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Transcriptional Regulation of a Human H4 Histone Gene is Mediated by Multiple Elements Interacting with Similar Transcription Factors: A Dissertation

Thomas J. Last
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TRANSCRIPTIONAL REGULATION OF A HUMAN H4 HISTONE GENE IS MEDIATED BY MULTIPLE ELEMENTS INTERACTING WITH SIMILAR TRANSCRIPTION FACTORS

A Dissertation Presented

By

Thomas John Last

Submitted to the Faculty of the

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of Biomedical Sciences, Worcester

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MAY 14, 1998

CELL BIOLOGY

TRANSCRIPTIONAL REGULATION OF A HUMAN H4 HISTONE GENE IS MEDIATED BY MULTIPLE ELEMENTS INTERACTING WITH SIMILAR TRANSCRIPTION FACTORS

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Thomas B. Miller Jr., Dean of the Graduate
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Department of Cell Biology
May 14, 1998

DEDICATION

This thesis is dedicated to my wife Rohini, who has inspired me to always seek the truth in nature and our society.

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Obtaining this doctorate in Philosophy is essential to my life purpose, that of serving the humanity through any contributions I might make towards the advancement of science. I would like to express my deepest gratitude towards Drs. Janet and Gary Stein and Dr. Jane Lian for allowing me to become part of their research group. Without their support, guidance and resources, the research presented in this thesis would not have been possible. I would also like to give special thanks to Andre van Wijnen and Ronald Ignotz for all their time and energy in assisting me with manuscripts and for many intellectual discussions.

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF A HUMAN H4 HISTONE GENE IS MEDIATED BY MULTIPLE ELEMENTS INTERACTING WITH SIMILAR TRANSCRIPTION FACTORS

By

Thomas John Last

May 1998

Thesis Advisor: Janet L. Stein
Department: Cell Biology

Synthesis of histone proteins occurs largely during the S phase of the cell cycle and coincides with DNA replication to provide adequate amounts of histones necessary to properly package newly replicated DNA. Controlling transcription from cell cycle dependent and proliferation specific genes, including histone H4, is an important level of regulation in the overall governance of the cell growth process. Coordination of histone gene transcription results from the cumulative effects of cell signaling pathways, dynamic chromatin structure and multiple transcription factor interactions. The research of this dissertation focused on the characterization and identification of transcription factors interacting on the human histone H4 gene FO108. I also focused on the elucidation of regulatory elements within the histone coding region. Our results suggest a possible mechanism by which a transcription

factor facilitates reorganization of histone gene chromatin structure.

The histone promoter region between -418 nt and -215 nt, Site III, was previously identified as both a positive and negative cis-regulatory element for transcription. Results of *in vitro* analyses presented in this dissertation identified multiple transcription factors interacting at Site III. These factors include H4UA-1/YY1, AP-2, AP-2 like factor and distal factor (NF-1 like factor). Transient transfection experiments show that Site III does not confer significant influence on transcription; however, there may exist a physiological role for Site III which would not be detected in these assay systems.

We analyzed the histone H4 gene sequences for additional transcription factor binding motifs and identified several putative YY1 binding sites. Using electrophoretic mobility shift assays (EMSA), we found that Site IV, Site I and two elements within the histone H4 coding region are capable of interacting with YY1. In transient transfection experiments using reporter constructs containing either Site III or one of the coding region elements as potential promoter regulatory elements, and an expression vector encoding YY1, we observed levels of expression up to 2.7 fold higher than from the reporters lacking these elements. Therefore, YY1 appears to interact at multiple regulatory sites of the histone gene and can influence transcription through these elements.

Prior to this study, the role of the coding region in histone gene expression was not known. To determine if the coding region is involved in regulating transcription, I constructed and tested a series of heterologous reporter constructs containing various sequences of the histone coding region.

Results from these experiments demonstrated that the histone coding region contains three repressor elements. Extensive *in vitro* analysis indicated that the three repressor elements interact with the repressor CDP/*cut*. Further analysis showed that CDP/*cut* interactions with the repressor elements are cell cycle regulated and proliferation specific. CDP/*cut* interactions increase during the cell cycle when histone transcription decreases. These observations are consistent with the hypothesis that CDP/*cut* is a cell cycle regulated repressor factor which influences transcription of the histone H4 gene as such.

The proximal promoter region of the histone H4 gene between -70 nt and +190 nt is devoid of normal nucleosome structure. This same region contains multiple CDP/*cut* binding sites. We hypothesized that CDP/*cut* is involved with chromatin remodeling of the histone gene. DNase I footprinting and EMSA results show purified recombinant CDP/*cut* interacts specifically with the histone promoter reconstituted into nucleosome cores. Thus, CDP/*cut* may facilitate the organization of chromatin of the histone gene.

In conclusion, the research presented in this dissertation supports the hypothesis that expression from the human histone H4 gene FO108 is regulated by multiple cis-regulatory elements which interact with several proteins. CDP/*cut* interacts with Site II, the three repressor elements in the histone coding region and at Distal Site I. YY1 interacts at Site IV, Site III, Site I, and twice in the coding region. ATF/CREB interacts with Site IV and Site I. Distal factor interacts with Site III and within the histone coding region. IRF 2 interacts with Site II and Distal Site I. Thus, histone gene expression is probably regulated by transcription factors CDP/cut, YY1, IRF 2 and ATF/CREB

interacting with multiple regulatory elements dispersed throughout its promoter and the coding region. Cell cycle regulation of these transcription factors may contribute to cell cycle dependent expression of the histone gene.

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CHAPTER I

INTRODUCTION

One of the fundamental fields of inquiry in modern molecular biology is the identification of factors and signaling pathways involved in mediating cell cycle and growth control. Every cell undergoes very complex series of regulatory events in order to properly proliferate or differentiate. Abnormal signal transduction and/or transcriptional regulation can result in tumorigenesis. Histone genes serve as excellent eukaryotic model systems for understanding molecular regulatory mechanisms involved in proliferation, differentiation and tumorigenesis. The histone proteins are essential for proper structural organization of all eukaryotic genomes by facilitating the compaction and condensation of their DNA. Histone expression is functionally related to cell growth and is temporally and functionally coupled with DNA synthesis. Transcriptional and post-transcriptional mechanisms are involved in controlling histone mRNA levels during the cell cycle. Regulation of histone gene transcription results from the cumulative effects of cell signaling pathways, dynamic chromatin structure and multiple transcription factor interactions. Elucidating histone gene regulatory mechanisms will enlighten and help us solve one of molecular biology's most vexing riddles.

Histone proteins

Histones are a family of small, basic proteins that are essential for proper

chromatin organization and transcriptional regulation in eukaryotic cells (reviewed in Stein and Stein, 1991; Grunstein, 1992). There are five major classes of histones which are further divided into two groups based on their function. The first group, the core histones (H2A, H2B, H3 and H4), form nucleosomes, the basic packaging unit of all eukaryotic DNA. The nucleosome is a strong, stable octamer complex of the core histones, each present in stoichiometric amounts, around which about 147 base pairs of DNA are wound in 1.7 turns. The second group, H1 linker histones, facilitate the organization of linear arrays of nucleosomes into 30 nm chromatin fibers by interacting with the DNA linker region between nucleosomes and with the adjacent nucleosomes.

Overall, the evolutionally conserved core histones are very similar between species, suggesting that their protein structures and functions are vital to all cells. Recently, key transcription factors of the TFIID initiation complex, $TAF_{II}20$, $TAF_{II}31$ and $TAF_{II}80$, were shown to have similar protein structures to the core histones (a long central α-helix flanked at both ends by shorter helixes) (Surridge, 1997; Hoffman et al., 1996), further emphasizing the important roles of these protein structures in chromatin architecture and gene regulation (reviewed in Hoffman et al., 1997). In comparison to the core histones, the H1 histones consists of many more variants which are not as highly conserved, thus suggesting the H1 variants may each have an unique function depending on cell type and species, which would account for the diversity in protein structures (reviewed in Stein et al., 1984).

Many genes are regulated by elaborate orchestration of transcription factors, intra- and extra-cellular signaling pathways, and chromatin structure, which involve histones. In certain genes, nucleosomes do not appear to be

randomly positioned. Instead, the location of a nucleosome dictates its functional role in transcriptional control of that gene (reviewed by Grunstein, 1992; Felsenfeld, 1992). Changes in post-translational modifications of histones, such as acetylation, may modulate the histones ability to influence transcription from the gene (reviewed in Bradbury, 1992; Matthews and Waterborg, 1985; Stein and Stein, 1991). Acetylation of histones has been associated with DNA replication and transcription (reviewed in Csordas, 1990; Allfrey, 1980). Regulation of histone acetylation appears to require certain transcription factors that can recruit histone acetyltransferase to the promoter of a gene. The recruited histone acetyltransferase then acetylates the amino terminal tail domains of the core histones, causing the nucleosomes to become destabilized, which in turn facilitates the transcriptional process (reviewed in Pennisi, 1997).

Cell cycle analysis of histone acetylation in *Physarum polycephalum* revealed three patterns of acetylation (reviewed in Bradbury, 1992; Matthews and Waterborg, 1985). One of the key observations was that all four core histones (H2A, H2B, H3 and H4) were maximally acetylated in S-phase. The next observation was that during G_2-phase, only H3 and H4 were hyperacetylated. The last pattern observed was that during M-phase, all four core histones were deacetylated.

Other post-translational modifications of histones, such as adenosine diphosphate-ribosylation, methylation, ubiquitination and phosphorylation, have also been correlated with nuclear processes (reviewed in Bradbury, 1992; Matthews and Waterborg, 1985; Stein and Stein, 1991). The lysines on C-terminal tails of H2A and H2B undergo cell cycle dependent ubiquitination

(Mueller et al., 1985). Furthermore, ubiquitinated histones are preferentially found associated with transcriptionally active DNA sequences (Nickel et al., 1989). Phosphorylation of histone H3 and H1 also appear to be cell cycle regulated (Langan and Chamber, 1987). For example, the histone H1 become phosphorylated through S-phase and G_2 phase. After mitosis, the hyperphosphorylated H1 histones were dephospholylated (Mueller et al., 1985(b)). Therefore, post-translational modifications of histones appear to be important for cell cycle and cell growth regulation.

Histone genes: organization and variants

There are significant differences between the genomes of various eukaryotic organisms in the number and organization of their histone genes (reviewed in 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 organized into quintets. Each quintet encodes one copy of each of the five histone classes. These quintets are usually arranged in tandemly repeated patterns in the genome. By contrast, humans, mice and chickens have only 10 to 40 copies of the histone genes, which are organized into complex clusters located within the genome in a largely random fashion (Carozzi et al., 1984; Heintz et al., 1981; Sierra et al., 1982; Sittman et al., 1981; Engel et al., 1981; Harvey et al., 1979; Harvey et al., 1981; Wilson et al., 1977; Albig et al., 1997). The human genome has histone clusters positioned in chromosomes 1 and 6 (Allen et al., 1991; Tripputi et al., 1986; Green et al.,1984). The histone H4 gene FO108, which is the primary gene of this study, belongs to a cluster located in chromosome 1 (Green et

al.,1984; Tripputi et al., 1986).

Each major class of histones is comprised of a variety of histone subtypes which can be categorized based on the regulation of their synthesis (reviewed in Old and Woodland 1984). Replication-dependent variants form the predominant category, which include the histone H4 gene FO108. Replication-dependent histone variants are generally proliferation specific and are synthesized simultaneously with DNA during S phase of the cell cycle. Another subtype of histone variants are the replication-independent variants (H2A.3 and H3.3). They are categorized as replication-independent because their expression is constitutive throughout the cell cycle and is not dependent on the proliferative status of the cell. The minor histone variants (also known as replacement histones) are primarily synthesized in non-proliferating somatic cells and are expressed at low levels in dividing cells. The last category of histone variants is that of the tissue-specific variants. Histone H5 found in avian erythocytes is an example of a tissue-specific variant. The expression of tissue specific variants is not replication dependent and is restricted to particular tissues.

Regulation of replication-dependent histone gene expression

The biosynthesis of replication-dependent histones is up-regulated in the S phase of the cell cycle by the cumulative influences from multiple regulatory mechanisms (reviewed in Marzluff and Pandey, 1988; Heintz, 1991; Osley, 1991). The synthesis of DNA and histones occurs simultaneously and transcriptional and post-transcriptional mechanisms ensure an appropriate level of histone proteins for proper packaging of the newly duplicated DNA. At

the peak of DNA synthesis in S phase, histone mRNA levels are 15-20 fold higher than basal levels outside of S phase (Baumbach et al., 1987; Plumb et al., 1983: Heintz et al., 1983). Part of the increase in histone mRNA levels results from altered post-transcriptional regulation. Two post-transcriptional pathways are involved. The first is histone mRNA stability in the cytoplasm. The histone mRNA half-life of 120 minutes in early S phase is reduced near the completion of DNA synthesis to 15 to 20 minutes (Morris et al., 1991). RNA processing is the other cell cycle regulated post-transcriptional pathway. The precursor histone RNA is transformed into a mature mRNA by a nuclear process involving specific endonucleolytic cleavage at the 3' end of the precursor (Gick et al., 1986; Krieg and Melton, 1984). The histone pre-mRNA processing is cell cycle controlled by being down-regulated in the G1 phase (reviewed in Osley, 1991; Marzluff, 1992). The rate of histone gene transcription is also coupled to DNA synthesis. In early S phase, transcription increases 2 to 5 fold over the basal level (Baumbach et al., 1987; Plumb et al., 1983; Heintz et al., 1983: Graves and Marzluff, 1984: Sittman et al., 1983). Our lab has shown that these changes in transcription during the cell cycle are in part due to transcriptional regulatory elements in the gene's promoter (Ramesy-Ewing et al., 1994).

Replication-dependent histone biosynthesis is also functionally linked to the growth status of the cell. Proliferating cells in non-S phases of the cell cycle have higher mRNA levels than do non-dividing cells (G_o). For example, histone H4 gene expression in differentiated 3T3-L1 pre-adipocytes is significantly lower when compared to level of expression in proliferating cells (Bortell et al., 1992; Ramsey-Ewing, 1991). Histone H4 levels are also down-regulated in growth-inhibited or differentiated osteoblasts (Shalhoub et al, 1989; Owen et al.,

1990), differentiated promyelocytic leukemia HL-60 cells (Stein et al., 1989) and in quiescent or senescent IMR-90 normal fibroblasts (Pang and Chen, 1994). Altered transcription factor binding and changes in chromatin structure are believed to be involved in the process of down-regulation of expression in non-dividing cells (Stein et al., 1989; van den Ent et al., 1993; Wright et al., 1992).

Regulatory organization of the histone H4 gene FO108

Basal and cell cycle regulated histone H4 gene expression levels are dependent on proper arrangement of chromatin structure and binding of regulatory molecules to gene elements. The chromatin structure of the FO108 histone H4 gene was analyzed in detail by monitoring the levels of nuclease sensitivity throughout the gene (Chrysogelos et al., 1989; Moreno et al., 1986; Pauli et al., 1988). During the cell cycle, there are significant changes in chromatin structure and nuclease sensitivity, in both the central promoter and the coding region of the histone H4 gene. DNase I and S1 nuclease sensitivity peaks in mid to late S phase, then decreases in M and G1 phases. In contrast, the DNase I and S1 nuclease hypersensitive sites in the proximal promoter and at -700 to -800 base pairs are present throughout the cell cycle (Chrysogelos et al., 1989). Micrococcal nuclease analysis demonstrated that the region of the gene between -70 bp to +190 bp lacks normal nucleosome structure throughout the cell cycle, and there are significant changes in nucleosome structure during the cell cycle within the region from +200 to +275 (Moreno et al., 1986; Moreno et al., 1988). These observations suggest that discrete regions in the gene undergo chromatin modifications which correlate with observed basal and cell cycle regulation of transcription.

Our laboratory has characterized and defined multiple regions involved in the transcriptional regulation of the histone H4 gene (Figure 1-1). Two elements in particular, Site I (-127 to -84 nt) and Site II (-67 to -17 nt), have been extensively studied *in vitro* and *in vivo* (van Wijnen et al., 1987; Pauli et al., 1987; Ramsey-Ewing et al., 1994). Site II, which is essential for proper initiation of transcription and cell cycle control, interacts with multiple factors (Kroeger et al., 1987; Ramsey-Ewing et al., 1994). The potentially oncogenic transcription factor IRF-2 (HiNF-M), interacts at the cell cycle control element on the distal side of Site II (Ramsey-Ewing et al., 1994; Vaughan et al., 1995). Phosphoprotein IRF-2 is believed to exert a positive effect on histone transcription at the onset of S-phase via Site II (Birnbaum et al., 1997; Vaughan et al., 1997). Another potential cell cycle regulated complex which interacts at Site II is HiNF-D. HiNF-D binding activity is cell cycle regulated in normal cells but is constitutive in tumor cells (Holthuis et al., 1990). The proliferation specific HiNF-D complex is composed of growth regulatory factors cyclin A, cdc-2, CDP/*cut* and an RB-related protein (van Wijnen et al., 1994; van Wijnen et al., 1996). The third factor that interacts at Site II is HiNF-P. HiNF-P appears to be identical to H4-TF2 (Dailey et al, 1988), but neither factor has yet been cloned or identified (van Wijnen et al., 1992). Site I assists in modulating transcriptional regulation. The Proximal Site I region interacts with transcription factors Sp1 and Sp3 (Birnbaum et al., 1995; Birnbaum et al., 1996). Distal Site I interacts with at least 3 transcription factors including HiNF-A, CREB and ATF-1 (van Wijnen et al., 1987; Wright et al., 1995; Guo et al., 1997).

Figure 1-1. <u>Schematic model of the histone H4 gene FO108.</u> DNase I
hypersensitive sites are indicated as large arrowheads and marked DNase H.S.
A micrococcal nuclease sensitive site is marked by a large box and is labeled
MNase H.S. The various identified transcription factors are indicated and
relative locations for H4UA-1, IRF-2 (IRF), CDP/*cut* (CDP), Sp1, ATF/CREB
(ATF) and HiNF-P (P) on the promoter are shown.
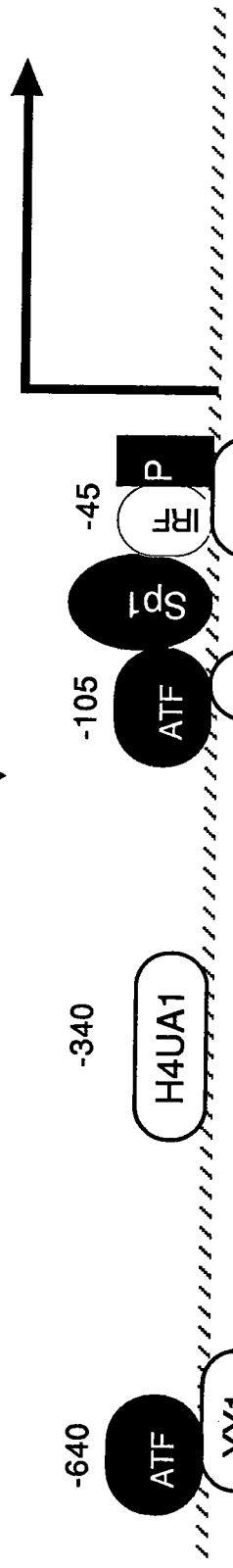
The distal promoter region is also involved in H4 gene regulation. There are two defined elements *in vitro*, Site III and Site IV. Site III (-418 to -215) has been shown by our laboratory to up-regulate transcription two-fold in transient transfections in mouse LTK⁻ cells and stable transfections in C127 cells (Kroeger et al., 1987). Other laboratories have observed that the Site III region is a repressor element in *in vitro* transcription systems using differentiated L6 myotube extracts or quiescent 3T6 fibroblast extracts (Larson et al.,1989; Zahradka et al., 1993). We have identified and characterized a protein-DNA complex, H4UA-1, *in vitro* by Electrophoretic Mobility Shift Assay (EMSA), DNase I footprinting and various fingerprinting techniques (Wright, 1990; van der Houven van Oordt et al., 1992). EMSA results show that nuclear extracts from differentiated osteoblasts have a faster migrating complex of H4UA-1 than that found in proliferating cells (van der Houven van Oordt et al., 1992). H4UA-1 does not appear to be cell cycle regulated (van der Houven van Oordt et al., 1992).

Site IV (-733 to -590) is a transcriptional activator element that binds preferentially to nuclear matrix protein extracts (Pauli et al., 1989; Dworetzky et al., 1992; Wright, 1990). Three characterized protein-DNA complexes form at Site IV (Wright, 1990). One of the complexes, Nuclear Matrix Protein 1 (NMP-1) has been identified as YY1 (Guo et al.,1995). The other complexes include ATF/CREB (Guo et al., 1997) and an unidentified complex, H4UA-3.

Transcription factors YY1 and CDP/*cut*

Transcription factors YY1 and CDP/*cut* have been implicated in the

regulation of histone H4 FO108 gene expression (Guo et al., 1995; van Wijnen et al., 1996). YY1 (UCRBP, δ, or NF-E1) is a multi-functional transcription factor that is ubiquitously expressed in mammalian cells (Shi et al., 1991; Flanagan et al., 1992; Hariharan et al., 1991; Park and Atchison, 1991). This 414 amino acid protein belongs to the GLI-Kruppel transcription factor family and contains four C_2H_2-type zinc fingers in the C-terminus (Shi et al., 1991). The N-terminus of YY1 contains an acidic region and a string of 11 histidine residues (Shi et al., 1991). YY1 is capable of modulating transcription by multiple mechanisms (for review see Shrivastava and Calame, 1994 and Hahn et al., 1992), including by its ability to interact with a vast array of regulatory factors, such as p300 (Lee et al., 1995), TAFii55 (Chiang and Roeder, 1995), TFIIB, TATA box-binding protein (Usheva and Shenk, 1994), CBP (Austen et al., 1997), SP1 (Seto et al., 1993; Lee et al., 1993), E1A, c-Myc (Shrivastava et al. 1993), ATF/CREB (Zhou et al., 1995), B23 (Inouye et al., 1994), YAF2 (Kalenik et al., 1997), RPD3 (Yang et al., 1996), cyclophilin A and FKBP 12 (Yang et al., 1995). YY1 has been shown to have positive, negative and neutral influences on transcription (for review see Shrivastava and Calame, 1994 and Hahn et al., 1992). In addition, YY1 is capable of initiating transcription with TFIIB and RNA polymerase II (Usheva and Shenk, 1994) and has been shown to influence transcription by its ability to bend DNA and thus regulate contact between elements (Natesan and Gilman, 1993).

CDP/*cut* was initially observed interacting with the promoter of a sperm histone H2B gene from the sea urchin *Psammechinus miliaris* (Barberis et al., 1987). Since then CDP/cut has been found to interact with the promoters of many histone genes; 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)). This factor is also involved in the repression of other human genes, including gp91-phox (Lievens et al., 1995; Skalnik et al., 1991), HPV6 LCR (Pattison et al.,1997), c-myc (Dufort and Nepveu, 1994), and gamma globin (Superti-Furga et al., 1988). CDP/*cut* is a large (from 180 to 190 kDa in size) multifunctional transcription factor with three cut repeat motifs and one homeodomain (Neufeld et al., 1992). Each of these four domains is capable of binding to DNA independently of one another (Andres et al.,1994; Auferio et al., 1994). Phosphorylation by protein kinase C and casein kinase II can regulate CDP/*cut* DNA binding activity (Coqueret et al., 1996; Coqueret et al., 1998). Furthermore, results from our laboratory have shown that CDP/*cut* can form higher order complexes with cyclin A, cdc-2 and RB-related proteins (van Wijnen et al., 1994; van Wijnen et al., 1996). Therefore, these observations suggest that CDP/*cut* plays an important role in controlling expression from cell growth regulated genes (including histones).

Overview of project

The goal of this research is to elucidate molecular mechanisms involved in the transcriptional regulation of the cell cycle dependent human histone H4 gene FO108. The initial focus of this reseach was on the functional characterization of the distal histone regulatory element Site III and the identification of the Site III protein-DNA complex, H4UA-1. This was followed by analyzing other histone FO108 gene sequences with homology to H4UA-1/YY1 binding motifs. Next, my research focused on examining the histone H4 coding region to determine whether it harbors any transcriptional regulatory elements.

Upon identification of three repressor elements within the coding region, I proceeded to identify a proliferation-specific, cell cycle regulatory transcription factor CDP/*cut*, which interacts at all three elements. I also investigated a possible molecular mechanism by which CDP/*cut* may be involved in cell cycle regulation by its ability to bind with nucleosomes.

CHAPTER II

MATERIALS AND METHODS

Plasmid construction

Histone H4 Site III constructs

pH4CARP (-210 to -418). The plasmid pH4CARP (Central Activating Region Plasmid) was constructed as an intermediate step cloning vector to be utilized for making other Site III related clones, including Proximal and Distal Site III site-specific mutants. The Sma I-Sma I promoter-CAT gene fragment was excised from Anna Ramsey's pFO005CAT (construct not published) and the plasmid was religated onto itself. The resultant vector therefore consists of the pUC backbone and Sma I (-210 bp) to Hind III (-418 bp) region of the histone H4 promoter.

Proximal Site III Mutant pH4CARP and Distal Site III Mutant pH4CARP.

A modified Long Primer Unique Site Elimination (LP-USE) method (Ray and Nickoloff, 1992) was used to generate site specific mutants of proximal or Distal Site III. AlwN I USE and Distal Site III mutant oligonucleotide primers or Xmn I USE and Proximal Site III mutant 1 oligonucleotide primers (see Table 2-1) were used in the PCR production of long primers. Long primers were synthesized using Vent DNA Polymerase in 35 PCR cycles of 1 minute at 90°C,

TABLE 2-1. Table of oligonucleotides used in this dissertation. Italics indicate linker sequences which were added to the ends of the oligonucleotides. Underlines indicate those nucleotides which are altered from the wild type sequence.

TABLE 2-1.

Site III related oligonucleotides

Full Site III (-368/-324): *GATC*TGGCGGCCTCCTGCCAGTCTCTGGCCTCCATTTGCTCTTCCTG

Distal Site III (-370/-341): GCGCTGGCGGCCTCCTGCCAGTCTCTGGCC

Dist. Site III Mut: GCGCTGGCGGCCTCCT<u>TC</u>A<u>CC</u>TCTCTGGCC

Proximal Site III (-349/-322): *GATC*TCTCTGGCCTCCATTTGCTCTTCCTGAG

Prox. Site III Mut. A: *GATC*TCTC<u>AC</u>GCCT<u>AGC</u>TTTGCTCTTCCTGAG

Prox. Site III Mut. B: GTCTCTGGCCT<u>AGC</u>TTTGCTCTTCCTGAG

Site III AP-2 site (-377/-356): GAATCCTGCGCTGGCGGCCTCC

Histone H4 coding region related oligonucleotides

+72/+110: CGCTAAGCGCCACCGCAAGGTCTTGAGAGACAACAT

+72/+125: CGCTAAGCGCCACCGCAAGGTCTTGAGAGACAACATTCAGGGCATCACCAAGCC

+72/+125: CGCTAAGCGCCACCG<u>GGGT</u>T<u>T</u>TCTTGAGAGAC<u>GGG</u>A<u>AAG</u>AGGGCATCACCAAGCC
(MUTANT 1)

+72/+125: CGCTAAGCGCCACCGCAAGGTC<u>GGT</u>AGAT<u>ATC</u>ACATTCAGGGCATCACCAAGCC
(MUTANT 2)

+110/+140 TCAGGGCATCACCAAGCCTGCCATTCGGCGTCTAGC

+170/+203: GGCCTCATTTACGAGGAGACCCGCGGTGTGCTG

+110/+160: TCAGGGCATCACCAAGCCTGCCATTCGGCGTCTAGCTCGGCGTGGCGGCGT

+123/+160: GCCTGCCATTCGGCGTCTAGCTCGGCGTGGCGGCG

+136/+184: CGTCTAGCTCGGCGTGGCGGCGTTAAGCGGATCTCTGGCCTCATTTACG

+154/+203: GGCGTTAAGCGGATCTCTGGCCTCATTTACGAGGAGACCCGCGGTGTGCT

EMSA oligonucleotides

TM-3 (-66/-26): CGCTTTCGGTTTTCAATCTGGTCCGATACTCTTGTATATCA

SUB-11: CGCTTTCGGTT<u>C</u>TCA<u>G</u>TT<u>C</u>GGTCCG<u>CC</u>ACTCT<u>T</u>GTATATCA

YY1: *GATC*CGCTCCGCGGCCATCTTGGCGGCTGGT

YY1 MUTANT: *GATC*CGCTCCGCG<u>ATT</u>ATCTTGGCGGCTGGT

E2F: ATTTAAGTTTCGCGCCCTTTCTCAA

E2F MUTANT: ATTTAAGTTTCG<u>AT</u>CCCTTTCTCAA

ATF: AGAGATTGCCTGACGTCAGAGAGCTAG

SP1: ATTCGATCGGGGCGGGGCGAGC

CTF/NF1: CCTTTGGCATGCTGCCAATATG

AP-2: *GATC*GAACTGACCGCCCGCGGCCCGT

PROXIMAL SITE I (-108/-96): *GATC*TTGTCGAGAGGGCGGGGACAATTG

DISTAL SITE I (-125/-101): *GATC*CGGAAAAGAAATGACGAAATGTCGAGA

DIST. SITE I MUT: *GATC*CGGAAAAGA<u>C</u>AT<u>C</u>AC<u>T</u>AAATGTCGAGA

FULL SITE I (-125/-96): CGGAAAAGAAATGACGAAATGTCGAGAGGGCGGGGACAATTG

FULL SITE I: CGGAAAAGA<u>C</u>AT<u>C</u>AC<u>T</u>AAATGTCGAGAGGGCGGGGACAATTG

(DISTAL MUT)

Special cloning oligonucleotides

Xmn I USE: TTGGAAAACGCTCTTCGGGGCG

ALWN I USE: CGCCACTGGCAGGGCCCACTGGTAACAGG

CR3 linker scanning mutations

(Antisense direction)

+203/+168 MUTANT 1:   CAGGAATTCGCGGGTCTCCTCGTAAATGAGGCCAGAGA

+203/+168 MUTANT 2:   CAGCACACGAATTCTCTCCTCGTAAATGAGGCCAGAGA

+203/+168 MUTANT 3:   CAGCACACCGCGGGGAATTCCGTAAATGAGGCCAGAGA

+203/+168 MUTANT 4:   CAGCACACCGCGGGTCTCCTGAATTCTGAGGCCAGAGA

(Sense strand)

+136/+177 PRIMER: CGTCTAGCTCGGCGTGGCGGCGTTAAGCGGATCTCTGGCCTC

3 minutes at 75°C and 3 minutes at 50°C and 1 cycle of 1 minute at 90°C and 10 minutes at 75°C. The PCR products were phenol/chloroform extracted, ethanol precipitated, washed twice in 70% ethanol and resuspended in 42.5 μl H_2O. The long primers were phosporylated by T4 polynucleotide kinase with 1 mM ATP for 1 hour at 37°C. Kinase reaction was terminated by incubating at 65°C for 30 minutes and free nucleotides were removed by passing through a G-25 Sephadex Quick spin column (Boehringer Mannheim, Indianapolis, IN.). The next step was to combine 34 μl of phosporylated long primers with 50 ng of pH4CARP and 4 μl 10 x reaction buffer (200 mM Tris, pH 7.4, 100 mM $MgCl_2$, 500 mM NaCl). The mixture was boiled for 5 minutes then gradually cooled to room temperature. The second strand synthesis reaction was allowed to proceed by adding 5 μl of mix buffer (16.6 mM Dithiothreitol (DTT), 8.33 mM ATP, 4.17 mM dNTP, 83.3 mM Tris pH 7.4), 3 μl ligase (New England Biolab. Beverly, MA.) and 1 μl of T4 DNA polymerase (Promega, Madison, WI.), then incubating at 37°C for 30 minutes. The reaction was terminated by the addition of 6 μl stop buffer (0.25% sodium dodecyl sulfate (SDS), 5 mM disodium ethylenediaminetetraacetic acid (EDTA), pH 8.0) and incubation at 65°C for 15 minutes. Competent E. coli strain BMH 71-18 mut S (gift from Dr. Jac A Nickoloff) were transformed with 10 μl of the final reaction mixture and grown in 10 mls of LB broth with ampicillin. Plasmid DNA was isolated using boiling minipreparation protocol. Distal Site III mutant H4CARP DNA was digested overnight at 37°C with AlwN I restriction enzyme. Proximal Site III mutant H4CARP DNA was digested overnight at 37°C with restriction enzyme Xmn I. Competent DH5α were transformed with 10 μl of overnight digested DNA and grown overnight at 37°C in 10 ml LB broth with ampicillin. Plasmid DNA was

The pBLCAT4 Site III series. -418 pBLCAT4, -418 Distal Mutant pBLCAT4 and -418 Proximal Mutant pBLCAT4 were designed to test the affect Site III had on transcription from a heterologous promoter. The Klenow blunted Hind III-Sma I fragments from pH4CARP and its derivatives Distal Site III Mutant pH4CARP and Proximal Site III Mutant pH4CARP, were cloned 5' to the thymide kinase promoter in the Klenow blunted Hind III Site In pBLCAT4.

-215 to +270 CAT and -418 to +270 CAT. Reporter plasmids -215 to +270 CAT (pFO108CAT Enhancer) and -418 to +270 CAT, both derivatives of Promega pCAT3-Enhancer vector, were designed to determine if any transcriptional synergy existed between the coding region and Site III of the histone H4 gene. Baruch Frenkel created -215 to +270 CAT (pFO108CAT Enhancer) by first deleting Hind III-Nco I fragment from pCAT3-Enhancer vector and ligating in the 41 bp Hind III-Xba I polylinker from pGEM 7. This intermediate clone was then digested with EcoR I and Nco I and the 500 bp EcoRI-Nco I fragment from pFO108 was ligated into the plasmid to create -215 to +270 CAT (pFO108CAT Enhancer). The vector -418 to +270 CAT was created by digesting -215 to +270 CAT (pFO108CAT Enhancer) plasmid with Sma I and inserting the blunted Sma I -Hind III fragment from pH4CARP.

