996a]. Consistent with these data, forced expression of CDP/Cux in transgenic mice resulted in enlargement of several organs including the heart, liver, kidneys, testes and seminiferous tubules, but the average body weight was not increased [Ledford et al., 2002]. Organomegaly observed in the kidneys was a result of hyperplasia in renal tubules and glomeruli, which was accompanied by downregulation of $p27^{kip1}$ expression. Interestingly, *Cutl1* ΔC mice that have minimal nuclear CDP/Cux function also exhibit myeloid hyperplasia [Sinclair et al., 2001]. Thus both increased and decreased CDP/Cux expression resulted in hyperplasia, albeit in different tissues.

It is possible that CDP has mitogenic functions in certain tissues and anti-mitogenic activities in other tissues.

Implications of CDP/Cux as a tumor suppressor

HiNF-D is a large multi-subunit complex that is composed of CDP/Cux and the cell cycle regulators pRB, CDK1 and cyclin A [van Wijnen et al., 1994; van Wijnen et al., 1996]. Because the C-terminus (CR2-Cterm) of CDP/Cux is an S phase-specific isoform that has greater DNA binding activity than the full-length protein [Moon et al., 2001], it was not surprising that the CDP/Cux C-terminus was observed to interact with the HiNF-D components cyclin A, CDK1 and pRB (Fig. 3-2). pRB has been reported to interact with more than 110 cellular proteins and many of these interactions are mediated by the pRB pocket domain [Morris and Dyson, 2001]. GST pull-down assays revealed that the pocket domain was sufficient for interaction with GST-tagged CDP/Cux (CR2-Cterm). Because hyperphosphorylation of pRB is concurrent with maximal levels of HiNF-D complex formation in S phase [van Wijnen et al., 1997], it was hypothesized that CDP/Cux interacts with hyperphosphorylated rather than hypophosphorylated pRB. However, results described in Chapter 3 show that CDP/Cux (CR2-Cterm) can interact with the hypophosphorylated pRB (Fig. 3-3A). Though it remains to be determined whether this interaction occurs under physiological conditions with endogenous concentrations of CDP/Cux and pRB proteins, these data suggest that the interaction of CDP/Cux with pRB is regulated in part by E2F-pRB complexes. It is very well possible that the sequestration of E2F by pRB in G_1 phase prevents pRB binding to

CDP/Cux until S phase. Since it is not known whether E2F binds to the entire cellular pool of pRB, some pRB may be free in G_1, though below threshold levels for interaction with CDP/Cux under physiological conditions (Fig. 3-10). CDP/Cux and pRB form complexes *in vivo* and at the single cell level, endogenous CDP/Cux and pRB exhibit significant colocalization (Figs. 3-5 and 3-6). These findings suggest that *in vivo* interaction between CDP/Cux and pRB are facilitated by close proximity of these two proteins within the nucleus.

Results presented in Chapter 3 show that CDP/Cux and pRB cooperate to repress histone gene transcription. It is known that the DNA binding activity of HiNF-D complex is maximal in mid to late S phase [van Wijnen et al., 1997]. The physical and functional interactions described in Chapter 3 suggest that CDP/Cux represses histone gene transcription by recruiting pRB to the promoter. Furthermore, transcriptional repression by HiNF-D may also be mediated by CDP/Cux recruitment of HDAC-1 and/or DNMT1, a DNA methyltransferase which functions to repress gene transcription [Robertson et al., 2000]. It will be interesting to determine whether *in vivo* complex formation and *in situ* association of CDP/Cux and pRB are cell cycle-dependent. CDP/Cux-pRB complex formation and colocalization at subnuclear domains in mid to late S phase would support the hypothesis that CDP/Cux interacts with the hyperphosphorylated form of pRB to repress histone gene transcription in mid to late S phase.

