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Functional and Structural Characterization of a Human H4 Histone Gene Promoter: a Thesis

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FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF A HUMAN H4 HISTONE GENE PROMOTER

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ABSTRACT

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Expression of the cell cycle dependent FO108 human H4 histone gene is regulated at both the transcriptional and post-transcriptional levels. We have investigated the 5' promoter elements mediating the transcriptional aspects of its regulation. A detailed *in vivo* and *in vitro* transcriptional analysis of promoter deletion mutants from this gene has identified three positive regulatory elements and two potentially negative regulatory elements within the first 1000 base pairs upstream of the transcription initiation site. In addition, the minimal promoter located within the first 70 base pairs is required for accurate transcription initiation and contains one of two *in vivo* identified protein-DNA interactions, Site II. Binding of the nuclear factor HiNF-D to this region was correlated with the turn-on of histone gene transcription following stimulation of quiescent normal diploid fibroblasts to re-enter the

proliferative phase. The most proximal positive regulatory element contains the other *in vivo* identified protein-DNA interaction, Site I. Results from a series of *in vitro* protein-DNA interaction studies revealed the binding of two nuclear factors to this element. One protein, HiNF-C, is indistinguishable from the transcription factor Sp1 while the other, HiNF-E, is a novel, potentially histone-specific member of the ATF transcription factor family. Binding of HiNF-C was required to stabilize the interaction of HiNF-E and together this region stimulated transcription 5 fold.

The near-distal transcription activator region lies between -418 and -213 base pairs and forms a single protein-DNA complex, H4UA-1. The interaction domain for H4UA-1 contains recognition sequences for both the thyroid hormone receptor and the nuclear factor CTF/NF-1. The far-distal activator region (-730 and -589 base pairs) was the strongest positive regulatory element identified in the H4 promoter. This region increased transcription 10 fold and contains three protein-DNA interactions. One of the factors, H4UA-2, is an ATF transcription factor closely related to the HiNF-E interaction in the proximal positive element.

These studies have defined the functional human H4 histone promoter to be a complex, modular structure extending at least 1000 base pairs.

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CHAPTER 1

INTRODUCTION

General Background

One of the requirements for cellular division is the replication of the entire genome of the cell, thereby producing up to two meters of new DNA which must be packaged into the nucleus. Histones are small, highly basic proteins principally responsible for the condensation of the DNA into chromatin of all eukaryotes. While structurally compacting the DNA the histones must also allow access to the genetic information encoded therein. Thus the positioning of the histones has the potential also to play a functional role in gene transcription. Expression of the majority of histone proteins is a multifaceted and tightly regulated event coupled to DNA replication because either an excess of unpackaged DNA or free histones is probably fatal to the cell. Regulation of histone expression is mediated at the transcriptional, post-transcriptional and post-translational levels and provides an important integrated model system to study eukaryotic gene expression. Furthermore, an understanding of the mechanisms regulating the growth related histone genes should provide insight into the molecular basis of cell growth control, differentiation and tumorigenesis.

Histone Proteins

There are five types of histones which are divided into the nucleosomal core histones (H2A, H2B, H3, and H4) and the H1 linker histones. The core histones are 100 to 150 amino acids in length and have a high level of evolutionary conservation (99). The classic example is the conservation of the H4 histones from pea plants to calf thymus which contain only two conservative amino acid substitutions out of 102 (50). This level of conservation demonstrates the critical role the histone protein structure plays in all eukaryotic cells. The H1 histones are slightly larger, about 225 amino acids and display a larger amount of diversity both between species and within different tissues of the same species (163).

Two copies of each of the four core histones assemble to form an 11 nm octameric nucleosome particle (110,135). The DNA is then wrapped about the nucleosome nearly two full turns to initiate the first level of chromatin formation. Approximately 145 base pairs of DNA encircle the nucleosome and each core particle is spaced about 60 base pairs apart. The H1 histone protein subsequently interacts with both the linker region DNA and the nucleosome particle to organize the nucleosomes into the basic 30 nm chromatin fiber.

Nucleosome positioning is not random in all regions of the genome. Studies using trace amounts of DNA digestive

enzymes reveal stretches of DNA which are hyper-sensitive to the enzymes indicating that these regions are not packaged by nucleosomes (60,136,210). Many of these hyper-sensitive regions lie at the upstream side of active genes suggesting that the presence or absence of nucleosomes in the promoter region may play a functional role by altering the availablity of the DNA for interaction with sequence specific DNA binding transcription factors.

Histone Gene Organization

Histones are encoded by a family of moderately reiterated genes consisting of 20 to 40 copies of the sequences coding for each of the five histone types (207). Initial studies of histone genes in sea urchin, *Drosophila* and other lower eukaryotes demonstrated a simple tandem repeat organization containing one of each of the five histone types (78,126,211). However, the histone genes of the higher eukaryotes including human, mouse and chicken are organized as random clusters containing mixtures of the core and H1 histone genes (29,61,86,90,181,184). In humans these clusters have been localized to at least three different chromosomes 1, 6, and 12 (76,200). Interestingly, the clusters are interspersed with repetitive elements which may

have played a role in gene duplication leading to the evolution of the multi-gene family (40).

Three classes of histone genes have been isolated; cell cycle regulated genes, cell cycle independent genes, and pseudo-genes. The cell cycle regulated genes are the most abundant class and have a very unusual and simple structure. These genes possess a very short 5' untranslated leader sequence, are not interrupted by introns, and have a short 3' untranslated sequence which terminates in a unique stem-loop structure instead of the typical polyadenylation site (1). Each of these characteristics makes the cell cycle regulated genes ideally suited to the large and rapid production of histone protein required during DNA synthesis. The cell cycle independent histone genes have a more typical structure containing both introns and a polyadenylation site (62,87). These genes may be important during cellular differentiation. Finally, pseudo-genes are non-functional copies which have frame-shifts in the coding region or deletions of the promoter sequences (133).

Cell Cycle Regulated Histone Gene Expression

The initial observations concerning the invariable 1:1 molar ratio of histone protein to DNA combined with the stringent requirement for histone protein to package newly replicated DNA while unbound histones can not be stored

suggested the hypothesis of a temporal and functional coupling between histone protein synthesis and DNA replication (2,161). Prescott (161) was able to demonstrate in cell cycle synchronized cultures of *Euplotes eurystomus* that histone protein synthesis occurred only during the period of DNA synthesis. Moreover, the amount of histone protein present within a cell doubled during S phase concomitant with the doubling of the cellular DNA content (20). These observations were followed by a series of studies which confirmed the temporal coupling between histone protein synthesis and DNA replication (169,191,194). Subsequently, isolation of polyribosomes from cells at different stages of the cell cycle indicated that histone encoding polyribosomes were selectively enriched during S phase (22).

The cloning of histone encoding cDNA and genomic DNA fragments allowed the level of histone mRNA to be monitored throughout the cell cycle. Cell cycle regulated histone genes were revealed to be expressed at a low, basal level through out the cell cycle and displayed a transient accumulation of the mRNA during DNA replication which paralleled the rate of DNA synthesis (13,89,160). A functional coupling of histone expression and DNA replication was suggested by the results of inhibiting DNA synthesis. When hydroxyurea or cytosine arabinoside was

administered to the cells to block DNA synthesis a concomitant drop in the amount of histone mRNA was observed (13,21,24,27). Furthermore, histone mRNA levels paralleled the changes in DNA synthesis when quiescent cells were stimulated to proliferate (51,75) or proliferating cells terminally differentiated (18,33,193).

The pattern of expression of the cell cycle regulated histone genes is mediated by regulation at both the transcriptional and post-transcriptional levels. Transcription of the histone genes was demonstrated to occur at a low level throughout the cell cycle combined with a transient 2 - 5 fold stimulation during the initial two hours of S phase (14,160,186). However, histone mRNA accumulated 15 - 20 fold during S phase and peaked simultaneously with DNA synthesis (13,89,160). Because the observed transcription could not completely account for this accumulation, the result demonstrated the presence of post-transcriptional mRNA regulation. DNA synthesis inhibitor studies revealed that the half-life of the histone mRNA was reduced from approximately 60 minutes to 10 - 15 minutes when the cells ceased DNA replication (14,160,185). These findings have demonstrated that it is a combination of the burst of transcription at the onset of S phase to produce a large amount of histone mRNA combined with and followed by

an increase in the stability of the mRNA which generates the observed pattern of histone mRNA expression.

RNA Polymerase II Transcription Initiation

The discovery of an RNA polymerizing activity in rat liver nuclei by Weiss and Gladstone in 1950 (203) initiated the still active research into the mechanics and regulation of eukaryotic RNA synthesis. Three distinct RNA polymerase activities have been defined (34,171). RNA polymerase I synthesizes ribosomal RNA precursors while RNA polymerase II transcribes the protein-encoding genes and RNA polymerase III transcribes the 5S and tRNA genes. Histone genes are transcribed by the RNA polymerase II enzyme (53). RNA polymerase II is a large (500 - 600 kDa), multi-subunit complex consisting of two large subunits and 6 - 10 small protein components (175). However, purified RNA polymerase II lacks the ability to initiate specifically at physiologic start sites (124). The development of *in vitro* transcription systems consisting of soluble cell extracts and cloned promoter template DNA has provided the means to dissect the components of site specific initiation (55,132). Five general transcription factors required for accurate initiation have been identified (41,42,59,66,166,167,172, 215). The function of only one of the factors, TFIID, has

been clearly defined. TFIID interacts directly with DNA over the region containing the TATA box sequence (155). In higher eukaryotes initiation always occurs 25 - 30 base pairs downstream of the TATA box and TFIID has been suggested to be required to align the initiation site (23). However, heterologous reconstitution experiments reveal that substitution of the mammalian TFIID with the yeast TFIID still directs initiation from the mammalian start site even though initiation in yeast occurs between 40 and 120 base pairs downstream from the TATA box (32,197). This indicates TFIID is not solely responsible for alignment of the initiation site.

Cis-acting DNA Elements and Trans-acting Transcription Factors

Although RNA polymerase II and its associated general transcription factors are sufficient to direct site specific initiation, most protein-encoding genes rely on auxiliary sequence elements and their associated transcription factors to augment and regulate transcription. The development of DNA manipulation techniques allowed the construction of promoter deletion mutants of cloned genes. Transcriptional studies with deletion mutants of a sea urchin H2A histone gene, the simian virus 40 (SV40) early promoter, the herpes simplex virus (HSV) thymidine kinase gene and α- and β-

globin genes revealed the presence of domains upstream of the TATA box which modulated the transcription efficiency (54,79,139,141,142). These findings were refined by high resolution point mutants and linker-scanning mutants which defined multiple discrete DNA domains capable of regulating transcription (140,149,213). Similar results have been found within the first few hundred base pairs of many other genes and present the picture that RNA polymerase II promoters are modular in structure. Furthermore, cis-acting DNA elements, referred to as enhancers, can stimulate transcription at distances greater than 1000 base pairs from the initiation site and can be located both upstream and downstream from the gene (10,16,68).

The development of techniques to assay sequence specific protein-DNA interactions directly has led to the description of a tremendous number of transcription factors interacting with promoters and enhancers (102,144,208). The findings have clarified a few of the questions concerning transcriptional regulation but have posed many more. Early studies by Tjian and co-workers led to the identification and purification of an RNA polymerase II sequence specific transcription factor, referred to as Sp1 (25,58). Sp1 was initially fractionated from HeLa cell soluble extracts based on its ability to activate transcription from the SV40 viral early promoter. DNase I protection studies demonstrated that

Sp1 selectively bound to the repeated GC rich boxes of the SV40 early promoter. Similar binding sites have been described, subsequently, in many viral and cellular promoters positioned upstream of the TATA box motif and have allowed the definition of a consensus recognition sequence, 5'-$^{G}_{T}$GGGCGG$^{G\ G\ C}_{A\ A\ T}$-3' (107). Point mutations within this consensus element have demonstrated that Sp1 binding mediates the functional aspects of this DNA domain (103). Isolation of the gene that encodes Sp1 revealed three $[cysteine]_2$-$[histidine]_2$ repeated motifs that have been suggested to interact with Zn^{+2} to form a "zinc finger" structure capable of interacting with DNA (106,143). The transcriptional activation by Sp1 is mediated primarily by a glutamine rich domain distinct from the DNA binding domain (44). The division of the DNA binding activity from the activating domain is a common theme repeated in other transcription factors.

In contrast to Sp1 which is a single protein interacting with a single conserved recognition sequence, several transcription factor families have been described which interact with partially divergent recognition sequences. One of the most interesting examples is the AP1 family of transcription factors. AP1 was originally defined as a sequence specific DNA binding activity that interacted with an element in the SV40 enhancer (120) and ensuing

studies identified the recognition sequence, 5'-TGACTCA-3', in many additional viral and cellular promoters and enhancers (5,121). AP1 has been the subject of intense research since the classification of the proto-oncogene c-*fos* as a member of this family (35,164,173). c-*fos* is an immediate early response gene whose expression can be triggered by a wide range of stimuli including growth factors (39,46,77) and thus its DNA binding activity represents a mechanism by which cells can convert external signals to changes in gene expression (56,199). Multiple AP1 proteins including c-*jun*, *jun*B, *jun*D, *fra*-1 and *fos* have been characterized and their shared DNA binding specificity is reflected in a conserved region referred to as the leucine zipper (111,174). The leucine zipper consists of an α-helix with 4 or more leucine residues spaced by seven amino acids and is preceded by a highly basic region. The zipper region is involved in dimerization and is required for DNA binding. It has been proposed that the interaction of two leucine zippers positions the adjacent basic regions in the proper orientation for interaction with the DNA helix (117). Selective and inducible dimerization between members of the *fos* and *jun* families and of *jun* with other *jun* proteins has been suggested as a mechanism by which AP1 binding can selectively regulate the many genes which contain the AP1

consensus recognition sequence (45). However, as yet little is known about the processes which promote the formation of specific dimers.

A second transcription factor family, ATF, recognizes DNA through a basic domain which is aligned by dimerization through the leucine zipper motif. ATF was initially characterized as a stimulatory activity in the adenovirus E4a promoter and as a cyclic AMP inducible factor in the promoter of the somatostatin gene (127,146). Purification and cloning of the ATF factors by a number of research groups has revealed the family to be made up of numerous proteins with molecular masses of 39 - 120 kDa (69,82,94,130). The ATF recognition sequence, 5'-GTGACGT$^{A}_{C}{}^{A}_{G}$-3' (127), has also been defined in many cellular and viral promoters and has been shown to be involved in the response to E1A (119), cAMP (145) and Ca^{2+} (179). The core ATF recognition sequence is only one nucleotide different from the AP1 binding site and studies have shown that each of the two factors can bind weakly to the other site (83). Purification of ATF and AP1 by DNA affinity chromatography demonstrated that the major species of both were almost identical in molecular weight (83). In addition, the two families were shown to be immunologically related and may belong to a super-family of leucine zipper containing transcription factors.

A third class of DNA binding proteins is defined by the presence of a helix-turn-helix structure required for binding. This structure was first described for the bacteriophage lambda proteins CRO and C1 and the CAP protein of *E. coli* (3,138,153). Primary sequence comparison to the highly conserved homeobox region of the *Drosophila* developmental regulatory genes indicated these genes also contained a helix-turn-helix motif which directed sequence specific DNA recognition (118,180). Because of the high sequence conservation of the homeobox region, homeotic genes have now been isolated from many species (30,123,137). However, identification and localization of the DNA binding sites for most of these factors are still under investigation. One type of homeobox containing factor which has been well defined is the octamer binding factors, OCT-1 and OCT-2 (122). These factors are distinct proteins but interact with the same consensus DNA recognition sequence, which is found in many promoters including histone H2B and the immunoglobulin promoter and enhancer (148,176,188). OCT-1 is a ubiquitous factor present in most tissues, while OCT-2 is found primarily in lymphoid tissue and is important for the lymphoid cell-specific transcription of the immunoglobulin genes. Interestingly, OCT-1 can not substitute for OCT-2 in promoting immunoglobulin

transcription (170). This result raises the interesting question of how the two factors discriminate between recognition sequences.

In addition to the examples described above numerous additional transcription factors and DNA binding activities have been described during the last few years. These include the steroid and thyroid hormone receptors and the CTF/NF-1 family. The hormone receptors utilize a zinc-finger DNA binding motif similar to Sp1 to translate hormone induction into the stimulation or repression of gene transcription (15). The CTF/NF-1 family is involved in both transcriptional activation and stimulation of DNA replication; however, its DNA binding motif has yet to be defined (80,105,150,165). Despite the volumes of information on specific transcription factors our understanding of the regulation of RNA polymerase II transcription is still very limited. Many questions remain regarding 1) how a specific binding site selects from a family of factors all with the same consensus recognition sequence, and 2) how transcription factors promote or repress transcription when located near the initiation site or when located hundreds or thousands of base pairs away. It will require an elucidation of the interaction of the factors with each other and with the general polymerase structure to allow an understanding of the control of gene expression.

Histone Gene Promoters

Although there has been much interest in the regulation of histone gene expression, only limited promoter sequence information is available (204). A comparison of the first 100 - 200 base pairs displays only a few obvious similarities (92,205). The TATA box motif is the only component found in each of the genes. In addition, many of the genes contain one or more sequences related to the CCAAT box recognition sequence (73,105). Several histone sub-type specific elements were also suggested by the alignment including the H1 element 5'-AAACACA-3', the H2B element 5'-CYTNATTTGCATAC-3', and the H4 element 5'-NRTCC-3'.

To functionally address the role of promoter elements in higher eukaryotic histone genes, several human histone genes were examined by *in vitro* transcription. Sierra *et al.* (182) constructed a series of promoter deletion mutants within the initial 200 base pairs of the FO108 human H4 histone gene. This study demonstrated that sequences extending 50 base pairs upstream of the initiation site were sufficient to direct site specific initiation. The defined minimal promoter contained both the TATA box and the H4 specific element. Furthermore, additional upstream sequences appeared to augment the initiation rate. A second human H4 histone gene, Hu4a, was independently isolated and analyzed

in a similar manner by Hanly *et al.* (84). The two H4 genes while distinct have a high degree of similarity over a 30 base pair region encompassing the TATA box and the H4 specific element. It was not surprising then to find that the minimal sequences required for *in vitro* transcription of this gene corresponded to the minimal promoter defined for the FO108 gene. In addition, sequences between -70 and -110 base pairs consisting of a G-C rich domain and a 5'-GATTTC-3' domain were required to achieve maximal transcription initiation. Fine mapping of the human H2B histone promoter by Sive *et al.* (187) confirmed the requirement of the TATA box in a minimal promoter and defined four discrete sequence elements within the first 120 base pairs required for maximal *in vitro* transcription. The H2B specific element located at -45 base pairs stimulated transcription several fold and site specific mutations of this region demonstrated the requirement of an octamer (OCT) binding site consensus.

An elegant study of the FO108 human H4 gene by Pauli *et al.* (157) structurally defined regions of protein-DNA interaction in the intact cell. Two 40 - 50 base pair interaction domains were located in the first 200 base pairs of the promoter. Site II encompasses the TATA box and extends approximately 25 base pairs upstream of the H4

specific element. The entire region of strong sequence similarity between the FO108 and Hu4a H4 genes is included within Site II suggesting that Site II may be a common regulatory element of human H4 genes. Site I is located approximately 20 base pairs upstream of Site II and contains at the proximal side a region with striking similarity to the Sp1 recognition sequence. With this background we initiated a series of studies to carefully define the specific promoter elements of the FO108 human H4 gene and to characterize the trans-acting factors mediating the promoter elements effect on transcription.

Overview of Project

Our research was divided into three sections each based on the development of a cell free transcription system capable of accurately and with fidelity initiating transcription from the FO108 human H4 histone gene promoter. The results have been schematically compiled in Appendix B. In the first section we functionally divided the *in vivo* Site I protein-DNA interaction into two stimulatory domains and characterized the nuclear factors responsible for the activation of transcription. We then extended our examination up to 1000 base pairs from the initiation site and defined two strong activator regions and two potential

repressor regions. Each of the activator regions was characterized as to the protein-DNA interactions involved and related to the factors present in Site I. Finally, we utilized the *in vitro* transcription system to functionally correlate the protein-DNA interactions at Site II with the turn-on of H4 histone gene transcription during stimulation of quiescent normal diploid fibroblasts.

CHAPTER 2

A NOVEL TRANSCRIPTION FACTOR, ATF-84, AND SP1 ARE INVOLVED IN THE TRANSCRIPTIONAL REGULATION OF A HUMAN H4 HISTONE GENE

Abstract

The promoter of the human H4 histone gene FO108 contains two regions of *in vivo* protein-DNA interaction, Site I and II. We have examined the transcriptional contribution of Site I (-124 to -90 bp) both *in vivo* and *in vitro* and have identified the two trans-acting factors mediating the transcriptional effect. Deletion mutants of the proximal promoter were assayed in a cell free transcription system or were fused to the chloramphenicol acetyltransferase (CAT) reporter gene and assayed in HeLa cells. Site I increased the *in vivo* basal level of transcription greater than 7 fold. This region was subsequently divided into two functional domains each contributing a 2 fold increase in cell free transcription. Two sequence specific transcription factors, HiNF-E and HiNF-C, have been demonstrated to interact with Site I with high affinity. The protein-DNA interactions have been defined by combining site-directed base substitution with the gel mobility shift assay, DNase I footprinting and DMS interference. Binding site mutation analysis of the proximally located HiNF-C factor demonstrates the

requirement of an intact Sp1 consensus recognition sequence for HiNF-C binding and supports earlier evidence that HiNF-C may be the transcription factor Sp1. HiNF-E interacts with the distal half of Site I and the guanine residue contact points lie within an element with identity to the core recognition sequence of the ATF transcription factor. Site directed mutagenesis of this element reveals it to be required for HiNF-E binding. HiNF-E and HiNF-C have overlapping DNase I footprints, suggesting that the two proteins are likely to be in contact. Deletion of the HiNF-C binding site and its protein-DNA interaction severely reduces the binding affinity of HiNF-E as measured by DNase I footprint protection. Additionally, HiNF-E is detected primarily as a complex with HiNF-C in the gel mobility shift assay. These results suggest a role for HiNF-C in stabilizing the HiNF-E/Site I interaction. The molecular mass of HiNF-E as determined by UV cross linking is 84 kDa. Since HiNF-E binds to an ATF consensus sequence, we suggest that HiNF-E is a new member of the ATF transcription factor family, termed ATF-84. These results demonstrate that a previously undescribed ATF transcription factor interacting with Sp1 at Site I contributes to the regulation of this human H4 histone gene.

Introduction

Eukaryotic gene transcription is a major focal point for the regulation of gene expression during normal growth and differentiation and in the loss of growth control in transformed and tumor cells. A clear understanding of the transcription process and its components is a prerequisite for understanding the broader questions of cell growth control. Recent advances in the study of protein-DNA interactions have revealed a complex array of trans-acting protein factors and cis-acting DNA elements (reviewed in (102)). Eukaryotic gene promoters are modular in structure, consisting of a series of regulatory sequence elements and corresponding sequence-specific DNA binding proteins. In addition the level of complexity is increased by the presence of multiple factors with the same DNA recognition sequence and single factors that recognize several different DNA binding sites. Elucidating these complex pathways of regulation supporting stringent growth control will require detailed analysis of specific genes and promoter factors. To this end, we have undertaken the study of the molecular mechanisms controlling transcription of a cell cycle regulated human H4 histone gene.

Human histone genes constitute a moderately repeated, multi-gene family which encode five histone sub-types critical for the packaging of newly replicated DNA into

chromatin (31,98,99). The human genes are organized in clusters of core (H2A, H2B, H3, H4) or core with H1 histone genes (29,90,125,133,181,217) and have been identified on at least three different chromosomes (76,200). The majority of histone genes are expressed in a cell cycle regulated manner, tightly coupled both temporally and functionally to DNA replication (178,195). The cell cycle dependent genes are constitutively transcribed at a basal level and exhibit a 2 - 5 fold induction of transcription during the initial period of DNA synthesis (14,89,160). This induction of transcription is accompanied by a disruption of the chromatin structure and nucleosome organization about the promoter region (37,147). Histone mRNA accumulates rapidly during S-phase to a level 20 - 100 fold higher than that detected in non S-phase cells (6,14,51,89,160). This demonstrates the major role of post-transcriptional control in regulating the cell cycle dependent expression of histone genes. The accumulation is mediated by an increase in histone mRNA stability at the onset of DNA replication followed by a selective and rapid turnover of the histone mRNA at the completion of S phase or following inhibition of DNA synthesis (13,185 and T. Morris, personal communication).

Transcription of the cell cycle regulated FO108 human H4 histone gene has been shown to be modulated by both

proximal and distal promoter sequence elements (74,112,182). We have previously shown that the proximal promoter consists of two regions of *in vivo* protein-DNA interaction, Site I and II (157). Site II lies between -24 and -64 base pairs upstream from the transcription initiation site and contains the TATA box, the histone specific element (5'-GGTCC-3') and a putative CCAAT box. Factor HiNF-D binds to the distal half of Site II (202), and occupancy of Site II *in vivo* and *in vitro* has been correlated with rendering the gene transcribable in proliferating cells (152,193). Furthermore, HiNF-D binding activity is stringently coupled to DNA replication in normal diploid cells suggesting a functional role for Site II in mediating the up-regulation of histone gene transcription during S phase (95). Site I is positioned at -90 to -124 base pairs upstream of the initiation site. Previously we have shown that HeLa cell nuclear factor HiNF-C binds *in vitro* to the proximal half of Site I over a consensus Sp1 recognition sequence and that factor HiNF-A is able to interact with sequences overlapping the distal end of Site I (202). In this study we have systematically characterized the protein-DNA interactions at Site I and have identified factors HiNF-E and HiNF-C as the functional components of this domain. HiNF-E is proposed to be a novel member of the ATF transcription factor family, ATF-84, with

a molecular mass of 84 kDa, while HiNF-C is shown to be equivalent to the transcription factor Sp1. Furthermore, we have directly determined the influence of these transcription factors on the specificity and level of H4 histone gene transcription.

Materials and Methods

Plasmid constructions. The FO108 histone promoter is numbered from the transcription initiation site. The histone promoter-chloramphenicol acetyltransferase (CAT) gene fusion construct, FO108/CAT was derived from the previously described construct FO002/CAT-A3 (112) by EcoRI endonuclease digestion followed by circularization through ligation of the proximal EcoRI site of the histone promoter (-213 bp) to the EcoRI site of the vector. The construct FP201/CAT is derived from FO108/CAT by deletion of the sequences between -204 (SmaI) and -70 (NaeI) by restriction digestion with the indicated enzymes and circularization through blunt-end ligation of the CAT containing DNA fragment. The 5' deletion mutants used for *in vitro* transcription were prepared by BAL-31 digestion as previously reported (182) and were cloned into a pUC19 vector in order to make the vector sequences uniform. The fusion points were confirmed by the chemical sequencing method of Maxam and Gilbert (134).

Plasmid DNA isolation and purification. Plasmid DNA was amplified in either the *E. coli* strain XL-1 Blue (Stratagene) or DH5α (Bethesda Research Laboratories) unless otherwise indicated. Large scale preparation (131) of plasmid DNA was initiated by inoculating 10 ml of TYN growth

medium [1% bactotryptone, 1% yeast extract, and 0.5% NaCl] containing 0.1% glucose and antibiotics (50 μg/ml ampicillin or 25 μg/ml tetracycline as required by the genotype of the plasmid or bacterium) with 40 μl of a bacterial glycerol stock and incubated for 12 - 16 hours at 37^{0}C. The culture was then used to inoculate 500 ml of TYN medium and incubated for an additional 12 - 16 hours. The bacteria were collected by centrifugation at 4400xg for 15 minutes. The pellet was resuspended in 10 ml of Solution 1 [25 mM Tris-HCl (pH 8.0), 50 mM glucose, and 10 mM EDTA (ethylenediaminetetraacetic acid, disodium)] for 5 minutes at 22^{0}C followed by the addition of 20 ml of Solution 2 [1% SDS (sodium dodecyl sulfate), 0.2 N NaOH] for 15 minutes on ice and 15 ml of 4^{0}C 5 M K-acetate (pH 4.8) for an additional 15 minutes on ice. The mixture was centrifuged at 31,000xg for 30 minutes at 4^{0}C and the supernatant was collected and warmed to 22^{0}C. The nucleic acids were precipitated with 2/3 volume of isopropanol and pelleted at 12,000xg for 30 minutes at 22^{0}C. After an excess 70% ethanol wash and resuspension of the pellet in 8 ml of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], 8 grams of $CsCl_2$ and 0.8 ml of 10 mg/ml ethidium bromide was added. The sample was then centrifuged for 36 hours at 180,000 x g at 22^{0}C and the plasmid DNA band was drained from the $CsCl_2$ gradient. The DNA was repeatedly extracted with 1-butanol to remove

the ethidium bromide followed by extensive dialysis against TE buffer (pH 8.0) to remove the $CsCl_2$ and the DNA was stored at -20^0C.

Single stranded M13 DNA was amplified in XL1-Blue bacteria unless otherwise indicated by inoculating 100 ml of TY medium [1.6% bactotryptone, 1.0% yeast extract, and 1.0% NaCl] with a single M13 plaque and 5 ml of an exponentially growing culture of bacteria. The culture was incubated at 37^0C with vigorous shaking for 6 - 18 hours. The cells were pelleted and discarded by centrifugation at 4400xg for 30 minutes. Phage particles were precipitated from the supernatant by the addition of 1 volume of 5xPEG/NaCl solution [15% (wt/vol) polyethylene glycol 8000 (Kodak), 2.5 M NaCl] and incubation at 0^0C for 1 - 12 hours. The phage was collected by centrifugation at 4400xg for 15 minutes, resuspended in 5 ml TE buffer and precipitated again with 5xPEG/NaCl. The pellet was then resuspended in 5 ml TE buffer with 1% SDS and repeatedly extracted with phenol:$CHCl_3$:isoamyl alcohol. The single stranded DNA was recovered by ethanol precipitation and stored in TE buffer at 4^0C.

<u>HeLa S3 cell culture and transfection.</u> HeLa S3 cells were maintained as a 37^0C suspension culture at a density of 3-6 x 10^5 cells/ml in Joklik-modified Eagle's minimal medium (SMEM) supplemented with 7% calf serum (Gibco), 100U/ml

penicillin, 100μg/ml streptomycin and 1 mM glutamine. Cells were prepared for transfection by seeding 3 x 10^6 cells into 100 mm culture dishes containing Eagle's-modified minimum essential medium (EMEM) supplemented with 5% fetal calf serum (Gibco), 5% horse serum (Gibco), 100 U/ml penicillin, 100μg/ml streptomycin and 1 mM glutamine and incubated 18 - 24 hours in a 37^0C, 5% CO_2 incubator. Calcium phosphate transfection of 20 - 40% confluent monolayers of HeLa cells was essentially as described by Gorman *et al.* (70). The DNA/calcium phosphate precipitate was prepared according to Graham and van der Eb (72). The DNA used in short term transient expression experiments consisted of a mixture of 10 μg of plasmid DNA and 10 μg of carrier salmon sperm DNA in 500 μl of 250 mM $CaCl_2$, while the DNA mixture used for the establishment of stable cell lines consisted of 20 μg of a 20:1 molar ratio of test plasmid to pSV2-*neo* plasmid (190). The DNA mixture was added dropwise to 500 μl of HEBS buffer [50 mM Hepes (pH 7.12±0.05), 280 mM NaCl, 1.5 mM Na_2HPO_4] with constant vortexing. Four hours prior to transfection the cells were refed with completed EMEM. The DNA/calcium phosphate precipitate was added evenly to the monolayer culture and incubated as above for 4 hours. The cells were then glycerol shocked by replacing the medium with 2 ml completed EMEM containing 15% glycerol for one

minute. The cells were washed with 10 ml of EMEM, refed with 20 ml completed EMEM and incubated at 37^{0}C ,5% CO_2 for 24 - 48 hours. Short term transient expression was analyzed by harvesting the cells at this point. To establish stable cell lines, the cells were split 1:10 and refed every 3 days with 20 ml completed EMEM containing 500 μg/ml Geneticin (G418, Gibco). The Geneticin selects for cells expressing the *neo* gene [an aminoglycoside phosphotransferase 3' (II) gene] as described by Southern and Berg (190) and resistant colonies became visible after 9 - 10 days. Polyclonal cell lines were developed for analysis by pooling individual colonies and adapting the cultures into suspension cultures.

Chloramphenicol acetyltransferase (CAT) assay.

Short term transient expression was measured by CAT assays according to Gorman *et al.* (71) and performed by Anna Ramsey-Ewing. The transfected HeLa cells were washed 3 times with 10 ml phosphate buffered saline and scraped into 1 ml harvesting solution [40 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl] with a rubber policeman. The cells were collected by centrifugation, resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8) and lysed by three freeze-thaw cycles (-70^{0}C to 37^{0}C). The cellular debris was pelleted by centrifugation in a microcentrifuge for 5 minutes at 4^{0}C and the freeze-thaw supernatant was decanted and saved for the

CAT assay reaction. The reactions were prepared by combining 70μl of 1 M Tris-HCl (pH 7.8), 30μl H_2O, 25 μl freeze-thaw supernatant, 2.5 μl ^{14}C-chloramphenicol (Amersham 40 - 50 mCi/mmole), and 20 μl 4 mM acetyl CoA (Pharmacia) and incubating the mixture at 37^{0}C for 10 - 60 minutes. The reaction was stopped and the chloramphenicol extracted by the addition of 1 ml ethyl acetate, vortexing, and brief centrifugation to separate the organic and aqueous phases. The organic phase, containing the chloramphenicol was transferred to a new microcentrifuge tube and dried to completion in a vacuum aspirator. The sample was resuspended in 30 μl ethyl acetate, spotted onto a silica gel thin layer chromatography plate, and placed in an equilibrated chloroform:methanol (95:5 volume/volume) ascending developing tank. The developed plate was exposed to Kodak XAR-5 film for 12 - 24 hours. Absolute values of CAT activity obtained were subsequently normalized to the efficiency of transfection as determined by DNA content in the Hirt supernatant (93).

Nuclear run-on transcription. Expression of the transfected genes in stably transformed cell lines was analyzed by nuclear run-on transcription reactions, performed by Anna Ramsey-Ewing essentially as described by Clayton and Darnell (38). All steps of the nuclei isolation were carried out at 4^{0}C. The cells were harvested by

centrifugation at 1000xg for 10 minutes and washed twice in isotonic buffer [30 mM Tris-HCl (pH 7.9), 125 mM KCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol]. The cells were finally resuspended in hypotonic buffer [30 mM Tris-HCl (pH 7.9), 10 mM KCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol] and allowed to swell for 15 minutes. The cells were transferred to a Wheaton dounce homogenizer and >90% were lysed with an type A pestle. The nuclei were collected by centrifugation at 1000xg for 10 minutes, resuspended in nuclei storage buffer [50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol] and stored in small fractions for use in the transcription reactions. The reactions contained 1 x 10^7 nuclei, 100 μCi α-^{32}P-UTP (3000 Ci/mmole, New England Nuclear), 1 mM ATP, 0.25 mM GTP and CTP in a final volume of 130 μl and were incubated for 30 minutes at 30^{0}C with periodic shaking. Radiolabeled RNA transcripts were isolated by adjusting the reaction solution to 50 mM Tris-HCl (pH 7.5), 600 mM NaCl, 20 mM $MgCl_2$ and incubating with 100 μg/ml DNase I (Boehringer Mannheim) at 20 - 22^{0}C for 15 minutes. The solution was adjusted again to 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 12.5 mM EDTA and incubated with 200 μg/ml Proteinase K (Boehringer Mannheim) at 37^{0}C for 30 - 60 minutes. Sodium acetate (pH 5.5) was added to 0.2 M and the nucleic acids extracted several times with hot phenol. The radiolabeled transcripts were

precipitated with 150 μg yeast RNA by the addition of 2.5 volumes 95% ethanol and incubation at -20^{0}C for 4 - 12 hours. After centrifugation the RNA pellet was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and an aliquot of each sample was precipitated with 150 μg yeast RNA and cold 10% TCA (trichloro-acetic acid). TCA-precipitable counts were determined by liquid scintillation spectrometry. Nitrocellulose southern blots (189) were prepared containing restriction endonuclease digested DNA fragments complementary to the RNA transcripts of interest and various control DNA fragments. The blots were pre-hybridized in 20 mM Tris-HCl (pH 7.4), 1 M NaCl, 2 mM EDTA, 0.1% SDS (sodium dodecyl sulfate), 5x Denhardt's [100x Denhardt's: 2% (w/v) ficoll 400, 2% (w/v) polyvinylpyrrolidone], 250 μg/ml *E. coli* DNA and 12.5 mM sodium pyrophosphate at 65^{0}C for 6 hours. Hybridization was with 5 x 10^5 - 1 x 10^6 TCA-precipitable counts of radiolabeled RNA per ml of hybridization solution [20 mM Tris-HCl (pH 7.4), 1 M NaCl, 2 mM EDTA, 0.1% SDS, 2.5x Denhardt's, 250 μg/ml *E. coli* DNA] at 65^{0}C for 72 hours. The blots were washed at 65^{0}C for 15 minutes in fresh pre-hybridization solution, 1 hour in 2x SSC/0.1% SDS [20x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0], overnight in 2x SSC/0.1% SDS, and 1 hour in

0.2x SSC/0.1% SDS. The washed blots were air dried and exposed to Kodak XAR-5 film.