Histone H4 coding region constructs

-10/+210 PBLCAT, -10/+75 PBLCAT, +75/+210 PBLCAT, and +110/+196 PBLCAT. The heterologous reporter CAT constructs were designed to determine if the coding region of the histone H4 gene harbors any

transcriptional regulatory elements. Inserts were cloned into a Klenow-filled Hind III site upstream of the thymidine kinase promoter using blunt end ligation. The following inserts were derived from a subclone of pFO108, pJUC 67: -10/+210 bp (Bbs I/Xmn I), -10/+75 bp (Bbs-I/Blp I),+75/+210 bp (Blp I/Xmn I) and +110/+196 (Hph I/Sac II).

<u>+72/+110 PBLCAT, +110/+140 PBLCAT, +72/+125 PBLCAT, +72/+140 PBLCAT, +136/+184 PBLCAT, +110/+160 PBLCAT, +123/+158 PBLCAT, +170/+204 PBLCAT, +154/+204 PBLCAT and +136/+204 PBLCAT.</u> A series of heterologous reporter CAT constructs containing oligonucleotides of the histone H4 coding region (see Table 2-1) were designed to delineate the exact locations of regulatory elements within the coding region. +72/+110 PBLCAT, +110/+140 PBLCAT, +72/+125 PBLCAT, +136/+184 PBLCAT, +110/+160 PBLCAT, +123/+158 PBLCAT, +170/+204 PBLCAT and +154/+204 PBLCAT were constructed by cloning their respective oligonucleotides into a Klenow-filled Hind III site 5' of the thymidine kinase promoter in pBLCAT4. +72/+140 PBLCAT was constructed by first ligating oligonucleotides +72/+110 and +110/+140 together, then ligating this product into pBLCAT 4. +136/+204 was constructed by first annealing the +136/+184 sense strand oligonucleotide with the +170/+204 antisense strand oligonucleotide, then filling in the overhangs using Klenow. The double stranded DNA was gel purified and cloned into Hind III site of pBLCAT 4.

<u>+136/+204 PBLCAT linker scanner mutation constructs.</u> Antisense Coding Region 3 oligonucleotides +204/+168 mutants 1, 2, 3 and 4 were

phosphorylated by T4 polynucleotide kinase in 30 µl reactions containing 2 µg of mutant oligonucleotide, 2 µg of the sense strand +136/+178 wild type oligonucleotide, 3 µl 10x NEB ligase buffer, 1 µl 100 mM ATP, 1 µl NEB T4 polynucleotide kinase and H_2O. The reactions were performed at 37°C for 1 hour and terminated by incubating at 65°C for 30 minutes. The oligonucleotides were annealed by boiling the sample for 5 minutes and gradually cooled to room temperature. The oligonucleotides were ethanol precipitated, washed in 70% ethanol, dried and resuspended in 15 µl H_2O. The overhanging single stranded areas were made double stranded by Klenow extension reaction by combining the annealed oligonucleotides with 2 µl 10xNEB DNA polymerase buffer, 1 µl 10 mM dNTP, 1 µl α ^{32}P-dCTP and 1 µl Klenow enzyme and incubating the reaction for 3 hours at room temperature (an extra 1 µl aliquot of Klenow enzyme added 1 hour into the reaction). The fill-in reaction was terminated by adding 4 µl 0.5 M EDTA and 180 µl H_2O then heat inactivating at 65°C for 30 minutes. The samples were extracted with phenol/chloroform, ethanol precipitated, washed twice with 70% ethanol, dried and resuspended in 30 µl TE (10 mM Tris, pH 7.8, 1 mM EDTA). The double stranded DNA was gel isolated on an 8% 0.75 mm native polyacrylamide gel in 1x TAE buffer (40 mM Tris-acetate, 5 mM sodium acetate, 1 mM EDTA). The gel was run at 200 Volts for 3 hours and autoradiographed wet. The bands were excised and soaked overnight in 1 ml TE. The eluted DNA was purified from gel contaminants by Elutip column chromatography. The final DNA product was resuspended in 100 µl H_2O. The DNA containing linker mutations were cloned into pBLCAT4 as above. All constructs were screened by restriction enzyme

digestion pattern analysis and only the positive clones were sequenced. Due to poor quality of oligonucleotide synthesis there are missing nucleotides in some of the clones.

Bacteria cell culture

Host strains and culture. Multiple E. coli host strains were utilized in these studies for various purposes. E. coli strains DH5α (Gibco BRL, Galthersburg, MD) and HB101 were used as host strains to clone and propagate plasmids. The K-12 derived E. coli host strain M15(pRep4) (Qiagen, Chatsworth, CA.) was used for production of His-tagged fusion proteins and the strain BMH 71-18 mut S (gift from Dr. Jac A. Nickoloff, Harvard University School of Public Health) was used to generate site directed mutants.

The bacteria were propagated either in liquid cultures or on Luria Bertani (LB)-agar plates (1.5% agar by weight). LB (1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl) or 2xYT (1.0% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl) liquid cultures (air to liquid ratio > 4:1) were grown at 37°C in a shaking platform incubator, whereas, bacterial plates were grown inverted in a 37°C incubator. When antibiotics were required, they were used at the following concentrations: Ampicillin (50 ug/ml), Kanomycin (25 ug/ml), Tetracycline (15 ug/ml) and Chloramphenicol (10 ug/ml).

Preparation of competent host bacteria. A seed culture was prepared by inoculating 10 ml of LB broth with 25 μl of glycerol stock and growing overnight at 37°C. A prewarmed flask containing 500 ml LB was inoculated with 5 ml of

the seed culture and allowed to propagate until an optical density at 595 nm of 0.4 was obtained. The culture was divided into eight 50 ml plastic conical tubes and chilled on ice for 10 minutes. The bacteria were then centrifuged at 4°C, at 3,000 g for 5 minutes. Each bacterial pellet was resuspended in 10 mls filter sterilized $CaCl_2$ buffer (60 mM $CaCl_2$, 10 mM PIPES, 15% glycerol, pH 7.0) and consolidated into two 50 ml tubes. The bacteria were again centrifuged at 4°C, at 3,000 g for 5 minutes and the supernatant was carefully removed. The pellet was resuspended in 10 ml of $CaCl_2$ buffer and incubated on ice for 30 minutes, then centrifuged as above. Finally, the pellets were resuspended in 2.5 ml of $CaCl_2$ buffer and divided into 100 µl aliquots. The aliquots of competent bacteria were stored at -70°C.

Bacterial Transformation. Competent cells were slowly thawed on ice. DNA (1 to 50 ng) was added to the bacteria, carefully mixed and then incubated on ice for 30 minutes. The bacteria were heat shocked by incubating at 42°C for 1 minute. After the heat shock, the bacteria were given 900 µl of LB broth and allowed to grow at 37°C in a shaking incubator for 45 minutes. Finally, the 50 µl of bacteria transformed by plasmid or all the bacteria (after centrifugation) transformed with ligate DNA were plated onto LB-agar plates containing appropriate antibiotics, then incubated overnight at 37°C.

Isolation and purification of DNA

Maxi-preparation of plasmid DNA Large-scale preparation of DNA was achieved using Plasmid Maxi kit (catalog number 12162) from Qiagen. E. coli bacterial strain DH5α was used as a host for growing all plasmids. A single bacterial colony was picked from a selective plate to inoculate Luria Bertani (LB) medium containing the appropriate antibiotic(s). Culture volumes for growing varied depending on vector being purified. Usually 100 ml culture volume was used for growing high copy number vectors, such as pUC19, pGEM and pBluescript. For low copy number vectors, such as pBR322, the culture volume size was 500 ml. Cultures were grown overnight in a shaking platform incubator at 37oC for 12 to 16 hours. Bacterial cells were harvested by centrifugation at 4oC for 15 minutes at 4,000g using a Beckman JA-10 or JA-14 rotor. The bacterial pellets were completely resuspended in 10 ml of buffer P1 (100 ug/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and transferred to 30 ml Corex tube. Next, 10 ml of buffer P2 (200 mM NaOH, 1% SDS) was added, gently mixed by inverting and incubated at room temperature for exactly 5 minutes, at which time 10 ml of buffer P3 (3.0 M KAc, pH 5.5) was added and gently mixed by inverting. The mixture was incubated on ice for 20 minutes, then centrifuged at 4oC for 30 minutes at 15,000g. The supernatant was applied to a Qiagen-tip 500 that had been equilibrated with 10 ml of QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% ethanol,0.15% Triton X-100). After supernatant finished entering resin by gravity flow, the Qiagen-tip 500 was washed three times with 30 ml buffer QC (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0). The plasmid DNA was eluted off the resin by adding 15 ml of

buffer QF (1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol, pH 8.5) into a 30 ml Corex tube. The plasmid DNA was then precipitated by adding 10.5 ml of isopropanol. Samples were centrifuged for 30 minutes at 4°C at 15,000 g and supernatant carefully removed. The DNA was washed with 15 ml of 70% ethanol, air dried for 5 minutes, redissolved in 400 µl of TE pH 8.0 and transferred to a 1.5 ml Eppendorf tube. The plasmid DNA was then precipitated by adding 20 µl 3 M sodium acetate and 1.0 ml of 95% ethanol. Samples were centrifuged, washed with 70% ethanol twice, air dried and finally resuspended in 200 µl sterile TE pH 8.0.

Alkaline lysis minipreparation of plasmid DNA Small-scale preparations of DNA was achieved by a modified alkaline lysis method described in Maniatis et al. (1989). A single bacterial colony was picked to inoculate 10 mls of LB medium containing the appropriate antibiotic(s) in a sterile, loosely capped 50-ml tube and allowed to grow overnight at 37°C. A total of 3 ml of overnight culture was centrifuge at 14000g for 1 minute and the medium was removed by aspiration using a Pasteur pipette attached to a vacuum line. The bacterial pellet was completely resuspended in 100 µl of ice cold Solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0) and 10 ug/ml lysozyme) by pipetting up and down multiple times. Solution II (0.2 N NaOH and 1% SDS) was prepared fresh and 200 µl of it was added to each tube. The mixture was neutralized by mixing in 150 µl of ice cold Solution III (5 M potassium acetate, pH 4.8) and placing on ice for 5 minutes. The tubes were centrifuged for 5 minutes at 4°C at 14,000g and the supernatant was transferred to a new tube. Proteinase K (15 µg) was added to the supernatant and

incubated at 37oC for 20 minutes. Next, each tube was extracted with phenol:chloroform, ethanol precipitated and washed with 70% ethanol. The DNA pellet was air dried then resuspended in 50 μl of TE (pH 8.0). 1 μl of RNase One (Promega) was added to each sample. Plasmid DNA was stored at 4oC.

Boiling minipreparation of plasmid DNA. Rapid boiling protocol was employed to purify small amounts (5-10 ug) of plasmid DNA. Bacteria harboring plasmid in 10 ml culture were grown overnight at 37oC. 1.5 ml of the overnight culture was centrifuged at 14,000 g for 5 minutes. The media was removed by vacuum asiparation and the pellet was resuspended in 300 μl STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris, pH 8.0) and 25 μl 10 mg/ml lysozyme dissolved in STET. The bacteria was lysed by incubating on ice for 5 minutes then boiling for 40 seconds. Samples were centrifuged for 10 minutes at 14,000g. The pellet was removed with a sterile tooth pick and equal volume of 2X PK buffer (1% SDS, 10 mM EDTA, 20 mM Tris, pH 7.8) and 10 μl of 20 mg/ml Proteinase K was added. The solution was incubated for 30 minutes at 37oC then phenol/chloroform extracted and ethanol precipitated. DNA pellets were washed in 70% ethanol, dried and resuspended in 30 μl of TE with 1.5 μl of 2 mg/ml RNase A. Plasmid DNA was stored at 4oC or -20oC.

DNA sequencing

All clones constructed in this study were verified by sequencing using the

Sequenase Version 2.0 DNA Sequencing Kit (USB). 10 μg of the plasmid was denatured for 5 minutes by addition of 0.4 M NaOH and 0.4 mM EDTA. After neutralizing the reaction with addition of ammonium acetate (pH 4.6) to 0.4 M, the DNA was precipitated by adding five volumes of 95% ethanol and placed on dry ice for 15 minutes then spun in a microcentrifuge for 15 minutes at 14,000 g. The pellet was washed in 70% ethanol and dried. After resuspending the pellet in 5 μl primer (1 pmole), 2 μl of 5X sequencing buffer and 3 μl deionized H_2O, the reaction mixture was incubated for three minutes at 65°C, then gradually cooled to room temperature to allow the primer to anneal to the plasmid DNA. While the samples cooled, 2.5 μl of each dideoxynucleotide termination mixes (ddG, ddA, ddT, ddC) was added to four separate tubes. The following were added to each reaction mix; 1 μl [^{35}S]-dATP (10 uCi/ul, 1000 Ci/mMol), 2 μl of 1:8 Sequenase 2.0 diluted 1:8 in enzyme dilution buffer, 1 μl of 0.1 M DTT and 2 μl of labeling mix diluted 1:5 with deionized water. The reaction mix was incubated at room temperature for 10 minutes then 3.5 μl of the reaction was transferred to each of the four termination tubes (ddG, ddA, ddT and ddC) and incubated for an additional 10 minutes at 37°C. Stop solution (4 μl) was added to each sample and heated at 95°C for 5 minutes prior to loading into shark's tooth wells on a 6% urea polyacrylamide Sequagel sequencing system (National Diagnostic, Georgia). The gels were run at 50 Watts until bromophenol blue reached the bottom. The gel was then vacuum dried and exposed to film overnight.

Quantitation of nucleic acids and proteins

Beckman DU-70 series spectrophotometer was used to quantitate nucleic acid solutions by measuring their absorbance at wavelength of 260 nm (A_{260}). Diluted nucleic acid samples were measured after the spectrophotometer was calibrated at A_{260} and A_{280} against the diluting solution. The nucleic acid concentrations of the original samples were calculated by multiplying the dilution factor with the observed optical density at A_{260} and the appropriate A_{260} conversion constant. The A_{260} conversion constants at an A_{260} of 1.0 for double stranded DNA is 50 µg/ml, RNA is 40 µg/ml and those for single stranded oligonucleotides determined by base composition. The absorbance at the wavelength of 280 nm (A_{280}) was also determined for each sample to examine the quality of the nucleic acid samples. The A_{260}/A_{280} ratio of a pure sample of DNA is 1.8 and 2.0 for a pure RNA sample. A lower ratio is indicative of contaminants (eg., protein and phenol) in the nucleic acid sample.

Protein concentration of nuclear extracts, whole cell extracts and affinity purified fusion proteins were determined by colorimetric Bradford assay. Coomassie Plus Protein Assay reagent (Pierce cat. # 23236) was pipetted into the wells of a 96 well plate with either Pierce bovine serum albumin standards or test samples. Samples and standards were measured using a Molecular Devices Corporation microplate reader at 570 nm.

Tissue culture and DNA transfection

Hela S3 cell culture and transfection. Suspension cultures of HeLa S3 cells (2.8-9 x 10^5 cells/ml) were maintained in Joklik-modified Eagle's minimal medium (SMEM) completed with 5% fetal calf serum, 2% Horse serum, 1 mM L-glutamine, 100 µg/ml streptomycin and 100U/ml penicillin. HeLa S3 cells were transiently transfected by BBS DNA/calcium phosphate method (Ausubel et al., 1994). 100 mm culture plates were seeded with 1 x 10^6 cells in 10 mls Eagle's modified minimum essential medium (DMEM) supplemented with 10% fetal calf serum, 100 ug/ml streptomycin, 100 U/ml penicillin and 1 mM L-glutamine. Cells were incubated 17-22 hours in a 37°C, 5% CO_2 incubator, then refeed with 9 mls completed DMEM. Three hours after being fed, DNA/BBS mixes were prepared and added to the cells. DNA/BBS mixes were prepared by combining a total of 20 µg of plasmid DNA plus 1µg of RSV luciferase plasmid diluted in 500 µl of 250 mM $CaCl_2$ with 500 µl of 2 x BBS solution (50 mM *N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; CALBIOCHEM), 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95). The mixes were incubated at room temperature for 20 minutes and added to the plated cells. The cells were incubated for 20 hours in the presence of $CaPO_4$-DNA precipitates. The plates were washed two times in 15 mls in phosphate buffered saline (PBS, 138 mM NaCl, 1.1 mM potassium phosphate monobasic, 8.1 mM sodium phosphate dibasic, 2.7 mM KCl, pH 7.0) to remove precipitates, then fed 10 mls of completed DMEM. Cells were harvested 72 hours after transfection by washing the cells twice in ice cold PBS and scrapping in 1 ml of TES (40 mM Tris-HCl, 1

mM EDTA, 150 mM NaCl, pH 7.4). Cells were pelleted in a 1.5 ml microcentrifuge tube and store at -20oC until needed for analysis.

IMR-90 normal human fetal lung fibroblasts. IMR-90 cells (from Coriell, Camden, NJ) were maintained in Basal Medium Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum, 100 ug/ml streptomycin, 100 units/ml penicillin and 2 mM glutamine. Cultures were passaged by detaching confluent cells with prewarmed Puck's EDTA-trypsin solution (0.02% EDTA, 0.04% trypsin) from the flask, inactivating trypsin with completed medium and diluting cells 1:3 of the original culture volume into new sterile tissue culture flask(s). Proliferating IMR-90 cells were passaged onto 100 mm plates and harvested 2 to 3 days after plating. Quiescent IMR-90 cells were passaged onto 100 mm plates, allowed grow to confluency, then maintained for 7 to 16 days without being refed.

Drosophila melanogaster Schneider's S-2 cell culture and DNA transfection. Drosophila melanogaster Schneider's S-2 cells were grown at room temperature in Schneider's insect medium (Sigma, St. Louis, MO) supplemented with 10% heat inactivated fetal calf serum. Cells were passaged into new flasks by diluting stocks 1:5, 1:10 or 1:20, depending on the rate of growth. Drosophila Schneider cells were plated at a density of $4X\ 10^5$ cells/well in six-well flat bottom plates (Corning) in 1.8 mL of completed Schneider's insect media. A modified transient transfection protocol (P. Paolo et al. 1983; Chen & Okayama, 1988; Di Nocera & Dawid, 1983) was used to introduce DNA into cultured Drosophila cells. Efficiency of transfection was

normalized by cotransfection with pRSV-Luciferase. Plasmid DNA was first diluted in 100 μl of 250 mM $CaCl_2$, then combined with 100 μl of 2 x BBS solution and allowed to sit at room temperature for 20 minutes. The total 200 μl of DNA/BBS mix was added to each well and left undisturbed after the addition of DNA until time of harvest. The transfected Drosophila cells were scraped off the plate 48-72 hr after transfection, centrifuged for 5 min at 12000 g, washed in 2 mL of cold PBS, and recentrifuged. The cell pellets were stored at -80°C until analyzed. Cells were lysed in 1 X Promega reporter lysis buffer, debris removed by centrifugation and supernatant assayed for chloramphenicol acetyltransferase (Gorman et al 1982) and luciferase activity.

Cell synchronization and whole cell extracts

HeLa cell synchronization. Healthy, exponentially growing cells were diluted with fresh media (SMEM + 5% CS + 2% HS) and 50 X Thymidine (final concentration of 2 mM) to a final density of 3.5×10^5 cells/ml. The first thymidine block lasted for 16 hours, then the cells were centrifuged for 5 min at 600 g and media discarded. The cells were washed once in 200 volumes of incomplete prewarmed media, pelleted, and cells resuspended to a final density of 3.5×10^5 cells/ml in completed media supplemented with 24 uM deoxycytidine. Nine hours later the cells were blocked for a second time for sixteen hours with thymidine then released. Cells were released by being pelleted, washed and resuspended to a final cell density of 3.5×10^5 cells/ml in completed media supplemented with deoxycytidine. The synchronized cells were grown at 37°C

until harvested.

Tritiated Thymidine monitoring of cell synchrony. Two samples of 2 mls of suspension cells placed in 15 ml conical tube with 10 µl of (^{3}H)Thymidine (1 uCi/ul) (20 Ci/mmol) on a spinning wheel for 30 minutes at 37oC. Cells were centrifuged for 5 min at 600g, media removed. Cells were then washed twice in ice cold PBS followed each time by centrifugation. Cell pellet were resuspended in 5 mls ice cold 10% trichloroacetic acid (TCA) and left on ice for 5 to 10 minutes. Cells were pelleted, resuspended in TCA and incubated on ice. Cells were pelleted then resuspended in 1 ml 10% SDS at room temperature for one minute and then transferred to a vial with 16 mls of Ecolume scintillation cocktail. Radioactivity levels were quantitated by scintillation spectrometer.

Hela whole cell extracts. 400 mls of suspension grown Hela cells (3.5 x 10 ^{5} cells/ml) were harvested per time point in cell synchrony (0, 2, 4, 6, 8, 11, 15, 24 & 28 hrs after release from a double thymidine block.). Cells were washed two times in 1xPBS. The cell pellets were resuspended in three volumes of WCE buffer (20 mM HEPES-HCl pH 7.5, 20% glycerol, 0.5 M KCl, 0.2 mM EDTA, 0.01% NP-40, 0.5 mM phenylmethyl-sulfonylfluoride (PMSF) and 0.5 mM DTT) in 15 ml conical tubes. The pellets were easily resuspended with a few stokes of mini pestle. The cells were immediately frozen in liquid nitrogen (stored at -70oC). The frozen cells were then thawed at 37oC in a water bath. Again cells were resuspended with a few strokes of mini pestle. Samples were split into 1.5 ml tubes and centrifuged 5 minutes at 15,000g at

4oC. The supernatant was carefully transferred to new tube, aliquotted, protein concentration determined, frozen in liquid nitrogen and stored at -70oC.

Chloramphenicol acetyltransferase (CAT) assay

To monitor the level of gene expression in transient transfection experiments, CAT assays were performed as according to Gorman et al.,1982. Transfected HeLa cells were washed twice with ice cold phosphate buffered saline solution and then scrapped in 1 ml of TES (40 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 7.4) with disposable cell lifter (Fisher). Cells were transferred into a microcentrifuge tube and centrifuged for 5 minutes at 14,000g. Supernatant was removed by vacuum aspiration and pellet was completely resuspended in 200 μl of 1X Promega reporter lysis buffer. After incubating in lysis buffer for 40 minutes, the debris was removed by centrifugation for 5 minutes and supernatant transferred into new tube. This supernatant was stored at -20oC until used in a CAT assay reaction.

The CAT assay reactions were prepared by combining transfected cell supernatant, 2.5 μl Chloramphenicol D-Thero (Dichloroacetyl-1,2-^{14}C) (57 mCi/mmol) (NEN), 20 μl 4 mM acetyl CoA (Sigma) and adding 1 M Tris-HCl (pH 7.8) to a final concentration to 0.25 M. Reactions were incubated at 37oC for 1 to 16 hours. To terminate the reaction, 1 ml of ethyl acetate was added, mixed, centrifuged and the organic layer transferred to a new tube. The samples were lyopholized by Speedvac centrifugation for 1 hour, resuspended in 20 μl ethyl acetate, spotted onto Whatman silica gel thin layer chromatography plates and chromatographed in a chamber equilibrated with chloroform:methanol (95:5

volume/volume). All experimental samples were analyzed directly from the developed plates by using a Beta emission analyzer, Betascope 603 (Betagen, Mountainveiw, CA). CAT activities were normalized for transfection efficiency using the luciferase data. Each CAT assay was performed in triplicate with at least two different plasmid preparations for a minimum of two independent experiments. Statistical significance was determined by using Dunnett (2-sided) t-test.

Luciferase assay

All cells that were transiently transfected with test CAT reporter constructs were also cotransfected with the luciferase reporter construct, RSV-LUC, to normalize for transfection efficiencies. Luciferase assay substrate and 10 ml of Luciferase Assay buffer from Promega's Luciferase assay system were combined to give a solution with the composition as follows; 270 uM Coenzyme A (lithium salt), 470 uM Luciferin, 530 uM ATP, 20 mM Tricine, 1.07 mM $(MgCO_3)_4 Mg(OH)_2 \cdot 5 H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, pH 7.8. Luciferase reactions were initiated by combining 100 µl of Luciferase Assay Reagent with 20 µl of transfected cell extract (both components at room temperature). The light produced from the reaction for the first 30 seconds was measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Preparation of nuclear extracts

Hela nuclear extracts. HeLa S3 cells were grown in suspension culture at 3.5 X10^5 cells/ml. Cells were harvested by centrifuging at 1,500 g for 5-10 minutes, washing the with cold PBS, transferring to 50 ml tubes and centrifuging again. The solution was removed and the pellets resuspended in 5 cell pellet volumes of buffer A (10 mM 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid (HEPES), 0.75 mM spermidine, 0.15 mM spermine, 10 mM KCl, 1 mM DTT, 1 mM [Ethylenebis(oxyethlenenitrilo)] tetra acetic acid (EGTA) and protease inhibitors). Cells were allowed to swell in the hypotonic buffer on ice for 10-30 minutes. The swollen cells were centrifuged for 10 minutes, resuspended in fresh buffer A and transferred to a dounce homogenizer. Ten strokes from the type A pestle in the dounce homogenizer were used to lyse the cells. The lysed cells were transferred to a 30 ml corex tube, centrifuged at 1,500 g for 15 min, supernatant removed, centrifuged at 10,000 g for 30 minutes and the remaining supernatant discarded. The remaining nuclei were resuspended in 1 pellet volume of buffer C (20 mM HEPES, 25% Glycerol, 0.42 M KCl, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT, 1 mM EGTA, 0.2 mM EDTA and protease inhibitors), Dounce homogenized with 5 strokes of type B pestle and transfer to 15 ml corex tube. The suspension was agitated with a magnetic stirrer on ice in cold room for 30 min. The extracted proteins were separated by centrifuging at 15,000g for 15 minutes. The nuclear extracts were carefully collected, transferred to new tubes, frozen in liquid nitrogen and stored at -70°C.

Proteases and their final concentrations used in these studies are as follows: PMSF, 0.5 mM; Leupeptin, 0.5 ug/ml; Pepstatin, 1.0 mg/ml; TPCK, 10

µg/ml; Trypsin inhibitor, 2 µg/ml; Bestatin, 4 µg/ml; Calpain, 17µg/ml; E64, 1 µg/ml.

Preparation of glutathione-S-transferase (GST) or His-tagged fusion proteins

His-tagged fusion proteins were affinity purified following a protocol similar to Shi et al. 1991, using Qiagen nickel resin kit. Vectors encoding the His-tagged proteins were first transformed into competent E. coli strain M15(pRep4). A single colony was picked to inoculate an overnight culture of 10 ml LB broth with ampicillin (100 µg/ml) and kanomycin (25 µg/ml). A larger culture was initiated by inoculating 100 ml LB broth (containing above antibiotics) with 2 ml of overnight culture. The culture was grown at 37°C until an optical density at 595 nm of 0.8 was observed then synthesis of the His tagged fusion protein was induced by the addition of isopropylthio-β-D-galactoside (IPTG) (final concentration 1mM). The bacteria were grown in the presence of IPTG for an additional 2 hours, then centrifuged for 10 minutes. The pellet was resuspended in phosphate buffered saline solution and centrifuged again. The washed pellets were resuspended in 6 M guanidine-HCl pH 8.0 and left on ice overnight to lyse. Bacterial debris was removed by centrifugation and supernatant transferred to new tube. Qiagen nickel chelate spin column was equilibrated 600 µl of 6 M guanidine pH 8.0 for 2 minutes at 1000 g. Bacterial supernatant was added to the column then spun. The columns were washed twice with 600 µl of 6 M guanidine pH 6.3. The his-tagged proteins were eluted by twice adding 200 µl 6 M guanidine pH 4.5. The

fusion proteins were renatured by dialyzing in colloidal bags. The proteins were first dialyzed for 3 hours against two exchanges of 1 M guanidine, 0.05 M Tris pH 8. The proteins were then dialyzed for 2 hours against two exchanges of 0.1 M guanidine, 0.05 M Tris pH 8.0, followed by two exchanges of ice cold PBS. Finally the fusion proteins were centrifuge concentrated using Amicon's Centricon 10. The concentrated proteins were aliquotted, frozen in liquid and stored at -70ºC.

Marleen de Ridder provided all the GST and GST/CDP (CR2-Cterm) fusion proteins used in these studies. She affinity purified the GST/CDP (CR2-Cterm) (Lievens et al., 1995), a glutathione-S-transferase (GST) protein fused to the C-terminal 100 kDa region of human CDP, from bacterial lysate after induction by isopropyl-b-D thiogalactoside, using glutathione beads.

Electrophoretic mobility shift assay (EMSA)

EMSA and probe preparation were performed essentially as described by van Wijnen et al. (1996). Binding reactions were performed by combining 10 µl of a protein mixture (in 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, and 25 mM Hepes, pH 7.5) with 10 µl of a DNA mixture (10 fmol ^{32}P-γ - ATP end labeled DNA probe and nonspecific DNA) for 20 minutes. The coding region probes used in methylation interference assays and EMSA experiments for identification of repressor complexes were dimethylsulfate treated. Competition assays were performed by adding 1 pmol of unlabeled specific oligonucleotide to the reaction. When antibodies were included in the binding reaction, 1 µl of antiserum was added to the 10 µl of

protein mixture and incubated on ice for 20 minutes to 2 hours prior to combining with DNA mix. The reaction mixtures were then electrophoresed on 4% polyacrylamide (80:1) gels or 4% polyacrylamide (40:1) gels in 0.5 X TBE (45 mM Tris-borate, 1.25 mM EDTA). Nucleosome binding EMSA experiments were performed using a modified protocol of Li et al. (1994).

DNase I footprint assay

A modified DNase I footprinting protocol was performed as described in Current Protocols, 1989. Nuclear extracts/recombinant protein and KN100 mix (20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, and 25 mM Hepes, pH 7.5) (total 25 μl) was combined with 1 μg non-specific DNA (poly dI-dC), 5 fmole of single end radiolabeled DNA probe and varying amounts of BSA and H_2O. Final volume of reaction was 50 μl. Binding reactions were incubated at room temperature for 20 minutes, after which time, 5 μl of DNase I digestion mix (1 μl DNase I (Promega), 1 μl of 0.4 $MgCl_2$ and H_2O) was added for 1 minute. The reaction was inhibited by the addition of 100 μl Stop buffer (1% SDS, 30 mM EDTA, 0.1 mg tRNA, 200 mM NaCl and 15 μg Proteinase K) and placed immediately on ice. Samples were then incubated at 37oC for 20 minutes, then phenol:chloroform:isoamyl alcohol extracted once. Supernatants were transferred to a new tube and ethanol precipitated. The samples were resuspended in formamide loading dye, heated at 90oC for 5 minutes and electrophoresed on a 6% denaturing urea polyacrylamide gel.

Methylation interference assay

Methylation interference assays were performed to identify the binding site and important G residue contacts of transcription factor interactions on the regulatory elements. EMSA probes were partially methylated by incubating probe with 1 µl of dimethylsulfate (DMS) in 200 µl of DMS reaction buffer (50 mM sodium cacodylate, 1 mM EDTA, pH 8.0) for 5 minutes at room temperature. The reaction was terminated by the addition of 50 µl of DMS stop solution (1.5 M sodium acetate, pH 7.0, 1 M β-mercaptoethanol, 10 µg poly(dI-dC) DNA) and 750 µl of cold 95% ethanol. Probe was precipitated by centrifugation at 14,000g for 20 minutes. Pellet was washed in 70% ethanol, air dried then resuspended in 100 µl of TE.

EMSA experiments were performed using the methylated probe to isolate protein/DNA complexes of interest. Normal EMSA experimental conditions were used except that 3 to 6 EMSA binding reactions were loaded into each lane on the EMSA gel. After electrophoresis, the wet gels were wrapped in plastic wrap and autoradiographed overnight at 4°C. The gel regions which aligned with the autoradiograph for the free probe and protein/DNA complexes were sliced out of the gel with a razor blade and immediately electroeluted using the Elutrap apparatus for 2 hours at 200 V. The electroeluted probe DNA was ethanol precipitated, washed twice in 70% ethanol, air dried, then resuspended in 100 µl of deionized H_2O. The samples were incubated at 90°C for 10 minutes, then 10 µl of 11 M piperidine was added to each. These G>A residue cleavage reactions were carried out for 30 minutes at 90°C and then immediately frozen on dry ice. The samples were lyophilized

in a Speedvac, resuspended in 100 µl dH$_2$O, placed on dry ice and again lyophilized. After samples were dried, they were resuspended in 100 µl H$_2$O, frozen on dry ice and lyophilized again. The pellet was then resuspended in 20 µl of formamide loading buffer and an aliquot was taken to determine radioactivity by scintillation counting. The G ladders were resolved on 6-8% urea polyacrylamide gels, with equal amounts of radioactivity loaded into each lane. The gels were vacuum dried and autoradiographed.

Histone purification

Core nucleosome histones were isolated by hydroxylapatite chromatography using the protocol by Workman et al. (1991). HeLa cells were maintained in suspension and harvested by centrifugation for 5 minutes at 1,500g and washed twice with cold phosphate buffered saline. The cell pellets were lysed by resuspending in 20 pellet volumes of lysis buffer (10 mM Tris-HCl, pH 8.0, 3 mM MgCl$_2$, 0.25 M sucrose, 1% NP40, 0.5 mM PMSF). Lysates were homogenized with several strokes with a type B pestle in a Dounce homogenizer. The nuclei were centrifuged for 20 min. at 3,600 g, washed twice in lysis buffer and then washed twice in rinse buffer (same as lysis buffer but omitting NP40). The nuclei (equivalent to 12 mg of genomic DNA) were resuspended in 50 ml of LSB (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM PMSF) and gently stirred for 15 min at 4°C. The suspension was centrifuged for 15 min at 3600 g and the pellet was washed with LSB. The concentration of resuspended nuclei was determined by diluting an aliquot in 2 M NaCl. Diluted samples were quantitated in a spectrophotometer by

measuring the asorbance at 260 nm. The nuclei were centrifuged and resuspended in 20 ml of MSB (0.6 M NaCl, 50 mM NaPO4 pH 6.8, 0.5 mM PMSF). The mixture was gently stirred to lyse the nuclei. While the nuclei were stirring, 5 to 20 g of dry Bio-gel HTP hydroxylapatite resin was slowly added to form a paste. MSB was added to the nuclei mix until a final volume of 40 ml and stirred slowly for 10 min at 0°C. The hydroxylapatite-immobilized chromatin was collected by low-speed centrifugation and washed five more times with MSB buffer to remove H1 histones. Core histones were eluted from the hydroxylapatite resin by resuspending each pellet in 40 mls of HSB (2.5 M NaCl, 50 mM $NaPO_4$ pH 6.8, 0.5 mM PMSF) and gentle stirring for 20 min at 0°C. Hydroxylapatite resin was collected by low speed centrifugation and the supernatant containing the core histones was transferred to a sterile container. The resin was washed by adding another 40 ml of HSB, the suspension was stirred for 20 min at 0°C and again centrifuged. The wash solution was combined with the supernatant and concentrated by ultrafiltration (Centricon-10, Amicon). The quality of the core histone were evaluated by 15% SDS-PAGE. The concentrated core histones were aliquoted, frozen in liquid nitrogen and stored at -70°C.