In addition to HiNF-D, HiNF-M (IRF-2) and HiNF-P interact with the histone Site II element and mediate transcriptional activation [Mitra et al., 2002b; Vaughan et al., 1995]. Two recent studies showed that NPAT is an additional link between the

cell cycle and histone gene transcription [Ma et al., 2000; Zhao et al., 2000]. NPAT is a substrate of cyclin E-CDK2 complexes and colocalizes with Cajal bodies, which are associated with histone gene clusters [Frey and Matera, 1995; Shopland et al., 2001]. NPAT activates transcription of histone H2B, H4 and H3 and this activation is dependent on promoter elements that confer cell cycle-regulation. Cyclin E-CDK2 enhances the NPAT-mediated activation of histone gene transcription. HiNF-P and HiNF-M (IRF-2) activate histone H4 transcription and co-expression of NPAT with HiNF-P resulted in cooperative activation [Mitra et al., 2002a]. Together these data support a model for the regulation of histone gene transcription during the cell cycle (Fig. 5-1). The growth factor dependent activation of cyclin E/CDK2 complexes releases pRB from E2F at the restriction (R) point and concurrent activation of NPAT by cyclin E-CDK2 supports HiNF-P induction of the histone H4 gene at the G_1/S phase transition [Mitra et al., 2002a]. The "free" pRB then interacts with CDP/Cux, and recruitment of cyclin A and CDK1 to the histone gene promoter results in the formation of the HiNF-D complex, which functions to repress histone gene transcription in later stages of S phase when the need for histone biosynthesis is decreased.

In the absence of HiNF-D complex, histone H4 FO108 mRNA levels are moderately reduced on *Cutl1* ΔC mice, however this reduction does not change the overall levels of histone H4 mRNA (Fig. 4-8). Increased expression by histone H4 genes that do not contain HiNF-D binding sites as well as increased histone mRNA stability may account for the unaltered H4 mRNA levels. These results are consistent with studies in which genetic deletion of histone genes did not cause changes in