Preparation of nuclear extracts. Nuclear extracts were prepared from $1x10^9$ exponentially growing HeLa S3 cells essentially as described by Dignam *et al.* (55), except 2 ml of 600 mM KCl was used to extract the nuclei. All steps were done at 4^0C and all buffers except the phosphate buffered saline contained fresh 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 5 μg/ml each of pepstatin A, antipain, leupeptin, and chymostatin (Sigma). The cells were harvested by centrifugation at 1000xg for 10 minutes, washed in 20 ml of phosphate buffered saline [137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.0)] and collected by centrifugation as before. The cells were resuspended in 20 ml Buffer A [10 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) (pH 7.9 with KOH), 1.5 mM $MgCl_2$, 10.0 mM KCl], allowed to stand for 10 minutes to swell, centrifuged as before, and resuspended in 8 ml of Buffer A. The cells were lysed in a 15 ml Wheaton dounce homogenizer with a type A pestle. Cell disruption was monitored by examination of aliquots under the microscope until less than 10% of the cells remained intact. The nuclei were collected by centrifugation as before, washed in 8 ml of Buffer A and re-collected by centrifugation at 25,000xg

for 20 minutes. The packed nuclear pellet was resuspended in 2 ml of Buffer C [20 mM Hepes (pH 7.9), 20% glycerol, 600 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA] by two gentle strokes of a B pestle in a 7 ml Wheaton dounce homogenizer. Extraction was for 30 minutes with constant stirring. The extracted nuclei were pelleted by centrifugation at 25,000xg for 30 minutes and the clear supernatant was dialyzed in a 2000 molecular weight cut-off dialysis bag (Spectra/Por, Spectrum Medical Industries, Inc.) against 2 changes of 500 ml Buffer D [20 mM Hepes (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA]. After dialysis the sample was cleared by centrifugation at 25,000xg for 20 minutes and 25 μl aliquots of the supernatant were quick frozen in liquid nitrogen and stored at -70^0C.

In vitro transcription. Transcription from linearized template DNA was essentially as described by Dignam *et al.* (55). The histone templates were linearized at the unique NcoI restriction site to produce a run-off transcript of 279 nucleotides. Transcription from covalently closed circular template DNA was performed by pre-incubating the DNA and 50 - 100 μg of crude nuclear extract in 21 μl at 4^0C for 15 minutes in a buffer containing 12 mM Hepes (pH 7.9 with KOH), 12% glycerol, 60 mM KCl, 8 mM $MgCl_2$, 0.3 mM DTT, 0.12 mM EDTA. Transcription was initiated by the addition of

280 μM of ATP, CTP, GTP, and UTP (Sigma) to bring the final volume to 25 μl and the reactions were placed at 30°C for 30 minutes. The reactions were stopped by making the sample 50 mM Na-acetate, 0.5% SDS, 0.5 mg/ml yeast t-RNA in a total volume of 100 μl. The nucleic acids were isolated by phenol:$CHCl_3$:isoamyl alcohol extraction, precipitated and resuspended in 49 μl of DNase I digestion buffer [40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$]. After digestion with 1 unit of RQ1 RNase free DNase I (Promega) for 15 minutes at 37°C the RNA transcripts were isolated by extraction as before and directly used for nuclease S1 protection analysis.

S1 nuclease protection. The S1 nuclease protection analysis was performed using a modification of the method of Berk and Sharp (17,125). The probe used was a single end-labeled DNA fragment overlapping the transcription initiation site (-418 - +279 base pairs), which yields a 279 nucleotide protected fragment corresponding to the accurately initiated FO108 H4 histone mRNA. The probe was labeled and isolated as described for the gel mobility shift assay. The RNA was precipitated with 10 ng of probe and 20 μg of glycogen by the addition of NaCl to 0.25 M and 3 volumes of ethanol. The pelleted nucleic acids were resuspended in 2.5 μl of 5x hybridization buffer [2 M NaCl, 0.2 M Pipes (pH 6.4), and 5 mM EDTA] and 10 μl of

recrystallized formamide, denatured by incubation at 100^{0}C for 10 minutes and transferred directly to 55^{0}C for 3 - 12 hours. The reactions were then placed on ice and digestion was initiated by the addition of 100 μl of S1 buffer [0.25 M NaCl, 0.03 M Na-acetate (pH 4.6), and 4 mM $ZnSO_4$], 10 μg salmon sperm DNA, and 440 units of S1 nuclease (Boehringer Mannheim). The digestion reactions were for 1 hour at 22^{0}C followed by 15 minutes on ice and were stopped by the addition of 10 μl 10% SDS and 4 μl 0.5 M EDTA. The protected nucleic acids were recovered by phenol:$CHCl_3$:isoamyl alcohol extraction followed by ethanol precipitation with 20μg of glycogen and 2.5 volumes of 95% ethanol at -20^{0}C overnight. The protected probe was recovered by centrifugation, resuspended in 5μL of loading dye (99.8% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol), heated to 100^{0}C for 3 minutes and resolved on a 6% polyacrylamide denaturing gel [0.3% *N,N'*-methylene-bisacrylamide, 7.75 M urea, 1xTBE (20XTBE: 89 mM Tris-HCl (pH 8.3), 89 mM boric acid, and 20 mM EDTA)] followed by autoradiography.

Gel mobility shift analysis. The gel mobility shift analysis (67) was performed essentially as described by Staudt *et al.* (192). DNA probes were prepared by digestion of the pD3 plasmid [contains only Site I (-204 to -72 bp) cloned into the SmaI-PstI sites of a pUC19 vector] with

HinDIII under the conditions recommended by the supplier followed by 5' end labeling 5 μg with ^{32}P in a 25 μl reaction mixture containing 100 μCi γ-^{32}P-ATP (3000 Ci/mmole, New England Nuclear), and 2.5 μl of 10x kinase buffer [0.5 M Tris-HCl (pH 7.6), 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, and 1 mM EDTA] and 15-30 units of T4 polynucleotide kinase (cloned, U.S.Biochemical). The reaction was allowed to proceed for 30 minutes at 37^{0}C then stopped by the addition of 2 μl of 0.5 M EDTA. The DNA was recovered by phenol:$CHCl_3$:isoamyl alcohol extraction and subjected to digestion with DdeI to generate the 99 base pair Site I probe. The probe was isolated by electrophoresis on a 4% native polyacrylamide gel (0.2% *N,N'*-methylene-bisacrylamide, 1xTBE), localized by autoradiography and electroeluted from the gel with the Elu-trap system (Schleicher & Schuell). Protein-DNA binding reactions were allowed to occur between 2 - 4 ng of ^{32}P-radiolabeled Site I DNA probe (-158 to -71 bp) and 1 - 10 μg of crude HeLa nuclear extract in a final volume of 20 μl containing 4 μg of poly(dI-dC)·(dI-dC), 12 mM Hepes (pH 7.9), 12% glycerol, 60 mM KCl, and 0.37 mM DTT. Binding occurred during a 30 minute incubation at 22^{0}C and samples were immediately loaded onto a high ionic strength 4% native polyacrylamide gel (0.1% *N,N'*-methylene-bisacrylamide, 50 mM Tris-HCl,

380 mM glycine, 2 mM EDTA, pH 8.5). Electrophoresis was at 200 V constant and the gels were maintained at <10^{0}C throughout the run. The electrophoresis was stopped when the marker bromophenol blue dye reach the bottom, the gels were vacuum dried onto Whatman 3MM paper and exposed to Kodak XAR film at room temperature for 12 to 16 hours. The specific oligonucleotide competitor for distal Site I is:

5'-GATCCGGAAAAGAAATGACGAAATGTCGAGA-3'
3'-GCCTTTTCTTTACTGCTTTACAGCTCTCTAG-5'

The ATF oligonucleotides were the generous gift of Dr. Michael Green (81,82) and the consensus Sp1 oligonucleotide has been described (206). The H3 histone distal site II oligonucleotide is:

5'-GATCTGCACAGAGATGGACCAATCCAAGAAGGG-3'
3'-ACGTGTCTCTACCTGGTTAGGTTCTTCCCCTAG-5'

Gel mobility shift analysis of the purified protein preparations was the same except the poly(dI-dC)·(dI-dC) was omitted and gels were exposed for 16 - 24 hours at -70^{0}C.

Site-directed mutagenesis. Selected base substitutions were introduced into the promoter of the H4 histone gene by oligonucleotide directed mutagenesis without phenotypic selection, essentially as described by Kunkel (113). Uracil containing single strand M13 DNA carrying the 1.85 kB EcoRI/HinDIII fragment of FO108, was prepared by amplification in the *E. coli* strain CJ236 (*dut* $^-$*ung* $^-$F') (a

gift of Dr. Barbara Bachmann, *E. coli* Genetic Stock Center, Yale University) in normal growth medium supplemented with 0.25 μg/ml uridine (Boehringer Mannheim). Mutations were introduced in complementary synthetic oligonucleotides containing the base substitutions in either distal Site I, 5'-GAGGAAAACAGAAAAGAcATcACtAAATGTCGAG-3', or proximal Site I, 5'-CGAGAGttCGGGGAC-3'. A 4 - 8 fold molar excess of oligonucleotide was 5' phosphorylated with 2 units of cloned T4 polynucleotide kinase (US Biochemical) in a 20 μl reaction mixture containing 2 μl of 10x kinase buffer [500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, and 50 mM DTT] and 1.25 mM ATP for 60 minutes at 37^{0}C. The reaction was stopped by the addition of 3 μl of 100 mM EDTA and heating to 70^{0}C for 10 minutes. The single strand DNA template (1 μg) was added to the oligonucleotide, the buffer was adjusted to 1xSSC [20x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0], and the mixture was heated to 80^{0}C and allowed to cool slowly over several hours. Synthesis and ligation of the second strand was initiated by the addition of 20 μl of 5x polymerase mix [100 mM Tris-HCl (pH 8.8), 10 mM DTT, 50 mM $MgCl_2$, 2.5 mM dATP, dCTP, dGTP, dTTP, and 5 mM ATP], 2.5 units T4 DNA polymerase (Boehringer Mannheim), 2.0 units T4 DNA ligase (Boehringer Mannheim) in a final volume of 100 μl. The reactions were incubated for 5 minutes at room

temperature, then 2 hours at 37^{0}C and were stopped by the addition of 3 μl of 0.5 M EDTA. The mutations were recovered by transfection of half of the final reaction volume into wild type *E. coli* strain XL1-Blue (Stratagene) which degrades the uracil containing parental strand and amplifies the mutagenized strand. Mutations were confirmed by enzymatic sequencing (Sequenase™, US Biochemical) and the 1.85 kB EcoRI/HiNDIII histone gene containing fragment was cloned back into pUC19.

DNase I protection. DNase I footprinting was performed as described by Augereau and Chambon (9) and used the same probe as in the gel mobility shift assay. Binding reactions in a final volume of 10 μl consisted of 1 μg poly(dI-dC)·(dI-dC), 0.3 mM DTT, and the remaining 6 μl was made up of the indicated amount of nuclear extract plus nuclear extract storage buffer (Buffer D). The reaction mixture was pre-incubated for 15 minutes on ice then 5 fmole of the single end radiolabeled DNA probe was added and the reaction was shifted to 20^{0}C for an additional 15 minutes. A 1 minute digestion was initiated by the addition of 0.5 μl $MgCl_2$ and 1.0 μl of a 62.5 μg/μl stock of DNase I (Boehringer Mannheim). The digestion was stopped by the addition of 50 μl of Stop Buffer (0.65% SDS, 20 mM EDTA) then the samples were extracted with phenol:$CHCl_3$:isoamyl alcohol and

ethanol precipitated. Resolution of the DNase I pattern was by electrophoresis on a 6% or 8% denaturing polyacrylamide gel with 7.75 M urea as described for S1 nuclease protection. Control lanes contained 3 - 20 μg of bovine serum albumin (BSA) and were treated the same except the amount of DNase I used was 5 fold less. The relative affinities of the factors for the binding sites were assessed by determining the degree of change in the intensity of the footprint protection as measured by densitometry.

UV cross-linking of nuclear proteins. Molecular mass determination by UV cross-linking was performed as described by Chodosh *et al.* (36). Uniformly labeled DNA probes were synthesized from a single stranded M13 template containing the Site I binding domain. Template DNA (10 μg) and an equimolar amount of the M13 17 base pair universal primer were annealed by heating to 100^{0}C followed by slow cooling over several hours in a 100 μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 100 μg/ml BSA. Synthesis of the second strand was initiated by the addition of 500 μCi α-^{32}P-dCTP (New England Nuclear, 3000 Ci/mmole), 50 μM dATP, 50 μM dGTP, 5 μM dCTP, 50 μM 5-bromo-2'-deoxyuridine 5'-triphosphate (Pharmacia) in the same buffer as before and adding 24 units of the Klenow

fragment of *E. coli* DNA polymerase I (Bethesda Research Laboratories). The reaction was allowed to proceed for 90 minutes at 16^{0}C and was stopped by heating to 68^{0}C for 10 minutes. The buffer was adjusted to the recommended concentrations for digestion with DdeI and HinDIII and digestion proceeded for 3 hours. The digested DNA was precipitated by the addition of 0.3 M ammonium acetate and 2 volumes of ethanol followed by centrifugation at room temperature. The uniformly labeled Site I probe was isolated on a 4% polyacrylamide gel as described above. Protein-DNA interactions were formed exactly as described for the gel mobility shift assay except the probe was approximately 250,000 cpm. The binding reactions were then subjected to UV light (302 nm, 7000 $\mu W/cm^2$) at a distance of 5 cm for 5 to 60 minutes. After cross-linking the excess DNA was digested by the addition of 1 μl of 200 mM $CaCl_2$, 4 units DNase I (Worthington) and 1 unit micrococcal nuclease (Boehringer Mannheim) for 30 minutes at 37^{0}C. The cross-linked proteins were analyzed by discontinuous (SDS) polyacrylamide gel electrophoresis as described below and autoradiographed.

DNA affinity chromatography. Site I DNA probes were prepared with biotin and ^{32}P using a T4 DNA polymerase replacement synthesis method essentially as described by Kasher *et al.* (108) and performed with the assistance of

Dr. Neil Aronin. Plasmid DNA (pD3, 50 μg) containing the Site I region was linearized at the HinDIII restriction site 31 base pairs 3' to the Site I footprint and incubated for 60 seconds at 37^{0}C in a final volume of 100 μl containing 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 100 μg/ml bovine serum albumin, 0.5 mM DTT and 10 units of T4 DNA polymerase (Boehringer Mannheim) to excise 10 - 20 nucleotides from the 3'-hydroxyl termini. Termination of the exonuclease reaction and initiation of the re-synthesis reaction was by the addition of 200 μM dATP and dGTP, 40 μM dCTP, 20 μM biotin-16-dUTP (Boehringer Mannheim), and 100 μCi α-^{32}P-dCTP (New England Nuclear, 3000 Ci/mmole) and continued incubation at 37^{0}C for 45 minutes. The extension was chased to completion for 20 minutes at 37^{0}C by the addition of an additional 20 μM dCTP and 20 μM dUTP and the reaction was stopped by heating the mixture to 75^{0}C for 15 minutes. The reaction mixture was adjusted to the buffer conditions recommended for digestion with DdeI and digestion was carried out for 2 hours followed by isolation of the probe as described previously. The binding of the crude nuclear extract to the probe was exactly as described in the gel mobility shift protocol except all components and the final volume were scaled up 50 fold. The protein-DNA complexes were recovered by addition of a 5 fold molar excess of streptavidin (1.0 μl of

1 mg/ml stock; Boehringer Mannheim) and incubation for 5 minutes at 22^{0}C followed by a 30 minute incubation at 4^{0}C on a slowly rotating wheel with 9 μl of biotin-cellulose (Pierce, pre-treated with 500 μg/ml insulin and 200 μg poly(dI-dC)·(dI-dC)). The protein-DNA-streptavidin-biotin-cellulose complex was then pelleted by a brief centrifugation at 4^{0}C and the pellet was resuspended in 500 μl of gel mobility shift binding buffer and transferred to a new tube. The mixture was gently mixed for 2 minutes and pelleted as before. The proteins were eluted from the DNA by resuspension of the pellet in 20 μl of elution buffer [20 mM Hepes (pH 7.9), 1 M KCl, 12% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 200 μg/ml insulin] and incubation for 20 minutes at 4^{0}C with gentle rocking followed by pelleting of the biotin-cellulose complex. The supernatant containing the proteins was recovered and half was immediately frozen at -70^{0}C while the other half was diluted 10 fold with KCl-free elution buffer and stored at -70^{0}C in aliquots for DNA binding studies.

Silver stain and western blot analysis. Purified proteins were resolved by 10% denaturing (SDS) discontinuous gel electrophoresis as described by Laemmli (116) and performed with assistance of Dr. Neil Aronin. The protein fraction was mixed at a 1:1 ratio with 2xSDS sample buffer [126 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 300 mM

β-mercaptoethanol, and bromophenol blue], heated to 90°C for 3 minutes and loaded onto a 4% polyacrylamide-SDS stacking gel [375 mM Tris-HCl (pH 6.8), 4% polyacrylamide, 0.1% *N,N'*-methylene-bisacrylamide, 0.1% SDS] and electrophoresed into a 10% polyacrylamide-SDS resolving gel [same buffer as stacking gel except pH = 8.8]. The gel was electrophoresed at 7 - 15 mA until the dye front was within 2 cm of the bottom. The silver staining (209) was accomplished by soaking the gel for 30 minutes in 100 ml of staining solution prepared by adding 4 ml of a 1.18 M $AgNO_3$ solution drop-wise to a mixture of 21 ml 0.36% NaOH and 1.4 ml NH_4OH and subsequently increasing the volume to 100 ml with H_2O. The gel was washed 3 times with H_2O for 5 minutes each and developed in 250 ml of a solution of 0.26 mM citric acid and 0.019% formaldehyde until the bands were clearly visible. The reaction was stopped by several quick rinses in water and soaking the gel for 1 hour in 50% methanol-10% acetic acid. Electrophoresis of the purified protein for western blot analysis was described as above except instead of staining the gel it was electrophoretically transferred to Immobilon-P membrane (Millipore) at 100 mA for 12 hours in a transfer buffer of 20 mM Tris-HCl (pH 8), 150 mM glycine, and 20% methanol. The blot was serially treated for 1 hour with 2% BSA, for 2 hours with anti-AP1 rabbit anti-serum

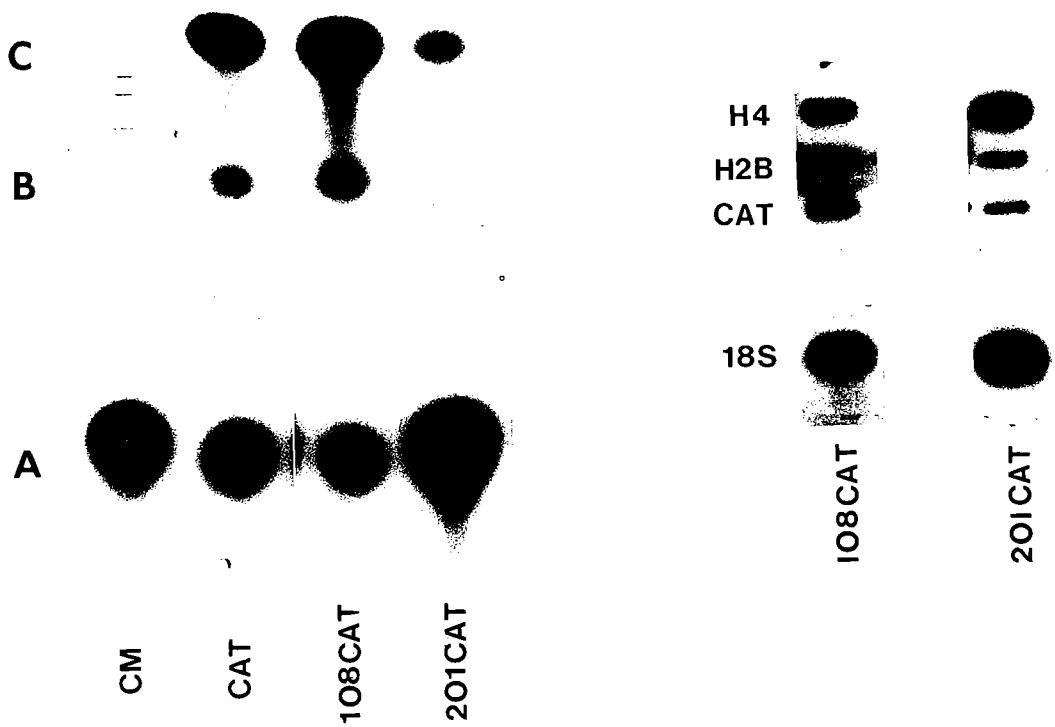
(diluted 1:1000, a gift of Dr. M. Green) and for 1.5 hours with alkaline phosphatase conjugated anti-rabbit anti-serum (diluted 1:3000). All dilutions and washes between anti-serum applications were with a buffer of 10 mM Tris-HCl (pH 7.6) and 150 mM NaCl. The alkaline phosphatase labeled bands were then visualized by treatment of the blot with commercially available Nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt (BCIP).

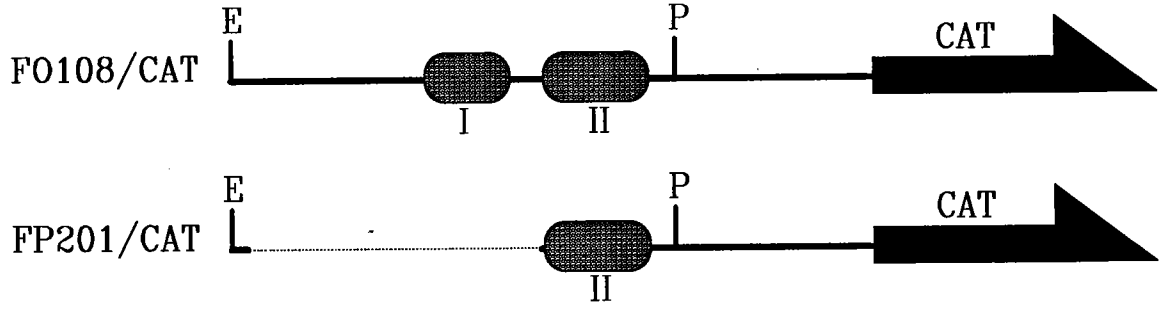
Results

The FO108 human H4 histone gene proximal promoter consists of two regions of protein-DNA interaction *in vivo*, Site I and II. We have determined the contribution of Site I in modulating the extent of H4 histone gene transcription by the construction and analysis, both *in vivo* and *in vitro*, of a series of 5' and internal deletion mutants. Furthermore, to understand the molecular mechanisms by which Site I influences the initiation rate, we have characterized the proteins interacting at this region.

Transcriptional contribution of Site I *in vivo*. The initial approach to understanding the role of Site I in transcription was to examine the effect of an internal deletion of Site I in a short-term transient expression system. Histone promoter deletions, spanning from -213 bp to -70 bp, were fused to the CAT structural gene 10 base pairs upstream of the histone transcription initiation site (Figure 2-1C). The constructs were transfected into HeLa S3 cells and 48 hours later whole cell freeze-thaw supernatants containing the expressed CAT protein (71) and Hirt supernatants of the transfected plasmid DNA (93) were prepared. The levels of CAT activity in the whole cell lysates were assayed by thin layer chromatography and

Figure 2-1. *In vivo* analysis of Site I deletion mutants. A) CAT assay of HeLa cells transfected with the indicated histone promoter-CAT fusion constructs. The un-acetylated and two acetylated forms of chloramphenicol are designated A, B and C respectively. Lanes: CM, control with no CAT enzyme; CAT, control with 0.9 units of purified CAT enzyme; 108CAT and 201CAT, extracts from cells transfected with the indicated construct. B) Nuclear-run transcription analysis of stable cell lines containing the indicated constructs. Radiolabeled RNA transcripts were hybridized to Southern blots containing immobilized plasmid DNA of H4 human histone gene (FO108), human H2B histone gene, CAT coding sequence, and 18S ribosomal gene. C) Schematic showing deletion constructs and quantitation of the CAT activity and nuclear run-on transcription. CAT activity was normalized to the transfection efficiency as determined from the Hirt supernatant (93) and nuclear run-on transcription was normalized to the endogenous 18S ribosomal transcript.


C
B
A
CM
CAT
108CAT
201CAT
H4
H2B
CAT
18S
108CAT
201CAT



	CAT Activity	Transcription
FO108/CAT	100	100
FP201/CAT	12	13

normalized for transfection efficiency by quantification of the plasmid DNA content in Hirt supernatants. The results (Figure 2-1A) indicate that deletion of the Site I protein-DNA interaction domain severely reduced the CAT activity (construct FO108/CAT vs FP201/CAT). After normalization with respect to the transfection efficiency, Site I was measured to increase CAT activity 7 fold over a minimal histone promoter. Further deletion of the promoter, which disrupts the Site II protein-DNA interaction domain, abolished all specific initiation (112) and reduced CAT activity to background levels (data not shown).

In order to demonstrate directly a role for Site I in modulating transcription and to address the effect of integration into a chromosomal structure, we established stable transformed cell lines containing each of the deletion constructs. The expression of each construct was assayed by nuclear run-on transcription and normalized to the level of 18S ribosomal gene expression as an internal control (Figure 2-1B). The findings were consistent with the transient expression results and demonstrate that Site I is responsible for a 7.0 to 7.5 fold increase in transcription from this histone promoter *in vivo*. The effect of Site I on transcription was independent of integration into a chromosomal structure, as the relative contribution of

Site I to transcription was approximately the same in both the transient transfection and stable cell line expression systems.

Site I contributes to the level of cell free transcription. To directly address the molecular mechanisms operative within the proximal promoter, we developed an *in vitro* transcription system capable of faithfully initiating transcription of the human H4 histone gene. Five 5' deletion mutants created by Bal 31 digestion (182) were cloned into a pUC19 vector and used to assess the contribution of the proximal promoter *in vitro*. Initially, linearized templates of the deletion mutants were employed in a standard run-off transcription assay (132). This system failed to detect any cis-acting elements upstream of the TATA box motif (data not shown). Because this result was in direct contrast to the *in vivo* transcription results, we examined the possibility that closed supercoiled templates were required to observe an effect by the cis-acting DNA elements. The transcription initiation complex was allowed to form by pre-incubating high salt extracted nuclear proteins with supercoiled DNA of the deletion mutants linked to the normal histone structural gene. Transcription was initiated by the addition of ribonucleoside triphosphates and the resulting mRNA was

quantified by densitometry of autoradiograms from S1 nuclease protection assays. The template DNA was relaxed during the initial pre-incubation period into a mixture of approximately 80% relaxed covalently closed circles and 20% with up to 8 supercoils (Figure 2-2). This mixture was maintained for up to 4 hours which is in great excess of the time required for the transcription reaction.

The results shown in Figure 2-3A and 2-3B indicate that the supercoiled cell free transcription system accurately reflects the *in vivo* transcription results. A comparison of the 5' deletion mutants JUC56 (-73 bp) and KUC8 (-155 bp) revealed that the addition of Site I sequences stimulated transcription greater than 5 fold. Further subdivision of this element at -99 bp (JUC50) demonstrated that Site I contains at least two distinct functional domains. Both halves contribute approximately equally to the activity of Site I. Additional sequences up to -185 bp (LUC14) and the distal half of Site II do not contribute significantly to the level of transcription. It is interesting to note, however, that Site II has been implicated as being required for correct initiation during transient transfection experiments (112) and unpublished results). These results establish that Site I is a functional cis-acting element capable of stimulating transcription of the human H4 histone

Figure 2-2. Structure of plasmid DNA after incubation with nuclear extracts. Maximally supercoiled plasmid DNA was incubated with HeLa nuclear extracts under the same conditions of the cell free transcription reactions except the nucleotides were omitted. Reactions were stopped by addition of 0.5% SDS and assayed on a 0.8% agarose gel. Lanes 1 - 8, increasing duration of incubation from 10 seconds to 30 minutes.

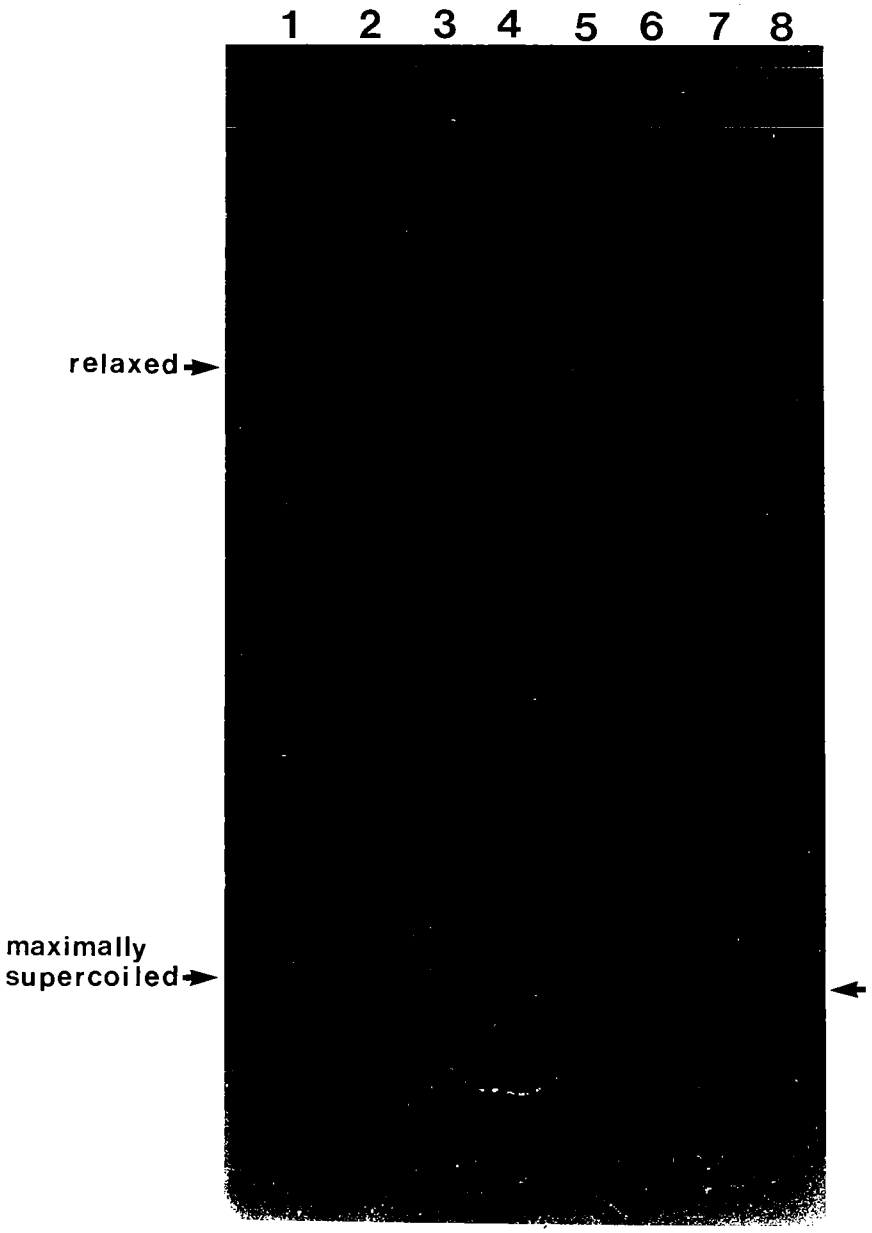
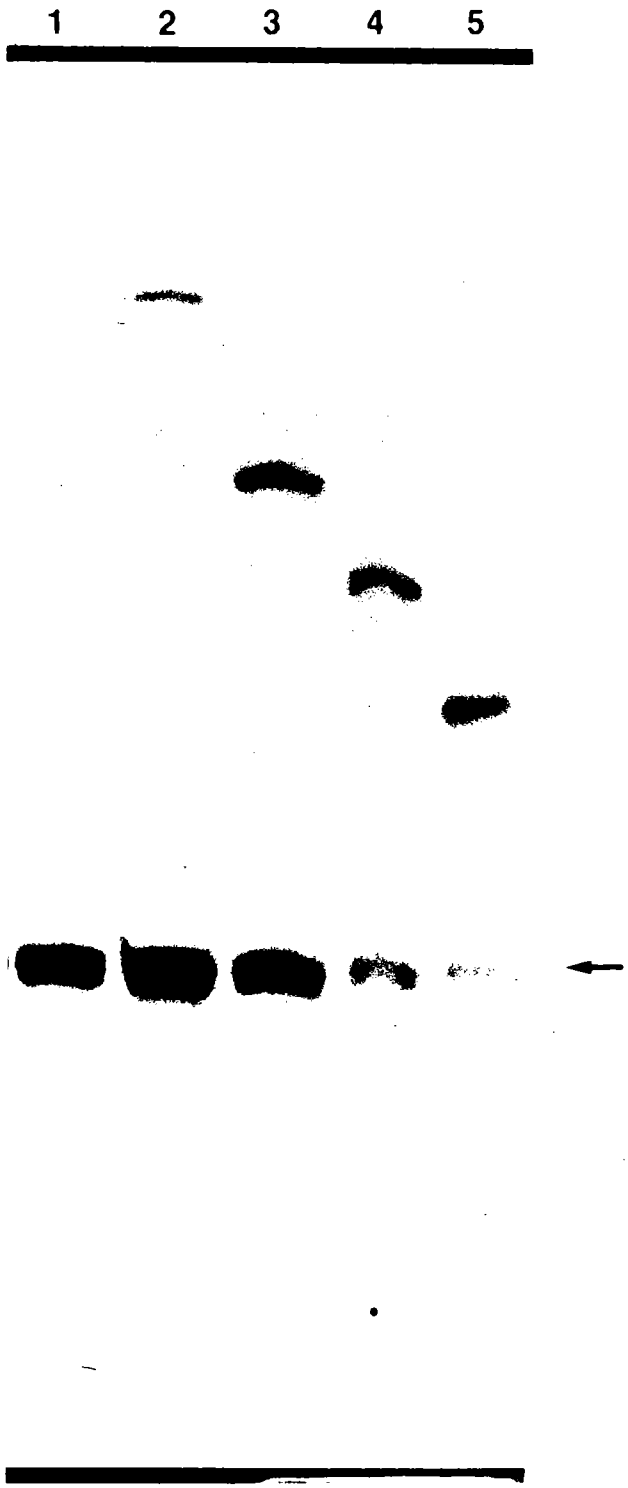
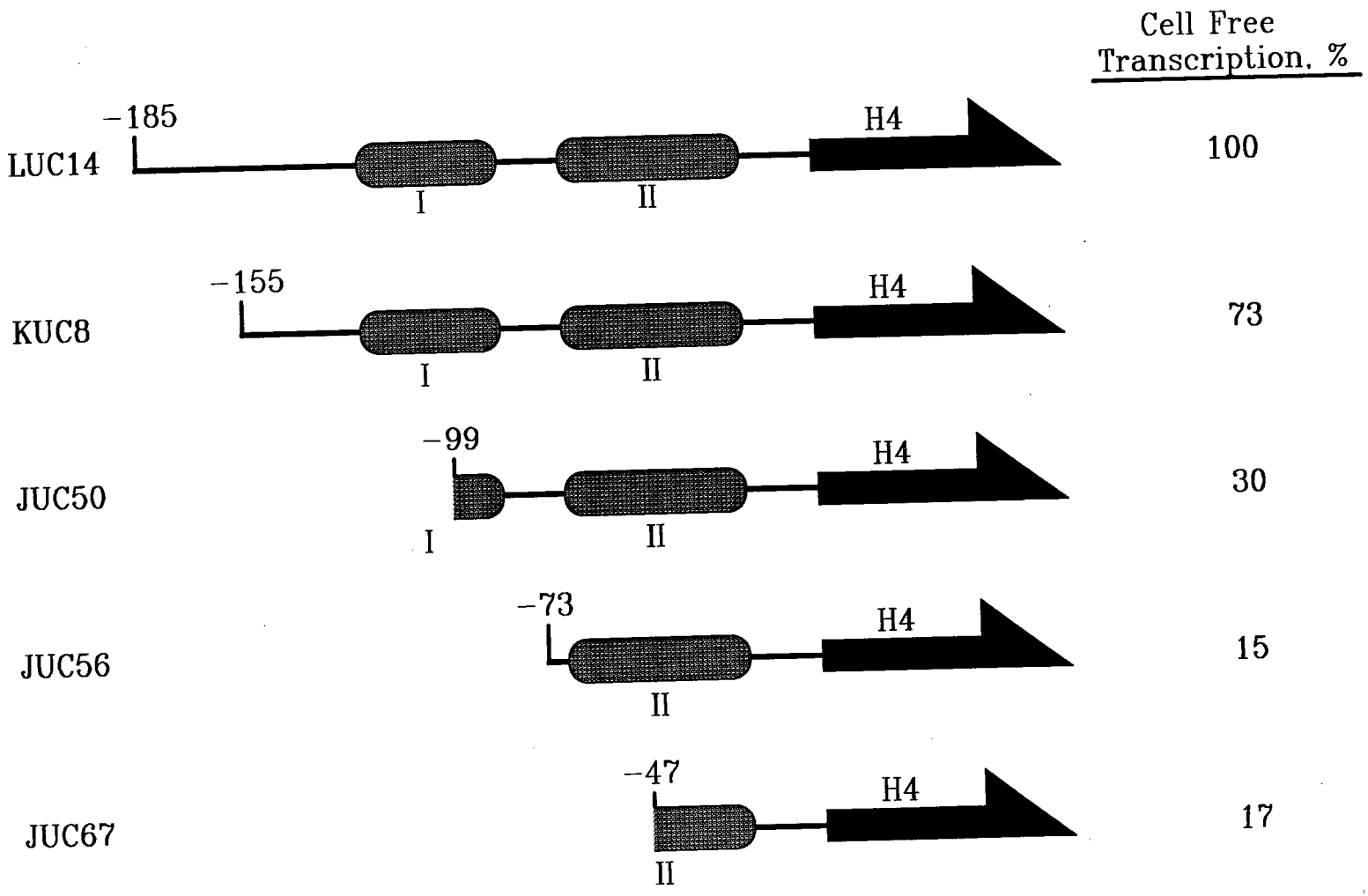

1 2 3 4 5 6 7 8
relaxed
maximally
supercoiled

Figure 2-3. Cell free transcription analysis of H4 histone promoter deletions. Supercoiled plasmid containing a series of promoter deletion mutants were transcribed in a nuclear extract system and transcripts were assayed by S1 nuclease protection. A) Autoradiograph of S1 analysis. lane 1 - LUC14, lane 2 - KUC8, lane 3 - JUC 50, lane 4 - JUC56, lane 5 - JUC67. Arrow indicates accurately initiated transcripts. B) Schematic of the promoter deletion constructs and quantitation of the average of at least five independent transcription experiments.