Nucleosome Reconstitution

Nucleosomes were reconstituted *in vitro* on Mfe I-Blp I end labeled histone H4 probe fragment using a modified protocol documented in Li et al. (1994). The reagents (75 µg of purified Hela core histones, 75 µg of salmon sperm and 200 ng of end labeled probe) were combined together in a final volume of 50 µl with a final concentration of 1 mg/ml bovine serum albumin, 2 M

NaCl, 10 mM Hepes pH 7.5, 1 mM EDTA and 1 mM 2-mercaptoethanol. The mixture was dialyzed for 2 hours against 200 ml of (2 M NaCl, 10 mM Hepes pH 7.5, 1 mM EDTA and 2 mM 2-mercaptoethanol) in 3 kD dialysis tubing. 600 ml dialysis buffer (10 mM Hepes pH 7.5, 1 mM EDTA and 2 mM 2-mercaptoethanol) was slowly added drop wise using a peristaltic pump in the cold room over a 15 hr time period to lower the salt concentration to 500 mM. Samples were then dialyzed for 2 hours against 200 ml of 250 mM NaCl, 10 mM Hepes pH 7.5, 1 mM EDTA, and 2 mM 2-mercaptoethanol). The reconstituted core particles were further purified from free and packaged DNA by separating samples on sucrose gradients. The samples were loaded on a 5 to 25% linear sucrose gradient that contains 100 mM KCl, 10 mM Hepes pH 7.5, 1 mM EGTA, 0.1% NP40 and 0.1 mM PMSF. Samples were centrifuged overnight in a Beckman SW55 rotor at 4oC at 34,500 rpm. Fractions were collected from the gradients and monitored for nucleosome complexes by determining cpm in a scintillation counter. Quality of the fractions were tested by native 4% PAGE using 0.5 X TBE as the running buffer. The peak fractions were pooled together, aliquoted into small tubes, frozen in liquid nitrogen, and stored at -80oC. Aliquots were thawed only once at the time of use. Binding reactions contained a mixture of 5 µl (25 fmol) nucleosome core or naked DNA probe in sucrose buffer.

CHAPTER III

CHARACTERIZATION OF SITE III OF THE HISTONE H4 PROMOTER

Abstract

The histone H4 promoter region Site III (-418 nt and -215 nt) has been characterized as both a positive and a negative regulatory element (Kroeger et al., 1987; Larson et al., 1989; Zahradka et al., 1993). One major *in vitro* protein/DNA complex, H4UA-1, has been shown to interact within this region (van der Houven van Oordt et al., 1992). The results presented in this chapter show that Site III interacts with other factors *in vitro*. These include distal factor (NF1-like), AP-2, and an AP-2 like factor. I have identified a Site III-like element within the coding region (+150 to +183 nt) which also interacts with distal factor and H4UA-1. Transient transfection analyses with three reporter construct series testing the role of Site III sequences on transcription show that the sub-region between -418 and -215 nt has a neutral effect on promoter activity. Potential physiological roles for Site III are discussed.

Introduction

The expression of cell cycle regulated histone genes is modulated by transcription factors interacting with multiple distinct regulatory elements in the

genes. Our laborarory has defined through both *in vitro* and in cellular analyses numerous transcriptional regulatory elements in the FO108 histone H4 gene promoter. These elements are arranged as follows: Site II (-20), Site I (-90), Site III (-320), and Site IV (-589).

Site II (-70 to -20 nt) has been defined as the minimal promoter required to direct site specific initiation and is necessary for cell cycle control. Site II consists of the TATA box and multiple protein/DNA complexes including HiNF-D (complex containing CDP/cut, cdc 2, cyclin A and RB), IRF 2 (HiNF-M) and HiNF-P (van Wijnen et al., 1991; van Wijnen et al., 1994; van Wijnen et al., 1996; Ramsey-Ewing et al., 1991; Vaughan et al., 1995; Vaughan et al., 1998). IRF 2 interacts with the cell cycle control element in Site II, activates transcription two-fold, and appears to be an essential cell cycle regulatory factor (Ramsey-Ewing et al., 1991; Vaughan et al., 1995). HiNF-D binding activity is regulated during the cell cycle in normal diploid cells, peaking in S phase. However in tumor cells, HiNF-D binding activity is constitutive throughout the cell cycle (Holthuis et al., 1990). The role of HiNF-D in cell cycle regulation of histone expression is not known. HiNF-D binding activity decreases upon differentiation (Stein et al., 1989). This correlates with the loss of occupancy of the H4 Site II within the cell during the onset of differentiation and down-regulation of H4 gene transcription (Stein et al., 1989).

Site I (-124 to -90 nt) functions as a positive regulatory element of histone transcription (Birnbaum et al, 1995). Transcription factors Sp-1, Sp-3, CREB, ATF and HiNF A (HMG related) interact with Site I (Birnbaum et al., 1995; Birnbaum et al., 1996; Wright et al., 1995; Guo et al., 1997; van Wijnen et al., 1987). A far distal activator region, Site IV (-730 to -589 nt), stimulates

transcription approximately 10-fold (Wright, 1990). YY1/NMP-1, ATF/CREB, H4UA-3, and H4UA-4 form protein/DNA interactions with Site IV (Wright, 1990; Guo et al., 1995; Guo et al., 1997). There may be other unidentified regulatory elements for the histone H4 gene.

Site III (-418 to -215) was initially observed as a positive transcriptional regulatory element by Kroeger et al. (1987). They constructed a 5' deletion series of the histone H4 gene FO108, which was either transiently transfected into mouse LTK⁻ cells or stably transfected into C127 mouse cells. Results from S1 nuclease protection analysis on RNA isolated from stable cell lines or from the transiently transfected cells showed that the construct FO 005, which contains 418 nucleotides upstream from the cap site, was capable of increasing expression 2.5-fold over that observed for the construct FO 108A (up to -215 nt). Wright (1990) tested a series of histone H4 promoter constructs for their efficiency of transcription *in vitro*. *In vitro* transcription assays were performed in a cell free transcription system utilizing supercoiled DNA templates and HeLa S3 nuclear extracts, which are competent for initiation of transcription accurately from the histone proximal promoter. Results of the S1 nuclease protection assays of the cell free transcription reactions showed that the addition of Site III (FO 005) resulted in a two-fold increase in the rate of transcription initiation above that observed for the proximal promoter construct (FO 108X).

The histone promoter region between -200 and -600 nt, which includes Site III, was postulated by our laboratory as a potential cell cycle control region. Chrysogelos et al. (1989) showed that the 5' flanking region up to approximately -600 base pairs is sensitive to both DNase I and S1 during mid S

phase, while during mitosis/G1 accessibility to these enzymes is greatly reduced. However, Ramsey-Ewing et al. (1994) did not observe any differences during the cell cycle when they compared levels of transcription of cells transfected with constructs that either contained or lacked the Site III region. These results do not completely rule out that Site III may respond to, rather than regulate the changes in expression of the histone H4 gene during the cell cycle.

Site III has been implicated by other laboratories as a transcriptional repressor element in quiescent or differentiated cells (Larson et al., 1989; Zahradka et al., 1993). According to Larson et al. (1989), *in vitro* transcription analysis using a pFO 005 construct and myotube extracts shows a level of transcription 60 % less than *in vitro* assays using myoblast extracts. The levels of transcription for the construct lacking -215 to -418, pFO 108A, were the same when myotube or myoblast extracts were used. They also observed that pST519, an histone H3 construct containing 900 bp 5' of initiation, also had decreased levels of *in vitro* transcription with myotube extracts. They suggest that a common repressor factor may be involved in both promoters. Zahradka et al. (1993) argue that the region between -215 and -418 is involved in transcriptional repression in 3T6 cells in a G_0 state. They compared pFO005 with pFO108A constructs using nuclear extracts from quiescent or proliferative 3T6 cells in *in vitro* transcription assays. Their EMSA results show differences in protein/DNA complexes when they compare extracts of cells from G_0, G_1 and S phase after release from serum starvation. The -215 to -418 EMSA probe formed one detectable band in G_0 extracts and two bands in G_1 and S extracts. These bands do not appear to be related to H4UA1, because H4UA1 has been shown to be constituative during the cell cycle (van der Houven van Oordt et al.,

1992).

H4UA-1 binding to Site III has been well characterized *in vitro* by our laboratory (Wright, 1990; van der Houven van Oordt et al., 1992). DNase I footprint analysis of H4UA-1 shows that H4UA-1 binds between -360 and -334 nt in Site III. Nucleotide residue contacts for H4UA-1 were determined by methylation interference and copper-phenanthroline protection analyses. The copper-phenanthroline footprint for H4UA-1 spans nucleotides -358 to -332 with two distinct regions protected (-358 to -346 nt and -341 to -332 nt). Enhanced susceptibility to cleavage of residues at either border of the footprint was also observed. Methylation interference analysis on the H4UA-1 complex indicated that the guanine residues -342, -341, -339, -338, -332 on the lower strand and -344 on the upper strand were in close proximity with H4UA-1. In van der Houven van Oordt et al. (1992) Site III was further defined as the region between -373 and -320 nt with Distal Site III region as -367 to -348 and H4UA-1 binding to the Proximal Site III region (-354 to -326 nt). This study also showed that the formation of the H4UA-1 complex is sensitive to acid phosphatases and low concentrations of zinc (0.75 mM). Furthermore, nuclear extracts from differentiated rat calvarial osteoblasts have less H4UA-1, but more H4UA-1 b (a related, faster migrating complex) binding activity than extracts from proliferating osteoblasts. Prior to the present study, no other *in vitro* protein/DNA complexes were characterized for this region.

The results presented in this chapter show that Site III does not alter transcription when compared to reporter constructs lacking Site III. I also identified a region within the coding region of the histone gene which has high sequence homology to nucleotides -366 to -333 within Site III, and forms similar

protein/DNA complexes *in vitro*. Furthermore, NF-1 and AP-2 related complexes were shown to interact with the distal half of Site III. The data presented in this study do not completely rule out Site III as a repressor element in non-proliferating cells.

Results

Multiple protein/DNA interactions with Site III *in vitro*.

Previous *in vitro* analysis of the histone regulatory element Site III by our laboratory identified H4UA-1 as the major protein/DNA complex within this region. To determine whether Site III can form other *in vitro* DNA/protein complexes, further analysis by EMSA was performed. Figure 3-1 shows that an oligonucleotide probe to a sub-region of Site III (-368 to -324 nt) forms complexes other than H4UA1. A set of closely migrating bands (named the distal factor) was specifically competed by an oligonucleotide for the distal side (-370 to -341) of Site III but not by the proximal side (-349 to -322) of Site III. The proximal site III mutant oligonucleotide appears to partially compete the distal factor. This is not surprising since proximal mutant oligonucleotides contain unmutated sequences which are necessary for distal factor binding (see Table 2-1). A slower migrating band (named proximal c) was specifically competed by an oligonucleotide for the proximal side of Site III. The predominant, fast mobility complex previously identified as H4UA-1 was specifically competed by an oligonucleotide for the proximal side of Site III. Competition with proximal oligonucleotide also causes an increase of distal factor binding. While these results confirm that H4UA-1 is the predominant

Figure 3-1. H4 Site III forms multiple protein/DNA complexes *in vitro*. To determine if Site III interacts with factors other than H4UA-1, EMSA analysis was performed using a Site III radiolabeled oligonucleotide probe (-368 to -324) and 5 μg HeLa nuclear extract. Oligonucleotide competition was performed to identify the specific complexes. Distal Site III (distal), Distal Site III mutant (distal mutant), Proximal Site III (proximal), and Proximal Site III mutant (prox. mutant) 100 fold excess oligonucleotide competions are marked above the lanes (1 pmol). Three major complexes are observed after separation on 4% native PAGE in 0.5 X TBE running buffer. The proximal c complex, distal factor and H4UA-1 are indicated.

Distal
Distal mutant
Proximal
Prox. mutant

<- prox. c

<- distal factor

<- H4UA-1

Site III probe
(-368 to -324)

complex, they also show other protein/DNA complexes can form on Site III. Furthermore, H4UA-1 and the distal factor may bind to Site III in a mutually exclusive manner.

Further characterization of the distal factor interaction with the Site III binding element was necessary to determine its functional relevance. Methylation interference assays were performed to identify which guanine residues the distal factor may contact. Partially purified distal factor was used in EMSA to avoid contamination of the closely migrating H4UA-1. The distal factor was partially purified from HeLa nuclear extracts first by P11 phosphocellulose chromatography (the distal factor eluted between 50 mM and 300 mM KCl) followed by hydroxyapitite chromatography (the distal factor eluted between 100 mM and 300 mM phosphate buffer). The distal factor complex on the dimethylsulphate (DMS) treated pGW-2 (van der Houven van Oordt et al., 1992) Hind III/Msp I (-418 to -278) promoter fragment probe was isolated from a preparative EMSA gel by electroelution. The probe was then purified, cleaved by piperidine and analyzed on a 6% denaturing urea polyacramide gel. Results from the methylation interference assays (Figure 3-2) show that the distal factor interacts with the guanine residues at -354 and -350 nt on the coding strand, and -347, -342 and -341 on the non-coding strand. The most striking conclusion from these results is that the distal factor binding element partially overlaps that of H4UA-1 (van der Houven van Oordt et al., 1992) (Figure 3-3). EMSA results (Fig. 3-1) show that H4UA-1 and distal factor do not to bind simultaneously to the Site III probe. Thus, the distal factor may compete with H4UA-1 for binding to Site III, which is consistent with the results from the methylation interference assays and EMSA analysis.

Figure 3-2. Methylation interference analysis of the distal factor interactions

with Site III. Binding reactions containing partially methylated MspI/Hind III (-

418 to -278 bp) upper or lower strand radiolabeled probes and partially purified

distal factor fraction were separated by EMSA. Free and distal factor bound

probes were electroeluted from the gel, purified, cleaved by piperidine and

resolved on 6% polyacrylamide sequencing gel. Separated cleaved free (F)

and distal factor bound (B) probes are shown. Guanine residue interactions are

indicated on the side of the gels by arrows. Sequence of Site III and the distal

factor interactions are shown below the methylation interference

autoradiographs.

Lower   Upper

F   B   F   B

-363

-->

-->   -333

<--

<--

-327

-368
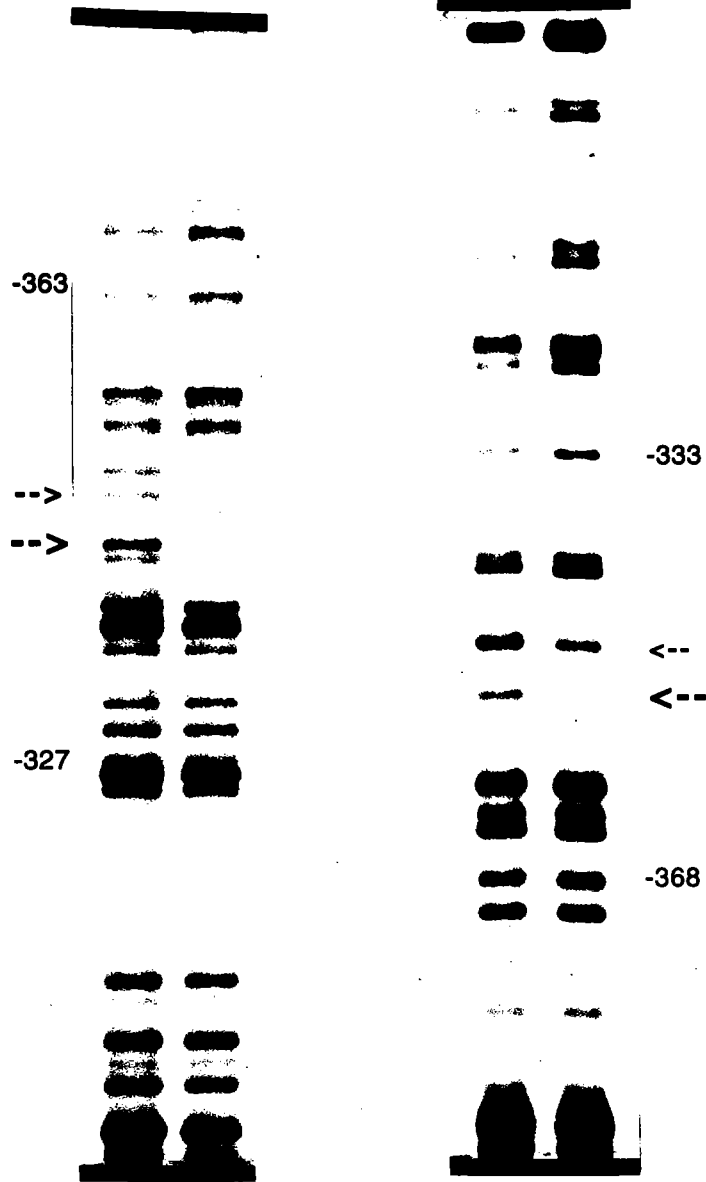
```
        v        v
GGCCTCCTGCCAGTCTCTGGCCTCC
CCGGAGGACGGTCAGAGACCGGAGG
          ^      ^^
```

Figure 3-3. The distal factor and H4UA-1 have overlapping binding sites.
Sequence for Site III (-368 to -337 nt) is shown with the distal factor and H4UA-
1 (van der Houven van Oordt et al., 1992) binding elements separately
underlined. Nucleotide interactions are indicated by "D" (distal factor only), "B"
(both H4UA-1 and distal factor) or "H" (H4UA-1 only) above or below the
nucleotides.

Distal factor and H4UA-1 have overlapping binding sites

-368

-337

```
          D  DD       H
GCTGGGCGGCCTCCTGCCAGTCTCTGGCCTCCATTGCTCTTC
CGACCGCCGGAGGACGGTCAGAGACCGGAGGTAAACGAGAAG
          DD   BB  HH        H
```

KEY:
D = Distal factor contacts
H = H4UA-1 contacts
B = Contacts that both Distal factor and H4UA-1 have in common

AP-2 and NF1 like proteins bind to Site III *in vitro.*

Although three protein/DNA complexes interacting at Site III were characterized, the exact identities of these factors were not known. In an attempt to identify the distal factor and H4UA-1, Site III sequences were compared against the transcription factor data base of Genetics Computer Group, Inc. (GCG) (Madison, Wisconsin). Results from the search identified many potential binding elements between -420 nt and -215 (Figure 3-4). A consensus TATA box element was identified between -420 and -410. Drosophila Zeste and Biocoid regulatory elements, ETS 1, Sp1, CP2, PEAE 3 and NFkB elements were also identified. These motifs do not align with any characterized *in vitro* complexes for Site III. Unfortunately, no matches for the H4UA-1 binding element were found. The distal factor element did match with a NF-1 consensus binding motif (T/CGGA/CN5-6GCCA). Also, an AP-2 motif which partially overlaps the 5' side of the NF-1 motif was identified. Therefore, the distal factor may be related to the NF-1 family of transcription factors.

To test whether the distal factor was related to NF-1, EMSA analyses using Site III and NF-1 consensus binding site oligonucleotide probes were performed. Results from EMSA using the Site III oligonucleotide probe show that the distal factor band was specifically competed by an excess of Distal Site III oligonucleotide and only partially competed by an excess of NF-1 consensus binding oligonucleotide (Figure 3-5A). These results appear to be reproducible in other experiments. The results in Figure 3-5B show that complexes formed on the NF1 oligonucleotide probe were competed by an excess of unlabeled NF1, Distal Site III and Site III oligonucleotides, though not by an excess of Distal Site III mutant oligonucleotide. These results suggest that the distal factor

Figure 3-4. <u>Potential transcription factor binding elements for Site III identified</u> <u>by search of GCG transcription factor data base.</u> Schematic of potential transcription factor binding Site In Site III. An asterisk indicates the relative location of either the NF1 or the AP-2 sites.

Potential transcription factor binding elements for site III identified by search of GCG transcription factor data base

TATA box

Biocoid

NF-1*

-340 nt

H4UA1

AP-2*

Zeste

SP-1

PEAE 3

SP-1

ETS 1

-260 nt

CP2

ETS 1

NF-KB

-420 nt

-215
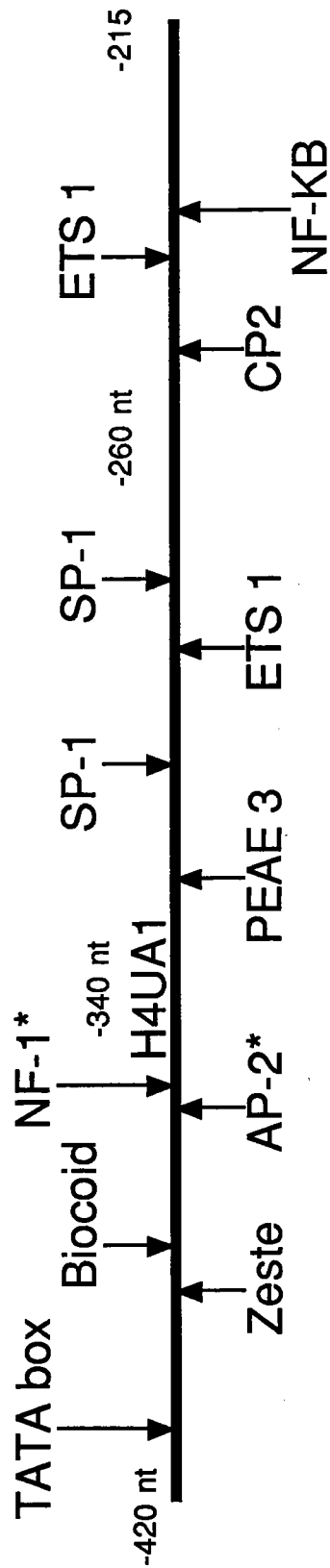
Figure 3-5. <u>NF1 may interact with Distal Site III.</u> (A) EMSA binding reactions using the Site III (-368 to -324 nt) oligonucleotide probe and 5 µg HeLa nuclear extracts were resolved on 4% native polyacryamide gel in 0.5 X TBE running buffer. (B) EMSA binding reactions using the NF1 oligonucleotide probe and 5 µg HeLa nuclear extracts were resolved on 4% native polyacryamide gel in 0.5 X TBE running buffer. Oligonucleotide competition with either 0.5 pmol (a) or 1.0 pmol (b) of unlabeled oligonucleotide added to binding reactions were performed. Site III (-368 to -324 nt), Distal Site III (distal), distal factor mutant binding site (distal mut.), Proximal Site III (proximal) and NF1 EMSA oligonucleotide competitions are marked above their lanes.

A

Site III | Distal | Proximal | NF1
a b | a b | a b | a b

Site III Probe

B

Distal | Distal mut. | NF-1 | Site III
a b | a b | a b | a
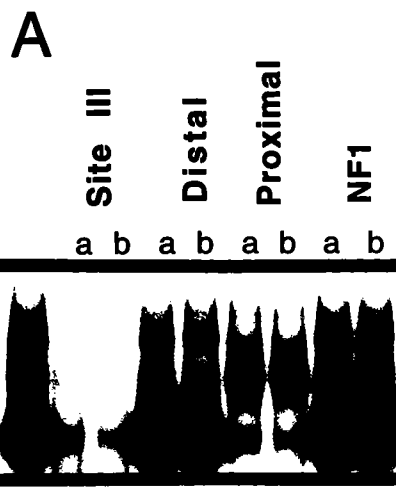
NF1 Probe

might be related to NF1 based on DNA binding competition. Further analysis

was difficult at the time, since there are at least 6 different NF-1 related proteins,

and the reagents (antibodies, purified proteins, etc.) to identify NF-1 were

unavailable.

Comparison of the Site III region against the GCG transcription factor

binding site data base also identified a potential AP-2 binding element (-366 to

-357). EMSA analyses were performed to determine whether Site III complexes

formed on an AP-2 probe (Fig. 3-6A) and whether purified AP-2 (Promega) can

specifically interact with the Site III pGW-2 HindIII/Msp-1 (-418 to -278) EMSA

probe (Fig. 3-6 B). A doublet of complexes were competed by both AP-2 and

Site III oligonucleotides and not by Distal Site III and one complex on the AP-2

probe was specifically competed by an excess of Site III, Distal Site III or AP-2

oligonucleotides (Fig. 3-6 A). However, none of these complexes were

recognized by a supershift antibody against AP-2. Therefore, these complexes

are not AP-2, but are factors that recognize similar binding elements. The

results in Figure 3-6 B show that purified AP-2 interacts with Site III probe, but

not at all with the Site II probe. The Site II EMSA probe was used as a control

for specificity. The AP-2 complex on Site III probe was competed by an excess

of AP-2 consensus oligonucleotide and only partially with an excess of the Site

III-AP-2 binding site oligonucleotide. The addition of an excess of Distal Site III

or distal mutant Site III oligonucleotide did not compete the complex. An

antibody against AP-2 (Santa Cruz) recognizes the AP-2 complex. The results

from both EMSA experiments suggest that purified AP-2 can interact with Site III

and that another AP-2-like factor can interact with Site III and AP-2 binding

element.

Figure 3-6. <u>AP-2 like factors may interact at Site III.</u> (A) EMSA reactions contained 10 μg HeLa whole cell extracts and end labeled AP-2 probe. (B) Purified AP-2 (Promega) interacts specifically with Hind III/Msp I end labeled Site III probe, but not with the Site II (EcoR I/Hind III p202 probe). Oligonucleotide competition with excess of unlabeled oligonucleotide (1 pmol) or recognition by α-AP-2 antibody (Santa Cruz) are indicated above the lanes. Sequences for oligonucleotides for AP-2, Distal Site III (distal III), Site III, Distal factor mutant binding site (distal mutant), GCG identified Site III AP2 site (Site III AP2) are listed in the methods chapter of this thesis.

A

no competition | AP 2 | distal III | site III | α-AP 2

AP-2 probe

B

no competition | AP 2 | α-AP 2 | distal III | distal mutant | site III AP 2 | no competition
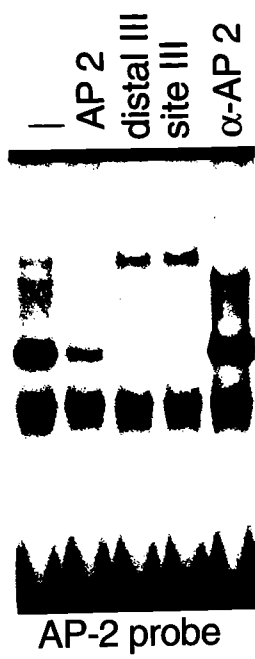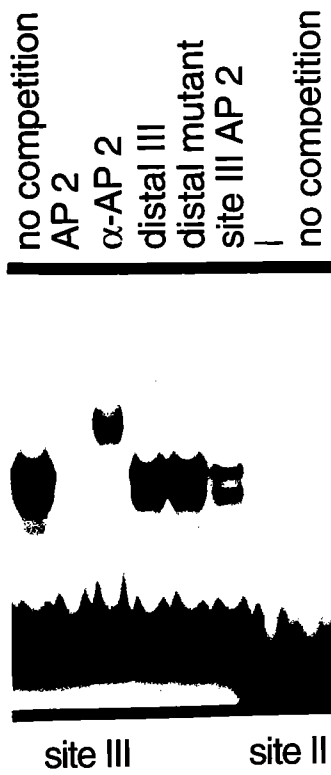
site III   site II

Both histone promoter and heterologous thymidine kinase promoter controlled chloramphenicol acetyltransferase reporters are unresponsive to the presence of the Site III region.

Previous transfection analysis of Site III by Kroeger et al. (1987) only partially characterized this element. To further delineate the Site III regulatory element and to determine if H4UA-1 and the distal factor binding sites were functionally relevant, a series of chloramphenicol acetyltransferase (CAT) reporter constructs were created to test the effect of Site III and its mutants on the basal histone promoter. Distal factor and H4UA-1 binding site mutants within the -418 to -215 context were created by long primer unique site elimination mutagenesis (Ray and Nickoloff, 1992) of a subclone (H4CARP) containing only the histone Site III fragment and pUC backbone. The wild type and the two mutant Site III fragments were excised from plasmids and cloned into the Sma I site 5' of Site I of the histone promoter-CAT reporter plasmid, 108CAT (-215CAT). The constructs were transiently transfected along with pRSV-Luciferase into HeLa cells for 48 hours then assayed for CAT activity and luciferase activity (control for transfection efficiency). HeLa cells transfected with -418CAT, Proximal Site III mutant of -418CAT or Distal Site III mutant of -418CAT had CAT activity levels comparable to the reporter construct lacking Site III (-215CAT) (Figure 3-7). These results suggest that Site III and the two mutants do not alter expression of the CAT reporter genes.

A second series of Site III reporter constructs were created to test whether Site III and its mutants had any effect on a heterologous promoter-CAT reporter construct. The -418 to -215 fragments of wild type, distal factor binding site mutant and proximal H4UA-1 binding site mutant of Site III were cloned into

Figure 3-7. <u>Site III and its mutants, in histone promoter context, are neutral</u> <u>transcriptional regulatory elements in transient transfection experiments in</u> <u>HeLa cells.</u> HeLa cells were transiently transfected with 20 μg CAT reporter plasmid and 1 μg RSV-luciferase plasmid by the calcium-phosphate-DNA precipitation method (Chen and Okayama, 1988) and harvested 48 hours later. Graph of percentage of -215 CAT CAT activities (-215 CAT = 100 %) are shown for constructs -215 CAT, -418 CAT, H4UA-1 binding mutant of -418 CAT (Prox. mutant) and distal factor binding site mutant (Dist. mutant) (n ≥ 8). Error bars designate the standard deviation. Schematic diagrams depicting the constructs -215 CAT and -418 CAT and the relative locations of Sites I, II and III are shown below the graph.
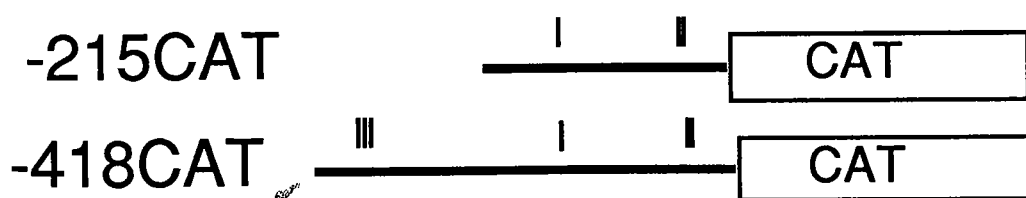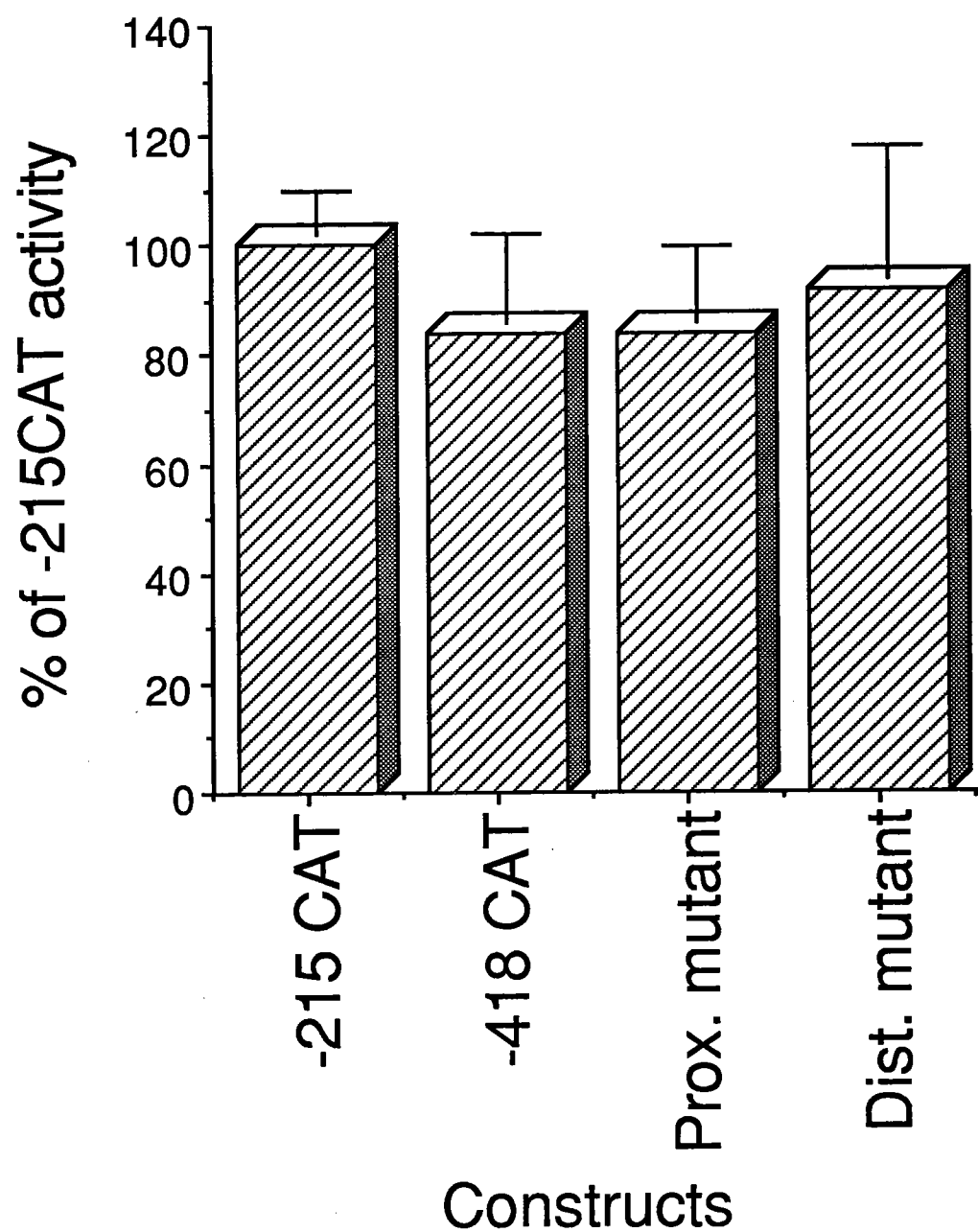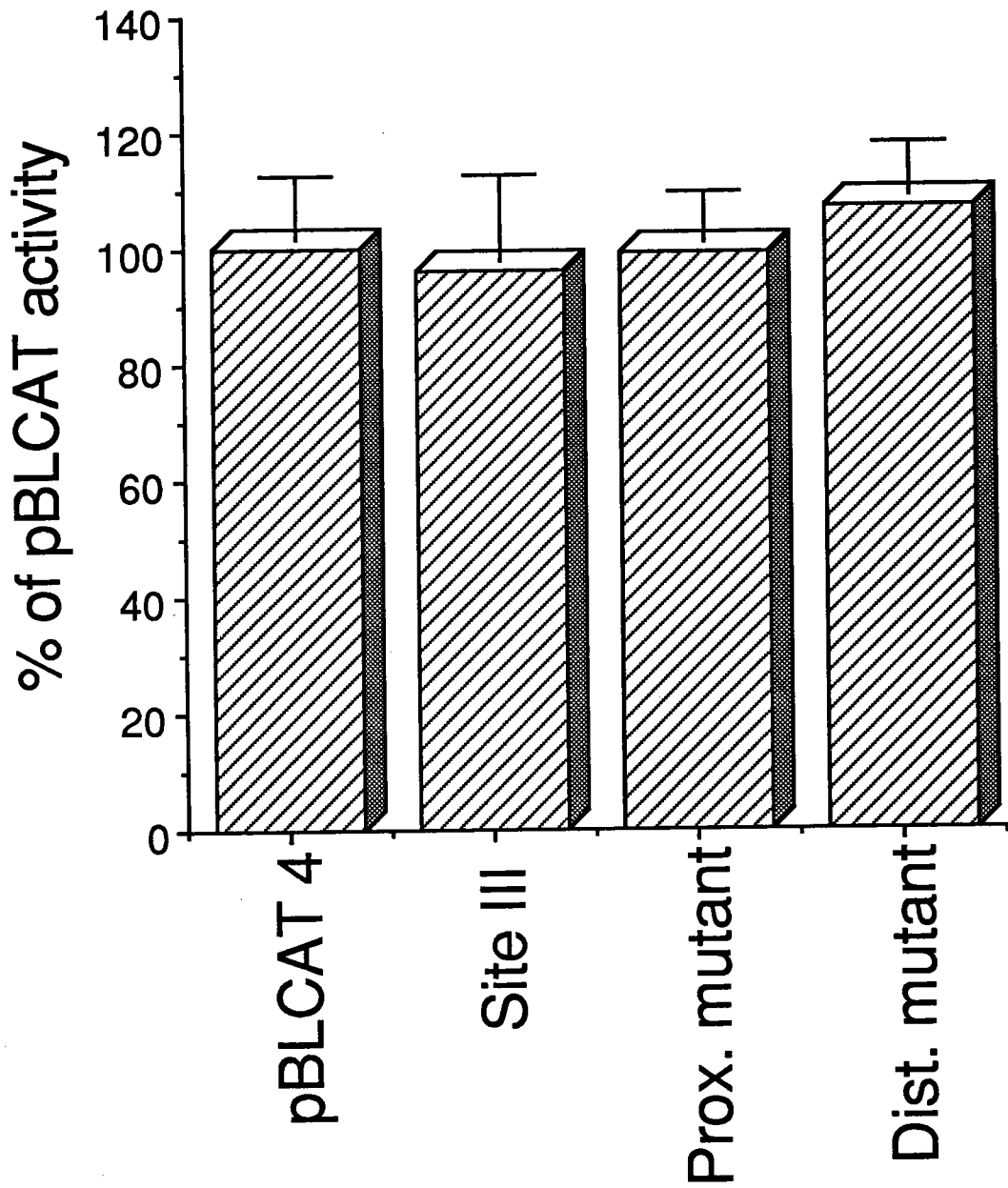
pBLCAT4 (Jonat et al., 1990), upstream of a thymidine kinase promoter which controls transcription from the CAT gene. These chimeric constructs were transiently transfected into HeLa cells and tested for CAT activity. The transcriptional activities of the reporter constructs containing the wild type, distal factor binding site mutant and proximal H4UA-1 binding site mutant Site III fragments were not significantly different from that of pBLCAT4 (Fig. 3-8). The results from transient transfection with the Site III heterologous reporter constructs corroborate the results observed in transient transfection experiments with Site III histone promoter CAT reporter constructs. Neither Site III nor either of its mutants appear to alter the levels of expression in transient transfection experiments.