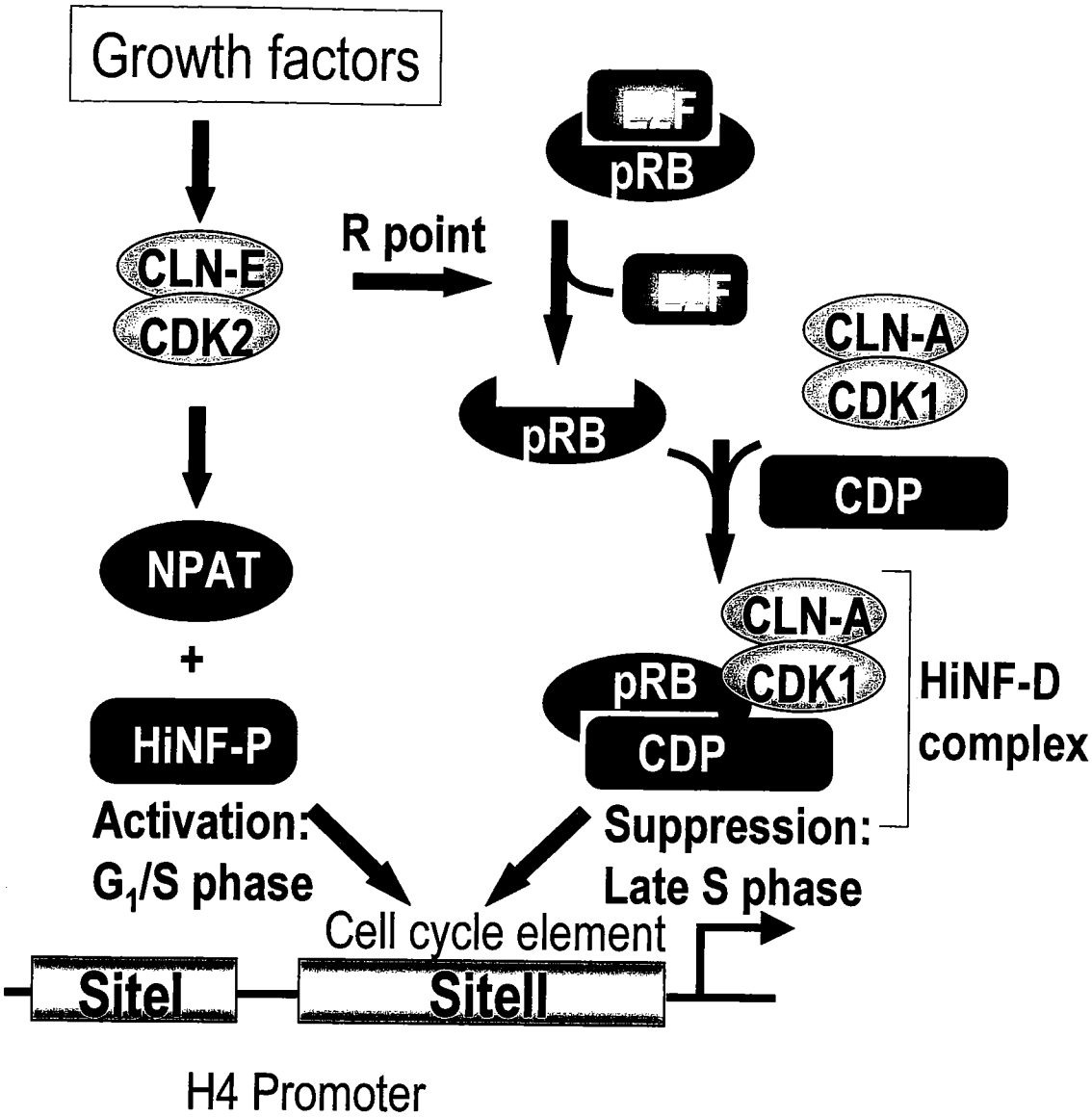

FIG 5-1. Model for CDP/Cux-pRB interaction in different cell cycle stages. HiNF-P and CDP/Cux (as part of the HiNF-D complex) interactions with the Site II cell cycle regulatory sequences of the H4 gene integrate temporally distinct cell cycle regulatory signals. The growth factor dependent activation of cyclin E/CDK2 kinase complexes releases E2F from pRB at the restriction (R) point. Concomitant activation of NPAT by cyclin E/CDK2 supports the HiNF-P dependent induction of the histone H4 gene at the G_1/S phase transition (Mitra et al., 2002). The pRB protein interacts with CDP/Cux, cyclin A and CDK1 to form the HiNF-D complex when cells progress through later stages of S phase.

histone mRNA levels due to compensation by the remaining histone genes [Fan et al., 2001; Takami et al., 1997].

By repressing expression of histone proteins that are essential for cell division, HiNF-D and CDP/Cux act in an anti-mitogenic manner similar to pRB. Indeed several studies suggest that the *Cutl1* gene, which encodes the CDP/Cux protein, is a candidate tumor suppressor gene. Loss of heterozygosity in the *Cutl1* gene occurs at a high frequency in uterine leiomyomas [Zeng et al., 1997], breast cancer [Zeng et al., 1999] and myeloid disorders [Tosi et al., 1999]. In addition, CDP/Cux has been shown to repress the expression of mouse mammary tumor virus [Zhu et al., 2000] and human papillomavirus Type 6 E6, E7, and E1 promoters [Ai et al., 1999]. Inducible expression of E7 increases protein levels of cdc25A, a tyrosine phosphatase that is involved in the regulation of the G_1/S phase transition by activating cyclin E/Cdk2 and cyclin A/Cdk2 complexes [Katich et al., 2001]. Dephosphorylation by cdc25A increases CDP/Cux DNA binding activity [Coqueret et al., 1998a] and may lead to repression of E7 expression (Fig. 5-2A). Furthermore, induction of mammary tumors and leiomyomas in transgenic mice expressing Polyomavirus large T antigen is associated with the ability of large T antigen to form specific complexes with pRB and CDP/Cux [Webster et al., 1998]. Altogether, these data suggest that CDP/Cux, like pRB, functions as a tumor suppressor protein and is a target for inactivation by viral oncoproteins (Fig. 5-2B).

