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Transcriptional Control of Human Histone Gene Expression: Delineation and Regulation of Protein/DNA Interactions: A Thesis

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TRANSCRIPTIONAL CONTROL OF HUMAN HISTONE GENE EXPRESSION: DELINEATION
AND REGULATION OF PROTEIN/DNA INTERACTIONS

A Thesis Presented

By

André John van Wijnen

Submitted to the Faculty of the
University of Massachusetts Graduate School
of Biomedical Sciences, Worcester
in partial fulfillment of the requirement for the degree of:

DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES

MAY 1991

CELL BIOLOGY

TRANSCRIPTIONAL CONTROL OF HUMAN HISTONE GENE EXPRESSION: DELINEATION AND REGULATION OF PROTEIN/DNA INTERACTIONS

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Thomas Miller, Dean of the Graduate
School of Biomedical Sciences

Department of Cell Biology
May   1991

DEDICATION

This thesis is dedicated to my parents

who have always shown me

unfailing love, support and understanding

ACKNOWLEDGMENTS

The intense enthusiasm and dedication of Drs. Gary and Janet Stein, as well as the zeal and loyalty of the many colleagues who have worked in their laboratory during the course of this project, have played a tremendous role in succesful completion of the experimental phase of this dissertation. I deeply appreciate this fine group of people for their personal encouragement, expert guidance and selfless cooperativity, as well as for consistently showing their strong commitment to the idealistic goals of science. I also would like to thank the members of my Thesis Committee who have volunteered their time and advice in the preparation of this dissertation.

The first part of my stay in the Stein's laboratory at the University of Florida, Gainesville was a most rewarding experience through the help and wisdom of Linda Green, as well as Drs. Farhad Marashi, Alan Shiels and Urs Pauli. I also appreciate the opportunity to have worked with fellow students Robert Massung and Jesse Hirschman, and with Dr. Charles Stewart. In addition, I have fond memories of the interactions with Martijn Gerretsen, Dick Schaap, Laurens Wilming and many other Dutch student-exchange visitors at the University of Florida campus. The Dutch contingent made this period a perfect blend between hard-core science and deserved relaxation in a joyful climate.

The stimulating environment created by the University of Florida graduate students was invaluable during the second part in the Stein's laboratory at the University of Massachusetts, Worcester. I would like to thank Paul Kroeger, Ken Wright and Anna Ramsey-Ewing for valuable

ACCOMPANYING THESES

(1)   "The most credible statement in animal-studies is the notion that the subjects are usually *irreversibly killed* as a result of retrieving vital organs by dissection." (journal club meeting at the University of Florida, 1984-1987)

(2)   "The term *repressor* in the study of gene regulation does not always refer to a molecular entity, but may also relate to the working attitudes of individual investigators." (laboratory meeting at the University of Florida, 1984-1987).

(3)   "The best way to avoid spilling radio-active materials on a laboratory bench is to cover the entire room from the *floor to the ceiling* with absorbing paper." (observation at the University of Florida, 1984-1987)

(4)   "The best fitting curve that describes a biological parameter measured by two single data-points is a line *perpendicular* to the line connecting these points." (laboratory meeting at the University of Florida, 1984-1987)

(5)   "Experimental attempts to obtain the DNA sequence of ^{32}P-gamma ATP are hopelessly doomed from the onset for *several* reasons." (observation at the University of Florida, 1984-1987)

(6)   "Incorporation of only one carbon atom in the structural formula

of ethanol, while answering questions during a qualifying

examination for the PhD degree in Biochemistry, is a deliberate

and *truly disrespectful* act towards the examinator."

(qualifying examinations at the University of Florida, 1984-1987).

(7)   "The best way to correct multiple data for experimental variation

is to normalize values with respect to a control parameter that is

reciprocally related to the test-parameter"

(observation at the University of Massachusetts, 1987-1991)

(8)   "The visual impact of scientific data is *more* important than the

meaning of the conclusions that can be drawn from these data"

(observation at the University of Massachusetts, 1987-1991)

(9)   "Foreigners usually have difficulties understanding why it is so

funny that because of their chemistry men can't make a vitamin,

but can make a hormone."

(conversation overheard at the University of Florida, 1984-1987)

(10)   "Keep up, or shut up" (quotation from an empathetic colleague,

University of Massachusetts, 1987-1991).

GENERAL ABSTRACT

TRANSCRIPTIONAL CONTROL OF HUMAN HISTONE GENE EXPRESSION: DELINEATION
AND REGULATION OF PROTEIN/DNA INTERACTIONS

By

André John van Wijnen

May 1991

Thesis Advisor: Gary S. Stein
Department: Cell Biology

Transcriptional regulation of cell cycle controlled genes is
fundamental to cell division in eukaryotes and a broad spectrum of
physiological processes directly related to cell proliferation.
Expression of the cell cycle dependent human H4, H3 and H1 histone genes
is coordinately regulated at both the transcriptional and post-
transcriptional levels. We have systematically analyzed the protein/DNA
interactions of the immediate 5′regions of three prototypical cell cycle
controlled histone genes, designated H4-FO108, H3-ST519 and H1-FNC16, to
define components of the cellular mechanisms mediating transcriptional
regulation.

Multiple biochemically distinct protein/DNA interactions were
characterized for each of these genes, and the binding sites of several
promoter-specific nuclear DNA binding activities were delineated at
single nucleotide resolution using a variety of techniques. These
findings were integrated with results obtained by others and revealed
that the *in vitro* factor binding sites in H4, H3 and H1 histone
promoters coincide with genomic protein/DNA interaction sites defined *in*

vivo for the H4-F0108 and H3-ST519 genes, and with evolutionarily conserved cis-acting sequences shown to affect the efficiency of histone gene transcription. Specifically, we have defined binding sites for Sp1, ATF, CP1/NF-Y, HiNF-D, HiNF-M, HiNF-P and HMG-I related factors. Based on sequence-similarities and cross-competition experiments, we postulate that most of these protein/DNA interaction elements are associated with more than one class of histone genes. Thus, the protein/DNA interactions characterized in this study may represent components of a cellular mechanism that couples transcription rates of the various histone gene classes.

Regulation of the protein/DNA interactions involved in transcriptional control of these H4, H3 and H1 histone genes was investigated in a spectrum of cell types using several distinct *in vitro* cell culture models for the onset of differentiation and quiescence, as well as cell cycle progression. Moreover, we studied control of histone gene associated DNA binding activities during hepatic development from fetus to adult in transgenic mice reflecting the onset of differentiation and quiescence *in vivo*. We show that the H4 histone promoter protein/DNA interaction mediated by factor HiNF-D is *selectively* modulated, and directly at the level of DNA binding activity, during the entry into, progress through and exit from the cell cycle in normal diploid cells, as well as during hepatic development. The regulation of this protein/DNA interaction occurs in parallel with analogous interactions occurring in H3 and H1 histone genes. Moreover, these proliferation-specific protein/DNA interactions are collectively

deregulated during the cell cycle in four distinct cell types displaying properties of the transformed phenotype. Hence, the cellular competency to coordinately transcribe distinct classes of histone genes during the cell cycle may be mediated by the intricate interplay of constitutively expressed general transcription factors and temporally regulated, cell growth controlled nuclear factors interacting specifically with cell cycle dependent histone genes.

Finally, we show that HiNF-D is represented by two electrophoretically distinct species. The ratio of these forms of HiNF-D fluctuates dramatically during the cell cycle of normal diploid cells, but remains relatively constant in tumor cells. Total HiNF-D binding activity embodied by both HiNF-D species is negatively influenced *in vitro* by incubation with exogenous phosphatase activity. These observations provide a first indication for the hypothesis that HiNF-D may exist in distinct post-translationally modified forms that are subject to a stringent cell growth control mechanism involving protein kinases and phosphatases. Such a cellular post-translational modification mechanism, which directly impinges on (or activates) the DNA binding activity of a key factor controlling histone genes, would provide a highly efficient means by which to influence the rate of transcription in rapid response to intra-cellular requirements for histone mRNA and extra-cellular cues signalling the onset and cessation of cell proliferation.

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CHAPTER 1

INTRODUCTION

1.0: Summary.

Production of histone proteins, structural components of chromatin, represents a paradigm for cell cycle control of eukaryotic gene expression during cell growth and development from embryonic expansion to senescence, as well as deregulation of stringent cell growth control during tumorigenesis. Five major DNA replication-dependent histone gene classes exist (designated H1, H2A, H2B, H3 and H4). Each class represents a functionally expressed multi-gene family whose members encode essentially the same or closely related histone proteins. Although control of histone gene expression is mediated at multiple regulatory levels, transcriptional regulation represents a prominent level of control. This is illustrated by the requirement for histone mRNA synthesis during the cell cycle, and downregulation of histone gene transcription during differentiation. Moreover, transcription of these five gene classes is coordinately regulated during entry into, progress through and exit from the cell cycle. Arguments are presented that provide the scope for transcriptional models of histone gene expression based on sequence specific protein/DNA interactions established in vitro. These models should take into account molecular parameters that mediate specific recognition of the mRNA start site, efficiency of initiation, selective discrimination of histone and other genes, and the extent by which transcription is coupled to specific stages of the cell cycle.

1.1: Critical role of histone gene expression in the life cycle of eukaryotes.

1.1.1: Histone proteins function in the packaging of DNA during the cell cycle.

Development from a totipotent zygote to an adult organism with a variety of specialized tissues is mediated by the intricate interplay of multiple gene regulatory programs. These programs coordinately control proliferation from a unicellular to a multicellular stage and the concomitant specialization of a series of distinct cell growth systems. Such individual, organized cell populations mutually influence each other both internally and externally and are generally characterized at the cell population level by a reciprocal relationship between expression of genes chracteristic of cells displaying either the proliferation- or differentiation-specific phenotype. At the cellular level, in the most orthodox abstraction, each cell can be considered a binary unit that can adopt two fundamentally different biological states: "cycling" (proliferating) versus "non-cycling" (quiescent or differentiated).

The proliferative phase (reviewed in Baserga, 1985; Cross et al., 1989; Murray & Kirschner, 1989; Pardee, 1989; Weinberg, 1989; Levine, 1990) is characterized by a sequential series of biochemical events that is first aimed at the duplication of the eukaryotic genome during S-phase, followed by the ordered segregation of multiple chromosomes into two daughter cells during and as a result of mitosis. The maintenance of nuclear architecture during the various phases of the cell division

cycle is considered critical in the orchestration of dual genome separation, and because of this appears to be a prerequisite for completion of the cell growth process. Structural and functional patterns of molecular organization within the nucleus at several scales of length accommodate the entire complement of linear chromosomal DNA in an orderly manner within the confines of the compact eukaryotic nucleus, even though each DNA molecule is several orders of magnitude larger than the nuclear diameter. The main levels of DNA condensation are mediated by histone proteins.

1.1.2: De novo histone synthesis during S-phase involves a prominent gene regulatory program.

Histones are a family of related and evolutionarily highly conserved proteins comprised of five major classes of polypeptides designated, respectively, H1, H2A, H2B, H3 and H4. Two copies of each of the core histones (H2A, H2B, H3 and H4) specifically aggregate into a nucleosome, the basic unit involved in facilitating the folding of linear DNA and the spiralling of approximately 0.2 kB around these octameric nuclear protein bodies. Higher order levels of chromatin organization are achieved via H1 histones (reviewed by McGhee & Felsenfeld, 1980; Kornberg & Klug, 1981).

The following example of calculation illustrates in rough approximation the magnitude of protein synthesis that is required for constructing chromatin with newly synthesized polynucleotide chains and histones. Newly replicated DNA is rapidly incorporated into nucleosomes.

Typical mammalian cells with, for instance, a 4 X 10^6 kB genome must assemble minimally 2 X 10^7 nucleosomes and synthesize at least 4 X 10^7 copies of each histone subtype, or 2 X 10^8 polypeptides in total. Actively dividing cells do not contain storage sites for histones other than those already utilized in formation of chromatin stucture necessitating de novo protein synthesis. The vast amount of protein required to package the genome reflects formation of 3 X 10^{10} peptide bonds (assuming on average 150 amino acids per histone). This is several-fold the number of nucleotides fused together by phosphodiester bonds during DNA replication.

The efficiency by which histone genes must be expressed to accommodate a rapid rate of histone protein synthesis is illustrated by the following: a minimum of approximately 4 X 10^7 copies of each histone subtype must be synthesized by a hypothetical cell during S-phase with a duration of, for example, 400 minutes. Hence, at least about 10^5 copies of each histone class must be synthesized per minute on average, or several thousands per second. This example indicates that the cellular machinery allocates a substantial portion of its gene-expression resources during S-phase to the production of histone structural proteins. The cell also uses this capability for synthesizing those multiple enzymatic activities required for maintaining appropiate levels of the various amino acid and nucleotide pools and those directly performing the process of DNA replication. (reviewed by Laskey et al., 1989; see also Kauffman & Kelly, 1991; Bradley et al., 1990; and references therein). However, enzymes can be used and recycled

frequently for the same function and modulated at the level of biochemical activity, whereas this does not apply to histone proteins. It is clear that control of histone genes represents a fundamental paradigm for the regulation of gene expression, directly at the level of protein-encoding nucleic acids, in the life-cycle of every eukaryotic organism.

1.1.3: Biological constraints on gene regulatory models of histone gene expression.

Models describing the gene regulatory program that regulates the timing and coordination of histone multi-gene expression must take into account a number of general biological and biochemical criteria, including the following: (I) histone gene expression occurs, or may occur, during all stages of development from embryonic expansion to senescence in cells of diverse ontogeny; (II) regulation of histone gene expression is at least indirectly responsive to extracellular cues (growth factors) signalling the initiation of cell division from quiescence, or cessation of proliferation during differentiation or re-entry into quiescence; (III) silencing of histone gene expression is reversible in non-proliferating, terminally differentiated cells during tumorigenesis; and (IV) histone gene expression is functionally and temporally coupled to the initiation and termination of DNA synthesis during the cell cycle.

Apart from this, in anticipation of a detailed molecular description, this regulatory program may be characterized by: (V) a

well-defined hierarchy in organization that allows continuous,

stoichiometric adjustments in the synthesis of the various histone

proteins at any of the multiple possible gene regulatory levels; (VI)

redundancy in gene control to prevent aberrant synthesis of histone

proteins in cells where this fine-tuning mechanism is not fully

operative; (VII) participation of control elements (or factors) at some

gene regulatory level in coordinating histone gene expression with

expression of other S-phase related genes, perhaps in this manner cross-

coupling several alternative, cell cycle specific gene regulatory

programs; and (VIII) efficient means by which to silence and activate

mutiple potential gene regulators, and ultimately histone gene

expression, as cells enter into, progress through and exit from the cell

cycle. Models of histone gene transcriptional control that invoke

sequence specific, regulatory protein/DNA interactions should fit within

these biological constraints and may have some of the above

characteristics.

The principles governing transcriptional regulation of prokaryotic

(Jacob & Monod, 1961; Gilbert & Muller-Hill, 1966; Emmer et al., 1970)

and eukaryotic viral genes (Tooze, 1981) have been firmly established.

The first promoter DNA elements of eukaryotic genes were identified by

sequence-alignments (Breathnach & Chambon, 1981) and mutational analyses

(Grosschedl & Birnstiel, 1980; McKnight & Kingsbury, 1982) of the region

immediately upstream of the mRNA start-site. Tjian and collaborators

identified the first cellular transcriptional activators with sequence-

specific DNA binding activity including Sp1 (Dynan & Tjian, 1983;

Kadonaga & Tjian, 1986; Briggs et al., 1986; Kadonaga et al., 1987; Pugh & Tjian, 1990) and CTF/NF-1 (Rosenfeld & Kelly, 1986; Jones et al., 1987). Subsequently, similar trans-activating factors (reviewed in Curran & Franza, 1988; Johnson & McKnight, 1989; Kadonaga et al., 1986; Struhl, 1987; Mitchel & Tjian, 1989; Luscher & Eisenman, 1990 a & 1990b; Wingender, 1990; Ziff, 1990) were isolated in many other laboratories (for example, Biedenkapp et al., 1988; Cavalini et al., 1988; Chodosh et al., 1988; Hoeffler, 1988; Levine & Hoey, 1988; Hai et al., 1989; Blackwell & Weintraub, 1990; Blackwood & Eisenman, 1990; England et al., 1990; Hooft van Huijsduijnen et al., 1990). These and other findings have led to the dogma that transcriptional regulation in eukaryotes is mediated by distinct, sequence-specific, trans-acting factors that interact with cis-acting elements that convey a modular organization to the 5′ flanking sequences of cellular genes. These trans-activating proteins function by promoter-selective recruitment of RNA polymerse II and accessory proteins (including the TATA-box factor) to the initiation site of mRNA synthesis.

At the initiation of this study (January 1986), human histone genes had been cloned recently (Heintz et al., 1981; Sierra et al., 1982; Carozzi et al., 1984; Zwollo et al., 1984). The availability of the cloned genomic DNA segments allowed expansion of previous studies (Stein et al., 1974 & 1975; Kleinsmith, 1975; Thomson et al., 1975) on histone gene regulation. Specific knowledge of transcriptional elements associated with histone genes was sparsely available (Grosschedl & Birnstiel, 1982; Sierra et al., 1983; Capasso & Heintz, 1985; Coles &

Wells, 1985; Hanly et al., 1985; Marashi et al., 1986; Osley et al., 1986). The first objective of this study was the delineation of protein/DNA interaction sites in vitro, as one of several approaches undertaken in this laboratory to identify potential transcription factors. Secondly, we studied the temporal regulation of these protein/DNA interactions during multiple biological processes, and initiated studies towards identification of mechanisms that may alter the activity of histone gene transcription factors.

1.2: Coordinate regulation of human histone gene expression.

1.2.1: Human histone gene chromosomal organization.

Human H1 and core histone genes comprise a set of genes that reside in multiple, polymorphically arranged clusters located on several different chromosomes (Green et al., 1984; Triputti et al., 1986). Although in lower eukaryotes histone gene clusters (reviewed in Stein et al., 1984) are contained within tandemly repeated arrays, the super-structure of human histone gene clusters has not been established. Human histone gene organization is probably not as homogeneous as in lower eukaryotes based on the variety of types of genomic histone gene containing DNA segments cloned thusfar (Heintz et al., 1981; Sierra et al., 1982; Carozzi et al., 1984; Zwollo et al., 1984).

Several trends in the clustering of human histone genes can be noted, such as (I) the presence of H4 and H3 histone genes in close proximity to each other, (II) the typical structure of H2A and H2B histone gene pairs arranged in opposite orientation, and (III) the

clustering of H1 histone genes either in the immediate vicinity of all members of the other classes or its isolated location in other genomic segments. Also, many if not all of these histone gene clusters are interspersed with one or more copies of a number of mammalian repetitive sequence elements (Collart et al., 1985). These structural features are consistent with a very limited role of long range histone gene organization in the expression of these genes.

Copies of each of the histone gene classes may encode (I) a DNA replication dependent histone mRNA, a short RNA molecule (400-600 nt) lacking a polyadenylation-sequence and which instead contains a secondary structure ("hairpin-loop") that specifies the 3' end of the mRNA ("type I"), (II) a DNA replication independent histone mRNA including conventional introns and polyadenylation sequences ("type II"), or (III) may not encode a functional mRNA as is the case with certain histone pseudo-genes. Recently, several laboratories have described (IV) partially DNA replication dependent genes displaying hybrid properties of both type I and type II genes (see also section 1.2.3). DNA replication independent histone mRNAs encode variant histone proteins with a broad spectrum of possible functions related to chromatin structure, but are not very abundant relative to the replication dependent mRNAs during S-phase. In this study we have examined three distinct, cell cycle regulated histone genes from three separate clusters that are designated, respectively H4-FO108, H3-ST519 and H1-FNC16.

Two aspects of histone gene organization are relevant to transcriptional regulation. First, histone coding sequences present in several clusters occur on opposite strands. This indicates that these genes are unlikely to be transcribed by means of a prokaryote-like polycistronic mRNA, but rather represent individually and divergently transcribed mRNA synthesis units. Second, the evolutionary conservation of multiple clusters containing one or more members of the various histone gene classes implies that each histone gene class represents a multi-gene family in itself. This allows for the possibility that rapid synthesis of histone mRNAs during S-phase may be accomplished by utilizing more than one transcription unit for each class. Finally, the divergent transcription of gene-pairs also indicates that histone 5'flanking regions have at least in some cases a finite length (e.g. the smallest intergenic regions are 0.2 kB in length), and may share cis-acting elements with genes in the immediate vicinity. Because many transcriptional elements can operate in two orientations (e.g. "enhancer" or "silencer" elements), shared promoter elements could provide histone genes with a pairwise level of transcriptional coordination.

1.2.2: Coordinate expression of the human histone multi-gene family.

Multiple human histone mRNA species can be detected during S-phase and are encoded by several distinct histone genes located in different clusters. Moreover, with the possible exception of an H2A/H2B gene pair that may represent two non-functional pseudo-genes, most histone genes

cloned and studied to date have been shown to be functionally expressed copies. The various histone mRNAs of each subtype encode proteins of essentially the same amino acid composition, despite divergence at the nucleotide level (in particular at the third codon position). These findings imply that each histone subtype is in fact encoded by a series of functionally expressed genes (Lichtler et al., 1982; Stein et al., 1984; Levine et al., 1988). Thus, models of histone gene transcription may incorporate cis-acting elements common to at least individual histone gene classes.

Expression of the multiple core and H1 histone gene-copies is coordinately controlled at the mRNA level during the cell cycle and tightly coupled to DNA replication. This has been established at the single gene level by various gene-specific hybridization techniques, including high resolution electrophoresis of in vivo labelled hybrid-selected mRNAs and S1 nuclease protection analysis. Coordinate regulation of entire histone gene classes has been demonstrated in a plethora of cell proliferation related processes by Northern blot analysis. However, differences have been noted in the extent to which individual histone genes are expressed: (I) within a given cell type, as reflected by differences in mRNA levels of individual histone genes of each class (for example, Levine et al., 1988; reviewed in Stein et al., 1984 & 1989b); and (II) differences in relative mRNA levels of the same histone gene in distinct cell types (Collart et al., 1988). Because the same gene in different cell types, and two distinct cell cycle dependent histone mRNAs in the same cell type, in both cases are presumably

subject to identical post-transcriptional regulation, these findings may reflect intra-class and inter-cell specific differences in transcription efficiencies (for example, Levine et al., 1988; see also Stein et al., 1984 & 1989b). Therefore, transcriptional models may allow for this possibility by including differences in the presence of auxiliary control elements that could selectively influence transcription in a wide spectrum of ontogenically distinct cells.

1.2.3: Multiple regulatory levels mediate histone gene expression.

Regulation of gene expression may occur at almost any level from genetic read-out to post-translational modification of the final gene product. Expression of histone genes occurs according to a classical multi-step process involving components at several molecular and cellular levels (Schumperli, 1984; Stein et al., 1984 & 1989; Stein & Stein, 1984; Marzluff & Pandey; 1988). First, access of putative transcription initiation factors to histone promoters may be influenced by modifications in chromatin structure (Elgin, 1981; Chrysogelos et al., 1985; Moreno et al., 1986), and possibly by regional localization of histone genes within the nucleus by putative nuclear matrix attachment sites (Fey et al., 1986; von Kries et al., 1991; Dworetzky et al., 1991). At least two histone genes have been shown to display broad cell cycle dependent changes in (hyper)sensitivity of 5'flanking sequences to several nucleases, including DNaseI, MNase, S1 nuclease and site specific endonucleases (Chrysogelos et al., 1985; Moreno et al., 1986; Pauli et al., 1989). These chromatin structural features are

generally, but not exclusively, associated with actively transcribed genes. Modifications in chromatin structure and nuclear matrix attachment may directly influence histone gene transcription by limiting accessibility and influencing local concentrations of trans-acting factors.

Second, putative trans-acting factors influence the rate and specificity of histone gene transcription. These factors operate at a rate-limiting step in histone gene expression and, therefore, may perform before key regulatory roles. Transcription factors that selective recognize histone genes in a sequence-specific manner are the focus of the results discussed in this dissertation (Chapters 2 to 5). The occupancy of histone gene promoters elements by trans-acting factors in principle may depend on a variety of transcriptional and post-transcriptional events acting on these entities, as well as combinatorial protein/DNA interaction events involving distinct trans-acting factors.

Third, initiation of transcription is followed by elongation and subsequent termination of histone nuclear precursor RNAs. It is generally assumed that all primary transcripts of cell cycle dependent histone genes are initially heterogeneous in length and subsequently trimmed to mRNAs with well defined lengths by specific 3′ end processing events. This mechanism involves a histone-specific U7 snRNP and several conserved histone mRNA 3′ trailer sequences (for example, Vasserot et al., 1989 and Harris et al., 1991 as well as references therein), including the hairpin-loop structure. This process has been shown to be

regulatory and in principle may prevent production of functional mRNAs in cells actively transcribing histone genes. Because of this regulatory redundancy, putative transcription factors involved in histone mRNA synthesis may be abundant in cells not requiring histone proteins, without immediately having a deleterious effect on overall control. The existence of a separate and histone-specific U7 snRNP that produces compact histone mRNAs, underscores the importance of efficiency in histone gene expression.

Recently, examples have been obtained that suggest that partially cell cycle regulated histone genes may be subject to alternative 3' end processing. This results in formation of either typical DNA replication dependent histone mRNAs with a hairpin-loop, or mRNAs similar to those of variant histones that are more extended and are polyadenylated (Collart et al., 1991). Note that alternative 3'end processing perhaps reflects an optional process to aid in the versatility of positively expressing histones in a variety of cellular situations rather than a rate-limiting and regulatory mechanism in preventing expression.

Processed histone mRNAs are transported to the cytosol. The question as to whether transport of mRNAs in general occurs by spontaneous diffusion or by means of an active (and potentially regulatory) mechanism is unresolved, although at least one case has been reported that could argue against spontaneous diffusion in the transport of mRNAs from nucleus to cytosol (Lawrence et al., 1989). Cytosolic histone mRNAs are translated on non-membrane bound polysomes throughout the cell (Zambetti et al., 1987; Lawrence et al., 1988). Translated

histone mRNAs are rapidly degraded in the absence of DNA synthesis by an exonuclease that selectively recognizes the 3'end and hairpin-loop of these transcripts, a process that may be directly coupled to translation by ribosomes (reviewed in Marzluff & Pandey; 1989). Cell cycle dependent differences in the half-lives of histone mRNAs are consistent with auto-regulatory models in which histone mRNA stability is directly influenced by the encoded proteins (Morris et al., 1991). Hence, histone mRNA stability is a major level of regulation in proliferating cells, and may represent the predominant mechanism by which cells temporally couple histone protein synthesis with DNA replication. However, the intrinsic instability and low level of histone mRNA outside S-phase, and the requirement for rapid accumulation of high levels of histone transcripts during S-phase suggests that a very efficient and selective process of histone mRNA synthesis is also important.

1.2.4: Transcriptional control of histone genes.

Transcription of human histone genes in HeLa S3 cervical carcinoma cells occurs constitutively during the cell cycle, and a transient and coordinate increase (approximately 2 to 3-fold) in transcription rates occurs at the G1/S phase boundary (reviewed in Stein et al., 1984; see also Baumbach et al., 1987 and references therein). Similarly, transcription is increased several-fold towards S-phase when cells enter the cell cycle from a quiescent state (G_o)(Wright et al., 1991a). Conclusive data on histone gene transcription during the cell cycle of cells resembling the normal diploid phenotype have not been published.

Irrespectively, at least one cis-acting control-element in histone promoters may exist that is involved in the enhancement of transcription at the G1/S-phase boundary that is observed in tumor cells.

Transcription of histone genes traditionally has been studied during the cell cycle with several distinct synchronization procedures (reviewed in Stein et al., 1984). These procedures enrich for cells at defined stages during each round of proliferation by using chemically induced blockades, separation protocols based on physical differences between cells, or growth factor and nutrient deprivation techniques. These methods may perturb physiological control mechanisms to various degrees, and studying control of histone genes during S phase with any of these techniques is subject to multiple technical considerations that qualify the interpretation of results. However, during the course of the studies described in this dissertation compelling evidence has been presented that an important aspect of cell cycle studies of histone gene regulation is the extent to which the cell of experimentation displays properties of the transformed phenotype (Ito et al., 1988; Holthuis et al., 1990). This may in principle influence the regulation of histone-specific transcription factors (for example, see Chapter 3) and cellular rates of histone gene transcription.

Histone gene transcription is quantitatively and coordinately downregulated at the induced cessation of proliferation and onset of differentiation in human HL60 promyelocytic leukemia cells, as demonstrated by the shutdown of total H4, H2B and H1 histone gene transcription and undetectable levels of H4 and H1 histone mRNAs

(Collart et al., 1988; Shalhoub et al., 1989; Stein et al., 1989). Similar observations have been made during induced differentiation of mouse 3T3-L1 adipocytes (Bortell et al., 1991) and rat myoblasts (Bird et al. 1985; Larson et al., 1989), as well as during the developmental sequence of differentiating rat primary calvarial osteoblasts in cell culture systems (Owen et al., 1990a & 1990b). Apart from this, fluctuations in histone gene transcription in vitro have been observed when human CF-3 foreskin fibroblasts switch from an actively proliferating mode to a quiescent state and vice versa (Wright et al., 1991a). Thus, regulatory protein/DNA interactions may exist that are dependent of the cell growth state, and are rate-limiting in the proliferation-specific transcription of these genes.

Transcription rates have also been measured in several strains of transgenic mice containing various reporter genes fused to the human H4-FO108 histone gene promoter (van Wijnen et al., 1991a). In general, histone gene transcription (as reflected by reporter gene expression) parallels histone gene expression at the mRNA level in several tissues of the adult mouse, in particular liver, kidney, spleen and thymus (ranking in order of increasing relative levels of expression). Because of the tight coupling between histone gene expression, DNA replication and cell proliferation, this indicates that histone gene transcription reflects the level of cell proliferation within these tissues. This supports the existence of proliferation-specific cis-acting elements influencing histone gene transcription.