1
2
3
4
5


Cell Free
Transcription, %
LUC14
−185
H4
I
II
100
KUC8
−155
H4
I
II
73
JUC50
−99
H4
I
II
30
JUC56
−73
H4
II
15
JUC67
−47
H4
II
17

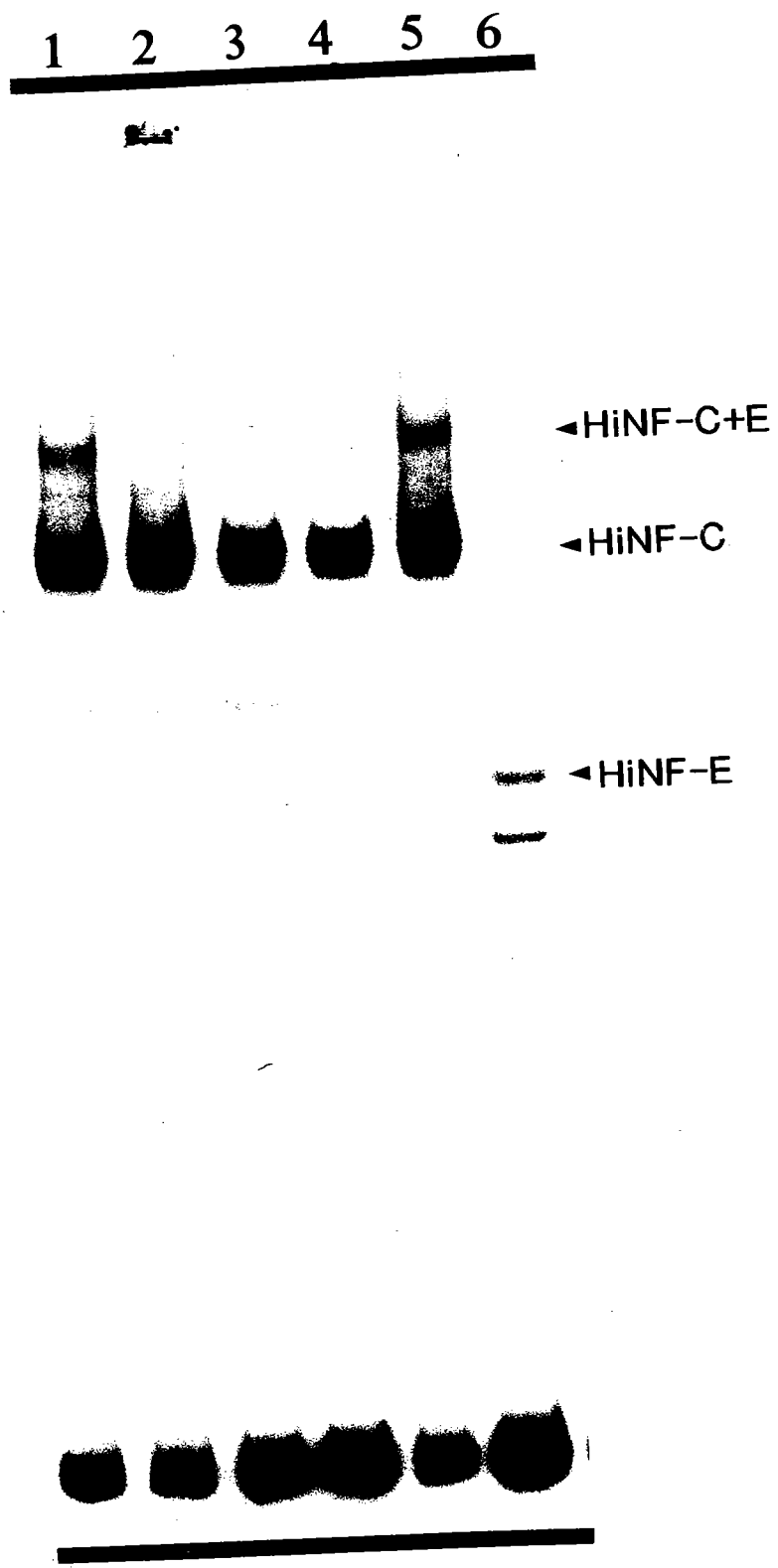
gene. In addition, the effectiveness of this element *in vitro* indicates that the necessary protein factors involved are contained in the high salt nuclear extract.

HiNF-E and HiNF-C are distinct binding events at Site I. The protein factors interacting at Site I were investigated to reveal the mechanisms by which Site I influences transcription. The initial approach was to employ the gel mobility shift assay to address the number and types of interactions occurring. A radioactive DNA probe spanning only Site I (-156 to -72 bp) was incubated with the high salt extracted nuclear proteins from HeLa cells. Site I sequences formed protein-DNA complexes resulting in two prominent shifted bands and several light bands (Figure 2-4). The specific interactions in each complex were defined by DNA binding site competition with synthetic oligonucleotides. An oligonucleotide representing the distal half of Site I (-125 to -101 bp) competed one of the lower complexes designated HiNF-E and partially competed the upper prominent complex, HiNF-C+E (Figure 2-5). Because Site I has a region of sequence similarity to the ATF transcription factor consensus site, we examined whether either a consensus ATF recognition sequence or an ATF binding site from the adenovirus E4a promoter would compete with the

Figure 2-4. Gel mobility shift analysis of the Site I protein-DNA interactions. A radiolabeled Site I probe (-158 to -71 bp) was incubated with increasing concentrations (0.5 to 8 μg) of HeLa nuclear extract and resolved on a polyacrylamide gel as described in (192).


HiNF-C+E
HiNF-C
HiNF-E
increasing protein

Figure 2-5. Competition of the Site I protein-DNA complexes with consensus ATF and Sp1 oligonucleotides. Gel mobility shift analysis of the Site I probe incubated with 4 μg of HeLa nuclear extract and 500 fold molar excess of specific oligonucleotides. Lanes: 1, no competitor; 2, distal Site I (DS-1); 3, consensus ATF (CATX3, 82); 4, ATF site from the E4 promoter (pE427X1, 81); 5, non-specific oligonucleotide from the histone H3 promoter (H3-SII); 6, Sp1 consensus dimer.


1 2 3 4 5 6
HiNF-C+E
HiNF-C
HiNF-E

Site I interactions. Both ATF recognition sites competed HiNF-E and HiNF-C+E binding, though not as effectively as the distal Site I competitor (Figure 2-5). A third band that migrated more rapidly than HiNF-E was also competed by the DNA fragments containing the ATF binding sites but is apparently unrelated to the ATF site in Site I, as mutations of the ATF recognition sequence (see below) did not disturb this interaction.

Because the proximal half of Site I contains a sequence with strong similarity to the Sp1 consensus binding site, we used a dimer of the Sp1 consensus as an oligonucleotide competitor. This oligonucleotide effectively competed both of the prominent shifted complexes, HiNF-C and HiNF-C+E (Figure 2-5). An unrelated oligonucleotide was unable to compete specifically for any of the bands. The results suggest that HiNF-C is related to Sp1 binding to the proximal half of Site I while HiNF-E is an interaction in the distal half related to an ATF consensus sequence. The ability of both specific oligonucleotides to compete for the upper prominent band indicates this band is related to binding of both HiNF-E and HiNF-C, and suggests that Site I is a bipartite structure with at least two distinct protein-DNA binding events.

HiNF-E requires an ATF consensus site for binding; HiNF-C requires an intact Sp1 consensus site. Site-directed base substitution is a highly specific method to define the binding site requirements of protein-DNA interactions. We created two independent mutations of Site I to resolve the binding sites of HiNF-E and HiNF-C. Previously, we have identified by *in vivo* genomic sequencing (157) and *in vitro* DMS fingerprinting (202), a series of highly specific protein/guanine residue contact points at Site I. These contacts are arranged into two groups, one in the proximal half over the region of similarity to the Sp1 consensus recognition site and the other group in the distal half about a potential ATF recognition sequence. The contact points were used as a guide for the construction of the site-directed mutations. Mutation 1 alters the two guanine contacts in the distal half of Site I as well as one adjacent nucleotide (Figure 2-6) and disrupts the ATF binding site consensus core, 5'-TGACG-3'. Examination of the protein-DNA interactions reveals that both the HiNF-E and HiNF-C+E complexes were abolished by the mutation (Figure 2-7). The gel mobility shift pattern is analogous to the wild type pattern after competition with the distal Site I or ATF consensus oligonucleotide and implies that all of the

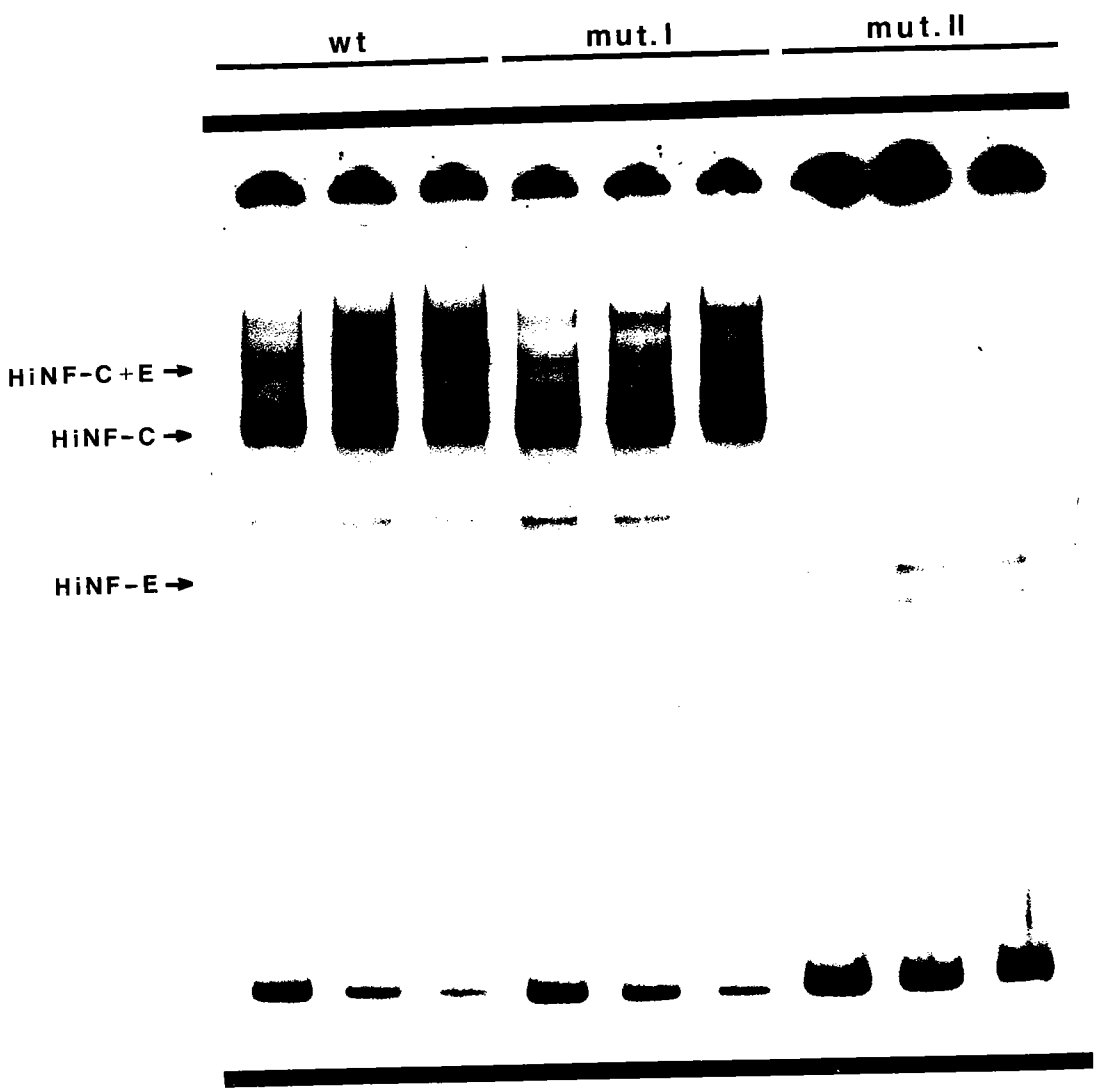
Figure 2-6. Schematic of the Site I domain. Dark lines indicate the extent of the *in vivo* DNase I footprint and filled circles denote the methylation protected guanine residues (157). Brackets define the *in vitro* Dnase I footprints of HiNF-E and HiNF-C determined in the absence of binding by the other factor. Filled boxes designate the ATF and Sp1 consensus recognition sequences. The base substitutions in Mutation 1 and Mutation 2 are indicated below.

Site I

Mut.1 -------C--C--T-------------------------

Mut.2 -------------------------TT----------

Figure 2-7. Effect of specific base substitutions on the Site I protein-DNA interactions defines two binding events. Gel mobility shift analysis of Site I probes containing either the wild type sequence (wt) or mutation 1 (mut.1) or mutation 2 (mut.2) as detailed in Figure 2-6. Each probe was incubated with three concentrations of HeLa nuclear extract (4, 6, 8 μg).


wt
mut.I
mut.II
HiNF-C+E
HiNF-C
HiNF-E

interactions in the distal half are due to binding at the ATF consensus site.

Mutation 2 substitutes two adjacent guanine nucleotides of the Sp1 consensus with thymine residues (Figure 2-6). This mutation abolishes purified Sp1 binding activity in other genes (103). The effect on the Site I gel mobility shift pattern (Figure 2-7) was to abolish both prominent bands while increasing the intensity of the HiNF-E band. This result mimics consensus Sp1 oligonucleotide competition of the wild type pattern and further confirms the presence of an Sp1/Site I interaction.

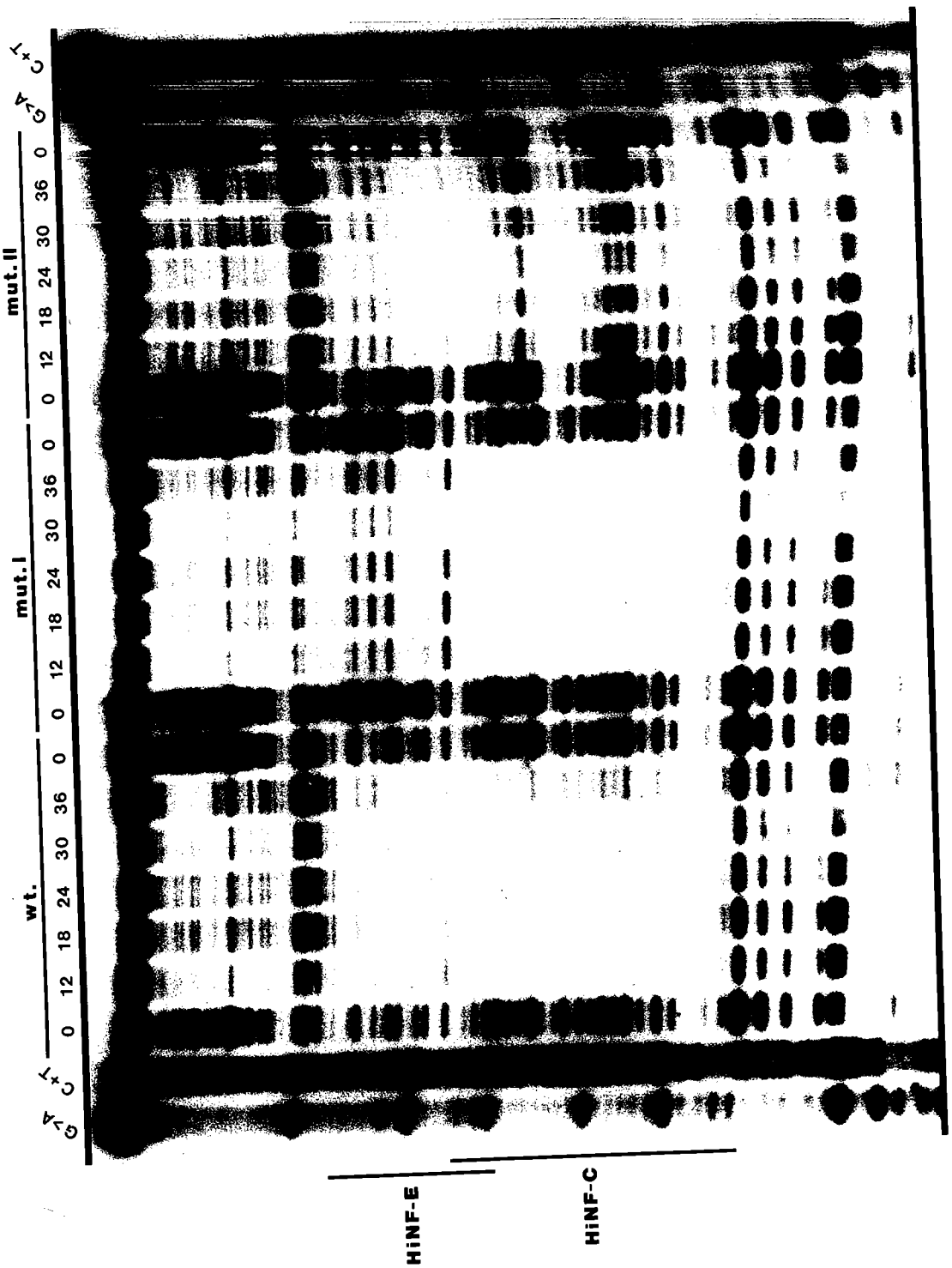
The upper prominent shifted band is detected only at high concentrations of protein extract, subsequent to the appearance of HiNF-E and HiNF-C and is eliminated by mutations to or competition with either the ATF consensus binding site or the Sp1 binding site. These results can be most directly interpreted to signify that this upper prominent band is a complex of both the Sp1 interaction, HiNF-C, along with the ATF interaction, HiNF-E.

HiNF-E/Site I interaction is stabilized by HiNF-C binding. Gel mobility shift analysis has demonstrated that HiNF-E and HiNF-C are capable of binding independently and simultaneously to Site I. To better understand this relationship, we examined the position and relative

affinities of the interactions by DNase I footprint analysis. Crude nuclear extract incubated with the wild type Site I probe protected a region of 36 base pairs (-122 to -87 bp) that spans the Sp1 and ATF-like recognition sequences (Figure 2-8). The boundaries of this region match the DNase I footprint of Site I determined *in vivo* (157) (Figure 2-6). Footprint analysis of the independent binding events within Site I is possible by using the site-directed mutants described above. Mutation 1, which abolished binding of HiNF-E, produced a footprint of reduced size (-108 to -87 bp) that was aligned over the Sp1 recognition site. The boundary on the proximal side was unchanged while the distal side was reduced by 14 nucleotides and excluded the ATF consensus binding site. Mutation 2 abolished the Sp1 consensus site and also produced a reduced size footprint. This footprint was unaltered on the distal side (-122 bp) but was withdrawn on the proximal side up to nucleotide -104. The footprint for HiNF-E was 19 nucleotides long and aligned over the ATF consensus similarity sequence.

The affinity of the HiNF-E interaction as measured by the intensity of DNase I cleavage pattern was substantially reduced by the mutation that abolished HiNF-C binding, even though the HiNF-E footprint clearly does not extend to the site of the Sp1 mutation. HiNF-E binding affinity was

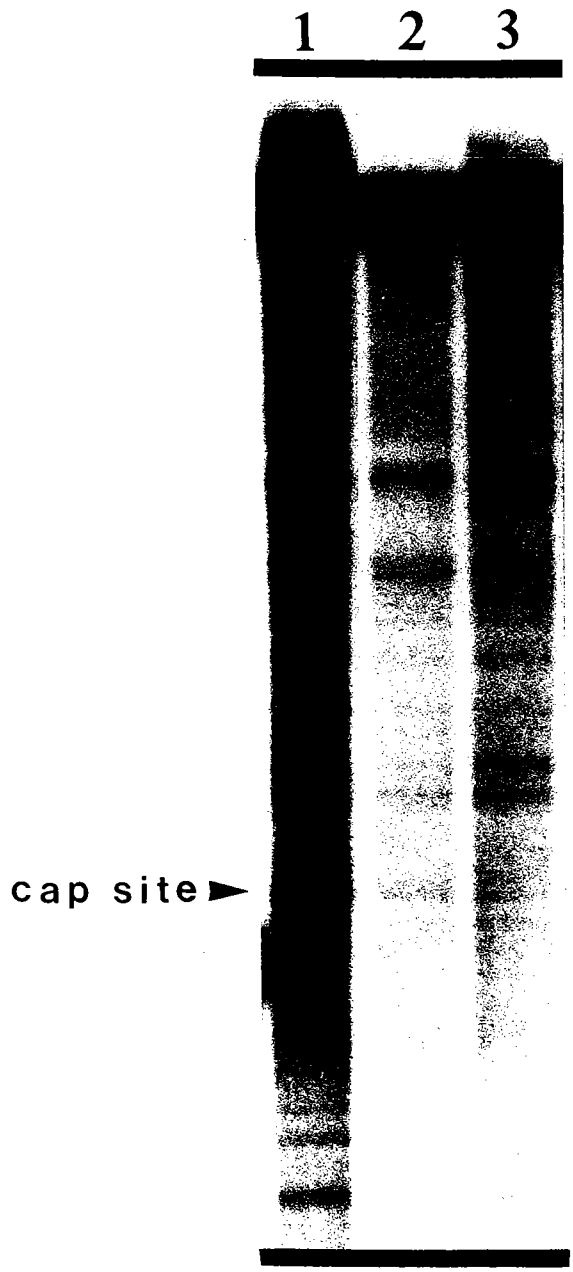
Figure 2-8. DNase I footprint of Site I protein factors: HiNF-E and HiNF-C. Amounts of nuclear protein extract present in the reaction are indicated above each lane. Boundaries of the footprints are indicated along the side and Figure 2-6. Mutations are those shown in Figure 2-6.


G>A
C>Y
wt.
0 12 18 24 30 36
mut.I
0 12 18 24 30 36
mut.II
0 12 18 24 30 36
G>A
C>Y
HiNF-E
HiNF-C

reduced greater than 60% by the loss of HiNF-C binding. However, no effect on the strength of the HiNF-C interaction was observed by the mutation eliminating the ATF recognition site. This finding suggests that the affinity of the HiNF-E factor for Site I is strengthened by the prior binding of HiNF-C to its adjacent recognition sequence. Thus, HiNF-C/Sp1 may play a dual role in this promoter; first as a general transcription factor stimulating transcription and secondly by stabilizing the adjacent binding of a second transcription factor, HiNF-E.

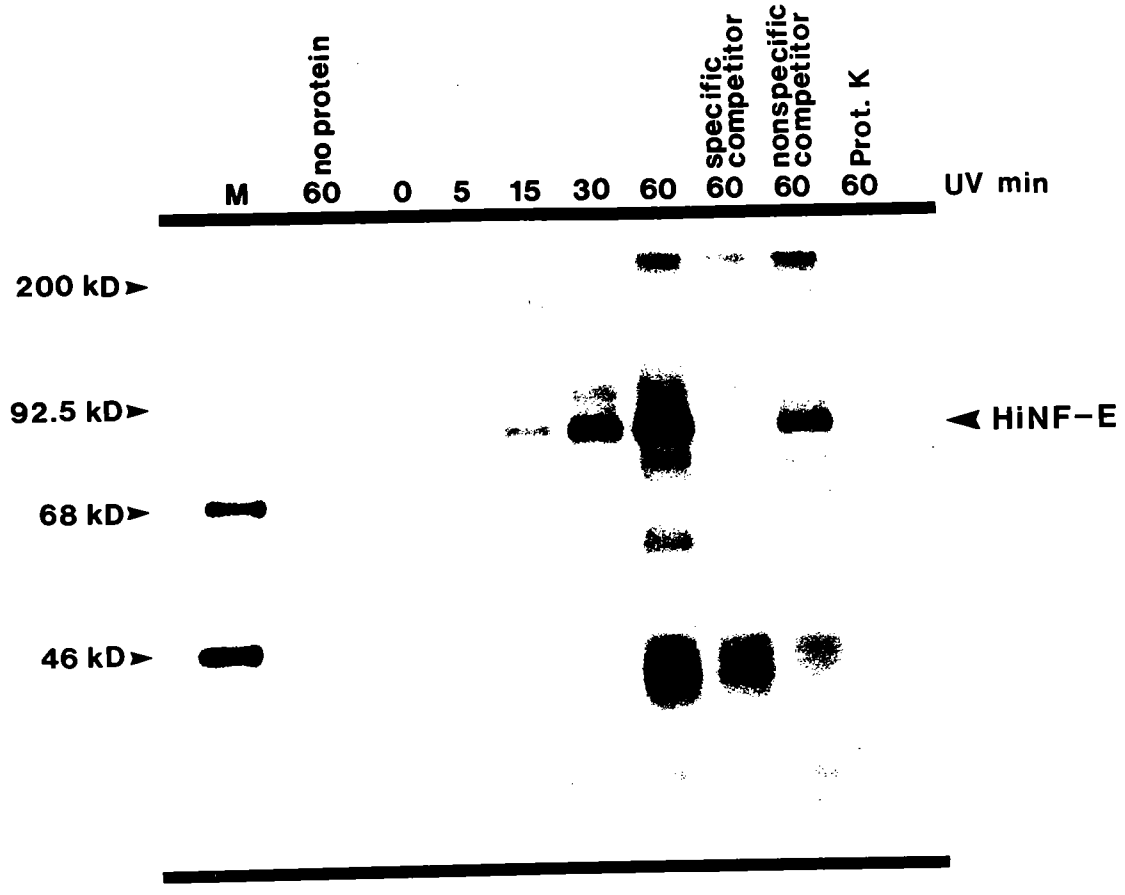
HiNF-E and HiNF-C are the functional components of Site I. To demonstrate that HiNF-E and HiNF-C are responsible for the stimulation of transcription exerted by Site I, we analyzed the competence of the site-directed mutant constructs to support cell free transcription of the H4 histone gene. The results shown in Figure 2-9 indicate that both HiNF-E and HiNF-C binding sequences contribute to the level of transcription. Elimination of either binding site interaction reduced transcription initiation approximately 2 fold. This evidence confirms that Site I can be divided into two functional domains and that HiNF-E and HiNF-C are the protein components mediating the activation of transcription.

Figure 2-9. Effect of mutations on cell free transcription. Comparison of the promoter activity from the wild type H4 histone promoter (KUC8) (lane 1) and KUC8 constructs containing the HiNF-E (lane 2) and HiNF-C (lane 3) binding site mutations. Cell free transcription was performed as in Figure 2-3. Arrowhead indicates the accurately initiated transcripts.


1 2 3
cap site

HiNF-E is a novel ATF-like factor with a molecular mass of 84 kDa. The requirement for an intact ATF recognition sequence for HiNF-E binding to Site I raised the possibility that HiNF-E is a member of the ATF transcription factor family (82,216). In order to address the relationship of HiNF-E to previously identified ATF transcription factors, we determined the molecular mass by UV cross linking. A uniformly labeled HiNF-E binding site probe containing bromodeoxyuridine was covalently attached to the HiNF-E factor by treatment with UV light follwed by DNase I digestion of the excess DNA and resolution by SDS polyacrylamide gel electrophoresis. The results shown in Figure 2-10 indicate that the primary protein interacting with distal Site I is of approximately 84 kDa. Other proteins of approximately 43 - 45 kDa are also detected after 60 minutes of UV treatment. All of the bands detected were dependent on the addition of nuclear extract and treatment with UV and could be eliminated by digestion with Proteinase K. This demonstrates that all of the resolved bands consist of protein cross linked to DNA and not probe contamination. Competition with the distal Site I oligonucleotide specifically eliminated the 84 kDa band while only minimally decreasing the signal of the 43 - 45 kDa bands. A non-specific oligonucleotide consisting of Sp1 consensus recognition sequences (Figure 2-10) or random

Figure 2-10. Molecular weight determination of HiNF-E by UV cross linking indicates HiNF-E is 84 kDa. UV cross linking to Site I was performed with BrdU-substituted, uniformly labeled Site I DNA probes. After DNase I digestion the samples were resolved on a 10% SDS-polyacrylamide gel and autoradiographed. Length of UV treatment is indicated above each lane. Specific competitor, DS-I; non-specific competitor, H3-DSII; Prot.K indicates sample was digested with Proteinase K prior to electrophoresis.


M
no protein 60
0
5
15
30
60
specific competitor 60
nonspecific competitor 60
Prot. K 60
UV min
200 kD
92.5 kD
68 kD
46 kD
HiNF-E

pUC19 fragments (data not shown) generally lowered the signal but did not specifically compete any of the bands. Interestingly, the size of the weakly competed bands is approximately the size of previously described ATF factors, 43 - 45 kDa (97,127,145) and indicates that these ATF factors have some affinity for distal Site I. These results suggest that an 84 kDa protein is preferentially interacting at the HiNF-E binding site and because HiNF-E has been demonstrated to require an ATF consensus sequence for binding, we define HiNF-E to be a novel member of the ATF transcription factor family and term it ATF-84.

Selective Purification of ATF-84 by DNA Affinity Chromatography. In order to confirm the presence of an 84 kDa protein interacting at Site I and to initiate the physical characterization of ATF-84 we partially purified the HiNF-E binding activity. Because previous studies have shown HeLa cells to contain several ATF factors in addition to ATF-84 we required a method of purification which could discriminate between these factors. Typical DNA affinity chromatography involves the synthesis and coupling of double stranded oligonucleotides containing multimers of the binding site to a stationary support. This method has been used successfully by several groups to purify the more abundant 43 - 47 kDa ATF factors (83,145). Therefore in order to selectively purify ATF-84 we utilized the high

binding affinity of Site I for ATF-84 to capture this protein. Crude HeLa nuclear extract was incubated with biotin labeled Site I DNA probe under the same conditions as used in the gel mobility shift assay. The protein-DNA complex was then separated from the excess proteins by forming a biotin-streptavidin precipitatable super-complex and followed by elution of the purified protein. The results shown in Figure 2-11 indicate that this procedure selectively purified approximately equal amounts of both the 84 kDa and the 45 kDa proteins. This purified preparation was tested for the presence of HiNF-E binding activity. Gel mobility shift analysis using the Site I probe detects a protein-DNA complex co-migrating with the HiNF-E/ATF-84 interaction from crude nuclear extracts (Figure 2-12). Furthermore, competition with either excess distal Site I sequences or consensus ATF sequences specifically blocked the formation of the HiNF-E interaction. An additional faster migrating complex was also detected in the purified preparation, however, this band is apparently unrelated to the HiNF-E interaction as it was not competed by any of the oligonucleotides. The level of HiNF-E binding activity in the purified preparation was less than expected for the amount of 84 kDa protein dectected on the silver stained gel. This suggests that during purifcation ATF-84 binding activity is lost perhaps due to modifications to the protein

Figure 2-11. Biotin/streptavidin affinity purification of proteins binding to Site I of the H4 Histone Gene. Hela cell nuclear extracts were bound to a 16-biotin-UTP-substituted H4 Site I probe in the presence of non-specific competitor poly (dI-dC). The protein/DNA complexes were isolated and the specifically bound proteins were eluted and electrophoresed on a 10% SDS-PAGE. Silver stain detection of proteins. Lane 1, MW markers; 2, crude Hela cell nuclear extract; 3, affinity purified protein. Prominent purified bands of molecular weight 40 and 80 kD are indicated. Proteins of 40 - 47 kD size are known to bind to consensus ATP sites. The 80 kD band is in about the same molecular weight as estimated by UV cross linking of crude extract with Site I.