Loss of pRB function has been shown to result in apoptosis due to activation of the p53 pathway [Morgenbesser et al., 1994]. Increased apoptosis is also observed in *Cutl1* ΔC mice, which have minimal CDP/Cux nuclear function. Levels of tumor

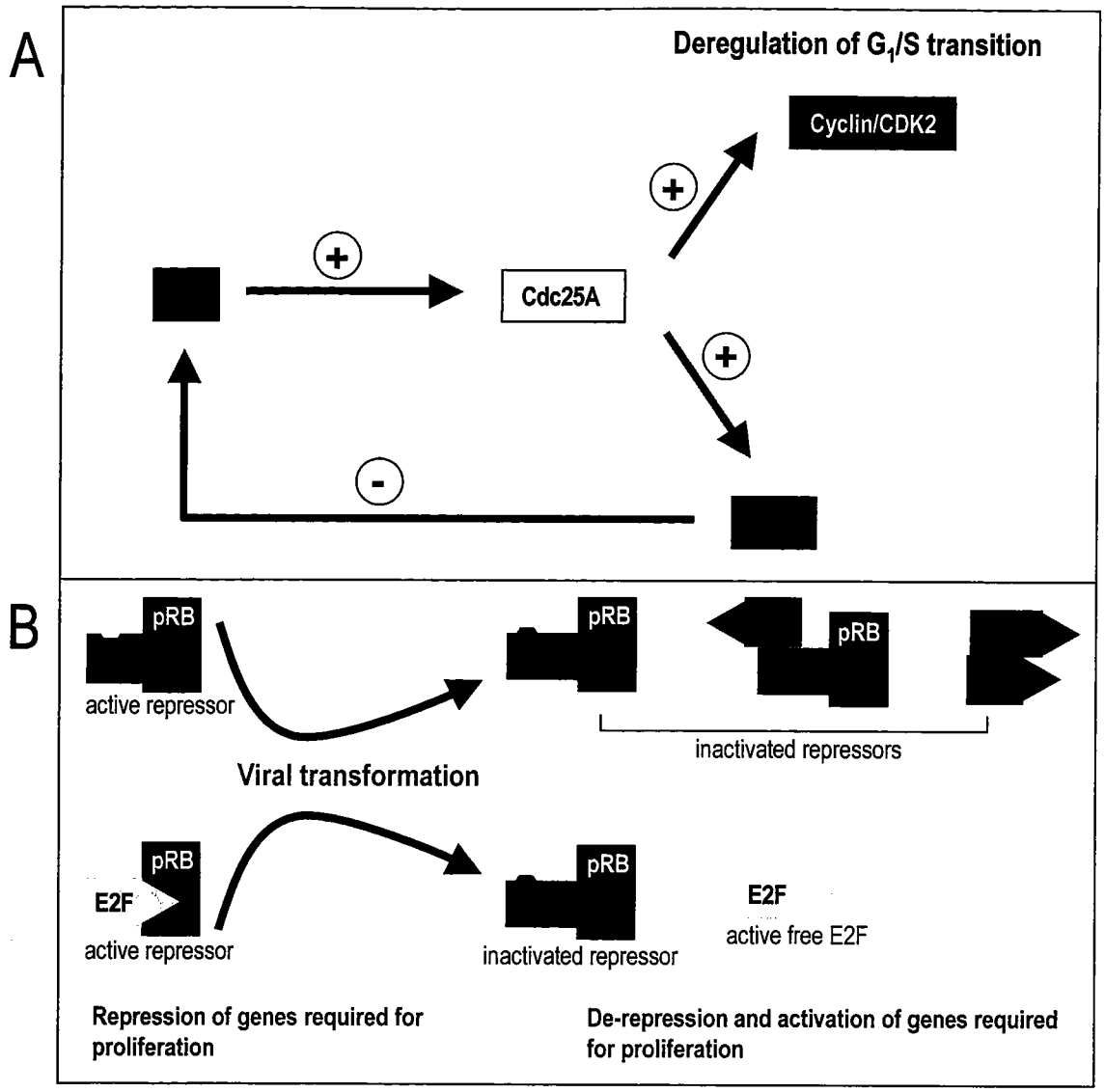

FIG 5-2. Model for CDP/Cux as a putative tumor suppressor protein. (A) CDP/Cux acts as a sensor for papillomavirus protein E7 and inhibits E7 gene expression. Induced expression of cdc25A by E7 (Katich et al., 2001) not only activates cyclin/CDK2 complexes but also increases CDP/Cux DNA binding activity (Coqueret et al., 1998). A negative feedback loop is formed when CDP/Cux in turn represses E7 gene expression. **(B)** Viral disruption of CDP/Cux-pRB and E2F/pRB repressor complexes during cellular transformation. pRB and CDP/Cux interact and cooperate to repress expression of histone genes which are required for cellular division. pRB interaction with E2F inhibits E2F transactivation of genes required for the G_1/S transition. Both CDP/Cux and E2F interact with pRB through the pocket domain. During viral transformation viral proteins such as large T antigen and E1A sequester pRB by binding the pocket domain (Kaelin et al., 1990, Larose et al., 1991). As a result CDP/Cux-pRB and E2F/pRB repressor complexes are disrupted, which leads to de-repression of histone gene expression and activation of genes required for proliferation.