High levels of reporter gene expression can be observed in adult brain despite the observation that histone mRNA levels are almost below the level of detection. This tissue contains a small population of cells with proliferative potential (e.g., glial cells), but consists primarily of non-proliferating neuronal cells that by definition do not require DNA replication dependent histones. These findings clearly suggest that, first, multiple levels of histone gene regulation are operative in the intact animal and, second, that redundancy in control mechanisms is functionally related to the prevention of aberrant histone synthesis. Therefore, it is not strictly necessary to invoke negative regulatory transcription factors in order to stringently control histone gene expression.

Downregulation of histone gene transcription as measured by reporter genes has been observed during murine hepatic development reflecting the onset of in vivo quiescence and differentiation (van Wijnen et al., 1991). These modifications in transcription correspond to similar decreases in histone mRNA levels. This indicates that transcriptional regulation is a primary mode of gene control during development. Because transcription rates may directly correlate with the presence of rate-limiting trans-acting factors, these findings provide a first indication for cell growth related, developmental regulation of histone gene transcription factors.

1.2.5: Regulatory elements that influence human histone gene transcription.

The first 200 base pairs upstream of the histone protein coding sequence are arbitrarily referred to as the proximal promoter, and sequences beyond that as the distal promoter. Several histone genes are located in divergently transcribed clusters, occasionally separated by a maximum of 0.2 kB DNA that represents two overlapping 5' flanking regions. Recombination and mutation events typically assumed for histone and other multi-gene families may recruit or create both distal and proximal regulatory elements over a period of time. However, because recombination frequencies (at least in theory) are expected to be proportional to the distance between promoter elements and the genes to which they are associated, it is possible that secondary recombination events will favor those elements in close proximity of the transcription initiation site to remain functionally conserved. Therefore, in this study we focus on histone proximal promoter regions, in which several apparently conserved sequence motifs have been identified (Wells et al., 1986; Wells & McBride; 1989).

The TATA-box is the first transcriptional element shown to be functionally involved in human histone gene transcription, because this sequence is sufficient for mediating synthesis of correctly initiated transcripts in vitro of the human H4-FO108 histone gene (Sierra et al., 1983). Studies using the analogous H4 histone gene Hu4A confirmed these findings and provided indications for auxiliary sequences capable of stimulating in vitro transcription (Hanly et al., 1985), and recently it

has been shown that the H4-FO108 histone gene has similar additional
stimulatory sequences (Wright et al., 1991b). These and other cis-acting
elements identified by in vitro transcription assays are discussed in
the next chapters, and related to the protein/DNA interactions occurring
at these sites in vitro.

   In vivo analysis of a series of deletion mutants spanning the
5′ flanking region of the H4-FO108 histone gene has revealed that the
region immediately upstream of the TATA-box is required for expression
of this gene (Kroeger et al., 1987). In these and parallel studies
(Helms et al., 1987), indications were also found for distally located
elements capable of influencing expression. Furthermore, protein/DNA
interactions in vivo have been established in the proximal promoter
regions of the human H4-FO108 (Pauli et al., 1987) and H3-ST519 (Pauli
et al., 1989) histone genes with genomic DNaseI footprinting assays
(using isolated nuclei) and genomic DMS fingerprinting assays (using
intact cells). Both genes appear to have two protein/DNA interaction
domains, designated H4-Site I, H4-Site II, H3-Site I and H3-Site II,
respectively. These domains most likely represent crucial cis-acting
control elements, and are located within the first 0.2 kB upstream of
the coding region which emphasizes the importance of histone proximal
promoters. The number of protein/DNA contacts at guanine residues
detected within these sites is consistent with each domain interacting
with more than one DNA binding protein. Although these in vivo data were
not available at the onset of this project, a major focal point of the

studies presented here is related to the characterization of the
putative factors binding to these domains in vitro.

CHAPTER 2

CHARACTERIZATION OF PROTEIN/DNA INTERACTIONS IN THE 5'FLANKING REGIONS

OF HUMAN H4, H3 AND H1 HISTONE GENES IN VITRO

2.0: Summary

Protein/DNA interaction sites in the proximal promoters of human H4(-FO108), H3(-ST519) and H1(-FNC16) histone genes were localized in vitro by a variety of DNA binding assays. Seven binding sites were established, albeit with various degrees of accuracy, and characterization of the cognate factors suggests that these sites interact with at least four distinct proteins. Factor HiNF-A is a relatively ubiquitous DNA binding protein that recognizes in all three promoters specific elements, that are comprised primarily of dA/dT sequences. Because this factor shows similarities with certain (HMG-I like) non-histone chromosomal proteins that also bind to dA/dT sequences, it is possible that the function of HiNF-A is related to chromatin structure. Factor HiNF-B interacts with a sequence adjacent to the TATA-box of the H1-FNC16 histone gene. Reconstitution experiments show that HiNF-B is a heteromeric protein. The HiNF-B consensus sequence is an extended CCAAT-box sequence that is present in the 5'region of most but not all histone gene classes, suggesting that HiNF-B may selectively recognize CCAAT-boxes associated with at least some histone genes. The Spl-like factor HiNF-C and the proliferation-specific factor interact with two distinct elements in the H4-FO108 histone promoter. Factor HiNF-D recognizes a putative H4 histone consensus element that is required for expression of the H4-FO108 histone gene. These findings

imply that coordinate transcriptional regulation of H4, H3 and H1 histone genes involves a complex interplay of DNA binding proteins that are selective for histone genes, and those that recognize a broad spectrum of genes.

2.1: Characterization of histone promoter factors HiNF-A and HiNF-B.

2.1.1: A nuclear protein with affinity for the 5′ flanking region of a human H4 histone gene.

André J. van Wijnen, Janet L. Stein, and Gary S. Stein (1987) Nucleic Acids Research **15**: 1679-1698.

The initial characterization of protein/DNA interactions in the human H4-F0108 histone gene in vitro was performed according to the following rationale: (I) the H4-F0108 histone gene expresses a prototypical cell cycle regulated mRNA, and was under intensive scrutiny by a number of parallel approaches relating to its transcriptional regulation, (II) information on specific transcriptional elements of this gene was not available, and (III) although a few Breathnach & Chambon consensus sequences (i.e. TATA- and CAAT-boxes) had been identified previously, the relevance of these elements in mediating protein/DNA interactions was uncertain. Thus, the experimental approach that was pursued was aimed at examining a broad DNA region of interest, with no other bias than the strategy of studying the immediate 5′ flanking region of this gene.

Techniques pertaining to the detection of protein/DNA interactions (reviewed in Pauli et al., 1990) in unfractionated nuclear extracts

(Dignam et al., 1983; Shapiro et al., 1988), as well as efficient methods for characterizing and purifying DNA binding proteins (Rosenfeld & Kelly, 1986; Kadonaga & Tjian, 1986) were being developed in several laboratories, but these protocols were not widely available. The recent emergence of the electrophoretic mobility shift assay (gel retardation assay)(Fried & Crothers, 1981; Garner & Revzin; 1981) provided a useful tool to study DNA binding proteins. A modification of this method was used as a means to scan a broad area of 5' flanking region for protein/DNA interactions, and simultaneously determine specificity of the corresponding DNA binding proteins.

A DNA fragment spanning the H4-FO108 proximal promoter region (nt -240 to nt -13) was radioactively labeled at one end, and incubated with nuclear protein in the presence of non-specific competitor DNA. The binding mixtures were subjected to gel electrophoresis and examination of the dried gel was accomplished by autoradiography. Binding of factors to this full-length DNA fragment was compared to binding events occurring to a set of DNA fragments that contained progressive deletions (introduced by site-specific endonucleases) at defined locations with approximately 30-50 bp intervals. Shortening of the intact probe results in faster migration of the free DNA and associated protein/DNA complexes yielding a stairway-like pattern on autoradiograms. More importantly, the deletion of certain DNA segments and concomitant loss of protein binding establishes the first boundary of a specific protein/DNA interaction domain at a predetermined position (i.e. endonuclease cleavage site). The reciprocal experiment in which the opposing end of

the full-length DNA fragment was labeled, was used to define the other

boundary. This procedure in which bidirectional analysis of site-

specific endonuclease induced DNA deletions is combined with gel

retardation assays is referred to as a stairway assay.

The H4-FO108 proximal promoter (nt -240 to -13) mediates formation

of several distinct protein/DNA complexes, and detection of these

interactions depends on the experimental conditions, in particular the

type of non-specific competitor DNA used in the binding reaction. We

focused initially on a protein designated HiNF-A that is detected with

random DNA (sonicated Escherichia coli DNA), but not with the synthetic

alternating co-polymer poly (dI-dC)*(dI-dC) DNA. The other protein/DNA

complexes are discussed in the next section (2.2).

The binding site of HiNF-A is located between nt -183 and -131,

with each of these boundaries coinciding with the position of a

restriction site, as revealed by stairway assays (Fig. 2-1). Parallel

experiments were performed with another upstream fragment of the H4-

FO108 histone gene (nt -440 to -240), as well as the proximal promoter

of the H3-ST519 histone gene (nt -200 to -20). Whereas HiNF-A binding is

not observed with the distal H4-FO108 fragment (data not shown), a

protein/DNA complex with similar relative migration requires a specific

DNA segment (nt -110 to -87) in the H3-ST519 histone promoter (Fig.

2.1). We have adopted the working hypothesis that this complex is also

mediated by HiNF-A, which is consistent with other biocemical

characteristics of this factor (summarized in van Wijnen et al., 1987).

Figure 2-1. <u>HiNF-A interacts with DNA segments containg 5'dATTT</u>

<u>elements.</u> Stairway assays of the proximal promoters of the H4-F108, H3

ST519 and H1-FNC16 histone genes. DNA fragments were ^{32}P-labelled at

endonuclease sites located at either the proximal (E1) or distal

terminus (E2) of the proximal promoters of each of these genes, and

these probes were each cleaved at secondary sites to yield two opposir

sets of deletion mutants. Panel I) H4-FO108 fragment labelled at nt -1

(ln 1-5)(E1 = Ban II; secondary enzymes, resp., EcoRI/-234, DdeI/-183,

TaqI/-130, ThaI/-94 and AvaII/-70), or at nt -234 (ln 6-9)(E2 = EcoRI

cleaved with, resp., Ban II/-13, ThaI/-95, TaqI/-133, or DdeI/-187).

H3-ST519 fragment labelled at nt -20 (ln 1-5)(E1 = HindIII; cut with,

resp., HpaI/-200, BstNI/-139, ThaI/-109, AvaII/-83, or HinfI/-63), or

nt -200 (ln 6-11)(E2 = HpaI; cut with, resp., HindIII/-20, MboII/-43,

HinfI/-67, ThaI/-110, AvaII/-129, or BstNI/-143). III) H1-FNC16 fragm

labelled at nt -78 (ln 1-3)(E1 = SmaI; cut with, resp., AluI/-234,

RsaI/-213, or HinfI/-156), or nt -213 (ln4-6)(E2 = RsaI; cut with,

resp., SmaI/-78, HaeIII/-138 or HinfI/-160). Numbers after enzymes re

to dsDNA sequences that remain associated with the labelled portion

after cleavage. Random DNA (from E.coli) was used as non-specific

competitor DNA. Arrowheads indicate the position of the HiNF-A comple

with the full length DNA fragment. Gel retardation conditions and the

origin of DNA fragments were as described (van Wijnen et al., 1987).

Binding reactions with HiNF-A were loaded on a 4% (30:1) PAA-gel.

^{32}P:−13 ^{32}P:−234 ^{32}P:−78 ^{32}P:−213

A▸   A▸
−234▸   −234▸
−183▸   −156▸
                                −95
−130▸   −13
−94▸   −78▸
−70▸   −133
                                −187

I) H4   III) H1

^{32}P:−20 ^{32}P:−200

A▸
−200▸   −20
−139▸   −43
−109▸   −67
−83▸   −110
−63▸   −129
                                −143

II) H3
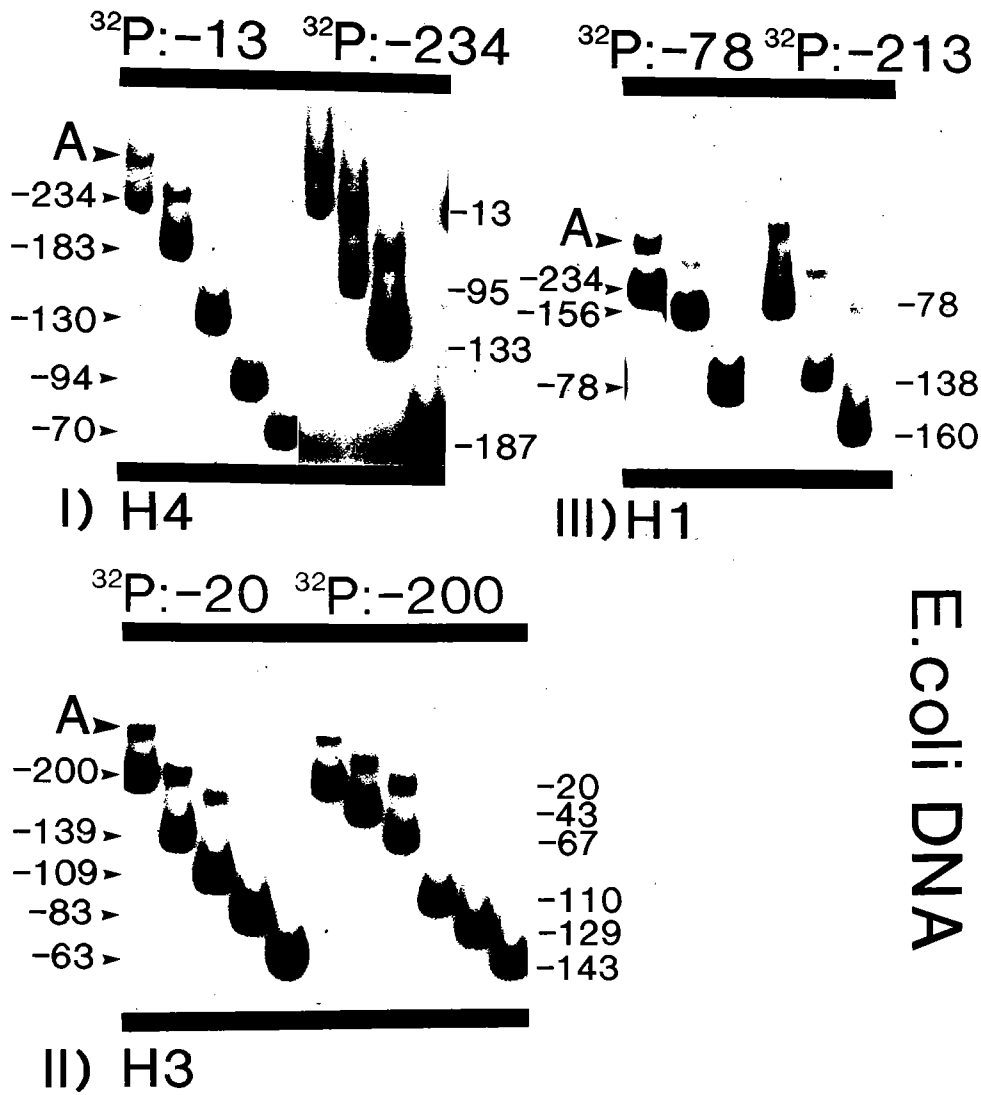
E.coli DNA

A consensus sequence was formulated based on the putative binding of HiNF-A to the H4-FO108 and H3-ST519 histone promoter segments. DNaseI footprinting experiments with the H4 histone DNA fragment using a chromatographic fraction enriched for HiNF-A binding activity revealed protection of a short dA/dT-rich sequence (nt -148 to -143) coinciding with this consensus sequence. This assignment of the HiNF-A binding site was further confirmed by direct binding of HiNF-A to a synthetic oligonucleotide spanning nt -152 to -128 of the H4-FO108 histone promoter (Fig. 2-2).

Although HiNF-A is probably not directly relevant to histone gene transcription (see below), these studies established a rapid assay to determine the boundaries of multiple specific protein/DNA interactions. Stairway assays (van Wijnen et al., 1991e) have been used in the intitial screening of binding events occurring with a number of other histone gene 5'flanking sequences (see next sections; Dworetzky et al., 1991; K.L. Wright, JLS & GSS, unpublished data). Despite that advances in protein/DNA interaction methodologies have resulted in multiple protocols to establish specificity of DNA binding proteins, stairway assays remain an attractive, inexpensive option to achieve first indications towards this objective.

Figure 2-2. <u>Summary of the HiNF-A binding sites in H4-F108, H3-ST519 and</u>

<u>H1-FNC16 histone genes.</u> Indicated are the sequences of restriction

fragments identified by stairway assays, BAL31 deletion mutants and

oligonucleotides used for direct binding studies (DS-I forms an

electrophoretically stable complex with HiNF-A, but DS-II does not), and

the approximate boundaries of the HiNF-A DNaseI footprint in the H4-

FO108 promoter (thin line above the sequence). Bold nucleotides

represents the putative HiNF-A consensus sequences 5'dATTT. The

sequences are aligned in the orientation of maximal similarity; i.e.,

either the sense or the anti-sense strand is depicted as the top-strand.

(See van Wijnen et al., 1987 for further details).

HiNF-A BINDING SITES IN THE H4-F0108, H3-ST519 AND H1-FNC16 PROMOTERS:

gene	fragment	sequence		binding
H4-F0108	TaqI/DdeI	5'	---AAAACAG AAAAGAAATGACGAAATGT ---TTTTGTC TTTTCTTTACTGCTTTACA gc	+
	K6-EcoRI/BanII	5'	a attCGAAATGACGAAATGT CGAGAGG--- GCTTTACTGCTTTACA GCTCTCC---	−
	K2-EcoRI/BanII	5'	aattCTGACGAAATGT CGAGAGG--- GACTGCTTTACA GCTCTCC---	−
	DS-I	5'	gatcCGG AAAAGAAATGACGAAATGT CGAGA GCC TTTTCTTTACTGCTTTACA GCTCTctag	+
	DS-II (inv)	5'ctagAGTATCGGACC AGATTGAAAACCGAAAG CTATAGCCTGG TCTAACTTTTGGCTTTCga tc		−
H3-ST519	ThaI/AvaII (inv)	5'	gacC CAAGAAAATTCAAAAATCC CCG G GTTCTTTTAAGTTTTTAGG GGC	+
H1-FNC16	RsaI/HinfI (inv)	5'	---TGTTAGT CCTCAAAATTTGATTAAAT GCTTAAA--- ---ACAATCA GGAGTTTTAAACTAATTTA CGAATTT---	+

possible HiNF-A consensus sequence: 5'dAAAT(NNNN)AAAT

2.1.2: Two target sites for protein binding in the promoter of a cell cycle regulated human H1 histone gene.

André J. van Wijnen, Kenneth L. Wright, Robert F. Massung, Martijn Gerretsen, Janet L. Stein and Gary S. Stein (1988) Nucleic Acids Research 16:571-590.

The human H4-FO108 and H3-ST519 histone genes are actively expressed in proliferating cells, and have been shown previously to contain a functional promoter. The H1-FNC16 histone gene was being characterized in the laboratory at the onset of this study, and its promoter appears to have similar properties. Comparison of protein/DNA interactions in three different histone promoters is a means by which to identify DNA binding activities capable of selectively recognizing histone genes. A shared promoter factor may have a high propensity to be functionally related to histone gene transcription, and most notably may have a coordinating role in regulating the various histone gene clases. Moreover, the presence of HiNF-A binding sites in human H4 and H3 histone proximal promoters suggested the attractive possibility that this factor may also have a binding site in the H1-FNC16 histone promoter.

The proximal promoter of the H1-FNC16 histone gene was selected for study and a DNA fragment was used that encompasses nt -234 to -78. Protein/DNA interactions were studied by stairway assays, DNaseI footprinting and DMS protection experiments of gel purified protein/DNA complexes, as well as methylation interference analysis. In addition, chromatographic separation procedures were designed that were aimed at

fractionating various putative histone promoter factors, and the effectiveness of these procedures was monitored by gel retardation assays.

A putative HiNF-A binding site was detected using stairway assays, and this site is located between nt -213 and -160 in a segment containing dA/dT-rich sequences similar to those found in the H4-F0108 and H3-ST519 histone proximal promoters (Fig. 2-1). Several similarities were noted between HiNF-A, monkey alpha-protein and high mobility group proteins such as HMG-I. Based on these similarities, it is unlikely that HiNF-A is a rate-limiting transcription factor and directly linked to histone gene expression. However, apparent conservation of detectable HiNF-A binding sites at defined locations in the distal part of three different human histone proximal promoters suggests a possible role for this factor, perhaps related to chromatin structure of the H4-F0108 and H3-ST519 histone genes.

Apart from HiNF-A, we established the binding site of a CCAAT-box binding protein designated HiNF-B using stairway assays (Fig. 2-3), and at the single nucleotide level using three different protein/DNA contact analysis techniques on both strands (Fig. 2-4). Several other DNA binding complexes were observed in vitro, but were not studied further. A putative HiNF-B CCAAT-box consensus element has been obtained by promoter sequence comparisons that show extensive similarities between the HiNF-B binding site and sequences in most, but not all, proximal promoters of H1, H2A, H2B and H3 histone genes, although this element was not frequently observed in the 5' flanking sequences of several H4

Figure 2-3. <u>HiNF-B interacts with the region immediately upstream of the</u> <u>TATA-box of the H1-FNC16 histone gene.</u> Stairway assays of the proximal promoter of the H1-FNC16 histone gene: termini of the H1-FNC16 5′region were labelled on the distal side (E1 = SmaI, nt -78)(ln 1-4) or proximal side (E2 =RsaI, nt -213)(ln 5-8)(part I). Enzymes similar to those in Fig. 2-1 were used for secondary cleavage (location of sites indicated in nucleotide numbers). Arrowhead indicates the position of the HiNF-B complex with the full length DNA fragment. (See van Wijnen et al., 1988a & 1988b for further details).

$^{32}P{:}{-}78$ $^{32}P{:}{-}213$

B▸

poly I/C DNA

−234▸
−213▸

|−78
−94

−156▸
−138▸

−138
−160
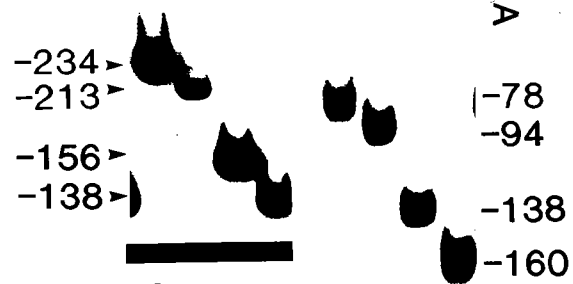
I) H1

Figure 2-4. HiNF-B is a CCAAT-box binding protein. Diagrammatic summary

of DNaseI footprinting, DMS fingerprinting and methylation interference

analysis of the HiNF-B binding site in the H1-FNC16 promoter (data shown

in van Wijnen et al., 1998a). Competition analysis is shown in Chapter

5. Lanes represent the cleavage products of chemical sequencing

reactions (G; G+A), free DNA (U) and the HiNF-B complex (B). Symbols

represent the maximal DNaseI footprint boundaries (brackets), DMS

protection (open circles) and enhancement (filled circles), methylation

interference contacts (thin arrows), and position of guanines (dashes

with nucleotide numbers relative to the histone ATG translational start-

codon). (See van Wijnen et al., 1988a for further details).

5′ G G T G G A T T G G A C G C T C C A C C A A T C A C A G G G C A G G C G C C G G C T T T A T A T A (TOP/+)

3′ C C A C C T A A C C T G C G A G G G T G G T T A G T G T C C C G T C G G G C C G A A A T A T A T (BOTTOM/−)

124 121

91 89

99

111 110

113

124 122

90 89

histone genes. The HiNF-B CCAAT-box (5′ dYYRRCCAAT(C/G)RRRR; Y = C and T, R = G and A) is more extended than the original Breathnach & Chambon CAAT-element. Competition analysis using a DNA fragment spanning the CCAAT-box of the H3-ST519 gene (see Chapter 5 and Fig. 2-9) shows that this non-identical but similar sequence competes for binding of HiNF-B. These results suggest that transcriptional regulation of a subset of the five histone gene classes may involve a CCAAT-box factor with HiNF-B binding activity.

2.1.3: Human H1 histone gene promoter CCAAT-box binding protein HiNF-B is a mosaic factor.

André J. van Wijnen, Robert F. Massung, Janet L. Stein and Gary S. Stein

The finding that HiNF-B recognizes a putative histone-specific CCAAT-box and that this factor could be involved in the coordinate transcriptional regulation of at least a subset of the multiple histone genes prompted further studies on HiNF-B. Thus, HiNF-B was investigated by analysis of its activity in nuclear extracts from exponentially growing mouse C127 cells (the cell type utilized in deletion analysis of transcriptional elements associated with the human H4-FO108 histone promoter), as well as from synchronized human HeLa S3 cells during the cell cycle (the procedure and cell type used to establish cell cycle dependency of human histone gene transcription). The results show that HiNF-B can be detected in proliferating C127 cells, as well as in HeLa S3 cells throughout the cell cycle (van Wijnen et al, 1988b). This

suggests that this factor is an evolutionarily conserved, constitutive

DNA binding activity during the cell cycle of cells that display

properties of the transformed phenotype. Interestingly, the

chromatographic behaviour of this factor is inconsistent with this

activity representing a single molecular entity.

Chromatographic fractions obtained in a standard separation

procedure using phosphocellulose contain substantially less HiNF-B

activity than expected from the amount of activity that was subjected to

the procedure (which was initially designed to efficiently separate

HiNF-A from other DNA binding proteins such as HiNF-B). Combination

experiments were performed with separate fractions and HiNF-B activity

was measured in gel retardation assays. The results demonstrate that

HiNF-B activity can be dramatically increased by the combination of two

different phosphocellulose fractions (Fig. 2-5). Several additional

experiments supported the suggestion that HiNF-B may be composed of a

minimum of two non-identical components.

At present, many heteromeric DNA binding activities have been

described (reviewed by Curran & Franza, 1988; Johnson & McKnight, 1989;

Mitchel & Tjian, 1989; Luscher & Eisenman, 1990a & 1990b; Wingender,

1990; Ziff, 1990), and these appear to be fairly common. However, at the

time the study of HiNF-B was pursued, the proposal that at least some

eukaryotic DNA binding proteins are mosaic factors was rather

unorthodox. The idea that the composite nature of CCAAT-box binding

proteins may facilitate the discriminate recognition of distinct CCAAT-

elements associated with diversely expressed genes, has been vindicated

39

Figure 2-5. <u>HiNF-B is a heteromeric DNA binding activity.</u> Gel

retardation assay showing reconstitution of HiNF-B DNA binding activity

using phosphocelulose fractions. Incubation with probe from the H1-FNC16

promoter (nt -213 to -78) with unfractionated nuclear proteins

(approximately 2 μg) or the flow-through of a DEAE-Sephacel column

(D200)(approximately 4 and 8 μg, respectively) results in visualization

of the HiNF-B complex (B, indicated with arrowhead). However, HiNF-B is

barely detectable in binding reactions with proteins from

phosphocellulose fractions (P200 flow-through: 1.2 and 2.4 μg; P350

fraction: 1.4 and 2.8 μg). However, combination of the P200 & P350

fractions (respectively, 1.2 & 1.4 μg, and 2.4 & 2.8 μg) results in

partial reconstitution of HiNF-B activity. (See van Wijnen et al., 1988b

for further details).

by elegant studies by Chodosh and others (Chodosh et al., 1988). The evolutionarily conserved nature of heteromeric CCAAT-box factors from yeast to human has recently been used in a clever cloning procedure to obtain the mammalian genes for the components of the composite CCAAT-box protein CP1/NF-Y (Hooft van Huijsduijnen et al., 1990). Finally, Heintz and colleagues (Gallinari et al., 1990) also have adopted the hypothesis that the extended HiNF-B CCAAT-box element may be selectively recognized by a specific CCAAT-box binding protein (H1-TF2) which at least is involved in human H1 histone gene transcription

2.2: Characterization of histone promoter factors HiNF-C and HiNF-D:

2.2.1: H4 histone gene transcription requires nuclear factor HiNF-D: auxiliary roles for HiNF-C (Sp1-like) and possibly HiNF-A (HMG-like).

André J. van Wijnen, Kenneth L. Wright, Jane B. Lian, Janet, L. Stein and Gary S. Stein (1989) Journal of Biological Chemistry 264:15034-15042.

The H4-FO108 histone proximal promoter has been shown to have two in vivo protein/DNA interaction domains, designated H4-Site I and H4-Site II, and each of these domains may interact with several distinct proteins (Pauli et al., 1987). The H4-FO108 histone promoter has been shown previously to interact with factor HiNF-A to a region coinciding with the distal part of H4-Site I, but several other factors also bind to this promoter in vitro (van Wijnen et al., 1987). Hence, further characterization of the complement of factors that binds to the H4-FO108 histone promoter is required to gain insight into its transcriptional

regulation.

Binding reactions were optimized for detection of factors different from HiNF-A by using the non-specific competitor DNA poly (dI-dC)*(dI-dC). Binding domains of two factors (HiNF-C and HiNF-D) were characterized by stairway assays, competition analysis and/or DNaseI footprinting experiments of gel-purified protein/DNA complexes. The physical properties of these DNA binding activities were analyzed with regard to temperature- and detergent stability, mono- and divalent cation requirements, as well as chromatographic properties.

The binding domain of HiNF-C is located between nt -130 and -99 as revealed by stairway assays (Fig. 2-6; see also van Wijnen et al., 1989). DNaseI footprinting experiments using the gel purified HiNF-C complex demonstrated nuclease protection of nt -134 to -113 on the sense-strand (summarized in Fig. 2-7; van Wijnen et al., 1989). Factor HiNF-C has a selective requirement for Zn^{2+} ions, is relatively unstable at physiological temperatures in vitro, and its binding activity is resistant to high ionic strength (van Wijnen et al., 1989). In addition, detection of HiNF-C is relatively independent of the type of non-specific competitor DNA that is included in excess in binding reactions (data not shown), indicating that HiNF-C is a highly specific DNA binding protein. This factor appears to be a relatively ubiquitous protein that can be detected with nuclear protein preparations from synchronized human HeLa S3 cells (in G1 and S-phases), in exponentially growing mouse C127 cells, as well as proliferating and differentiated human HL60 cells (see section 2.2.2).