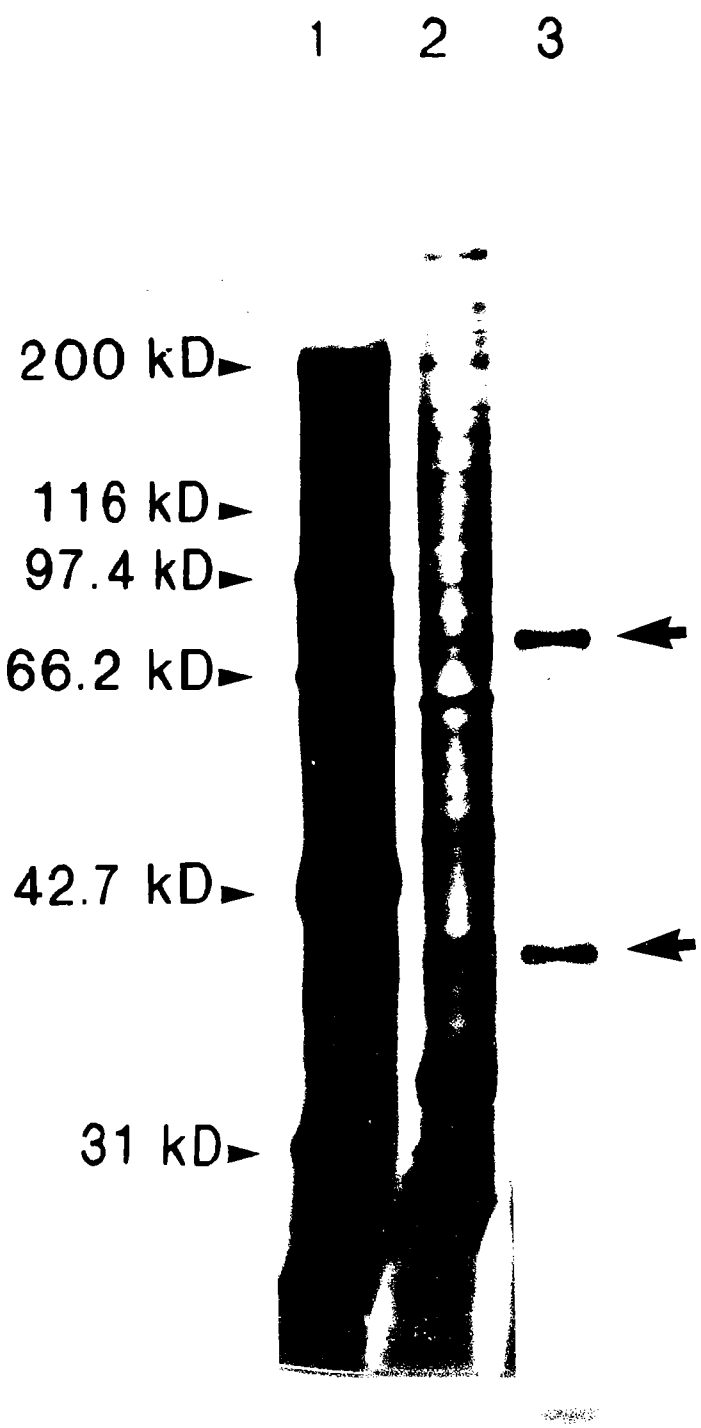
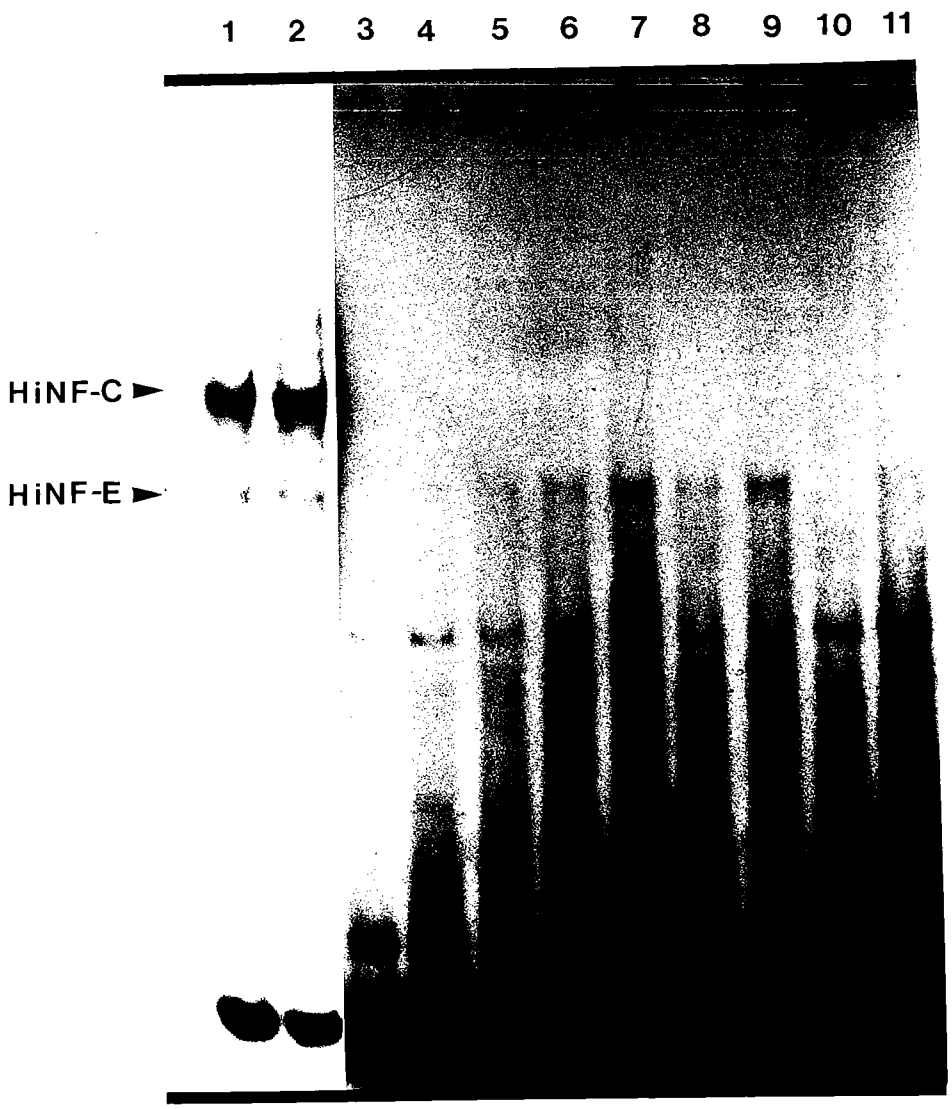
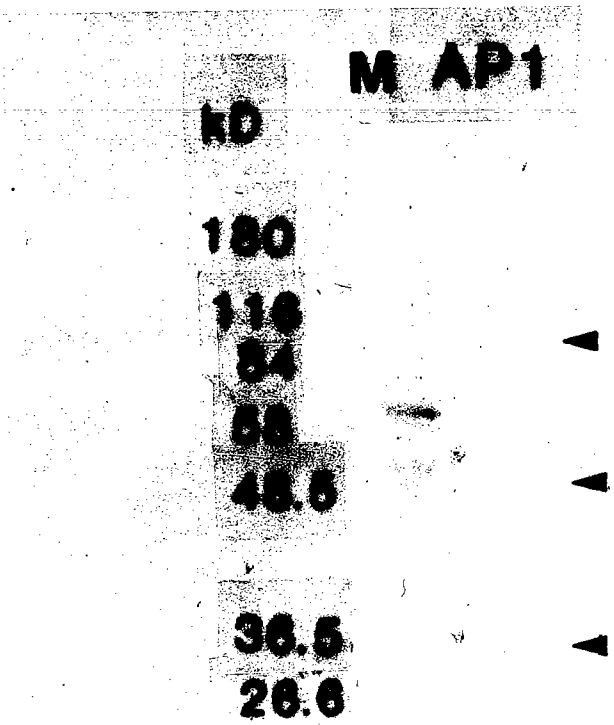

1
2
3
200 kD
116 kD
97.4 kD
66.2 kD
42.7 kD
31 kD

Figure 2-12. HiNF-E binding activity is present in the purified protein preparation. Gel mobility shift analysis of the DNA affinity purified protein preparation with Site I. Lanes 1 - 2, crude nuclear extract; Lanes 3 - 7, increasing amounts of purified protein; Lanes 8 - 11, oligonucleotide competition, 8 - distal Site I oligo, 9 - consensus dimer Sp1 oligo, 10 -consensus ATF oligo (CATX3, 82), 11 - ATF site from the E4 promoter (pE427X1, 81). Lanes 3 - 11 were exposed 6 times longer than Lanes 1 and 2.


1 2 3 4 5 6 7 8 9 10 11
HiNF-C
HiNF-E

or due to separation from an essential component. To assess the physical relationship between ATF-84 and other ATF family members, weexamined their immunochemical cross-reactivity. Previous studies have shown that antiserum prepared against either the ATF-43/47 factors or the AP1 transcription factors detect proteins from both families due to the strong similarities in the leucine zipper and DNA recognition domains (83). We obtained both ATF-43/47 and AP1 antiserum (a gift of Dr. M. Green) and utilized them to detect proteins in the ATF-84 purified preparation. Several bands were detected on a Western blot of the purified fraction (Figure 2-13). These included proteins in the size range of 84 and 47 kDa which were seen in the silver stained gel and several other bands which were not detectable by silver staining. While this blot does not provide definitive proof that the 84 kDa band is immunochemically related to ATF-43/47 it is very suggestive of this. Interestingly, the antiserum interacted with the band corresponding to ATF-84 very weakly as compared to the other bands suggesting that while ATF-84 is an ATF consensus site recognition protein and thus an ATF family member its structure has diverged substantially from the more common 43 - 47 kDa ATF transcription factors.

Figure 2-13. Western blot analysis of partially purified extracts. Proteins were blotted to Immobilon and incubated with rabbit antiserum directed to the leucine-zipper region of AP1 (antisera were generously provided by Dr. Michael Green). The blots were then treated with alkaline phosphatase conjugated Ig G antiserum and the protein patterns were detected with NBT/BCIP The 84 - 90 kD protein and the 43 - 47 kDa protein of the partially purified extract is recognized by the antisera directed against the leucine-zipper.


M
AP1
kD
180
116
84
58
48.5
36.5
26.6

Discussion

The FO108 human H4 histone gene contains two regions of *in vivo* protein-DNA contact, Site I and II, located within the first 200 base pairs upstream of the transcription initiation site (157). The Site II binding factors described previously (202) consist of the proliferation-specific, cell cycle regulated transcription factor HiNF-D and a TATA box binding factor. The results presented in this report characterize the interactions at Site I and contribute to the structural understanding of this histone gene proximal promoter.

Site I (-124 to -90 bp) is a bipartite element consisting of the proximal and distal binding sites HiNF-C and HiNF-E, respectively. The HiNF-E binding site contains a sequence with strong similarity to the ATF consensus recognition element, 5'-GTGACGT$^{A}_{C}{}^{A}_{G}$-3' (127), and mutations of this consensus in the histone promoter reduce the level of transcription approximately 50%. The ATF sequence has been identified in several cellular and adenovirus gene promoters and is referred to as both ATF and CREB (cAMP responsive element) (127,146). A number of protein-DNA binding activities have been described which interact with this sequence and the factors have been implicated in the induction of transcription by E1A (119), cAMP (145) and Ca^{2+}

(179). The isolation of several cDNA clones corresponding to these factors has confirmed that ATF is a large transcription factor family (69,82,94,130). The unifying characteristics of the family are the requirement for binding of at least the pentamer core, 5'-TGACG-3', and the presence of a leucine zipper motif assumed to be important for dimerization and DNA binding (216).

ATF transcription factors have shown substantial heterogeneity in their molecular mass. The originally characterized ATF and CREB factors were of 43 to 47 kDa (97,127,145); however, recently additional mammalian and rodent factors binding to the ATF consensus have been identified with molecular mass of 54.5 (130), 65 (43), 72 (43), and 120 (4) kDa. Molecular mass determination by UV cross linking measured HiNF-E to be 84 kDa. It is unlikely that this represents a dimer of the 43 to 47 kDa forms since similar UV cross linking studies with the adenovirus E4 promoter detected an ATF factor of 45 kDa (127). In addition, we purified HiNF-E by DNA affinity chromatography and have shown by western blot analysis that HiNF-E reacts to some extent with antibodies prepared to ATF-43/47 (a gift of Dr. Michael Green). Thus, we suggest that HiNF-E is a novel member of the ATF transcription factor family and term it ATF-84.

It is intriguing that the H4 histone promoter preferentially binds ATF-84 and not the previously characterized ATF factors even though extracts from the same cell type as used in these experiments have been shown to contain 43 - 47 and 120 kDa ATF factors (4,83). Andrisani *et al.* (4) initially co-purified an 80 kDa protein along with the 120 kDa ATF factor but were unable to detect sequence specific binding to the ATF consensus site. Thus, the flanking sequences of the HiNF-E binding site may define the affinity of this site for the ATF-84 factor. In addition, other factors present in the histone H4 proximal promoter may assist in the selective binding of ATF-84.

As yet it is not clear why ATF-84, but not other ATF factors is required in this H4 histone promoter. Notably, the ST519 human H3 histone gene promoter also contains an ATF consensus sequence element that is involved in protein-DNA interactions both *in vivo* and *in vitro* (156 and A. van Wijnen, personal communication). No other histone gene has been described to bind ATF, however, a search of the published histone gene sequences reveals that a number of these promoters contain potential ATF binding sites. It will be important to determine if the ST519 H3 ATF factor and other histone ATF binding sites selectively interact with

ATF-84 and thus provide a point to coordinately regulate the histone gene family.

The HiNF-E/ATF-84 interaction is stabilized by the binding of HiNF-C adjacent to it. HiNF-C binding characteristics are indistinguishable from Sp1 and previous studies in our laboratory have shown HiNF-C binding to be dependent on metal ions, in particular Zn^{2+} (202). The human Sp1 transcription factor also displays a metal ion dependence for DNA binding (106). The conserved $[cysteine]_2$-$[histidine]_2$ repeated motif contained in Sp1 is assumed to complex with Zn^{2+} to form a zinc finger DNA binding structure (reviewed in (109). Furthermore, HiNF-C and Sp1 behave similarly during wheat germ agglutinin affinity chromatography. Wheat germ agglutinin binds with high affinity to proteins such as Sp1 which contain *N*-acetylglucosamine moieties and can be used to selectively purify for this proteins (100). Thus HiNF-C and Sp1 are modified similarly with *N*-acetylglucosamine moieties providing further evidence consistent with HiNF-C being equivalent to Sp1. Sp1 recognition sites were first described in the SV40 promoter and since have been identified in many viral and cellular promoters (107). However, no multiplicity of the Sp1 factor has been described. Thus, these findings strongly suggest that HiNF-C

and Sp1 are identical. Furthermore, mutation of the binding site resulted in a 50% drop in the transcription efficiency, suggesting that HiNF-C/Sp1 is the protein component mediating the stimulation of transcription by the proximal half of Site I.

In vivo Site I is a key element in the expression of this H4 histone gene, up-regulating transcription approximately 7 fold. Moreover, a promoter consisting of only Site I and upstream sequences is capable of supporting significant levels of transcription *in vivo*, in the absence of a TATA box and the HiNF-D binding site (112). Thus Site I is sufficient for directing the formation of a pre-initiation complex, while the TATA box sequence in Site II may only be required for site specific initiation. The HiNF-D interaction which is cell cycle regulated may not be necessary for the basal level of transcription activity. Interestingly, recent reports have demonstrated that ATF interacts directly with TFIID, the TATA box binding factor, and facilitates the formation of the pre-initiation complex (81,96). This may be the function of ATF-84 in this H4 histone gene promoter. Furthermore, ATF-84 may function to position TFIID in the absence of the TATA box sequence as described for the Site II deleted promoter construct. This may be a mechanism by which naturally occurring RNA

polymerase II, TATA-less promoters assemble the pre-initiation complex.

In conclusion, the proximal promoter of the FO108 human H4 histone gene displays a complex architecture in which the factors stimulate transcription both directly by interaction with the basic initiation factors and indirectly by interacting with other transcription factors to stabilize their association with the promoter.

CHAPTER 3

DISTALLY LOCATED ACTIVATORS AND REPRESSORS MODULATE HUMAN H4 HISTONE GENE TRANSCRIPTION - A STRUCTURAL AND FUNCTIONAL STUDY

Abstract

The extent to which distal promoter sequences contribute to H4 histone gene transcription was examined and the protein-DNA interactions involved were identified. *In vivo* and cell free transcription analysis of a series of 5' deletion mutants of the FO108 human H4 histone gene promoter identified two strong activator elements located at -418 to -213 bp (near-distal activator) and -730 to -589 bp (far-distal activator) as well as two potential repressor elements whose detection was assay dependent. The far-distal activator stimulates transcription approximately ten fold and is involved in the formation of three protein-DNA interactions, H4UA-2, -3, and -4 (Histone H4 Upstream Activator). H4UA-2 requires an intact ATF consensus recognition sequence for binding and can be competed by other unrelated ATF binding sites. Site directed base substitution of the H4UA-2 binding site resulted in a two fold drop in the transcription initiation rate. Thus, these data strongly suggest that H4UA-2 is a member of the ATF transcription factor family. Molecular mass analysis has indicated that the H4UA-2 recognition sequence

preferentially binds an 84 kDa factor but has only limited affinity for the originally characterized 43-47 kDa ATF factors. An ATF-84 factor has been previously shown to interact with the proximal promoter of this H4 histone gene, suggesting the possibility that a single member of the ATF transcription factor family might be associated with the histone gene promoter. H4UA-3 and H4UA-4 interact adjacent to H4UA-2 at novel recognition sequences. The near-distal activator stimulated transcription greater than two fold. Protein-DNA interaction mapping of this region identified a single complex, H4UA-1, with a recognition sequence characterized by similarity to both the NF1/CTF recognition consensus and the thyroid hormone response element. These studies demonstrate a complex modular structure of the human H4 histone gene promoter, consistent with the requirement of histone expression to be responsive to alterations in cell growth.

Introduction

Histone genes encode a set of structural proteins stringently required for the packaging of newly replicated DNA into chromatin (31,98). The human histone genes constitute a heterogenous, multi-copy family arranged as clusters of core histone genes (H2A, H2B, H3, H4) or core with H1 histone genes located on at least three different chromosomes (29,76,90,125,133,181,200,217). Expression of the major class of histone genes, replication dependent genes, is tightly coupled to DNA synthesis, exhibiting a 20 - 100 fold increase in expression during S-phase (178,195). The stimulation of replication dependent expression is mediated at both the post-transcriptional and transcriptional levels. The post-transcriptional regulation is accounted for by an increase in histone mRNA stability during DNA replication and a selective and rapid degradation of the mRNA at the completion of S phase or following inhibition of DNA synthesis (6,14,48,51,89,160). The transcriptional component of the cell-cycle regulation is a 2 - 5 fold induction of transcription above the constitutive basal level during the initial period of DNA synthesis (14,89,160). This stimulation of transcription is accompanied by alterations in the chromatin structure and nucleosomal organization in the promoter region (37,147).

Promoter sequence elements required for the accurate initiation of transcription have been identified within the first 213 base pairs of the replication dependent FO108 human H4 histone gene (157,202),and Chapter 2 of this thesis). The proximal promoter contains two regions of *in vivo* protein-DNA contact, Site I and II (157). Site II, which interacts *in vitro* with factor HiNF-D, consists of the TATA box and the highly conserved histone specific element (202). Occupancy of this site *in vivo* and *in vitro* has been correlated with rendering the gene transcribable in proliferating cells (193). Site I is positioned 26 base pairs upstream of Site II and is required to achieve the high level of transcription detected from this histone gene (112), and Chapter 2 of this thesis). Site I consists of two *in vitro* protein-DNA binding sites, HiNF-E and HiNF-C (202), and Chapter 2 of this thesis). HiNF-E contains an ATF transcription factor recognition sequence and binds a novel member of the ATF family termed ATF-84. HiNF-C binds an Sp1 transcription factor whose binding activity stabilizes the interaction of ATF-84 adjacent to it.

Distal promoter elements have also been suggested to play a role in transcription of this histone gene. Kroeger *et al* demonstrated that the addition of sequences from -213 to -418 base pairs to the proximal promoter

resulted in a 10 fold stimulation of transcription (112). Additionally, a sequence element located 6.5 kB upstream of the initiation site has been proposed to function as an enhancer (91). However, we have a poor understanding of the role these or other distally located elements might play in regulating histone gene transcription. In this study we have examined the FO108 human H4 histone gene promoter and defined a series of activator and repressor sequence elements located between -231 and -1038 base pairs. Furthermore, we have identified and partially characterized the trans-acting protein factors responsible for mediating the activator effects.

Materials and Methods

Promoter constructs. The FO108 human H4 histone promoter is numbered from the transcription initiation site. Promoter deletion constructs (Figure 3-1) were prepared according to standard cloning procedures. FO005 and FO108A have been described previously (112). FO002 was constructed from the original λHHG41 phage clone isolated by Sierra *et al.* (181) and FO007 is a sub-clone of FO002 derived by partial EcoRI digestion. FO002D1 was constructed by partial DraI digestion of the PstI fragment of FO002 followed by isolation of the histone gene containing 2.16 kB DraI/PstI fragment and cloning into a pUC19 vector. FO002E9 was constructed similarly by partial Eco0109 digestion of the XbaI fragment of FO002 followed by isolation of the histone gene containing 1.63 kB Eco0109/XbaI fragment and cloning into a pUC19 vector. FO001 is an internal deletion of the EcoRI fragment (-213 to -589 bp) and was generated by fusing the 2.7 kB EcoRI fragment (-3300 to -589 bp) of FO003 (112) to the 5' EcoRI site (-213 bp) of FO108A in the original orientation. FO108X is a 3' deletion of FO108A constructed by removal of the 770 bp XbaI/HindIII fragment by digestion with the indicated restriction enzymes and circularization.

Construction of stable cell lines and short-term transient expression. *In vivo* expression studies were performed by Dr. Paul Kroeger. Stable cell lines were established by cotransfection of C127 mouse cells with 10 μg of the indicated histone construct and 10 μg of pSV2neo (190) by the $CaPO_4$ precipitate/glycerol shock method (70,72) followed by selection for neomycin resistance with Geneticin (G418, Gibco) (190). The integrated copy number of the human histone gene construct was measured by preparation of genomic DNA followed by Southern Blot analysis (189). Densitometric quantitation of the human histone gene was normalized to the mouse 18S ribosomal gene. Transfections for short-term transient expression analysis were performed as described above except that the pSV2neo plasmid was not included. The cells were harvested 36 - 48 hours after glycerol shock. Total cellular RNA was prepared from both the stable cell lines and the short-term transient transfection as previously described (19,159) and assayed by S1 nuclease protection as described in Chapter 2.

Preparation of nuclear extracts. Nuclear extracts were prepared from $1x10^9$ exponentially growing HeLa S3 cells as described in Chapter 2.

In vitro transcription. Transcription from covalently closed circular template DNA was performed as described in Chapter 2.

Gel mobility shift analysis. The gel mobility shift analysis was performed as described in Chapter 2 except 3 μg of poly(dI-dC)·(dI-dC) was used as the non-specific competitor and the radioactive probes spanned from -418 to -213 bp (near-distal activator) and -730 to -589 bp (far-distal activator). Synthetic oligonucleotides were all used at 500 fold molar excess. The ATF-84 oligonucleotide has been described in Chapter 2 and is referred to therein as the distal Site I oligonucleotide. The consensus Sp1 dimer recognition site oligonucleotide has been described previously (206). The H4UA-2 oligonucleotide is:

5'-GATCTGGGATTCGCTGACGTCCATGAGAAAG-3'
3'-ACCCTAAGCGACTGCAGGTACTCTTTCCTAG-5'

Methylation interference and methylation protection. Methylation interference was performed essentially as described by Staudt *et al.* (192) except that the entire preparative gel mobility shift gel was electrotransferred to NA45 DEAE membrane (Schleicher & Schuell) as described by Singh *et al.* (183). The probes used were as described for the gel mobility shift assay and were partially methylated by a 5 minute 22^{0}C treatment with 1 μl of dimethylsulfate (DMS, Kodak) in a 200 μl reaction volume containing 50 mM

Na-cacodylate (pH 8.0) and 1 mM EDTA. The reaction was stopped by the addition of 40 μl of DMS stop buffer [1.5 M Na-acetate (pH 7.0), 1 M β-mercaptoethanol], 10 μg yeast t-RNA, and 600 μl ethanol and immediately placing the reaction tube in a dry ice-ethanol bath. The DNA was pelleted, resuspended in 250 μl of 0.3 M Na-acetate and precipitated again with 3 volumes of ethanol. The precipitation was repeated for a third time and the DNA was resuspended and subjected to a standard gel mobility shift assay. After electrophoresis the entire gel was electrophoretically transferred to DEAE membrane (NA45, Schleicher & Schuell) at 200 mA for 2 hours at 4^0C. The blot was then autoradiographed and the regions corresponding to the free and bound DNA were isolated and the DNA eluted with 200 μl of a solution of 1.0 M NaCl, 0.1 mM EDTA, and 20 mM Tris-HCl (pH 8.0) at 68^0C for 45 minutes. The DNA was precipitated, resuspended in 100 μl of 10% piperidine and incubated for 30 minutes at 90^0C. The reaction was then lyophilized and serially resuspended in 50 and 25 μl H_2O and lyophilized to dryness each time. The samples were then resolved on an 8% polyacrylamide gel as described for the S1 protection analysis (Chapter 2). Methylation protection of the same probes as used in the methylation interference assay was performed as described previously (9). Binding reactions were as described for the DNase I protection and

followed by treatment of the reactions with 0.5% final DMS for 45 seconds at 22^{0}C. The reactions were stopped with 3.2 μl DMS stop buffer and 48 μl ethanol and immediately placed in a dry ice-ethanol bath. The DNA was precipitated twice followed by piperidine treatment and electrophoresis as described above.

Site-directed mutagenesis. Selected base substitutions were introduced into the promoter of the H4 histone gene by oligonucleotide directed mutagenesis without phenotypic selection, as described in Chapter 2. Uracil containing single strand M13 DNA carrying the 834 bp BamHI/SmaI promoter fragment from FO002 was prepared by amplification in the *E. coli* strain CJ236 (*dut* $^-$*ung* $^-$F') (a gift of Dr. Barbara Bachmann, *E. coli* Genetic Stock Center, Yale University). Mutations were introduced in complementary synthetic oligonucleotides containing the base substitutions in the H4UA-2 binding site:

5'-GGGATTCGCTGAtcTCCATGAG-3'

The mutations were recovered by transfection into wild type *E. coli* strain XL1-Blue (Stratagene) and were confirmed by enzymatic sequencing (Sequenase^{TM}, US Biochemical). The mutated 834 bp BamHI/SmaI promoter containing fragment was cloned back into FO002 to generate FO002ΔNMP.

DNase I protection. DNase I footprinting was performed as described in Chapter 2.

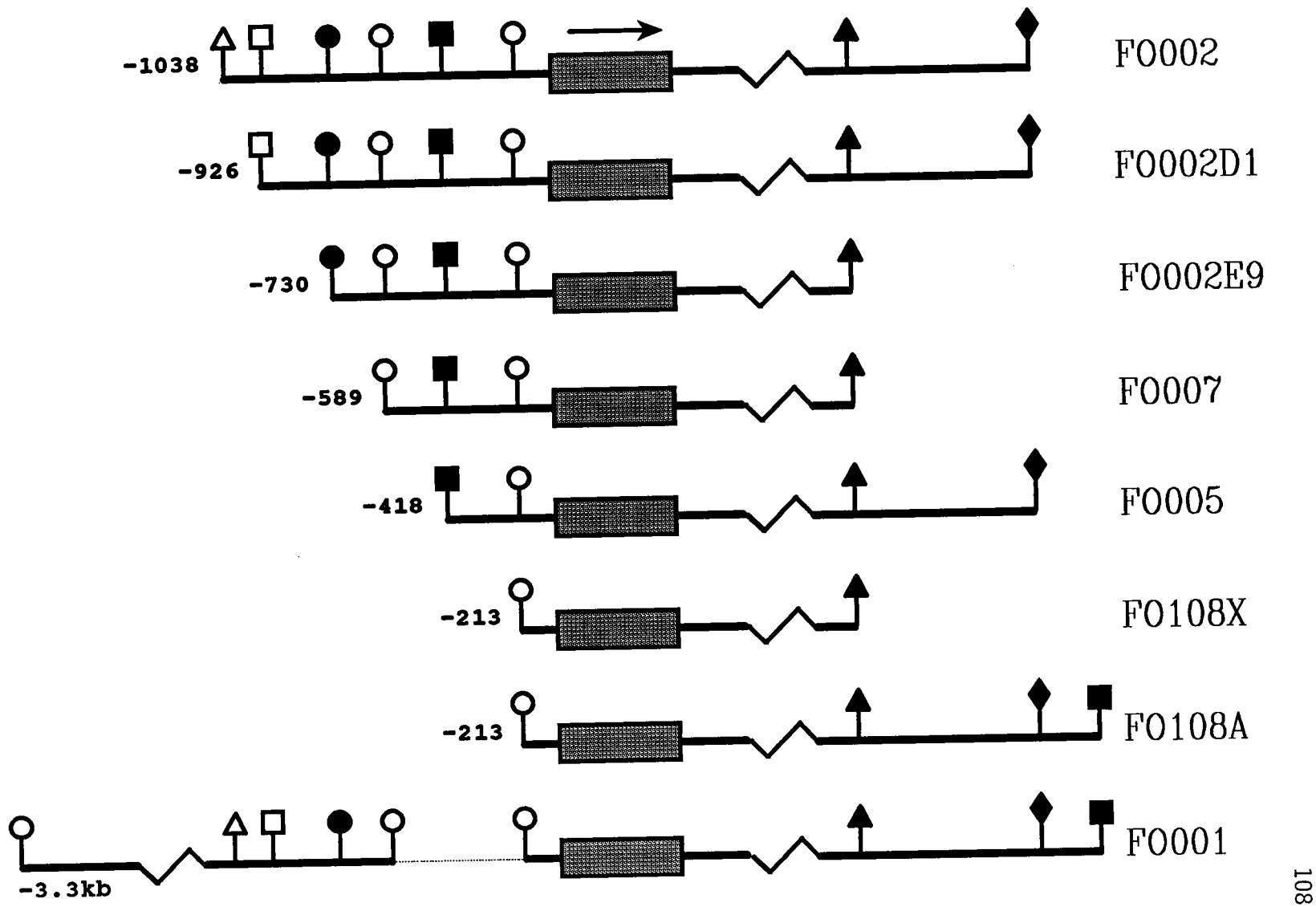
UV cross-linking of nuclear proteins. Molecular mass determination by UV cross-linking was performed as described in Chapter 2 except the probe contained the entire far-distal activator region (-730 to -589 bp).

Results

The human H4 histone gene FO108 contains at least three transcriptional regulatory elements in the proximal promoter region which have been described previously (112,157,202),and Chapter 2 of this thesis), and additional elements located further upstream in the distal promoter (112). To address the function and structure of the distal promoter we have scanned the region with a detailed set of 5' deletion mutants for activators or repressors of transcription and subsequently have investigated the protein-DNA interactions involved in the activator domains.

Transcription analysis of 5' deletion mutants. We created a series of six 5' promoter deletion constructs encompassing the region from -213 bp to -1038 bp which divided the distal promoter into 150 - 200 base pair segments (Figure 3-1). Each construct was subsequently assayed for efficiency of transcription both *in vitro* and *in vivo*. Transcription *in vitro* was performed in a cell free transcription system utilizing supercoiled DNA templates and HeLa S3 nuclear extracts which was shown previously to support accurate transcription initiation from this histone H4 proximal promoter (-213 bp) (38,190). *In vivo* expression was measured after transfection of the constructs into C127

Figure 3-1. Schematic representation of the human H4 histone gene distal promoter 5' deletion mutants. Construction of the deletions is detailed in the Materials and Methods. Shaded box represents the location of the histone gene transcribed region and arrow indicates the direction of transcription. Symbols for restriction endonuclease sites: ▵ ,BamHI; ▫ ,DraI; ● ,Eco0109; ○ ,EcoRI; ■ ,HindIII; ▲ ,XbaI; ♦ ,PstI.


-1038
F0002
-926
F0002D1
-730
F0002E9
-589
F0007
-418
F0005
-213
F0108X
-213
F0108A
-3.3kb
F0001

or LTK$^-$ mouse cells as either short-term transient expression or as stably integrated cell lines. The resultant transcripts from both the *in vitro* and *in vivo* experiments were assayed by S1 nuclease protection.

In vivo transcription analysis of the region between -418 bp and -213 bp with both transient expression assays and cell lines containing stably integrated constructs identified a positive element within these 205 nucleotides. Addition of the -418 to -213 bp sequences (FO005) to the proximal promoter (FO108X or FO108A) resulted in a 2.5 - 4 fold higher expression in stable cell lines, while addition of 3' flanking sequences had no effect on expression (FO108X vs FO108A) (Table 3-1). In all cases results for the stable cell lines were corrected for the number of copies per cell. Furthermore, transient expression experiments in both C127 and LTK$^-$ mouse cells showed that addition of the -418 to -213 sequences resulted in expression above proximal promoter levels (FO108A) (Figure 3-2, Table 3-1). The stimulatory nature of these sequences was further confirmed by cell free transcription analysis. Addition of this element (FO005 vs FO108X) increased the rate of cell free transcription initiation two fold above that of the proximal promoter (Figure 3-3 and Table 3-1), although data from the FO005 construct were variable and

Table 3-1. Transcriptional activity of the human H4 histone gene distal promoter deletion constructs. Values represent the average of at least five independent experiments. Levels of transcription in the stable cell lines has been normalized for the copy number. ND - no data.

TABLE 3-1.

CONSTRUCT	IN VITRO	IN VIVO	
		TRANSIENT	CELL LINE
F0002	71.4 ± 44.5	52.1 ± 13.5	4.5 ± 5.3
F0002D1	69.0 ± 16.9	31.8 ± 16.2	ND
F0002E9	54.4 ± 31.5	100	ND
F0007	5.3 ± 4.9	42.8 ± 20.7	ND
F0005	44.3 ± 47.8	41.1 ± 22.3	20.1 ± 14.0
F0108X	23.7 ± 13.5	ND	4.9 ± 4.2
F0108A	ND	2.6 ± 1.1	7.9 ± 6.1
F0001	81.7 ± 36.7	0.7 ± 0.3	ND

Figure 3-2. Expression of the histone promoter deletion constructs in transiently transfected C127 and LTK$^-$ mouse cells. Total cellular RNA was isolated and assayed by S1 nuclease protection. Arrows indicate the location of the accurately initiated transcripts of the transfected human H4 histone gene and the endogenous mouse H4 histone gene. The construct transfected is indicated above each lane. HeLa, control human RNA; C127 and LTK$^-$ control untransfected mouse cell RNA.