Histone H4 gene coding sequence has a region homologous to Site III.

In an attempt to resolve differences between my observations and those of Kroeger et al. (1987), I carefully reexamined the differences in the experimental design. One of the key differences between the transfection experiments was that the constructs used by Kroeger et al. (1987), included histone coding region sequences, whereas the CAT reporter constructs I used did not. The histone Site III element may require a coding region element to function properly. Upon analyzing the coding region sequence, it became apparent that the sequences between nucleotides +150 and +183 have very high sequence homology with the Site III sequences between nucleotides -366 and -333 (Fig. 3-9). Both the coding region element and Site III have the AP-2, distal factor and H4UA-1 binding motifs. The identification of a Site III-like element in the coding region supports the hypothesis that Site III may need this

Figure 3-9. <u>Sequence homology between Site III and the histone H4 coding region sequences.</u> Schematic of Site III (-366 to -333 nt) and histone H4 coding region (+150 to +183 nt) sequences that are highly homologous. AP-2, distal factor and H4UA-1 binding elements are indicated either above or below the sequences.

Sequence homology between site III and the histone H4 coding region

Distal factor

-366   -333

TGGCGGGCCTCCTGCCAGTCTCTGGCCTCCATTTG
ACCGCCCGGAGGACGGTCAGAGACCGGAGGTAAAC

AP-2   H4UA-1

Distal factor

+150   +183

TGGCGGGCGTTAAGCGGATCTCTGGCCTCATTTAC
ACCGCCCGCAATTCGCCTAGAGACCGGAGTAAATG

AP-2   H4UA-1

Figure 3-10. <u>Distal factor and H4UA-1 interact with the Site III-like element in the histone coding region.</u> Binding reactions using the Cel II/Xmn I (+75 to +200 nt) radiolabeled probe and 5 µg HeLa nuclear extracts were resolved on 4 % polyacrylamide gel in 0.5 X TBE running buffer. Oligonucleotide competitions are indicated above the lanes. 1 pmol of Site III, Distal Site III (distal), distal factor mutant binding site (distal mutant), Proximal Site III (H4UA1 WT) or YY1 mutant binding site (H4UA1 mut.) was added to the EMSA binding reactions.

Site III
Distal
Distal mutant
H4UA1 WT
H4UA1 mut.
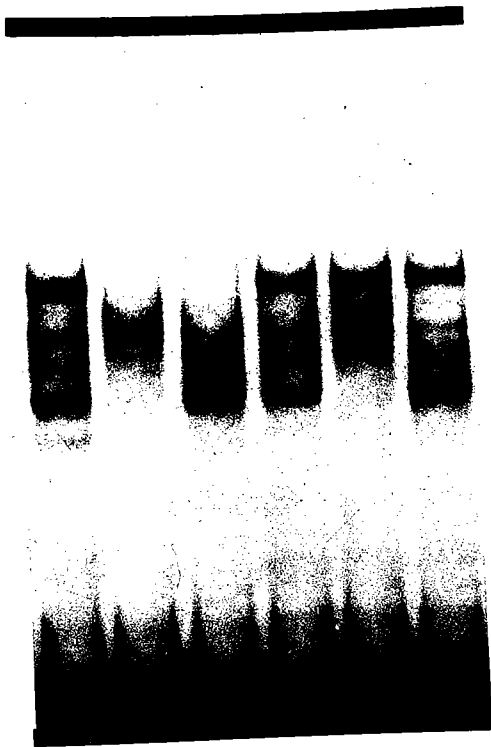
PROBE: +75 TO +200

coding region element to have an effect on transcription.

Although the Site III-like element in the histone coding region is highly homologous in sequence and arrangement to Site III in the promoter, further characterization of the coding region was required to prove that both elements indeed interact with similar transcription factors. I performed EMSA analysis to determine if the distal factor and H4UA-1 bind to the histone coding region element. Results presented in Figure 3-10 clearly show that H4UA-1 and the distal factor complexes interact with the coding region probe (+75 to +200). A slow-migrating complex was competed with an excess amount of unlabeled Site III or Distal Site III oligonucleotides, but not by the other oligonucleotides. This slow-migrating complex is therefore likely to be related to the Site III distal factor. A fast migrating complex was specifically competed by only the Site III and H4UA-1 wild type (Proximal Site III) oligonucleotides. This fast-migrating complex is therefore likely to be related to the Site III factor H4UA-1. However, the affinity for H4UA-1 binding to the coding region appears to be weaker than that observed for Site III. EMSA results suggest that the histone coding region does indeed harbor an element very similar to Site III and that both elements interact with H4UA-1 and the distal factor.

Site III in context of the histone promoter and coding region does not influence gene expression.

The identification and characterization of a histone H4 coding region element, which is remarkably similar to Site III supports the hypothesis that Site III requires a coding region element to influence transcription from the histone gene and may explain the differences between the results observed in this

Figure 3-11. <u>Site III in a histone promoter and coding region context is a</u> <u>neutral transcriptional regulatory element in transient transfection experiments</u> <u>in HeLa and C127 cells.</u> **(A)** HeLa cells were transiently transfected with 20 µg CAT reporter plasmid and 1 µg RSV-luciferase plasmid by calcium-phosphate-DNA precipitation method (Chen and Okayama, 1988) and harvested 48 hours later. Graph of percentage of -215 to +270 CAT CAT activities (-215 to +270 CAT = 100 %) are shown for the -418 to +270 CAT construct. Error bars designate the standard deviation. n=16 for each construct.

A

Figure A. Bar graph showing % of -215 to +270 CAT activity for constructs -215 to +270 and -418 to +270.
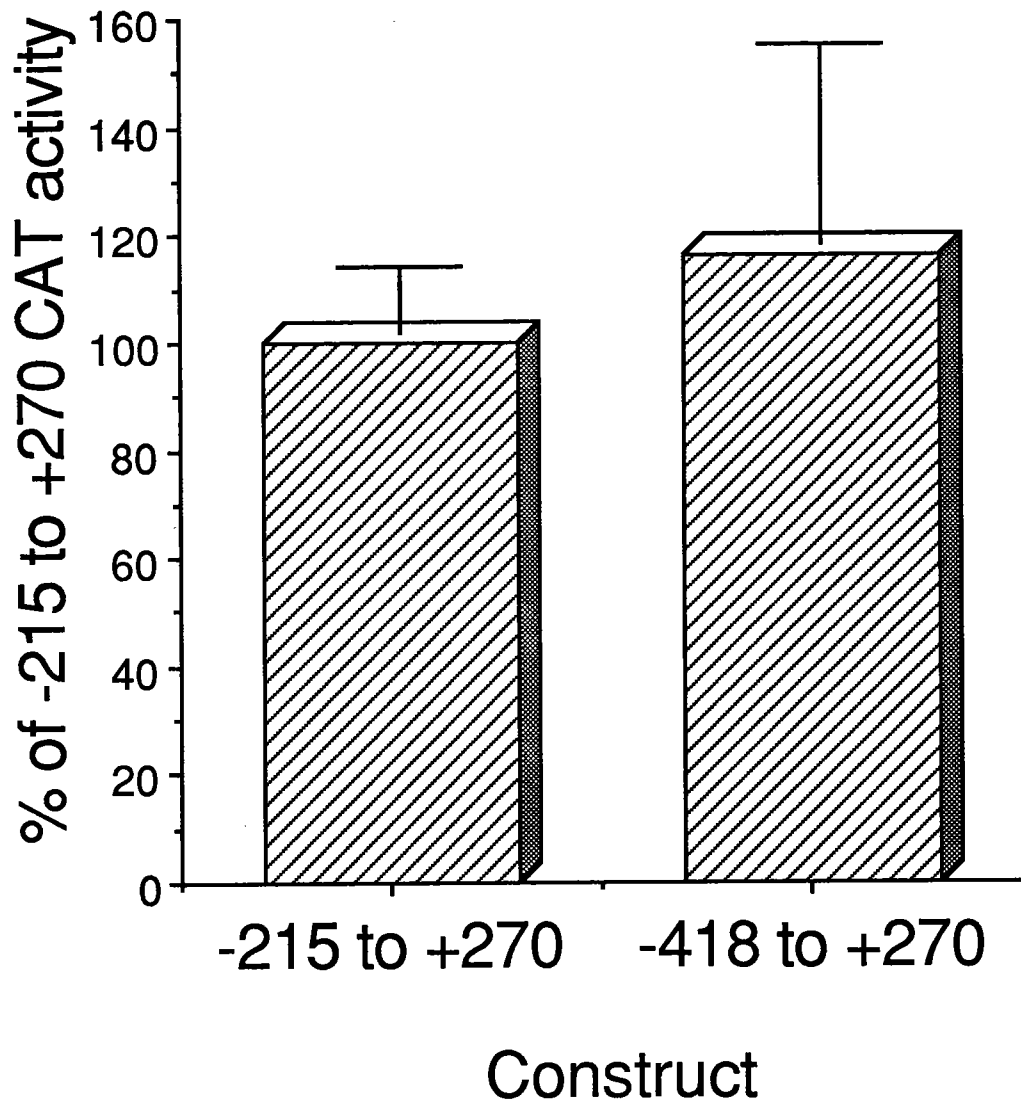
Figure 3-11 (continued). Panel (B) C127 mouse cells were transiently transfected with 20 µg CAT reporter plasmid and 1 µg RSV-luciferase plasmid by calcium-phosphate-DNA precipitation method (Chen and Okayama, 1988) and harvested 48 hours later.

B
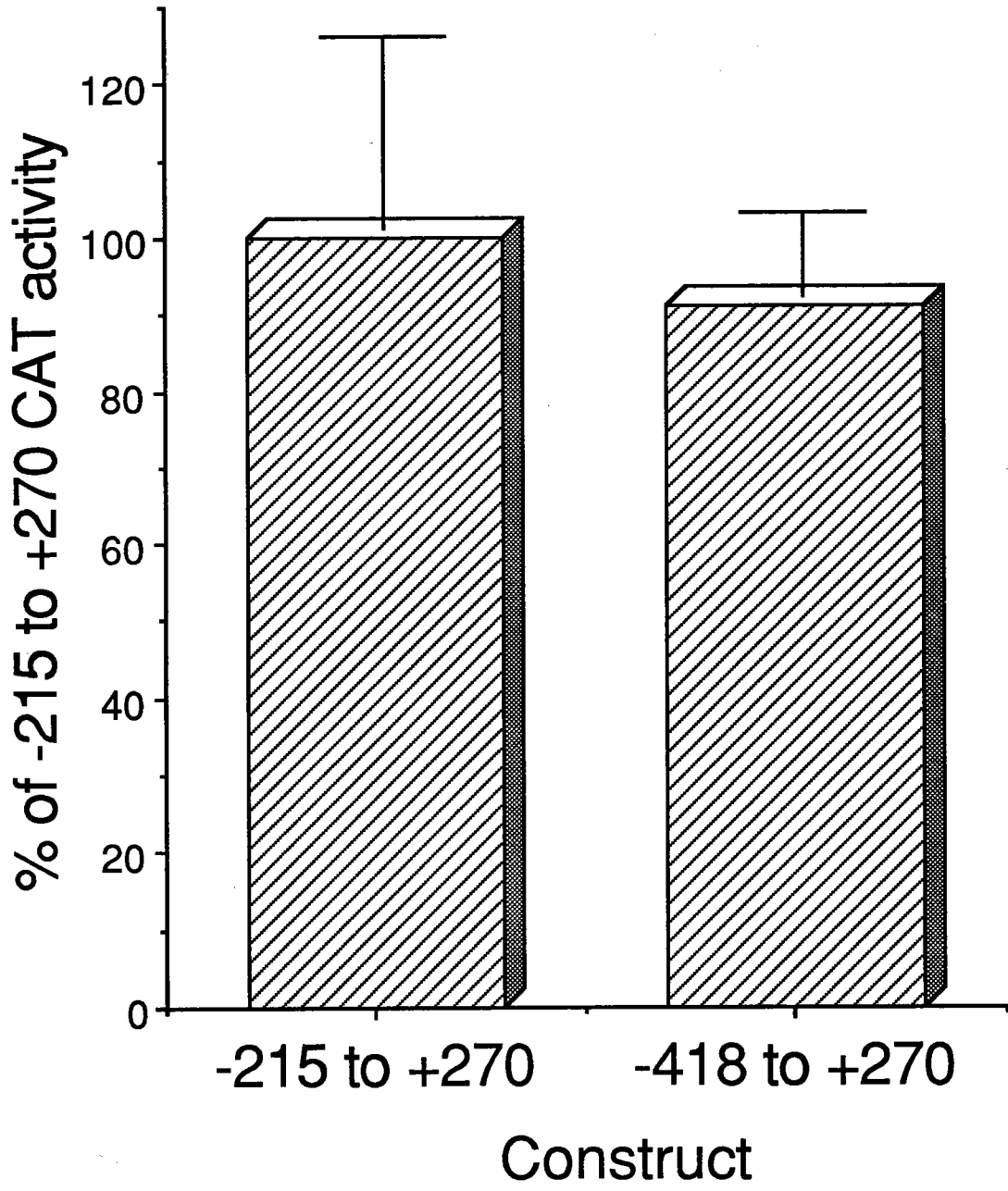
study and those by Kroeger et al. (1987). To test this hypothesis, we created two histone promoter-gene-CAT reporter fusion constructs (-215 to +270 CAT and -418 to +270 CAT). The -215 to +270 CAT, which lacks Site III, has the -215 to +270 bp (EcoR I/ Nco I) fragment of the histone H4 gene cloned in frame with the CAT reporter gene. The -418 to +270 CAT, which has Site III, was constructed by cloning the Hind III/Sma I fragment of H4CARP into the Sma I site 5' of Site I of the -215 to +270 CAT construct. These two constructs were transiently transfected into HeLa cells and analyzed for CAT activity. Results in Figure 3-11A show that both -215 to +270 CAT and -418 to +270 CAT have similar levels of expression. Kroeger et al. (1987) observed the positive effect on transcription in transfected C127 and LTK- mouse cells. In this experiment, I used HeLa S3 cells. To rule out that differences in transcriptional activity were cell line specific, I transiently transfected C127 cells with the -215 to +270 CAT and -418 to +270 CAT constructs (Figure 3-11 B). Again, I did not observe any activation by Site III. -418 to +270 CAT showed very similar CAT activity levels with -215 to +270 CAT. These transient transfection results do not support the hypothesis that Site III needs the coding region element to influence transcription.

Discussion

In this chapter, I further characterized and attempted to identify transcription factors which interact within a sub-region (-368 to -324 nt) of Site III (-418 to -215 nt)*in vitro*. A heretofore uncharacterized Site III protein/DNA

complex, the distal factor, was observed by EMSA analysis. Results from methylation interference assays identified guanine residue contacts for the distal factor with Site III. The distal factor binding element partially overlaps that identified for H4UA-1 and it is possible that the two complexes compete with each other for binding in a mutually exclusive manner. H4UA-1 has been extensively characterized by our laboratory as the predominant transcription factor complex of Site III. A GCG transcription factor data base search identified many potential binding motifs. However, only the NF 1 binding motif may match an established *in vitro* complex, that of the distal factor. Two other potentially important motifs identified were a strong TATA box (-415 nt) and an AP-2 binding motif (-360 nt). I also show by EMSA analysis that AP-2, an AP-2-like factor and NF1 can interact with Site III.

AP-2 is involved in mediating the transcriptional regulation of genes responsive to numerous signal transduction pathways, such as cyclic AMP, phorbol esters and retinoic acid (Williams et al., 1988). These pathways are intimately involved in cellular differentiation and growth regulation. The AP-2 family includes two alternatively spliced proteins (Buettner et al., 1993) and ERF-1/AP2γ (McPherson et al., 1997). Although the larger 52 kD protein (AP-2A) is capable of binding to DNA, the alternatively spliced form (AP-2B) does not bind DNA and can inhibit the binding of AP-2A (Buettner et al., 1993). AP-2 is one of the few transcription factors known that is capable of interacting with mitotic chromatin (Martinez-Balbas et al., 1995). The role of AP-2 in regulating histone H4 transcription has not been determined. Although purified AP-2 can specifically interact with Site III, the AP-2 immunoreactive band on the AP-2 probe was only slightly competed by Site III oligonucleotides. However, a faster

migrating band on the AP-2 probe, which was not recognized by the antibody was competed by Site III oligonucleotides. This Site III competable complex could be another form of AP-2 or another transcription factor which can recognize similar sequences. There are multiple reports of genes containing AP-2-like repressor elements which interact with factors that are not AP-2 (Ye et al., 1996; Tamai et al., 1994; LaPres and Hudson, 1996; Hu et al., 1996; Okamuro et al., 1997). Whether Site III physiologically interacts with this class of AP-2 sequence binding factors is not known.

The NF1/CTF family of transcription factors is involved in initiation of DNA replication and regulation of gene expression (Jones et al., 1987). Multiple NF1/CTF polypeptides, ranging in size from 52 to 66 kilodaltons, have been purified and cloned from human cells (Rosenfeld and Kelley, 1986; Santoro et al., 1988). The distal factor binding to Site III may be a member of the NF1 family. EMSA competition analysis suggests that a common set of factors interacts with Distal Site III and NF1 binding sequences. The possibility of a transcription factor involved both in the DNA replication pathway and in transcription regulation, interacting with the histone H4 promoter is very intriguing. Factors such as NF1 could possibly link cell cycle regulation of transcription from the histone H4 gene and the DNA replication process. However, further evidence is necessary to confirm that the distal factor is an NF1 family member and that it is functionally relevant.

In the search to identify a potential element within the histone coding region that may function with Site III, I found that the sequences between nucleotides +150 and +183 were highly homologous to Site III. Results from EMSA experiments show that the Site III complexes H4UA-1 and the distal

factor bind to the Site III-like element in the coding region. It is not known whether the AP-2-like factors can also interact with the coding region. The ability of both elements to interact with similar factors supports the concept that they work together, possibly through mechanisms involving similar transcription factors. Other transcription factors e.g. Sp1(Su et al., 1991; Mastrangelo et al., 1991) have been shown to form a single complex which interacts at multiple sites, causing the DNA to form a loop structure. A similar mechanism could be envisioned for the histone gene, where Site III and the Site III-like element in the coding region may form a loop structure with the assistance of the distal factor, H4UA-1, or even AP-2.

Although Site III interacts with multiple factors *in vitro*, it does not appear to influence the rate of gene expression in transient transfection experiments. CAT reporter constructs containing the wild type, distal factor mutant or the proximal H4UA-1 binding site mutant -418 to -215 region were tested by transient transfection analysis and found to be transcriptionally neutral on both the histone promoter and heterologous thymidine kinase promoter. The Site III region was also tested in the context of the histone promoter with most of the histone coding region, and again was observed to be transcriptionally neutral. Therefore, Site III does not coordinate transcriptional upregulation with the coding region in transient transfection experiments and may not even be transcriptionally relevant.

An unresolved difference still exists between the data presented in this study and the data presented by Kroeger et al. (1987). I attempted to address this difference at multiple levels. Because pFO 005 and pFO 108A have histone coding sequences, I created constructs with Site III in the context of the histone

coding region (-418 to +270 CAT). No transcriptional effect of Site III and the coding region was observed when the constructs were transfected into HeLa cells. In comparing the -215 to +270 CAT with the -418 to +270 CAT constructs in the C127 mouse cells, the same cell line used by Kroeger et al. (1987), no effect from Site III was observed. I designed the pBLCAT4 and histone promoter CAT reporter construct series so that the only differences between constructs in a series would be in sequences related to Site III. The two plasmid constructs, pFO 005 and pFO 108A, tested by Kroeger et al. (1987), have other sequence differences as well. The backbone plasmid of pFO 005 is pUC 13, whereas that of pFO 108A is pBR322. Furthermore, while pFO 005 lacks the 3' Pst I to Hind III fragment, pFO 108A has it. In the *in vitro* transcription assays analysis by Wright (1990), pFO 005 (pUC 13 backbone) was compared to pFO 108X (pUC 19 backbone). pFO 108X lacks the 3' Xba I to Pst I fragment, whereas pFO 005 has it. It is possible that these other sequence differences are responsible for the positive effect on transcription.

The Site III region of the histone H4 promoter may be a promoter for a potential open reading frame in the opposite direction. Based on a search of the histone promoter sequence, I identified two possible open reading frames in the noncoding strand starting at nucleotide -440 and ending at -773, and an open reading frame in the coding strand starting at nucleotide -810 and ending at -380. No matches for the open reading frames were found to other proteins or protein motifs in the data base. Transcription from either open reading frame would be possible because of the strong consensus TATA box located at nucleotide -410 and many TA rich sequences 5' of -810 nt. The protein product on the noncoding strand would have a molecular mass of 12,344 daltons and

the product on the coding strand would have a molecular mass of 16,286 daltons (histone H4 protein has a molecular mass of 11,367 daltons). Previous experiments from our laboratory, as well as those presented in this study, were not designed to address the possibility of such open reading frames. Further analysis is necessary to determine whether the open reading frames are transcribed, and if so, whether Site III regulates the transcription of that gene.

The results presented in this study neither support nor disprove the observations that Site III represses transcription in non-proliferating cells (Larson et al., 1989; Zahradka et al., 1993). It is possible that one of the factor complexes or sequences characterized in this study could be involved. Site III also has an E box sequence (CANNTG) between nucleotides -338 and -333 (CATTTG), which is a potential candidate element for such regulation. However, there are no data to support such a claim. Site III may function as a mediator of growth control of the histone gene expression.

Site III could have a functional role in transcriptional regulation of the histone H4 gene which requires normal chromatin context. Transient transfection experiments with plasmid DNA would not be able to address this possibility. Previously, our laboratory has observed differing results between transient and stable transfection experiments (Frenkel et al., 1996). Another possible way Site III might function is that DNA of Site III may under appropriate conditions form cruciform structures which could be altered depending on the binding of transcription factors (distal factor or H4UA-1). Such a situation was observed in the human enkephalin enhancer (Spiro et al., 1993). Again, the experimental systems used in this study would not detect these possibilities.

In conclusion, while Site III interacts with many factors *in vitro*, it is a

neutral transcriptional element in transient transfection experiments in proliferating cells.

CHAPTER IV

MULTIPLE INTERACTIONS OF THE TRANSCRIPTION FACTOR YY1 WITH HUMAN HISTONE H4 GENE REGULATORY ELEMENTS

Abstract

Multiple regulatory elements and intricate protein/DNA interactions mediate the transcription of the human histone H4 genes in a cell growth dependent manner. Upon analysis of the regulatory elements of the FO108 histone H4 gene, I identified several potential YY1 binding sites. In this study, I have analyzed the ability of the YY1 transcription factor to interact at these sites *in vitro* by using electrophoretic mobility shift assays in combination with oligonucleotide competition and antibody immunoreactivity. I show that YY1 specifically binds transcriptional regulatory elements at -340 nt (Site III), -100 nt (Site I) and at least two domains within the coding region of the histone H4 gene. To test if these elements were functionally responsive to YY1, I performed transient expression experiments in Drosophila S-2 cells transfected with heterologous reporter gene constructs driven by histone H4 gene segments fused to the thymidine kinase promoter. Co-expression of YY1 stimulated promoter activity of these constructs relative to the reporter construct lacking histone H4 gene fragments. Our results suggest that YY1 contributes to

transcriptional regulation of the histone H4 gene via interactions at multiple regulatory elements.

Introduction

The five classes of replication-dependent histone genes; H1, H2A, H2B, H3 and H4, encode abundant and highly conserved basic proteins required for nucleosomal packaging and proper transcriptional regulation of chromatin (reviewed in Osley 1991 and Stein et al., 1992). Expression of histone genes is tightly linked to DNA synthesis and peaks in S phase (Plumb et al., 1983; Heintz et al., 1983; Graves et al., 1984; Baumbach et al., 1987). Transcriptional expression is also dependent on the growth status of the cell. Quiescent or post-proliferative differentiated cells have a basal level of histone gene transcription which is significantly lower than that of replicating cells (reviewed in Stein et al., 1992). Consequently, histone gene promoter activity is responsive to cellular cues related to cell cycle progression and proliferation (reviewed in Stein et al., 1992). Transcriptional control of histone genes involves cell signaling pathways converging through multiple transcription factors which interact with composite regulatory elements in the promoter and coding sequences of the gene. The expression of the human histone H4 gene FO108 is controlled by an array of transcription factors, such as ATF-1, CREB, IRF-2 and CDP/cut, which are components of gene regulatory signaling pathways that control cell growth and cell cycle progression (Wright et al., 1995; Guo et al., 1997; Vaughan et al., 1995; van Wijnen et al., 1996). Recently, our

laboratory showed that the nuclear matrix protein NMP-1, which interacts with the histone H4 distal regulatory element Site IV, is identical to YY1 (Guo et al., 1995). Hence, YY1 may represent a critical regulatory factor involved in modulating transcription of the histone H4 gene.

YY1 (UCRBP, δ, or NF-E1) is a multi-functional zinc finger transcription factor that is ubiquitously expressed in mammalian cells (Shi et al., 1991; Flanagan et al., 1992; Hariharan et al., 1991; Park and Atchison, 1991). YY1 is capable of modulating transcription by multiple mechanisms (for review see Shrivastava and Calame, 1994 and Hahn et al., 1992), including its ability to interact with a vast array of regulatory factors, such as p300 (Lee et al., 1995), TAFII55 (Chiang and Roeder, 1995), TFIIB, TATA box-binding protein (Usheva and Shenk, 1994), CBP (Austen et al., 1997), SP1 (Seto et al., 1993; Lee et al., 1993), E1A, c-Myc (Shrivastava et al. 1993), ATF/CREB (Zhou et al., 1995), B23 (Inouye et al., 1994), YAF2 (Kalenik et al., 1997), RPD3 (Yang et al., 1996), cyclophilin A and FKBP 12 (Yang et al., 1995). YY1 has been shown to have positive, negative and neutral influences on transcription (for review see Shrivastava and Calame, 1994 and Hahn et al., 1992). In addition, YY1 is capable of initiating transcription with TFIIB and RNA polymerase II (Usheva and Shenk, 1994) and has been shown to influence transcription by its ability to bend DNA and thus regulate contact between elements (Natesan and Gilman, 1993). Therefore, elucidation and characterization of YY1 responsive genes such as the histone H4 gene, is essential to our understanding of this versatile transcription factor.

In this study, I provide evidence that YY1 is functionally involved in the regulation of histone H4 gene expression. First, YY1 interacts with multiple

gene regulatory elements *in vitro*. Furthermore, expression of YY1 in Drosophila cells influences promoter activity through the histone H4 regulatory elements suggesting that these elements are functional YY1 sites. I discuss potential regulatory mechanisms and models by which YY1 is intimately involved in histone gene regulation.

Results

H4UA1 is identical to transcription factor YY1

Histone H4 gene transcription is modulated in a cell growth and cell cycle dependent manner. The regulatory element Site III (nt -418 and -215) has been shown to influence histone transcription and may facilitate regulation of cell growth (Kroeger et al., 1987; Zahradka et al., 1993; Larson et al., 1989). Our laboratory has previously identified H4UA1 as a protein-DNA complex that interacts within this region (van der Houven van Oordt et al., 1992). The sequences important for H4UA1 binding (5'-GGCCTCCATTTGC-3') are also homologous to a consensus YY1 binding site (see Table 4-2). To test if H4UA1 is YY1, I performed Electrophoretic Mobility Shift Assay (EMSA) analysis together with oligonucleotide competition and specific antibody recognition experiments. Figure 4-1 and Table 4-1 show the relative locations or the sequences of EMSA probes used. Results in Figure 4-2A show that the H4UA1 interaction with the Site III EMSA probe (-368 to -323 nt) is specifically competed by an excess of an oligonucleotide encoding the wild type H4UA1 binding site (lane 2) or the YY1 (lane 4) consensus element, but not with an

Table 4-1. Sequences of oligonucleotides used in this study.

Sequences of oligonucleotides used either as EMSA probes or as unlabeled

EMSA competitors are shown.

TABLE 1:

Full length site III:

5′ CGCTGGCGGCCTCCTGCCAGTCTCTCTGGCCTCCATTGCTCTTCCTG 3′

Proximal site III:

5′ TCTCTGGCCTCCATTTGCTCTTCCTGAG 3′

Prox. site III mutant:

5′ TCTCTGGCCTagcTTTGCTCTTCCTGAG 3′

Distal site III:

5′ GCGCTGGGGCCTCCTGCCAGTCTCTGGCC 3′

Dist. site III mutant:

5′ GCGCTGGGGCCTCCTTCACCTCTCTGGCC 3′

YY1 wild type:

5′ CGCTCCGCGGCCATCTTGGCGGCTGGT 3′

YY1 mutant:

5′ CGCTCCGCGattATCTTGGCGGCTGGT 3′

Distal Site I:

5′ CGGAAAAGAAATGACGAAATGTCGAGA 3′

Table 4-2. Potential YY1 binding sequences in the histone H4 gene. Histone regulatory sequences for Sites IV, III, I and histone H4 coding region are shown aligned with the YY1 consensus sequences (Shrivastava and Calame, 1994).

TABLE 2:

```
SITE IV  (-630nt) :  TGACGTC  CAT  GAGA
SITE III (-340nt) :  TGGCCTC  CAT  TTGC
SITE I  (-120 nt) :  ATTTCGT  CAT  TTCT
H4 GENE  (+30) :     CAGCGGT  CAT  GTCC
H4 GENE  (+130) :    AGCCTGC  CAT  TCGG
H4 GENE  (+180) :    CTGGCCT  CAT  TTAC

YY1 consensus:       CGC  CAT  NTT
                     GTT       AG
                     A         C
```

Figure 4-1. <u>A schematic of probes and gene fragments used for studying</u> <u>YY1 interactions on the H4 histone gene FO108.</u> Dashed lines represent DNA fragments cloned into blunt Hind III site of PBLCAT4. Solid line under gene map represents the location of the oligonucleotide used in this study. "*" represents that the DNA fragment or oligonucleotide was used as a EMSA probe.