Figure 2-6. <u>Localization of HiNF-C and HiNF-D binding sites in the H4-</u>

<u>F0108 histone gene.</u> Stairway assays of the proximal promoter of the H4-

F108 histone gene were performed using DNA fragments labelled at a

plasmid (pKUC8) derived EcoRI site (nt -182)(panels I, II & III) or at a

Ban II site (nt -13)(panel IV). These probes were cleaved with secondary

enzymes at the indicated nucleotide positions. DNA fragments in panels

II & III represent identical sets, but were incubated with different

types of non-specific competitor DNA. Binding reactions were performed

with nuclear proteins from human HeLa S3 (panel I) or mouse C127 cells

(panel II, III and IV). Non-specific competitor DNA substrates added in

the reaction were poly I/C DNA (panel I, II and IV) or random DNA (panel

III). The complexes mediated by factors HiNF-A, -C and -D are indicated

by arrowheads. A unidirectional analysis of the mouse HiNF-A binding

site (panel III) is shown to indicate differences in the detection of

protein/DNA complexes when a different non-specific competitor DNA is

used in the binding reaction. Note that the stairway patterns of human

and mouse proteins are indistinguishable consistent with cross-species

similarities in these factors. (See van Wijnen et al., 1989 for further

details).

H4 promoter

human
^{32}P:-182

mouse
^{32}P:-182 ^{32}P:-182 ^{32}P:-13

I) poly I/C DNA

II) poly I/C DNA

III) E.coli DNA

IV) poly I/C DNA

−13
−36
−74
−95
−99
−133

−182
−130
−96
−94
−70
−35

D
C
A
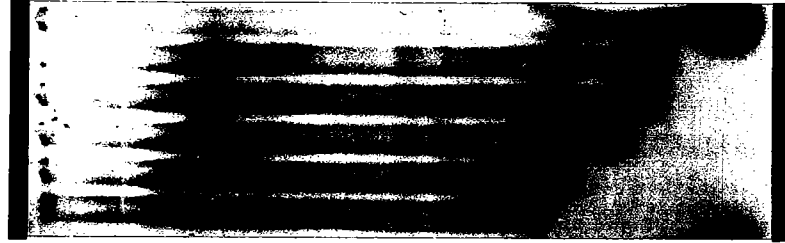
Figure 2-7. <u>Factor HiNF-C interacts with the Sp1 consensus sequence and</u>

<u>HiNF-D with an H4 histone consensus sequence.</u> Diagram depicting proximal

promoter sequences of the FO108 H4 histone gene (see van Wijnen et al.,

1989 for further details). Lines underneath the sequence represent the

in vivo DNaseI footprints of sites I and II, and circles represent the

combination of in vivo DMS fingerprints of guanines on the top and the

bottom strand (data from Pauli et al., 1987). Dashes between the strands

represent various consensus sequences as detailed in the text, the

distal part of site I contains both an AT-rich repeat (5′dAAAT[N4]AAAT)

and an AAATGACG element that partially overlap.

Solid brackets show the DNaseI footprint boundaries of HiNF-C and

HiNF-D. The DNase I protection results for HiNF-C and HiNF-D were

obtained using the gel-purified protein/DNA complexes (data shown in van

Wijnen et al., 1989). DMS protection experiments were performed by

direct analysis of piperidine-cleavage products of DMS treated binding

reactions performed with excess nuclear protein. The pattern of DMS

protection attributed to HiNF-C (data shown in van Wijnen et al., 1989)

is indicated by open circles above the sequence. The conditions used for

these experiments were as described in van Wijnen et al., 1989.

Similarly, putative DMS fingerprints for HiNF-D were obtained (not

indicated on diagram) showing DMS protection and enhancement attributed

to binding of HiNF-D near the AvaII site (i.e., DMS protection at dG^{69}

and DMS enhancements at dG^{80} and dG^{76})(data not shown). The conditions of

these experiments was as described in van Wijnen et al., 1991b (see

Chapter 4).

-170

HiNF-E?

HiNF-C

-110

HiNF-A

AGTGGAGGAGGAGGGAGAGGAGGAAGAAACAGAGAAAAGAGAAAATGACGAAATGTCGAGAGAGGCGGGGACAATTGAGAACGCTT
TCACCTCCTCCTCCCTCTCCTTTGTCTTTTCTTTTACTGCTTTACAGCTCTCTCCGCCCCTGTTAACTCTTGCGAA

Site I

TaqI

TATA-factor?

-40

-100

HiNF-D

CCCGCCGGGCGGCGCTTTCGGTTTTCAATCGGTCCGATACTCTTGTATATCAGGGGAAGAGACGGTGCTCGCCTTGAC
GGGCGGCCGCGCGGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTTCTGCCACGAGCGGAACTG

MspI ThaI

AvaII

Site II

MboII

Figure 2-8. Competition analysis of human histone gene transcription factors. Competition experiments for HiNF-D and HiNF-C (middle panel) were performed using a standard binding reaction with 0.5 ng probe (EcoRI/TaqI fragment of pFP-1), 2 μg poly (dG-dC)*(dG-dC), 4 μg nuclear protein, and a 200-fold molar excess of specific competitor oligonucleotides. These oligonucleotides contain consensus sequences for HiNF-D (DS-II), Myb, Spl, ATF- (DS-I) and CCAAT-related (H3-II) DNA binding activities (systematic name designated in brackets; summarized in Chapter 4) as indicated above the lanes. Arrowheads indicate complexes of HiNF-C and -D, respectively. The probe used here was derived from pTP-1 (H4-FO108 5'region from nt -130/-38; EcoRI/TaqI) with proteins from human HeLa S3 cells (5 μg) and poly I/C DNA as non-specific competitor.

In the middle panel, a band is observed below the HiNF-C complex that competes with oligonucleotides H4-I and H4-II (not indicated). It is possible that this protein/DNA complex is most likely identical to the HiNF-M complex (see Chapter 4). The left and right panel show competition results for, respectively, HiNF-B and HiNF-E (discussed in Chapter 5).

poly I/C DNA

H1

B

I)
CTRL
CCAAT
HiNF-D
MYB

poly I/C DNA

H4

C
D

II)
CTRL
ATF(1)
Sp1
HiNF-D
CCAAT

poly I/C DNA

H3

F

III)
CTRL
ATF(1)
ATF(2)
MYB
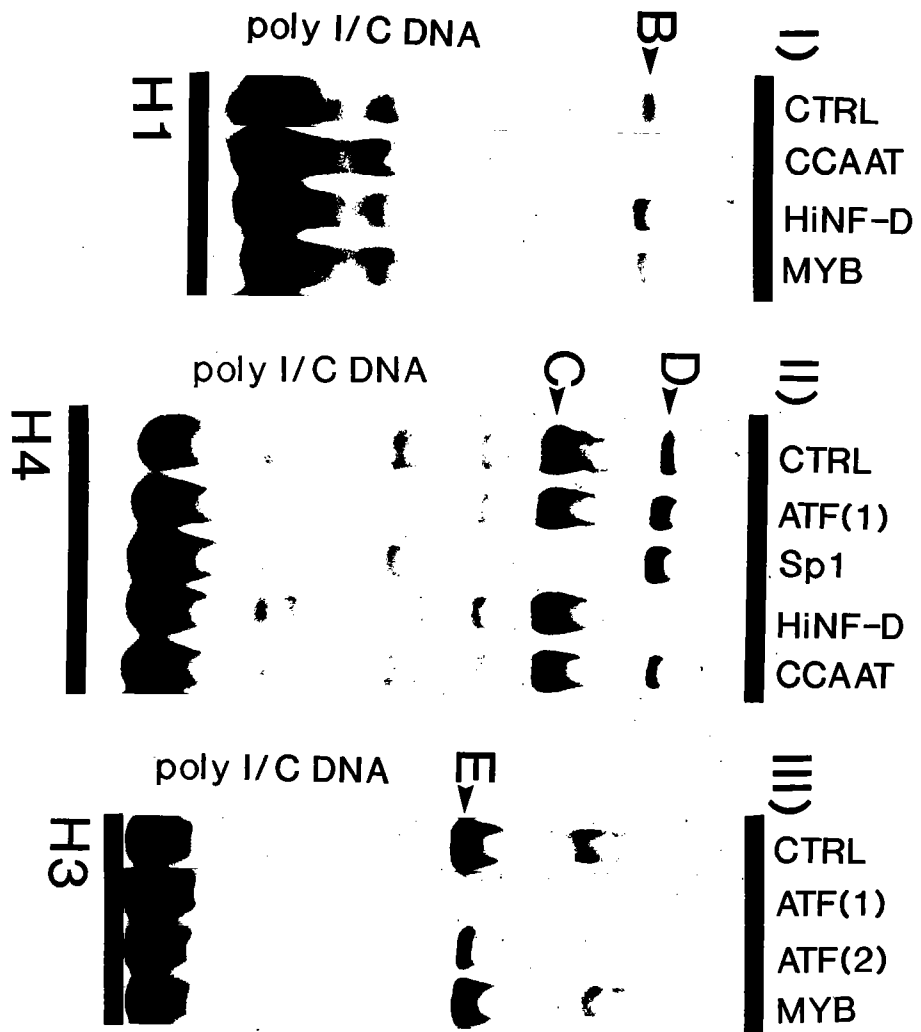
The HiNF-C binding site coincides with the DNA binding consensus sequence of the trans-activating DNA binding protein Spl (Kadonaga etal., 1986), and the above properties are consistent with a similarity between these factors. Results of later studies, including competition analysis (Fig. 2-8) and specific binding to wheat germ agglutinin (which indicates that HiNF-C, similar to Spl (Jackson & Tjian, 1989), is N-acetyl-glucosaminylated; data not shown), have strongly supported the assignment that HiNF-C is identical to Spl. Subsequent mutational analysis of the HiNF-C binding site is also consistent with this conclusion (Wright et al., 1991b). Regardless of identity with Spl, the available evidence indicates that HiNF-C is the protein that binds to the proximal part of in vivo protein/DNA interaction domain H4-Site I, and that HiNF-C is an auxiliary Spl-like transcription stimulatory factor.

The binding site of HiNF-D was located by means of stairway assays between nt -94 and -36 (ThaI and MboII sites, respectively)(Fig. 2-6; and data in van Wijnen et al., 1989), and sequences both downstream and upstream of an AvaII site (nt -74 to -71) are required for HiNF-D binding. The assignment of the binding site location was confirmed by competition analysis (Fig. 2-8). DNaseI footprinting experiments of the gel purified HiNF-D complex revealed protection of a number of phosphodiester bonds between nt -90 and -66 (summarized Fig. 2-7), but it has not been possible to define clear footprint boundaries in these experiments. These results demonstrate that HiNF-D is a sequence-specific DNA binding protein that recognizes sequences of the in vivo

protein/DNA interaction domain H4-Site II. The distal part of H4-Site II

contains an H4 histone gene consensus sequence (5'dGGTYYTCAATCNGGTCCG;

with Y = C or T, and N = A, C, T or G) derived by sequence comparison of

several H4 histone genes from diverse mammalian species. Hence, HiNF-D

may interact with and could be involved in regulation of multiple

functionally expressed H4 histone genes.

Results relating to the physical properties of HiNF-D indicate

that the factor is strongly temperature-sensitive in vitro above 48°

(Fig. 2-8), and sensitivity for the divalent cation chelator 1,10-

phenanthroline is observed only at elevated temperatures, which could

imply that the factor has a tightly anchored divalent cation. These and

other properties distinguish HiNF-D from factor H4-TF2, a DNA binding

activity that binds to the analogous region of the H4-Hu4A histone gene

characterized by Heintz and collaborators (Dailey et al., 1988).

Interestingly, HiNF-D binding in vitro is, unlike HiNF-A, -B and -C

binding, only stable at moderate ionic strength (van Wijnen et al.,

1989). A possible interpretation of this finding is that HiNF-D binding

to DNA is largely electrostatic in nature, and that specific binding of

HiNF-D in vivo may require interaction with other components.

The HiNF-D binding site overlaps with a DNA segment that is

necessary for in vivo expression of the human H4-FO108 histone gene in

mouse C127 cells (Kroeger et al., 1987), and it has been shown that

HiNF-D binding activity can be detected in these cells (confirming the

cross-species compatibility of histone gene trans-acting factors in

mammalians)(Fig. 2-6). Thus, HiNF-D may represent a DNA binding activity

required for H4 histone gene transcription. The establishment of binding sites for HiNF-D and the Sp1-like factor HiNF-C indicates that regulation of H4 histone gene transcription involves a complex interplay of putative histone gene specific DNA binding proteins and activities recognizing a broad spectrum of gene promoters.

2.2.2: Altered binding of human histone gene transcription factors during the shutdown of proliferation and onset of differentiation in HL60 cells.

Gary S. Stein, Jane B. Lian, Janet L. Stein, Robert Briggs, Victoria Shalhoub, Kenneth L. Wright and André J. van Wijnen (1989) Proceedings of the National Academy of Sciences U.S.A. **86**:1865-1869.

Genomic DMS fingerprinting experiments by Urs Pauli showed that the transcriptional downregulation of H4 histone gene transcription during differentiation of human HL60 promyelocytic leukemia cells coincides with a selective loss of occupancy of H4-Site II by the corresponding DNA binding activities in vivo, whereas no modifications were observed in the interaction of factors with the in vivo protein/DNA interaction domain H4-Site I of the H4-F0108 histone gene (Pauli et al., 1987). The extent to which these findings relate to protein/DNA interactions in vitro was explored by monitoring H4 histone gene promoter activities in nuclear protein preparations of proliferating and differentiated HL60 cells.

Binding activities HiNF-A and HiNF-C, proteins previously shown to interact with H4-Site I sequences, were detected in extracts of both

proliferating and differentiated HL60 cells, whereas HiNF-D binding to

H4-Site II was detected only in extracts of actively dividing cells

(Stein et al., 1989). The down-regulation of HiNF-D, and constitutive

presence of HiNF-A and HiNF-C, at the cessation of proliferation during

HL60 differentiation parallels the in vivo results. These and other

findings were incorporated in a tentative working model of H4 histone

gene transcription (Stein et al., 1989), with H4-Site I representing a

constitutive and auxiliary protein/DNA interaction domain, and H4-Site

II embodying a putative, proliferation-specific on/off switch. Another

proposal accompanying this model is that HiNF-D is involved in the

coordinate regulation of the proliferation-specific H4 histone multi-

gene subfamily during the cell cycle, and may become a rate-limiting

factor during the shut-down of histone gene transcription at the onset

of terminal differentiation.

CHAPTER 3

REGULATION OF PROTEIN/DNA INTERACTIONS IN THE PROXIMAL PROMOTERS OF HUMAN H4, H3 AND H1 HISTONE GENES DURING THE CELL CYCLE, DIFFERENTIATION, TUMORIGENESIS AND DEVELOPMENT

3.0: Summary

Regulation of protein/DNA interactions in histone genes was investigated during several cell cycle related processes. We focused on the proliferation-specific H4 histone gene DNA binding protein HiNF-D, because (I) this factor is downregulated in conjunction with histone gene transcription during HL60 cell differentiation (van Wijnen et al., 1989). Several collaborative experiments were performed in which HiNF-D DNA binding activity was measured during various biological processes, e.g., cell cycle progression and mammalian development. We also examined protein/DNA interactions in H3 and H1 histone promoters. The results show that (II) factor HiNF-D DNA binding activity is downregulated during the onset of quiescence, and upregulated in a cell density dependent manner during re-entry into the cell cycle in growth factor stimulated normal CF-3 diploid fibroblasts (Wright et al., 1990a). Also, (III) HiNF-D activity is modulated during the cell cycle and is at maximal levels during S-phase (Holthuis et al., 1990). This modulation is coupled to the entry into and exit from S-phase, but is not directly associated with continued DNA synthesis (Holthuis et al., 1990). Interestingly, (IV) cell cycle changes in HiNF-D activity are not observed in several transformed and tumor derived cells suggesting

derangement of cell cycle regulation of HiNF-D activity during

tumorigenesis (Holthuis et al., 1990). Consistent with these results

obtained in cell culture systems, (V) HiNF-D activity is downregulated

in conjunction with histone gene transcription during murine hepatic

development, reflecting the onset of in vivo quiescence and

differentiation (van Wijnen et al., 1991a). Analysis of protein/DNA

interactions of human H3 and H1 histone genes during several biological

processes shows (VI) a coupling between the HiNF-D:H4-Site II

protein/DNA interaction and analogous protein/DNA interactions in H3 and

H1 histone genes (van Wijnen et al., 1991c). This suggests that

protein/DNA interactions of all three genes are collectively subject to

stringent cell growth regulation, and HiNF-D is a component of this

putative coordinate control mechanism. Because modulations in the HiNF-

D:H4-Site II interactions correlate with modifications in histone gene

transcription and histone gene expression during several biological

processes (Chapter 1), these data suggest that this interaction performs

a key role in H4 histone gene regulation.

**3.1: Multiple mechanisms regulate the proliferation-specific histone
gene transcription factor HiNF-D in normal human diploid fibroblasts.**
Kenneth L. Wright, Robert T. Dell'Orco, André J. van Wijnen, Janet L.
Stein and Gary S. Stein (1991) Biochemistry, in revision.

The involvement of the proliferation specific nuclear factor HiNF-

D in human H4 histone gene transcription has led to the hypothesis that

HiNF-D binding activity may be regulated during several cell growth

related processes. In order to test this proposal, HiNF-D activity was monitored during the exit from the cell cycle into quiescence, and re-entry into an actively proliferating mode using normal diploid human foreskin CF-3 fibroblasts.

The cell growth states of these cells were modulated in culture by varying cell density (confluent versus pre-confluent cells) and/or the concentration of nutrients and growth factors (serum-stimulated versus serum-deprived cells)(experiments by R.T.D.). Nuclear protein preparations were tested for the presence of HiNF-D binding activity using gel retardation assays (experiments by A.J.v.W. and K.L.W.). In addition, in vitro transcription assays were performed using these unfractionated nuclear extracts (experiments by K.L.W., data not shown; Wright et al., 1990a).

The results show that overall HiNF-D binding activity is down-regulated when cells cease to divide in culture at quiescence (data not shown; Wright et al., 1990a). This decrease in activity occurs when exponentially growing cells have become growth arrested during serum-deprivation, and when cells have proliferated to confluency, become contact-inhibited and are subject to serum deprivation. Interestingly, the extent to which total HiNF-D activity is induced at the cell population level upon serum-stimulation appears to depend strongly on initial cell density. This suggests that individual cells may regulate HiNF-D activity at two levels, one dependent on the current proliferative state of the cell ("cycling" versus "non-cycling"), and the other related to proliferative potential.

3.2: Tumor cells exhibit deregulation of the cell cycle histone gene promoter factor HiNF-D.

J. Holthuis, T.A. Owen, A.J. van Wijnen, K.L. Wright, A. Ramsey-Ewing, M.B. Kennedy, R. Carter, K.C. Cosenza, K.J. Soprano, J.B. Lian, J.L. Stein, G.S. Stein (1990) Science **247**: 1454-1457.

The cell growth related downregulation of HiNF-D activity during the initiation of differentiation in human HL60 promyelocytic leukemia cells, and during the onset of quiescence in normal diploid human CF-3 foreskin fibroblasts, suggested that HiNF-D may be involved in the transcription of H4 histone genes during the cell cycle. This also suggested the possibility that HiNF-D itself may be regulated during the cell cycle, at the level of either HiNF-D DNA binding activity and/or perhaps a putative intrinsic transcription stimulatory activity. Initial results were obtained with synchronized, heteroploid human HeLaS3 cervical carcinoma cells that were isolated at multiple hourly intervals after release from a thymidine blockade at the G1/S-phase boundary. The cell synchronization was induced by the administration of excess thymidine at two strategically coupled time-points during previous rounds of cell proliferation. To ensure that this synchronization procedure was not creating a biological artifact, HiNF-D activity was monitored both during entry into the first S-phase, and after the ensuing mitosis, during progress through G1 phase and re-entry into a second S-phase. This experiment clearly showed that the total level of HiNF-D binding activity is not dramatically altered, but rather constitutive during the cell cycle of these tumor-derived cells (data

not shown; discussed in van Wijnen et al., 1989).

Additional cell synchronization experiments were performed by Joost Holthuis (and others) with several distinct normal diploid, as well as tumor-derived and transformed cell types using various chemically induced blockades. Nuclear extracts were prepared from cells at several stages during the cell cycle and HiNF-D binding activity was monitored using gel retardation assays (experiments by J.H. and T.A.O.). The extent by which HiNF-D activity is coupled to exit from S-phase was addressed by parallel experiments in which synchronized cells were treated with the DNA synthesis inhibitor hydroxyurea (experiments by J.H. and T.A.O.; also discussed in section 3.4). The presence of HiNF-D binding activity was confirmed by specific competition analysis using synthetic oligonucleotides (Fig. 3-1; data not shown in Holthuis et al., 1990).

Comparable amounts of HiNF-D activity were detected in the S and G1 stages of human HL60 promyelocytic leukemia cells, similar to the observations made with HeLa S3 epithelial cervical carcinoma cells. In contrast, nuclear extracts derived from normal diploid human WI38 lung fibroblasts and primary rat calvarial osteoblasts contain high levels of HiNF-D activity only in cells progressing through S-phase, whereas this activity is barely detectable in extracts from cells outside S-phase. These results demonstrate that HiNF-D activity is cell cycle regulated in cells displaying growth characteristics resembling those of the normal diploid phenotype.

Figure 3-1. <u>Competition analysis of HiNF-D isolated from distinct</u>

<u>species and cell types with different cell growth characteristics.</u> HiNF-

D can be detected in nuclear extracts from proliferating cells of

various mammalian species supporting the cross-species similarities in

trans-acting factors regulating histone gene transcription. Binding

reactions were performed with nuclear proteins from human HeLa S3 and

WI-38 cells, as well as rat primary osteoblast and osteosarcoma cells

(approximately 2 μg protein each). Reactions were performed incubated in

the absence of competitor DNA (C), or the presence of the non-competing

H4-I oligonucleotide (N; systematic designation DS-I, see Chapter 4) or

the specific H4-II oligonucleotide (S)(systematic designation DS-I).

These specific competitor DNA fragments were present in approximately

500-fold molar excess. The H4-probe used is the HindIII/TaqI fragment of

pFP-1.

normal diploid

tumor derived

rat
C N S

human
C N S

rat
C N S

human
C N S

D

C

H4 promoter

ROB

WI38

ROS

HeLa
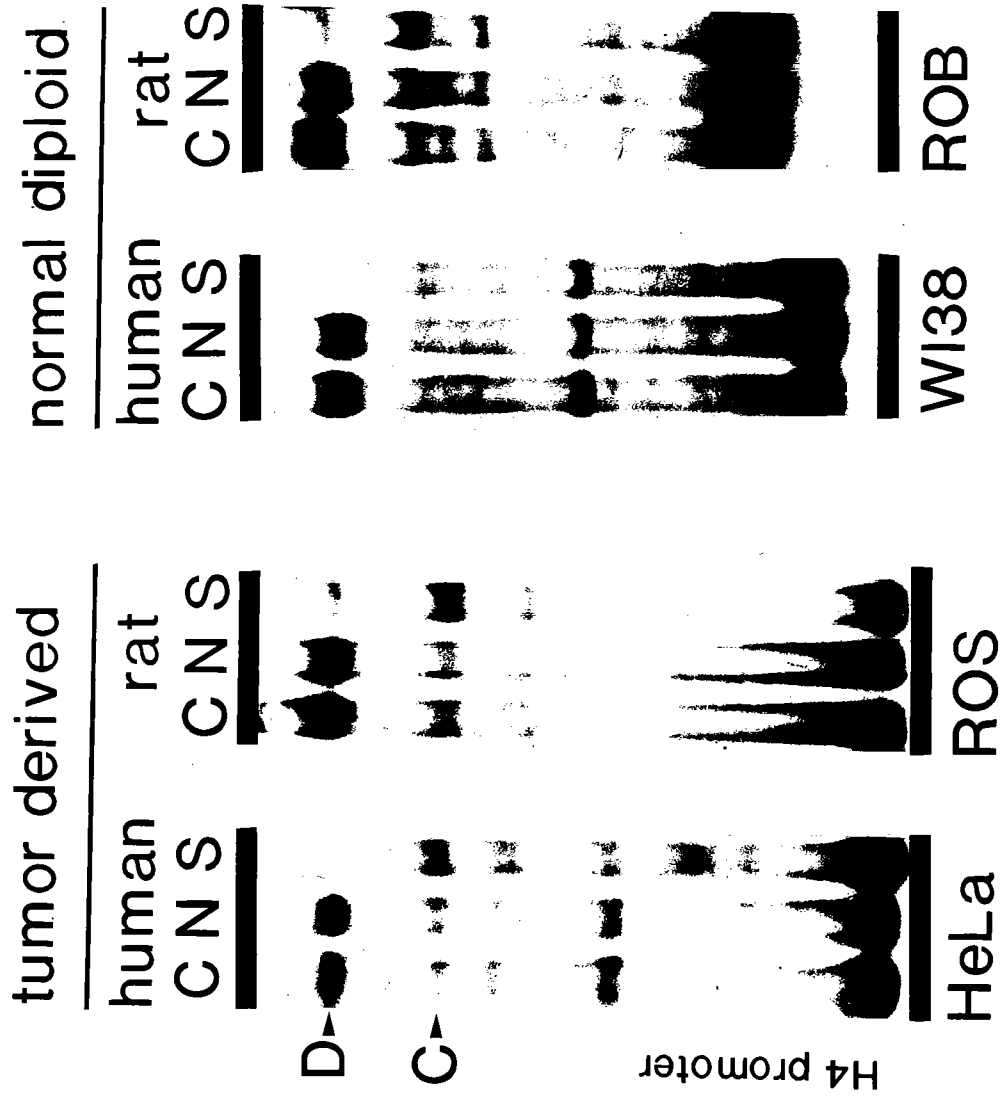
Cell cycle stage specific nuclear extracts from SV40 virally
transformed WI38 cells and rat osteosarcoma cells were also assayed
(Holthuis et al., 1990). The results show that HiNF-D activity is
abundant both during and outside S-phase in both cell lines. These cell
types represent transformed or tumor-derived counterparts of,
respectively, normal diploid human WI38 fibroblasts and rat osteoblasts.
Thus, direct comparison of the results obtained with these four cell
types indicates that HiNF-D is cell cycle regulated in normal diploid
cells that are subjected to stringent cell growth regulation. However,
several transformed and tumor-derived cells exhibit deregulation of
HiNF-D binding activity resulting in constitutive presence of this
factor throughout the cell cycle. We conclude that control of HiNF-D
binding activity is intimately associated with cell growth mechanisms
regulating the progress through S-phase, and that deregulation of HiNF-D
is related to the loss in stringent cell growth control that accompanies
neoplastic transformation.

3.3: Involvement of the cell cycle regulated nuclear factor HiNF-D in cell growth control of a human H4 histone gene during hepatic development in transgenic mice.

Andre J. van Wijnen, Theodore K. Choi, Thomas A. Owen, Kenneth L.
Wright, Jane B. Lian, Rudolf Jaenisch, Janet L. Stein and Gary S. Stein
(1991) Proc. Natl Acad. Sci. U.S.A. 88:2573-2577.

Regulation of the cell cycle controlled H4 histone promoter factor
HiNF-D has been extensively studied using a variety of cell types with

diverse cell growth characteristics. However, our understanding of cell cycle and cell growth control of this factor during the cessation of proliferation and intitiation of differentiation is restricted to results obtained from cell culture models. Therefore, the mechanism associated with regulation of HiNF-D binding activity was studied during the onset of quiescence and differentiation in the intact animal. In this biological model system, development of tissue organization is influenced by physiological mediators, and normal cell growth- and developmental control mechanisms functionally related to the cell division cycle are operative.

Nuclear extracts were prepared from several adult mouse tissues, including liver, spleen, thymus and brain, as well as from fetal liver and brain. HiNF-D binding activity was measured in gel retardation assays optimized for detection of this factor. The identity of HiNF-D in preparations containing this activity was confirmed by competition analysis using specific and non-specific synthetic oligonucleotides (see section 3.4).

The results show that HiNF-D binding activity can be detected in vitro in protein preparations derived from tissues, such as spleen and thymus, containing actively proliferating cells and expressing DNA replication dependent histone genes (van Wijnen et al., 1991a). Factor HiNF-D could not be detected in adult liver where cell cycle dependent H4 histone gene expression is below the level of detection. Interestingly, adult brain contains high levels of HiNF-D activity, but histone gene expression in this tissue is barely detectable. The finding

that cells in this tissue actively transcribe H4 histone genes (as measured by reporter gene expression of chimeric constructs containing the H4-F0108 histone promoter fused to several different reporter genes; experiments by Theodore Choi), is consistent with the abundance of HiNF-D in this tissue.

The correlation between cell proliferative activity, histone gene expression and HiNF-D binding activity is consistent with a role for this factor in the transcription of H4 histone genes during the cell cycle of dividing cells in the intact animal. The parallel between abundance of HiNF-D activity and active H4 histone gene transcription in brain, with limited expression of H4 histone genes at the mRNA level, supports this observation, but also indicates that both transcriptional and post-transcriptional mechanisms are operative in this tissue.