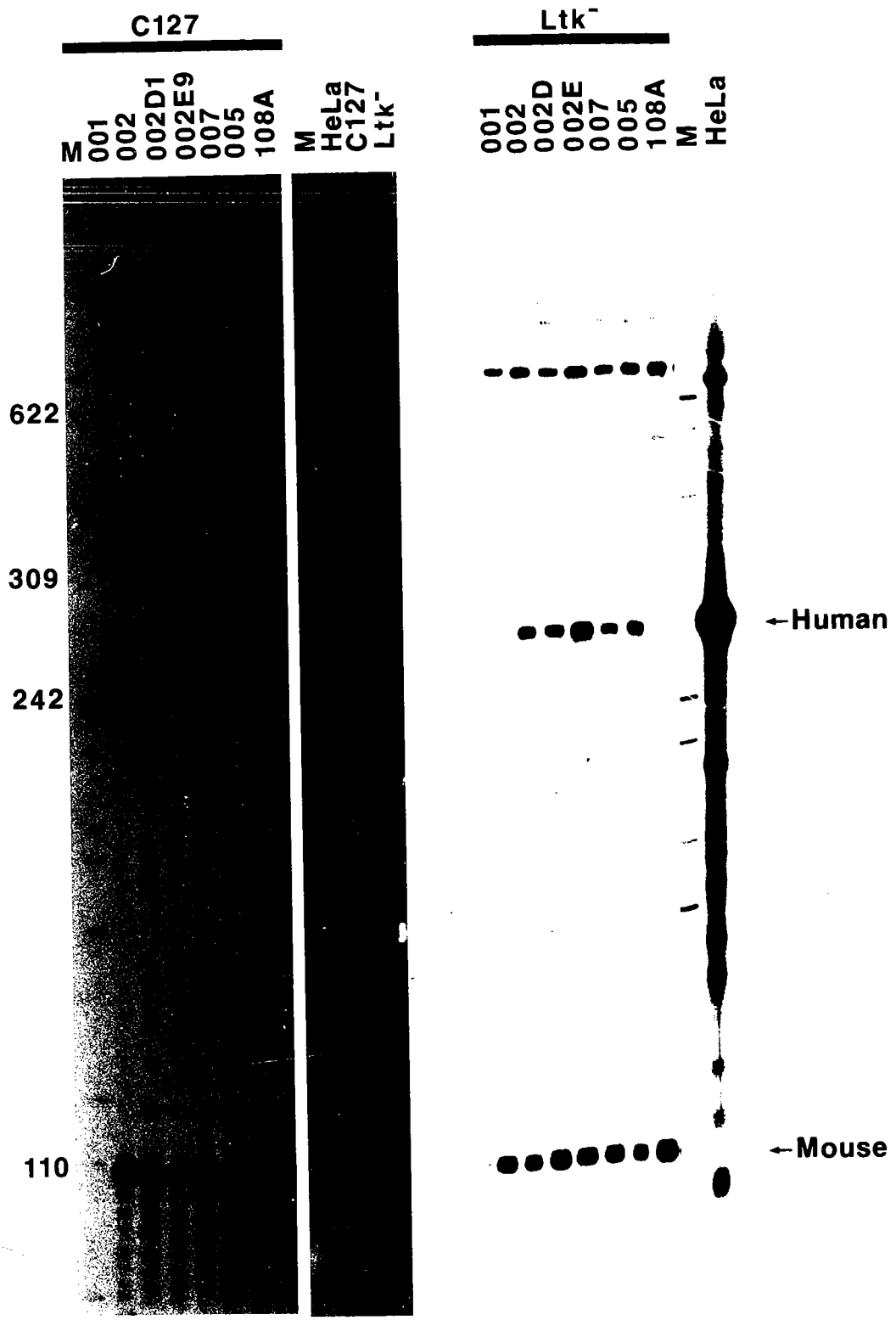
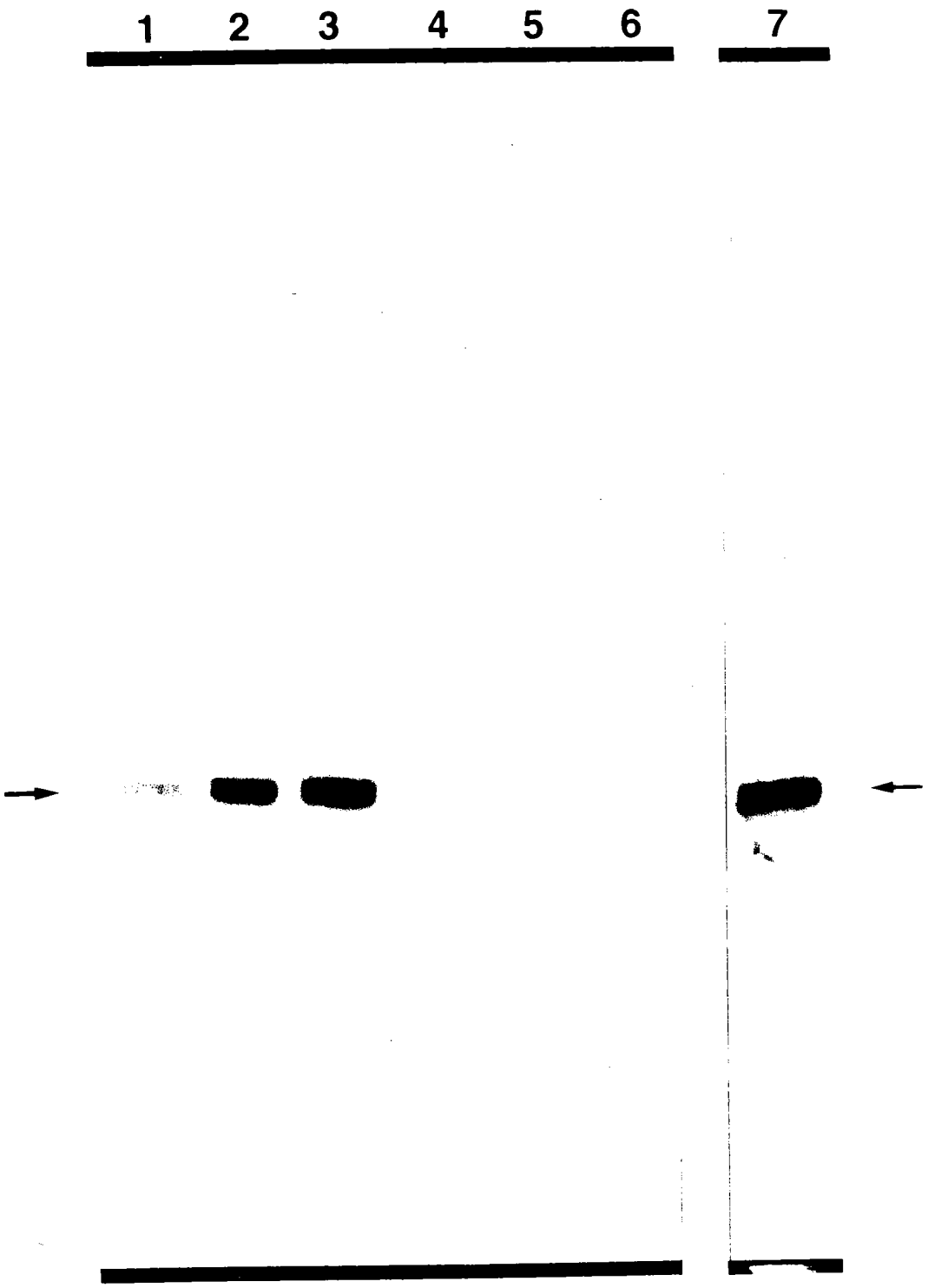

C127
Ltk⁻
M
001
002
002D1
002E9
007
005
108A
M
HeLa
C127
Ltk⁻
001
002
002D
002E
007
005
108A
M
HeLa
622
309
242
110
←Human
←Mouse

Figure 3-3. Cell free transcription analysis of the H4 histone promoter deletions. Supercoiled plasmids containing the series of distal promoter deletion mutants was transcribed in a HeLa nuclear extract system and transcripts were assayed by S1 nuclease protection. Arrow indicates the accurately initiated transcripts. Lanes: 1, FO002; 2, FO002D1; 3, FO002E9; 4, FO007; 5, FO005; 6, FO108X; 7, FO001.


1
2
3
4
5
6
7

inconclusive after statistical analysis. The combination of each of these expression studies clearly indicates that this region has a stimulatory effect on transcription and it has been termed the near-distal activator.

The next deletion mutant, FO007, includes an additional 171 base pairs up to -589 bp. This region represses transcription *in vitro* 4.5 fold below the proximal promoter rate (FO108X) but interestingly does not have a significant effect *in vivo* (Figures 3-2 and 3-3, Table 3-1). The difference may imply species specificity for the repressor since the *in vivo* experiments are performed in mouse cells while the *in vitro* experiments use nuclear extracts from human cells. It is also possible that differences in the structure of the DNA template or association of the DNA with structural elements *in vivo* may affect the interaction and availability of specific factors which contribute to the repressor activity.

A second activator region was identified between -730 bp (FO002E9) and -589 bp (FO007). A comparison of the cell free transcription initiation rate reveals FO002E9 is greater than 10 fold more active than FO007 (Figure 3-3, Table 3-1). This is the strongest activator detected in the histone promoter and has been termed the far-distal activator. This 141 base pair region is able to overcome

fully the repression of FO007 *in vitro* and stimulates transcription 2.3 fold above the proximal promoter (FO108X). Two additional constructs, FO002D1 (-926 bp) and FO002 (-1038 bp), are indistinguishable from FO002E9 in transcription efficiency. The function of the far-distal activator was further demonstrated by construction of an internal deletion which positions the activator adjacent to the proximal promoter. This construct, FO001, displayed the highest level of transcription *in vitro* of any construct tested, 3.5 fold higher than the proximal promoter alone.

The far-distal activator is also highly active *in vivo*. Transient expression of FO002E9 (-930 bp) was 2.3 fold higher than the next smaller construct, FO007 (-589 bp) (Figure 3-2). The absence of a repressor activity *in vivo* in FO007 allows for a cumulative effect of the near-distal activator and the far-distal activator. This is revealed by an approximately 40 fold stimulation of expression of FO002E9 as compared to FO108A (Table 3-1). Interestingly, additional sequences contained in FO002D1 (-926 bp), which had no effect *in vitro*, were able to repress transcription substantially *in vivo*. This result is supported by the expression of the internal deletion construct, FO001, which is missing the near-distal activator (-418 to -213 bp) and thus has the far-distal activator and the *in vivo* repressor

positioned upstream of the proximal promoter; the result is a poorly expressing construct. Furthermore, stable cell lines of FO002 containing the entire promoter up to -1038 bp expressed at a lower level than FO005 (-418 bp), indicating that the activity *in vivo* of the far-distal activator can be masked by sequences upstream from it or perhaps is functionally linked to the adjacent sequences.

Multiple protein-DNA interactions in the far-distal activator element. To address the molecular mechanisms mediating the transcriptional contribution of the far-distal activator element (-730 to -589 bp), we systematically investigated the number and characteristics of the protein-DNA interactions involved in this region. Initial characterization by the gel mobility shift assay identified two strong interactions, Histone H4 Upstream Activator - 2 (H4UA-2) and H4UA-3, and one weak interaction H4UA-4 (Figure 3-4). H4UA-2 and H4UA-3 were equally represented at low nuclear extract concentrations, 50ng/μl, while H4UA-4 was detectable only at high concentrations, >200 ng/μl. The binding site regions were localized within the far-distal activator by bi-directional deletion analysis combined with the gel mobility shift assay (Figure 3-5A and 3-5B). In this technique the full length fragment is radiolabeled at one

Figure 3-4. Gel mobility shift analysis of the far-distal activator region detects three protein-DNA interactions, H4UA-2, -3, -4. A radiolabeled BanII/EcoRI probe (-730 to -589 bp) was incubated with increasing concentrations (0.5 to 8 μg) of HeLa nuclear extract and resolved on a polyacrylamide gel as described previously (192).

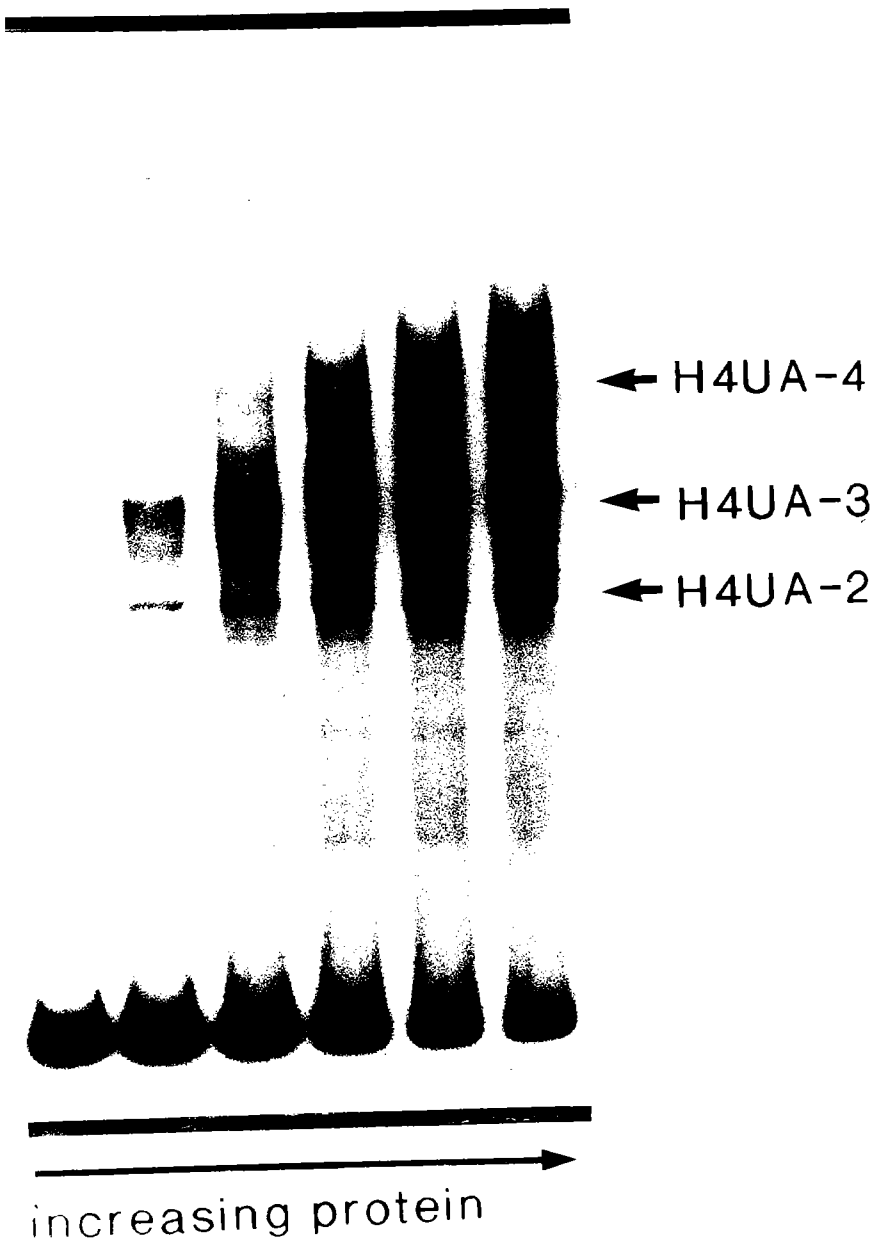
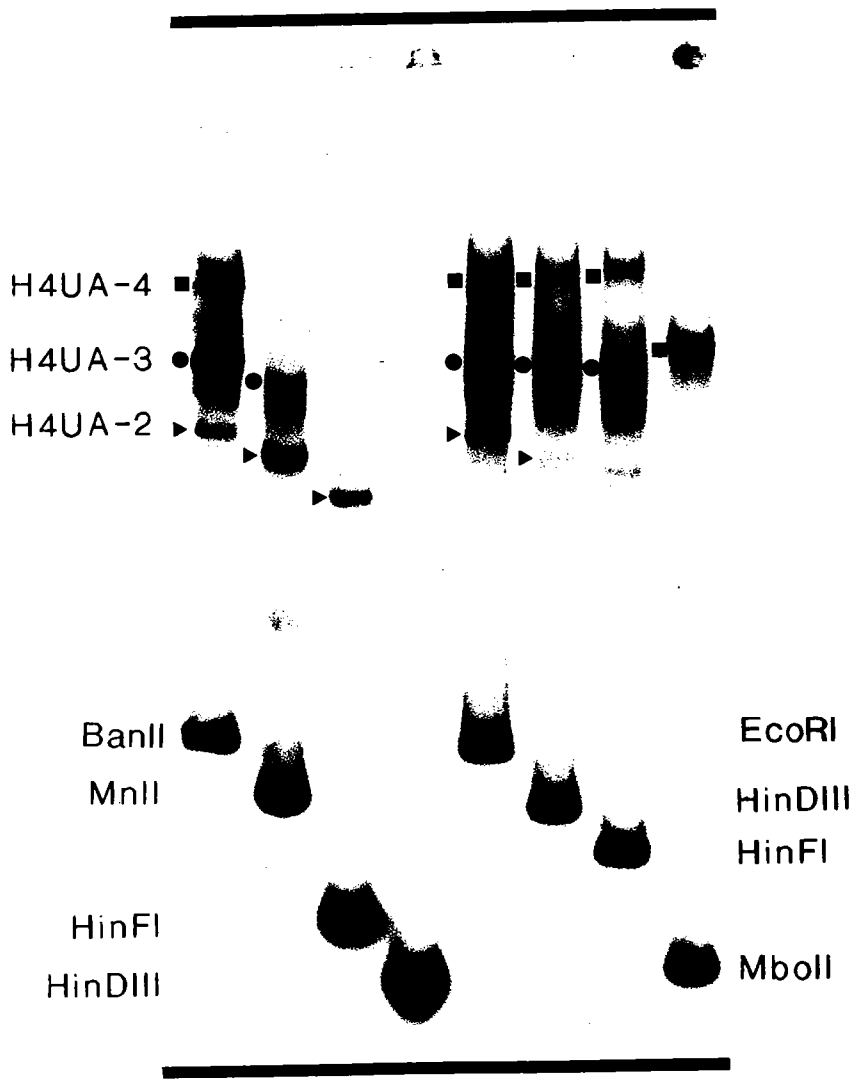
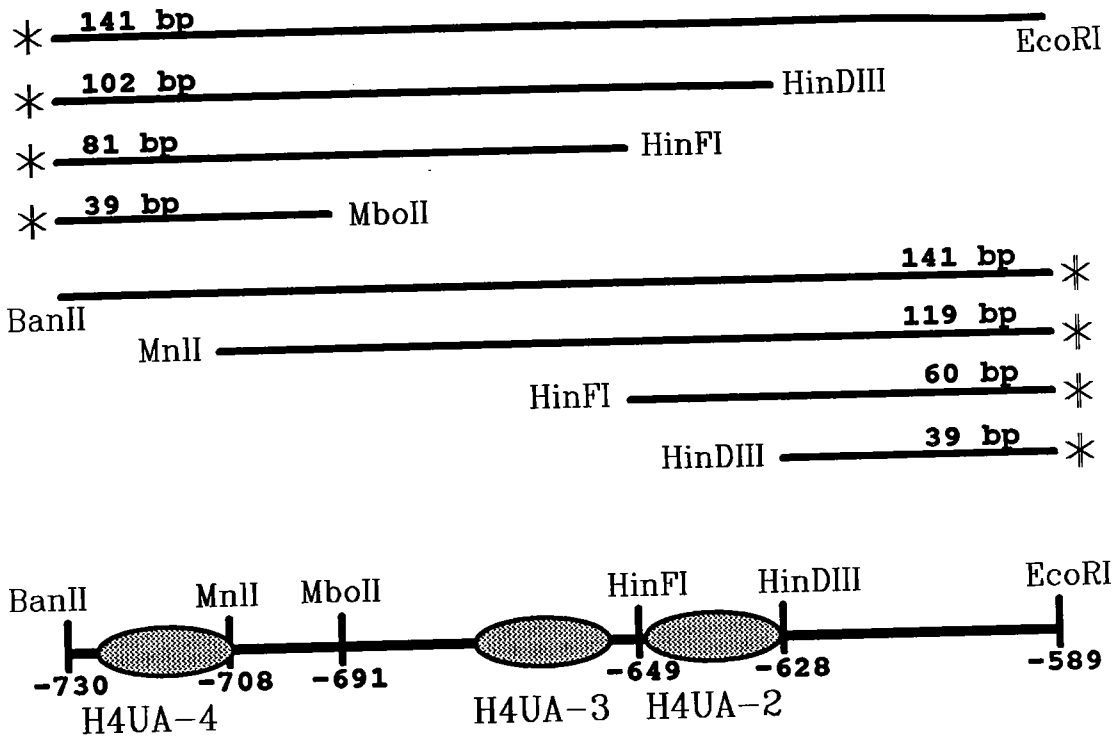

H4UA-4
H4UA-3
H4UA-2
increasing protein

Figure 3-5. Bi-directional deletion analysis of the far-distal activator region defines the location of H4UA-2, -3, -4. A) Autoradiograph of gel mobility shift assay performed with a series of progressively shortened probes. Probes used are indicated at the side and the symbols used are: ▸ ,H4UA-2; ● ,H4UA-3; ■ ,H4UA-4. B) Schematic representation of the bi-directional deletion probes and results. Hatched ovals indicate approximate positions of the protein-DNA interactions.


H4UA-4
H4UA-3
H4UA-2
BanII
MnlI
HinFI
HinDIII
EcoRI
HinDIII
HinFI
MboII


141 bp
EcoRI
102 bp
HinDIII
81 bp
HinFI
39 bp
MboII
141 bp
BanII
119 bp
MnlI
60 bp
HinFI
39 bp
HinDIII
BanII
MnlI
MboII
HinFI
HinDIII
EcoRI
-730
-708
-691
-649
-628
-589
H4UA-4
H4UA-3
H4UA-2

end and then cleaved with the appropriate restriction enzymes to produce a series of progressively shorter radiolabeled fragments. These fragments are subsequently assayed for protein binding by altered electrophoretic mobility. Deletions are generated from both ends to define the maximal 5' and 3' boundaries of the interaction sites. H4UA-2 binding activity was maintained as the probe was deleted from the 5' end (BanII, -730) down to the HinfI restriction site (-649 bp). Further deletion from the 5' end (HindIII) abolished H4UA-2 binding. Deletion from the 3' end of the probe to the HindIII restriction site (-628 bp) resulted in a 90% loss of H4UA-2 binding activity. Additional 3' deletions completely abolished binding. These results define the maximal boundaries of H4UA-2 to between -649 bp (HinfI) and -628 bp (HindIII). Because cleavage at the HindIII restriction site dramatically destabilized the interaction without abolishing it, the H4UA-2 recognition sequence is likely to be just upstream of this site.

H4UA-2 binding requires an intact ATF recognition site.

The H4UA-2 binding site was confirmed and refined by determination of the methylation interference fingerprint. In this technique the radiolabeled DNA fragment was treated with DMS prior to the formation of the protein-DNA complex. The free probe and H4UA-1 bound probe were separated by the

gel mobility shift assay and isolated. After treatment according to the Maxam and Gilbert guanine sequencing protocol (134) the free and bound DNA are resolved on a 6% denaturing polyacrylamide gel. The absence of a band in the bound lane indicates methylation at this guanine residue was sufficient to block formation of the protein-DNA interaction. A comparison of the interference patterns of the free probe and the H4UA-2 bound probe reveals six guanine contact points (Figure 3-6). The lower strand contains three closely spaced contacts at -636, -637, and -640 bp, while the three upper strand contacts extend to both sides at -633, -639, and -642 bp. Sequence analysis of this domain identified a region of strong similarity to the consensus ATF recognition site, 5'-CGTCA-3' (Figure 3-7). The prototype ATF consensus is an overlapping indirect repeat of this pentamer and is proposed to be involved in binding of a dimerized ATF molecule. Each of the guanine residues in this consensus element was implicated as important for binding of H4UA-2 by the DMS fingerprint. Furthermore, this fingerprint matches with published interference patterns for known ATF binding sites (119).

To assess directly the relationship between H4UA-2 binding and the ATF consensus element, we carried out oligonucleotide competition analysis with an unrelated consensus ATF recognition site. Competition with a 31 bp

Figure 3-6. Methylation interference analysis of the H4UA-2 and H4UA-3 interactions. A partially methylated BanII/EcoRI (-730 to -589 bp) probe, uniquely labeled at the 5' end of the upper or lower strand, was used in the HeLa nuclear extract binding reactions. Free and H4UA-2 bound or H4UA-3 bound probe were separated by altered electrophoretic mobility, isolated and analyzed on 8% polyacrylamide sequencing gels after piperidine cleavage (134). Relative sequence position is indicated next to the C+T and G>A sequencing ladders. Symbols: ▸ ,H4UA-2; ● ,H4UA-3.

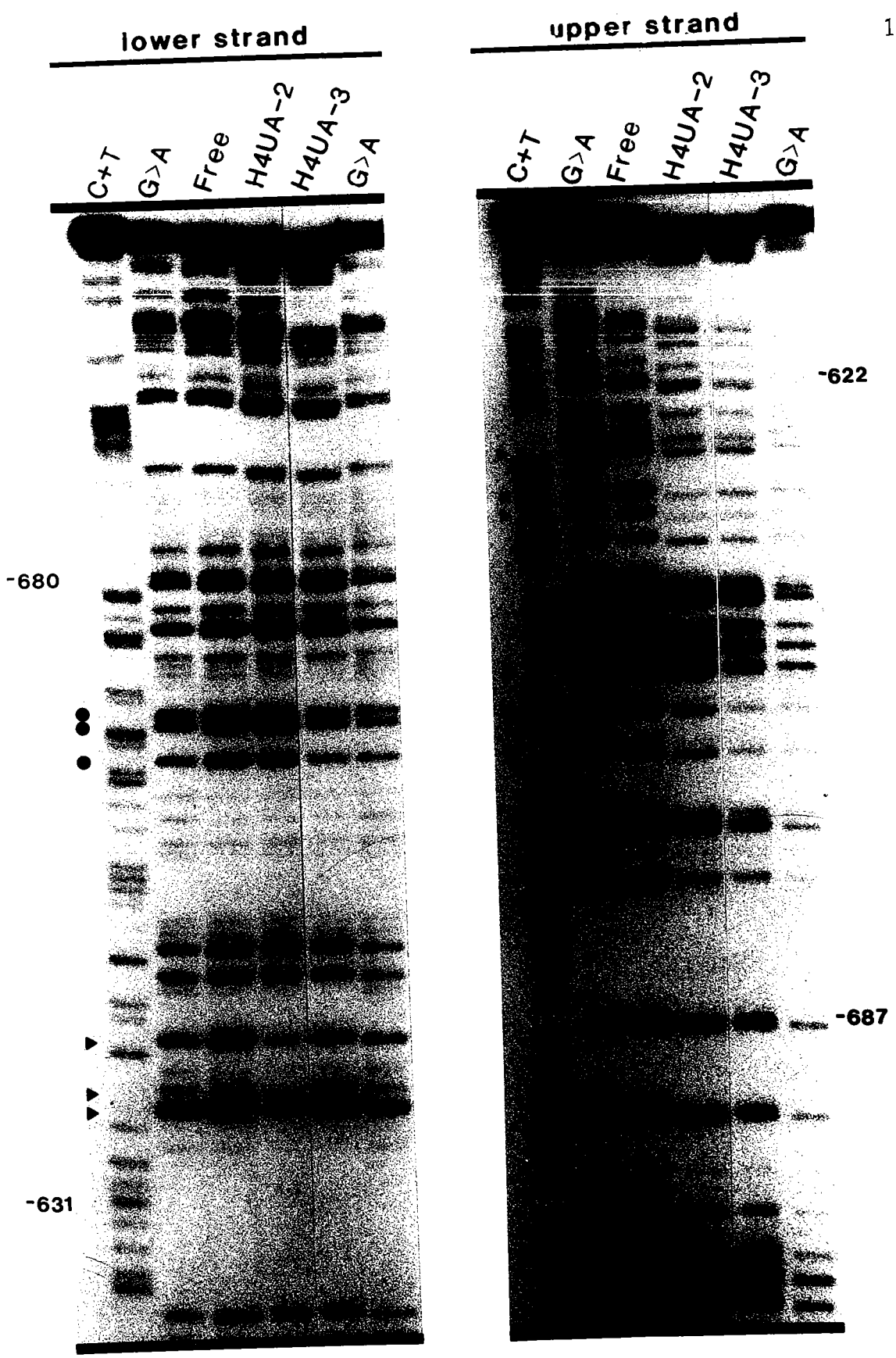
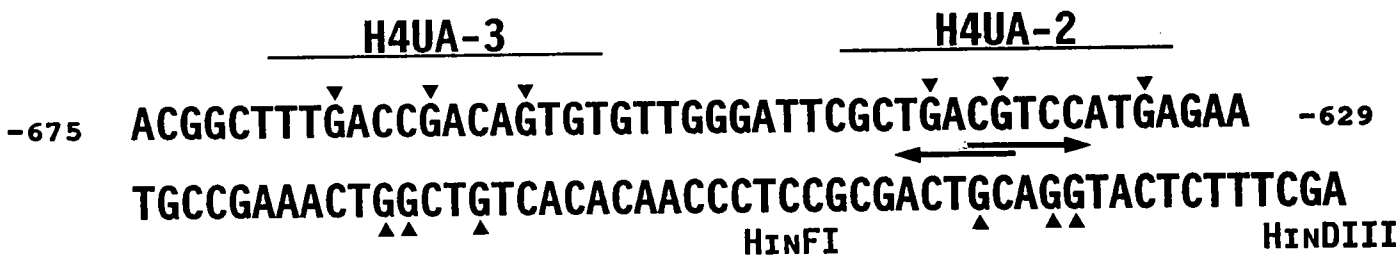

lower strand
upper strand
C+T
G>A
Free
H4UA-2
H4UA-3
G>A
C+T
G>A
Free
H4UA-2
H4UA-3
G>A
-680
-631
-622
-687

Figure 3-7. Sequence schematic of the far-distal activator region. Positions of H4UA-2 and H4UA-3 are over-lined, ▲ marks the guanine methylation interference contact points. The ATF consensus element is indicated by the overlapping arrows. The H4UA-2 binding site mutant is defined below.

FAR-DISTAL ACTIVATOR REGION ---



MUTANT

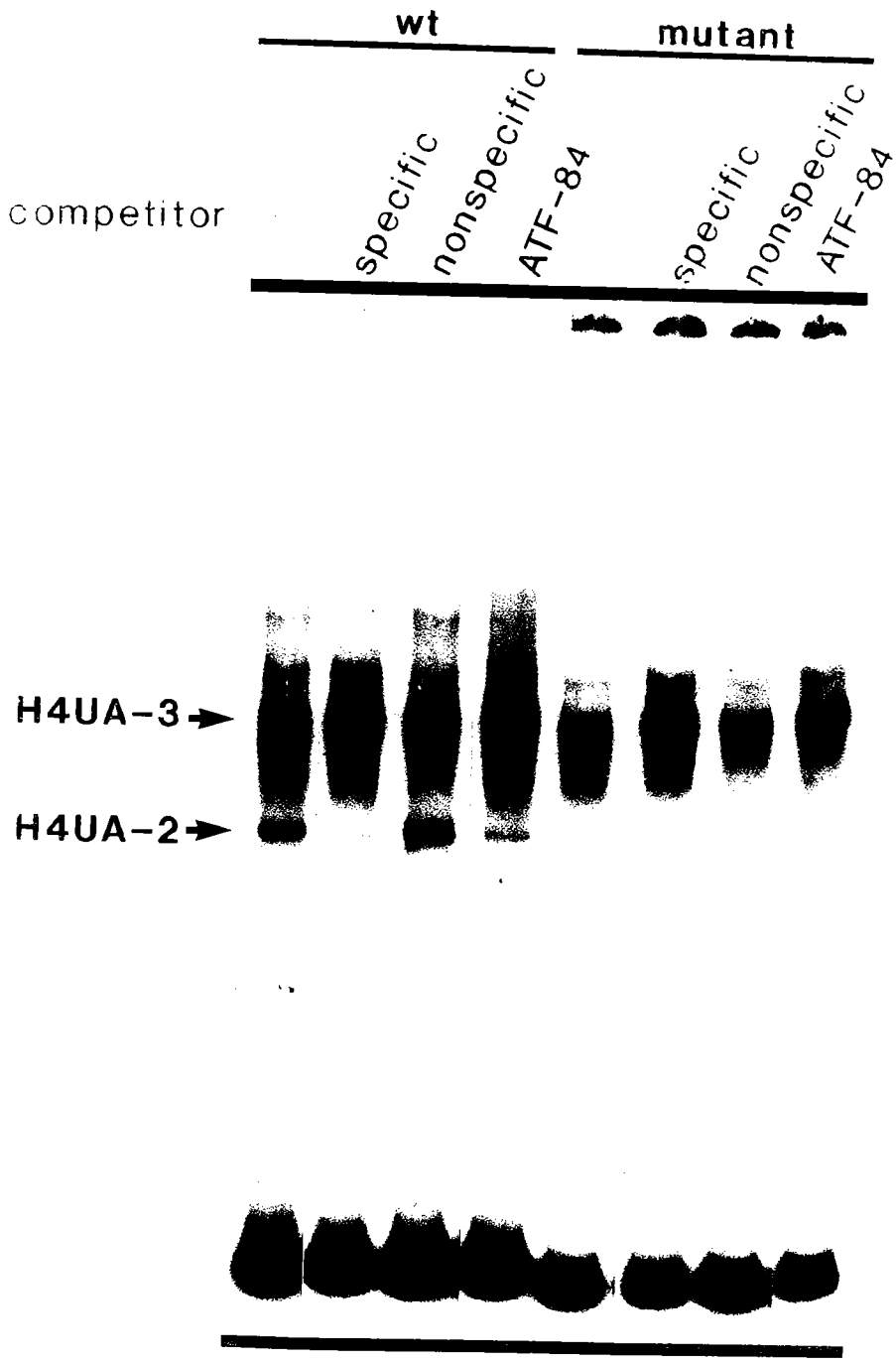
----------TGATCTCC-------

----------ACTAGAGG----------

synthetic oligomer spanning the H4UA-2 binding domain effectively competed H4UA-2 binding, as expected (Figure 3-8A), while H4UA-3 binding was unaffected. Sequences corresponding to a known ATF binding site (ATF-84) from the proximal promoter of this human H4 histone gene were also able to compete specifically for H4UA-2 binding although the ATF-84 oligomer is only related to the H4UA-2 binding domain in the 5 base pair core ATF recognition sequence. An unrelated sequence was ineffective as a competitor of either H4UA-2 or H4UA-3 binding activity. These results suggest that H4UA-2 binding is directly related to the presence of an ATF consensus binding site and demonstrate that H4UA-2 is capable of binding to other ATF consensus elements.

Further evidence supporting the relationship between H4UA-2 and ATF is derived from studies of site directed mutants of the binding domain. The ATF consensus sequence was disrupted by a two base pair substitution using mismatched oligonucleotide directed mutagenesis (Figure 3-7). The mutated sequence was subsequently employed in the gel mobility shift assay to determine the effect on the protein-DNA interactions (Figure 3-8A). The mutated probe maintained the H4UA-3 binding activity but was unable to form any detectable H4UA-2 interactions. These results demonstrate that H4UA-2 binding requires an intact ATF

Figure 3-8. Competition and mutation of the H4UA-2 binding site. A) Gel mobility shift analysis of the wild type (wt) and H4UA-2 binding site mutant (mutant) far-distal activator region probes. Competition was at 500 fold molar excess of the indicated oligonucleotide: specific, H4UA-2; nonspecific, H3-DII; ATF-84, oligonucleotide of the proximal ATF site in the histone promoter. B) Effect of the H4UA-2 mutation on cell free transcription from the histone promoter. Transcriptions were performed as in Figure 3-3. Lane 1, FO002; Lane 2, FO002ΔNMP. Arrowhead indicates the accurately initiated transcripts.


wt
mutant
competitor
specific
nonspecific
ATF-84
specific
nonspecific
ATF-84
H4UA-3
H4UA-2


1
2
cap site

consensus pentamer and confirms a close relationship between H4UA-2 and ATF.

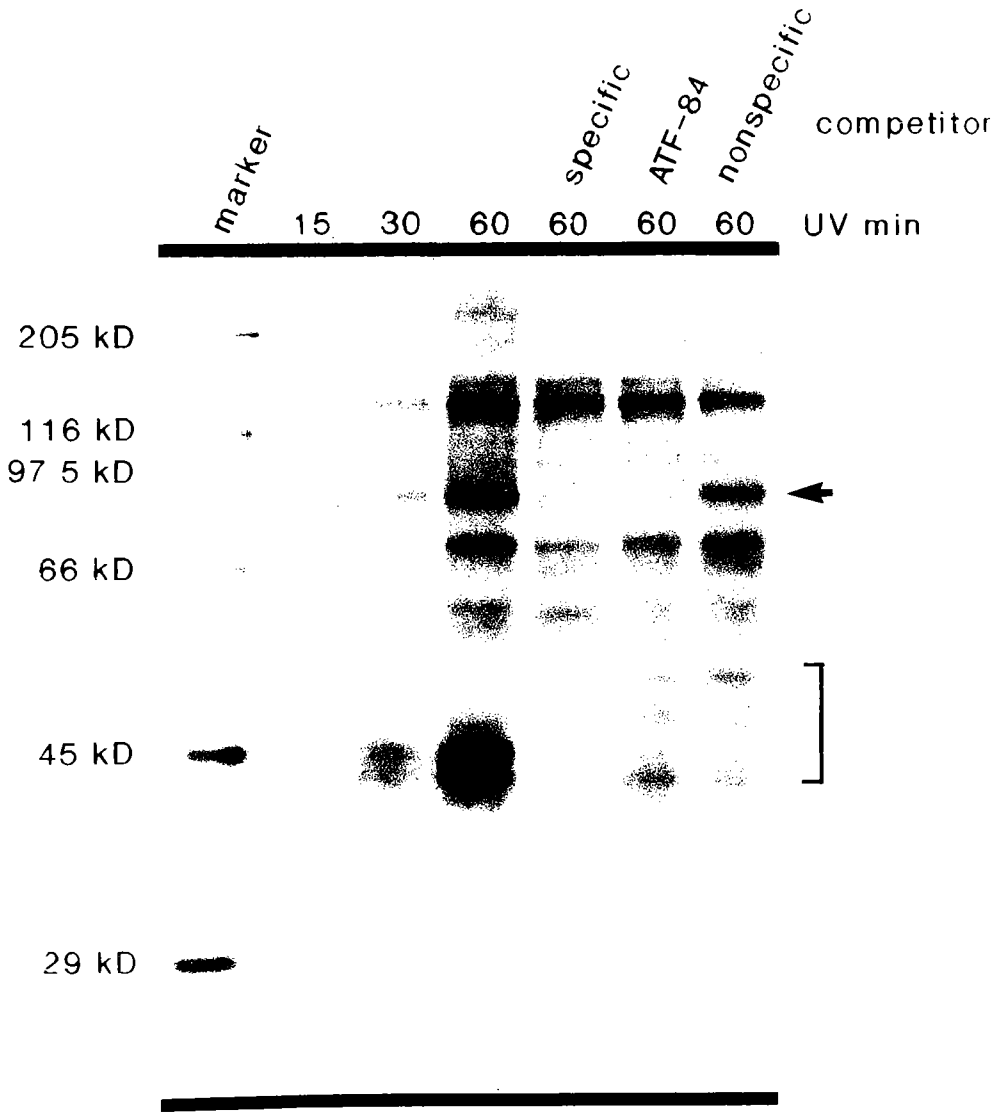
H4UA-2 is a transcriptional activator. The availability of the site directed mutant that specifically abolished H4UA-2 binding activity provided a means of directly establishing the contribution of H4UA-2 to transcription. The mutation was placed into the parent construct FO002 (-1038 bp) to create FO002ΔNMP. Both of these constructs were assayed in a cell free transcription system to measure the relative efficiency of transcription. The results shown in Figure 3-8B reveal that mutation of the ATF consensus sequence and therefore elimination of H4UA-2 binding reduced the transcription rate approximately two fold. These findings strongly suggest that H4UA-2 is at least one of the protein components mediating the stimulatory effect of the far-distal activator region. Secondly, this result identifies H4UA-2 as a functional member of the ATF family of transcription factors.