Hind III
-418

Sma I
-210

Bbs I Blp I Xmn I
-10 +75 +210

Histone H4

IV
-640

III
-340

II
-105

I
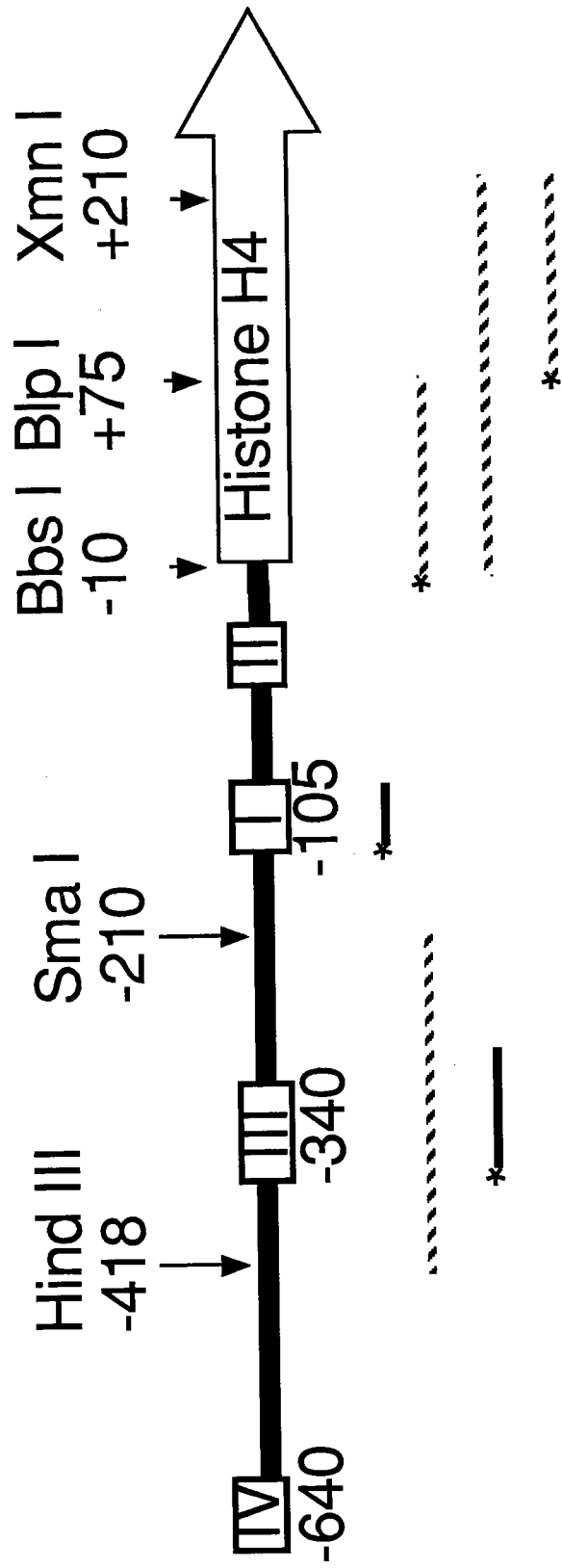
oligonucleotide containing mutated H4UA1 or YY1 sites (lanes 3 and 5). The remaining binding activity represents a comigrating non-specific band (van der Houven van Oordt et al., 1992). Furthermore, the H4UA-1 complex was recognized by an anti-YY1 antibody (lane 6). Thus, these results suggest that the Site III binding factor H4UA1 is YY1.

To confirm that YY1 interacts specifically with the H4UA1 binding site, I tested the ability of Site III oligonucleotides to compete for YY1 interactions on a YY1 binding site EMSA probe. Results in Figure 4-2B show that oligonucleotides spanning the H4UA1 binding site (lanes 3 and 6) compete for the YY1 interaction on the YY1 probe, whereas competition with oligonucleotides lacking the H4UA1 binding site (lanes 4 and 5) do not. The YY1 complex is recognized by an antibody against YY1 (lane 2) whereas a control antibody against AP-2 does not recognize the YY1 complex (lane 7). Further evidence that YY1 interacts specifically at Site III is presented in Figure 4-2C, which shows that recombinant His-YY1 fusion protein interacts on the Site III oligonucleotide probe. His-YY1 fusion protein binding to Site III is competed by the consensus YY1 oligonucleotide (lane 2), but not by the corresponding mutant YY1 oligonucleotide (lane 3). Consistent with these results, the His-YY1 complex co-migrates with the H4UA-1 complex (data not shown) and is also recognized by the YY1 antibody (lane 4). Thus, the data presented in Figures 2A, B and C establish that the previously defined Site III binding protein H4UA1 is the transcriptional regulator YY1.

Transcription factor YY1 interacts at Site I

Site IV (-600 nt) and the distal region of Site I (-110 nt) of the FO108

Figure 4-2. <u>The H4 Site III binding protein H4UA1 is YY1.</u>

(A.) EMSA was performed using HeLa nuclear extract (3 µg/reaction) and the

Full Site III oligonucleotide probe (Lanes 1-6) to determine if H4UA1 is YY1.

The H4UA1 complex is competed by an excess of either unlabeled Proximal

Site III (Lane 2) or YY1 binding site (Lane 4) oligonucleotide but not by an

equivalent amount (2.5 pmole (250 fold molar excess)) of either the Proximal

Site III mutant (Lane 3) or YY1 mutant binding site (Lane 5) oligonucleotide.

Incubation with 1 µl of YY1 antibody results in a block-shift of the H4UA1

complex (Lane 6).

PROX 3
PROX 3 MUT
YY1
YY1 MUT
ANTI-YY1
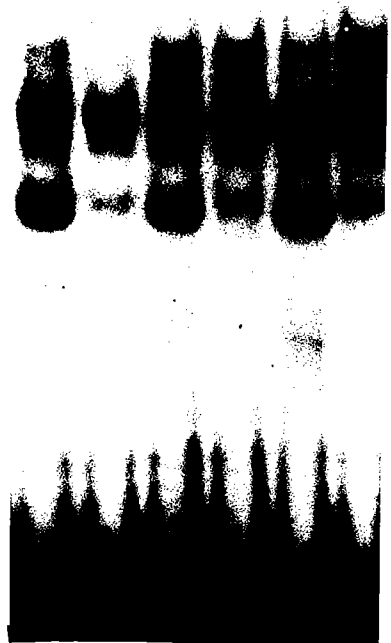
<-H4UA1

1 2 3 4 5 6

Figure 4-2 (continued). Panel (B) EMSA was performed using HeLa nuclear extract (3 µg/reaction) and the YY1 oligonucleotide probe (Lanes 1-7). YY1 interaction on the YY1 probe is competed by H4UA1 binding site oligonucleotides (Proximal Site III oligonucleotide (Lane 3) and Full Site III oligonucleotide (Lane 6)), but not by an equivalent amount (2.5 pmole) of the oligonucleotides lacking H4UA1 binding site (Distal Site III (Lane 5) and Proximal Site III mutant (Lane 4)). Incubation with YY1 antibody (Lane 2) results in a block-shift of the YY1 complex, whereas incubation with an AP 2 antibody (Lane 7) did not.

ANTI-YY1
PROX 3
PROX 3 MUT
DIST 3
FULL 3
ANTI-AP2
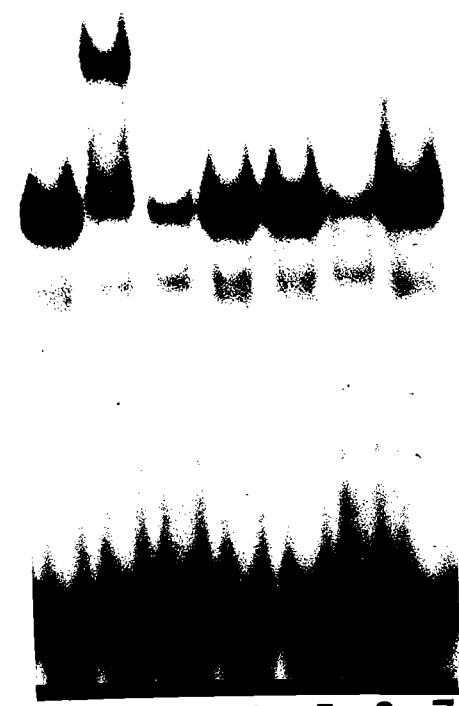
1 2 3 4 5 6 7

Figure 4-2 (continued). **Panel (C)** EMSA was performed to determine if purified recombinant fusion protein His-YY1 interacts specifically with full Site III probe (Lane 1-4). His-YY1 complex is competed by an excess of unlabeled YY1 binding site oligonucleotide (Lane 2) but not by an equivalent amount of unlabeled YY1 mutant binding site (Lane 3). Incubation with the YY1 antibody (Lane 4) results in a block-shift of the His-YY1 complex.

YY1   YY1 MUT   ANTI-YY1

1   2   3   4

histone H4 gene bind common factors, including ATF and CREB (Guo et al., 1998). The nuclear matrix associated protein NMP-1 interacts specifically at Site IV, and has been identified as YY1 (Guo et al., 1995). YY1 binds in an overlapping arrangement with ATF/CREB (See Fig. 4-6 below). I performed EMSA experiments to test whether YY1 interacts with Site I in a similar overlapping arrangement. Figure 4-3 demonstrates that YY1 binds to an oligonucleotide probe (-125 to -101 nt) spanning Distal Site I. The Site I-YY1 complex is specifically recognized by the YY1 antibody (lane 1) and is competed by YY1 binding site oligonucleotides (lane 3 and 5). Oligonucleotides lacking the YY1 binding site have no effect on the YY1 complex (lane 4, 6 and 7). The slower migrating ATF/CREB complexes interacting on the Distal Site I probe are only competed by the ATF/CREB consensus oligonucleotide (lane 7) and this oligonucleotide does not compete for the YY1 complex. Furthermore, purified His-YY1 fusion protein interacts with the Distal Site I probe in EMSA experiments (data not shown). These results show that YY1 interacts specifically with histone H4 Distal Site I.

YY1 interacts within the coding region of the histone H4 gene

I have recently shown that the coding sequences between nt -10 and +200 of the histone H4 gene contribute to transcriptional control (Last et al., manuscript submitted). Analysis of these coding sequences (nt -10 to +210) identified potential YY1 binding sites at +30, +130 and +180 (Table 4-2). EMSA experiments were performed to determine whether YY1 can interact with these putative YY1 elements. Figure 4-4A demonstrates that an EMSA probe spanning nt -10 to +75 of the coding region forms a complex with either His-YY1

Figure 4-3. <u>YY1 can interact with distal site 1.</u>   EMSA was performed using HeLa nuclear extract to determine if YY1 interacts with Distal Site I probe (Lanes 1-7) . A fast-mobility complex (marked) is specifically competed by an excess of oligonucleotides which interact with YY1 (YY1 binding site(Lane 3) and Proximal Site III (Lane5)), but not an equivalent amount (2.5 pmole) of oligonucleotides lacking YY1 binding sites (YY1 mutant (Lane 4), Proximal Site III mutant (Lane 6) and ATF consensus (Lane 7)). Incubation with the YY1 antibody results in a block-shift of the complex (Lane 1).

ANTI-YY1

YY1

YY1 MUT

PROX 3

PROX 3 MUT

ATF

<-YY1

1 2 3 4 5 6 7

Figure 4-4. <u>YY1 can interact with H4 histone coding region.</u> **(A)** EMSA results showing that the -10 to +75 H4 coding region probe interacts with purified His-YY1 (Lane 1 and 2) and with YY1 in HeLa nuclear extract (Lane 3-6). The YY1 complex (marked) was competed by an excess of the YY1 binding site oligonucleotide (Lane 3) but not by an excess of the YY1 mutant oligonucleotide (Lane 4). Incubation with the YY1 antibody results in a block-shift of either the His-YY1 complex (Lane 2) or the YY1 complex formed with HeLa nuclear extract (Lane 5).

ANTI-YY1 YY1 YY1 MUT ANTI-YY1

<-YY1
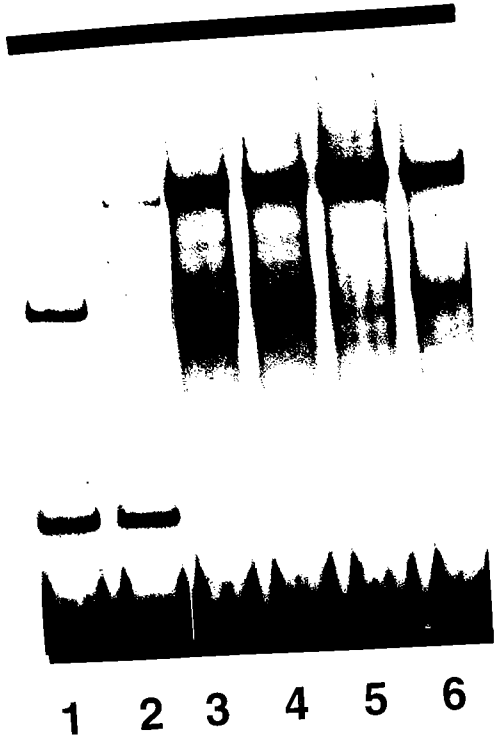
1 2 3 4 5 6

Figure 4-4 (continued). **Panel (B)** The +75 to +210 H4 coding region probe interacts with purified His-YY1 (Lane 1 and 2) and with YY1 in HeLa nuclear extract (Lane 3-6). The YY1 complex (marked) was competed by an excess of the YY1 binding site oligonucleotide (Lane 3) but not by an excess of the YY1 mutant oligonucleotide (Lane 4). Incubation with the YY1 antibody results in a block-shift of either the His-YY1 complex (Lane 2) or the YY1 complex formed with HeLa nuclear extract (Lane 5).
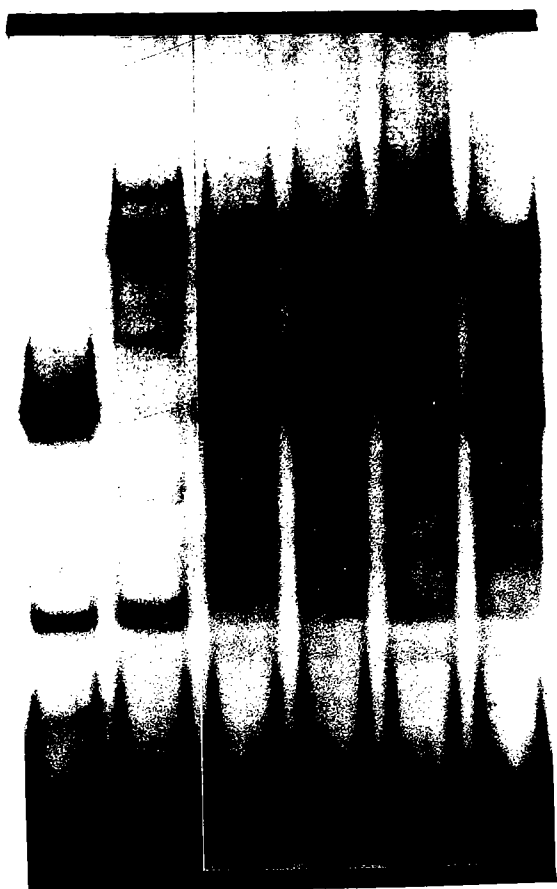
ANTI-YY1
YY1
YY1 MUT
ANTI-YY1

<-YY1

1   2   3   4   5   6

fusion protein (lane 1) or with YY1 from HeLa cell nuclear extract (lane 6). Both the His-YY1/coding region complex (lane 2) and the YY1/coding region complex from HeLa extract (lane 5) are recognized by an antibody against YY1. Lanes 3 and 4 show that the YY1 complex in HeLa nuclear extract is specifically competed by a consensus YY1 oligonucleotide but not by a mutant YY1 binding site. We also observed that YY1 complex in HeLa nuclear extract co-migrates with the His-YY1 complex in the same gel.

The +75 to +210 coding region probe also binds YY1 (Figure 4-4B). Purified His-YY1 interacts specifically with the nt +75 to +210 region (lane 1) and a YY1 complex was also detected in HeLa nuclear extract (lane 6). Both the His-YY1 complex (lane 2) and the YY1 complex from HeLa extract (lane 5) are recognized by an antibody against YY1. Lanes 3 and 4 show that the YY1 complex in HeLa nuclear extracts is specifically competed by wild type but not by the mutant YY1 oligonucleotides. The results presented in Figures 4-4A and B demonstrate that YY1 interacts with at least two regulatory sites within the histone H4 coding region *in vitro*.

Histone H4 gene fragments are responsive to YY1 expression

To study the effects of YY1 on histone H4 gene transcription within the cell, several segments of the histone gene were cloned upstream of a thymidine kinase promoter and chloramphenicol acetyltransferase reporter gene. These H4 gene segments span one or two of the YY1 binding sites characterized in this study and are located in previously defined regulatory domains (nt -10 to +200 of the coding region (Last et al., manuscript submitted) and Site III (Kroeger et al., 1987). Schneider's S-2 cells were chosen for transfection

experiments because these cells are devoid of endogenous YY1 (Shi et al., 1991). The reporter construct pBLCAT4, which lacks histone gene sequences, was unresponsive to YY1 expression. In contrast, reporter gene constructs containing the histone gene segments nt -10 to +210, nt -10 to +75, nt +75 to +210 or nt -418 to -210 are responsive up to 2-fold (Fig. 4-5A). The construct containing two YY1 binding sites (-10 to +210 PBLCAT) did not have higher expression than the coding region constructs containg only one YY1 site (-10 to +75 PBLCAT and +75 to +210 PBLCAT). Therefore, the effect of YY1 is not additive or synergistic. Figure 4-5B shows that promoter activity from the -10 to +210 nt PBLCAT4 or the -418 to -215 nt (Site III) PBLCAT4 constructs cotransfected with increasing amounts of YY1 expression vector is dose-dependent. These results suggest that YY1 can functionally stimulate transcription up to 2.7-fold through histone H4 regulatory elements.

Figure 4-5. <u>Functional evidence showing that YY1 expression up-regulates transcription through the histone H4 regulatory elements.</u> **(A.)** Graphic representation of the relative CAT activity of test constructs in Drosophila Schneider's S-2 cells co-transfected in the presence or absence of YY1. The heterologous reporter CAT constructs (pBLCAT4, -10/+75, +75/+210, -10/+210 and Site III) were individually co-transfected with either the expression plasmid pACT YY1 (+) or pACT vector (lacking YY1) (-). Transfected cells were harvested after 48-72 hours and analyzed for CAT and luciferase activity (n≥3). CAT activities were normalized for transfection efficiency using luciferase results. Error bars designate standard deviation. Results are expressed as percent of the reporter vector CAT activity (100%) without any pACTYY1 cotransfected.

A

CAT Activity

250 — 200 — 150 — 100 — 50 — 0

pACTYY1   - +   - +   - +   - +   - +   - +   - +   - +

pBLCAT4   Site III   -10/+210   -10/+75   +75/+210
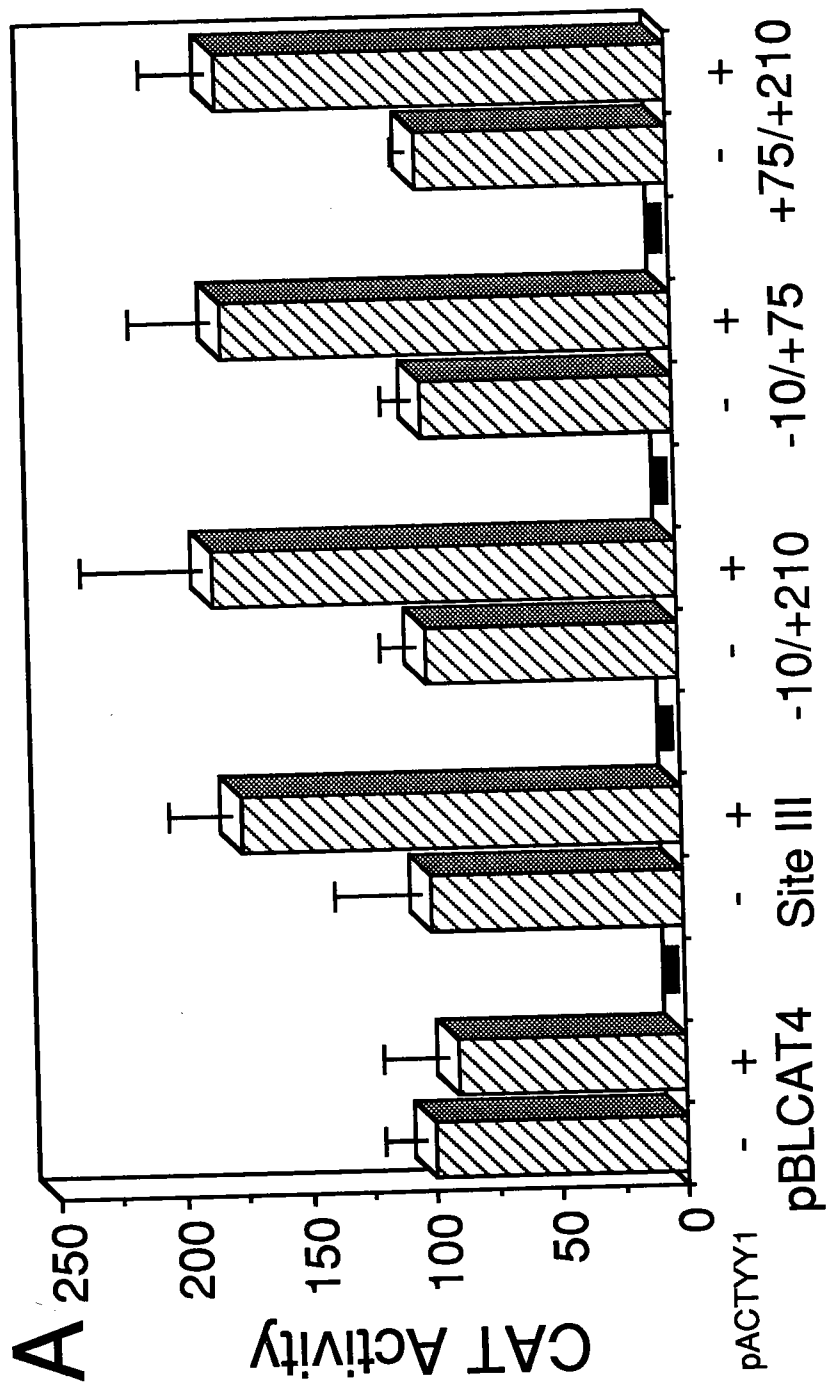
Figure 4-5 (continued). Panel (B) The effect of YY1 expression on

-10/+210 and on Site III reporter constructs is dose dependent. Increasing

amounts (0, 3.5, 7, 14 µg) of pACT YY1 expression plasmid were cotransfected

with CAT reporter plasmids (-10/+210, Site III or PBLCAT4) into Drosophila

Schneider's S-2 cells grown on 100 mm plates (n≥2). Error bars designate

standard deviation.

B

CAT Activity

300 250 200 150 100 50 0

Amount
pACTYY1 (ug): 0 3.5 7 14 - 0 3.5 7 14 - 0 3.5 7 14 - 0 3.5 7 14

-10/+210   pBLCAT4   Site III
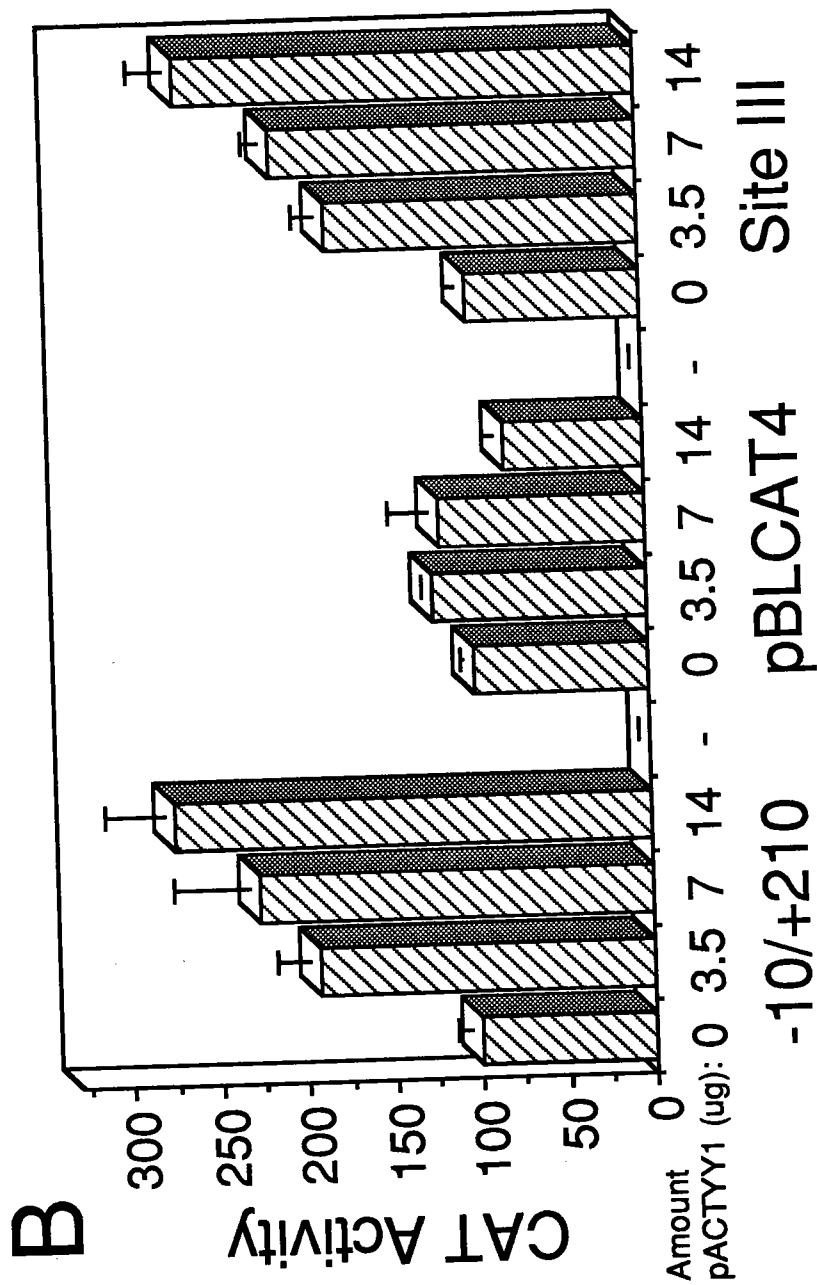
Figure 4-6. <u>Schematic model of the multiple transcription factors interaction</u> <u>of the histone H4 gene FO108.</u> DNase I hypersensitive sites are indicated as large arrowheads and marked DNase H.S. A micrococcal nuclease sensitive site is marked by a large box and is labeled MNase H.S. The various identified transcription factors are indicated and relative locations for IRF-2 (IRF), CDP/*cut* (CDP), Sp1, ATF/CREB (ATF) and HiNF-P (P) on the promoter are shown. Overlapping YY1 and ATF/CREB binding motifs for Sites I and IV are also shown.

DNase H.S.

DNase H.S.

S1 H.S.

YY1 –340

ATF YY1 –640

YY1 ATF Sp1 RF P CDP –105 ... –45

YY1 +30

YY1 +200

ATF/CREB
CGCTGACGTCCATGAG
YY1

Site IV

ATF/CREB
AAATGACGAAATGTC
YY1

Site I

Discussion

The cell growth and cell cycle regulation of histone H4 gene expression is mediated by multiple regulatory elements located throughout the gene. Although significant progress has been made in understanding molecular mechanisms involved in the control of histone transcription, the identity of some of the factors and pathways involved still need to be elucidated. The purpose of this study was to determine if the ubiquitous transcription factor YY1 interacts with functional histone H4 gene regulatory elements.

The data presented in this study suggest that the versatile transcription factor YY1 has a functional role in regulating human histone H4 gene transcription. Transient expression experiments in eukaryotic cells lacking YY1 demonstrate that YY1 functionally interacts at Site III in the histone H4 promoter and within the coding region. Protein/DNA interaction experiments show that both endogenous YY1 in HeLa extracts and affinity-purified recombinant YY1 interact specifically with multiple histone gene regulatory elements, including Site I, Site III and coding region elements. Hence, several lines of evidence support the intimate involvement of YY1 in histone gene regulation.

YY1 may regulate histone gene expression by mediating protein-protein interactions with other gene regulatory factors, or by altering the responsiveness of other factors, perhaps by mutually exclusive binding. For example, YY1 may compete with other H4 gene transcription factors (e.g., ATF1/CREB (Wright et al., 1995; Guo et al., 1997) for binding to the H4 gene

promoter. The regulatory element Site IV (nt -643 to -636) and Site I (nt -117 to -110), both contain functionally relevant ATF/CRE motifs which overlap with YY1 binding sites (Figure 4-6). Modulation of histone H4 gene transcription may depend on critical threshold levels in the binding activities of YY1 and the opposing factors. There are many examples of YY1 influencing gene expression by competing with DNA binding factors of cell signaling pathways (Guo et al., 1998 ; reviewed in Shrivastava and Calame, 1994). Interestingly, the multi-functional YY1 forms protein/protein interactions with either transcription factor ATF/CREB or Sp1 (Seto et al., 1993; Lee et al., 1993; Zhou et al., 1995). Both ATF/CREB and Sp1 have been shown to regulate histone H4 gene expression (Guo et al., 1997; Birnbaum et al., 1995). Therefore, YY1 might also modulate histone transcription by altering ATF/CREB or Sp1 activity.

YY1 may participate in a process which remodels the local chromatin of the histone H4 gene. Both Sites I and IV reside near DNase I hypersensitive sites (Chrysogelos et al., 1989). Site III and coding region elements are within segments of the gene that change in accessibility to nucleases during the cell cycle (Chrysogelos et al., 1989). Thus, chromatin structure near YY1 binding sites are modified in a cell cycle stage-specific manner. I have previously observed that both YY1 DNA binding activity and overall protein levels remain constitutive during the cell cycle (Last et al., 1998; see Fig. 5-7D). Thus, if YY1 contributes to cell cycle control of the histone H4 gene it is probably by a mechanism mediated by protein-protein interactions with other gene regulatory factors, or by altering the responsiveness of these other factors. YY1 is known to interact with co-activators and repressors that alter the acetylation-status of histones (Austen et al., 1997; Lee et al., 1995; Yang et al., 1996; reviewed in

Pazin and Kadonaga). ATF/CREB family members have also been shown to interact with analogous cofactors (Lundblad et al., 1995). Notably, both YY1 and ATF/CREB interact with CBP/ p300, which represent a class of histone acetylases (Austen et al., 1997; Lee et al., 1995). Therefore, YY1 may have a critical role in recruiting factors which modify chromatin structure of histone H4 gene regulatory elements in conjunction with other sequence specific factors and histone-modifying enzymes.

Regulating nuclear matrix attachment is another transcriptional mechanism by which YY1 might be involved in modifying histone H4 gene architecture. Because YY1 is an established nuclear matrix protein (Guo et al., 1995; McNeil et al., 1998), YY1 may support association of the H4 gene with the nuclear matrix in a transient open chromatin state. This association might enhance promoter accessibility and allow other transcription factors to interact with the H4 promoter to drive transcription, although the details of the mechanism by which YY1 influences histone H4 transcription needs to be established. The data presented in this study suggest that this mechanism involves YY1 interacting at multiple regulatory elements of the gene.

CHAPTER V

REPRESSOR ELEMENTS IN THE CODING REGION OF THE HUMAN HISTONE GENE INTERACT WITH CELL CYCLE REGULATED CDP/*cut* HOMEODOMAIN PROTEIN COMPLEXES

Abstract

The coding region of the human histone H4 gene FO108 undergoes dynamic changes in chromatin structure which correlate with modifications in gene expression. Such structural alterations generally reflect transcription factor interactions with gene regulatory sequences. To test for regulatory elements within the coding region, I performed transient transfection experiments in HeLa cells using constructs with histone H4 sequences fused upstream of a heterologous thymidine kinase promoter and CAT reporter gene. H4 gene sequences from -10 to +210 repressed transcription 4.8-fold. Further deletion and mutational analysis delineated three repressor elements within this region. Using oligonucleotide competition analysis and specific antibody recognition in electrophoretic mobility shift assays, as well as methylation interference and DNase I footprinting analyses, I have identified the CCAAT displacement protein (CDP/*cut*) as the factor that interacts with these three repressor elements. CDP/*cut* binding to these repressor sites is proliferation-specific and cell cycle regulated, increasing in mid to late S phase. Our results

indicate that the proximal 200 nucleotides of the histone H4 coding region contain transcriptional regulatory elements that may contribute to cell cycle control of histone gene expression by interacting with repressor complexes containing CDP/*cut* homeodomain transcription factors.

Introduction

The histone gene family encodes a set of abundant and highly conserved basic proteins required for packaging DNA into nucleosomes and for the proper transcriptional regulation (reviewed in Osley, 1991; Stein et al., 1992). In proliferating cells, histone H4 gene expression is temporally and functionally linked to DNA synthesis. In early S phase, levels of transcription increase three to five-fold (Heintz et al., 1983; Plumb et al., 1983; Graves and Marzluff, 1984); then upon replication of the gene in mid S phase, there is a return to basal levels of transcription. However, in non-proliferating cells, whether in a state of quiescence or post-proliferative differentiation, the basal level of H4 gene transcription is severely diminished (Stein et al., 1994 (a)). For these reasons, the study of histone gene regulation provides an excellent model for elucidating cellular mechanisms involved in the control of gene expression both during the cell cycle and in the process of differentiation.

Transcriptional control of the human histone H4 gene FO108 results from cumulative influences of a dynamic chromatin structure and multiple composite regulatory elements located throughout the promoter (Stein et al., 1992; Chrysogelos et al., 1989). Extensive evidence in cellular and *in vitro* shows that the proximal region of the gene promoter contains two highly complex

domains of protein-DNA interactions (designated Site I and Site II) that are directly involved in regulating basal and cell cycle transcription levels (Pauli et al., 1987; Ramsey-Ewing et al., 1994; van Wijnen et al., 1989) . Transcription factors Sp1, ATF 1 and other ATF/CREB family members are able to interact with and regulate transcription via Site I (Wright et al., 1995; Birnbaum et al., 1995). Site II, the region necessary for cell cycle control and proper initiation of transcription, interacts with IRF-2 (Vaughan et al., 1995; Vaughan et al.,,1997), HiNF-D (a complex of CDP/cut, cyclin A, cdk and Rb related protein) (van Wijnen et al., 1994; van Wijnen et al., 1996) and HiNF-P. The distal promoter contains at least two other regulatory elements involved in modulating transcription levels: Site III, located between -370 and -320 (Kroeger et al., 1987) and Site IV, located at -651 to -625 bp each of which interact with YY1, ATF 1 and CREB (Guo et al.,, 1995; Dworetzky et al., 1992; Guo et al., 1997).