The relationship between HiNF-D activity and the onset of in vivo quiescence and differentiation during hepatic development was studied by direct comparison of the presence of this factor in nuclear extracts derived from fetal and adult liver, as well as from fetal and adult brain (see section 3.4). Whereas high levels of factor HiNF-D can be detected in both fetal and adult brain, as well as in fetal liver, this DNA binding activity is below the level of detection in adult liver. The physiological significance of the constitutive presence of HiNF-D during brain development is unclear, but could perhaps be related to the presence of mitogenic factors in this tissue throughout development. More importantly, these findings indicate that HiNF-D is down-regulated during hepatic development in conjunction with histone gene

transcription (as measured by reporter gene expression) and expression of these genes at the histone mRNA level. This suggests that HiNF-D is a predominant regulatory factor that may determine the competency of the H4 histone gene 5′ flanking region to promote transcription, and therefore may be a rate-limiting factor in the accumulation H4 histone gene transcripts.

3.4: Coordination of protein/DNA interactions in the promoters of human H4, H3 and H1 histone genes during the cell cycle, tumorigenesis and development.

Andre J. van Wijnen, Thomas A. Owen, Joost Holthuis, Jane B. Lian, Janet L. Stein and Gary S. Stein (1991) J. Cell. Physiol. in press.

The previous studies have resulted in the following findings: (I) the establishment of multiple protein/DNA interaction sites in human H4, H3 and H1 proximal promoters (Chapter 2); (II) the identification of one protein/DNA interaction site in the H4 histone promoter involving a DNA binding activity designated HiNF-D that is regulated during entry into, progress through and exit from the cell cycle (sections 3.1, 3.2 and 2.2.2); and (III) the observation that HiNF-D interacts with a proliferation-specific protein/DNA interaction domain designated H4-Site II (Chapter 2), and the integrity and occupancy of this domain, encompassing both a histone specific element and a TATA-box, is required for the expression of H4 histone genes (Chapter 1). Because of this, it is conceivable that analogous regulated interactions may occur in the promoters of other histone genes. Interestingly, the H3-ST519 histone

gene interacts with a factor (HiNF-D3) that appears to be downregulated in conjunction with HiNF-D during differentiation of (tumor-derived) HL60 cells (discussed in manuscript accompanying section 2.2.2). The following experiments elaborate on this finding.

DNA fragments spanning the proximal promoters of the human H3-ST519 (nt -200 to -20) and H1-FNC16 (nt -213 to -78) histone genes were used as probes in gel retardation assays. Binding reactions were performed with DNA binding proteins present in cell cycle- and developmental stage specific nuclear extracts. These nuclear protein preparations were derived from various cultured cell lines and a spectrum of mouse tissues, and are identical to those used to establish regulation of HiNF-D during various cell growth related processes (sections 3.2 and 3.3).

These experiments reveal that both the H3-ST519 and H1-FNC16 histone proximal promoters interact with sequence-specific DNA binding activities, respectively designated HiNF-D3 and HiNF-D1, that are cell cycle regulated in normal diploid cells in parallel with HiNF-D (Figs. 3-2 and 3-3; cell cycle data on HiNF-D in Fig. 3-3 were obtained by Holthuis et al., 1990 and are shown for reference only). Similar to HiNF-D, activation of these DNA binding proteins requires progress into S-phase and the concomitant initiation of DNA synthesis as evidenced by low levels of these factors in cells chemically blocked at the G1/S-phase boundary by excess thymidine. In addition, downregulation of these factors is not dependent on the cessation of DNA replication, but does require the exit from S-phase (and perhaps progress through other cell

Figure 3-2. <u>Binding sites of HiNF-D related protein/DNA complexes in</u> <u>human H4-F0108, H3-ST519 and H1-FNC16 histone genes.</u> Deletion analysis of the proximal promoters of these three genes shows sequence-specific protein/DNA complexes with similar relative mobility. Stairway assays were performed as described (Chapter 2). Promoter fragments were uniquely labelled at endonuclease cleavage sites in H4, H3 and H1 histone promoters (respectively, BanII of pKUC8, HindIII of pTP-1 and SmaI of pOX001) at the indicated positions (abbreviated ^{32}P: nt#). These probes were progressively shortened by endonuclease cleavage at the indicated positions (from left to right, respectively: H4 = EcoRI, TaqI, ThaI and AvaII; H3 = BstNI, ThaI, AvaII and HinfI; H1 = RsaI, HinfI and HaeIII) with the minus-sign of nucleotide numbers (dash) pointing at the free DNA of each probe. Binding reactions were performed in parallel for each set using chromatography fractions containing HiNF-D activity: respectively, P0-200 (H4), P100-400 (H3) and D0-200 (H1) (approximately 1 μg protein each). The HiNF-D (H4), HiNF-D3 (H3) and HiNF-D1 (H1) complexes are indicated with arrow-heads. The darkest band (relative migration of approximately 0.4) in the H1-series represents the HiNF-B complex (binds to nt -122 to -90) and this protein/DNA complex occurs with all three probes (not indicated). (See van Wijnen et al., 1991c for further details).

H1

D1→

−213
−150
−139

^{32}P:−78

H3

D3→

−142
−110
−77
−67

^{32}P:−20

H4

D→

−183
−130
−94
−70
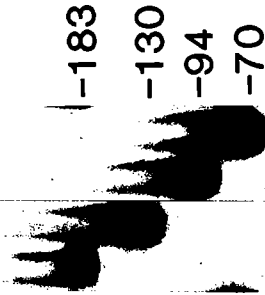
^{32}P:−13
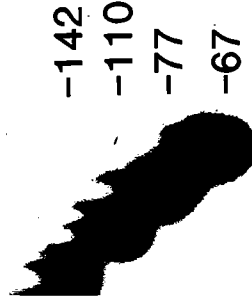
Figure 3-3. <u>Coordination of protein/DNA interactions in H4, H3 and H1</u>
<u>histone promoters during the cell cycle.</u> Nuclear proteins were isolated
from rat primary calvarial osteoblasts synchronized by double thymidine
block and analyzed using gel retardation assays. Each panel shows
results with proteins derived from cells, respectively, blocked with 2
mM thymidine before release in the cell cycle (PR), traversing through
S-phase and harvested 4 hr after release (S), and progressing through
early G1 and harvested 12 hr after release (G1); cells in mid-S phase
were treated with the DNA synthesis inhibitor hydroxyurea and harvested
2 hr (S+HU2) and 8 hr (S+HU8) after treatment. The upper panel shows
results obtained with the H4 promoter probe using, respectively 2, 4, 6,
9 and 12 μg protein for each sample (Holthuis et al., 1990) and is shown
for reference only. The middle (H3 promoter probe) and lower (H1
promoter probe) panels show results using, respectively 2, 4, 6, 8 and
10 μg protein. The non-specific competitor DNA was 2 μg poly I/C DNA.
The position of the HiNF-D, HiNF-D3 and HiNF-D1 protein-DNA complexes
are indicated by arrowheads.

Cell cycle analysis

PR   S   G1   S/HU1   S/HU8

H4 promoter — D▶

H3 promoter — D3▶

H1 promoter — D1▶
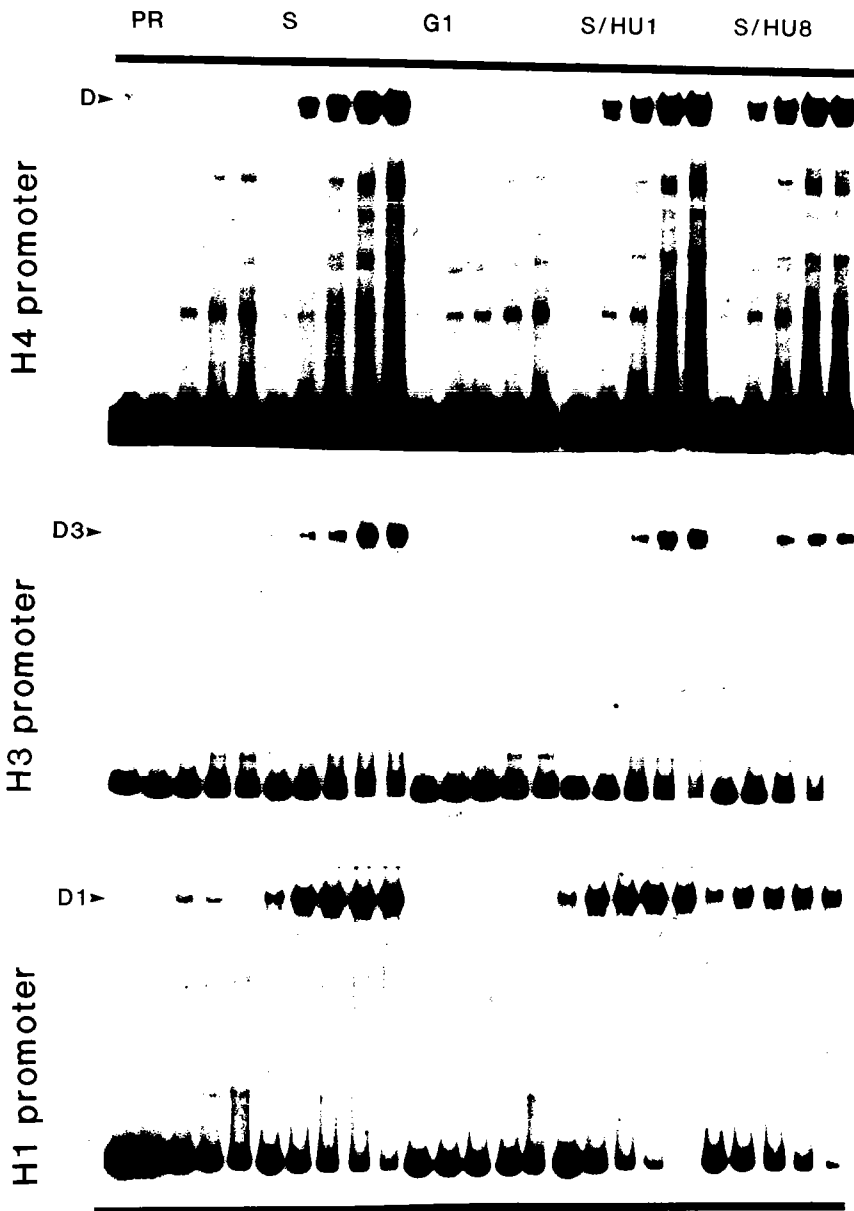
Figure 3-4. <u>Collective deregulation of protein/DNA interactions in H4,</u> <u>H3 and H1 histone promoters during tumorigenesis.</u> Cell cycle analysis was performed with nuclear proteins derived from WI38 human normal diploid fibroblasts, SV40 transformed WI38 cells, rat primary calvarial osteoblasts (ROB) and rat 17/2.8 osteosarcoma cells (ROS), human HeLa S3 heteroploid cervical carcinoma cells and HL60 promyelocytic leukemia cells and analyzed using gel retardation assays. Upper panels and lower left panel show results of S-phase and G1-phase specific extracts from the indicated cell types with the H3 promoter probe and, respectively, 2, 4, 6 and 8 μg protein added. The lower right panel shows same for the H1 promoter probe, using 4 μg protein only for each sample. Poly G/C DNA (2 μg) and poly I/C DNA (200 ng) were used as non-specific competitors. Deregulation of HiNF-D activity (H4-F0108 gene) in tumor cells has been presented elsewhere (Holthuis et al., 1990).

Cell cycle analysis

osteosarcoma
osteoblast
H3 promoter
D3

H1 promoter
D1
ROS
ROB
WI38/SV40
WI38
HL60
HeLa
S G1 S G1 S G1 S G1 S G1 S G1

S G1 S G1

WI38/SV40
WI38
H3 promoter
D3
S G1 S G1

HL 60
HeLa S3
H3 promoter
D3
S G1 S G1
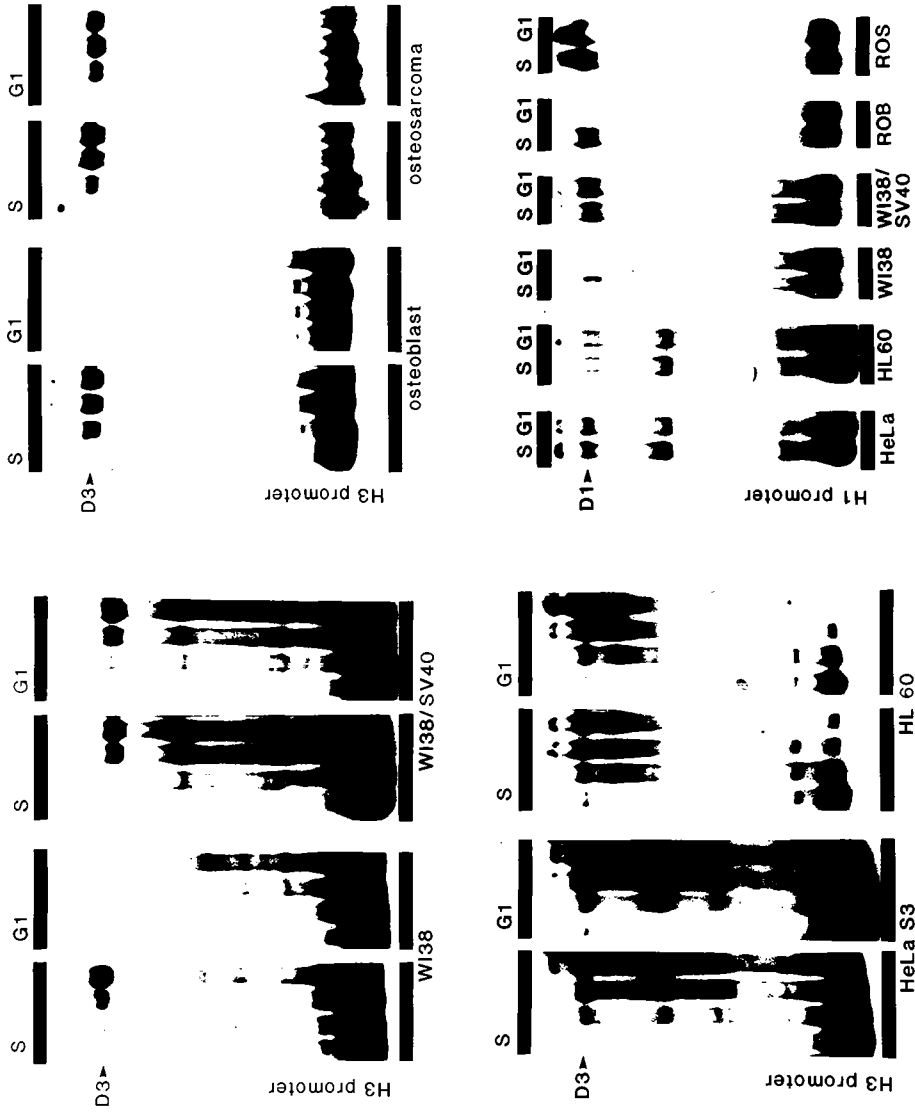
cycle stages) as shown by the presence of HiNF-D, HiNF-D3 and HiNF-D1 in cells blocked in mid-S phase for a prolonged period of time with the DNA synthesis inhibitor hydroxyurea. A simple interpretation of these findings is accomplished by postulating an oscillatory molecular mechanism that regulates specific protein/DNA interactions of three different histone subtypes in concert. This hypothetical mechanisms monitors cellular events at two distinct transition points related to the entry and exit from S-phase.

Interestingly, results obtained with four distinct transformed and tumor-derived cell types indicate that the H3 and H1 histone promoter factors, analogous to HiNF-D, are constitutively present during the cell cycle of these cells (Fig. 3-4). Comparison of the results obtained with normal diploid and tumor cells shows that the difference in cell cycle dependency of these three DNA binding activities correlates with the extent to which a cell displays cell growth properties of the transformed phenotype. This suggests that deregulation of the putative control mechanism that modulates these DNA binding activities simultaneously during the cell cycle is a very frequent event during aberrations in stringent cell growth control during tumorigenesis.

Experiments with mouse nuclear proteins derived from fetal and adult liver, as well as fetal and adult brain, show that the DNA binding activities of HiNF-D3 and HiNF-D1 are downregulated in conjunction with HiNF-D during murine hepatic development (Fig. 3-5). In contrast, constitutive levels of HiNF-D, HiNF-D3 and HiNF-D1 are observed during brain development from fetus to adult. The parallel in the regulation of

Figure 3-5. <u>Coordination of protein/DNA interactions in H4, H3 and H1</u>
<u>histone promoters during hepatic development.</u> Gel retardation analysis
of nuclear factors with nuclear proteins from mouse liver (Lv) and brain
(Br) in both the fetal (F) and adult (A) developmental stages. Assays
were performed using the H4 promoter probe (first panel), H3 promoter
probe (second panel) or the H1 promoter probe (third panel).
Respectively 2, 3 and 4 μg protein was added per binding reaction.
Arrowheads in the upper panel indicate two forms of HiNF-D binding
activity (which is related to phosphatase- sensitive post-translational
modifications) and another H4-Site II DNA binding protein designated
HiNF-M (van Wijnen et al., 1991b).

Mouse development:
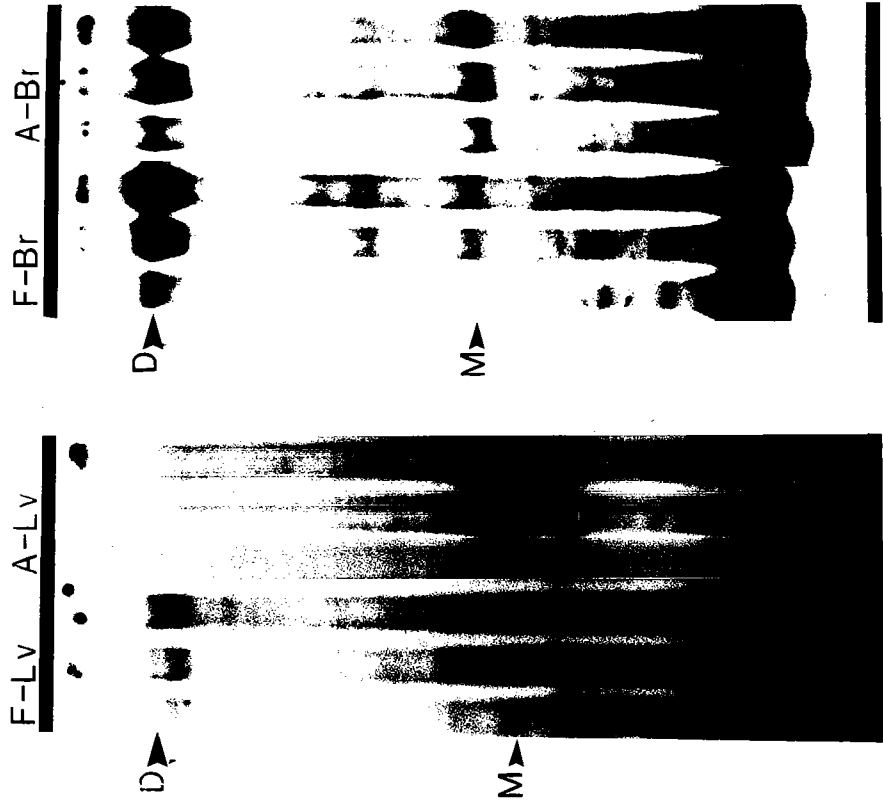
H4 promoter

Mouse development

F-Lv A-Lv   F-Br A-Br   F-Lv A-Lv   F-Br A-Br

D3►   D1►

B3►   B►

H3 promoter   H1 promoter
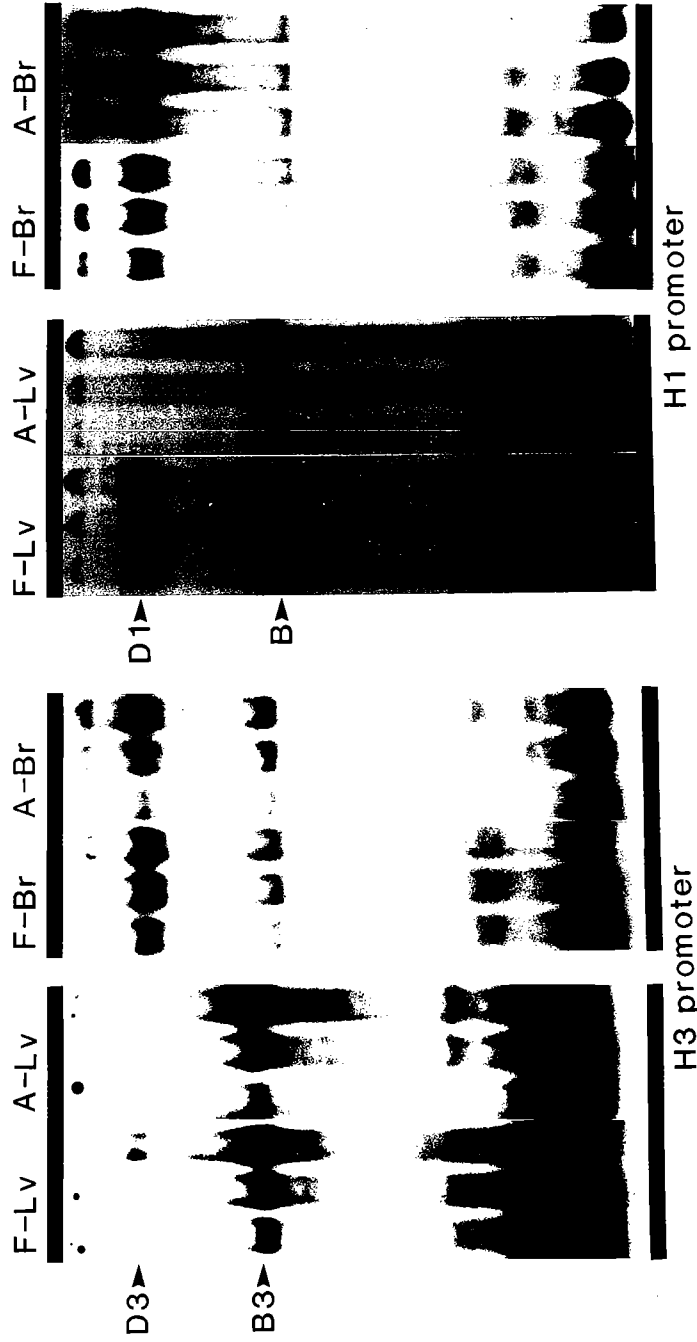
protein/DNA interactions in H4, H3 and H1 histone gene promoters during the cell cycle and development, as well as the putative collective deregulation of these interactions during tumorigenesis supports the proposal that the corresponding factors are subject to a shared mechanism that coordinately modulates the level of these DNA binding activities.

The possibility that this coordinate control mechanism is related to shared molecular components intrinsic to these DNA binding activities was addressed initially by temperature stability experiments, as well as directly at the level of DNA binding activity by specific competition analysis. These are among a handful of meaningful experiments that can be performed at present in the absence of efficient purification procedures and/or specific molecular probes for any of these factors (except the ability to detect DNA binding activities).

The results show that the temperature inactivation profiles of human HiNF-D, -D3 and -D1 are indistinguishable (data not shown; van Wijnen et al., 1991c), and are consistent with previous studies that indicated that HiNF-D activity is inactivated above 48^0C (section 2.2.1). Temperature stability chacteristics of four histone promoter factors (HiNF-A, -B, -C and -D) are very distinct, and this same physical property has been used previously to discriminate between individual members of a heterogeneous family of CCAAT-box and other DNA binding proteins. Thus, HiNF-D, HiNF-D3 and HiNF-D1 are characterized by similar relative migration rates in gel retardation assays, as well as indistinguishable biological regulation and temperature inactivation

profiles.

A striking example of the specificity of temperature inactivation experiments for a given factor is the following. Parallel experiments were performed with nuclear proteins from rat osteoblasts. Rat HiNF-D binding activity is thermally inactivated at temperatures lower than human HiNF-D (approximately 10°C lower relative to human HiNF-D with the same assay conditions)(A.J.v.W., unpublished observations). However, the three analogous rat DNA binding activities (HiNF-D, -D3 and -D1) are inactivated at identical temperatures. Although the reason for the difference in inactivation temperatures of rat and human HiNF-D is unclear, this observation may exemplify intra-species similarities in the heat-stability of HiNF-D, -D3 and -D1, in further support of a direct molecular relationship between these activities.

Competition experiments using specific oligonucleotides reveal that an oligonucleotide spanning the HiNF-D binding site in the H4-FO108 histone gene competes for HiNF-D binding to the H4-FO108 histone promoter and HiNF-D3 binding to the H3-ST519 histone promoter (Fig. 3-6), but not for HiNF-D1 binding to the H1-FNC16 histone promoter. Other oligonucleotides spanning only a subset of the HiNF-D binding site sequences do not compete. Sequence comparisons with these proximal promoters do not indicate obvious extended sequence similarities. However, cross-competition between HiNF-D and HiNF-D3 clearly suggests that these factors may be directly related by a shared DNA binding activity that may not recognize an extended primary DNA sequence, but perhaps short permutated sequences or a particular DNA conformation.

Figure 3-6. <u>Cell cycle regulated protein/DNA interactions in H4 and H3</u>

<u>histone genes involves a shared DNA binding activity.</u> Competition

analysis of protein-DNA complexes was performed with the H4 (page 78,

lower panels), H3 (page 79) and H1 (page 80) promoter probes using 500-

fold molar excess of synthetic oligonucleotides spanning H4 (oligo's H4-

II and DD-1) and H3 (oligo H3-II) histone gene promoter sequences. All

lanes contain 4 μg protein from, as indicated, human HeLa S3 cells,

fetal mouse liver and brain, and adult mouse liver and brain cells.

Arrowheads indicate the positions of the HiNF-D3 and HiNF-D1 complexes,

and complexes mediated by CCAAT-box binding activities (B and B3). The

upper panel on page 78 shows HiNF-D activity in adult mouse tissues as

discussed in this chapter and as described by van Wijnen et al., 1991a.