H4UA-2 and ATF-84 are related. Because this human H4 histone gene contains a second previously identified ATF binding site located in the proximal promoter (-122 to -104 bp), we were interested in determining the relationship between the two factors. The proximal ATF binding site

selectively interacts with a novel ATF transcription factor family member, termed ATF-84, which was identified by its unique molecular mass, 84 kDa. The molecular mass determination of H4UA-2 was performed by indirectly radiolabeling the factor by UV cross-linking the bound proteins to a uniformly radiolabeled, bromo-deoxyuridine substituted DNA binding site. The unbound DNA sequences were removed by DNase I digestion and the covalently cross-linked protein-DNA complexes were resolved on SDS-polyacrylamide gels.

We used the entire far-distal activator region as the UV cross-linking probe which will potentially allow for the detection of H4UA-2, H4UA-3, and H4UA-4. Treatment of the binding mixture with UV light for up to 60 minutes resulted in the appearance of several bands from 40 - 130 kDa (Figure 3-9). Control experiments demonstrated that all of these bands were dependent on the presence of nuclear extract and treatment with UV light (data not shown). In order to define which of the bands were related to H4UA-2 binding, we added a 500 fold molar excess of specific or non-specific competitor oligomers as described above. The specific competitor, spanning the H4UA-2 binding domain, selectively competed the band at 84 kDa and a group of bands at 40 - 50 kDa. Most ATF factors thus far identified have a molecular mass in the range of 43 to 47 kDa (97,127,145)

Figure 3-9. Molecular weight determination of H4UA-2 by UV cross linking detects specific proteins at 84 kDa and 40 - 50 kDa. UV cross linking to the far-distal activator was performed with a BrdU-substituted, uniformly labeled DNA probe. After DNase I digestion the samples were resolved on a 10% SDS-polyacrylamide gel and autoradiographed. Length of UV treatment is indicated above each lane. Specific competitor, H4UA-2 oligonucleotide; non-specific competitor, H3-DSII; ATF-84, oligonucleotide of the additional ATF binding site in the histone proximal promoter.


marker
15
30
60
60
60
60
UV min
specific
ATF-84
nonspecific
competitor
205 kD
116 kD
97 5 kD
66 kD
45 kD
29 kD

suggesting that the H4UA-2 binding domain is capable of forming interactions with these ATF factors. Additionally, the presence of an 84 kDa band that can be competed specifically indicates H4UA-2 may be related to the histone H4 promoter factor, ATF-84. Competition with the highly selective ATF-84 binding site oligomer specifically abolished the 84 kDa band while only partially affecting the interactions in the 40 to 50 kDa range. A non-specific competitor consisting of a dimerized Sp1 binding site had no effect on the 84 kDa band but did significantly reduce the signal at 40 to 50 kDa. This may signify that the 40 to 50 kDa bands are either very weak ATF interactions with the H4UA-2 binding domain or that these bands are non-specific interactions with probe. Thus, these results indicate that H4UA-2 is a member of the ATF transcription factor family and that its binding domain has preferential affinity for the histone H4 ATF-84 transcription factor and weak affinity for the common ATF factors (43 - 47 kDa).

Analysis of H4UA-3 and H4UA-4 recognition sequences.

Analysis of the remainder of the far-distal activator has defined the binding domains for H4UA-3 and H4UA-4. The H4UA-3 protein-DNA interaction mapped to the upstream side of H4UA-2 by bi-directional deletion analysis (Figure 3-5A and 3-5B). H4UA-3 binding activity was abolished by either

5' or 3' deletion of the fragment between the MboII and HinfI restriction sites. H4UA-4 binding was detectable with even the largest 3' deletion (BanII-MboII probe) and conversely was abolished with the first deletion from the 5' end (MnlI-EcoRI probe). Thus these results demonstrate that H4UA-3 lies between -691 and -649 bp (MboII to HinfI) and that H4UA-4 is positioned between -730 and -691 bp (BanII to MboII). Moreover, H4UA-4 requires sequences overlapping or 5' of the MnlI site (-708 bp).

The sequence requirements for H4UA-3 binding were determined through an analysis of the methylation interference pattern (Figure 3-6). Six guanine residues located 16 base pairs upstream of the H4UA-2 binding site were found to be in close association with the H4UA-3 protein. The upper strand contact points were at -659, -663, and -667 bp while the lower strand was in contact at -661, -664, and -665 bp. These six contacts form a cluster representing all of the guanine residues in a nine base pair domain. Extensive search of published binding sites has revealed no similarity to known transcription factor recognition sequences. H4UA-4 has eluded further analysis due to its weak interaction with this probe.

Protein-DNA interactions in the near-distal activator element. Investigation of the structural components of the

Figure 3-10. Resolution of only one protein-DNA interaction, H4UA-1, in the near-distal activator region. Gel mobility shift analysis of the entire HindIII/EcoRI (-418 to -213 bp) DNA fragment with increasing amounts of HeLa nuclear extract (0.5 - 8 μg).

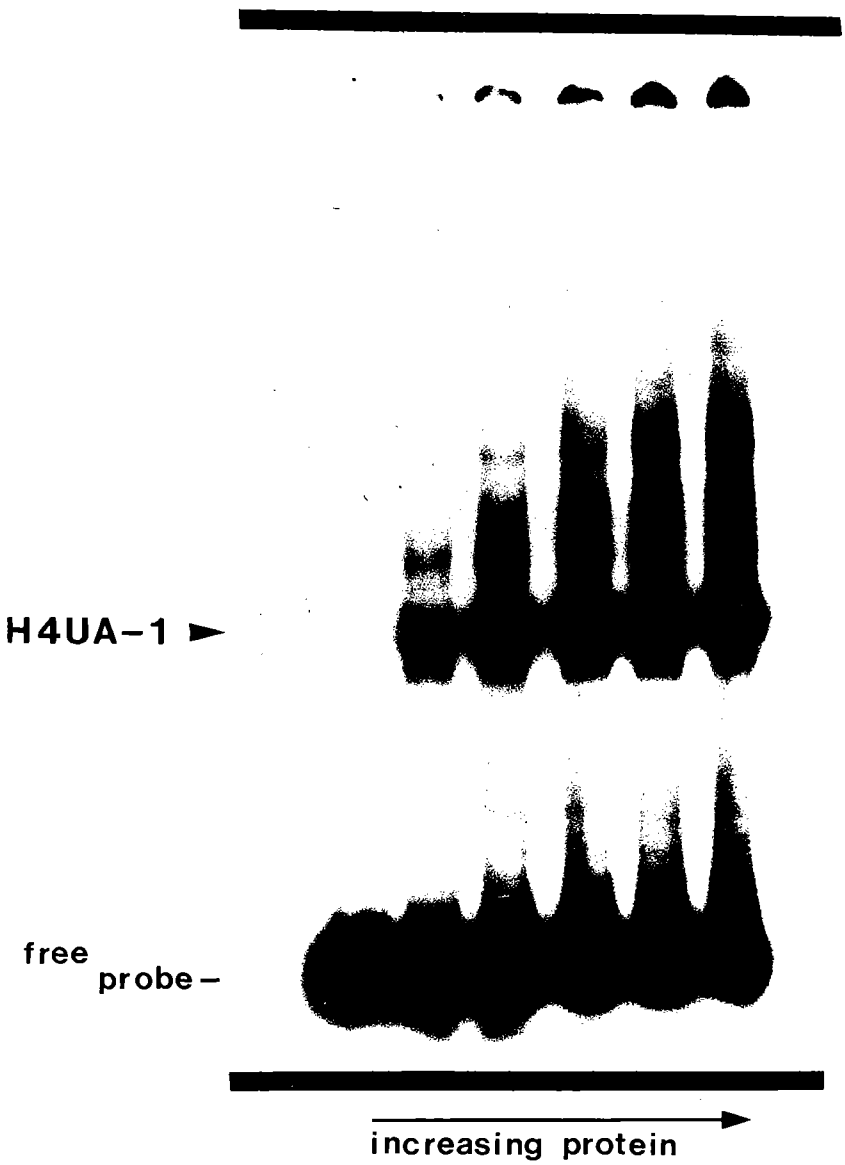
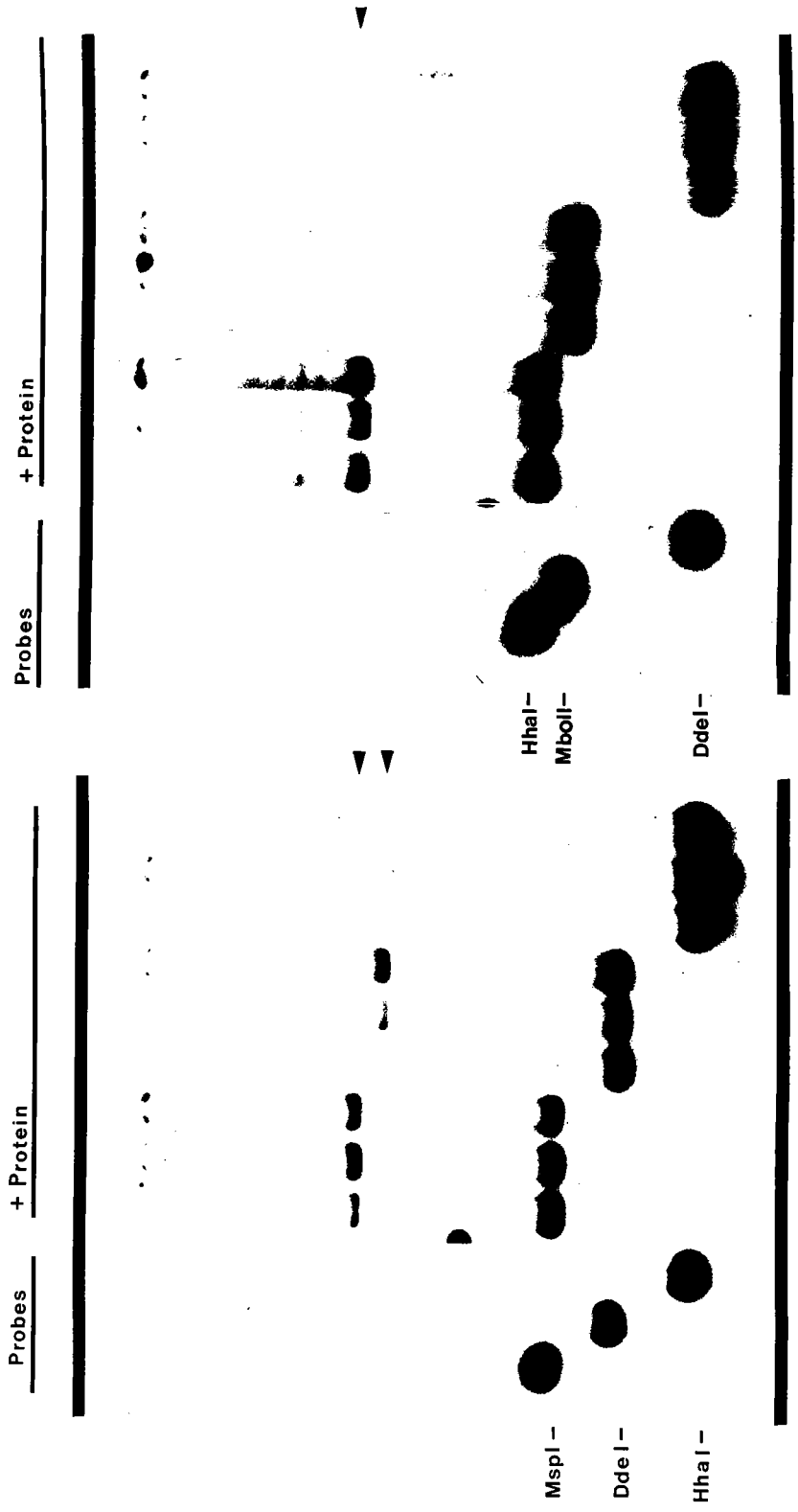
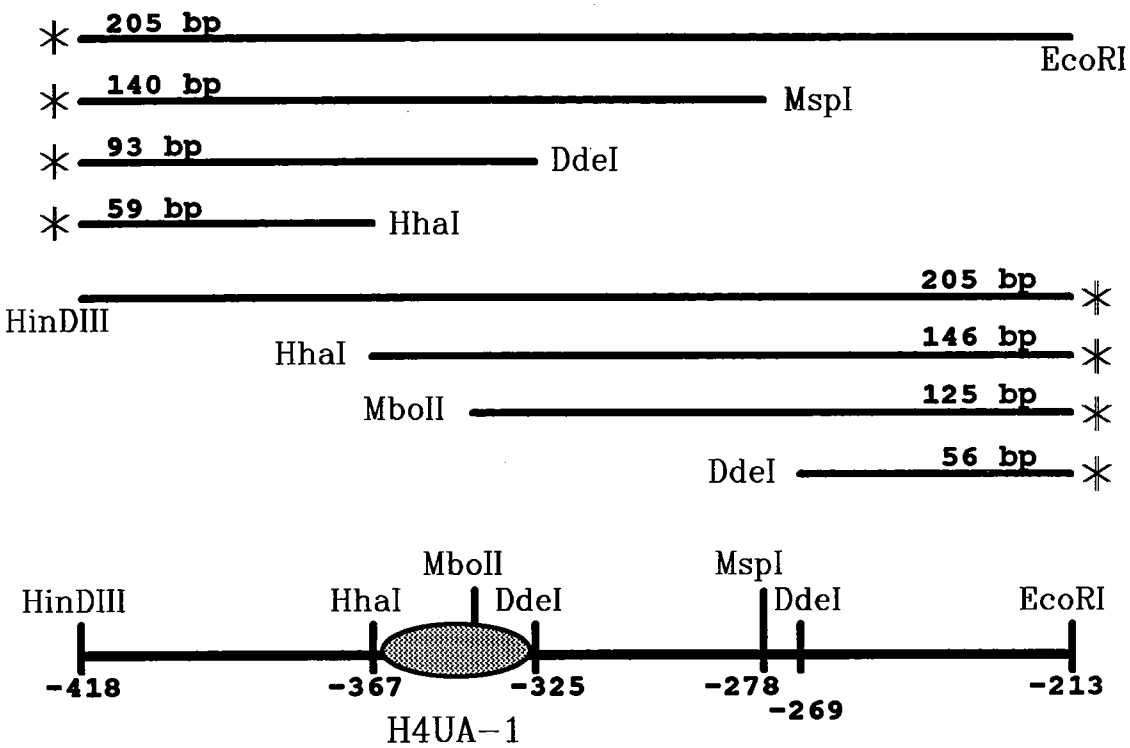

H4UA-1
free probe
increasing protein

Figure 3-11. Bi-directional deletion analysis of the near-distal activator region defines the location of H4UA-1. A) Autoradiograph of gel mobility shift assay performed with a series of progressively shortened probes. Probes used are indicated at the side and the arrowheads indicates the H4UA-1 complex. B) Schematic representation of the bi-directional deletion probes and results. Hatched oval indicates approximate position of the protein-DNA interaction.


Probes
+ Protein
Probes
+ Protein
MspI–
DdeI–
HhaI–
HhaI–
MboII–
DdeI–


205 bp
EcoRI
140 bp
MspI
93 bp
DdeI
59 bp
HhaI
205 bp
HinDIII
146 bp
HhaI
125 bp
MboII
56 bp
DdeI
MboII
MspI
HinDIII
HhaI
DdeI
DdeI
EcoRI
-418
-367
-325
-278
-269
-213
H4UA-1

Nucleotide resolution of the H4UA-1 binding site.

Mapping of the H4UA-1 interaction to a small segment of the H4 promoter facilitated a closer determination of the interaction boundaries by DNase I footprint analysis. Crude nuclear extract was incubated with a full length radiolabeled near-distal activator fragment under conditions which allow formation of the protein-DNA interactions. After brief treatment with DNase I, the DNA was recovered and resolved on a 6% denaturing polyacrylamide gel (Figure 3-12). Comparison of the control lanes (no protein) to the lanes with increasing amounts of nuclear extract reveals a 27 nucleotide region protected from DNase I cleavage. This region spans from -334 to -360 bp on the upper strand and is positioned within the maximal boundaries predicted from the bi-directional deletion analysis.

The sequence requirements for the H4UA-1 interaction were resolved in more detail by an analysis of the guanine residues in very close contact with the protein. Two DMS fingerprinting approaches were undertaken simultaneously, DMS interference and DMS protection. In the first approach the lower strand displayed five residues which interfered with binding of H4UA-1, -332, -338, -339, -341, and -342 bp, while the upper strand contained only one contact at -344 bp (Figure 3-13). Interestingly, all six of these methylation interference sites lie in the 3' half of the H4UA-1

Figure 3-12. DNase I footprint of the H4UA-1 interaction. The HindIII/EcoRI (-418 to -213 bp) probe was uniquely labeled at the EcoRI site and incubated with 12, 24, 36, and 48 μg of HeLa nuclear extract (lanes 1-4). Control lanes were incubated with 20 μg of BSA. After partial DNase I digestion the DNA was recovered an analyzed on an 8% polyacrylamide sequencing gel. The footprint is indicated by the bar and relative nucleotide numbers.

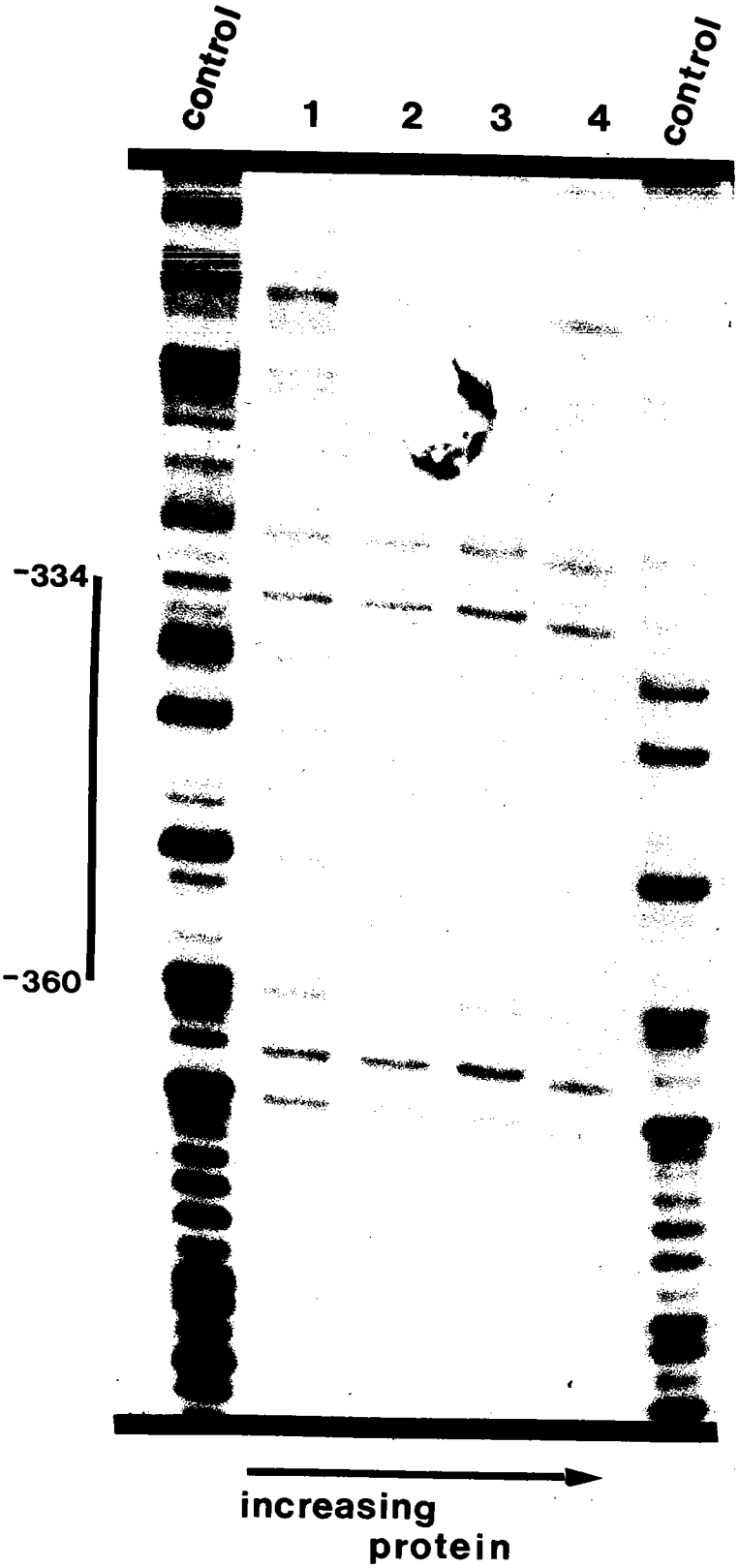
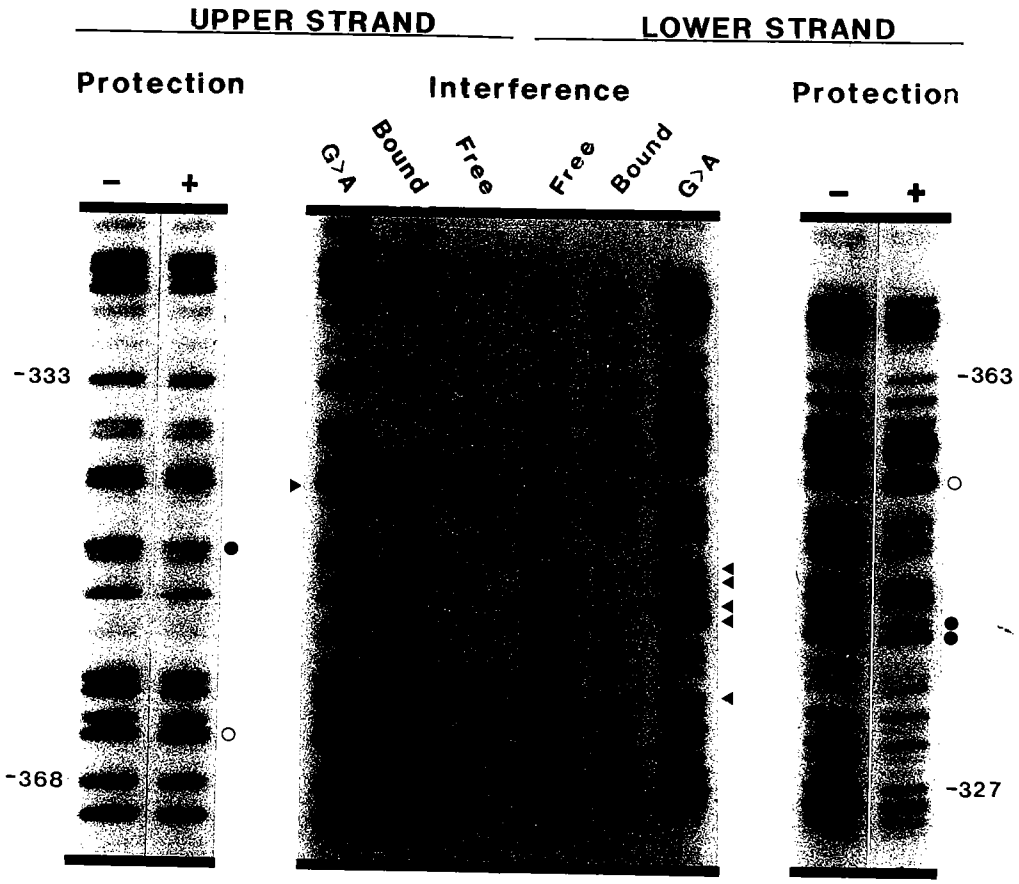

control
1
2
3
4
control
-334
-360
increasing
protein

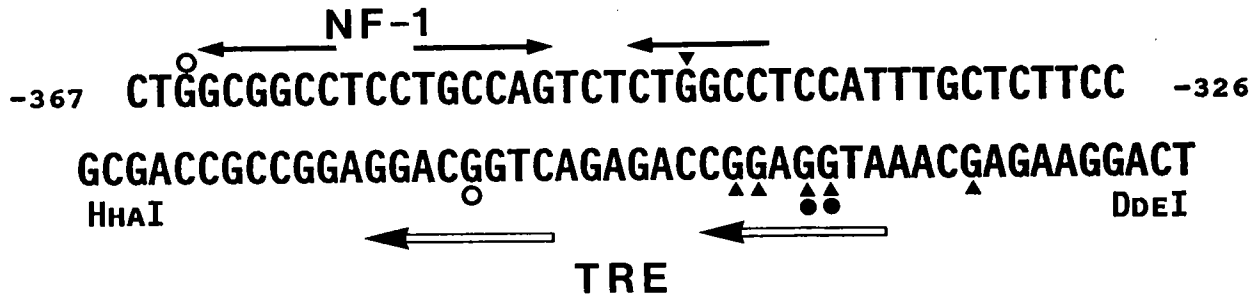
Figure 3-13. Methylation interference and methylation protection of the H4UA-1 interaction. The HindIII/MspI (-418 to -278 bp) DNA fragment was uniquely labeled on the 5' end of either the upper or lower strand. Methylation interference: Partially methylated probes were incubated with 6 μg of HeLa nuclear extract and free and H4UA-1 bound probe were separated by altered electrophoretic mobility followed by piperidine cleavage and analysis on an 8% polyacrylamide sequencing gel. The points of methylation interference are indicated by ▸ . Methylation protection: The H4UA-1 binding site probes were incubated with nuclear extract and then partially methylated by DMS. The DNA was purified and analyzed as above. Lanes: (-), 20μg BSA; (+), 36 μg of nuclear extract. Filled circles indicate protections. Open circles indicate enhancements.


UPPER STRAND
LOWER STRAND
Protection
Interference
Protection
G>A
Bound
Free
Free
Bound
G>A
-
+
-
+
-333
-368
-363
-327

footprint. We also examined the element for additional guanine contacts that might only be detectable with the direct DMS protection assay in which the protein-DNA complexes are formed prior to treatment with DMS. The results shown in Figure 3-13 indicate several additional points of DNA contact with H4UA-1. DMS protection of the lower strand revealed strong protections at -338 and -339 bp. Furthermore, there is an enhancement of methylation at -352 bp. The cause of enhancements is not fully understood but it is believed to be the result of the protein forming a hydrophobic pocket around the guanine residue. The pocket stabilizes the very labile DMS molecule leading to an enhancement of methylation. However, both protections and enhancements indicate a close association of the protein with that guanine residue. The upper strand contains one protection at -350 bp and one enhancement at -365 bp. The five methylation protections and enhancements are located throughout the H4UA-1 binding domain. A compilation of all of the fingerprint data (Figure 3-14) and an analysis of the sequences within this region reveal two potential homologies to known transcription factor recognition sequences. In the distal two thirds of the DNase I footprint are three copies of the NF1/CTF consensus recognition sequence, 5'-YTGGCA-3'. Two are arranged in the typical indirect repeat form while the third is spaced three

Figure 3-14. Sequence schematic of the near-distal activator region. Guanine residue contact points are indicated: ▲ ,interference; ● ,protection; ○ ,enhancement. Regions of similarity to the NF-1 consensus recognition sequence are indicated by the dark arrows. Regions of similarity to the thyroid hormone receptor binding site are indicated by the open arrows.

NEAR-DISTAL ACTIVATOR REGION --- H4UA-1



base pairs down stream. This sequence has been shown to be present in many different viral and cellular promoters and is functionally important in both transcription and replication (104). Additionally, there is a potential thyroid hormone response element consensus sequence. An imperfect direct repeat encompassing 6 of the 9 guanine contact points bears striking similarities to the high affinity thyroid hormone receptor binding site 5'-CTGGAGGT-3' described in the human α-myosin heavy chain gene promoter (65).

Discussion

The functional promoter of the replication dependent FO108 human H4 histone gene has been shown to extend at least 1000 base pairs upstream of the initiation site. Previous studies from this laboratory have defined two domains within the proximal promoter (-213 to 0 bp); one is required for site specific transcription initiation and one has a stimulatory activity (157,193,202), and Chapter 2 of this thesis). In this report we have extended the molecular characterization of the elements that determine the specific level of H4 histone gene transcription and the response to a spectrum of physiological signals.

The far-distal activator region (-730 to -589 bp) possesses the strongest stimulatory activity detected in this promoter, increasing the initiation rate greater than 10 fold. Investigation of the protein-DNA interactions occurring within this region revealed three binding sites, H4UA-2, H4UA-3, H4UA-4. Specific elimination of the H4UA-2 binding activity by site directed mutagenesis demonstrated that H4UA-2 was at least one of the protein components contributing to the stimulatory activity of the far-distal activator as deletion of this binding site resulted in a greater than 50% drop in transcription. H4UA-2 binds to a sequence with strong similarity (7/8 match) to the consensus ATF/CREB recognition site 5'-TGACGTCA-3'. Previously

described ATF factors have been shown to bind to degenerate forms of this sequence but the invariant pentamer core, 5'-TGACG-3', is always required (85,127). Site directed mutagenesis of the pentamer core of the H4UA-2 binding site completely abolished binding activity, demonstrating that H4UA-2 is an ATF consensus site binding factor. Many cellular and viral gene promoters have been shown to contain ATF consensus binding sites and these sites have been implicated in mediating induction by adenovirus E1a protein (119), cyclic AMP (145), and calcium (179). A number of protein factors have been identified which bind to these sites. ATF transcription factors have thus far been found in various eukaryotic tissues and transformed cell lines, yeast and *Xenopus* oocytes and have molecular mass spanning from 43 kDa to 120 kDa (4,43,97,127,128,130,145,168). Multiple ATF family members have been identified in the same cell, raising the interesting questions of what is the role of multiple factors and how are they resolved *in vivo*. Furthermore, while it is apparent that these factors have similar binding characteristics, the function of each individual ATF factor may be quite distinct (129).

The histone H4 gene promoter we have studied has an additional ATF transcription factor binding site, HiNF-E, located within the proximal promoter (see Chapter 2). This

binding site is more widely divergent from the prototype consensus than the H4UA-2 binding site but still maintains the invariant pentamer core. A single ATF factor of molecular mass 84 kDa preferentially binds to this site. H4UA-2 also displayed affinity for the ATF-84 factor. ATF-84 has not been detected by other laboratories using HeLa cell extracts and different promoters; this presents the intriguing possibility that ATF-84 may be directed specifically to histone gene promoters. Another ATF binding site has been localized in the proximal promoter of the ST519 human H3 histone gene (A. van Wijnen personal communication). Characterization of the ATF factor binding to this histone gene may provide additional information on the nature of ATF-84.

The nuclear matrix contains a factor, NMP-1, which exhibits binding characteristics identical to H4UA-2 (Dr. S. Dworetzky personal communication). NMP-1 was isolated from HeLa cell nuclear matrix prepared by the double detergent method of Fey *et al* (63) followed by solubilization in 7.0 M urea and dialysis back into typical nuclear extract storage buffer. Gel mobility shift analysis with the entire far distal activator region detected only one interaction and localized it to the H4UA-2 binding site. Fingerprint analysis and site directed mutagenesis

identified NMP-1 as an ATF family member. However, molecular mass determination revealed NMP-1 to consist of only 54 and 39 kDa proteins. Thus, H4UA-2 and NMP-1 are apparently distinct members of the ATF transcription factor family which compete for binding in the far distal activator region. It is unclear at this point which factor is binding to the far distal activator region *in vivo* or if there is some sort of switching between the soluble ATF-84 and attachment to the matrix through NMP-1. Analysis of these two proteins during changing conditions of histone gene transcription may begin to provide direct insight into the relationship between matrix attachment and transcription of nearby genes. Furthermore, the presence of two additional soluble transcription factors, H4UA-3 and H4UA-4, adjacent to the H4UA-2/NMP-1 binding site suggests a mechanism by which distally located transcription factors could be sequestered to stimulate the assembly and activation of the transcription complex. Tethering of the factors at a matrix attachment site would provide the structural means to loop out the intervening DNA sequences between the initiation complex and the distal activator region and allow the proteins to come in contact to effect a change in transcription.

The FO108 human H4 histone gene contains an additional positive element located at -213 to -418 base pairs. The near-distal activator is capable of forming only one strong protein-DNA interaction, H4UA-1. The DNase I footprint H4UA-1 contains consensus recognition sequences for the ubiquitous factor NF1/CTF. NF1/CTF has been described to possess two functions. NF1 was originally detected binding to the origin of replication of adenovirus 2 and shown to be involved in the initiation of DNA synthesis (80,165). CTF was identified in several cellular and viral promoters as a member of the CCAAT box binding factor family which was involved in the activation of transcription (105,150). A biochemical analysis of these two factors has suggested that they are identical (104). Thus, H4UA-1 may be related to NF1/CTF, however, early studies of this H4 histone gene promoter demonstrated this region could not function as an origin of replication (91). It is interesting to note that while the NF1/CTF recognition elements are toward the distal side of the footprint the strong DMS interference points lie to the proximal side of the footprint. Overlapping the proximal NF1/CTF sites is a recognition sequence for the thyroid hormone receptor. A consensus thyroid hormone receptor binding sites has not been well defined, however, one previously characterized TRE consists of an 8 base pair imperfect direct repeat in the α-myosin heavy chain gene

promoter (65). The H4UA-1 binding site contains an 11/16 match with this sequence suggesting the potential to interact with the thyroid hormone receptor. It has been observed previously that hormone response elements tend to be clustered with binding sites for other transcription factors (177). An arrangement such as this with structural similarities to the near-distal activator has been described for the mouse mammary tumor virus promoter. The long terminal repeats contain adjacent NF1/CTF and hormone response element binding sites which compete for binding (26). This could provide a direct mechanism to regulate histone gene transcription in response to changes in growth due to hormone induction. Further competition and binding site mutation studies will be required to sort out the relationship of H4UA-1 to these factors.

In conclusion we have demonstrated that the histone H4 promoter is a complex modular structure extending at least 1000 base pairs upstream of the initiation site. Transcription is modulated with respect to a series of positive and negative elements. The two distally located activator regions have the potential to interact with general transcription factors, hormone response elements, nuclear matrix proteins and two previously uncharacterized factors.

CHAPTER 4

MULTIPLE MECHANISMS REGULATE THE PROLIFERATION SPECIFIC HISTONE GENE TRANSCRIPTION FACTOR, HINF-D, IN NORMAL HUMAN DIPLOID FIBROBLASTS

Abstract

The proliferation-specific transcription factor complex, HiNF-D, interacts with sequence specificity in a proximal promoter element of the human H4 histone gene FO108, designated Site II. The occupancy of Site II by HiNF-D has been implicated in proper transcription initiation and as a component of the cell cycle regulation of this gene. In the present study we have investigated the role of the HiNF-D/Site II interaction in controlling the level of H4 histone gene transcription during modifications of normal cellular growth. HiNF-D binding activity is present at high levels in rapidly proliferating cultures of human diploid fibroblasts and is reduced to less than 2% upon the cessation of proliferation induced by serum deprivation of sparsely populated fibroblast cultures. Density dependent quiescence also abolishes the HiNF-D binding activity. Down regulation of transcription from the H4 gene occurs concomitant with the loss of the HiNF-D/Site II interaction, further suggesting a functional relationship between Site II occupancy and the capacity for transcription. Serum stimulation of quiescent pre-confluent

cells results in an up-regulation of HiNF-D binding activity as the cells are resuming DNA synthesis and H4 histone gene transcription. Density inhibited quiescent cells respond to serum stimulation with only a minimal up-regulation of the HiNF-D binding activity, 30% of maximal levels. However, H4 histone gene transcription is stimulated to a level equal to that detected in extracts of the sparsely populated serum stimulated cultures. These results suggest that there is a threshold level of HiNF-D binding activity necessary for the activation of H4 histone gene transcription. Additionally, these findings suggest that there may be a mechanism repressing HiNF-D binding activity in the density inhibited cultures which is not operative in the sparsely populated, serum deprived cultures. Density inhibited cultures may have reached a state analogous to the initial steps of differentiation and have invoked a series of mechanisms to down-regulate expression of proliferation-specific factors. Serum stimulation is able to overcome the one mechanism down-regulating HiNF-D in both sparsely populated and density inhibited quiescent cultures but is unable to reverse the repression of proliferation-specific factors which occurs in density inhibited cultures. These results are consistent with the presence of at least two levels of control over the HiNF-D/Site II interaction which are responsive to and reflect the proliferative state of the

cell and the extent to which the cell exhibits properties associated with differentiation.