During the cell cycle, there are significant changes in chromatin structure and nuclease sensitivity both in the promoter and coding region of the histone H4 gene. DNase I and S1 nuclease sensitivity peaks in mid to late S phase, then decreases in M and G1 phases (Chrysogelos et al., 1989; Moreno et al., 1988). The region of the gene between -70 bp to +190 bp displays modified nucleosome structure throughout the cell cycle (Moreno et al., 1988). There are also significant changes in nucleosome structure during the cell cycle within the region from +200 to +275. Recently, transcriptional regulatory elements have been found in the coding regions of several histone genes (Fei and Childs, 1993; Gomez-Cuadrado et al., 1992; Herrero et al., 1996; Tung et al., 1990) including a potential cell cycle regulatory element in the mouse H3.2 histone gene (Hurt et al., 1991; Kaludov et al., 1996). These combined observations led

us to examine the proximal histone H4 coding region for transcriptional regulatory elements.

In this report, I provide evidence that the coding region of the histone H4 gene FO108 contains at least three elements that can function to repress transcription. All three of these elements interact with proliferation specific complexes containing the CCAAT displacement protein (CDP/*cut* homeodomain protein). Furthermore, the DNA binding activity of the CDP/*cut* complexes changes during the cell cycle, increasing in mid to late S phase. These data suggest that CDP/*cut* is involved in cell cycle and cell growth regulatory pathways.

Results

Functional identification of repressor elements within the coding region of the human H4 histone gene FO108

To identify potential transcriptional regulatory elements within the nucleosome "free" area of the histone H4 coding region (see Figure 5-1A), I constructed a series of heterologous reporter plasmids. Fragments from the FO108 human histone H4 coding region were cloned into pBLCAT4, upstream of a thymidine kinase promoter which controls transcription from the CAT gene. These chimeric constructs were transiently transfected into HeLa cells and tested for CAT activity. The transcriptional activity for the reporter construct containing the entire proximal coding region (-10/+210 pBLCAT) was 4.8-fold lower than that observed for control (Fig. 5-1B). Further analysis with

Figure 5-1. <u>Identification of three regulatory elements within the histone H4 coding region.</u> **(A)** An illustration showing the boundaries of the FO108 human histone H4 gene regulatory elements. The areas of the histone promoter which are "free" of normal nucleosome structure and have altered nuclease sensitivity during the cell cycle are shown below the gene map.

A

Histone H4 transcription

Histone H4

+310

+30

1

−45

II

−105

I

−340

III

−640

IV

Nucleosome "free" area

Nuclease sensitivity altered during cell cycle
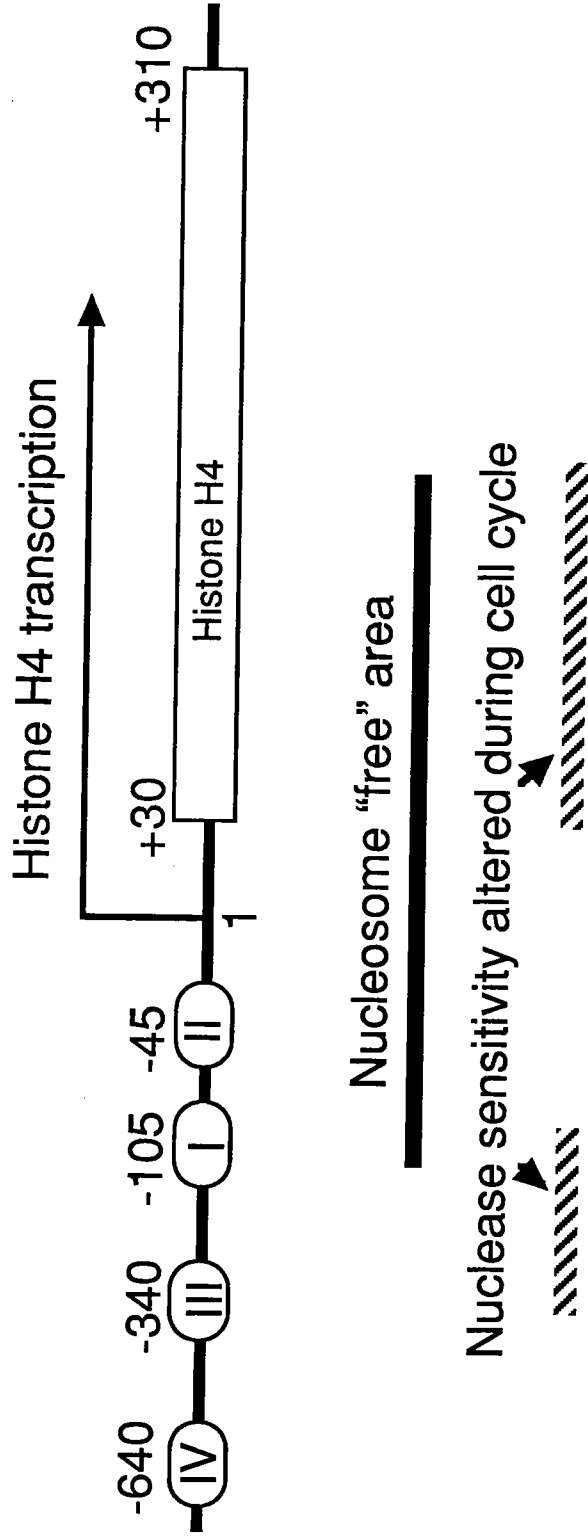
Figure 5-1 (continued). Panel (B) Relative CAT activities of H4 coding region/pBLCAT 4 reporter constructs transiently transfected in HeLa cells. The corresponding histone gene fragment of each construct is illustrated to the left. Results are expressed as percent of pBLCAT4 CAT activity with standard error. Fold repression for each construct is shown in parentheses. Each result represents multiple transfection experiments using multiple plasmid preparations (total $n \geq 32$). Constructs were considered to display repressor activity if they had greater than two fold difference in CAT activity compared with pBLCAT 4 and p values were < 0.005.

B

Constructs

% CAT activity
(fold repression)

pBLCAT 4   TK|CAT 100 ± 4

-10/+210   TK|CAT 21 ± 4 (4.8)

-10/+75   TK|CAT 39 ± 7 (2.6)

+75/+210   TK|CAT 40 ± 2 (2.5)

+72/+140   TK CAT 46 ± 9 (2.2)

+110/+140   TK CAT 92 ± 13 (1.1)
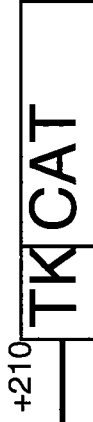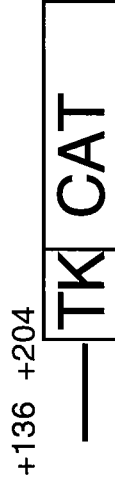
+136/+204   TK CAT 48 ± 5 (2.1)

subfragments of this region identified repressor activity in three separate elements; -10 to +75 bp (Coding Region 1 (CR1)) (39% of control), +72 to +140 bp (CR2) (46%) and +136 and +204 bp (CR3) (48%).

Delineation of motifs in the Coding Region 2 element required for repressor activity

To define sequence motifs that contribute to the activity of the CR2 repressor element (+72 to +140), I cloned synthetic oligonucleotides for the subfragments +72 to +110 bp, +110 to +140 bp, +72 to +125 bp and two sets of nucleotide substitutions in the +72 to +125 context into pBLCAT4. CAT activity of these constructs was assayed after transient transfection into HeLa cells. The results of these mutations and deletions are shown in Figure 5-2A. The +72/+125 pBLCAT exhibits repressor activity equivalent to that of full length CR2 (+72/+140) (46% of control). Further 3' deletion of CR2 as tested in +72/+110 pBLCAT resulted in reduced repressor activity (62 % of control), while the companion construct +110/+140 pBLCAT displayed none. Thus, CR2 activity is located within the sequences between +72 to +125. I designed two mutants of +72/+125 pBLCAT (Fig. 5-2B) to identify specific nucleotides necessary for repressor activity. Mutant 1 was based on sequence homology with a known repressor element in the human T-cell receptor Vβ2.2 gene (Dombret et al., 1996). Mutant 2 was designed to disrupt a potential factor binding element. The results show that there is a complete loss of repressor activity for Mutant 1 (Fig. 5-2A), while the nucleotide substitutions in Mutant 2 had essentially no effect on repressor activity. These data delineate the region

Figure 5-2. <u>Functional analysis of histone H4 Coding Region 2.</u>

(A) Graphic representation of relative CAT activity for the Coding Region 2

(CR2)-pBLCAT4 series transiently transfected into HeLa cells. Each result

represents multiple transfections using multiple plasmid preparations (total n ≥

32). Results are expressed as percent of pBLCAT4 CAT activity; error bars

designate the standard error. An asterisk above the bars for constructs

+72/+125 PBLCAT and +72/+125 mutant 2 PBLCAT indicate repressor activity

based on the criteria described for Figure 1.

A

Figure 5-2 (continued). **Panel (B)** Sequence of CR2 mutations cloned into pBLCAT4 constructs. Mutations were cloned in the +72/+125 context, but only the sequences between +85 and +115 are shown. The mutated residues are indicated by lower case letters under the wild type sequence.

B

+72/+125
PBLCAT

Sequence

+85

CGCAAGGTCTTGAGAGACAACATTCAGGGCA +115   WT

--gggtt----------ggg-aag----   Mutant 1

----------ggt---t-tc------   Mutant 2

between +72 and +125 nt as necessary for CR2 repressor activity and the underlined sequences within this region CCG<u>CAAGG</u>TCTTGAGAGACA<u>ACA</u><u>TTC</u>AGG in particular are essential.

Coding Region 3 is a bipartite regulatory element

To localize more precisely the repressor element within CR3 (+136 to +204), I constructed multiple clones containing subfragments of the region inserted into pBLCAT4 upstream of the heterologous thymidine kinase promoter. These reporter constructs were then transfected into HeLa cells and CAT activities analyzed. The results in Figure 5-3A show that clone +154/+204 pBLCAT (34% of control) retains repressor activity of the full length CR3 (+136/+204). The 3' and 5' deletion constructs +136/+184 pBLCAT (82%) and +170/+204 pBLCAT (70%) have less than maximal repressor activity. These results indicate that both regions from +154 to +170 and +184 to +204 are required for repressor activity in CR3 since constructs lacking either of these sequences had significantly reduced ability to repress gene expression. Next, I created a set of linker scanning mutants in the +136 to +204 nt context (Figure 5-3B) focusing on the region between +179 and +204 to identify which sequences are essential. The results from this mutation analysis are shown in Figure 5-3C. +136/+204 PBLCAT Mutants 1, 2 and 3 (Fig. 5-3B and C) have lost repressor activity, suggesting that all three sets of sequences mutated are required and confirming that the region between +186 and +201 is necessary for CR3 repressor activity. Because +136/+204 Mutant 4 still retains repressor activity, the sequences between +179 to +184 appear to be dispensable. Collectively, the results obtained from the deletion and mutation series suggest

Figure 5-3. <u>Functional analysis of histone H4 Coding Region 3.</u>

(A) Graphic representation of relative CAT activity for the Coding Region 3

(CR3)-pBLCAT4 deletion series transiently transfected into HeLa cells. Results

are expressed relative to pBLCAT4 CAT activity, with error bars designating the

standard error. An asterisk above the corresponding bar in the graphs indicate

repressor activity based on the criteria described in Figure 1. Each result

represents multiple transfections of multiple plasmid preparations ($n \geq 8$).
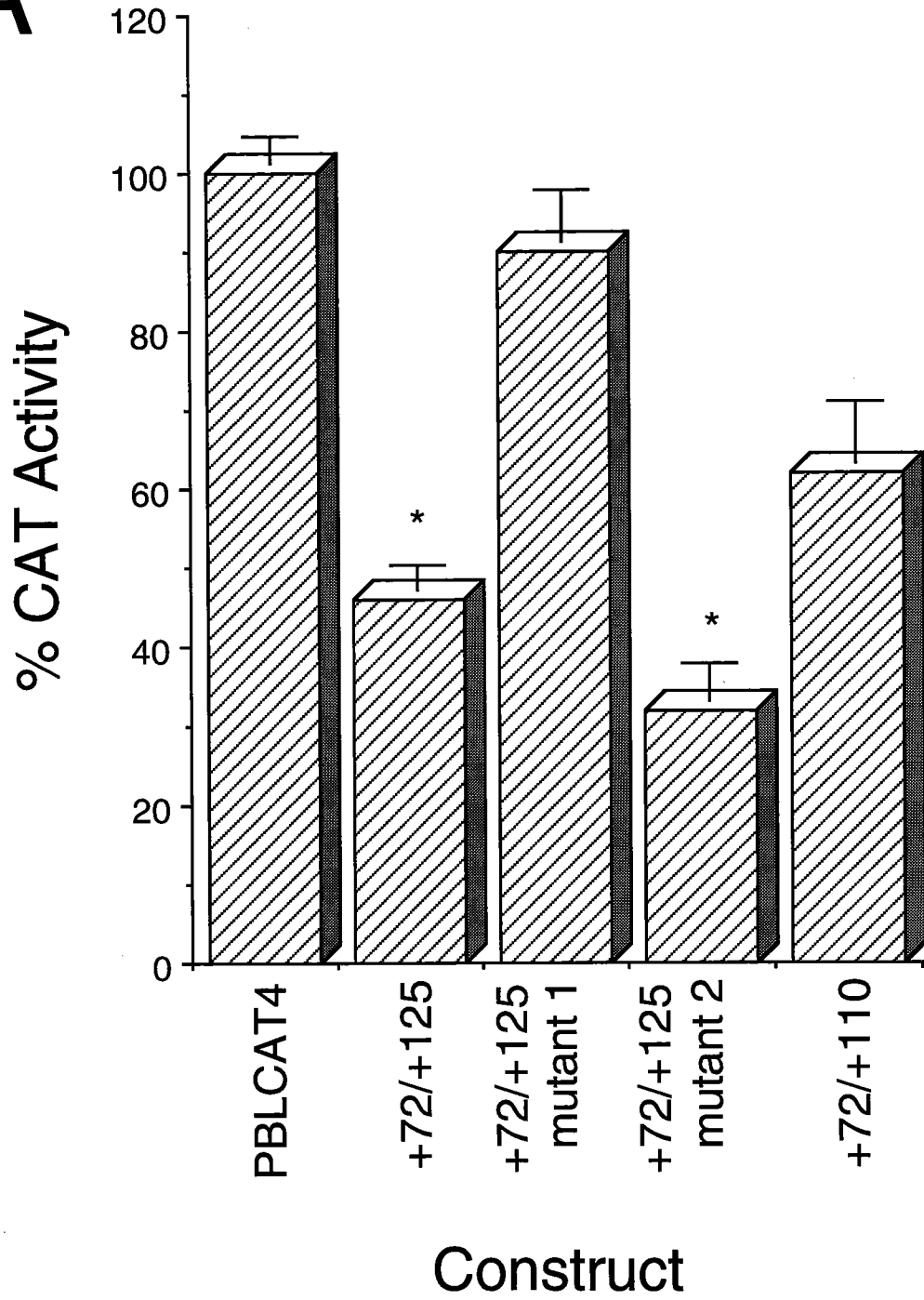
A
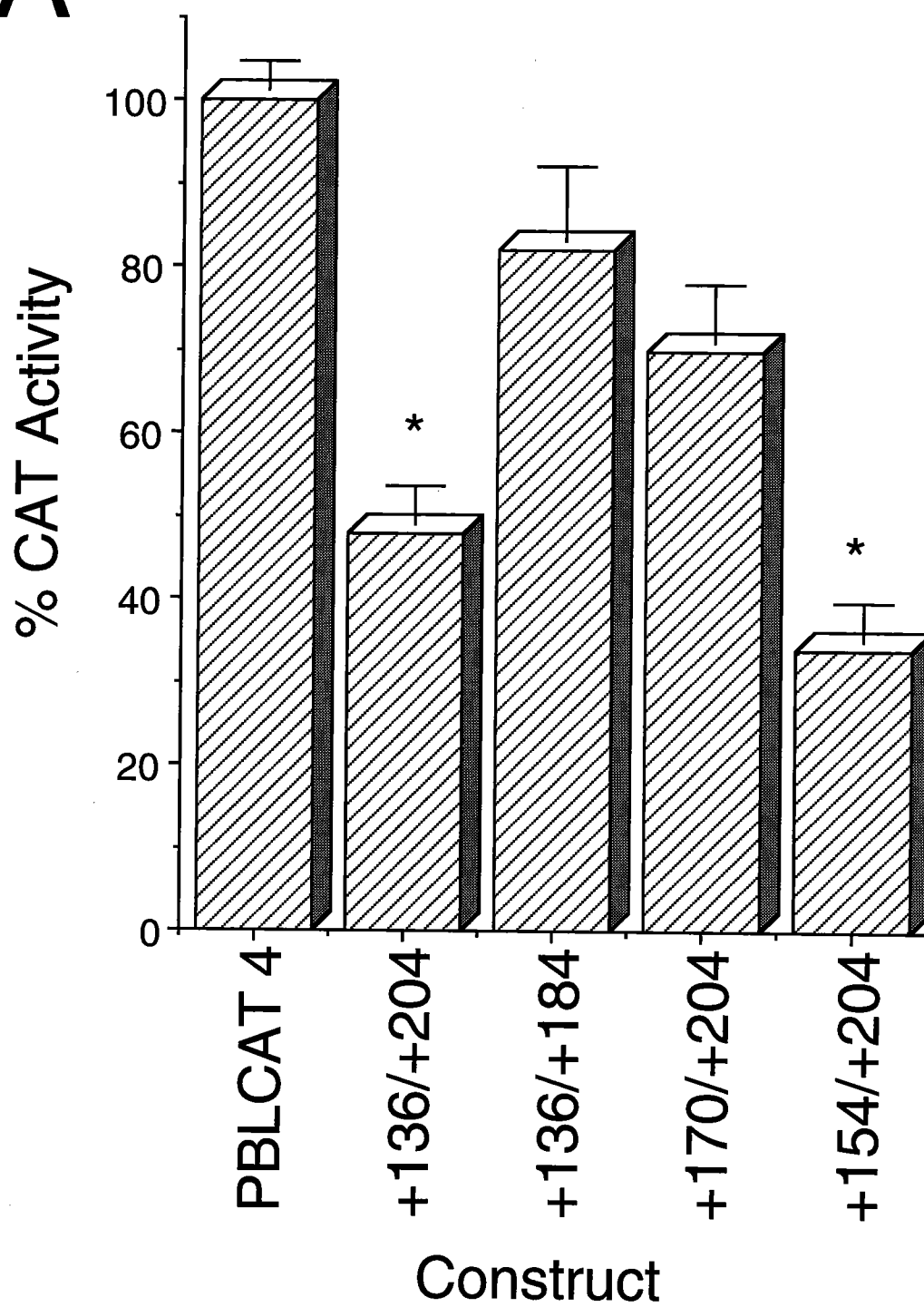
Figure 5-3 (continued). **Panel (B)** Sequence of CR3 mutations cloned into pBLCAT4 constructs. Mutations were constructed in the +136/+204 context, but only the sequences between +163 and +201 are shown. The mutated residues are indicated by lower case letters. The asterisk indicates missing nucleotides.

B

136/204
PBLCAT

Sequence

WT   +163   +201

WT   GGATCTCTGGCCTCATTTACGGAGGAGACCCGCGGTGTG

Mut1 -------------------------aattc

Mut2 -------------------*-gaattc-----

Mut3 ---*-------------gaattc-----

Mut4 -*------------gaattc-----

Figure 5-3 (continued). **Panel (C)** Graphic representation of relative CAT activity for the CR3 mutants 48 hours after transient transfection into HeLa cells.

C
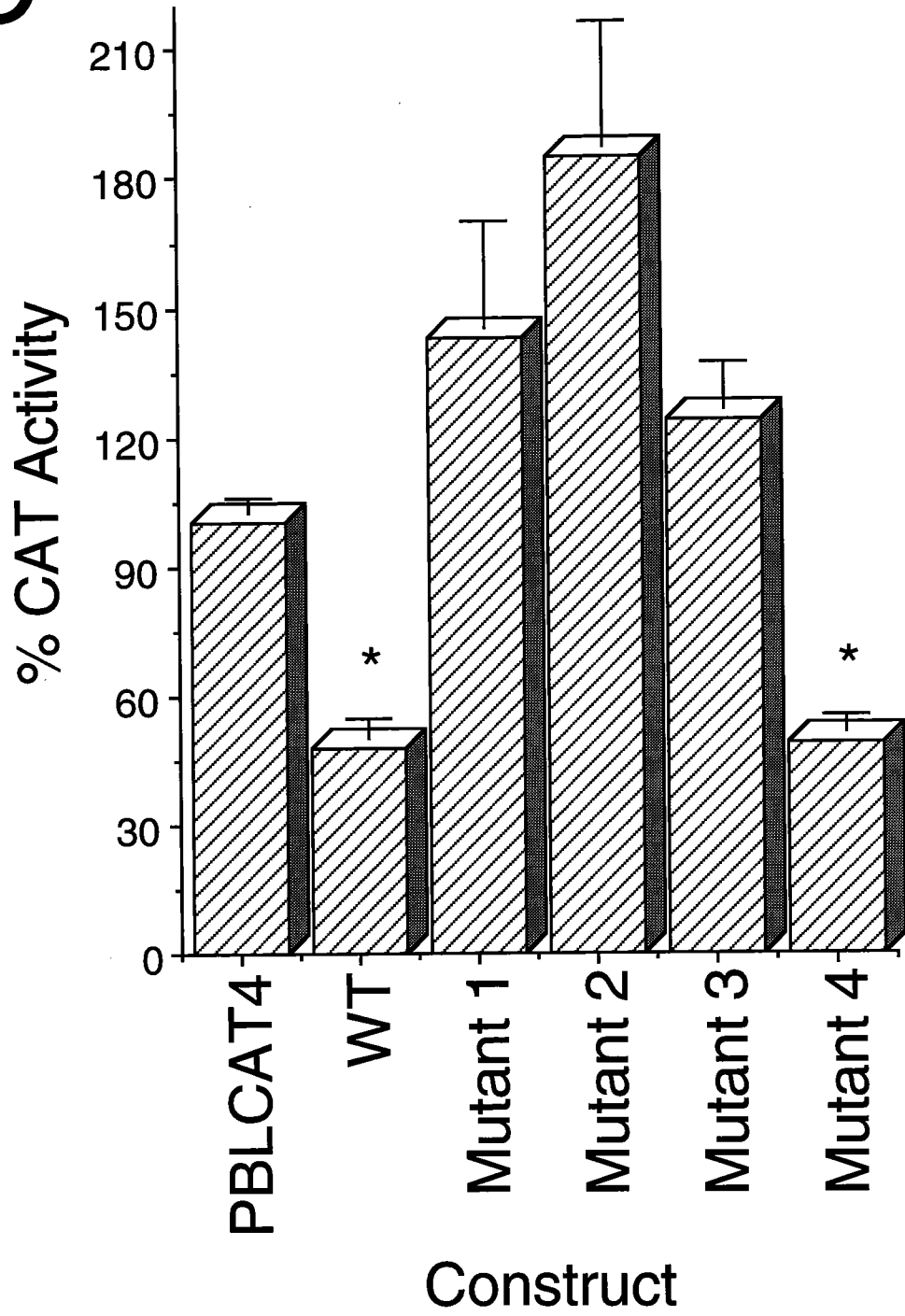
that a bipartite repressor element exists in CR3 because nucleotides +154 to +170 and +186 to +201 are necessary for full repressor activity.

Identification of CDP/cut binding sites within histone H4 coding region

I performed EMSA analysis to identify transcription factors which interact with the identified repressor elements in the coding region of the histone H4 gene. CDP/cut was tested as a candidate because it is an established repressor protein (Lievens et al., 1995; Superti-Furga et al., 1988; Barberis et al., 1987; Dufort and Nepveu, 1994; Skalnik et al., 1991) that can bind to other histone gene regulatory elements. Furthermore, there is sequence homology between established CDP/cut-binding sites and the coding region of the H4 gene; the sequence +10 to +17 is homologous to a known CDP/cut binding motif in Site II of the H4 histone promoter (van den Ent et al., 1994).

Radio-labelled EMSA probes for CR1, CR2, and CR3 (Fig. 5-4) were incubated with nuclear extracts from HeLa cells. Slowly migrating complexes are observed with all three probes, a characteristic of CDP/cut complexes (van Wijnen et al., 1996). Results in Figure 5-4 show that these complexes are specifically competed by a 100-fold excess of a CDP/cut DNA binding site oligonucleotide (lane 4) but not by 100-fold excess of the corresponding binding site mutant (lane 5). These same complexes are blocked by preincubation with an antibody against CDP/cut (lane 2) but not by the pre-immune serum (lane 3). Purified GST-CDP/cut DNA binding domain fusion protein (CR2-Cterm) can also interact specifically with these probes in EMSAs (data not shown). Taken together, these data show that the transcriptional repressor protein CDP/cut interacts with all three repressor elements in the

Figure 5-4. <u>CDP/*cut* complexes interact with CR1, CR2 and CR3.</u> EMSA probes for CR1 (-10 to +75), CR2 (+72 to +125) and CR3 (+154 to +204) were used to identify specific protein/DNA interactions with HeLa nuclear extracts. Lanes 1, HeLa nuclear extract. Lanes 2, extract incubated with anti-CDP/*cut* antibody. Lanes 3, extracts incubated with pre-immune guinea pig serum. Lanes 4, extract incubated with an 100-fold excess of unlabelled CDP/*cut* binding site oligonucleotide. Lanes 5, extract incubated with an 100-fold excess of unlabelled mutant CDP/*cut* binding site oligonucleotide. The position of the CDP/*cut* complexes is indicated with an arrow.

CDP->

Probe: CR1   CR2   CR3
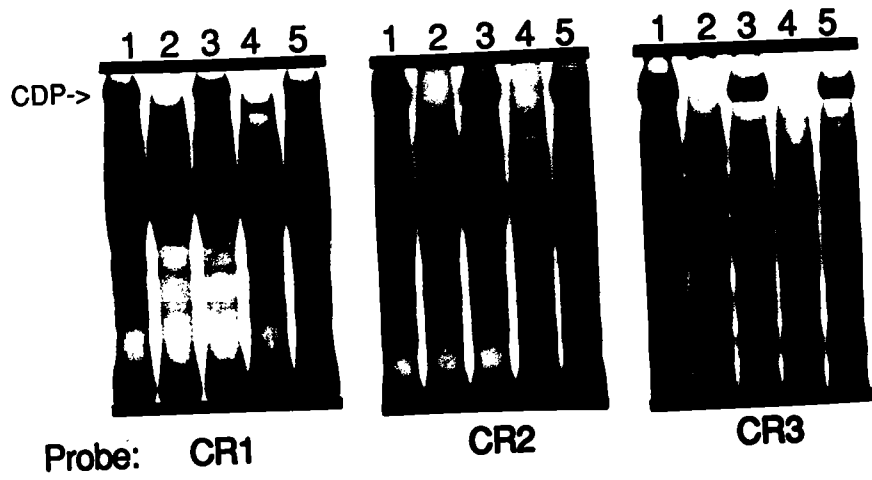
histone H4 coding region.

I performed methylation interference assays on the CDP/*cut* complexes formed with the CR1 (Fig. 5-5A), CR2 (Fig. 5-5B) and CR3 (Fig. 5-5C) probes. Figure 5-5A shows that the G residues of the bound CR1 complex at +9 and +13 nt are partially reduced, whereas the G residues at +5 and +17 nt are enhanced. The CDP/*cut* complex of CR2 (Figure 5-5B, bound lane) clearly shows enhancement of G residues at +74, +87, +93 and +113. The G residues at +102 and +109 are partially reduced. Similar alternating patterns of methylation interference and enhancements at G residues have been observed for CDP/*cut* interactions with the proximal promoters of H1, H3 and H4 histone genes (van den Ent et al., 1994). Results from DNase I footprinting (Fig. 5-6) provide additional evidence supporting that CDP/*cut* can interact with CR1 and CR2. Coding region probe was digested with DNase I after incubation with or without purified GST-CDP/*cut* DNA binding domain. The bound lane shows that GST-CDP/cut protects an area approximately from -4 to +26 nt in CR1 and an area from +100 to +127 in CR2. Therefore, the DNase I footprinting results corroborate the methylation interference data (Fig. 5-5) indicating that CDP/*cut* interacts with specific nucleotides in CR1 and CR2. Methylation interference assay of the CDP/*cut* complex on CR3 shows that G residues at +170 and +201 are enhanced, whereas the G residues at +163 and +183 are partially reduced. These results are consistent with sequences which were identified either by deletion or mutational analysis as being necessary for CR3 repressor activity (Fig. 5-3) (also see Fig. 5-9). Thus, for each of the repressor elements, CDP/*cut* interactions *in vitro*

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Figure 5-5. <u>Methylation interference patterns for CDP/*cut* complexes on CR1,</u> <u>CR2 and CR3.</u> To determine guanine residue contacts for CDP/*cut* complexes within the histone H4 coding region, I performed methylation interference analysis. The non-coding strand was labelled for all three probes. **(A)** CR1 EMSA probe (-10 to +75). Lane "F", Free Probe. Lane "B", DNA bound by the CDP/*cut* complex. Lane "I", Input. Results were analyzed by densitometry and G residues displaying significant changes between bound and free are indicated.

A

F B

+5 ->>
+9 ->>>
+13 ->>>
+17 ->>

CR1
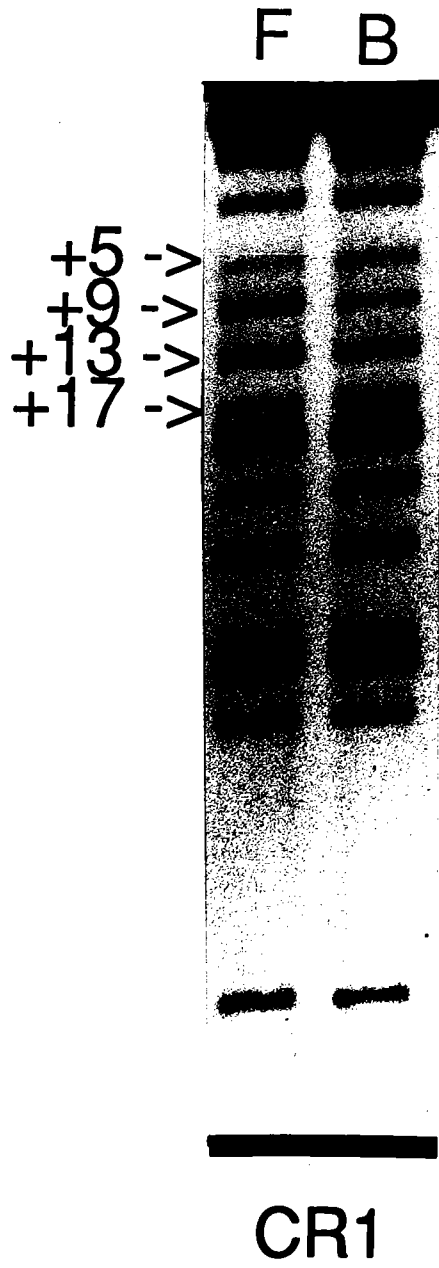
Figure 5-5 (continued). **Panel (B)** Methylation interference analysis using the CR2 EMSA probe (+72 to +125). Lane "F", Free Probe. Lane "B", DNA bound by the CDP/*cut* complex. Lane "I", Input. Results were analyzed by densitometry and G residues displaying significant changes between bound and free are indicated.

B

F B I

+74 ->

+87 ->

+93 ->

+102 ->

+109 ->

+113 ->

CR2

Figure 5-5 (continued). Panel (C) Methylation interference analysis using the CR3 EMSA probe (+154 to +204). Lane "F", Free Probe. Lane "B", DNA bound by the CDP/*cut* complex. Lane "I", Input. Results were analyzed by densitometry and G residues displaying significant changes between bound and free are indicated.

C

F　B　I

+163 ->
+170 ->

+183 ->

+201 ->
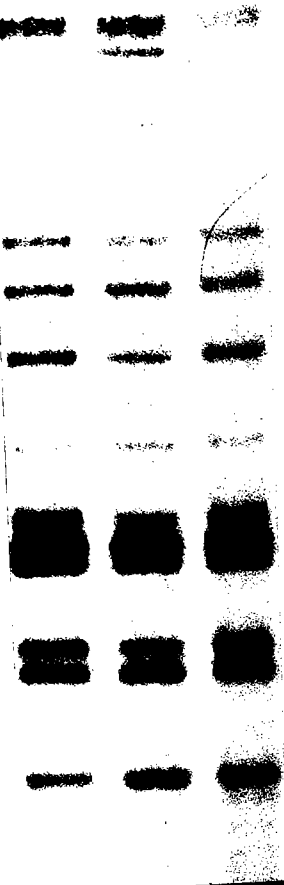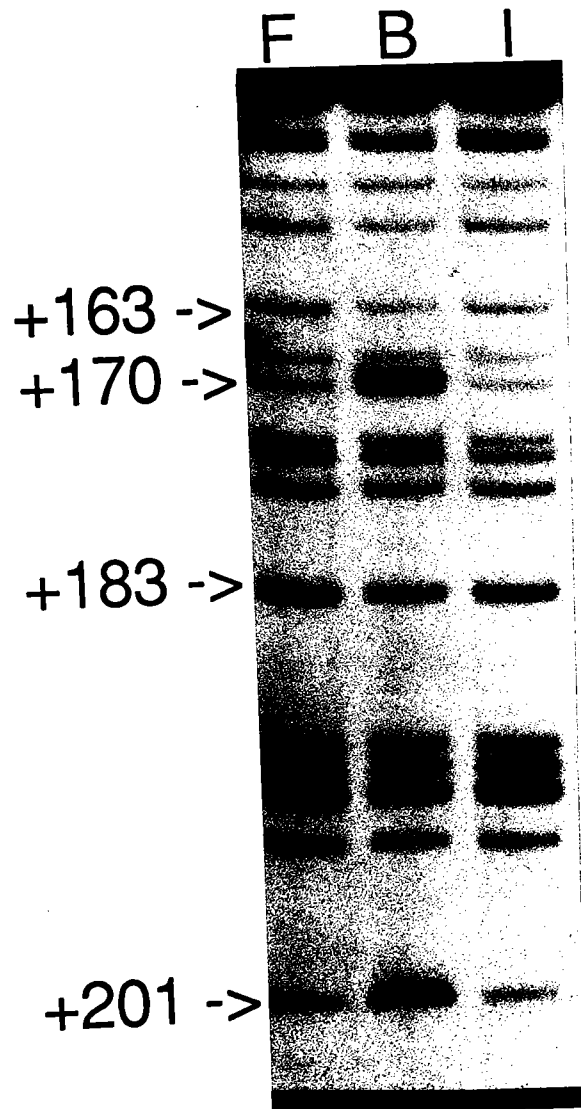
CR3

Figure 5-6. DNase I footprint analysis of GST-CDP/*cut* with CR1 and CR2.