Mouse tissue-distribution

HeLa   A–Lv   A–Br   A–Sp   A–Th

D►

M►
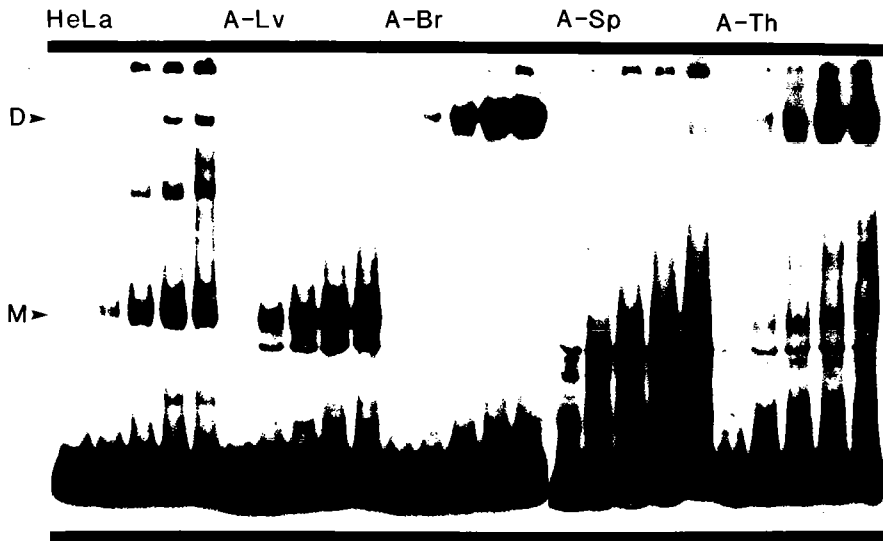
H4 promoter

Competition analysis

H4 promoter

F–Lv   F–Br   A–Lv   A–Br

Competition analysis

H1 promoter

HeLa — C, H3-II, H4-II, DD-1

A-Br — C, H3-II, H4-II, DD-1

A-Lv — C, H3-II, H4-II, DD-1

B, D1
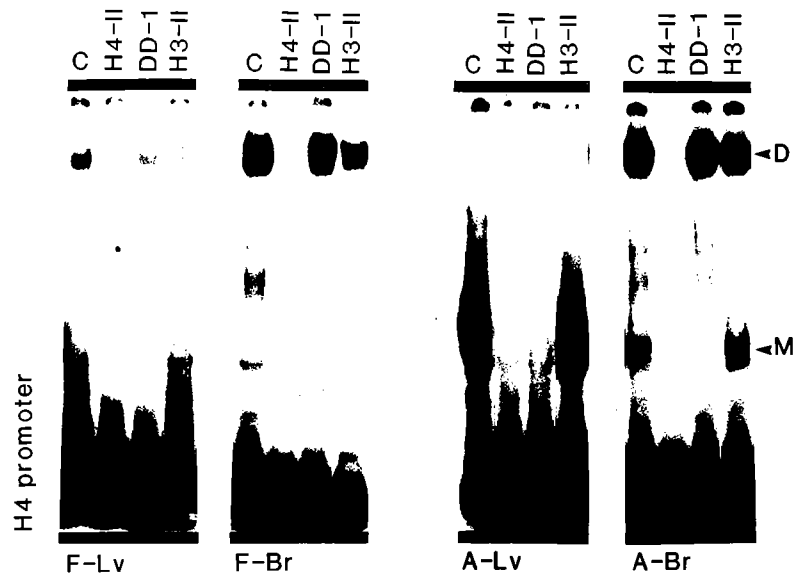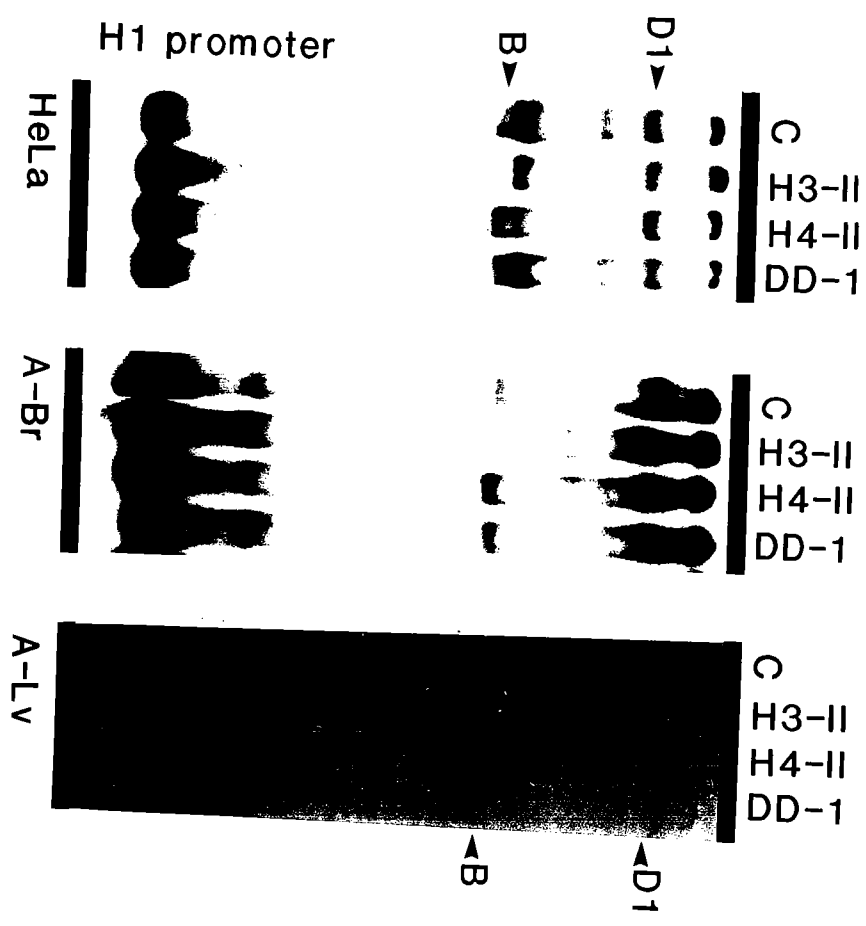
Competition analysis
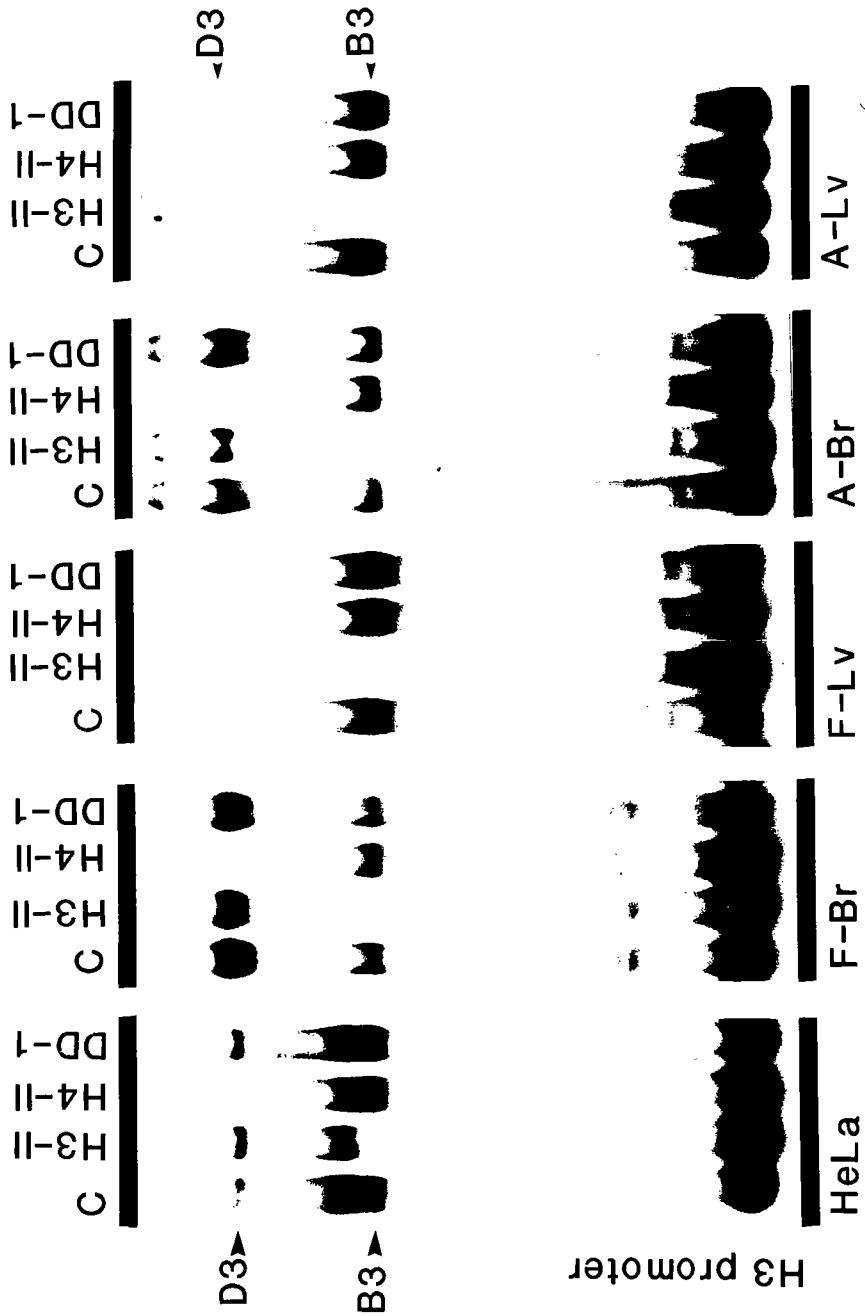
H3 promoter

The finding that analogous protein/DNA interactions in H4, H3 and H1 histone promoters are collectively regulated at several transition points during the cell cycle, as well as during cell growth and development clearly suggests that transcriptional control directly at the level of DNA binding activity of trans-activating factors is a primary means by which cells may coordinately regulate histone gene expression during the cell cycle in normal diploid cells. Moreover, four distinct tumor and transformed cell types display a loss in coupling with the cell cycle of the mechanism that coordinately controls these protein/DNA interactions. This suggests that this coordinating mechanism is intimately associated with stringent cell growth control and a frequent target of deregulation during tumorigenesis.

CHAPTER 4

TRANSCRIPTIONAL ELEMENT H4-SITE II OF CELL CYCLE REGULATED HUMAN H4 HISTONE GENES IS A MULTIPARTITE PROTEIN/DNA INTERACTION SITE FOR FACTORS HiNF-D, HiNF-M AND HiNF-P: INVOLVEMENT OF PHOSPHORYLATION

Andre J. van Wijnen, Anna L. Ramsey-Ewing, Rita Bortell, Thomas A. Owen, Jane B. Lian, Janet L. Stein, Gary S. Stein (1991). J. Cell. Biochem. 46: 1-16

4.0: Summary

Cell cycle regulated gene expression was studied by analyzing protein/DNA interactions occurring at the H4-Site II transcriptional element of H4 histone genes using several approaches. We show that this key proximal promoter element interacts with at least three distinct sequence-specific DNA binding activities, designated HiNF-D, HiNF-M and HiNF-P. HiNF-D binds to an extended series of nucleotides, whereas HiNF-M and HiNF-P recognize sequences internal to the HiNF-D binding domain. Gel retardation assays show that HiNF-D and HiNF-M each are represented by two distinct protein/DNA complexes involving the same DNA binding activity. These results suggest that these factors are subject to post-translational modifications. Dephosphorylation experiments in vitro suggest that both electrophoretic mobility and DNA binding activities of HiNF-D and HiNF-M are sensitive to phosphatase activity. We deduce that these factors may require a basal level of phosphorylation for sequence specific binding to H4-Site II and may represent phosphoproteins

occurring in putative hyper- and hypo-phosphorylated forms. Based on
dramatic fluctuations in the ratio of the two distinct HiNF-D species
both during hepatic development and the cell cycle in normal diploid
cells, we postulate that this modification of HiNF-D is related to the
cell cycle. However, in several tumor-derived and transformed cell types
the putative hyper-phosphorylated form of HiNF-D is constitutively
present. These data suggest that deregulation of a phosphatase-sensitive
post-translational modification required for HiNF-D binding is a
molecular event that reflects abrogation of a mechanism controlling cell
proliferation. Thus, phosphorylation and dephosphosphorylation of
histone promoter factors may provide a basis for modulation of
protein/DNA interactions and H4 histone gene transcription during the
cell cycle and at the onset of quiescence and differentiation.

4.1: Introduction

Cell cycle regulation of gene expression is fundamental to cell
growth control during embryonic development and throughout the life of
an organism. During tumorigenesis aberrations in stringent regulation
result in deregulation of the proliferative process. The histone multi-
gene family is a paradigm for regulatory mechanisms operative during the
eukaryotic cell cycle. Synthesis of histone proteins is a prerequisite
for the assembly of newly replicated DNA into chromatin, and is
essential for the ordered progression through the cell division cycle.
Histone mRNAs are among the most highly abundant gene transcripts
expressed during S-phase. Histone mRNA levels are coordinately regulated

and tightly coupled to DNA replication and histone protein synthesis. Histone gene expression is regulated at multiple levels with an important contribution of transcriptional control (Marzluff & Pandey, 1988; Stein et al., 1989b).

Transcriptional cis-acting elements involved in regulation of histone genes, and the cognate trans-acting factors, have been defined in some detail in diverse eukaryotic species. The human H4 histone gene FO108 contains a proliferation-specific protein/DNA interaction site (H4-Site II) that is essential for its transcription (Kroeger et al., 1987; Pauli et al., 1987; Stein et al., 1989a & 1989b), and interacts with the cell cycle regulated factor HiNF-D (van Wijnen et al., 1989; Holthuis et al., 1990). The involvement of HiNF-D in rendering this gene competent for transcription is suppported by downregulation of this factor during hepatic development in transgenic mice (van Wijnen et al., 1991a). This downregulation is coincident with the cessation of H4 histone gene transcription and the onset of in vivo quiescence and differentiation. Moreover, HiNF-D appears to be a component of a mechanism that coordinately modifies protein/DNA interactions in the promoters of human core (H4 and H3) and H1 histone genes during the cell cycle, tumorigenesis and development (van Wijnen et al., 1991c). Recently, Dailey et al. (Dailey et al., 1988) have characterized a different factor (H4-TF2) that interacts with the analogous region of the human H4 histone gene Hu4A. The role of H4-TF2 in cell cycle regulation of the H4-Hu4a gene and its relationship with HiNF-D are unclear at present.

In the present study, we have assessed the complement of factors that can interact with H4-Site II of the human H4 histone gene FO108 in vitro. Our results show that apart from HiNF-D two other distinct DNA binding activities (HiNF-M and HiNF-P) interact with H4-Site II. Based on sequence-specificity and conditions required for detection, it is possible that HiNF-P is directly related, or may be identical, to H4-TF2. The characterization of three distinct H4-Site II binding proteins for the H4-FO108 histone gene, and a single factor for the analogous element of the H4-Hu4a histone gene (Dailey et al., 1988) could imply heterogeneity in transcriptional regulation of individual H4 histone genes. Alternatively, these data suggest that multiple distinct factors from HeLa S3 cervical carcinoma cells recognize H4-Site II sequences in vitro, and perhaps in vivo. Interestingly, HiNF-D and HiNF-M are each represented by two distinct protein/DNA complexes, and binding of these factors to H4-Site II is sensitive to phosphatase activity in vitro. These and other findings suggest a viable mechanism for modulation of these factors during the cell cycle based in part on post-translational modifications that influence DNA binding activity.

4.2: Materials & Methods

4.2.1: DNA fragments and in vitro protein/DNA interactions

Plasmid FP202 was derived from the pUC19-based and H4-Site II containing construct pFP201 (insert DNA fragment from nt -97 to -38; measured from the H4 histone translational start codon) by cleaving with PstI, blunt-ending with T4 polymerase, and ligating with T4 ligase in

the presence of excess unphosphorylated BglII-linkers (5'dGAAGATCTTC)
according to standard procedures (Ausubel et al., 1987; Sambrook et al.,
1989). The EcoRI/BglII fragment of pFP202 was inserted into EcoRI and
BamHI sites of pFP202 to yield pFP203, which as a result contains a
unidirectional duplication of the H4-Site II fragment. Plasmid pFP204
contains the AvaII/Pst fragment (nt -74/-38) of pFP201 and the SmaI/PstI
vector fragment of pUC19.

Probes for protein/DNA interactions were derived from pFP201 by
EcoRI cleavage, calf intestinal phosphatase (CIP) treatment and T4
kinase 5'end-labelling, followed by HindIII digestion to obtain labelled
sense-strand (EH-probe; nt -97/-38). The order of the enzymes EcoRI and
HindIII was reversed for labelling of the anti-sense strand (HE-probe).
The EA- and AH-probes (coinciding with EcoRI/AvaII (nt -97/-74) and
AvaII/HindIII (-71/-38) fragments of pFP201, respectively) represent H4-
Site II deletion mutants and were prepared in an analogous manner. One
DNA fragment was internally labelled at an AvaII site (AIL-probe) and
prepared from pFP203 by separate actions of AvaII, CIP and T4 kinase.
The resulting mixture of DNA fragments was ligated using T4 ligase and
cleaved with XhoII (or BstYI). All probe fragments were isolated by
electrophoresis. The synthetic oligonucleotides (Nucleic Acid Facility
of the University of Massachusetts Medical School) used in this study
are summarized in the results section (see Fig. 4-3). Doublestranded
oligonucleotide ALRM-5 (Fig. 4-3) was designed by A.L.R.-E. for PCR-
mediated pointmutagenesis of H4-Site II. Based on this design,
oligonucleotide ALRW-4 was synthesized; this DNA fragment spans the same

H4-Site II sequences as ALRM-5, but without the mutations incorporated into ALRM-5.

Gel retardation assays and competition analysis, DNaseI and DMS protection analysis, as well as methylation- and depurination interference experiments were performed essentially as described (van Wijnen et al., 1988b; Pauli et al., 1990). Protein/DNA binding reactions (for detection of HiNF-D and HiNF-M) were performed by combining 10 μl of a protein mixture (in KN100 buffer; see below) with 10 μl of a DNA mixture containing probe DNA (25 pg/μl) and non-specific competitor DNA substrates at the following concentrations: 0.1 μg/μl poly (dG-dC)*(dG-dC)(=GC-DNA), 0.01 μg/μl poly (dI-dC)*(dI-dC)(Pharmacia)(=IC-DNA) and 0.01 μg/μl of crude Sp1 binding site oligonucleotide (van Wijnen et al., 1991b). Optimal conditions for detection of HiNF-P were similar to those of H4-TF2 (Dailey et al., 1988): binding reactions were performed as above with the exception that GC-DNA was replaced with salmon sperm DNA (0.1 μg/μl), and divalent cations were added, i.e., 0.1 mM $ZnCl_2$ and 0.5 mM $MgCl_2$ (to quench EDTA present in DNA and protein preparations).

4.2.2: Protein preparations and chromatography.

Nuclear extracts prepared according to Dignam et al.(Dignam et al. 1983) were obtained from HeLa S3 cells (density 7-9 X 10^5 cells/ml) as described previously (11,23,24), but magnesium salt was replaced in all buffers by 1 mM EGTA, 0.2 mM EDTA, 0.75 mM spermidine and 0.15 mM spermine according to Shapiro et al. (Shapiro et al., 1987), and a broad spectrum protease inhibitor cocktail was used (PMSF, leupeptin,

pepstatin, trypsin inhibitor, TPCK, EDTA and EGTA; Boehringer). Also, desalting was not performed by dialysis but by dilution with storage buffer without KCl (20% glycerol, 0.2 mM EDTA, 0.01% NP40, 1 mM DTT; KNO buffer). Alternatively, samples were desalted by gel filtration using PD-10 columns (P-L Biochemicals), or ultrafiltration using Centricon-10 units (Amicon). Final protein concentrations were adjusted with storage buffer (KNO buffer with 100 mM KCl = KN100 buffer).

Separation of H4-Site II DNA binding activities was performed by batch absorption of nuclear proteins to phosphocellulose resin pre-equilibrated with KN100. Proteins were eluted by a step-gradient using KNO buffer with, respectively, 100 mM, 500 mM and 1000 mM KCl to yield P0-100, P100-500 and P500-1000 fractions. Fractions obtained in similar procedures using heparin-agarose ("H"-fractions) and DEAE Sephacel ("D"-fractions) received analogous designations. Samples were also derived from an alternative fractionation scheme in which crude nuclear protein was separated using phosphocellulose and eluted with KN-buffers containing, respectively, 100 mM, 300 mM, 500 mM and 1000 mM KCl. The P300-500 fraction obtained in this way was used as a partially purified HiNF-M preparation in some experiments. Nuclear protein preparations derived from synchronized cells (Holthuis et al., 1990) and from mouse tissues (van Wijnen et al., 1991b) were identical to those generated by previous procedures.

4.2.3: **Protein phosphatase assays.**

Calf intestinal phosphatase (AlkP; Boehringer) and sweet potato acid phosphatase (AcP; Sigma) were stored at 4°C in buffers recommended by the suppliers, and diluted in KN100 buffer containing fresh protease inhibitor cocktail immediately prior to use. To use high amounts of AlkP (supplied in 3M NaCl) it was necessary to reduce excess salt (which may interfere with HiNF-D binding)(van Wijnen et al., 1989) by dilution of AlkP aliquots with KNO buffer (containing protease inhibitors) and concentration with Centricon-10 units. Absence of protease activity in phosphatase preparations was examined by SDS-PAGE of treated and untreated samples (data not shown).

Phosphatase assays were performed with various protein fractions by incubating with increasing units of AcP or AlkP for 10 minutes at 20°C in KN100 buffer in a 10 μl volume. Binding reactions were initiated by adding 10 μl of a mixture containing 2 μg poly (dG-dC)*(dG-dC), 0.2 μg poly (dI-dC)*(dI-dC) and 0.5 ng of the AIL-probe and incubating the resulting mixture for 10 minutes at 20°. Samples were then directly subjected to electrophoresis as described (van Wijnen et al., 1987 & 1991c).

4.3: **Results**

4.3.1: **Two distinct DNA binding activities, HiNF-M and HiNF-D, interact with H4-Site II sequences.**

To investigate systematically the complement of factors capable of interacting with H4-Site II sequences (nt -97 to -47), we analyzed

binding of nuclear proteins to DNA fragments spanning this element in the immediate proximal promoter of the H4 histone gene FO108. Protein/DNA complexes are observed that are attributed to binding of HiNF-D and HiNF-M, respectively (Fig. 4-1). HiNF-D interacts with the entire H4-Site II containing fragment EH (nt -97/-38), but in agreement with previous findings (van Wijnen et al., 1989) HiNF-D binding is not observed with fragments truncated at an AvaII-site internal to H4-Site II, i.e., fragments EA (nt -97/-74) and AH (nt -71/-38). HiNF-M binds to both the EH (nt -97/-38) and the EA (nt -97/-74) fragments, but not to the AH (nt -71/-38) fragment. These data demonstrate a novel sequence-specific protein/DNA interaction at H4-Site II involving HiNF-M.

To explore the possibility that complexes mediated by HiNF-M and HiNF-D are directly related by intermolecular association of more than one protein on the same DNA template, we analyzed binding of HiNF-D and HiNF-M using fractionated nuclear proteins (van Wijnen et al., 1991b). The results show that chromatography procedures using phosphocellulose, heparin-agarose and DEAE-Sephacel successfully separate a number of H4-Site II binding proteins. More importantly, HiNF-D binding is observed in the absence of HiNF-M binding. This suggests that the corresponding protein/DNA complexes are not in a direct, rapidly fluctuating association in vitro. These factors elute at different salt concentrations from heparin-agarose resin and are present in two different fractions designated H100-300 (HiNF-D) and H300-1000 (HiNF-M). Similar differences were also observed when these factors were eluted from phosphocellulose using step-gradients: HiNF-M elutes at

Figure 4-1. <u>HiNF-M binds to the distal part of H4-Site II.</u>

A) Gel retardation assay using increasing amounts of total nuclear protein with three different DNA fragments (EH-, EA- and AH-probes; respectively, ln 1-3, ln 4-6 and ln 7-9)(summarized in Fig. 3). Each DNA fragment was incubated with (from left to right) 4,6 and 8 μg protein. A mixture of different DNA fragments was used as non-specific competitor DNA (see Materials & Methods).

D►

M►

EH   EA   AH
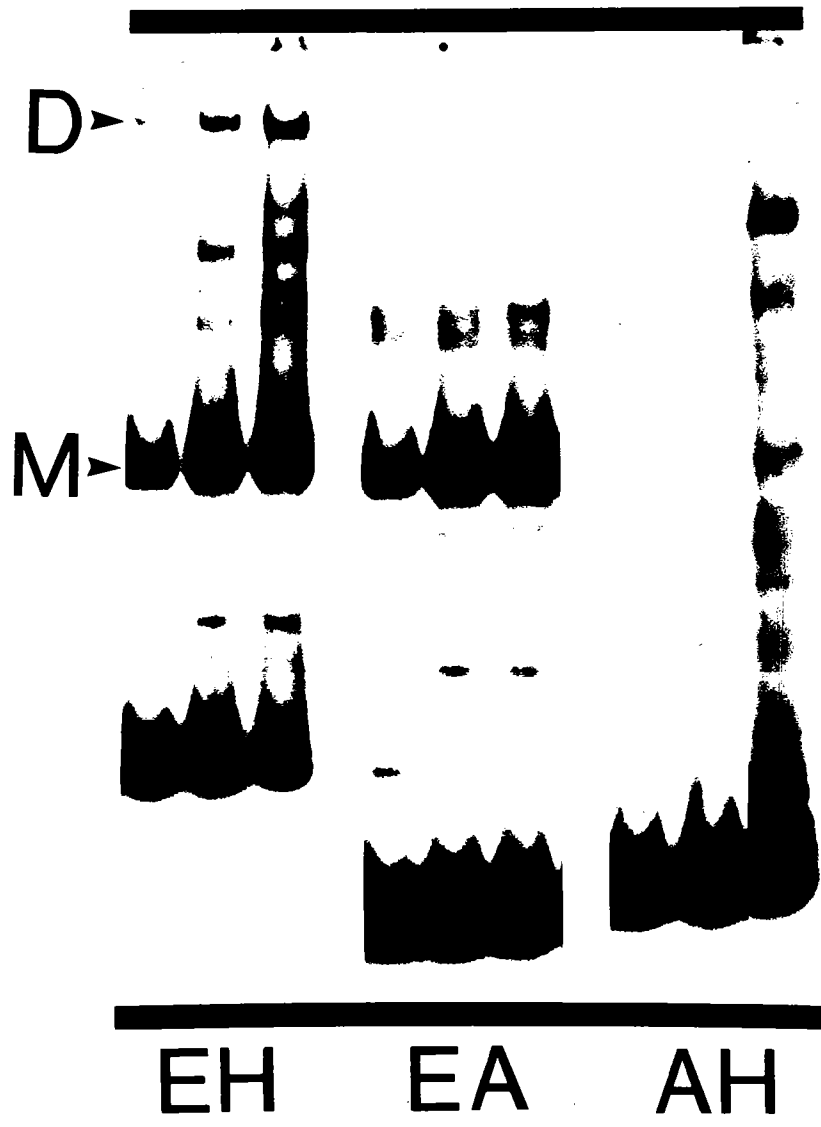
substantially higher salt concentrations than HiNF-D (data not shown).
Thus, HiNF-D and HiNF-M are distinct entities with different
electrophoretic and chromatographic properties.

4.3.2: Protein/DNA recognition analysis and regulation of HiNF-M

To define the binding site of HiNF-M at single nucleotide
resolution, we performed several experiments. DNaseI protection analysis
(Fig. 4-2) using partially purified HiNF-M preparations shows that this
factor protects sequences at nt -95 to -78 on the sense-strand and nt
-97 to -82 on the anti-sense strand. The specificity of nuclease
protection was confirmed by competition with oligonucleotides: inclusion
of an H4-Site II specific fragment (DS-II: nt -91 to -64) decreased
nuclease protection, whereas inclusion of a non-specific DNA fragment
did not (Fig 4-2). Depurination interference assays show that
depurination of either guanines or adenines between nt -92 to -83 in
both the sense- and anti-sense strands is inhibitory for binding (van
Wijnen et al., 1991b), consistent with the DNaseI footprinting results.
Methylation interference analysis (Fig. 4-2) on sense- and anti-sense
strands of the gel purified HiNF-M protein/DNA complex shows a pattern
of both methylation interference (dG^{87}, dG^{85}, dA^{84} and dA^{83}) and striking
methylation enhancement (dG^{86} and dA^{89}). The pattern of methylation
interference indicates that the heptameric sequence 5'dTTCGGTT (or
5'dAACCGAA) represents a minimal recognition sequence for HiNF-M.

Binding of HiNF-M to oligonucleotides containing this heptamer
(DD-1: nt -93/-80; DS-II: nt -91/-64) can be directly demonstrated and
confirmed by cross-competition (van Wijnen et al., 1991b and Fig. 4-3).

Figure 4-2. <u>Recognition site analysis of HiNF-M.</u>

(see Fig. 4-3 for summary and description of oligonucleotides).

Panel A) DNaseI protection analysis of the sense (left panel) and anti-sense (right panel) strands of H4-Site II using P300-500 protein. Brackets indicate the regions of DNaseI protection (see also Fig.3). Left panel: ln 1 and 8, G>A reaction of sense-strand; ln 2-5, respectively, 0 (=C), 12, 25 and 50 μl protein (=P) was added to a 100 μl binding reaction prior to DNaseI digestion; the binding reaction of ln 6 contained 500-fold molar excess of non-specific (N) DNA fragment (H3-II), and that of ln 7 contained the same excess of specific (S) H4-Site II competitor fragment (DS-II). Right panel: ln 1, G>A reactions of anti-sense-strand; ln 2-3, respectively, 0 (=C) and 50 μl protein added; ln 4, same as ln 3, but DS-II (S) oligonucleotide added; ln 5, as ln 3 but H3-II (N) fragment added.
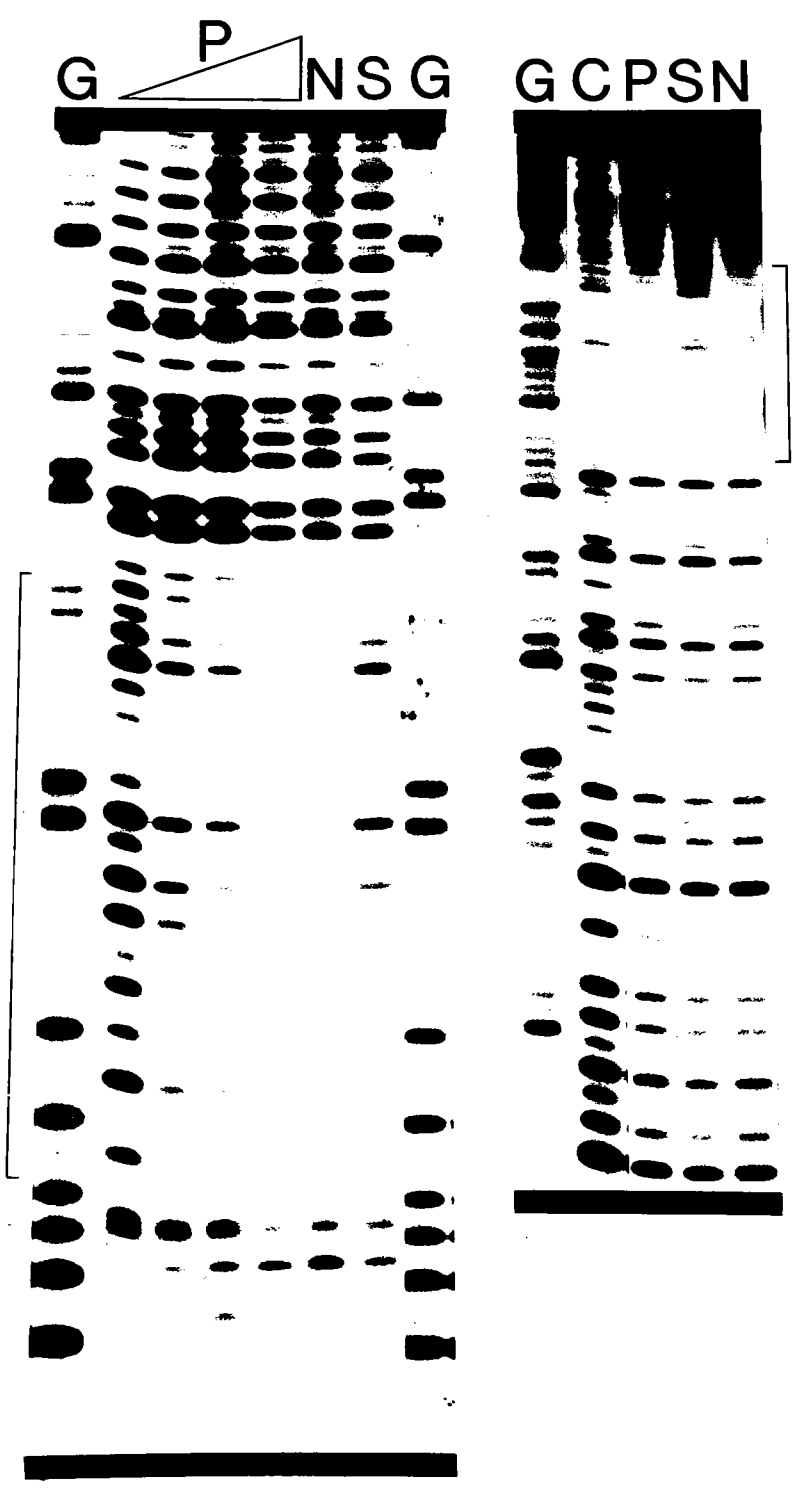
Figure 4-2 (continued). Panel B) Methylation interference analysis of the HiNF-M protein/DNA complex. Left panel: ln 1 and 4, G>A reaction products of input probe DNA from anti-sense strand (G), as well as of free (F) and complexed (M) probe DNA. Right panel, same abbreviations for sense-strand.