necrosis factor were increased in these mice [Sinclair et al., 2001] but the status of the p53 pathway was not determined. In addition to lymphoid apoptosis, *Cutl1* ΔC mice also exhibit myeloid hyperplasia [Sinclair et al., 2001]. Since CDP/Cux has been shown to repress c-myc expression, hyperplasia in these mice may be due to increased expression of this oncogene. Taken together, these data suggest a role for CDP/Cux in cellular growth suppression.

CDP/Cux regulates differentiation

Absence or severe reduction of nuclear CDP/Cux function in mice results in hair follicle deformities [Ellis et al., 2001; Luong et al., 2002], reduced fertility [Luong et al., 2002] and delayed lung development [Ellis et al., 2001] (Fig. 5-3). Hair follicles from both *Cutl1* ΔC and ΔCR3-Cterm mice have many abnormalities including kinky and twisted characteristics, which may result from the observed impaired differentiation of the inner root sheath that guides the hair shaft as it emerges from the immature cortex cells [Ellis et al., 2001; Fuchs and Byrne, 1994; Luong et al., 2002]. *Cutl1* ΔC mice have reduced fertility and although normal numbers of spermatids were observed in the testes and seminiferous tubules of *Cutl1* ΔC mice, it is possible that these mice have abnormal spermiogenesis that may result in defects in sperm motility and development of the acrosome, which is involved in sperm-egg fusion [Yoshinaga and Toshimori, 2003]. *Cutl1* ΔCR3-Cterm mice lack nuclear CDP/Cux function and die from respiratory failure due to delayed initiation and/or incomplete execution of the alveolar differentiation pathway [Ellis et al., 2001]. These findings indicate that CDP/Cux is involved in execution and regulation