Introduction

The control of growth related genes is central to maintaining stringent cell cycle control, regulating the transition between proliferation and a commitment to differentiate, and maintaining the ordered sequence of events leading to the development of a differentiated phenotype. Understanding molecular mechanisms operative in regulating expression of cell cycle and cell growth related genes should provide insights into the loss of growth control in transformed and tumor cells. The human H4 histone gene FO108 (181) is a cell cycle dependent, growth related gene for which expression is temporally and functionally coupled to DNA synthesis (160). Regulation of this coupling is mediated at both the transcriptional and post-transcriptional levels (14). This H4 histone gene exhibits a basal level of transcription throughout the cell cycle with a two to three fold enhanced level of transcription during the early stages of S phase (14 and A. Ramsey-Ewing, personal communication). This is accompanied by an accumulation of histone MRNA which is rapidly and selectively destabilized at the conclusion of DNA replication (14,51,89).

Two *in vivo* protein-DNA interactions, Site I and II, have been delineated within the first 200 nucleotides

upstream of the transcription initiation site (157,158). At least three independent protein factors have been detected to bind to these domains *in vitro* (202) and Chapter 2 of this thesis). HiNF-C, an Sp1-like protein, and HiNF-E, an ATF related transcription factor, bind adjacent to each other in the Site I domain. The interactions at Site I do not change during the cell cycle nor during the complete shutdown of H4 histone gene transcription that occurs during differentiation (152,157,193). However, deletion of Site I reduces transcription five to seven fold in actively proliferating cells (112,202 and Chapter 2 of this thesis). This implies that Site I is key in regulating the level of H4 histone gene transcription but is not a rate limiting step in the process of initiation.

Factor HiNF-D interacts specifically with Site II sequences at -20 to -70 nucleotides upstream from the transcription initiation site. The region of interaction includes the histone specific element (5'-GGTCC-3'), a CAAT box, and lies adjacent to the TATA box (202). Deletion of this protein-DNA interaction site abolishes transcription *in vivo* and indicates a critical role for Site II in the regulation of this gene (112). Furthermore, HiNF-D binding activity is down-regulated outside of S phase in normal diploid cells implying an involvement in the cell cycle

control of histone gene expression. The level of HiNF-D binding activity is un-coupled from the cell cycle in tumor cells where constitutively high binding activity is detected (95). In addition, *in vivo* Site II interactions and HiNF-D binding activity are specifically lost at the onset of differentiation of HL60 cells into monocytes, concomitant with the shutdown of H4 histone gene transcription (193). A similar correlation between HiNF-D interaction with Site II and H4 gene transcription is seen at the onset of differentiation in primary cultures of rat calvarial osteoblasts (152). HiNF-D binding and H4 histone gene transcription are lost simultaneously as the cells reach a transition point where growth related genes are down-regulated and tissue specific gene expression is initiated.

These observations prompted us to address further the relationship between histone gene expression and cell growth control. Human diploid fibroblasts provide a system in which proliferation can be reversibly shut down without inducing differentiation (12,57,154,162). Normal fibroblasts will cease to proliferate and enter a quiescent state if placed in medium containing very low serum (0 - 0.5%) or if allowed to become density inhibited. Sparsely populated cultures maintained in serum free medium become arrested in the G_1 phase of the cell cycle due to a lack of nutrients. However,

density inhibited fibroblasts become arrested in the G_1 phase and enter G_0 only after being maintained at reduced serum concentrations (57). Serum deprived quiescent cultures of either pre-confluent or confluent cells can be stimulated to re-enter the proliferative phase upon replenishment of the medium with 10% serum.

In these studies we have manipulated the proliferative state of the cells and examined the extent to which the level of HiNF-D binding at Site II is coupled to H4 histone gene transcription. A strict correlation between proliferation, the presence of the HiNF-D binding activity, and the ability of the H4 histone gene to be transcribed has been established. However, stimulation of the density inhibited cultures induced a significantly lower level of HiNF-D binding activity than stimulation of the sparsely populated cultures despite approximately equivalent levels of induction of H4 histone gene transcription. These results suggest that the density inhibited cells are competent to generate a threshold level of HiNF-D necessary to activate H4 histone gene transcription upon serum stimulation but have a mechanism restricting the accumulation of the quantities of HiNF-D normally present in actively proliferating cells. This mechanism may be related to the limited proliferative activity displayed by serum stimulated density inhibited cells which in turn may be a reflection of

the similarity between the density inhibited state and the initial steps of differentiation.

Materials and Methods

Human diploid fibroblast cell culture. The cells used in these experiments were human diploid fibroblasts originally derived from newborn foreskin and designated CF-3 (52). These cells have an *in vitro* life span of 65 +/- 10 population doublings and were used before 50% of this life span had been completed. Cells were subcultured into 150 cm^2 plastic culture vessels (Costar) with McCoy's Medium 5a (Gibco) containing 10% fetal bovine serum (Hyclone) and antibiotics as previously described (57). Cells were in exponential growth phase three days after subculture and formed confluent monolayers after seven days. Stock cultures were determined to be free of mycoplasma contamination by the method of (11). In some experiments, cells were induced to enter a quiescent state by replacing the medium containing 10% serum with serum-free medium 24 hours following subculture. These sparsely populated cultures were maintained with serum-free medium for 72 hours at which time the cells were stimulated to enter the division cycle by refeeding with medium containing 10% serum. In other experiments, a quiescent state was induced in confluent populations by maintaining the cells for 16 days with medium containing 0.1% serum which was replaced twice weekly. These

cells were also stimulated to enter the division cycle by refeeding with medium containing 10% serum.

DNA Synthesis. DNA synthesis was estimated by the incorporation of ^{3}H-thymidine into acid precipitable material as described previously (194). Cells were pulse labeled for 30 minutes with 0.5 μCi/ml of ^{3}H-thymidine (New England Nuclear, 82.7 Ci/mmol). Labeled cells were scraped into phosphate buffered saline, pelleted by low speed centrifugation, and resuspended in high salt buffer (0.05 M Na_2HPO_4, 2.0 M NaCl, pH7.4). Trichloro-acetic acid was added to 10% (wt/vol) and TCA precipitable material was collected on 0.45 μm nitrocellulose filters. The filters were washed 3 times with 5 ml cold 10% TCA and dissolved in 1 ml ethylene glycol monoethyl ether (cellusolve). Radioactivity was determined by liquid scintillation spectrometry in 10 ml of scintillation cocktail [72% toluene, 24% (vol/vol) cellusolve and 4.2% (vol/vol) Liquifluor (Research Products International)]. An aliquot of the cell suspension was also taken for the determination of DNA by the method of Labarca and Paigen (114).

Nuclear Extract Isolation. The isolation of nuclear extracts from normal diploid cells was essentially as described in Chapter 2 except the cells were lysed by expelling the swollen cell suspension through a 22 gauge

needle 10 times. All volumes were reduced to a minimum to aid in the recovery of highly concentrated extracts.

Gel Mobility Shift Assay. Binding reactions were as described in Chapter 2 except 2 μg of poly (dI-dC)·(dI-dC) was used as the non-specific competitor. The electrophoresis buffer conditions for the gel mobility shift assay were changed to a low ionic strength buffer [6.6 mM Tris-HCl (pH 7.9), 3.3 mM Na-acetate, and 1 mM EDTA] as described (67).

mRNA Isolation. Total cellular mRNA was isolated essentially as described previously (212). Cells from 2 - 4 T150 flasks were collected by centrifugation and stored at -70^{0}C. Nucleic acids were isolated by resuspending the frozen pellet in 400 μl of Lysis buffer [2 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 μg/ml polyvinylsulfate (PVS, Kodak), 2% SDS and 500 μg/ml Proteinase K (Bethesda Research Laboratories)] and incubating for 30 minutes at 22^{0}C. NaCl was added to 0.25 M and the reaction was repeatedly extracted with phenol:$CHCl_3$:isoamyl alcohol followed by ethanol precipitation. The nucleic acid pellet was resuspended in 300 μl of TCM buffer [10 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, 10 mM $MgCl_2$, and 10 μg/ml polyvinyl sulfate] and digested with 30 μl of DNase I Solution for 2 hours at 37^{0}C. DNase I was prepared by resuspending 3.4 mg of crude DNase I (2000 units, Sigma) in 1 ml of 20 mM

Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and incubating at 37^{0}C for 20 minutes followed by the addition of Proteinase K to 1 mg/ml and incubating the reaction an additional 2 hours at 37^{0}C. The DNase I Solution was then stored on ice until used. RNA was recovered by making the solution 1.7% SDS and repeatedly extracting and ethanol precipitating as before. The RNA was resuspended in 150 μl H_2O and stored at -70^{0}C.

S1 Nuclease Analysis. The S1 nuclease analysis was as described in Chapter 2.

In Vitro Transcription. *In vitro* transcription from circularized templates was as described in Chapter 2. Plasmids containing the first 536 base pairs of the adenovirus major late promoter (pMLH1) have been described previously (88).

Results

Cessation of proliferation is associated with the down-regulation of HiNF-D binding activity and H4 histone gene transcription. Human diploid fibroblasts derived from newborn foreskin (52) were examined during exponential growth and two modes of induced quiescence: i) serum deprivation and ii) density dependent quiescence. The proliferative state of the cultures was monitored by the level of DNA synthesis detected by ^{3}H-thymidine incorporation. Sparsely populated, proliferating cultures were incubated for three days in serum free medium. DNA synthesis in these serum deprived cultures was reduced to 2.5% of the proliferating rate, indicating that the cells had entered a quiescent state (Table 4-1). Density dependent, quiescent populations were also prepared by maintaining cultures in normal growth medium without sub-cultivation. Eight days after initial seeding the cultures had reached confluence and were quiescent as reflected by a 94% reduction in the rate of DNA synthesis. Maintaining the cultures at confluence for an additional one or two days (Table 4-1, Day 9 and Day 10) did not further reduce the rate of ^{3}H-thymidine incorporation into the DNA.

HiNF-D binding activity was measured in the quiescent fibroblasts as the loss of the HiNF-D/Site II interaction has been shown to be correlated with the down-regulation of

Table 4-1. DNA synthesis. Cells were pulse labeled with ^{3}H-thymidine for 30 minutes and incorporation into acid precipitable material was used to estimate DNA synthesis as described in the Experimental Procedures.

TABLE 4-1.

SAMPLE	^{3}H-TDR DPM/μG DNA
EXPONENTIALLY GROWING	2262
PRE-CONFLUENT - SERUM DEPRIVED	58
PRE-CONFLUENT - SERUM STIMULATED, 5 HRS	89
PRE-CONFLUENT - SERUM STIMULATED, 24 HRS	2845
CONFLUENT - 8 DAYS	143
CONFLUENT - 9 DAYS	158
CONFLUENT - 10 DAYS	119
CONFLUENT - SERUM DEPRIVED	99
CONFLUENT - SERUM STIMULATED, 5 HRS	30
CONFLUENT - SERUM STIMULATED, 24 HRS	3039

H4 histone gene transcription occurring at the onset of differentiation (152,193). High salt nuclear extracts were prepared from proliferating cells and from quiescent cells induced by serum deprivation or density dependent inhibition. Gel mobility shift assays were utilized to detect the level of HiNF-D binding to Site II of the FO108 H4 promoter in each of the nuclear extracts. The rapidly proliferating cells contained a high level of HiNF-D binding activity (Figures 4-1 and 4-2). However, cells which had ceased to proliferate due to three days of serum deprivation lost 98% of the HiNF-D interaction with Site II, to nearly undetectable levels (Figure 4-1). Similarly, the density dependent quiescent fibroblasts contained only 15% of the HiNF-D binding activity as compared to the level in the initial rapidly proliferating cultures (Figure 4-2).

The effect of the loss of interaction between HiNF-D and Site II on histone mRNA levels and gene transcription was determined in these cultures. H4 mRNA levels were detected by S1 analysis of total cellular RNA in order to resolve the FO108 H4 gene from the other members of the H4 multi-gene family. The mRNA levels paralleled HiNF-D binding activity and DNA synthesis as would be expected of a cell cycle regulated histone gene (Figures 4-3 and 4-4). The high levels of H4 mRNA present in the proliferating fibroblasts were completely down-regulated in quiescent cells. More

Figure 4-1. Analysis of HiNF-D binding activity in pre-confluent CF-3 normal diploid fibroblasts. A radiolabeled DNA fragment (-155 to +18 bp) containing the Site II domain of the human H4 histone gene was used in a gel mobility shift assay to probe for HiNF-D binding activity in nuclear extracts. Nuclear extracts prepared from proliferating normal diploid fibroblast cultures, cultures arrested for 72 hours in serum-free medium, and cultures 5 and 24 hours after stimulation with medium containing 10% serum. Each three lanes represent increasing concentrations of nuclear extract added to the binding reactions. Arrow indicates position of the HiNF-D/Site II complex.

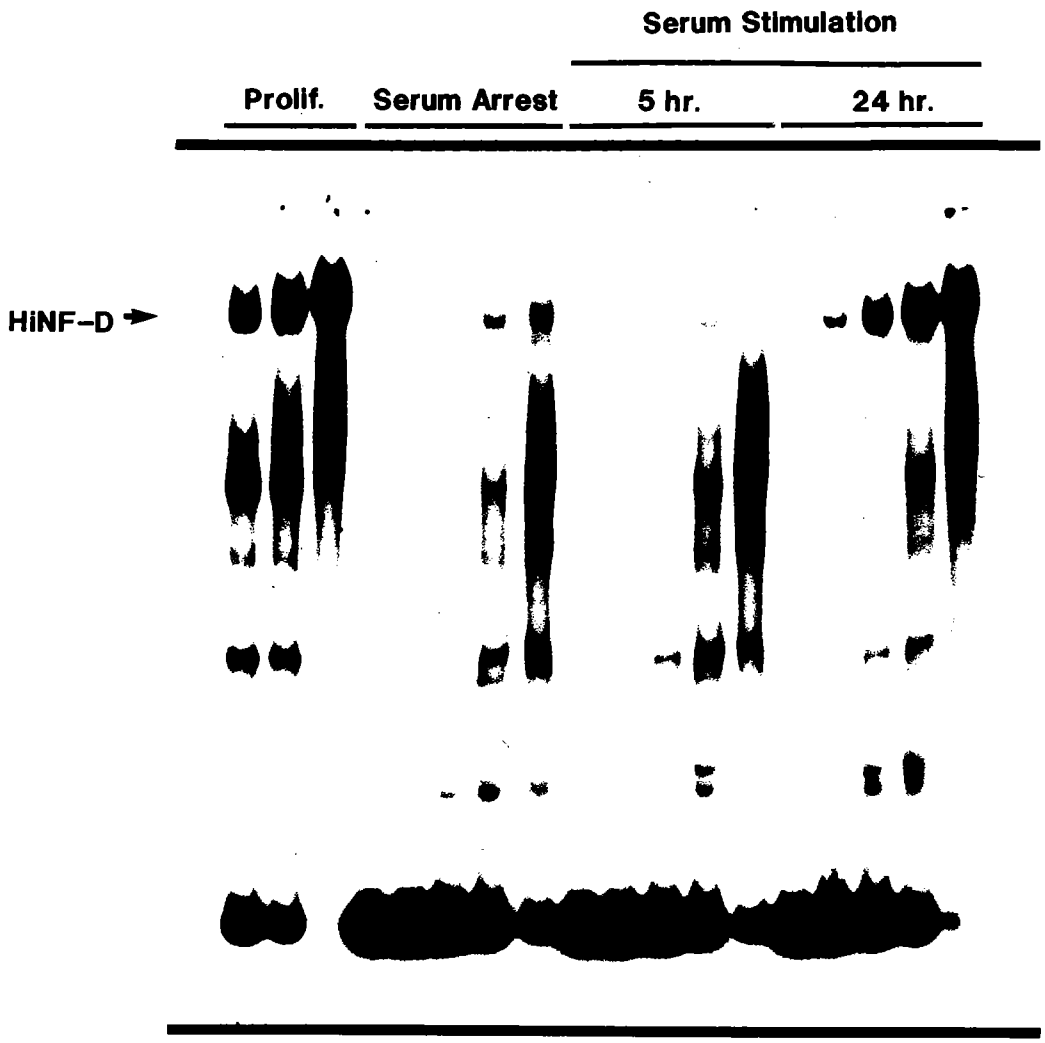

Serum Stimulation
Prolif.
Serum Arrest
5 hr.
24 hr.
HiNF-D

Figure 4-2. Analysis of HiNF-D binding activity in density inhibited CF-3 normal diploid fibroblasts. Gel mobility shift analysis conditions are the same as in Figure 4-1. Nuclear extracts were prepared from proliferating cultures, quiescent cultures 8, 9, and 10 days after initial plating, cultures arrested in 0.1% serum containing medium for 16 days after reaching confluence, and cultures 5 and 24 hours after stimulation with 10% serum containing medium. Each three lanes represent increasing concentrations of nuclear extract added to the binding reactions. Arrow indicates position of the HiNF-D/Site II complex.

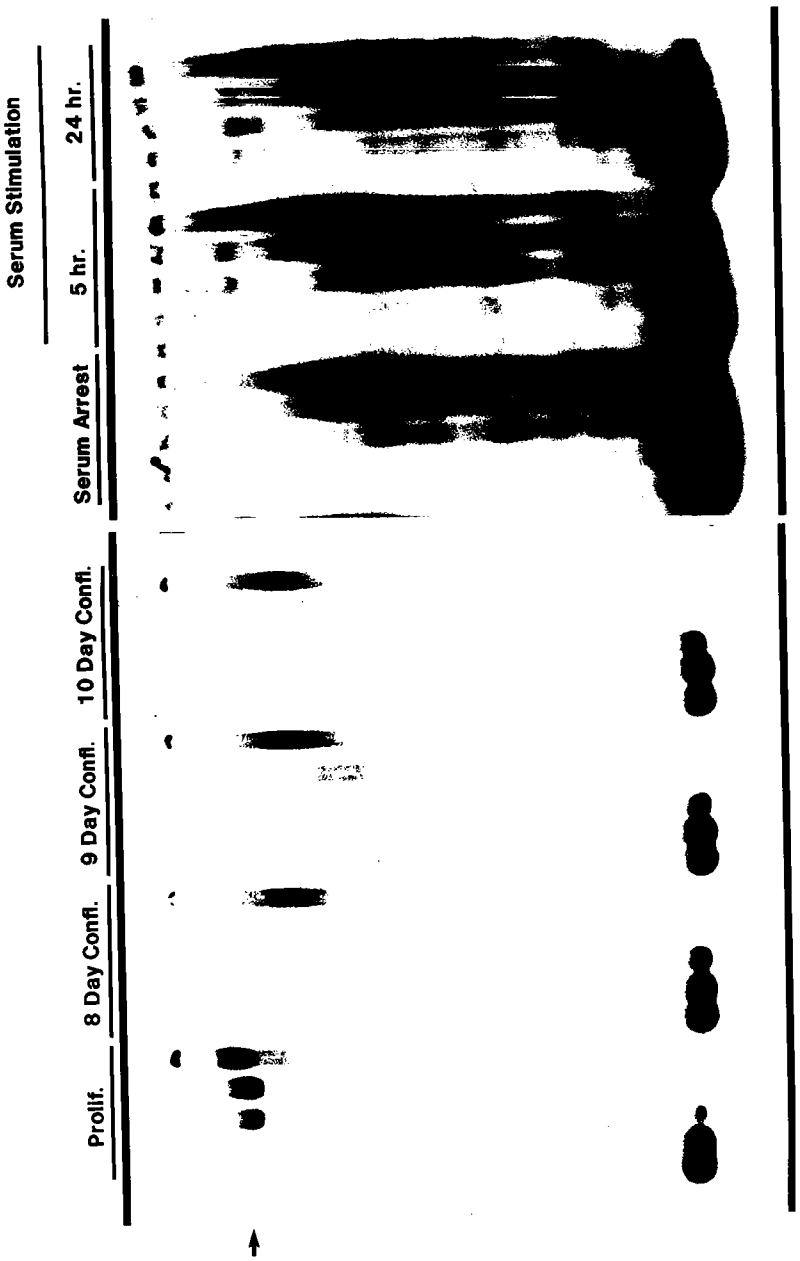

Serum Stimulation
Serum Arrest
5 hr.
24 hr.
Prolif.
8 Day Confl.
9 Day Confl.
10 Day Confl.

Figure 4-3. Expression of human H4 histone mRNA in pre-confluent normal diploid fibroblasts as a function of serum arrest and stimulation. Cultures were maintained as described in Figure 4-1 and total cellular RNA was isolated from proliferating, serum arrested and serum stimulated cultures as described in the Experimental Procedures. The FO108 H4 histone mRNA was specifically assayed by S1 nuclease protection. Arrow head indicates accurately initiated H4 histone mRNA.

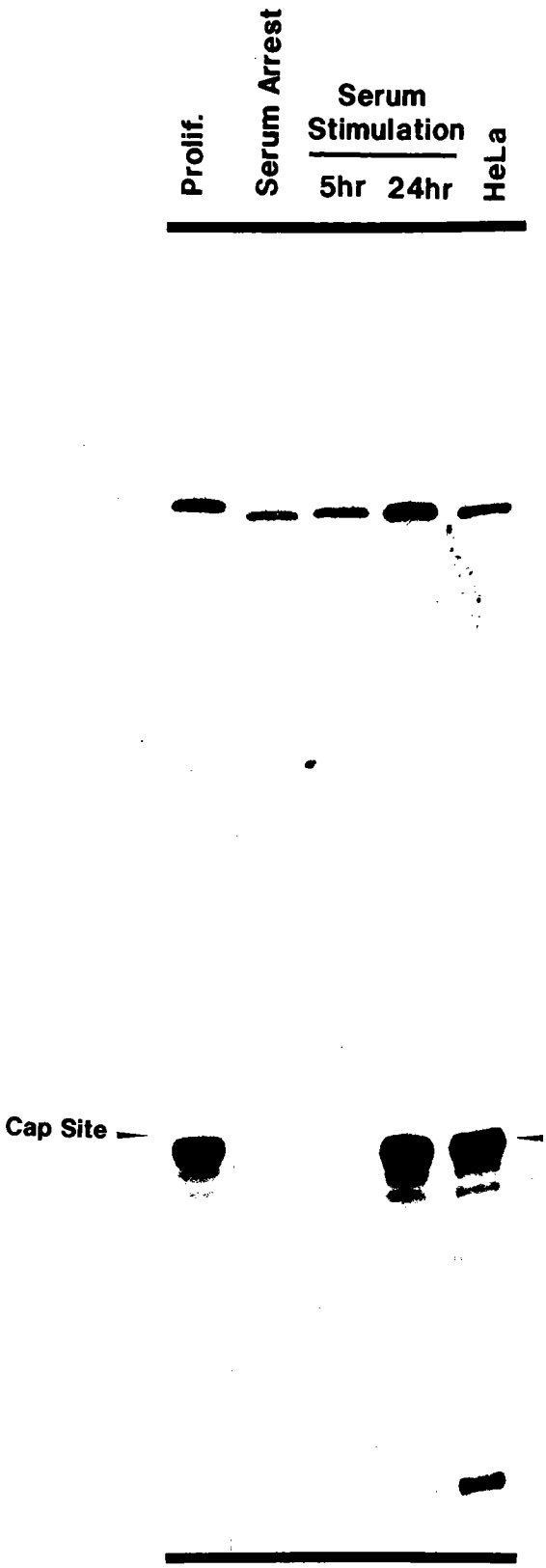
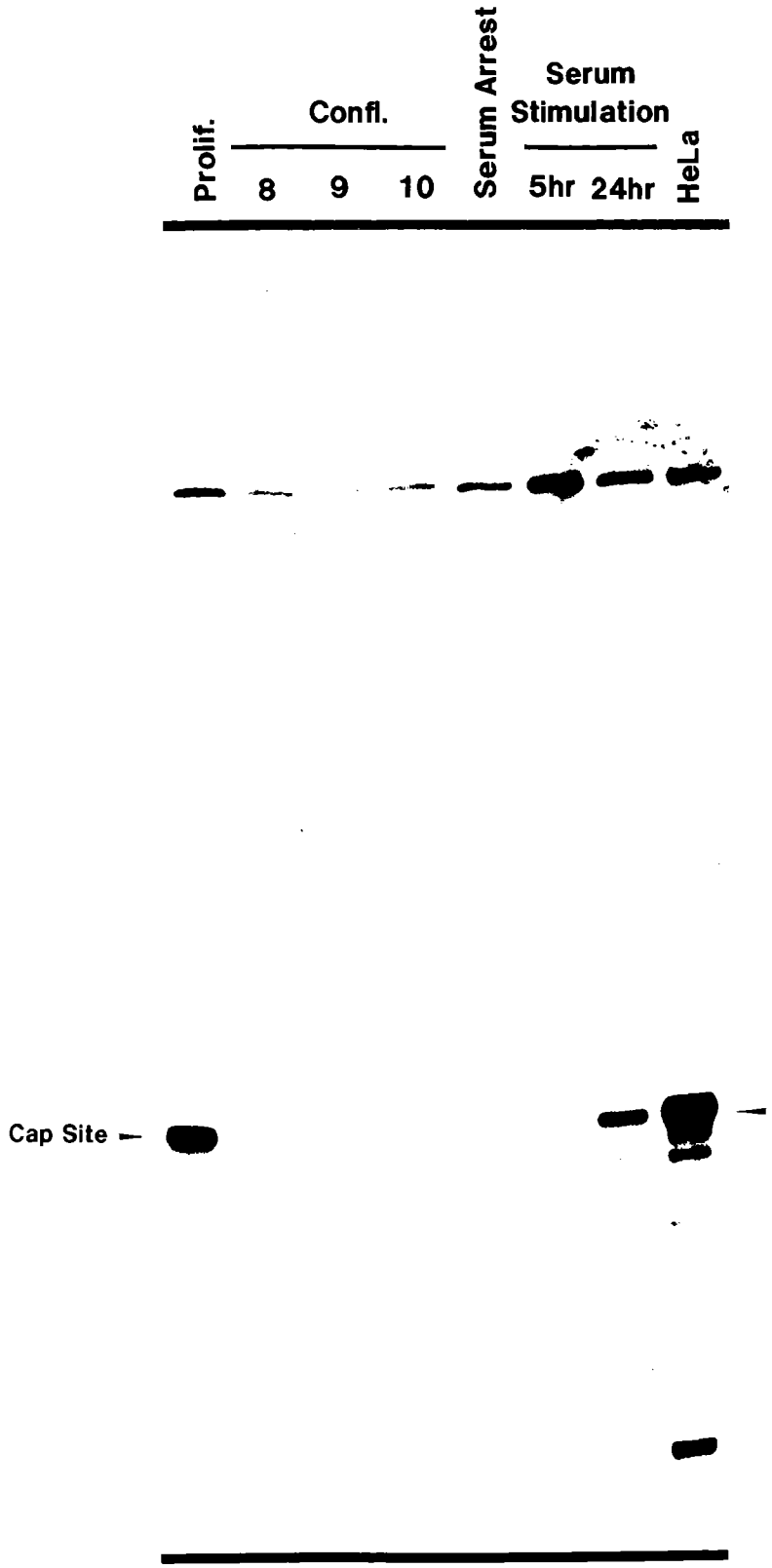

Prolif.
Serum Arrest
Serum Stimulation
5hr
24hr
HeLa
Cap Site

Figure 4-4. Expression of human H4 histone mRNA during density inhibition, serum arrest, and serum stimulation of normal diploid fibroblasts. Cultures were maintained as in Figure 4-2 and total cellular RNA was isolated from proliferating, confluent cultures 8, 9, and 10 days after plating, confluent cultures arrested in 0.1% serum for 16 days and 5 and 24 hours after serum stimulation. The arrowhead indicates the accurately initiated human H4 mRNA as detected by S1 nuclease protection.


Prolif.
Confl.
8
9
10
Serum Arrest
Serum Stimulation
5hr
24hr
HeLa
Cap Site

importantly, *in vitro* transcription of this gene by the same nuclear extracts used to monitor HiNF-D binding revealed transcription also paralleled the HiNF-D/Site II binding activity and DNA synthesis (Figures 4-5 and 4-6). Nuclear extracts from proliferating cells were able to initiate transcription from the H4 histone gene promoter. However, nuclear extracts from serum deprived and density dependent, quiescent cells, which contained very low levels of HiNF-D binding activity, were unable to support detectable levels of *in vitro* transcription of the H4 histone gene.

Because quiescent cells may exhibit a reduced level of general RNA polymerase II activity, each of the nuclear extracts was assayed for *in vitro* transcription activity on the adenovirus 2 major late promoter. While prolonged quiescence resulted in a gradual decay of the general polymerase activity, the quiescent cell extracts which were devoid of H4 histone gene transcription activity still exhibited a significant level of adenovirus transcription (Figure 4-7). These results suggest that the quiescent cells have selectively down-regulated transcription of the histone gene. Furthermore, these findings support previous observations demonstrating a requirement for the occupancy of Site II by HiNF-D in the initiation of H4 histone gene transcription (193). They also indicate a common site of

Figure 4-5. Assessment of specific H4 histone gene transcriptional activity in pre-confluent human diploid fibroblasts. Cell free transcription analysis was performed as described in the Experimental Procedures and resultant RNA transcripts were assayed by S1 nuclease protection. Nuclear extracts were the same as used in the determination of HiNF-D binding activity as described in Figure 4-1. The arrowhead indicates the accurately initiated human H4 mRNA.

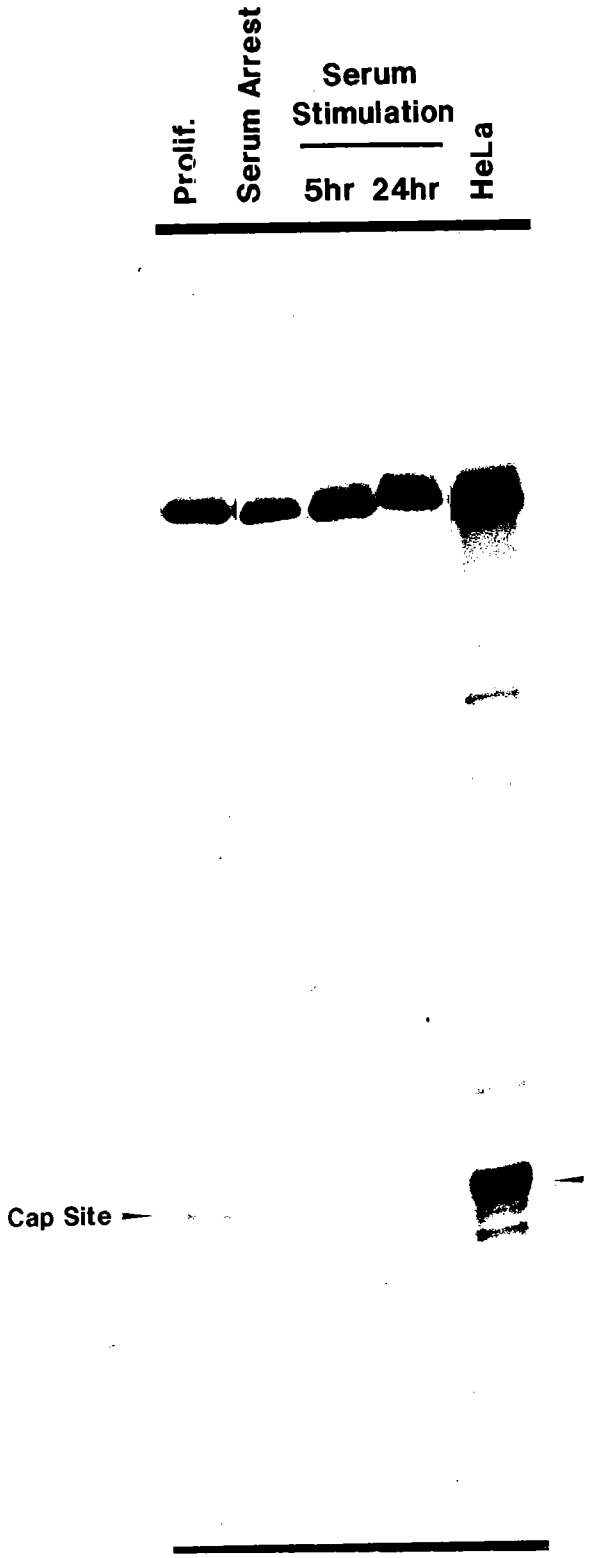

Prolif.
Serum Arrest
Serum Stimulation
5hr 24hr
HeLa
Cap Site

Figure 4-6. Assessment of specific H4 histone gene transcriptional activity during density inhibition, serum arrest and stimulation of confluent normal diploid fibroblasts. Nuclear extracts were the same as used in the determination of HiNF-D binding activity as described in Figure 4-2. Cell free transcription was performed as described in the Experimental Procedures. The arrowhead indicates the accurately initiated human H4 mRNA.