Protein-DNA binding reactions contained ^{32}P-end labelled DNA probe (-90 to +270) and protein as indicated. Lane "F", free DNA incubated with 25 µg BSA. Lane "B", bound DNA incubated with 1 µg of GST-CDP/*cut* (CR2-Cterm) and 20 µg BSA. Lane "G", G ladder. GST-CDP/*cut* footprints are indicated by brackets.

F B G

+127
+100

+26

-4
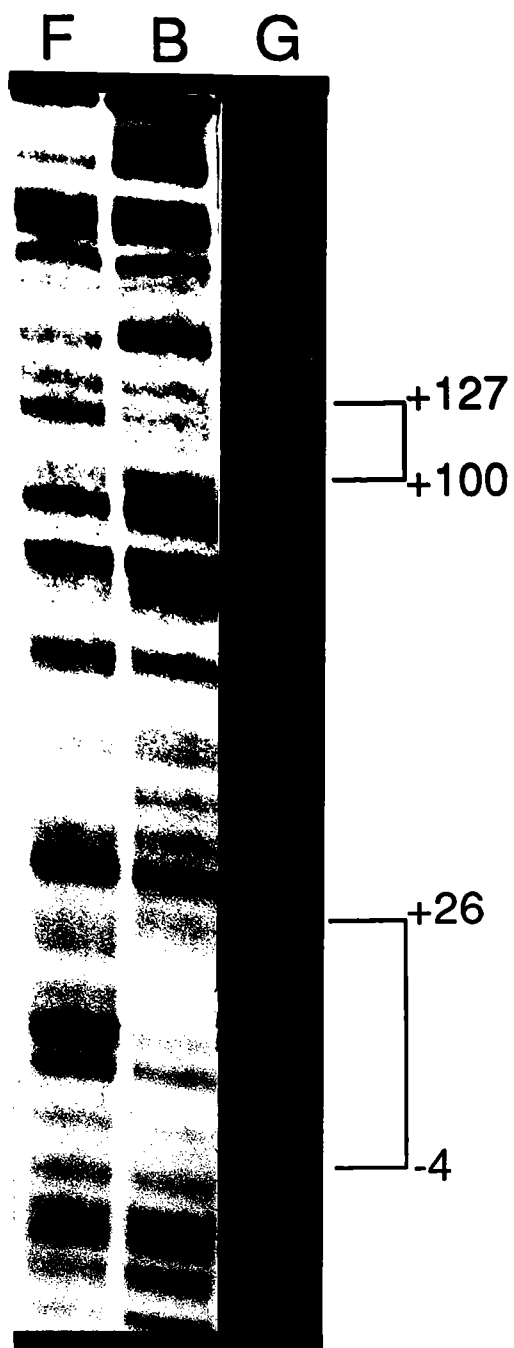
correlate very well with sequences found to be essential for repressor activity in our functional transfection analyses (Fig. 5-9).

Coding region CDP/*cut* complexes are cell cycle regulated and proliferation specific

I investigated the CDP/*cut* complexes within the histone H4 coding region to determine whether they are regulated during the cell cycle. HeLa cells were synchronized by double thymidine block and whole cell extracts prepared at various times following release from the block. DNA synthesis, as shown in Figure 5-7B, peaked at 5 hours and continued until 7 hours. Only low levels of CR1, CR2 and CR3 CDP/*cut* complexes could be detected in extracts of pre-release and early S phase cells (Fig. 5-7 A, 0 and 2 hours), whereas the CDP/*cut* complex formation increased at mid S (4 hours) and remained elevated in G2/M (8 hrs). CDP/*cut* activity was also detected in G1 phase (11 and 15 hours). CDP/*cut* protein levels do not correlate with DNA binding activity (Fig. 5-7C) and appear to be constitutive during the cell cycle. YY1 and E2F binding probes were used as controls to test the quality of extracts from the synchrony (Fig. 5-7A). The E2F binding pattern changed in a cell cycle dependent manner different than that observed for CDP/*cut*, whereas YY1 binding activity remained constant. Results from western blot analysis on YY1 protein levels (Fig. 5-7D) show that YY1 protein levels appear constant through out the cell cycle as well. The EMSA data indicate that the H4 coding region-CDP/*cut* complexes are cell cycle regulated, with increasing amounts of DNA binding activity as S phase progresses. Results from the western blot analyes suggest that the changes in CDP/*cut* DNA binding are not the result of changes

in overall CDP/*cut* protein levels, but may result from post-translational modification(s) or altered co-factor(s) interactions.

To determine whether coding region CDP/*cut* complex formation changes with cell growth status, I tested extracts from proliferating and quiescent IMR-90 normal diploid human fibroblasts, IMR-90. Figure 5-8 clearly shows a marked reduction in CDP/*cut* complex binding to coding region repressor elements CR1, CR2 and CR3 in extracts from quiescent compared with proliferating cells. The lower bands in comparative lanes did not change significantly; therefore, the changes in CDP/*cut* binding were not due to differences in quality of the extracts. As a positive control of growth status, I used an E2F EMSA probe. Results in figure 5-8 show that proliferating extracts have an E2F/cyclin A complex (complex 1), which is not found in quiescent extracts, whereas extracts from quiescent cells have more E2F/RB-family complexes (complex 2) (Reichel, 1992). These data suggest that the interactions of CDP/*cut* complexes with the coding region are cell growth regulated.

Figure 5-7. Cell cycle analysis of CDP/*cut* complex formation on CR1, CR2, and CR3. Whole cell extracts prepared from HeLa cells synchronized by double-thymidine block were collected at various points after release. **(A)** EMSA results using the CR1 (-10 to +75), CR2 (+72 to +125) and CR3 (+154 to +204), E2F and YY1 EMSA probes. EMSA reactions had equal amounts of protein for each time point tested. Extracts were quantitated in multiple assays. The positions of the CDP/*cut* and E2F complexes are indicated with an arrow. Time after release is indicated at the bottom of each figure.

A

CDP-> [gel image] CR1

CDP-> [gel image] CR2

CDP-> [gel image] CR3

-> [gel image] E2F

[gel image] YY1

0 2 4 8 11 15
HOURS

Figure 5-7 (continued). Panel (B) Cell cycle analysis of DNA synthesis. [3H]thymidine incorporation was measured at the indicated times after release from double thymidine block as described in Methods and Materials.

B

DNA Synthesis vs Hours bar chart
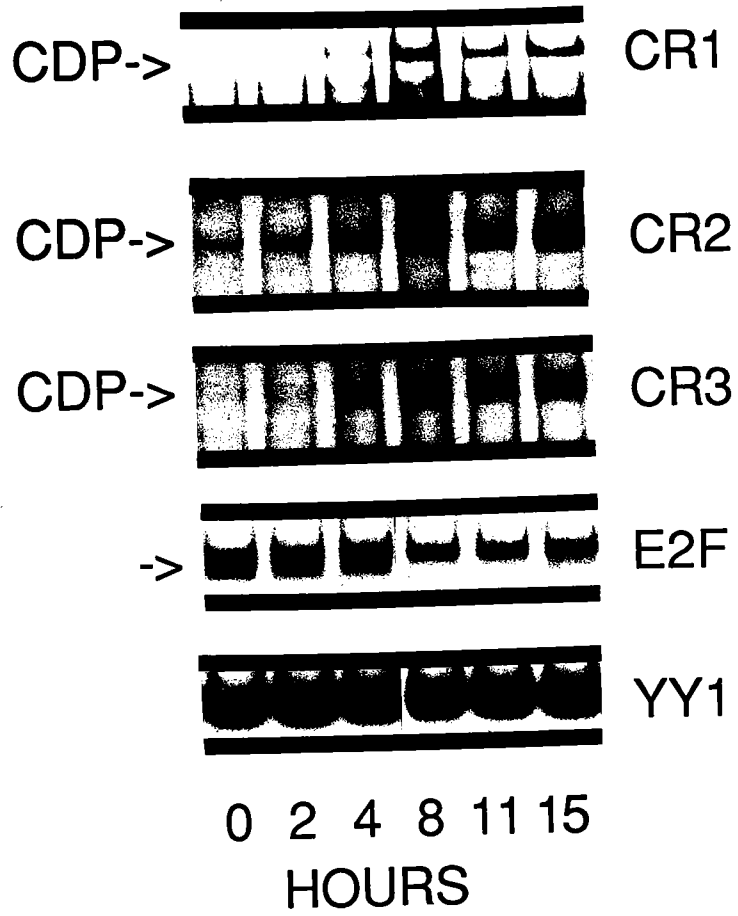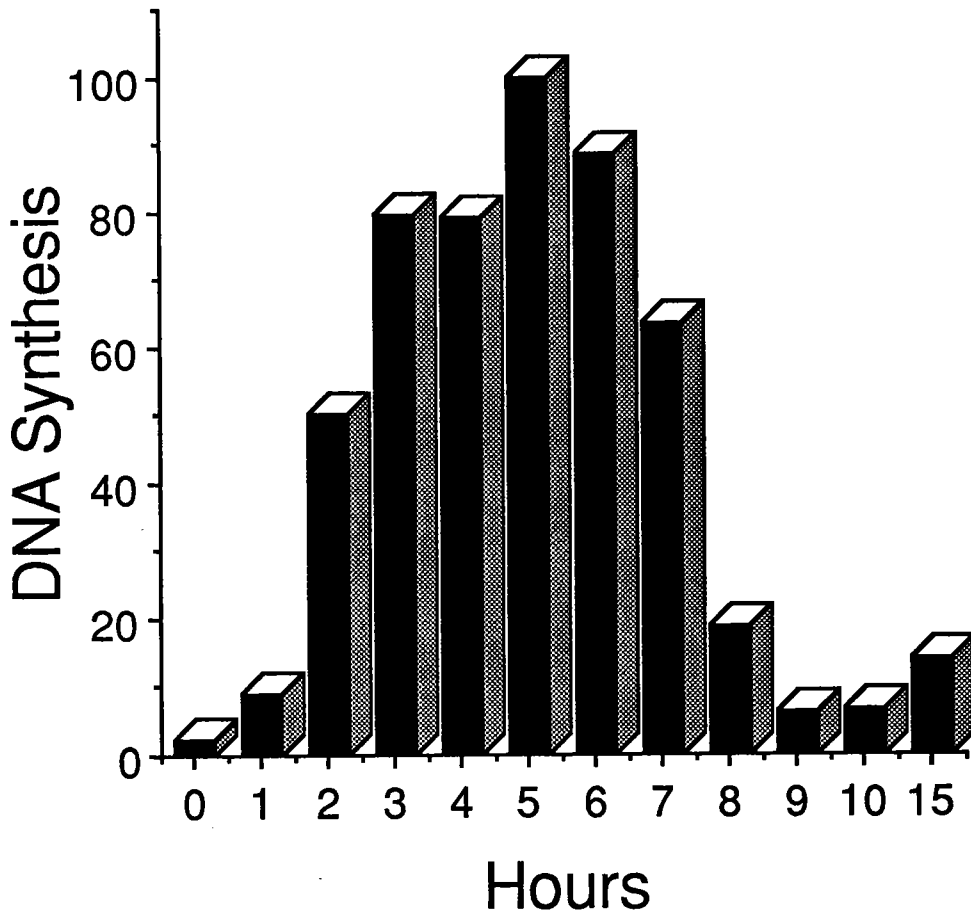
Figure 5-7 (continued). Panel (C) Western blot analysis on whole cell

extracts prepared from HeLa cells synchronized by double-thymidine block

were collected at various points after release. Equal amounts (20 μg) of protein

for each time point tested were fractionated by SDS-PAGE, electroblotted onto

nitrocellulose and probed with a primary antibody against CDP/*cut*. The

position of the CDP/*cut* protein is indicated with an arrow. Time after release is

indicated at the top of the figure. Western blot analysis of CDP/*cut* was

performed by Marleen de Ridder.

CDP→ 0 2 4 6 8 11 15 hrs after release
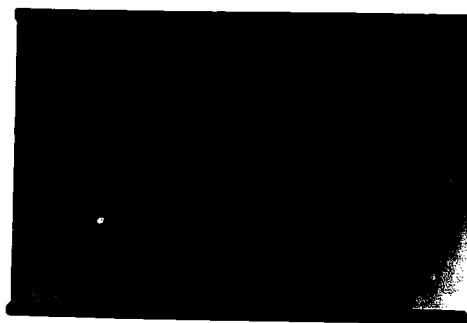
Figure 5-7 (continued). Panel (D) Whole cell extracts prepared from HeLa cells synchronized by double-thymidine block were collected at various points after release. Equal amounts (20 µg) of protein for each time point tested were fractionated by SDS-PAGE, electroblotted onto nitrocellulose and probed with a primary antibody against YY1 (Santa Cruz). Time after release is indicated at the top of the figure. Western blot analysis of YY1 was performed by Mark Birnbaum.

0 2 4 6 8 11 15 24 28 **HOURS AFTER RELEASE**

1 2 3 4 5 6 7 8 9

Figure 5-8. <u>CDP/*cut* interactions with CR1, CR2 and CR3 are growth</u> <u>regulated.</u> CDP/*cut* interactions with CR1 (-10 to +75), CR2 (+72 to +125) and CR3 (+154 to +204) probes were analyzed by EMSA. E2F oligonucleotide probe was used as a control. Extracts from proliferating (P) or confluent (C) IMR-90 fibroblasts were incubated with probe and separated by gel electrophoresis. The position of the CDP/*cut* and E2F complexes are indicated with arrows. E2F complex 1 is an E2F/cyclin A complex and E2F complex 2 is an E2F/Rb-family complex.

CR1   CR2   CR3   E2F
P C   P C   P C   P C

CDP->
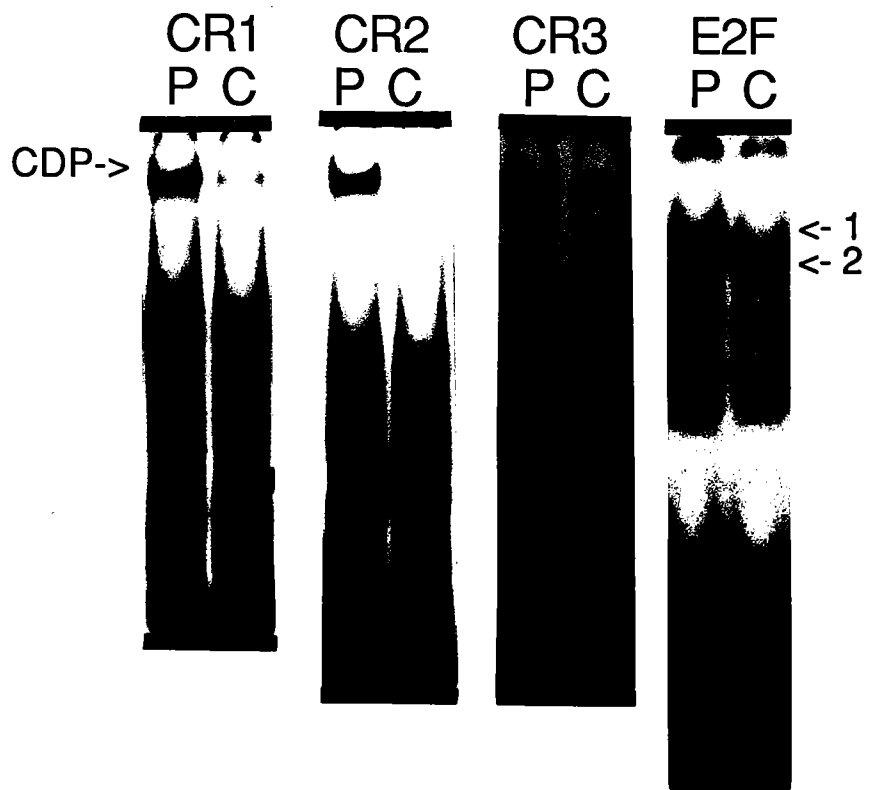
Figure 5-9. <u>Schematic diagram of the histone H4 coding region regulatory</u> <u>elements.</u> Nucleotides neccessary for transcriptional repression and CDP/*cut* contacts are shown.

CR1 | CR2 | CR3

CR1: $^{-4}$ GACAGAAGCTGTCTATCGGGCTCCAGCGGTCATG $^{+30}$

CR2: ^{74} CTAAGGCGGCCACCGCAAGGTCTTGAGAGACAACATTCAGGGCATCACCAAGC ^{124}

CR3: ^{163} CGGATCTCTGGCCTCATTTACGAGGAGACCCGCGGGTGTGC ^{201}

Ṅ = CDP/cut methylation enhancement or interference

N = Nucleotides critical for repressor function

■ = CDP/cut DNase I Footprint

Discussion

In this study, I examined the first 200 nucleotides of the FO108 histone H4 coding region for transcriptional regulatory elements. This region undergoes significant changes in nuclease sensitivity during the cell cycle and appears to be devoid of normal nucleosome structure (Chrysogelos et al., 1989; Moreno et al., 1988). My transfection experiments using heterologous promoter-CAT constructs in HeLa cells identified functional repressor activity within this region. Further dissection delineated three separate transcriptional repressor elements, CR1(-10 to +75), CR2 (+72 to +125) and CR3 (+154 to +204). EMSA in conjunction with immunological reagents revealed that CDP/*cut* interacts specifically with each of the three elements. DNase I footprinting and methylation interference assays showed that CDP/*cut* contacts nucleotides which are necessary for optimal repressor activity in functional transfection assays. Therefore, my results implicate CDP/*cut* as the factor responsible for the transcriptional repressor effect of all three coding region elements.

The intragenic repressor elements elucidated in this study are different than the CRAS elements (coding region activating sequences) that have been characterized for all four nucleosomal classes of mouse histone genes (Bowman et al., 1996). Unlike the human histone H4 coding region elements (CR1, CR2 and CR3) identified here, the CRAS elements play a positive role in transcription. Furthermore, the CRAS elements reside 3' to CR3 within the

homologous mouse histone H4 gene. Recently, mouse histone variant H1b has been identified as a factor which can interact *in vitro* with one of these CRAS elements (Kaludov et al., 1997). Two other unidentified specific binding factors for CRAS appear to be cell cycle regulated *in vitro*, with DNA binding peaking in G1, and then decreasing in S phase (Kaludov et al., 1996). In contrast, CDP/*cut* DNA binding activity to CR1, CR2 and CR3 increases in mid S phase, a pattern that is opposite to that observed for CRAS binding factors. Thus, the transcriptional coding region elements identified in this study are novel for histone gene regulation.

While this is the first report indicating that CDP/*cut* interacts with coding region elements in histone genes, CDP/*cut* has been found to interact with the promoters of many histone genes (sea urchin sperm histone H2B-1 (Barberis et al., 1987), 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)). This factor is also involved in the repression of other human genes, including gp91-phox (Lievens et al., 1995; Skalnik et al., 1991), HPV6 LCR (Pattison et al.,1997), c-myc (Dufort and Nepveu, 1994), and gamma globin (Superti-Furga et al., 1988). CDP/*cut* is a large (from 180 to 190 kDa in size) multifunctional transcription factor with three cut repeat motifs and one homeodomain (Neufeld et al., 1992). Each of these four domains are capable of binding to DNA independently of one another (Andres et al.,1994; Auferio et al., 1994). CDP/cut DNA binding activity is dependent on it's phosporylation status (Coqueret et al., 1996; Coqueret et al., 1998). Mailly et al. (1996) have shown that CDP/*cut* can repress gene expression by two distinct mechanisms: active repression and competition for binding site occupancy. In the gp91-phox

proximal promoter, CDP/*cut* appears to compete with multiple transcriptional activators for binding (Lievens et al., 1995). Whether or not one or both mechanism are involved in histone gene regulation remains to be determined.

I propose that transcriptional cell cycle regulation of the histone H4 gene results from the cumulative effects of positive influences of IRF-2 at Site II, and possibly the factors interacting at the CRAS elements in late G1/early S phase, and negative influences of the CDP/*cut* complexes interacting at CR1, CR2 and CR3 beginning at mid S phase. Furthermore, El-Hodiri and Perry (1995) have proposed that CDP/*cut* may repress histone transcription in cycling cells outside S phase by displacing positive acting factors. The interplay of multiple opposing elements in modulating transcriptional activity has been observed for other histone genes, including H3 and H4 in yeast (Freeman et al., 1992), H2A and H2B in yeast (Osley et al., 1986), and early H4 histone gene in sea urchins (Tung et al., 1990). Therefore, these observations of histone gene regulatory mechanisms in conjunction with our CDP/*cut* cell cycle results support the concept that the human FO108 histone H4 gene is regulated by multiple opposing regulatory elements.

Our results show that the higher order CDP/*cut* complexes interacting with the H4 coding region increase during the cell cycle as histone transcription decreases. These cell cycle regulated CDP/*cut* interactions may be mediated by a particular subset of CDP/*cut* complexes. For example, there are at least two protein iso-forms of CDP/*cut*, for which there may be divergent roles (Neufeld et al., 1992). Furthermore, we have reported that CDP/*cut* can form different higher order complexes with distinct cell cycle and cell growth regulatory factors (van Wijnen et al., 1994). Recently, Coqueret et al. (1998)

have shown that casein kinase II (CKII) phosphorylates a mouse homologue of CDP/*cut* resulting in down-modulation of DNA binding activity. CKII has been implicated in cell cycle regulation (Pepperkok et al., 1994). Thus, CDP/*cut* DNA binding activity may be modulated during the cell cycle by altered phosphorylation. I suggest that CDP/*cut* functions as a proliferation-specific repressor which is regulated during the cell cycle and interacts with histone gene coding sequences to control histone gene transcription.

CHAPTER VI

NUCLEOSOME BINDING BY THE CELL CYCLE REGULATORY TRANSCRIPTION FACTOR, CDP/*Cut*

Abstract

Alterations in chromatin structure of proliferation specific and developmentally regulated genes are associated with the growth status of the cell. The transcription factor CDP/*cut* has been implicated in the regulation of differentiation-specific and cell cycle controlled genes. To test the potential link between CDP/*cut* and chromatin structure, I analyzed the ability of CDP/*cut* to bind to the histone H4 gene promoter (-90 to +75) which was reconstituted *in vitro* into nucleosome cores. In electrophoretic mobility shift assays and DNase I footprinting experiments, I observed that affinity purified GST-CDP/*cut* specifically interacts with the CDP/*cut* binding element in a nucleosomal context without displacing the nucleosome core. The ability of CDP/*cut* to interact with nucleosomes and possibly facilitate chromatin remodeling may suggest a general mechanism by which CDP/*cut* could influence expression of multiple types of genes.

Introduction

The regulation of cellular proliferation and differentiation involves coordinate transcriptional control of many genes through the influence of multiple cell signaling pathways, arrays of key transcription factors and dynamic chromatin structure. Local promoter architecture of a gene can be modified by nuclear signalling pathways induced by physiological regulatory cues. Such pathways include transcriptional co-factors that function by phosphorylation, reversible acetylation or deactylation of nucleosomes and transcription factors. Some of these co-factors, such as GCN5P (Brownell et al., 1996), CBP/ p300 acetylases (Ogryzko et al., 1996) and RPD3 deactylase (Yang et al., 1996) lack sequence specificity and require transcription factors for recruitment to their target promoters and to modulate DNA accessibility in chromatin. Another mechanism which alters chromatin structure involves recruitment of the SWI/SNF complex to a gene as part of the Pol II holoenzyme that alters nucleosomes in an ATP-dependent manner (Imbalzano et al., 1994; Cote et al., 1994; Wilson et al., 1996; Owen-Hughes et al., 1996; reviewed in Wolffe and Pruss, 1996; Struhl, 1996). Many inactive genes exist in tightly packaged chromatin that inhibit transcription by blocking the binding and/or acessibility of transcription factors (Pina et al., 1990; Blomquist et al., 1996; Taylor et al., 1991) and the basal transciption apparatus to gene promoters (Knezetic and Luse, 1986; Workman and Roeder, 1987; Lorch et al., 1988). Induction of chromatin remodeling requires transcription factors that are capable of binding to

nucleosome cores. However, to date only a limited number of transcription factors, including GAL4 (Workman and Kingston, 1992), glucocorticoid receptor (Perlmann and Wrange, 1988), Max (Wechsler et al., 1994), TFIIIA (Rhodes, 1985) and Sp1 (Li et al., 1994) have been shown to interact with nucleosomal DNA.

The multifaceted nuclear protein CDP/*cut* participates in the regulation of many cell cycle regulated or differentiation-specific genes, including sea urchin sperm histone H2B-1 (Barberis et al., 1987), human histones H1, H3 and H4 (van den Ent et al., 1994; van Wijnen et al., 1996), H1, H2A, H2B, H3 and H4 genes in Xenopus (El-Hodiri and Perry, 1995), gp91-phox (Lievens et al., 1995; Skalnik et al., 1991), HPV6 LCR (Pattison et al.,1997), c-myc (Dufort et al., 1994), and gamma globin (Superti-Furga et al., 1988). CDP/*cut* is a large (from 180 to 190 kDa in size) transcription factor with four independent binding domains (three cut repeat motifs and one homeodomain) (Neufeld et al., 1992; Andres et al.,1994; Auferio et al, 1994). CDP/*cut* has been shown to repress gene expression by two distinct mechanisms: active repression and competition for binding site occupancy (Mailly et al., 1996). It has been proposed that CDP/*cut* may function by altering the ability of the mouse CD8a gene to associate with the nuclear matrix (Banan et al., 1997). We have previously shown that depending on promoter context, CDP/*cut* forms various complexes with cyclins, cdc 2 and Rb-related proteins (van Wijnen et al., 1996). The cell cycle controlled histone genes form a CDP/*cut* complex that is distinct from the CDP/*cut* complexes formed on the differentiation-specific human cytochrome b heavy chain (gp91-phox) gene (van Wijnen et al., 1996). We have also shown that CDP/*cut* DNA binding to the histone regulatory elements is cell cycle

regulated and proliferation specific (Holthuis et al.,1990; van Wijnen et al., 1997; Last et al., 1998). Because CDP/*cut* interacts with both proliferation and differentiation specific genes and is associated with cell growth regulatory factors, this factor may participate in several distinct physiological pathways.

CDP/*cut* interacts on a diverse set of promoters, suggesting that CDP/*cut* may function by a general mechanism involved in altering chromatin. Chromatin reorganization by acetylation and phosphorylation pathways may require transcription factors to target the promoter to properly regulate gene transcription. These transcription factors would need to recognize chromatin in order to initiate the process of remodeling. CDP/*cut* is a transcription factor likely to be involved in modifying chromatin due to its inherent ability to influence multiple types of growth regulated genes and to form various complexes with key cell signalling factors. To evaluate this hypothesis it is necessary to determine the ability of CDP/*cut* to interact with nucleosome cores.

In this study, I present evidence that CDP/*cut* binds to the histone H4 proximal promoter packaged in a nucleosome core without causing displacement. Furthermore, I show that CDP/*cut* specifically recognizes the Site II regulatory sequences in a nucleosomal context. These results support the hypothesis that CDP/*cut* may be involved in pathways which alter higher order chromatin organization.

Results

The proximal promoter of the histone H4 gene can be reconstituted into nucleosome cores.

To analyze the ability of affinity purified transcription factors to interact with the proximal histone promoter reconstituted into nucleosomal cores, it is necessary to establish that the H4 promoter can form a stable nucleosomal complex. I reconstituted mono-nucleosomes onto the H4 histone proximal promoter gene fragment (-90 to +75 nt) spanning a CDP/*cut* binding site. The results in figure 6-1A show that incubation of purified HeLa histone core proteins with the -90 to +75 histone H4 promoter probe results in the reconstitution of an electrophoretically stable mononucleosome complex. To assess the proper rotational orientation of the probe on the nucleosome core, I performed DNase I digestion analysis. Figure 6-1B shows that the 165 bp nucleosomal probe displays a 10 bp ladder upon DNase I digestion. However the free "naked" DNA probe does not. Because the 10 bp ladder is the hallmark of DNA in the correct rotational frame on a nucleosome core, our data indicate that the histone octamer forms specific nucleosomal arrangements with the proximal promoter of the H4 gene.

GST-CDP/*cut* interacts specifically with nucleosome cores

To assess whether CDP/*cut* is capable of interacting with nucleosomes, I performed binding reactions in which the mono-nucleosome probe complex was incubated with affinity purified CDP/*cut* and analyzed by EMSA (Fig. 6-2). Increasing amounts of affinity purified GST-CDP/*cut* (CR2-Cterm) were added to

Figure 6-1. *In vitro* reconstitution of the -90 to +75 histone H4 proximal promoter probe into nucleosome cores. (A) Naked -90 to +75 histone promoter probe and purified nucleosome core probe were analyzed by EMSA to confirm that the mononucleosomes were reconstituted on the promoter probe.

A

Naked DNA
Nucleosomal

Figure 6-1 (continued). Panel (B) Naked DNA probe and the reconstituted nucleosome probe were digested with increasing amounts of DNase I purified, then analyzed on a 6 % sequencing gel. Naked DNA probe was digested with 0, 0.25, 0.5, 1 or 2 units of DNase I. The nucleosomal probe was digested with 0, 0.25, 0.5, or 2 units of DNase I. DNase I digestion of the nucleosomal probe produces a repeated 10-11bp ladder which is not observed for the naked DNA probe.

-90 to +75
Naked DNA probe
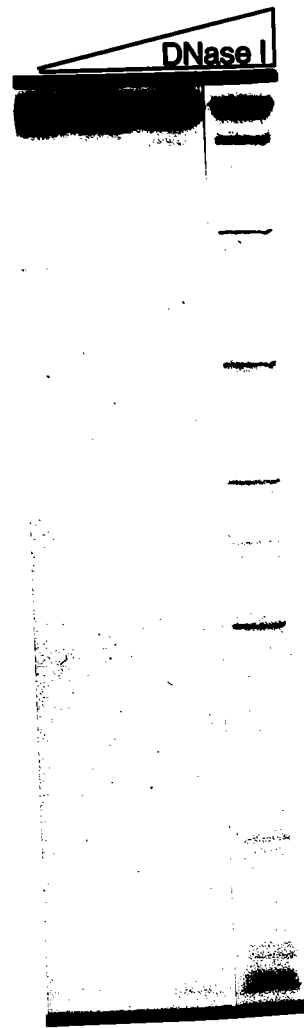
-90 to +75
Nucleosomal probe

Figure 6-2. <u>CDP/*cut* specifically interacts with nucleosome cores.</u> EMSA was performed using increasing amounts of affinity purified GST-CDP/*cut* (CR2-Cterm) on both the -90 to +75 naked DNA and nucleosomal probes. Amount of GST-CDP/*cut* in naked DNA probe reactions, 0, 0.02, 1 and 2 µg. Amount of GST-CDP/*cut* in nucleosomal probe reactions, 0, 0.5, 1 and 2 µg. To test for specificity of binding, competition experiments were performed by adding 1 pmol of CDP binding site oligonucleotide (CDP oligo.) or nonspecific (n.s. oligo) to EMSA binding reactions. Naked DNA free probe (f.p.), nucleosomal probe (nucl.) and CDP-nucleosome complexes (CDP+nucl.) are marked. EMSA and probe preparation were performed as described in Li et al. (1994). Autoradiographs were analyzed by densitometry.

-90 to +72

Naked DNA   Nucleosome

GST-CDP   GST-CDP

<- CDP
+nucl.

<- nucl.

<- f.p.
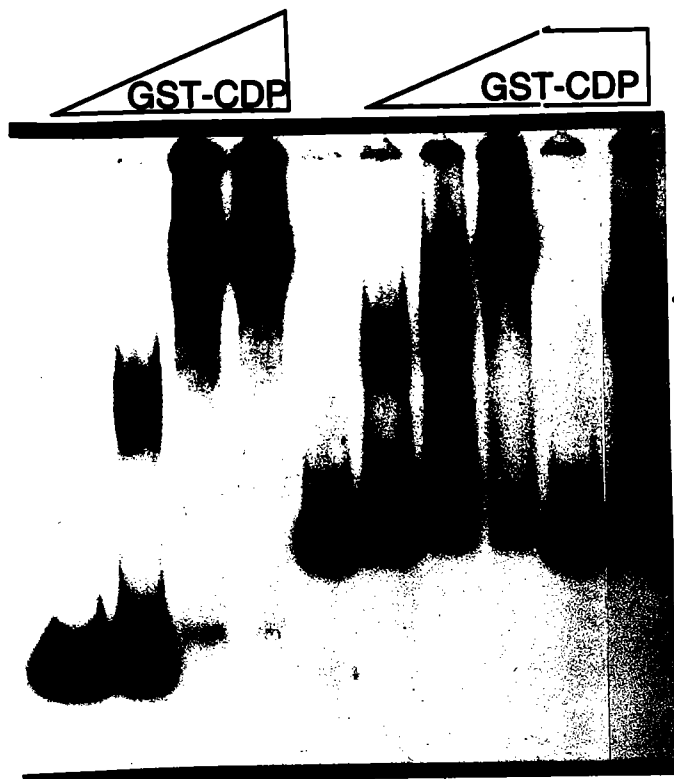
n.s. oligo
CDP oligo.

either naked free probe or reconstituted nucleosomal probe. EMSA results show that GST-CDP/*cut* forms a ternary complex with mono-nucleosome bound DNA. Results from densitometry analysis show that the affinity of the recombinant GST-CDP/*cut* (CR2-Cterm) for nucleosome cores is about 20 fold less than that for naked DNA. Oligonucleotide competition assays were used to establish the specificity of the CDP/*cut* interaction with nucleosome bound DNA. CDP/*cut* binding was competed by an oligonucleotide containing a wild type CDP/*cut* binding element and not by an oligonucleotide lacking a CDP/*cut* binding site. Specific competition with the CDP/*cut* binding site abolished the CDP/*cut*-nucleosome complex and the unbound reconstituted nucleosomes reappeared. Results from control EMSA experiments show that the purified glutathione-S-transferase protein, by itself, does not bind to the -90 to+75 nucleosome core probe (data not shown). These results indicate that CDP/*cut* is capable of interacting with DNA tightly bound into nucleosome cores.