98
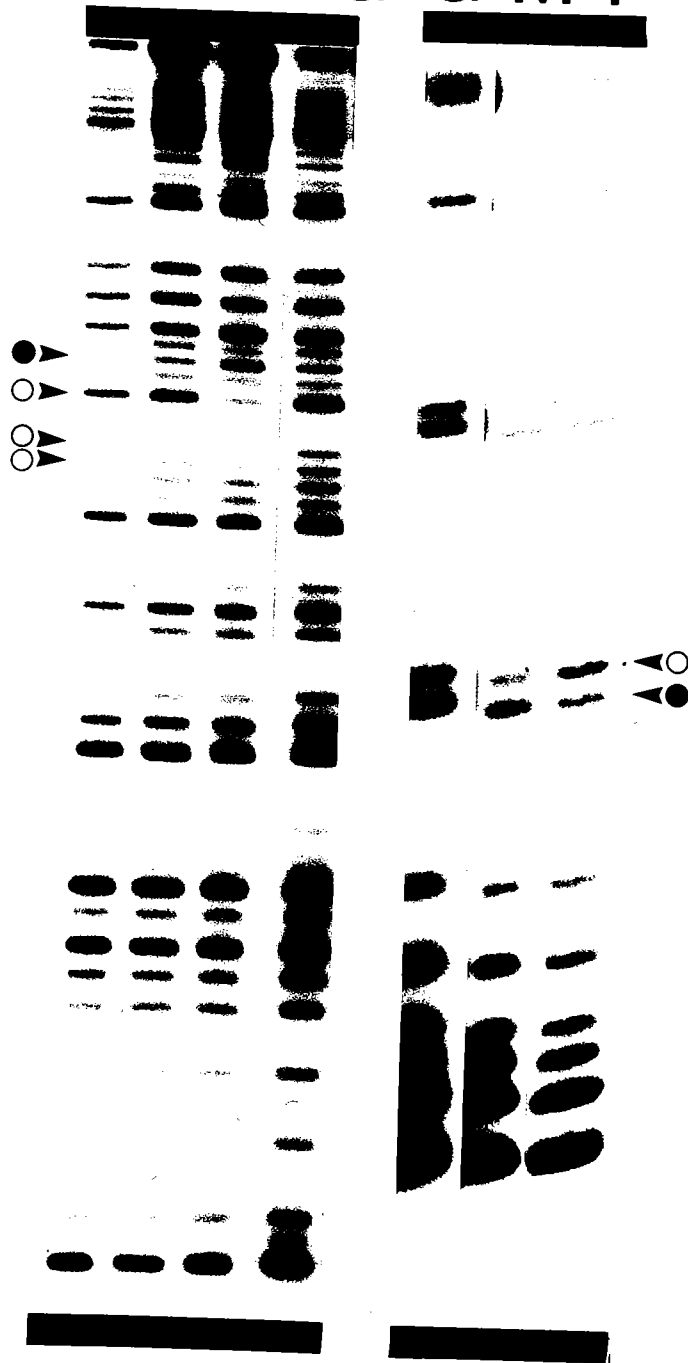
Figure 4-3. Figure 4-3: **Summary of H4-Site II protein/DNA interaction data.** (see Fig. 1 to 5 and data not shown).

Panel A) Deletion analysis with plasmid derived fragments (see Materials and Methods) and a nested set of oligonucleotides prepared by 5'to 3'polymerase or 3' to 5' exonuclease action of T4 DNA polymerase. The effect of deletions on binding of HiNF-D, HiNF-M and HiNF-P (abbreviated D, M and P, respectively) is represented as follows: +, strongest binding; +, weaker binding; $\pm$/-, binding barely detectable; -: no binding observed. Single letters in pointed and round brackets refer to the endonuclease sites at the termini of the plasmid derived probes (E=EcoRI, H=HindII, P=PstI and A=AvaII). The EP-probe is the EcoRI/PstI insert of pFP204 which contains sequences derived from the pUC19 EcoRI/SmaI polylinker (nucleotides printed in lowercase depict altered nucleotides relative to H4-Site II sequences). The sequence at the top shows the region of H4-FO108 histone proximal promoter spanning in vivo protein/DNA interaction domain H4-Site II; indicated are genomic DNaseI (lines above and underneath sequence) and DMS (open circles) protection patterns, the nucleotide numbering relative to the protein coding region, and the mRNA start site.

Deletion analysis:

```
                        NaeI              AvaII        MboII
                         ▽                ▽▽ ▽          ▽
H4-SiteII: 5'-TCCCGCCGGCCGCCTTTCGGTTTCAATCTGGTCCGATACTCTTGTATATCAGGGGAAGACGGTGCT-
             -AGGGCGGCCGGCGGGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTTCTGCCACGA-
              ▲         ▲       ▲    ▲   ▲   ▲      ▲     ▲
             -100      -90      -80  -70  -60 -50    -50   -40
                        00       00   0  0      00
                                         0  0
```

			D	M	P
EH	:	<E>-GGGCGCGCTTTCGGTTTCAATCTGGTCCGATACTCTTGTATATCAGGGGAAGACGGTGCT- -CCGCGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTTCTGCCACGA-<H>	+	+	+
(pFP201)					
EA	:	<E>-GGGCGCGCTTTCGGTTTCAATCTG -CCGCGCGAAAGCCAAAAGTTAGACCag (=A)	−	+	
(pFP201)					
AH	:	(A=) gtcCGATACTCTTGTATATCAGGGGAAGACGGTGCT- GCTATGAGAACATATAGTCCCCTTCTGCCACGA-<H>	−	−	
(pP201)					
EP	:	(E=) aattCGagcTcggtaCccGTCCGATACTCTTGTATATCAGGGGAAGACGGTGCT- GCtcgAgccatGggCAGGCTATGAGAACATATAGTCCCCTTCTGCCACGA-<P>	−	+	
(pP204)					
TM-3	:	gatcCGCTTTCGGTTTCAATCTGGTCCGATACTCTTGTATATCA GCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCtag	+	+	
TT	:	GatcCGCTTTCGGTTTCAATCTGGTCCGATACTCTTGTATATCAGatc CtagGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCtag	+	+	
TA	:	GatcCGCTTTCGGTTTCAATCTGGTCCGATACTCTTGT CtagGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACA	+/−	+	
AA	:	GGTTTTCAATCTGGTCCGATACTCTTGT CCAAAAGTTAGACCAGGCTATGAGAACA	−	−	−
AT	:	GGTTTTCAATCTGGTCCGATACTCTTGTATATCAGatc CCAAAAGTTAGACCAGGCTATGAGAACATATAGTCtag	+/−	−	+
ALRW-4	:	GGTTTTCAATCTGGTCCGATACTCTTGT gCCAAAAGTTAGACCAGGCTATGAGAACA	−	−	−
ALRM-5	:	GGTTTTCAATCTtcTaCGATACTCTTGT gCCAAAAGTTAGAagAtGCTATGAGAACA	−	−	−

100

Figure 4-3 (continued). Panel B) Competition results with the synthetic oligonucleotides used in this study. Shown on the right are qualitative assessments of the competition results using these oligonucleotides in gel retardation assays (+: band increase directly proportional to molar ratio of competitor and probe DNA; ±: competition only observed at higher ratio of competitor and probe DNA; ±/-: marginally specific competition; see text for details). Sequence at the top represents H4-Site II as described in part A. Single-stranded overhangs are represented by lowercase letters. DD-1 and PD-2 represent duplications of elements within H4-Site II (duplicated segment in bold and underlined lettering in each case). The nucleotide substitutions in ALRM-5 relative to ALRW-4 are depicted with underlined, lowercase lettering. Sequences in H3-II and MYB(291b) that display similarity with H4-Site II sequences are indicated by bold, underlined lettering.

Competition analysis:

```
              NaeI          AvaII         MboII
               ▽           ▽▽ ○          ▽
                          ○○ ○           ○
H4-SiteII: 5'-TCCCGCCGGCGCGTTTCGGTTTTCAATCTGGTCCGATACTCTTGTATATCAGGGGGAAGACGGTGCT-
           -AGGGCGCCGCGCGCGAAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTTCTGCCACGA-
              ▲          ▲      ○ ○    ○   ▲         ▲         ▲        ▲
            -100        -90    -80  -70     -60       -50       -40
```

		D	M	P
TM-3 :	gatcCGCTTTCGGTTTCAATCTGGTCCGATACTCTTGTATATCA GCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCtag	+	+	+
DD-1 :	gatcCGCTTTCGGTTTTCT GCGAAAGCCAAAAGCGCTTTCGGTTTTCT GCGAAAGCCAAAAGCGCAAAAGACtag	−	+	−
PD-2 :	gatcTCAATCTGGTCCGATTCAATCTGGTCCGAT AGTTAGACCAGGCTAAGTTAGACCAGGCTActag	+/−	−	+
ALRW-4:	GGTTTTCAATCTGGTCCGATACTCTTGT gCCAAAAGTTAGACCAGGCTATGAGAACA	+/−	−	+
ALRM-5:	GGTTTTCAATCTtcTaCGATACTCTTGT gCCAAAAGTTAGAAgAtGCTATGAGAACA	+/−	−	−
DS-II :	ctagCTTTCGGTTTTCAATCTGGTCCGATACT GAAAGCCAAAAGTTAGACCAGGCTATGAgatc	+/−	+	+
DS-I :	gatcCGGAAAAGAAAATGACGAAATGTCGAGA GCCTTTTCTTTACTGCTTTACAGCTCTCtag	−	+/−	−
H3-II :	gatcTCACAGAGATGGACCAATCCAAGAGGG AGTGTCTCTACCTGGTTAGGTTCTCCCctag	+/−	−	−
MYB : (291b)	gatcAGTAATCCAAACTGCCACAGTTCATAAG TCATTAGGTTTGACGGTGTCAAGTATTCcatg	−	−	−
NMP-1 :	gatcTGGGATTCGCTGACGTCCATGAGAAAG ACCCTAAGCGACTGCAGGTACTCTTTCctag	−	−	−

Figure 4-3 (continued). Panel C) Recognition site analysis of H4-Site II
binding proteins. Summarized are the results obtained for both strands:
in vitro DNaseI footprints of HiNF-M (bracketed lines), methylation
interference of HiNF-M (triangles closest to the sequence: open =
interference, filled = enhancement), depurination interference of HiNF-M
(open squares), and methylation interference of HiNF-D (triangles
connected with small arrows). The three stars refer to nucleotide
substitutions in the ALRM-5 oligonucleotide that interfere with binding
for HiNF-P. The thick lines with the designations HiNF-M and HiNF-P
indicate the minimal elements capable of competing for these factors;
the thick line with designation HiNF-D depicts the HiNF-D core sequence
with the 5′ and 3′ extensions that each contribute to HiNF-D binding
indicated by the dotted part of this line. Thin lines immediately above
and below the H4-Site II sequence (bold) represent the boundaries of in
vivo genomic DNaseI footprints; smallest dots indicate nucleotide
numbering.

Multipartite protein/DNA interaction domain H4–Site II:
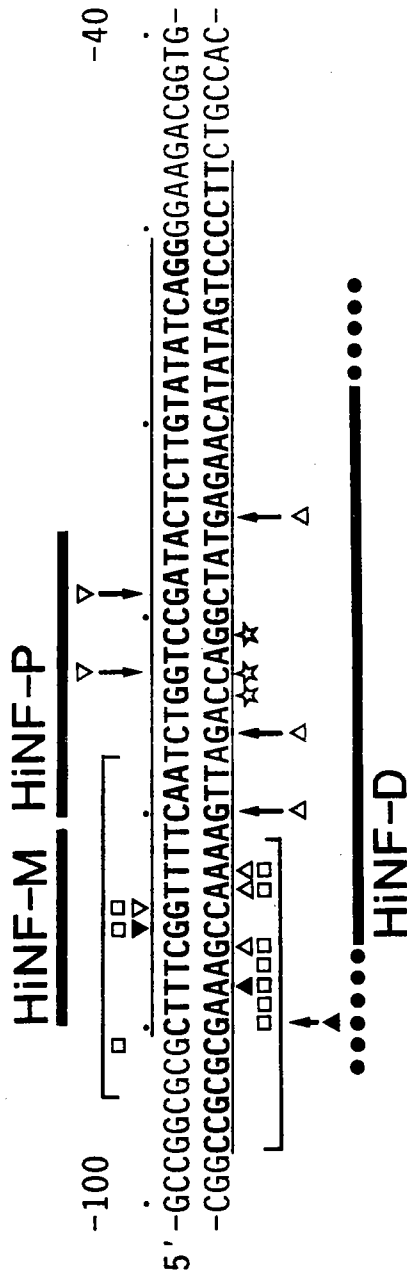
Furthermore, HiNF-M is specifically retained on Sepharose-CL2B chromatography resins containing these oligonucleotides during DNA affinity chromatography (data not shown). However, DNA fragments containing the CCAAT-box motif, such as CTF/NF-1 or CP1/NF-Y binding sites (5'dRRCCAAT; R = G or A)(Jones et al., 1987; Chodosh et al., 1988; Hooft van Huijsduijnen et al., 1990), and the oncoprotein MYB DNA binding consensus sequence (5'dYAACKG; Y = C or T, K = G or T)(Biedenkapp et al., 1988) do not compete for binding. These results further emphasize the importance of the heptamer element for HiNF-M binding.

To investigate the biological regulation of HiNF-M binding activity, we examined whether modulation of this activity occurs in relation to cell proliferation. HiNF-M was monitored in nuclear protein preparations derived from both tissue-culture cells and mammalian tissues. The HiNF-M protein/DNA complexes were identified by competition analysis (data not shown; van Wijnen et al., 1991a). Comparable levels of HiNF-M were observed in several cell types such as HeLa S3 cervical carcinoma cells, HL60 promyelocytic leukemia cells, as well as normal diploid and SV40 transformed WI38 lung fibroblasts, both during S and G1 phases of the cell cycle (data not shown).

Comparable levels of HiNF-M were also found in nuclear extracts from murine tissues, including adult liver, spleen, thymus and brain (van Wijnen et al., 1991a). More importantly, the levels of HiNF-M in cells from murine tissues do not correlate with those of HiNF-D. For example, the abundance of HiNF-D is in part proportional to the

proliferative state of cell populations in these tissues and is
downregulated during hepatic development; in contrast, HiNF-M binding
activity is constitutively expressed during liver development (van
Wijnen et al., 1991a). These observations suggest that these DNA binding
activities are subject to different modes of regulation.

4.3.3: Binding of HiNF-D and HiNF-M to overlapping elements of H4-Site II

To define the protein/DNA contacts of HiNF-D relative to those of
HiNF-M, we performed methylation interference analysis of the gel
purified HiNF-D protein/DNA complex (Fig. 4-4) on the sense- and anti-
sense strands. Methylation interference contacts were observed only on
guanine residues (dG^{80}, dG^{76}, dG^{73}, dG^{69}, dG^{65}) and one methylation
enhancement could be detected (dG^{91}). This result shows that the HiNF-M
heptamer contacts are contained within the HiNF-D binding domain and are
distinct from, and complementary to, those contacts mediated by HiNF-D.
This suggests that the HiNF-M and HiNF-D interactions represent binding
events to overlapping elements in the evolutionarily conserved H4-Site
II sequences.

The differences in binding activities of HiNF-M and HiNF-D were
further investigated by competition analysis (Fig. 4-3). As noted above,
HiNF-M competes specifically and very efficiently with both the DD-1 (nt
-93/-80) and DS-II (nt -91/-64) oligonucleotides, but not with DNA
fragment ALRW-4 (nt -86/-59), which represents a truncation of the HiNF-
M heptamer. However, HiNF-D displays a more heterogeneous competition
behaviour and shows a reciprocal relationship between band intensity and

Figure 4-4. <u>Recognition site analysis of HiNF-D.</u>

Panel A) Methylation interference analysis of the HiNF-D protein/DNA complex. Left panel: ln 1, G reaction of bottom-strand (G); ln 2, G reaction of HiNF-D complex (D). Right panel: G>A reaction of top-strand (G), free DNA (F) and the HiNF-D complex (D); the reaction products of the HiNF-M complex (M) are shown for reference.
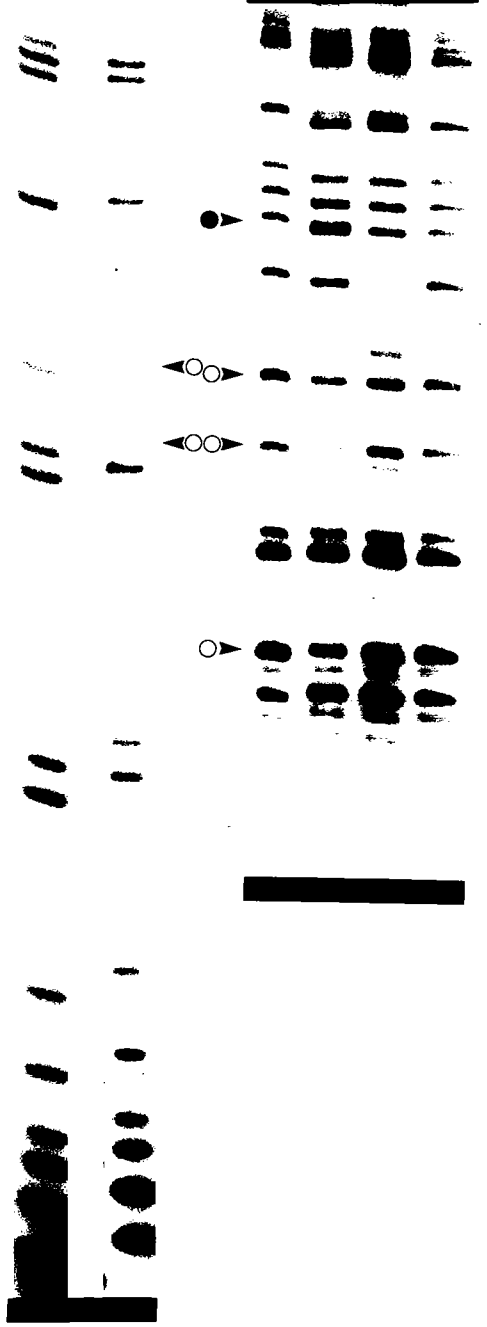
Figure 4-4 (continued). Panel B) Competition analysis using the EH-probe (0.5 ng) and unlabelled synthetic oligonucleotides as indicated above each group of five lanes. The amounts of competitor added were in each case, respectively 0, 50, 100, 200 and 400 ng.

DS-II ALRW-4/DD-1

D▷

M▷
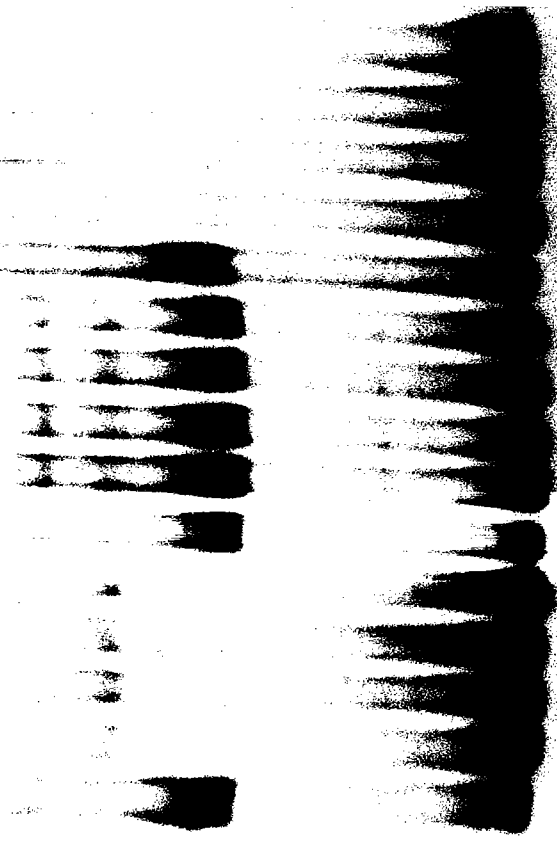
EH

Figure 4-4 (continued). Panel C) Gel retardation assay with D100-250

protein (10 μl each) and oligonucleotides (0.5 ng each) representing a

nested set of H4-Site II deletion mutants (see Fig. 3). The HiNF-D

complex was identified by competition analysis (100-fold excess) with

the following fragments, respectively, control (ln 1,6,11,16 and 21),

TM-3 (ln 2,7,12,17,22), ALRW-4 (ln 3,8,13,18,23), DD-1 (ln

4,9,14,19,24), and NMP-1 (ln 5,10,15,20,25). The NMP-1 oligonucleotide

spans the unrelated binding site of the nuclear matrix protein NMP-1 (S.

Dworetzky, JLS and GSS; unpublished data) and is used as a non-specific

competitor DNA.
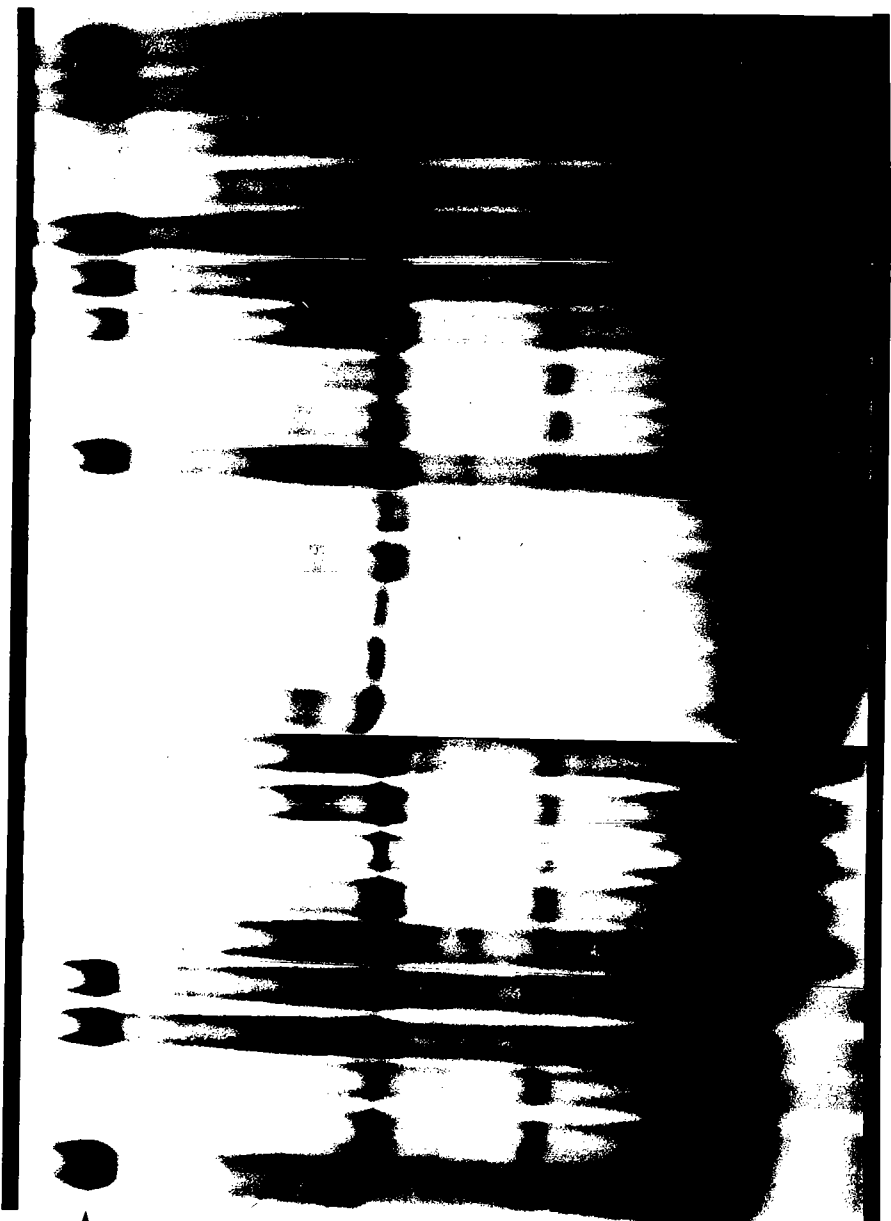
molar ratio of competitor DNA only at high oligonucleotide concentrations. Specific competition is observed at high concentrations of the DS-II (nt -91/64) and ALRW-4 (nt -86/-59) oligonucleotides. DNA fragments PD-2 (nt -82/-66) and H3-II (sequence similarities with nt -80 to -70), which contain subsets of the H4 histone gene consensus sequence, compete in a marginally specific manner, whereas the MYB binding site 291b (Biedenkapp et al., 1988) does not compete even at extremely high molar ratios (approximately 2000-fold) of competitor DNA (Fig. 4-3).

Several lines of evidence support the conclusion that the protein/DNA interactions involving HiNF-M and HiNF-D are independent binding events. Abolishment of HiNF-M activity by inclusion of excess specific competitor DNA does not influence HiNF-D binding, nor does competition of HiNF-D influence HiNF-M binding in vitro (Fig. 4-4). This indicates that HiNF-M is not a direct intermediate of the HiNF-D protein/DNA complex. Moreover, these factors have distinct chromatographic behaviour (Fig. 4-1), are subject to different modes of regulation (van Wijnen et al., 1991a), and mediate complementary protein/DNA contacts (Fig. 4-2 and 4-4). Taken together, the results are consistent with these factors collectively mediating regulatory protein/DNA interactions.

To define the DNA sequences that represent a minimal HiNF-D binding domain, we constructed a series of radio-labelled oligonucleotides that embody a nested set of H4-Site II deletion mutants (Fig. 4-4 and data not shown). The identity of the HiNF-D protein/DNA

complex was confirmed by competition analysis using both unfractionated nuclear proteins and partially purified HiNF-D preparations. Binding of HiNF-D to the AA-fragment (nt -86/-59) which spans the entire H4-Site II consensus element (5'dGGTYYTCAATCNGGTCCG; Y = T or C, N = any nucleotide; van Wijnen et al., 1989) was below the level of detection. Extension towards the 5' direction of this core sequence by several nucleotides (to nt -93; TA-fragment), with inclusion of the most distal HiNF-D contact, resulted in a low, but detectable level of HiNF-D binding. Notably, extension of the 3' terminus (to nt -53; AT-fragment) resulted in a stronger signal for the HiNF-D:H4-Site II interaction. Most efficient binding was observed using DNA fragments spanning nt -93 to -53 (TT and TM), consistent with the contribution of auxiliary sequences both at the 5' and 3' termini of the core sequence to HiNF-D binding.

Competition analysis with a spectrum of oligonucleotides using the TM-3 (nt -93/-53) fragment as a probe shows that HiNF-D binding is virtually abolished with a 100-fold molar excess of the unlabelled TM-3 oligonucleotide (Fig. 4-4). However, only a several-fold decrease in HiNF-D binding is observed upon inclusion in the binding reaction of the same molar excess of the oligonucleotides DS-II (nt -91/64), ALRW-4 (nt -86/-59) and ALRM-5 (as ALRW-4 but containing three point mutations; see Fig. 3 and below). The difference in competition potential between TM-3 (nt -93/-53) and the shorter oligonucleotides can be directly attributed to the requirement for HiNF-D to bind to an extended DNA sequence.

Based on these data we can not discriminate whether these extensions of the core sequence provide additional protein/DNA contacts, facilitate conformational changes of the DNA, or contribute to initial non-specific binding to the target DNA prior to stabilization at the putative HiNF-D recognition sequence. The combined results of methylation interference and competition analysis, as well as deletion analysis, strongly indicate that the HiNF-D binding site spans an extended polynucleotide sequence (between 27 and 41 nucleotides based on deletion analysis, with protein/DNA contacts distributed over 28 nucleotides) that is substantially larger than binding sites for both HiNF-M and most other eukaryotic DNA binding factors (reviewed by Mitchell & Tjian, 1989).

4.3.4: H4-Site II is a multipartite protein/DNA interaction site for factors HiNF-D, HiNF-M and HiNF-P

The establishment of specific binding sites for HiNF-D and HiNF-M that overlap the H4-Site II in vivo protein/DNA interaction domain does not exclude the possibility that additional factors may bind to these sequences. Using modified in vitro binding conditions, in particular, by replacing the non-specific competitor GC-DNA with random DNA (from salmon sperm), we do not observe the HiNF-D complex, and HiNF-M is barely detectable (data not shown). However, we do observe a third protein/DNA complex interacting with H4-Site II, and this complex has a migration rate faster than HiNF-M (Fig. 4-5). The specificity of this interaction is readily demonstrated by competition analysis: TM-3 (nt -93/-53) and ALRW-4 (nt -86/-59) compete, but DD-1 (nt -93/-80) does not.

Hence, the factor involved (designated HiNF-P) is distinct from HiNF-M.

To address a potential relationship between HiNF-P and HiNF-D, we performed the following experiment. The ALRW-4 oligonucleotide spans an AvaII site that coincides with a pentameric element (5'dGGTCC; nt -74/-70) that is most strongly conserved in the mammalian H4-SiteII consensus sequence (van Wijnen et al., 1989). The mutant oligonucleotide ALRM-5 (Fig. 4-3) which contains three substitutions within this pentamer is, unlike the ALRW-4 fragment, not capable of competing for HiNF-P (Fig. 4-5). However, both fragments compete with about equal (albeit moderate, relative to TM-3) efficiency for HiNF-D binding (Fig. 4-4). The difference in the competition results for HiNF-D and HiNF-P suggests that these factors represent distinct DNA binding activities.

Our assay conditions for detection of HiNF-M and HiNF-D using the full length H4-Site II probe fragment (and GC-DNA as non-specific competitor) do not allow detection of HiNF-P in gel retardation assays because, among other variables, the probe becomes saturated with HiNF-D and HiNF-M at relatively low protein concentrations. However, if we use the shorter ALRW-4 oligonucleotide (lacking the HiNF-M binding site) as a probe, and compare binding events with this fragment to those of the analogous mutated fragment ALRM-5, we can observe a very minor sequence specific protein/DNA complex at high protein concentration with a migration rate and competition properties identical to the HiNF-P complex (Fig. 4-5). Moreover, HiNF-P is not only competed by the ALRW-4 fragment but also by the DS-II (nt -91/64) and PD-2 (nt -82/-66) oligonucleotides. This establishes that the minimal binding site capable

Figure 4-5. <u>Detection of a novel H4-Site II protein/DNA interaction involving HiNF-P.</u> The HiNF-P complex is indicated by the arrowhead.