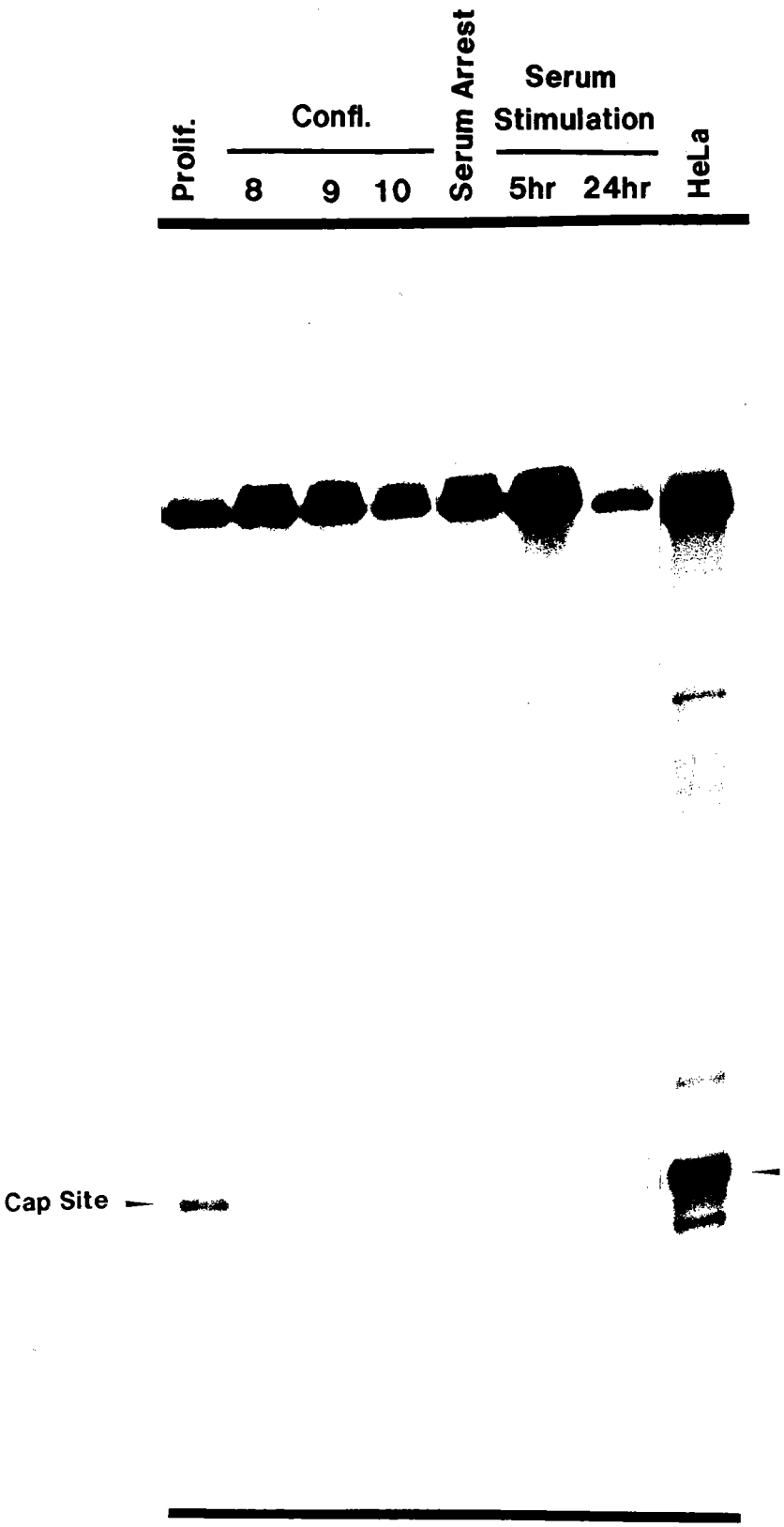
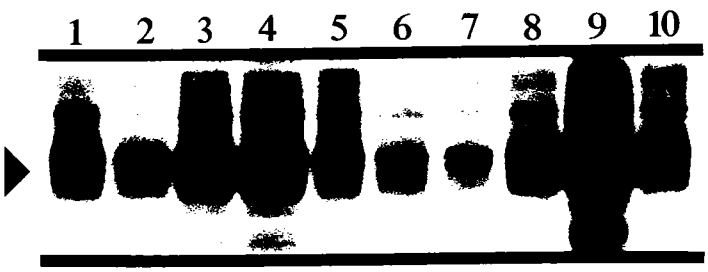
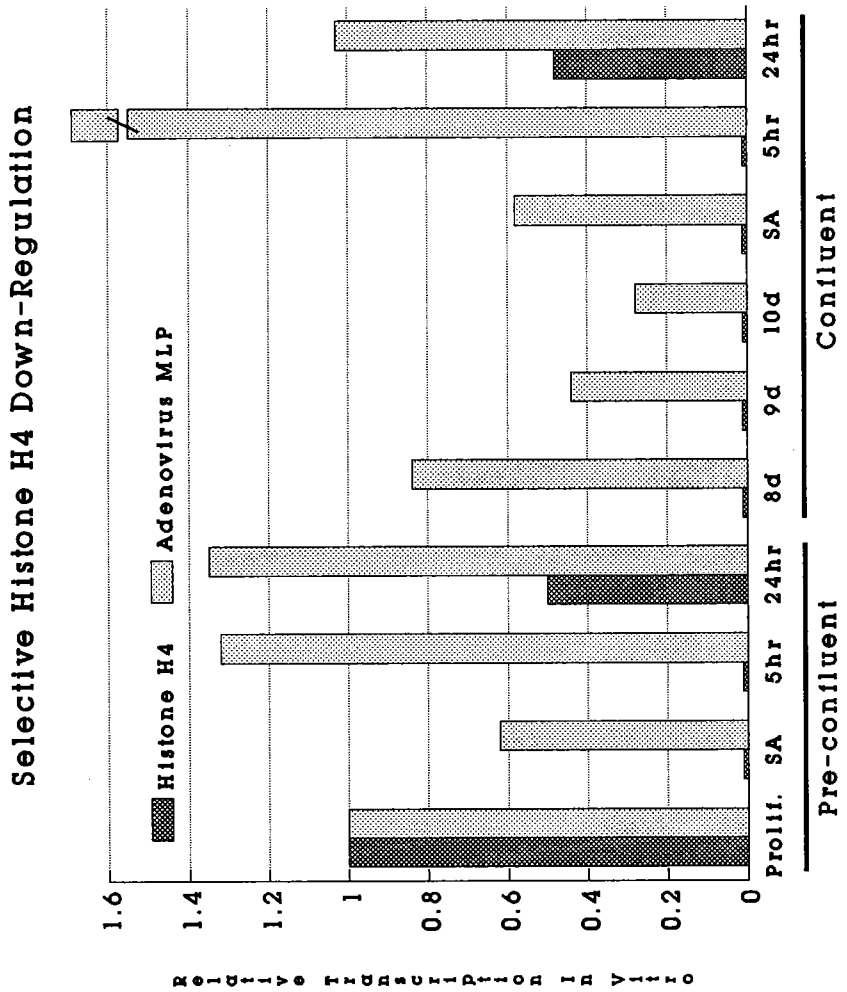

Prolif.
Confl.
8
9
10
Serum Arrest
Serum Stimulation
5hr
24hr
HeLa
Cap Site

Figure 4-7. Estimation of the general RNA polymerase II activity present in the nuclear extracts. The level of cell free transcription from the adenovirus 2 major late promoter (plasmid construct pMLH-1) (88) was used to demonstrate general RNA polymerase II activity in the nuclear extracts which have specifically down-regulated human H4 histone gene transcription. A) Proliferating normal diploid fibroblasts (lane 1), pre-confluent serum arrested cultures (lane 2), pre-confluent serum stimulated cultures 5 hours (lane 3) and 24 hours (lane 4) after stimulation, density inhibited cultures 8 (lane 5), 9 (lane 6), and 10 days (lane 7) after initial plating, density inhibited, serum arrested cultures (lane 8), and density inhibited, serum stimulated cultures 5 hours (lane 9) and 24 hours (lane 10) after stimulation. RNA transcripts were assayed by S1 nuclease protection as described in the Experimental Procedures. Broad arrowhead indicates the accurately initiated transcripts. B) Graphical comparison of the general RNA polymerase II activity (Adenovirus) with the specific histone H4 transcriptional activity. The samples are presented in the same order is described in part A.


1 2 3 4 5 6 7 8 9 10


Selective Histone H4 Down-Regulation
Histone H4
Adenovirus MLP
Relative Transcription in Vitro
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Prolif.
SA
5hr
24hr
8d
9d
10d
SA
5hr
24hr
Pre-confluent
Confluent

action (HiNF-D/Site II) for the down-regulation of transcription occurring at the onset of differentiation, serum deprived quiescence, or density dependent quiescence.

Serum stimulation of sparsely populated fibroblast cultures up-regulates HiNF-D binding activity and H4 histone gene transcription. Quiescent fibroblasts, in contrast to terminally differentiated cells, can be stimulated to re-enter the proliferative cycle (214). This system provides the opportunity to address the molecular mechanisms operative during the re-activation of histone gene expression and the role of the HiNF-D/Site II interaction in this process. Sparsely populated cultures were maintained in serum free medium for three days to induce the cells to enter a quiescent state and to down-regulate histone gene transcription and HiNF-D binding activity. The cultures were then stimulated by replacing the depleted medium with fresh medium containing 10% serum. The cells required approximately fourteen hours to exit the G_1 phase and enter the DNA replication period. The maximal rate of DNA synthesis occurred twenty four hours post-stimulation as reflected by the rate of ^{3}H-thymidine incorporation into DNA (Table 4-1).

The onset of DNA synthesis requires histone protein synthesis to package the newly replicated DNA and the

initiation of S phase is marked by a large increase in histone mRNA levels and elevated transcription in cycling cells (160). The same temporal and functional coupling of histone mRNA levels and DNA replication was demonstrated following serum stimulation of quiescent, serum deprived cells. Total cellular mRNA was isolated at five and twenty four hours after stimulation and assayed for FO108 H4 histone mRNA. H4 histone mRNA was not detectable in the quiescent cells or cells five hours after stimulation (Figure 4-3) but then accumulated dramatically in conjunction with maximal DNA synthesis at twenty four hours post-stimulation to levels found in rapidly proliferating populations.

Transcription of this H4 histone gene was monitored during serum stimulation and correlated with the presence of HiNF-D binding activity. High salt nuclear extracts were prepared from serum deprived cells and cells five and twenty four hours after serum stimulation. Nuclear extracts from the quiescent cultures and cultures five hours post-stimulation were unable to support detectable levels of *in vitro* transcription from the FO108 H4 histone gene (Figure 4-5). At twenty four hours after stimulation, when the fibroblasts had initiated DNA replication and histone

mRNA levels were elevated, the capacity for *in vitro* transcription of the H4 gene was recovered.

Each of the high salt nuclear extracts used for *in vitro* transcription was assayed for the presence of HiNF-D/Site II binding activity. The quiescent and five hour post-stimulation fibroblasts had down-regulated HiNF-D binding activity to less than 2% of levels found in proliferating cells, paralleling the down-regulation of proliferation and loss of H4 histone gene transcription. When the fibroblasts re-entered S phase and up-regulated H4 gene transcription, there was a concomitant up-regulation of the HINF-D binding activity (Figure 4-1). These results suggest that not only is HiNF-D involved in the down-regulation of H4 histone gene transcription occurring at quiescence, but it is also a critical element in the re-activation of transcription during serum stimulation.

Serum stimulation of density inhibited fibroblasts up-regulates H4 histone gene transcription but induces only threshold levels of HiNF-D binding activity. Human diploid fibroblasts will naturally cease to proliferate and enter an extended quiescent state when the cultures are allowed to become confluent. The density inhibited cultures can then be induced to undergo limited proliferation by serum

stimulation before returning to the quiescent state (12). Density dependent quiescence may be more analogous to conditions present in normal tissues. Thus, it is important to determine whether the same mechanisms regulating histone gene transcription are operational under these conditions.

Density inhibited, quiescent CF-3 fibroblasts were maintained in medium containing 0.1% serum for sixteen days. The fibroblasts were then stimulated by replacing the medium with fresh medium containing 10% serum. The time after stimulation at which the fibroblasts entered S phase was detected by ^{3}H-thymidine incorporation into DNA. The density inhibited cultures exhibited the same time course of stimulation as the sparsely populated, serum deprived cultures, with maximum DNA synthesis occurring twenty four hours post-stimulation (Table 4-1). H4 histone mRNA levels paralleled DNA synthesis during stimulation and were indistinguishable from the time course observed when sparsely populated fibroblast cultures were stimulated (Figure 4-4).

The relationship between HiNF-D binding activity and H4 histone gene transcription was examined during the stimulation of the density inhibited fibroblasts. High salt nuclear extracts were prepared from the quiescent cultures and cultures five and twenty four hours after serum stimulation. Only nuclear extracts prepared from fibroblasts

twenty four hours after stimulation were able to support detectable levels of *in vitro* transcription (Figure 4-6). H4 histone gene transcription was up-regulated at twenty four hours to 70% of the maximal level detected in rapidly proliferating cells. Both density inhibited and sparsely populated quiescent cells exhibited approximately the same rate of H4 histone gene transcription during the peak in DNA synthesis after serum stimulation.

Interestingly, the stimulation of HiNF-D binding activity in the density inhibited fibroblasts is not the same as in the sparsely populated cultures. HiNF-D binding activity is reduced to undetectable levels in the density inhibited, serum deprived cultures. However, at twenty four hours after stimulation, when H4 histone gene transcription has returned to near maximal levels, HiNF-D binding activity is up-regulated to only 30% of the level present in rapidly proliferating cells (Figure 4-2). The small stimulation of HiNF-D binding activity is in contrast to the near 100% stimulation induced in the sparsely populated fibroblast cultures twenty four hours after stimulation.

Discussion

The interaction between the proliferation specific transcription factor, HiNF-D, and the H4 histone gene cis-acting DNA element, Site II, is required for initiation of H4 histone gene transcription and has been demonstrated to play a critical role in the down-regulation of transcription occurring at the onset of differentiation. In the present study we have investigated the role of this interaction in regulating H4 histone gene transcription within the context of fibroblast quiescence and serum stimulation of proliferation.

Rapidly proliferating normal diploid CF-3 fibroblasts, derived from newborn human foreskin, have high levels of HiNF-D binding activity and H4 histone gene transcription. When sparsely populated cultures become quiescent due to serum deprivation or density inhibition, H4 histone gene transcription is down-regulated simultaneously with a loss of HiNF-D binding activity. The parallel between the specific down-regulation of HiNF-D binding activity and H4 histone gene transcription during quiescence is the same as demonstrated for the cessation of proliferation occurring at the onset of differentiation of HL60 cells and osteoblast cells (152,193). These results suggest that regulation of the HiNF-D/Site II interaction is an important event in the down-regulation of H4 histone gene transcription. Moreover,

regulation of this interaction may impinge on a common mechanism associated with the down-regulation of cell growth that accompanies differentiation, serum deprived quiescence and density dependent quiescence.

Sparsely populated cultures of quiescent fibroblasts can be serum stimulated to re-enter the proliferative cycle. This provides the opportunity to observe the re-activation of H4 histone gene transcription and the role of the HiNF-D/Site II interaction in this process. HiNF-D binding activity is undetectable in the quiescent populations but is up-regulated in parallel with the activation of H4 histone gene transcription as the cultures resume proliferation. This suggests that HiNF-D may not only be functionally related to the down-regulation of H4 histone gene transcription during the onset of quiescence or differentiation but, additionally, may be a key element in the up-regulation of transcription occurring during serum stimulation of normal quiescent cells.

Normal human fibroblasts, when allowed to become density inhibited, down-regulate proliferation and enter a G_0 resting state. This may be more analogous to the conditions present in tissues. Serum stimulation of density dependent, quiescent fibroblasts initiates proliferative activity and the required H4 histone gene transcription. However, HiNF-D binding activity is up-regulated to only 30%

of maximal levels. Because H4 histone gene transcription is activated to near normal levels, the 30% of HiNF-D binding activity present may represent a threshold level of HiNF-D required by the fibroblast. Furthermore, the difference in activation of HiNF-D binding activity between serum stimulation of sparsely populated quiescent fibroblasts and density dependent quiescent fibroblasts indicates two different mechanisms may be operational in the down-regulation of HiNF-D binding activity.

One possible model for the regulation of the HiNF-D binding activity observed incorporates a primary mechanism controlling HiNF-D binding activity in actively proliferating fibroblasts and fibroblasts which are serum arrested but capable of proliferation. This may be the same mechanism controlling the cell cycle regulation of HiNF-D observed by Holthuis et al (1990) in normal diploid cells. Upon density dependent quiescence and entry into the G_0 state, a second mechanism is activated to repress HiNF-D binding activity. Serum stimulation is not able to completely overcome this second level of repression. One can speculate that the function of a second mechanism down-regulating HiNF-D binding activity would be to control the replication dependent H4 histone gene transcription in cells which are programmed to remain quiescent or to undergo only a limited amount of proliferation before re-entering a

quiescent state. The appearance of an extracellular matrix and cell to cell communication analogous to that found in an intact tissue may be the means by which the cell is signaled to enter a deeper quiescent state and activate a second mechanism to down-regulate proliferation specific factors such as HiNF-D. A process akin to the serum stimulation of density inhibited cultures may be the limited proliferative activity observed in dermal fibroblasts during wound healing (201).

In conclusion, the HiNF-D/Site II interaction plays a critical role in the regulation of H4 histone gene transcription. It is important during both the down-regulation of proliferation at the onset of differentiation and quiescence and the up-regulation of proliferation due to serum stimulation of quiescent fibroblasts. HiNF-D binding activity is regulated minimally at two levels dependent on the proliferative state of the cell and the programmed future requirements for H4 histone gene transcription.

CHAPTER 5

DISCUSSION

Expression of the growth related FO108 human H4 histone gene is regulated in response to both cell cycle progression and the proliferative state of the cell. The control of expression is mediated at both the transcriptional and post-transcriptional levels. The investigation presented in this dissertation has focused on the transcriptional aspects of histone gene regulation.

We developed a cell free transcription system capable of accurately initiating transcription from the H4 histone gene promoter and utilized it to assess the functional contribution of upstream sequence elements. Our results (for a schematic reveiw of all of the results see Appendix B) identified the *in vivo* protein-DNA interaction domain Site I as a bipartite transcriptional activator element. Furthermore, two distal activator elements and two potential repressor elements were defined between -1000 and -200 base pairs upstream of the initiation site. Employing a myriad of *in vitro* techniques designed to detect protein-DNA interactions we characterized the trans-acting factors mediating the transcriptional stimulation from the activator elements. The functionally bipartite Site I element (-124 to -90 base pairs) formed protein-DNA complexes with two

distinct transcription factors, HiNF-E and HiNF-C. HiNF-E bound to the distal side of Site I and interacted with a consensus recognition sequence for the ATF transcription factor family. Binding site competition and mutagenesis confirmed HiNF-E is a member of the family of ATF transcription factors. The originally characterized ATF transcription factors were 43 to 47 kDa, however, molecular weight analysis of the HiNF-E binding site interaction detected an 84 kDa protein. This is a novel molecular weight for the ATF transcription factor family and thus we termed it ATF-84. Additionally, purification by DNA affinity chromatography and preliminary immunochemical analysis suggested ATF-84 is structurally related to ATF-43/47. HiNF-C binds adjacent to HiNF-E in Site I and requires an Sp1 like recognition sequence for binding. The binding characteristics of HiNF-C, including a dependence on Zn^{2+}, are indistinguishable from the transcription factor Sp1. Because extensive studies of Sp1 have not shown it to be part of a family of related factors, these observations strongly suggest HiNF-C and Sp1 are identical. In addition to the stimulation of transcription exerted by HiNF-C/Sp1, binding of this factor to Site I dramatically stabilized the adjacent interaction of the HiNF-E/ATF-84 factor. This interaction may be a key to the preferential binding of the 84 kDa ATF factor to the H4 histone promoter and may be an

important aspect in the regulation of this gene. Recently, Tjian and co-workers have described an Sp1-Sp1 interaction which can synergistically activate transcription and Janson & Pettersson (101) have characterized a cooperative binding interaction between Sp1 and OTF-1. These results together suggest that protein-protein interactions involving Sp1 may be a common mechanism for the regulation of transcription (44).

The near-distal activator region (-418 to -213 base pairs) stimulated transcription 2 - 4 fold and formed a single protein-DNA complex, H4UA-1. The binding site for H4UA-1 contained recognition sequences for both the CTF/NF-1 transcription factor and the thyroid hormone receptor (TRE). Interestingly, hormone response elements are commonly clustered with recognition sites for other transcription factors and both simultaneous and competitive binding of the factors has been observed (15,26,177,196). However, as yet little is known about the role of dual binding affinities at hormone response elements. The presence of a hormone response element in the human H4 histone gene suggests a direct mechanism by which histone gene transcription can rapidly respond to changes in growth due to hormone induction.

The far-distal activator region (-730 to -589 base pairs) was the strongest activator detected, stimulating

transcription 10 fold. A complex pattern of protein-DNA interactions consisting of at least three factors was detected in this region. Two of the factors, H4UA-3 and H4UA-4, bound to previously undescribed recognition sequences, however, H4UA-2 interacted at a second ATF recognition sequence. Binding site competition studies between HiNF-E and H4UA-2 demonstrated that they interacted with similar factors and a molecular weight determination of the factors binding at H4UA-2 detected an 84 kDa protein. Because ATF-84 has not been detected by laboratories investigating ATF binding activities in other promoters, these results suggest that ATF-84 may be directed specifically to histone promoters. An ATF binding site has been localized in the first 200 base pairs of the ST519 human H3 histone gene promoter (A. van Wijnen, personal communication) and in a wheat H3 histone gene promoter (198). Furthermore, a search of published histone promoter sequences detects several potential binding sites for ATF. It will be important to determine if ATF-84 is selectively used in these histone promoters as well, which would provide the first evidence of a mechanism to coordinately regulate different types of histone genes.

The FO108 H4 human histone gene promoter contains a second *in vivo* protein-DNA interaction domain, Site II.

Characterization of this domain by van Wijnen *et al.* (202) has identified the nuclear factor HiNF-D and initial *in vivo* and *in vitro* studies revealed the Site II/HiNF-D interaction to be preferentially lost during differentiation (193). Our findings extended these results to normal diploid cells and demonstrated that HiNF-D is a proliferation-specific transcription factor required to switch on H4 histone gene transcription. HiNF-D binding activity paralleled the loss of histone gene transcription as normal diploid fibroblasts entered a quiescent state. Furthermore, the binding activity was activated in concert with the return of histone gene transcription when the cells were stimulated to re-enter the proliferative cycle. Recently, Holthuis *et al.* (95) has shown HiNF-D binding activity to be regulated during the cell cycle of normal diploid cells, suggesting this factor is important for cell cycle control of transcription. Additionally, promoter fusion constructs containing only the Site II domain are transcribed in a cell cycle manner (A. Ramsey-Ewing, personal communication). However, the HiNF-D/Site II interaction does not change during the cell cycle of transformed cells despite the well documented cell cycle regulated transcription of histone genes in these cells. These results could be explained by a cell cycle regulated modification in the activation domain of HiNF-D

which in normal diploid cells would cause a loss of the binding activity but in transformed cells would not effect the binding activity. A second, more intriguing possibility is that the H4 histone gene contains multiple cell cycle elements which can compensate for the loss of any single element. Due to the procedures (5' deletion analysis) by which cell cycle elements have been investigated, only the last most proximal element would be detected, in this case HiNF-D/Site II. A possible candidate for the second cell cycle element is the HiNF-E/ATF-84 interaction at Site I. The stimulatory activities of several of the ATF transcription factor family members are responsive to phosphorylation by the cAMP dependent protein kinase and one has been described to respond to fluctuations of Ca^{2+} inducible phosphorylation. It will be interesting to determine if the activity of ATF-84 is also regulated by phosphorylation and whether the changes in phosphorylation occur in a cell cycle regulated manner.

The effects of the distal half of Site II (the HiNF-D binding site) on transcription *in vivo* and *in vitro* are slightly different. *In vivo*, as assayed in stable cell lines, the H4 histone promoter containing sequences only up through the TATA box (proximal half of Site II) is incapable of site specific initiation of transcription, however, the addition

of the remainder of Site II allows a low level of correctly initiated transcription to occur. In contrast, the level and specificity of *in vitro* transcription is indistinguishable between the 5' deletion constructs either with or with out the distal Site II region. This result suggests that the *in vitro* system has overcome the requirement for the HiNF-D/Site II interaction and perhaps indicats HiNF-D plays a role in opening up the initiation site for polymerase entry but due to the relaxed torsional stress present *in vitro* as compared to *in vivo* this activity is not required. However, if a promoter containing Site II and additional upstream elements is assayed for transcription using a nuclear extract deficient in HiNF-D/Site II binding activity (serum arrested nuclear extracts) *in vitro* transcription activity is not observed. One explanation for the requirement HiNF-D in a promoter containing upstream elements but not in a promoter without upstream elements suggests HiNF-D may also be serving as a target for the action of the distally located transcription factors present only in the larger promoter.

A comparison of our results to those with other vertebrate histone genes reflects limited similarities. The primary focus of most research has been the identification of cell cycle elements. Several groups have clearly

demonstrated that the 5' promoter region is sufficient to direct cell cycle regulation of initiation (7,28,74,151). The highly conserved sub-type specific elements of the H1 and H2B histone genes are required for maintaining the S phase specific stimulation of transcription (49,64,115). Interestingly, the H2B element is a member of the ubiquitous octamer binding transcription factor family, while the H1 histone element appears to be found only in H1 histone genes. Partial characterization of a 32 base pair region in the hamster H3 histone gene has identified a third cell cycle element (8). These results combined with our data on the H4 histone gene suggest that each of the histone gene sub-types may have distinct cell cycle elements.

A second human H4 histone gene (Hu4a) has been studied by Dailey *et al*. and two transcription factors which bind within the first 120 base pairs have been partially purified (47). H4TF-2 binds at the upstream side of the TATA box in a region which is highly similar to the Site II domain of the FO108 H4 histone gene. Similar patterns of DNA contact points have been described for H4TF-2 and HiNF-D, yet the factors behave differently during purification. Although these factors are undoubtablly related, the exact relationship has yet to be determined. One intriguing possibility is that H4TF-2 is a sub-component of a much

larger HiNF-D complex which is not formed with the Hu4a promoter. H4TF-1 is the second factor characterized as binding to the Hu4a histone gene promoter and is positioned at -113 to -90 base pairs. This factor binds to a G-C rich element 5'-GGGGGAGGG-3' similar to the HiNF-C binding site. However, the H4TF-1 binding site does not interact with Sp1, in contrast to the HiNF-C/Sp1 interaction we described in the FO108 H4 histone gene promoter. Interestingly, there is an Sp1 binding site between the H4TF-1 and H4TF-2 elements, though interactions at this site have not been identified. Thus it appears that the promoters of human H4 histone genes have diverged substantially with the exception of the region around the TATA box defined by Site II. Additional studies of the proximal and distal promoters of other human H4 histone genes will be required to understand fully the mechanisms regulating coordinated and cell cycle controlled transcription of H4 histone genes.

In summary, we have characterized several of the cis-acting sequence elements and trans-acting transcription factors mediating regulated transcription of a cell cycle regulated human H4 histone gene. Our results have described a promoter with a complex modular architecture extending at least 1000 base pairs upstream of the transcription initiation site and have defined both common and potentially

histone specific transcription factors mediating the regulation of transcription.

APPENDIX A

COMPLETE SEQUENCE OF THE FO108 HUMAN H4 HISTONE GENE

```
¦002
GGATCCGTAGAAACGGATGCACAGAATATCCCTCAGTCTTCTCTATGTAGCAGGCCCTCCA
                                  -1000
CCTAGGCATCTTTGCCTACGTGTCTTATAGGGAGTCAGAAGAGATACATCGTCCGGGAGGT

                                                          ¦D1
TATACGCGGGTTCCCCAAGACCGAAAATATTAAACAAATGAATTTCTTTTTTAAAAAAAA
                        -950
ATATGCGCCCAAGGGGTTCTGGCTTTTATAATTTGTTTACTTAAAGAAAAAATTTTTTTT

GTACAACAAAAGATAGTAAAAATAAAAACAGTTAAACAATTACTTACATAGCTTTACACA
              -900
CATGTTGTTTTCTATCATTTTTATTTTTGTCAATTTGTTAATGAATGTATCGAAATGTGT

CTGGATTGGTGTTCGAAGTAATTTGAGCTTATTTAAAGTACACGGGAGGATGTGCATAGT
     -850
GACCTAACCACAAGCTTCATTAAACTCGAATAAATTTCATGTGCCCTCCTACACGTATCA

TATGTGCAAATACTACCCCACTTTCTATGAGAGACTTGAGCAACCTGGATTTTGGTATCG
                                                  -750
ATACACGTTTATGATGGGGTGAAAGATACTCTCTGAACTCGTTGGACCTAAAACCATAGC

      ¦E9
GCGGGGGCCCTGGACCAATCCCCTCTCAGTTCTACCGAGGGAGAACTGTTTTGTTTCTTC
                                  -700
CGCCCCCGGGACCTGGTTAGGGGAGAGTCAAGATGGCTCCCTCTTGACAAAACAAAGAAG

CAGCACGGCTTTGACCGACAGTGTGTTGGGATTCGCTGACGTCCATGAGAAAGCTTGGCA
                        -650
GTCGTGCCGAAACTGGCTGTCACACAACCCTAAGCGACTGCAGGTACTCTTTCGAACCGT
```

```
                                  ¦007
GCATGCTGAGACCAATTTTCCCAGGGCCAGAATTCTCCTGTGTGAGCTAAAATACAGTGC
                 -600
CGTACGACTCTGGTTAAAAGGGTCCCGGTCTTAAGAGGACACACTCGATTTTATGTCACG


GTCGGTCCAACAAAACAGAGCCTGGAGCCAGGAATTATGGCGAACCTGCTCCCTCCCGTC
         -550
CAGCCAGGTTGTTTTGTCTCGGACCTCGGTCCTTAATACCGCTTGGACGAGGGAGGGCAG


CTCCCTTGGCGCACAGATCCCTGGCGCCGTCCTTGAGGTCGCCTTCGGTGTTGACCTCAT
                                          -450
GAGGGAACCGCGTGTCTAGGGACCGCGGCAGGAACTCCAGCGGAAGCCACAACTGGAGTA


                      ¦005
CGTCGGAACGGCGCTTCCTGAAGCTTTATATAAGCACGGCTCTGAATCCGCTCGTCGGGA
                                 -400
GCAGCCTTGCCGCGAAGGACTTCGAAATATATTCGTGCCGAGACTTAGGCGAGCAGCCCT


TTAAATCCTGCGCTGGCGGCCTCCTGCCAGTCTCTGGCCTCCATTTGCTCTTCCTGAGGC
                           -350
AATTTAGGACGCGACCGCCGGAGGACGGTCAGAGACCGGAGGTAAACGAGAAGGACTCCG


TCCCTCCAGAGACCTTTCCCTTAGCCTCAGTGCAATGCTTCCGGGCGTCCTCAGACCAGA
            -300
AGGGAGGTCTCTGGAAAGGGAATCGGAGTCACGTTACGAAGGCCCGCAGGAGTCTGGTCT


                                                  ¦108
CACAGCCAAAGCCACTACAGAATCCGGAAGCCCCGGTTGGGATCTGAATTCTCCCGGGGA
         -250
GTGTCGGTTTCGGTGATGTCTTAGGCCTTCGGGGCCAACCCTAGACTTAAGAGGGCCCCT


               ¦L14                               ¦K8
CTGTGGCGTCAGGTTAAAAAAAAAAAAGAGTGAGAGGGACCTGAGCAGAGTGGAGGAGGA
                                                -150
GACACCGCAGTCCAATTTTTTTTTTTTCTCACTCTCCCTGGACTCGTCTCACCTCCTCCT
```

J50

GGGAGAGGAAAACAGAAAAGAAATGACGAAATGTCGAGAGGGCGGGGACAATTGAGAACG

-100

CCCTCTCCTTTTGTCTTTTCTTTACTGCTTTACAGCTCTCCCGCCCCTGTTAACTCTTGC

J56 J67

CTTCCCGCCGGCGCGCTTTCGGTTTTCAATCTGGTCCGATACTCTTGTATATCAGGGGAA

-50

GAAGGGCGGCCGCGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTT

GACGGTGCTCGCCTTGACAGAAGCTGTCTATCGGGCTCCAGCGGTCATGTCCGGCAGAGG

+1

CTGCCACGAGCGGAACTGTCTTCGACAGATAGCCCGAGGTCGCCAGTACAGGCCGTCTCC

AAAGGGCGGAAAAGGCTTAGGCAAAGGGGGCGCTAAGCGCCACCGCAAGGTCTTGAGAGA

50

TTTCCCGCCTTTTCCGAATCCGTTTCCCCCGCGATTCGCGGTGGCGTTCCAGAACTCTCT

CAACATTCAGGGCATCACCAAGCCTGCCATTCGGCGTCTAGCTCGGCGTGGCGGCGTTAA

150

GTTGTAAGTCCCGTAGTGGTTCGGACGGTAAGCCGCAGATCGAGCCGCACCGCCGCAATT

GCGGATCTCTGGCCTCATTTACGAGGAGACCCGCGGTGTGCTGAAAGTGTTCTTGGAGAA

200

CGCCTAGAGACCGGAGTAAATGCTCCTCTGGGCGCCACACGACTTTCACAAGAACCTCTT

TGTGATTCGGGACGCAGTCACCTACACCGAGCACGCCAAGCGCAAGACCGTCACAGCCAT

250

ACACTAAGCCCTGCGTCAGTGGATGTGGCTCGTGCGGTTCGCGTTCTGGCAGTGTCGGTA

GGATGTGGTGTACGCGCTCAAGCGCCAGGGGAGAACCCTCTACGGCTTCGGAGGCTAGGC

300

CCTACACCACATGCGCGAGTTCGCGGTCCCCTCTTGGGAGATGCCGAAGCCTCCGATCCG

GCCGCTCCAGCTTTGCACGTTTCGATCCCAAAGGCCCTTTTTGGGCCGACCACTTGCTCA

350

CGGCGAGGTCGAAACGTGCAAAGCTAGGGTTTCCGGGAAAAACCCGGCTGGTGAACGAGT

TCCTGAGGAGTTGGACACTTGACTGCGTAAAGTGCAACAGTAACGATGTTGGAAGGTAAC

450

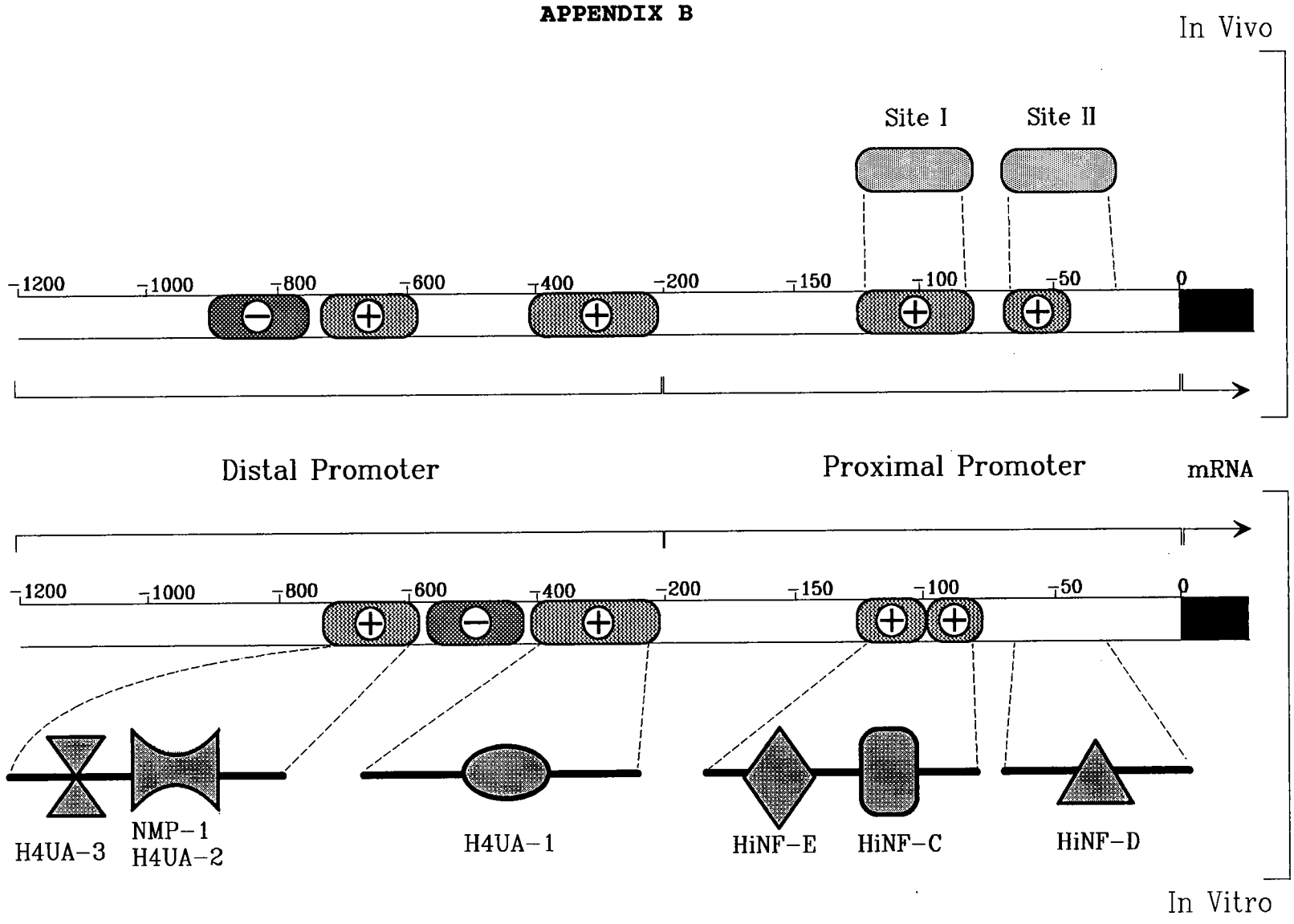
AGGACTCCTCAACCTGTGAACTGACGCATTTCACGTTGTCATTGCTACAACCTTCCATTG

TTTGGCAGTGGGGCGACATTCGGATCTGAAGTTAACGGAAAGACATAACCGC

500

AAACCGTCACCCCGCTGTAAGCCTAGACTTCAATTGCCTTTCTGTATTGGCG

APPENDIX B


In Vivo
Site I
Site II
-1200
-1000
-800
-600
-400
-200
-150
-100
-50
0
Distal Promoter
Proximal Promoter
mRNA
H4UA-3
NMP-1
H4UA-2
H4UA-1
HiNF-E
HiNF-C
HiNF-D
In Vitro

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BIOGRAPHICAL SKETCH

Kenneth Lynn Wright was born on March 12, 1962 in Tampa, Florida. He attended Brandon High School and graduated in the spring of 1980 in the top 2% of his class. He then entered the University of Florida and received a Bachelor of Science degree in Chemistry in the spring of 1984. In the fall of 1984, he joined the laboratory of Drs. Gary and Janet Stein in the Department of Biochemistry and Molecular Biology at the University of Florida and began the study of the regulation of human histone gene expression. In August of 1987, he transferred to the University of Massachusetts, Worcester in order to continue his research with Drs. Gary and Janet Stein, who had joined the Department of Cell Biology at the University of Massachusetts, Worcester. Kenneth married Gabriëla Marie Joseé Schiks of The Netherlands on April 5, 1986 and together they have a beautiful daughter, Sarah Grace Wright, born on February 6, 1989.