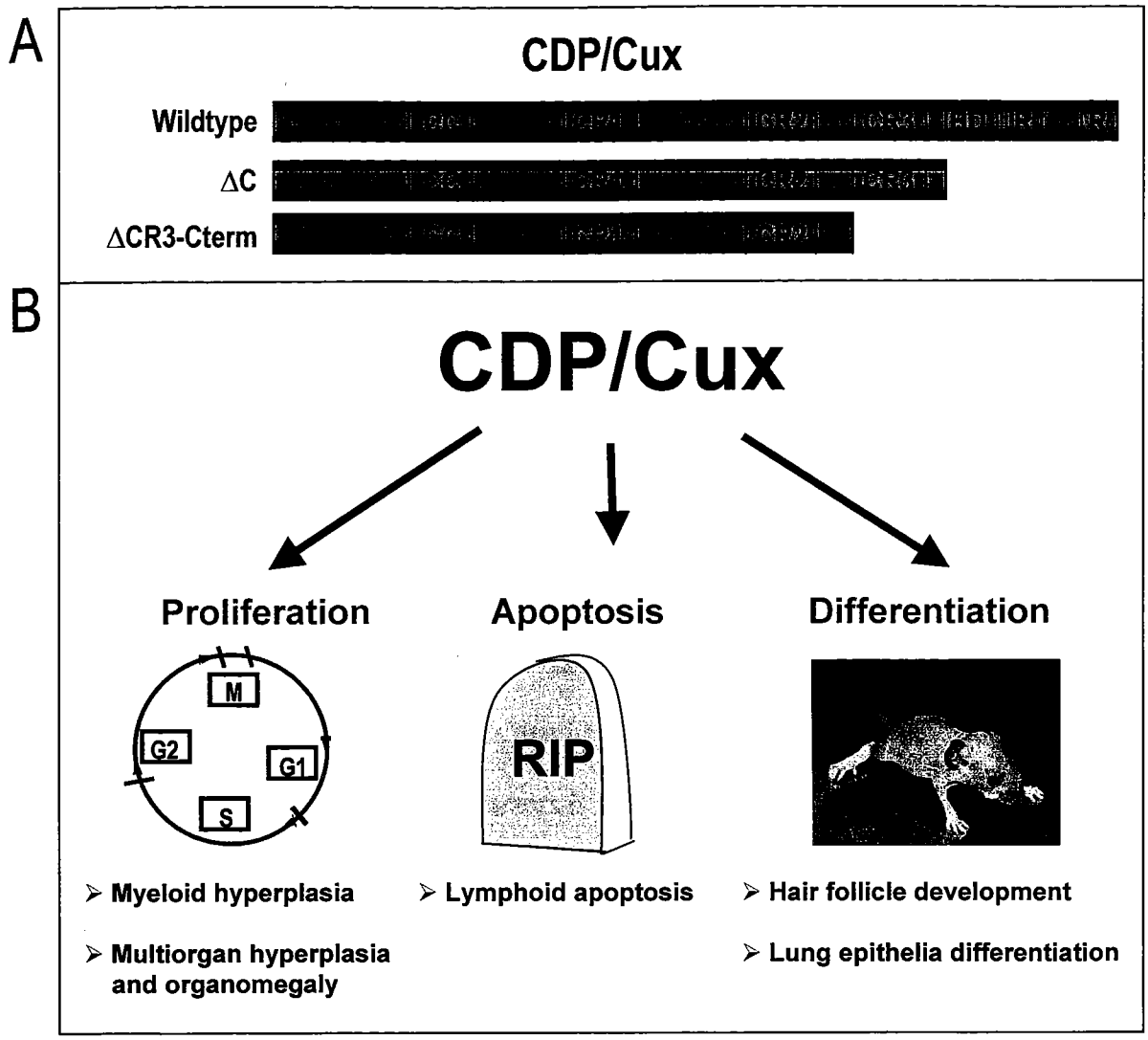

FIG 5-3. CDP/Cux is involved in the regulation of proliferation, apoptosis and differentiation. (A) Three *Cutl1* mouse models have been generated and characterized. In one model, wildtype CDP/Cux was expressed from a CMV promoter. In the other models, the mice express from the *Cutl1* locus truncated CDP/Cux proteins that lack either the homeodomain to the C-terminus (ΔC) or Cut repeat 3 to the C-terminus (ΔCR3-Cterm). **(B)** Forced expression of CDP/Cux in mice results in the enlargement of several organs such as the heart, liver, kidneys and reproductive organs (Ledford et al., 2002). *Cutl1* ΔC protein has minimal nuclear function and ΔC mice exhibit myeloid hyperplasia and lymphoid apoptosis (Luong et al., 2002, Sinclair et al., 2002). Together these results indicate that normal expression and function of CDP/Cux are essential for the regulation of cell growth and programmed cell death in specific tissues. Both the *Cutl1* ΔC and ΔCR3-Cterm mice exhibit hair follicle deformities (Ellis et al., 2001, Luong et al., 2002). In addition, deletion of CR3 to the C-terminus leads to exclusive cytoplasmic localization and delayed differentiation of the lung epithelia (Ellis et al., 2001). Thus CDP/Cux nuclear function is required for differentiation processes in dermal tissues and lung epithelia.

of tissue-specific differentiation programs. Cessation of proliferation is required for the onset of differentiation. CDP/Cux may play a role in the critical balance between proliferation and differentiation by repressing the proliferation marker, histone H4, thereby triggering the cessation of proliferation.

Thus the studies described in this thesis provide new insight into the involvement of CDP/Cux in the regulation of differentiation and proliferation *in vivo*. Furthermore, the results presented in this dissertation add new insight into the transcriptional regulation of the histone H4 FO108 gene. Mechanisms that mediate the regulation of histone gene expression include protein-protein interactions, protein-DNA interactions, and subnuclear localization. These mechanisms are facilitated by a large number of transcription factors that interact with the histone genes. In addition, the function of these transcription factors is influenced by the cell cycle, cell signaling pathways, post-translational modifications and biochemical alterations of DNA. Therefore, the coordinate control of histone gene transcription during the cell cycle results from the integration of many regulatory pathways.

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