Panel A) Binding and competition analysis of unfractionated and undialyzed nuclear extract proteins (25 μg in each case). The EH-probe was used and the non-specific competitor DNA was salmon sperm DNA (2 ug); divalent cations were also added (0.1 mM $ZnCl_2$, 0.5 mM $MgCl_2$). The following oligonucleotides were present in 100-fold molar excess: In 1-6, respectively, no specific competitor, TM-3, ALRW-4, ALRM-5, DD-1, NMP-1.

C TM-3 ALRW-4 ALRM-5 DD-1 NMP-1

P▸

NE

EH
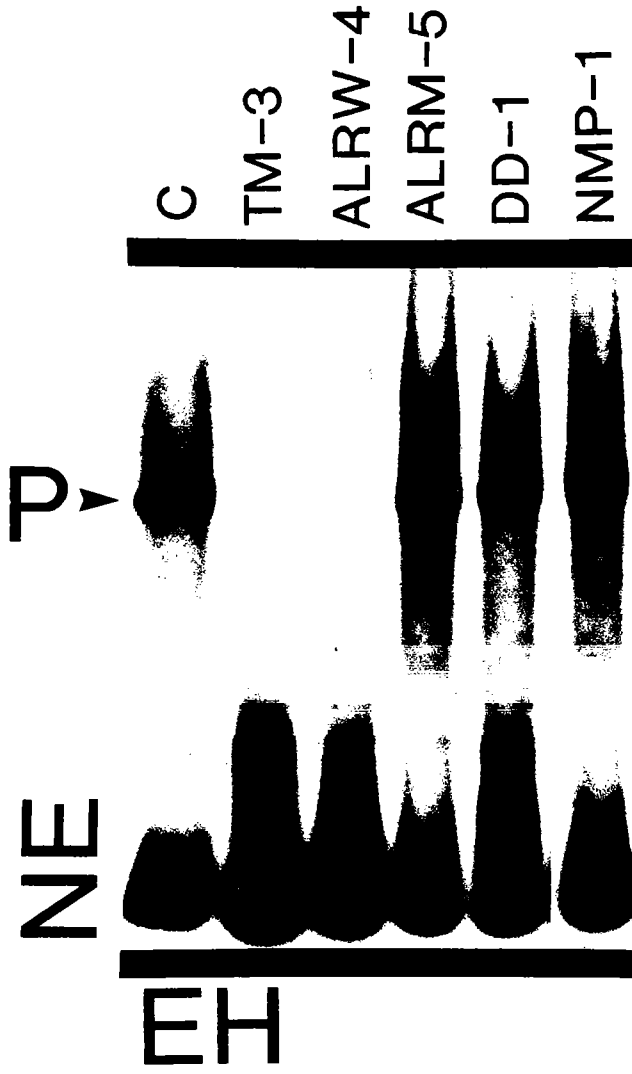
Figure 4-5 (continued). Panel B) Detection of HiNF-P binding activity using oligonucleotides ALRW-4 (left panel: ln 1-4) and ALRM-5 (left panel: ln 5-8) as probes. The binding reactions contain a mixture of DNA fragments (2 μg GC-DNA, 0.2 μg IC-DNA and 0.2 μg crude Sp1 oligonucleotide). The ALRW-4 and ALRM-5 fragments were in each case incubated with increasing amounts of nuclear protein (ln 1-4: from left to right, 20, 30, 40 and 50 μg protein; ln 5-8: same).

Panel C) Competition analysis of the HiNF-P complex using the ALRW-4 fragment as a probe with 25 μg nuclear protein, and 100-fold molar excess of the following oligonucleotides: ln 1-7 (right panel, respectively, no specific competitor DNA, TM-3, DS-II, PD-2, ALRW-4, ALRM-5 and MYB(291b).

P▶

NE

ALRW-4 | ALRM-5

C TM-3 DS-II PD-2 ALRW-4 ALRM-5 MYB

P▶

NE

ALRW-4
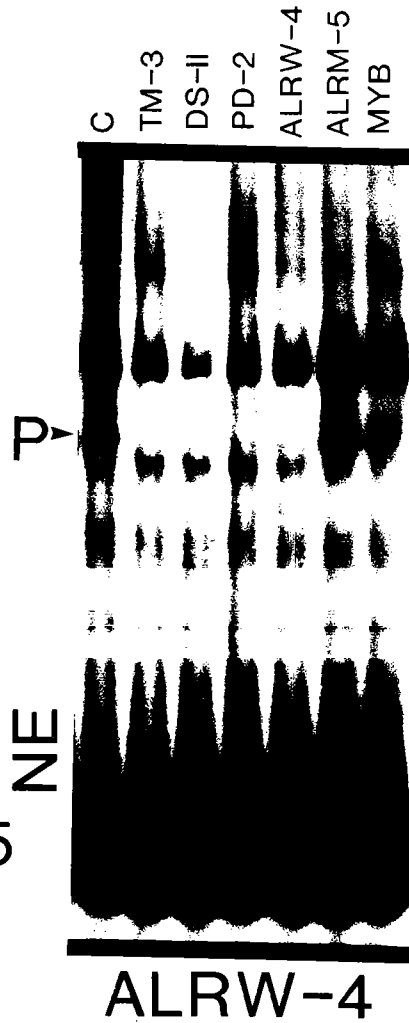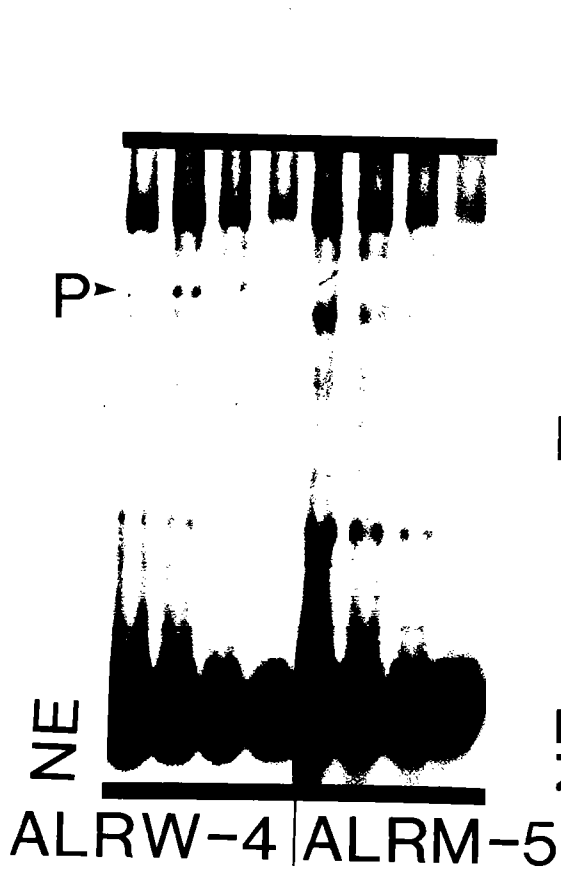
of competition spans the element 5'dTTCAATCT<u>GG</u>T<u>C</u>CGAT (nt -82 to -66; mutated nucleotides inhibitory for binding underlined) surrounding the 5'dGGTCC-pentamer element. The establishment of interaction sites for three distinct DNA binding activities clearly indicates that H4-Site II is a multi-partite protein/DNA interaction site.

4.3.5: Dephosphorylation-dependent interactions of factors HiNF-D and HiNF-M with the H4-Site II element

The protein/DNA complexes of HiNF-D and HiNF-M each appear as a doublet band on autoradiograms of several gel retardation assays. We therefore explored the possibility that this may be attributable to post-translational modifications. Nuclear proteins were enzymatically dephosphorylated by incubation with increasing amounts of calf intestinal phosphatase (AlkP) or sweet potato acid phosphatase (AcP) (Fig. 4-6) to examine the effect of phosphate groups on the formation of the HiNF-D protein/DNA complex. Both AlkP and AcP are broad spectrum phosphomonoesterases that are capable of removing the phosphate moiety of phosphorylated amino acids.

Incubation of unfractionated nuclear protein or partially purified HiNF-D preparations with low concentrations of AcP results in the disappearance of the upper band of HiNF-D, whereas both HiNF-D bands are abolished at higher concentrations of AcP (Fig. 4-6). The same results were observed when a fixed concentration of AlkP was used in the assay at various different temperatures (van Wijnen et al., 1991b). Note that in the absence of AlkP both HiNF-D species are irreversibly inactivated within an identical temperature interval (van Wijnen et al., 1991b).

Interestingly, low concentrations of AlkP (at fixed temperature) appear to shift the ratio of upper and lower bands of HiNF-D, while not dramatically influencing the combined binding represented by both complexes, yielding an apparent net increase in the formation of the lower HiNF-D complex (van Wijnen et al., 1991b). Results similar to those with HiNF-D were obtained for HiNF-M (Fig. 4-6): limited phosphatase treatment resulted in the appearance of a faster migrating protein/DNA complex and more extensive treatment abolished HiNF-M binding.

Competition experiments were performed using nuclear proteins treated with phosphatases (Fig. 4-6). These results demonstrate that the two sets of closely co-migrating species, corresponding to HiNF-D and HiNF-M respectively, each have indistinguishable competition behaviour, and we define each of these sets of species (in the simplest explanation) as post-translationally modified forms of the same DNA binding activity.

The possibility must be considered that alterations in the formation of these protein/DNA complexes are not directly related to dephosphorylation, but could reflect an effect of another enzymatic activity present in our phosphatase reaction mixtures, for instance the presence of contaminating protease activity. Protease activity would be fairly specific and cleave both HiNF-D and HiNF-M into discrete DNA binding products that exhibit faster migration of the corresponding protein/DNA complexes. We consider this unlikely because phosphatase treatment was carried out in the presence of a broad spectrum protease

Figure 4-6. <u>Dephosphorylation of HiNF-D and HiNF-M in vitro influences binding to H4-Site II.</u> Fractionated HeLa nuclear proteins were incubated with increasing amounts (dashed arrow) of sweet potato acid phosphatase (AcP) or calf intestinal alkaline phosphatase (AlkP). The AIL-fragment was used as probe. (See van Wijnen et al., 199c for further details).

Panel A) Incubation of P100-500 protein (4 μg) containing both HiNF-M and HiNF-D activity (ln 1-5) or H100-300 protein containing primarily HiNF-D activity (ln 6-10) with increasing amounts of acid phosphatase (units added in ln 1-5 and ln 6-10 in each case, respectively, 0 [=C], 0.05, 0.1, 0.2 and 0.5). The two sets of co-migrating complexes (doublets) corresponding to HiNF-D and HiNF-M are indicated by arrowheads.

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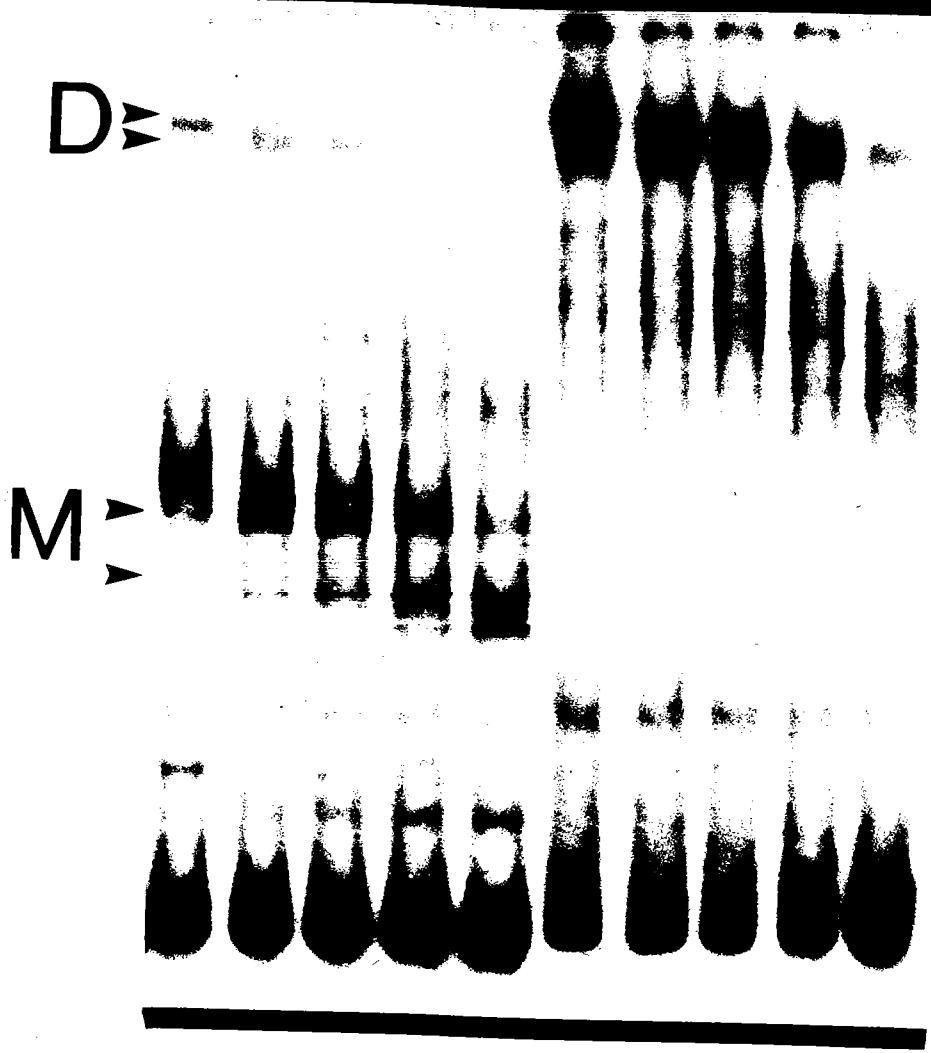
Figure 4-6 (continued). Panel B) Competition analysis of protein/DNA complexes using phosphatase treated nuclear proteins. Incubations occurred in the absence of competitor DNA and phosphatase (C), or in absence of competitor DNA and presence of acid phosphatase (C+AcP), or in the presence of both 500-fold molar excess of competitor DNA (as indicated above the gels) and phosphatase (remaining lanes). Left panel: H100-300 protein incubated with 0.05 units acid phosphatase (ln 2-5). Right panel: P100-500 protein incubated with 0.05, 0.1, 0.2 and 0.5 units acid phosphatase.
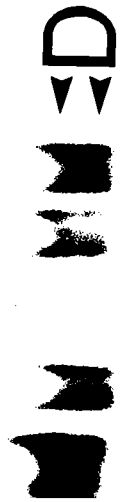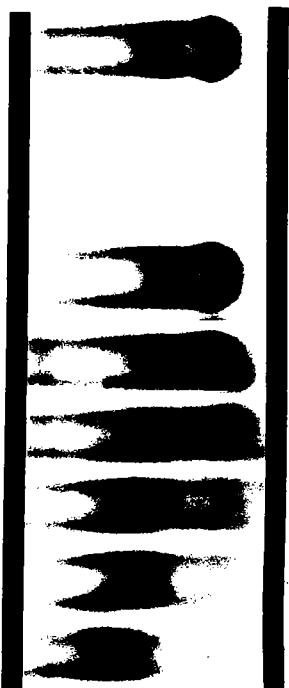
inhibitor cocktail, several distinct chromatography fractions, and with two entirely different enzymatic preparations. Moreover, we did not observe protein degradation upon examination of phosphatase treated-versus untreated protein samples using SDS/PAGE (data not shown).

We interpret these findings to indicate that both HiNF-D and HiNF-M are phosphoproteins. Based on the finding that extensive dephosphorylation abolishes binding of HiNF-D, the fast-migrating HiNF-D species must be at least partially phosphorylated, and therefore correspond to a putative hypo-phosphorylated species. The slower migrating form of HiNF-D appears to be a hyper-phosphorylated species. A similar rationale can be applied to the results for HiNF-M. Obviously, these electrophoretic mobility assays do not discriminate between limited dephosphorylation creating a long range structural effect on the protein conformation of a single polypeptide or (perhaps more likely) the dissociation of a loosely bound secondary molecule, both of which could contribute to a global effect on protein structure that may cause alterations in electrophoretic mobility. Extensive dephosphorylation ultimately affects protein domains that specify the DNA binding activity resulting in loss of binding. We conclude that the extent of dephosphorylation, and by deduction the state of phosphorylation, of HiNF-D and HiNF-M influences the ability to bind to DNA and alters the nature of H4-Site II protein/DNA interactions in vitro.

4.3.6: Alterations in post-translational modification of HiNF-D during the cell cycle and development

Post-translational modification of DNA binding proteins may represent a level of regulation in the control of histone gene transcription. To address differences in the extent to which HiNF-D is post-translationally modified, we evaluated HiNF-D binding activity during the cell cycle of normal diploid cells (Holthuis et al., 1990). Inspection of these shows that cells blocked at the G1/S phase boundary contain the two HiNF-D species in approximately equal quantities (van Wijnen et al., 1991b). Upon release into S phase, total HiNF-D binding activity increases along with the ratio of the two species. In contrast, cells in G1 phase contain lower amounts of total HiNF-D activity and only the faster migrating species can be detected. Similar results can be observed with synchronized populations of normal diploid WI38 fetal lung fibroblasts (data not shown; Holthuis et al., 1990). Hence, these results show that the phosphatase-sensitive post-translational modification of HiNF-D changes during the cell cycle in normal diploid cells.

To further explore the post-translational modification of HiNF-D during biological processes, we examined HiNF-D activity in mice during hepatic development (Fig 4-7; van Wijnen et al., 1991a). We observe that both forms of HiNF-D are present in fetal liver in mid- to late gestation, and the downregulation of HiNF-D (van Wijnen et al., 1991a) occurs in conjunction with a decrease in the ratio of slower to faster migrating forms (Fig. 4.7). However, no changes were observed with

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Figure 4-7: Cell cycle dependent and developmental modulations in the two electrophoretic species of HiNF-D.

Electrophoretic migration of the two forms of HiNF-D during liver (top part) and brain (lower part) development. Each panel shows the HiNF-D complex detected with, respectively, 2, 4 and 6 μg protein. Top part: first three panels, total nuclear protein from fetal liver (FL) at approximately day 14, day 16 and day 18 of gestation; right (and fourth) panel, protein from adult liver (AL). Bottom part, as top part but using protein from fetal brain (FB) and adult brain (AB).
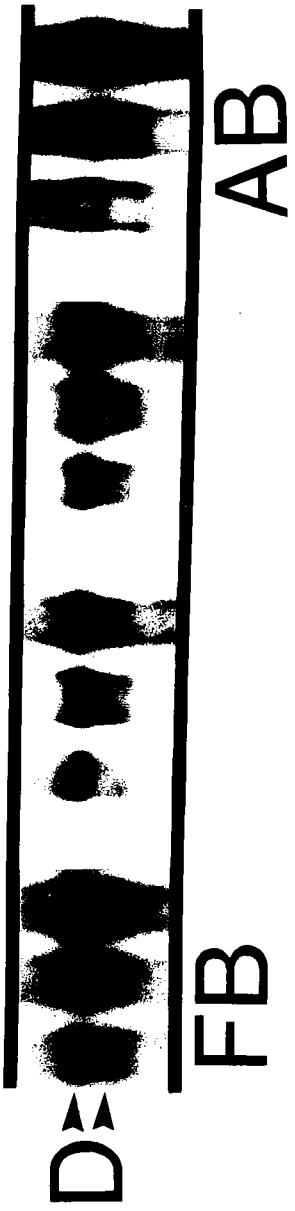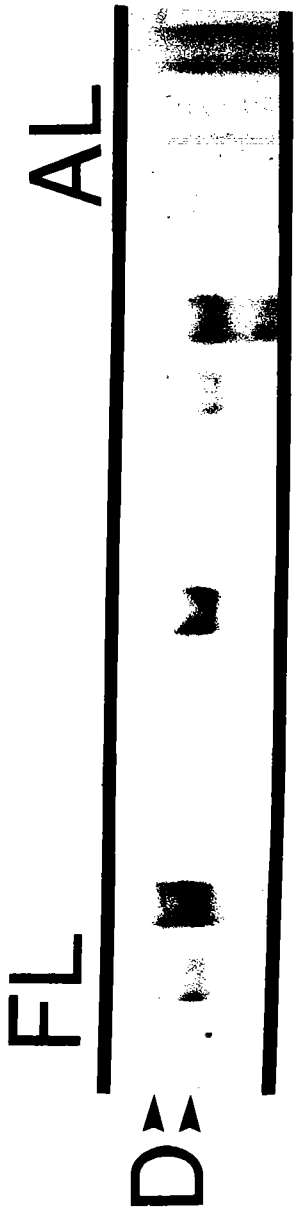
HiNF-D during brain development (discussed in van Wijnen et al., 1991a).
Thus, these observations indicate that the phosphorylation state of
HiNF-D changes during hepatic development coincident with the onset of
cellular quiescence and differentiation in this tissue, further lending
support to the physiological significance of multiple forms of HiNF-D.

4.4: Discussion

We have performed a systematic analysis of H4-Site II protein/DNA
interactions by the combined use of gel retardation deletion and
competition analysis, DNaseI footprinting, methylation interference and
depurination interference. These data suggest that at least three
factors (HiNF-D, HiNF-M and HiNF-P) exhibit interactions with H4-Site II
in a sequence specific and independent fashion, reflecting the
multipartite nature of this protein/DNA interaction domain. The
detection of three different factors binding to H4-Site II in vitro is
consistent with our previous estimates of the number of H4-Site II
factors that may interact with this regulatory element in vivo (Pauli et
al., 1987).

To date, we have not obtained indications that factor H4-TF2
characterized by Dailey et al. (Dailey et al., 1988), which binds to an
analogous human H4 histone gene, is identical to either HiNF-M or HiNF-
D. We note that H4-TF2 has been characterized in vitro using salmon
sperm DNA as non-specific competitor, and that HiNF-D can not be
detected under conditions used for detection of H4-TF2. Moreover,
differences in methylation interference contacts of HiNF-D, HiNF-M and

H4-TF2 are in agreement with the assessment that these proteins are different (see also van Wijnen et al., 1989). However, factor HiNF-P has interesting similarities with H4-TF2, because it requires very similar conditions for optimal detection and binds to a sequence in the H4-F0108 histone gene that is analogous to the H4-TF2 binding site in the H4-Hu4a histone gene (Dailey et al., 1988). The detection of at least three different DNA binding activities that specifically recognize the evolutionarily conserved H4-Site II sequences of H4 histone genes, suggests that a multiplicity of H4-Site II binding proteins exists. The regulation of these binding activities should occur in a stringent and well-balanced manner to render the H4-Site II proximal promoter element competent for selective occupancy in vivo by biologically relevant DNA binding factors.

The functional significance of the HiNF-D:H4-Site II interaction in regulating H4 histone gene transcription is indicated by the positive correlation of biological regulation of HiNF-D binding activity, histone gene transcription, and occupancy of H4-Site II in vivo in a variety of biological processes. Factor HiNF-P recognizes specific sequences internal to the HiNF-D binding domain, indicating a role in conjunction with HiNF-D. The third protein, HiNF-M, binds independently of HiNF-D interactions at H4-Site II. The dissimilar distribution of these factors in a broad spectrum of cell types suggests that these DNA binding activities are differentially regulated. However, similar to HiNF-D, HiNF-M is subject to analogous post-translational modifications. Hence, the binding of post-translationally modified forms of HiNF-M and HiNF-D

to overlapping sequences in H4-Site II may be functionally related to an extra regulatory dimension to accomodate cell types of diverse ontogeny in the modulation of H4 histone gene transcription during the cell cycle or the onset of quiescence and differentiation.

Phosphorylation of transcription factors involved in cell cycle regulation of histone gene expression has been proposed previously (Thomson et al., 1976). Here, we have used the cloned human H4 histone gene FO108, whose regulation has been well documented (Kroeger et al., 1987, Pauli et al., 1987; van Wijnen et al., 1989; Wright et al., 1991b). We show that the interactions of factors HiNF-M and HiNF-D at the key proximal promoter element H4-Site II are influenced by phosphatase-sensitive post-translational modification, and deduce that an apparent basal level of phosphorylation is required for these two DNA binding activities. At least one of these, HiNF-D, displays cell cycle dependent fluctuations in this post-translational modification. Hence, the present results are in support of the proposal that protein phosphorylation is involved in cell cycle regulation of histone gene transcription (Thomson et al., 1976).

The phosphatase-sensitive post-translational modification of HiNF-D changes during the cell cycle in normal diploid cells. Interestingly, tumor-derived or transformed cells not only have constitutively elevated levels of this activity during the cell cycle (Holthuis et al., 1990), but the major species that can be detected is the putative hyper-phosphorylated form of HiNF-D. Hence, the accumulation of this alternative form of HiNF-D in the G1-phase of four different tumor-

derived and transformed cell types is consistent with modulation of HiNF-D by a fundamental oscillatory mechanism involving protein kinases and phosphatases, that is a frequent (and perhaps invariable) target of deregulation in the process of tumorigenesis.

Phosphorylation of proteins has been shown to have a role within the context of cell cycle control of gene transcription. For instance, cyclins and yeast cell cycle mutant derived CDC-genes specify components of a hypothetical molecular oscillator mediating a cascade of cell cycle dependent phosphorylation and dephosphorylation events (reviewed by Cross et al., 1989; Murray & Kirschner, 1989). In this regard, the putative hyper-phosphorylated HiNF-D species is constitutively present throughout the cell cycle in four distinct tumor-derived or transformed cell lines, whereas in normal diploid cells we clearly observe cell cycle dependent fluctuations in two forms of HiNF-D. Others have reported similar cell cycle stage specific changes in phosphorylation states of proteins that are intimately associated with cell growth control, including tumor suppressors and oncoproteins such as RB, p53, MYC and MYB (reviewed in Weinberg, 1989; Levine, 1990; Luscher & Eisenman, 1990a & 1990b). Establishing the possible links between histone promoter factor HiNF-D and the genes encoding the specific oncoproteins, tumor-suppressor and/or CDC-related proteins that may be directly or indirectly associated with HiNF-D regulation will be a challenging task. This task will depend critically on the availability of genetic information and immunologic reagents for HiNF-D.

CHAPTER 5

PROTEIN/DNA INTERACTIONS INVOLVING ATF/AP1-, CCAAT- AND HiNF-D RELATED FACTORS IN THE HUMAN H3-ST519 HISTONE PROMOTERS: CROSS-COMPETITION WITH TRANSCRIPTION REGULATORY SITES IN CELL CYCLE CONTROLLED H4 and H1 HISTONE GENES

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5.0: Abstract

Protein/DNA interactions of the H3-ST519 histone gene were analyzed in vitro. Using several assays for sequence-specificity, we establish binding sites for ATF-, CCAAT- and HiNF-D related DNA binding proteins. These binding sites correlate with two genomic protein/DNA interaction domains previously established for this gene. We show that each of these protein/DNA interactions has a counterpart in other histone genes: H3-ST519 and H4-FO108 histone genes interact with the same ATF- and HiNF-D related binding activities, whereas H3-ST519 and H1-FNC16 histone genes interact with the same CCAAT-box binding activity. These factors may function in regulatory coupling of the expression of different histone gene classes. We incorporate these results into a promoter consensus model of established and putative protein/DNA interaction sites in mammalian histone genes. This model suggests that heterogeneous permutations of protein/DNA interaction elements, which involve both general and cell cycle regulated DNA

binding proteins, may govern the cellular competency to express and coordinately control multiple distinct histone genes.

5.1: Introduction

The orchestrated expression of groups of genes specifying the synthesis of structural and enzymatic proteins is required for the execution of global cellular functions. Duplication of the genome during the cell cycle occurs in every eukaryotic organism and involves expression of genes that are directly or indirectly associated with DNA replication. The regulated expression of this S-phase related gene-program is initially controlled at the transcriptional level.

Five classes of cell cycle dependent histone genes (H1, H2A, H2B, H3 and H4) are coordinately expressed during S-phase and are required for the assembly of DNA into chromatin. Each class represents a functional multi-gene family encoding the same or closely related proteins (Stein et al., 1984). Transcriptional regulation of individual histone genes has been studied in diverse organisms ranging from yeast to man (for example, Osley et al., 1986; Lai et al., 1988; Tung et al., 1989; Lee et al., 1991; Sharma et al., 1989; Sive et al., 1986; Sive & Roeder, 1986; Marashi et al., 1986; Grimes et al., 1987; Tabata et al., 1989; Seiler-Tuyns & Paterson, 1987; Heintz, 1988; Schumperli, 1986). Nonetheless, our understanding of how these genes are jointly rendered competent for transcription in proliferating cells from diverse cell lineages in higher eukaryotes is limited. The principles that govern the transcriptional competency of this group of structurally and

functionally related genes are elementary to the intricate mechanisms regulating batteries of cell type specific genes during development.

Parallel studies on in vitro transcription and protein/DNA interactions of several distinct human H1 (van Wijnen et al., 1988a & 1998b; Gallinari et al., 1989), H2B (Fletcher et al., 1987; LaBella et al., 198825-26), and H4 (Hanly et al., 1985; Dailey et al., 1988; van Wijnen et al., 1987 & 1989) histone genes in different laboratories have resulted in the definition of cis-acting elements that are functionally involved in transcription of these genes and represent sites for transcription factor binding in vitro. Elements have been described for Sp1, OTF-1, ATF-, and CCAAT-box related DNA binding proteins. Recently, the available repertoire of mammalian histone gene 5' flanking sequences has been expanded (Wells & McBride, 1989), which allows analysis of the evolutionary conservation of these elements in the 5' regions of closely related species. Moreover, we have performed an in-depth analysis of regulatory sequences and protein/DNA interactions required for in vivo and in vitro transcription of the human H4 histone gene designated FO108 during the cell cycle and differentiation (reviewed in Stein et al. 1989b), as well as during development in transgenic mice (van Wijnen et al., 1991a). We have also established genomic protein/DNA interaction domains in vivo for the human H4-FO108 and H3-ST519 histone genes (Pauli et al., 1987 & 1989).