DNase I footprinting of GST-CDP/*cut* interaction with core nucleosomes.

I have established that CDP/*cut* binds specifically to nucleosomal cores. To determine whether CDP/*cut* is bound to its correct binding site on the nucleosome, I performed DNase I footprinting experiments on the CDP/*cut*-nucleosome core complex. Results from densitometry analysis of figure 6-3 show that GST-CDP/*cut* interacts specifically with its recognition element from -53 to -35 nt from the transcriptional start site. Interestingly, we have previously shown that the same GST-CDP/*cut* protein mediates a larger DNase I footprint at Site II on free DNA probe which extends from nucleotides -58 to -31

Figure 6-3. Position of CDP/*cut* binding to -90 to +75 nucleosome core.

DNase I footprinting experiment was performed to determine where CDP/*cut* interacts on the nucleosomal probe. Increasing amounts (0, 1, 5, 7.5 and 12.5 µg) of affinity purified GST-CDP/*cut* (CR2-Cterm) were added to the binding reactions prior to DNase I digestion (2 units). After digestion, the DNA was purified and analyzed on a 6 % sequencing gel. The first lane (G) contains the probe's guanine ladder. Nucleotide numbers are marked next to guanine ladder. GST-CDP/*cut* footprint is indicated in figure as residing between nucleotides -53 and -35, which is within the region of the 10 bp ladder.

G   -   CDP

+1 ->

-25 ->

GST-CDP

-58 ->
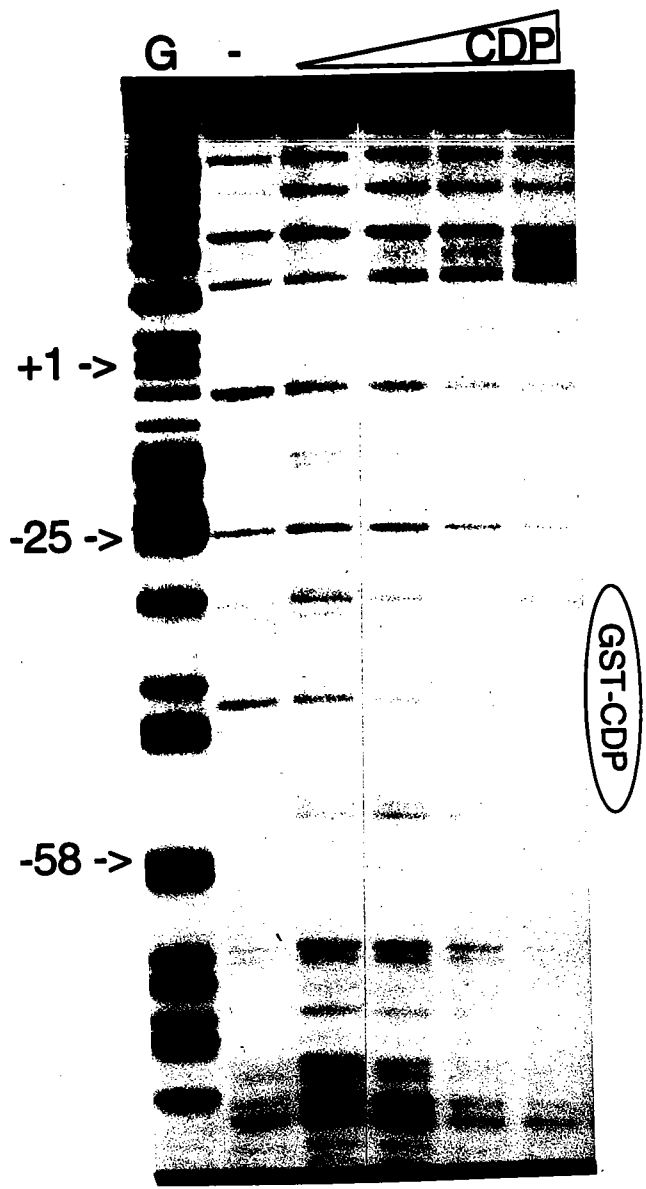
-90 to +75
Nucleosomal probe

(van Wijnen et al., 1996). However, little if any CR1-CDP/cut nucleosome

binding was observed. Our results indicate that GST-CDP/*cut* recognizes the

essential binding motif of Site II in a chromatin context in which the

conformation of the CDP/*cut* binding site is modified due to nucleosomal

packaging.

GST-CDP/*cut* interacts with core nucleosomes without displacing nucleosomes.

Although CDP/*cut* interacts specifically with nucleosomes, it may

displace the nucleosome core. My data show that this does not appear to be

the case. In figure 6-2, the amount of free probe was not altered and no

complex with CDP/*cut* and free probe was observed in binding reactions with

nucleosomal probe. These results suggest that CDP/*cut* binding to the

nucleosome core does not cause displacement of the nucleosome. To assess

the potential of CDP/*cut* to form a nucleosomal complex without causing

nucleosome core displacement, I analyzed CDP/*cut*-nucleosome complexes

after competition with excess amount of plasmid DNA (figure 6-4). A similar

approach was used by Workman and Kingston (1992) to show that GAL4 forms

a metastable ternary complex which displaces nucleosomes upon plasmid DNA

competition. If nucleosome cores are displaced, competition with plasmid DNA

will give rise to a complex of free DNA and CDP/*cut* because the histones will

bind to the excess competitor DNA. If on the other hand, plasmid DNA

competition results in detection of only the nucleosomal probe complex, this

result would imply that CDP/*cut* was competed by the plasmid DNA and did not

disrupt the nucleosome core. Because only the nucleosomal probe complex is

observed by DNA competition (Fig. 6-4), the results indicate that CDP/*cut* forms

Figure 6-4. <u>CDP/*cut* binding does not disrupt nucleosome core.</u> EMSA experiment using the -90 to +75 nucleosomal probe with affinity purified GST-CDP/*cut* (CR2-Cterm). The amount of GST-CDP/*cut* (CR2-Cterm) added to EMSA reactions was 0, 0.1, 0.2, 0.5 and 1 µg. To test if CDP/*cut* binding disrupts nucleosome cores, 1 and 2 µg of pUC 19 plasmid was added to the EMSA reactions. Nucleosome disruption would have caused an increase of free probe. Free probe (f.p.), nucleosomal probe (nucl.) and CDP-nucleosome complexes (CDP+nucl.) are marked.

-90 to +72
Nucleosome probe

GST-CDP

<- CDP
+nucl.

<- nucl.

<- f.p.
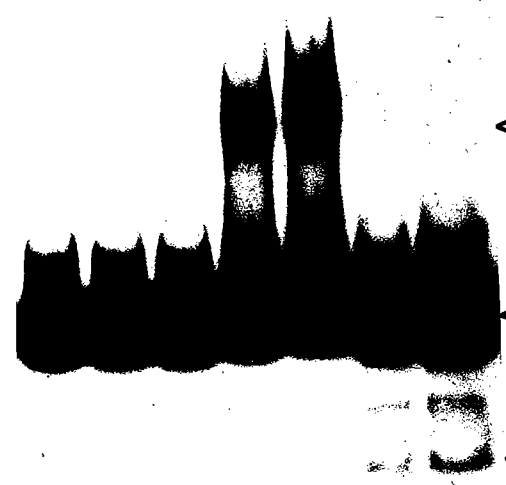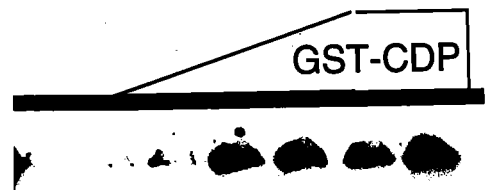
2γ plasmid
1γ plasmid

a complex with nucleosome cores without causing displacement.

Discussion

The local chromatin environment of a gene is important for proper gene expression and is influenced by physiological cell-signaling pathways. Key transcription factors and transcriptional co-factors are intimately involved in these gene regulatory pathways. In this study, I have shown that the transcription factor CDP/*cut* binds to Site II of the histone H4 promoter reconstituted into nucleosome cores. EMSA results show that GST-CDP/*cut* (CR2-Cterm) specifically interacts with these nucleosome cores without causing disruption of the nucleosome. Furthermore, DNase I footprinting results show that GST-CDP/*cut* (CR2-Cterm) interacts with Site II of the histone promoter, which we have previously characterized as the binding element of CDP/*cut* on DNA without nucleosome. Therefore, CDP/*cut* could be a key transcription factor necessary for initiating chromatin remodeling in the H4 promoter.

Although recombinant CDP/*cut* binds with a lower affinity to nucleosomal DNA, my data clearly show that GST-CDP/*cut* interacts with nucleosome cores at a similar protein concentration as other nucleosomal binding transcription factors (Adams and Workman, 1995; Workman and Kingston, 1992; Li et al., 1994) . Indeed, several DNA binding proteins including HSF, NF-kB and USF can not interact alone with nucleosome cores at the same concentration as CDP/cut (Taylor et al., 1991; Chen et al., 1994; Adams and Workman, 1995). Because CDP/*cut* can associate with other gene regulatory proteins and binds to composite elements which interact with other DNA binding factors, it would

not be surprising to find that these auxiliary proteins may modulate the affinity of CDP/cut interactions on nucleosome cores. It has been observed that the ability of some transcription factors to interact with nucleosome cores is greatly enhanced by the simultaneous binding of another factor or with the assistance of a co-factor (Chen et al., 1994; Adams and Workman, 1995; Taylor et al., 1991; Walter et al., 1995). In addition to this, CDP/cut may undergo endogenous posttranslational modifications (e.g., phosphorylation) that could alter its affinity and ability to interact with nucleosomes. However, the principal finding of our study is that CDP/cut does have the intrinsic ability to interact by itself with nucleosome core structure.

My results suggest that CDP/cut may be involved in pathways regulating chromatin structure. Alterations in chromatin structure directly influence expression of genes and may be necessary for correct developmental gene regulation (reviewed in Felsenfeld, 1992; Wolffe, 1991; Getzenberg et al., 1991; Gross and Garrad, 1988). CDP/cut may play a role in setting the proper chromatin context for histone expression in the quiescence to proliferation transition (Fig. 6-5A). The proximal gene region (-140 to +190) displays modified nucleosome formation in proliferating cells which is not present in non-proliferating cells (Moreno et al., 1988). This altered active chromatin architecture correlates with proliferation specific occupancy of Site II (Pauli et al., 1987; Stein et al., 1989). We have identified four CDP/cut binding elements within this region (Last et al., 1998). CDP/cut may function by interacting with these elements within the proximal promoter in nucleosome cores, enabling its accessory proteins (e.g., cdc 2, RB, cyclin A) and perhaps other H4 promoter associated factors, to perform biochemical modifications that may alter local

Figure 6-5. <u>Model of mechanism whereby CDP/*cut* is involved in chromatin remodeling.</u> (A) Inactive gene arranged in nucleosome is rearranged upon exiting G_0 in a process involving CDP/*cut*. A complex containing CDP/*cut* binds to and then modify the nucleosomes. (B) CDP/*cut* may also have a role in cell cycle control. During early S phase when CDP/*cut* binding levels are lower, transcription increases. In late S phase and G_2/M, when CDP/*cut* binding levels are higher, transcription decreases. CDP/*cut* function is to facilitate proper chromatin structure and to repress transcription in late S phase and G_2/M.
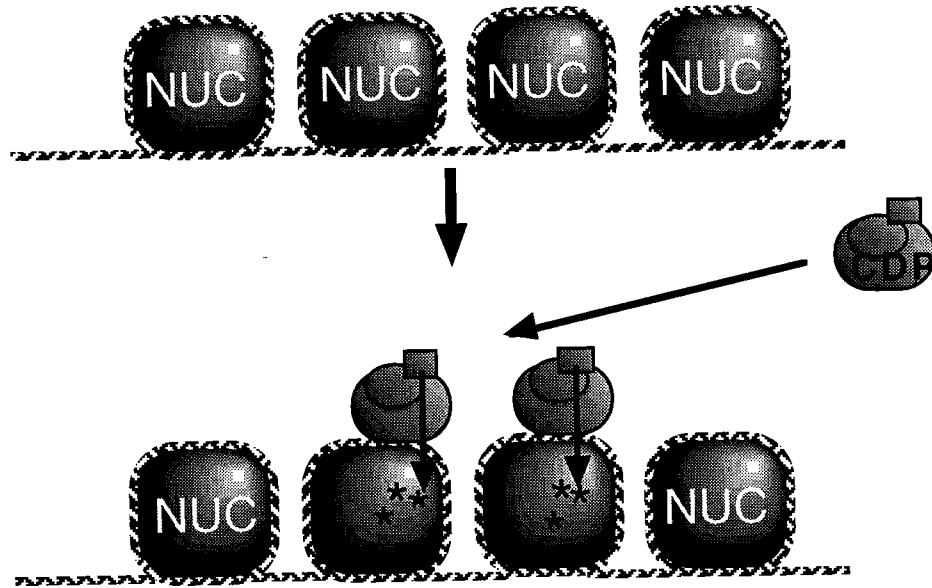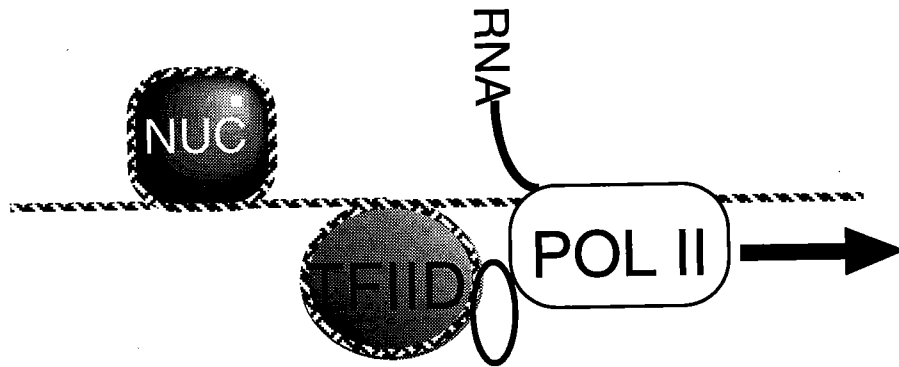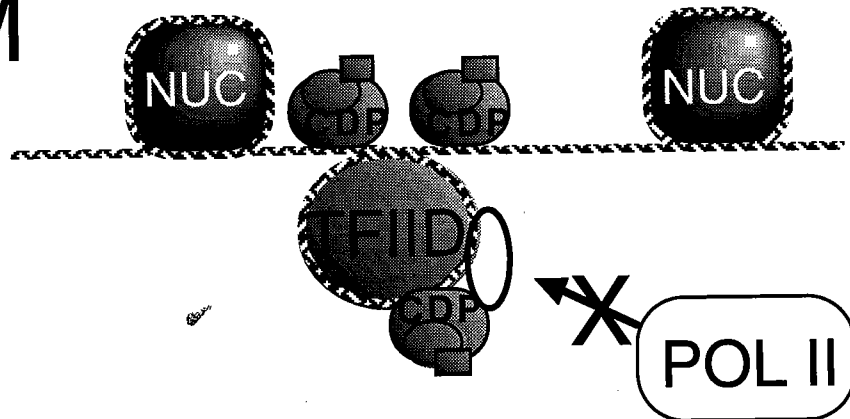
chromatin structure.

I propose that CDP/cut is an architectural protein that interacts at multiple regulatory elements of the histone H4 gene where it assists in modulating the local chromatin structure. It is probably more than a coincidence that the five CDP/cut binding elements are positioned in a segment of the gene which displays a modified nucleosomal organization and cell cycle altered nuclease hypersensitivity (Last et al., 1998; Chrysogelos, 1989; Moreno, 1988). Nuclease hypersensitive sites have been proposed to arise by several different mechanisms including displacement of nucleosomes by transcription factors binding (Gross and Garrad, 1988). Lucchini and Sogo (1995) have shown that replication of transcriptionally active chromatin results in temporary nucleosome formation on both daughter genes which must be disrupted prior to regeneration of active chromatin. Thus, a mechanism must exist for chromatin reorganization of active replication-dependent genes. CDP/cut may be one of the architectural transcription factors which is necessary for binding on nucleosome cores to initiate this process.

I have previously observed that the levels of CDP/cut complexes binding to the histone coding region repressor elements were lowest in early S phase and then increase until late S/G$_2$ boundary (Chapter 5; Last et al., 1998). Histone transcription peaks 3 to 5-fold in early S phase and DNA replication of the gene occurs about mid S phase (Plumb et al., 1983; Iqbal et al., 1984). The cell cycle regulated CDP/cut factor could function by first initiating the removal of the nucleosomes from the daughter genes immediately after DNA replication and then repressing transcription (Fig. 6-5B). This model is supported by the observed timing of DNA replication of this histone H4 gene (Iqbal et al., 1984)

and the inverse relationship between CDP/*cut* binding to repressor elements and histone transcription in the cell cycle (Plumb et al., 1983; Last et al., 1998). Furthermore, this concept is consistent with the requirement to remove newly placed nucleosomes after DNA replication and the ability of CDP/*cut* to bind to nucleosome cores and cell growth regulatory proteins.

CHAPTER VII

DISCUSSION

The cell cycle control of biosynthesis of the replication-dependent histones is mediated by transcriptional and post-transcriptional mechanisms. Transcription from the histone H4 gene FO108 is regulated by at least four promoter elements (Sites I, II, III and IV) (Kroeger et al., 1987; Ramsey-Ewing et al., 1994) which interact with an array of transcription factors such as CDP/*cut*, Sp1, IRF-2 and ATF/CREB (van Wijnen et al., 1996; Birnbaum et al., 1995; Vaughan et al., 1995; Guo et al., 1997). The primary purpose of my dissertation research was to further define molecular mechanisms involved with transcriptional control of the cell cycle regulated histone H4 gene FO108. My research focused on the functional analysis of Site III, regulatory elements in the histone coding region, and the identification of factors which interact with these elements. In this chapter, I will summarize the results of my research as well as present a model of histone H4 gene regulation which incorporates my observations as well as those of other investigators.

The initial focus of my research was on the functional characterization of the distal histone regulatory element Site III and the cloning or identification of the Site III protein-DNA complex, H4UA-1 (van der Houven van Oordt et al., 1992). Although Site III was previously identified as both a positive (Kroeger et al., 1987; Wright,1990) and negative (Larson et al., 1989; Zahradka et al.,1993)

transcriptional regulatory element, the promoter element needed to be further delineated and tested. The goals of my initial focus have been achieved. H4UA-1 has been identified as the multifunctional transcription factor YY1. Furthermore, I have shown that expression of YY1 in Drosophila S-2 cells transfected with a reporter gene construct increases CAT activity 2.7-fold via Site III. In addition to the identification of YY1 interaction at Site III, I have shown that other factors can interact with Site III. Purified AP-2 and distal factor (NF1-related) bind to Site III *in vitro*. Distal factor and YY1 also interact with an element (+150 to +183 nt) in the histone coding region which is highly homologous to Site III. Site III and the Site III-like element may function together by some mechanism involving the binding of similar transcription factors. However, results from transient transfection experiments in mammalian cells show that Site III, the YY1 binding site mutant or the distal factor binding site mutant do not alter the level of transcription. Site III region was tested in the context of chloramphenicol acetyltransferase reporter constructs under the control of either the histone promoter, the histone promoter and its coding region, or a heterologous thymidine kinase promoter. In all three series, those constructs containing Site III had little effect on transcription. Therefore, further evidence is necessary in order to prove a physiological role for Site III in human histone H4 gene expression. Otherwise, Site III appears to be a transcriptionally neutral regulatory element.

Although a physiological role for Site III has not been confirmed by this study, there still exists the possibility that Site III is indeed relevant. It is possible that Site III needs to be in proper chromatin context in order to function properly in the cell. Transient transfection experiments with plasmid DNA would

not be able to address this possibility. Previously, our laboratory has observed differing results between transient and stable transfection experiments (Frenkel et al., 1996). Site III may be involved in transcriptional control of the histone H4 gene in a cell growth dependent manner as suggested by others (Larson et al., 1989; Zahradka et al.,1993). The results presented in this study neither support nor disprove these possibilities. However, Site III might not have a direct role in regulating histone transcription but could be involved in regulating transcription from two other potential open reading frames 5' of Site III (as discussed in chapter 3). Whether the two open reading frames are actually transcribed needs to be determined. It is also possible that even though Site III is not directly involved in H4 transcription, it may be involved in organizing higher order gene architecture. YY1 is a nuclear matrix associated protein (Guo et al ., 1995). The role of YY1 at Site III may be to facilitate the association of the histone gene with the nuclear matrix. Dalton et al. (1986) have shown that chicken histone genes are associated with the nuclear matrix throughout the cell cycle. The same could hold true for the human histone H4 gene, and if so, Site III is a likely region, along with Site IV (Pauli et al.,1989; Dworetzky et al.,1992) for mediating this association.

YY1 interacts with and maybe transcriptionally functional through multiple regulatory elements of the histone H4 gene. I analyzed the histone FO108 gene sequences with sequence homology to YY1 binding motifs for their ability to interact with YY1, because both NMP-1 and H4UA-1 were found to be identical to YY1. Distal Site I was a likely candidate element for interacting with YY1. Wright (1990) observed that Distal Site I forms a complex that displays DNA binding behavior very similar to the H4UA-2 (NMP-1). EMSA results show

that YY1 does interact specifically with Site I. Further, both Site I and Site IV have overlapping ATF/CREB and YY1 binding sites. I also identified multiple YY1 interactions in the histone coding sequences. YY1 interacts with CR1 at a YY1 element at +30, and within the sequences between +75 and +200 nt. Expression of YY1 in Drosophila S-2 cells increases CAT activity up to 2.7-fold for constructs containing either the coding region sequences between -10 to +75 nt or sequences between +75 and +210. These results implicate YY1 in histone gene expression.

The next major focus of this dissertation research was to determine if the histone H4 coding region harbors any transcriptional regulatory elements. Although the four histone H4 promoter elements have been characterized extensively, the transcriptional role of the coding region was not known prior to this study. Observations from my analyses of YY1 interactions and the Site III-like element in the coding region initiated the process of addressing the role of the coding region on histone transcription. To test for regulatory elements within the coding region, I performed transient transfection experiments in HeLa cells using constructs with wild type and mutated histone H4 coding sequences fused upstream of a heterologous thymidine kinase promoter and CAT reporter gene. The results show that the histone gene has three repressor elements: CR1 (-10 to +75 nt), CR2 (+72 to +125 nt) and CR3 (+154 to +201 nt). The results also suggest that the Site III-like element is transcriptionally neutral in this assay system. Extensive *in vitro* analysis showed that the transcription factor CDP/*cut* specifically interacts at these three elements and correlates with repressor activity. Furthermore, I observed that the CDP/*cut* complexes binding on the coding region repressor elements were proliferation specific and cell

cycle regulated. The CDP/*cut* DNA binding complexes increase qualitatively during mid to late S phase of the cell cycle when histone transcription decreases. These observation are consistent with CDP/*cut* functioning as a repressor of histone transcription during the cell cycle by interacting with coding region regulatory elements.

The exact mechanism(s) by which CDP/*cut* functionally represses histone gene transcription in the native chromatin context has not been elucidated. However, Mailly et al. (1996) have shown that CDP/*cut* can repress gene expression by at least two distinct mechanisms: active repression and competition for binding site occupancy. One or both, could be involved in regulating histone expression. CDP/*cut* might also carry out nuclear functions on the histone gene other than that of a transcriptional repressor. For example, CDP/*cut* may function by facilitating chromatin remodeling of the histone gene. The placement of the CDP/*cut* binding sites (Site I, Site II, CR1, CR2 and CR3) coincides with the "nucleosome free" and nuclease sensitive promoter region (Chrysogelos et al., 1989; Moreno et al., 1988). Furthermore, CDP/*cut* DNA binding activity increases during S phase and correlates with DNA replication. I postulate that CDP/*cut* binds to nucleosomes in this region, either immediately after the histone gene undergoes DNA replication or in the transition out of quiescence, and then recruits enzymes or other factors which can modify or displace the nucleosomes. By EMSA and DNase I footprinting analysis using affinity purified GST-CDP/*cut* and reconstituted nucleosome cores of the proximal histone promoter, I was able to prove that CDP/*cut* does indeed interact with nucleosomes. These data partially support my model that CDP/*cut* may be involved in a chromatin remodeling pathway during the cell cycle.

The histone H4 gene clearly has a defined sequence structure with duplication of its regulatory elements. Site III (-366 to -333 nt) has a homologous element (+150 to +183) within the histone coding region and both elements interact with YY1 and distal factor. Site IV and Distal Site I both interact with ATF/CREB and YY1. There is sequence homology between the cell cycle control element (CCE) in Distal Site II (-64 to -53 nt) (5'-CTTTCGGTTTTC-3') and a region located 5' of Distal Site I (-132 to -121) (5'-TTTTCTGTTTTC-3'). The Site II CCE is the regulatory element defined by our laboratory as being essential for mediating cell cycle control of histone gene transcription. The Site I CCE-like element and the Site II CCE both interact with CDP/*cut* and IRF 2 (data not shown). The Proximal Site II, 5'-GTCCGATA-3' (-46 to -39 nt), is homologous to CR 1, 5'-GCCCGATA-3'(+10 to +17 nt). CDP/*cut* has been shown to specifically contact at both of these elements. Therefore, the histone gene appears to be regulated by multiple duplicated regulatory elements.

The results presented in this dissertation may provide insights into the control of gene expression in general. There probably exist other genes which are regulated by multiple complex regulatory elements, like those found in the histone gene. For example, CDP/*cut* competes with multiple transcriptional activators, including IRF, for binding to four sites in the proximal gp91phox promoter (Luo and Skalnik, 1996). The data from my analyses of CDP/*cut* interactions with histone coding region regulatory elements support the idea that there exists an E2F-independent pathway (proposed by van Wijnen et al., 1996) for controlling cell cycle and growth regulated genes. I anticipate that in the near future, investigators will show that CDP/*cut* interacts and regulates

other genes in a similar manner. Furthermore, I would not be surprised if CDP/*cut*'s ability to interact with nucleosomes is found to be a general mechanism for pathways involved in controlling growth regulated genes. The research presented in this dissertation does identify previously unknown histone regulatory mechanisms and possibly provides a new foundation for future research in understanding tumorigenesis, cell cycle and cell growth control.

Histone gene regulation - The "BIG" picture.

Cell growth and cell cycle control of the histone H4 gene results from the cumulative effects of multiple influences and higher level organization. Changes in the rate of transcription, in addition to altered histone mRNA stability and processing, have been shown to be important for modulating the level of histones during the cell cycle. Here I will only present a "Big picture" model of histone gene transcription which will address the multiple layers of regulation observed thus far. The highest level of regulation includes the association of the histone gene chromosomal loci with nuclear bodies and the nuclear matrix. Additional regulatory levels include modifications in chromatin structure and the interrelationship with nuclear proteins recruited to the gene. Thus, histone expression results from a dynamic process that nature has designed to be subordinate to a complex symphony of regulatory mechanisms.

The first level of regulation includes the localization of the histone gene within the organization of the nucleus. The nucleus consists of multiple

compartments that may function in specialized tasks. For example, the nucleolus is responsible for ribosomal RNA biosynthesis and processing. The nucleus also contains perichromatin fibrils, interchromatin granule clusters and nuclear bodies (reviewed in Spector, 1993; Lamond and Earnshaw, 1998). One type of nuclear body (the coiled body) has been shown to associate with the histone H4 FO108 gene locus in 32% of interphase HeLa-ATCC cells (Frey and Matera, 1995). Frey and Matera (1995) suggest that associations of coiled bodies with chromosomal loci are not stable and that coiled bodies are motile. Their results suggest that transcriptional expression of the histone H4 gene FO108 may be regulated through altering nuclear localization and associations during the cell cycle.

Nuclear matrix association of the histone gene is another possible mechanism of regulation (Pauli et al.,1989; Dworetzky et al.,1992). The nuclear matrix is a complex molecular network within the nucleus, which contributes to chromatin organization, regulation of DNA replication and gene expression (Berezney, 1991; Freeman and Garrad, 1992). The chicken histone genes have been shown to be associated with the nuclear matrix throughout the cell cycle (Dalton et al., 1986). Because most classes of replication-dependent histones are highly conserved among species, the human histone H4 gene might also interact with the nuclear matrix. Furthermore, the histone H4 gene can associate with YY1 and possibly other transcription factors in the nuclear matrix (Guo et al., 1995; van Wijnen et al., 1993). YY1 is a component of nuclear bodies (coiled bodies and the nucleolus (Guo et al., 1995)) (personal communications Sandra McNeil, Paul Odgren and Janet Stein) and may function by influencing expression of genes which associate with these bodies.

CDP/*cut* has been shown to interact at nuclear matrix-associated regions of two different genes and it has been suggested that CDP/*cut* acts as a repressor by inhibiting gene association with the nuclear matrix in a cell growth dependent manner (Banan et al., 1997; Liu et al., 1997). Whether CDP/*cut* functions in a similar manner with the histone gene is not known. My observations of YY1 and CDP/*cut* interacting with the histone H4 gene, and their relations to the nuclear matrix may provide a link between regulation by subnuclear localization and chromatin structure.

Chromatin remodeling is another aspect of the regulation of the histone gene. Chromatin structure is inextricably coupled to gene expression (reviewed in Felsenfeld, 1992; Kingston et al., 1996; Wu, 1997). The histone H4 gene is arranged such that nucleosomes are found 5' of Site I and 3' of CR3 (Moreno et al., 1988). The Tha I to Tha I region (-70 to +190 nt) of the histone gene is devoid of normal nucleosome structure during most of the cell cycle in HeLa cells (Moreno et al., 1988). I hypothesize that CDP/*cut* initiates a process which removes nucleosomes from the histone gene after it undergoes DNA replication. Other transcription factors which interact with the histone gene, such as YY1 and ATF/CREB, may recruit additional co-factors (p300/CBP) that can modify local chromatin structure.

Transcription factors, RNA polymerase II holoenzyme and TFIID complex are critical for histone gene expression. TFIID interacts at the TATA box, which is found in most genes, and facilitates the formation of initiation competent complexes on the promoters by recruiting RNA polymerase II holoenzyme. TFIID is a complex of TBP and seven or eight TBP-associated factors (TAFs) (Reviewed in Tansey and Herr, 1997). Several TAF's have protein structures

similar to histones, which could indicate that the TFIID complex could form a nucleosome like structure (Surridge,1996). The transcription factors Sp-1, ATF/CREB, IRF-2, CDP/*cut*, YY1, HiNF-P, the distal factor, C/EBP β, CRP1 and AP-2 interact at various cis-regulatory elements located throughout the gene (Figure 7-1) (Birnbaum et al., 1995; Guo et al., 1997; van Wijnen et al., 1996; data not shown) and may influence TFIID interactions with the promoter. Some transcription factors, like IRF 2 (Vaughan, et al., 1997; Birnbaum et al., 1997) and CDP/*cut* (Coqueret et al., 1998; Coqueret et al., 1996) are targets of cell-signaling pathways which result in posttranslational modifications that alter the activity of the factors.

Data presented in this dissertation in conjunction with results from other investigators (Ramsey-Ewing et al., 1994; van Wijnen et al., 1996), support a model that transcription factors are intimately involved in cell cycle regulation of histone H4 expression. I have shown that CDP/*cut*-DNA interactions with the histone coding region regulatory elements are cell cycle dependent, increasing in mid to late S phase. IRF-2 binding at Site II has been implicated as a positive regulatory influence on histone transcription at the G1/S phase boundary of the cell cycle (Vaughan et al.,1995; Vaughan et al., 1997).

Two other mechanisms which may regulate histone transcription include cell cycle regulated displacement of transcription factors and altered RNA polymerase II activity. During mitosis, there appears to be a general mechanism of displacement of transcription factors from chromatin (Marinez-Balbas et al., 1995), which could affect histone transcription. Parson and Spencer (1997) observed that the activity of RNA polymerase II is repressed

Figure 7-1. <u>Final schematic model of the histone H4 gene FO108.</u> The various identified transcription factors are indicated and relative locations for YY1, AP-2, distal factor (df), IRF-2 (IRF), CDP/*cut* (CDP), Sp1, ATF/CREB (ATF), C/EBP β and HiNF-P (P) on the promoter are shown.

H4 coding region

ATF YY1 −640

Site IV

df YY1 −340

Site III

CDP IRF −140 Sp1 ATF YY1 −70 IRF P CDP

Site I

CDP YY1 CDP CEBP β +75 +125

Site II

df YY1 +200 CDP

CR1 CR2 CR3

during mitosis and suggest that this plays a role in gene regulation during the cell cycle and in differentiation. It is conceivable that there are other cell cycle regulatory mechanisms involving protein/DNA interactions which could affect histone gene expression. The results presented in this dissertation add new insights into the transcriptional regulation of the histone H4 gene FO108 and possibly other genes at the level of protein/DNA interactions.

Subnuclear localization, nuclear matrix association and chromatin structure are three levels at which expression of the histone H4 gene may be regulated. These mechanisms of regulation are facilitated by the vast array of transcription factors interacting with the histone gene. Furthermore, the function of these transcription factors is influenced by the cell cycle, cell signaling pathways, posttranslational modifications and biochemical alterations of DNA. Therefore, the control of histone gene transcription results from the coordinate regulation at multiple levels.

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BIOGRAPHICAL SKETCH

Thomas J. Last was born on September 25, 1967 in Boston, Massachusetts. He attended Xaverian Brothers High School in Westwood, Massachusetts and graduated in 1986. He entered Rensselaer Polytechnic Institute in Troy, New York in the fall of 1986 and obtained a B.S. in biology in 1989. In June of 1989, he entered the graduate school of biomedical sciences at the University of Massachusetts and joined the laboratory of Janet and Gary Stein in the department of Cell Biology in the summer of 1991. On July 13, 1996 Thomas married Rohini Puri. He has a son named Jason.