These detailed analyses of molecular and cellular parameters that regulate several human histone genes raise a key issue in histone gene expression: What are the determinants that mediate coordinate

transcriptional regulation of multiple histone genes in cells of diverse ontogeny. Direct identification of protein/DNA interactions in additional histone gene promoters may contribute to our understanding of this problem. In the present study, we show that the H3-ST519 histone gene mediates multiple protein/DNA interactions in vitro that correlate with two genomic protein/DNA interaction domains previously established for this gene (Pauli et al. 1989). One of these involves the cell cycle regulated DNA binding activity HiNF-D3 (van Wijnen et al., 1991c). Competition analysis suggests that this protein/DNA interaction involves a DNA binding activity that also binds to three different H4 histone genes. We have also established binding sites for ATF- and CCAAT-box binding proteins. Competition experiments and sequence alignments using extended consensus sequences indicate that each of these protein/DNA interactions has a counterpart in other, but not all, histone gene classes. Our results are discussed within the context of established and putative protein/DNA interaction sites in mammalian histone genes. Current data suggest that heterogeneous permutations of protein/DNA interaction elements, which involve both general and cell cycle regulated DNA binding proteins, may govern the cellular competency to express, and perhaps coordinately control, multiple distinct histone genes.

5.2: Materials & methods

5.2.1: Gel retardation assays, stairway assays and competition analysis

Nuclear extraction and chromatographic fractionation procedures as well as gel retardation assays have been documented previously (van Wijnen et al., 1991b and references therein). Radio-labelled DNA fragments (5′ end-label) were prepared from pST519ΔH or pTP-1. These probes span the proximal promoter of the human H3 histone gene ST519 and are uniquely labelled at one DNA terminus, and were prepared by site-specific endonuclease cleavage, dephosphorylation using calf intestinal phosphatase, and subsequent ^{32}P-labelling using T4 kinase. DNA fragments were then cleaved using a secondary restriction enzyme and purified by gel electrophoresis.

The following probes were derived from pST519ΔH using the pair of restriction endonucleases indicated in brackets. The terminus created by the first enzyme is ^{32}P-labelled. Nucleotides are measured relative to the protein coding region and represent dsDNA sequences associated with the labelled fragment: respectively, HX- and XH-probes (nt -256/-20; HindIII/XmnI(=Asp700) fragment), and HHp- and HpH-probes (nt -200/-20; HindIII/HpaI fragment). The other probes were derived from pTP-1: respectively, EH- and HE-probes (nt -200/-20; EcoRI/HindIII fragment), EB-probe (nt -200/-143; EcoRI/BstNI fragment), and HB-probe (nt -139/-20; HindIII/BstNI fragment). Deletion analysis (stairway assay) was performed by shortening the HX-, EH- and HHp-probes using a panel of restriction enzymes (indicated in the figure legends). Protein/DNA interactions mediated by each set of deletion mutants were analyzed by

electrophoresis of binding reactions in parallel in native
polyacrylamide gels.

Competition analysis was carried out with a panel of
oligonucleotide (summarized in Results) and several unlabelled plasmid
DNA fragments isolated from pST523B (a 0.74 kB EcoRI/NcoI fragment
containing the 5′ portion of this H4 histone gene and a 1.1 kB
NcoI/EcoRI fragment containing the 3′ portion) and pST512 (1.0 kB
NcoI/NcoI fragment).

5.2.2: DNaseI footprinting, DMS fingerprinting and methylation interference analysis

DNaseI and dimethylsulphate (DMS) protection analysis, as well as
methylation interference assays were executed as described previously
(van Wijnen et al., 1988a; Pauli et al., 1990). The probes used for
these experiments are indicated in the figure legends, the non-specific
competitor in all cases was poly (dI-dC)*(dI-dC)(=poly I/C DNA). Results
with DNaseI nuclease and DMS protection experiments were obtained by
direct analysis on denaturing gels of the processed reaction products
derived from binding mixtures containing fractionated nuclear proteins.
Methylation interference analysis was performed by electrophoretic
separation on native polyacrylamide gels of free and complexed DNA, and
examination of processed reaction product on denaturing gels.

5.3: Results

5.3.1: Deletion analysis of binding sites for multiple factors interacting with the H3 histone gene promoter

The proximal promoter (nt -260 to nt -40) of the human H3 histone gene ST519 comprises two in vivo domains of DNA/protein interaction (Pauli et al., 1989). One of these, designated H3-Site I (nt -223 to nt -174), coincides with a genomic footprint. We have defined H3-Site II in this and a previous study (Stein et al., 1989a) as the region between nt -138 and -50, based on sequence similarities with short consensus elements (e.g., 5'dGGTCC, 5'dCCAAT and 5'dTATA) that are present in the analogous region, designated H4-Site II of the human H4-F0108 histone gene. The distal portion of H3-Site II coincides with a well-defined genomic DNaseI footprint (nt -138/-112), whereas the proximal part of H3-Site II displays a more heterogeneous, but altered reactivity pattern for dimethylsulphate (DMS) and DNaseI in vivo (Pauli et al., 1989). To examine the factors of HeLa S3 cells interacting with the H3-ST519 promoter in vitro, we analyzed protein binding to the HX-probe (nt -256/-20) in gel retardation assays (Fig. 5-1). The results show a variety of protein/DNA complexes that are observed using either unfractionated or chromatographically fractionated nuclear proteins. We have designated these complexes I, Ia, Ib, II and III, x, y and z, with complex Ib representing a set of five, and focus here on the complexes with Roman numerals.

Localization of the sequences required for binding of protein/DNA complexes I to III was accomplished by deletion analysis (stairway

assay)(Fig. 5-2; summarized in Fig. 5-3). Sets of deletion mutants were derived from either distally labelled DNA fragments (XM-probe, nt -256/-20; HpH-probe, nt -200/-20)(parts A-C), or proximally labelled DNA fragments (Hp- and HE- probes, both comprising nt -200/-20)(parts D-F). These proximal and distal series of deletion mutants were each incubated with fixed amounts of protein from various chromatographic fractions, and electrophoresed in parallel in native polyacrylamide gels. The pattern of complexes upon deletion of specific DNA segments was assessed to define sequences that are either required or sufficient for formation of protein/DNA complexes.

The results of these experiments show that complexes I (Fig. 5-2; ln C5-C6 & F2-F3), Ia (ln A5-A6 & D1-D2) and Ib (van Wijnen et al., 1991d) require sequences between -200 and -143. Also, although sequences between nt -256 and -143 mediate binding formation of complexes I and Ia, a DNA fragment spanning nt -256 to -190 does not. Deletions perturbing binding of complexes I, Ia and Ib coincide with the proximal part of H3-Site I (nt -223/-174). These results suggest that complexes I, Ia and Ib represent distinct H3-Site I protein/DNA interactions. These results suggest that complexes I, Ia and Ib represent distinct H3-Site I protein/DNA interactions.

Formation of complex II requires sequences between nt -139 and nt -67 (ln B3-B4 & E3-E4). Binding was below the level of detection when sequences between nt nt -139 and -110 (ln E4) or -109 and -67 (ln B4) were removed. This suggests that formation of complex II requires an extended DNA sequence that overlaps with the entire in vivo DNA/protein

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Figure 5-1. <u>Multiple protein/DNA interactions in the H3-ST519 histone</u> <u>promoter.</u> Gel retardation assay were performed with the XH-probe (Asp700/HindIII; nt -256/-20) and unfractionated (ln A1-A6) or fractionated nuclear proteins from HeLa S3 cells (ln B1-G6) using poly I/C DNA (2 μg) as non-specific competitor. Electrophoresis was performed using a 4% (80:1) polyacrylamide gel. Amounts of protein added per reaction range between 1-10 μg. Arrows indicate the positions of the various complexes. The bracket indicates the position of a series of complexes jointly designated Ib in ln E1-E5; these interaction events clearly resolve into five distinct complexes upon electrophoresis in 5% (30:1) polyacrylamide gels (data not shown).
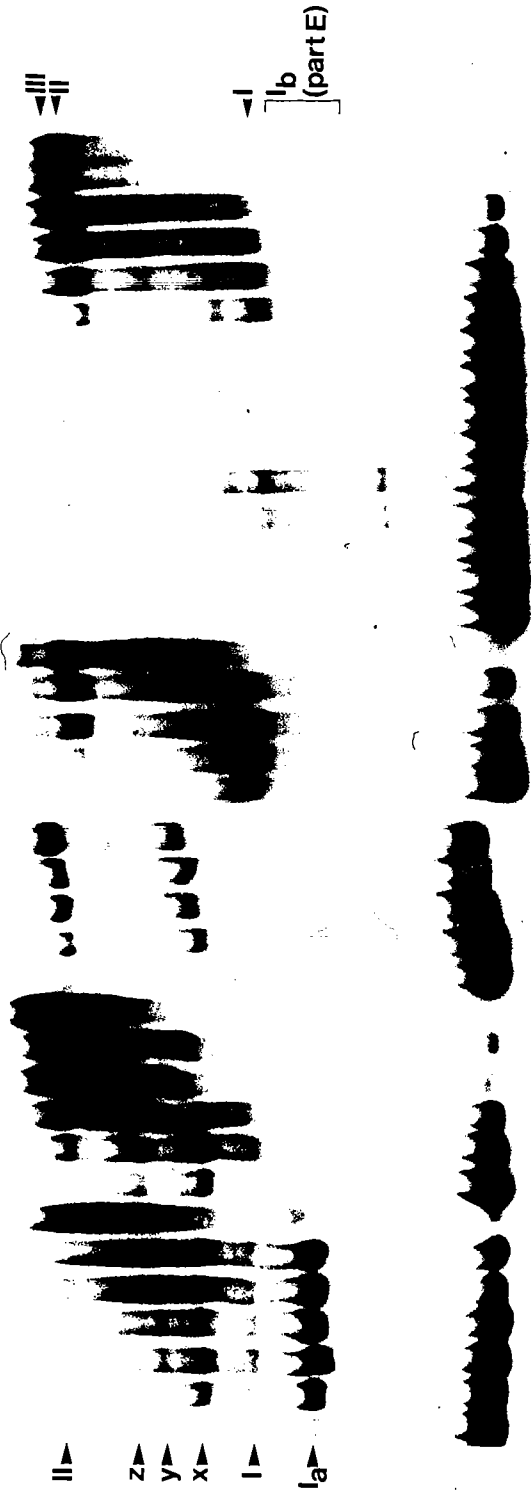
Figure 5-2. <u>Localization of protein/DNA interaction sites in the H3-</u>
<u>ST519 histone promoter.</u>

    Panels A, B & C: stairway assay with fragments derived from the
H3-ST519 promoter and labelled at the Asp700 site (nt -256)(XH-probe)
and digested with HindIII/-20 (ln 1), MboII/-43 (ln 2), HinfI/-67 (ln
3), Tha I/-110 (ln 4) AvaII/-129 (ln 5), BstNI/-143 (ln 6), AatII/-192
(ln 7), HpaI/-201 (ln 8), AluI/-210 (ln 9). The position of the cleavage
sites of these endonucleases is indicated in nucleotides relative to the
histone protein coding region, and refers to the dsDNA portion that
remains associated with the labelled portion of the probe. Each reaction
contains approximately 2 µg nuclear protein and 2 µg poly I/C DNA.
Arrowheads indicate the position of complexes I, II and III.

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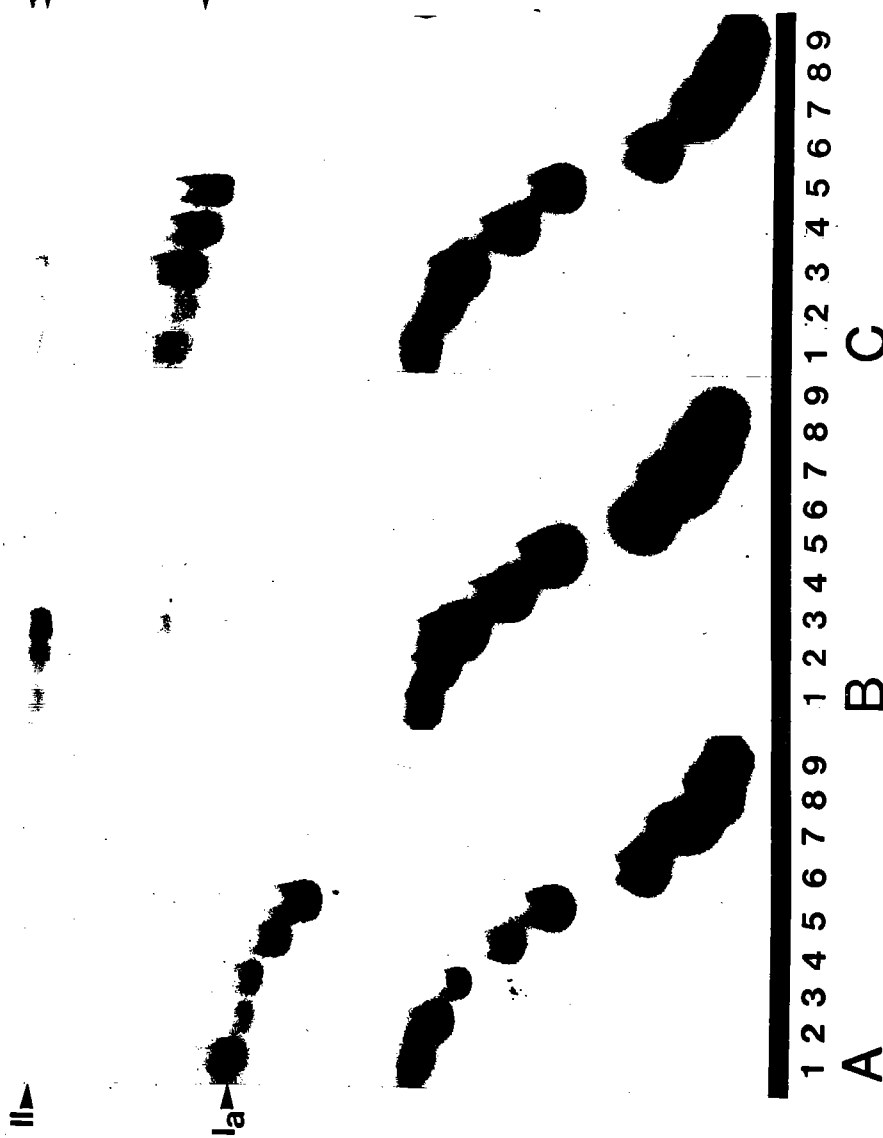
Figure 5-2 (continued). Panels D, E & F: as above, but stairway assays were performed with a fragment labelled at the Hind III site (nt -20) (HX-probe) and digested with EcoRI/-200(+ polylinker sequences)(ln 1), HindII/-200 (ln 2), BstNI/-139 (ln 3), ThaI/-109 (ln 4), AvaII/-83 (ln 5) and HinfI/-63 (ln 6). The small panel shown above the larger autoradiogram represents a lighter exposure of the top portion of the same gel. The EcoRI site is derived from the pUC8 polylinker and the HindII site originated from fusion of SmaI/pUC8 and HpaI/pST519ΔH sites during construction of

pTP-1.

NE   P0-200   P200-350

II▸   ◂≡II

II▸   ◂≡II

                                             ◂I

Ia▸

1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
D   E   F

148
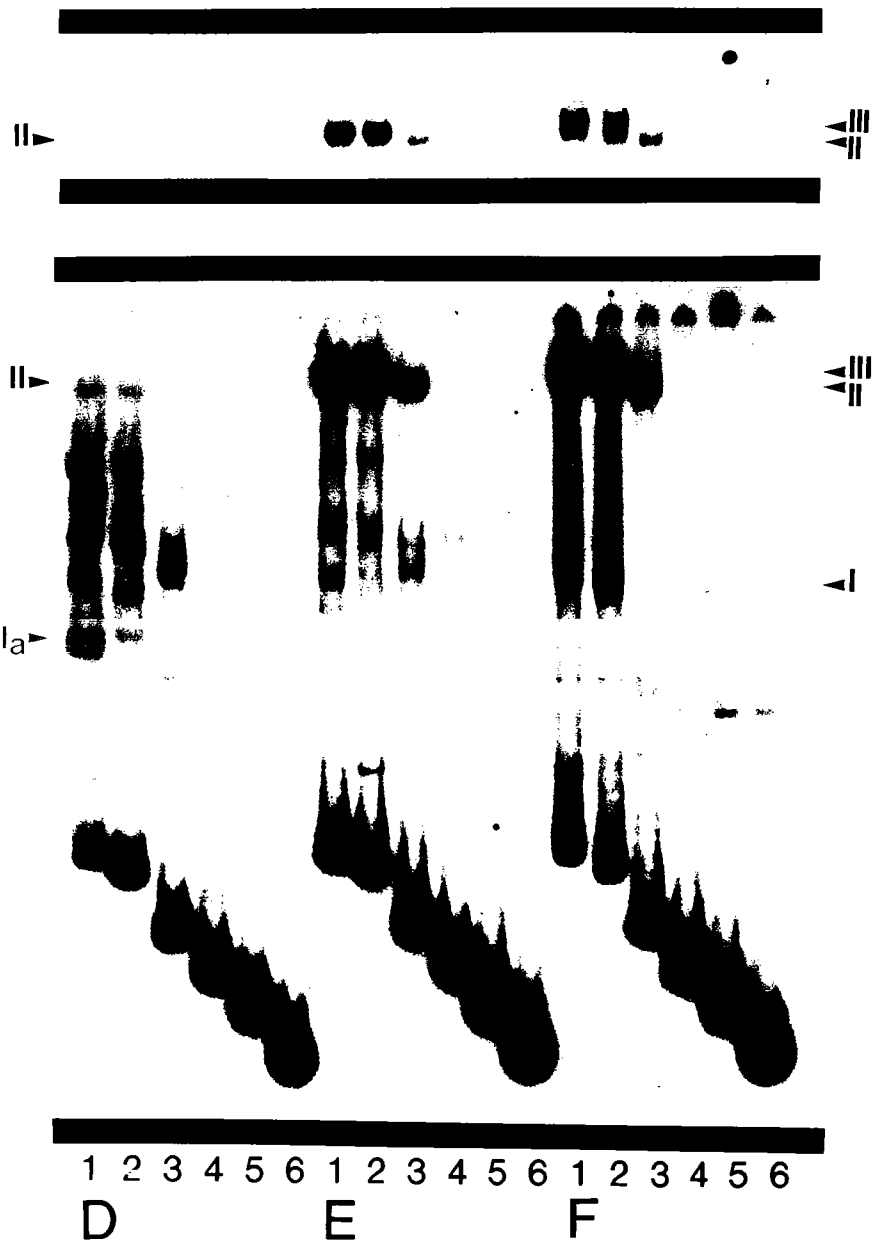
Figure 5-3. <u>Summary of protein/DNA interactions in the H3-ST519 histone promoter.</u> Panel A) Summary of stairway assay results. Horizontal lines depict restriction fragments (restriction enzymes above sequence) used in binding reactions; brackets denote a small segment of polylinker sequences derived from pUC8. The table on the right indicate the ability of the various DNA fragment to form the specific complexes I to III ("+": binding, "-": no binding, ±: decreased binding). Double lines beneath and above the sequence reflect in vivo genomic DNAseI footprints of H3-Site I and H3-Site II; 46) whereas the single lines beneath and above the sequence represent the general area that we refer to as H3-Site II that is analogous to H4-Site II. The DNA sequence of the H3-ST519 histone gene (63) has been revised at nt -140, -118, -92 and -79 using chemical sequencing data obtained with pTP-1. The revised sequence differs at nt -121 from the genomic DNA (46).

Complex formation
I Ia Ib II III

Site I

```
             AluI DdeI    HpaI  AatII                                                BstNI
      -240       -220              -180              -160
TACAAGACTTGTCCACA GTGGATATAGCAGCTAAGGGGTTAACAAAATGACGTCAGAGTAGCTACGG TAATGGGCAGGAGCCTCTCTTAATCTGCAACCAGG
ATGTTCTGAACAGGTGT CACCTATATCGTCGATTCCCCAATTGTTTACTGCAGTCTCATCGATGCC ATTACCCGTCCTCGGAGAGAATTAGACGTTGGTCC
```

[polylinker]

```
+ + + - -
- - - - -
- - - - -
- - - - -
- - - - -
| | | + + -
| | | + + +
| | | + + -
| | | + +|-
| | | + + -
```

Site II

```
BstNI        -120   ThaI    -100      AvaII  -80       HinfI -60   MboII
CCAGG CACAGAGATGGACCAATCCAAGAAGGCGCGGGATTTTGAATTTTCTTGGGTCCAATAGTTGGTGTCTGACTCTATAAAGAAGAG TAGCTCTT
GGTCC GTGTCTCTACCTGGTTAGGTTCTTCCCGCGCCCTAAAAACTTAAAGAACCCAGGTTATCAACCACCAGACTGAGATATTTCTTCTC ATCGAGAA
```

```
+ + | | | | | |
+ + | + | + | |
+ + + + | | | |
+ + + | | | | |
+ + + + | | | |
```

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Figure 5-3 (continued). Panel B) Summary of the results obtained for complexes I, Ia, B3 and D3 using specific competitor oligonucleotides ("+": competition, "-": no competition, ±: intermediate competition). Indicated in bold and underlined letters are sequences similar to the ATF (5'TGACG) and CP1/NF-Y (5'dCCAAT) binding sites.

Competition analysis:

	I	Ia	B3	D3
DS-I : gatcCGGAAAAGAAATGACGAAATGTCGAGA GCCTTTTCTTTACTGCTTTACAGCTCTCctag	+	+	-	-
NMP-1 : gatcTGGGATTCGCTGACGTCCATGAGAAAG ACCCTAAGCGACTGCAGGTACTCTTTCctag	+	+	-	-
DS-II : ctagCTTTCGGTTTTCAATCTGGTCCGATACT GAAAGCCAAAAGTTAGACCAGGCTATGAgatc	-	-	-	+
DD-1 : gatcCGCTTTCGGTTTTCGGCGTTTCGGTTTTCT GCGAAAGCCAAAAGCGGCGAAAGCCAAAAGActag	-	-	-	-
PD-2 : gatcTCAATCTGGTCCGATTCAATCTGGTCCGAT AGTTAGACCAGGCTAAGTTAGACCAGGCTActag	-	-	-	-
H3-II : gatcTCACAGAGATGGACCAATCCAAGAGGG AGTGTCTCTACCTGGTTAGGTTCTCCCctag	-	-	+	-
MYB (291b) gatcAGTAATCCAAACTGCCACAGTTCATAAG TCATTAGGTTTGACGGTGTCAAGTATTCCcatg	-	-	-	-

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Figure 5-3 (continued). Panel C) Sequence similarity between in vivo
DNA/protein interaction sites in the human H4 (Pauli et al., 1987) and
the H3 (Pauli et al., 1989) promoters. Indicated are sequence
similarities between the distal part of H4-FO108 Site I and the proximal
part of H3-ST519 Site I. The following symbols are used for in vitro
data: sequences protected from DNaseI digestion (bracketed lines),
guanines protected during DMS fingerprinting experiments (open
circles)(Wright et al., 1991 and van Wijnen et al., 1991d). In vivo
DNAseI footprints are indicated by lines immediately above and
underneath the sequence, whereas DMS fingerprints are indicated by bold
lettering (Pauli et al., 1987). The ATF-consensus sequence is shown for
reference (see text for references).

HiNF-E BINDING SITES IN THE H4-F0108 AND H3-ST519 PROMOTERS:

gene	sequence	binding

```
                        -201              HiNF-E           -173
H3-ST519
XmnI/HindIII  5'  ----GGTT|AACAAAATGACGTCAGAGTAGCTACGG|TAATGGGCAGG---        +
                  ----CCAA|TTGTTTTACTGCAGTCTCATCGATGCC|ATTACCCGTCC---

                             in vivo H3-I

XmnI/AatII    5'  ----GGTT|AACAAAATGACgtc
                  ----CCAA|TTGTTTTACT

HpaI/HindIII  5'  AACAAAATGACGTCAGAGTAGCTACGG|TAATGGGCAGG---                +
                  TTGTTTTACTGCAGTCTCATCGATGCC|ATTACCCGTCC---

H4-F0108
DS-I          5'  gatcCGGA|AAAGAAATGACGAAATGTCGAGA                          +
                      GCCT|TTTCTTTACTGCTTACAGCTCTctag

                  -151                                       -123
H4-I pm       5'  ----CAGA|AAAGAcATcACtAAATGTCGAGAGGGC|GGGGACAATTG---       -
                  ----GTCT|TTTCTgTAgTGaTTACAGCTCTCCCG|CCCCTGTTAAC---

DdeI/NaeI     5'  ----CAGA|AAAGAAATGACGAAATGTCGAGAGGGC|GGGGACAATTG---       +
                  ----GTCT|TTTCTTTACTGCTTTACAGCTCTCCCG|CCCCTGTTAAC---

                             in vivo H4-I
```

HiNF-E consensus sequence   5' AAATGACGWMA   with W = A or T, M= A or C, R = G or A

ATF consensus sequence   5' RTGACGTMR   bold sequence = match with consensus

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Figure 5-3 (continued). Panel D) Sequence similarity between DNA/protein interaction sites in the human H3 and H1 promoters. Indicated are, respectively, sequences of H3-ST519 Site II and the H1-FNC16 promoter binding site of CCAAT-box binding protein HiNF-B (van Wijnen et al., 1988b). The following symbols are used for in vitro data: sequences protected from DNaseI digestion (bracketed lines) and methylation interference contacts (open triangles)(van Wijnen et al., 1988 & 1991d). In vivo DNAseI footprints for the H3 promoter are indicated by lines immediately above and underneath the sequence, whereas DMS fingerprints are indicated by bold lettering (Pauli et al., 1989). The histone CCAAT-box similarity is shown for reference.

HiNF-B BINDING SITES IN THE H3-ST519 AND H1-FNC16 PROMOTERS:

gene   sequence   binding

                     -124   HiNF-B   -96
H1-FNC16   5' --GGTGGA|TTGGACGCTCCACCAATCACAGGGCAG|CGCCGGCTTATATA-   +
(RsaI/SmaI)   --CCACCT|AACCTGCGAGGTGGTTAGTGTCCCGTC|GCGGCCGAATATAT-
                                        ●ΔΔ   ○
                                         ○○

             5' --GGTGGA|TTGGACGCTCCACCAATCACAGGGCAG|CGC   +|
(RsaI/MspI)   --CCACCT|AACCTGCGAGGTGGTTAGTGTCCCGTC|GCGgc

H3-ST519   ·5' gatcT|CACAGAGATGGACCAATCCAAGAAGGG|ctag   +|
(H3-II)   A|GTGTCTCTACCTGGTTAGGTTCTTCCC|

             in vivo H3-II

             5' cagG|CACAGAGATGGACCAATCCAAGAAGGG|CGCGGGGATTTTTG-   +
(BstNI/HindIII) C|GTGTCTCTACCTGGTTAGGTTCTTCCC|GCGCCCCTAAAAAC-
                                 ΔΔ
                 -139   -111

HiNF-B consensus sequence: 5' KSYYRRCCAATSRRRR

with: K = T or G
         S = C or G
         Y = C or T
         R = A or G

interaction region H3-Site II (nt -138/-50). Formation of complex III

was abolished upon deletion of H3-Site II (ln C3-C4) or H3-Site I

sequences (ln F1-F2; top exposure). These findings are consistent with

complex III representing binding events involving factors that interact

with both H3-Site I and H3-Site II.

In summary (Fig. 5-3), these results show that several sequence-

specific protein/DNA interactions occur in the H3-ST519 proximal

promoter. The proximal part of H3-Site I that is required for formation

of complexes I, Ia and Ib, contains several short palindromic sequences

(e.g., 5'TGACGTCA). This suggests that this region interacts in vivo,

and in vitro (see below), with symmetrical factors (e.g., dimeric

proteins). Almost the entire H3-Site II region is required for complex

II suggesting that the factor interacts with an elongated sequence.

5.3.2: Competition between H3 and H4 histone genes for a cell cycle regulated factor

We have previously assigned gel retradation complex II to a DNA

binding activity designated HiNF-D3 (van Wijnen et al., 1991b). This

factor competes with an oligonucleotide that spans sequences of the

analogous in vivo protein/DNA interaction domain H4-Site II of the H4-

FO108 histone gene, and the same oligonucleotide also competes for the

H4-Site II:HiNF-D interaction (Fig. 5-4). Factors HiNF-D3 and HiNF-D can

be detected in a wide variety of mammalian cell types that actively

transcribe histone genes (van Wijnen et al., 1989 & 1991c; Holthuis et

al., 1990). Moreover, these analogous H3 and H4 histone gene protein/DNA

interactions are regulated in parallel during differentiation (Stein et

al., 1989a), the cell cycle, development and tumorigenesis (van Wijnen et al., 1991c). To determine whether the H3-Site II:HiNF-D3 and H4-Site II:HiNF-D protein/DNA interactions have counterparts in other H4 histone genes, we performed competition experiments using DNA fragments spanning the H4-ST523 and H4-ST512 histone genes (Fig. 5-4). Indeed, specific competition is observed for both HiNF-D3 and HiNF-D with these analogous H4 histone genes. This suggests that these H3 and H4 histone genes have corresponding proliferation-specific protein/DNA interactions, which may involve a common DNA binding activy.

5.3.3: Protein/DNA interactions with the ATF-consensus element of H3-Site I

The H3-Site I sequence 5'dTGACGTCA represents an ATF consensus sequence. ATF binding activity is mediated by dimeric proteins comprised of distinct subunits encoded by members of the ATF multigene family (reviewed in Ziff, 1990). Thus, some of the multiple complexes detected here may represent distinct ATF factors. We systematically analyzed chromatography fractions for ATF-like DNA binding activities using DNaseI protection analysis (summarized in Fig. 5-3, see van Wijnen et al., 1991d). The use of chromatographic fractions facilitates direct detection of DNA binding activities mediating DNaseI footprints. Stairway assays were performed with these same fractions (data not shown) to allow initial correlation between the gel retradtion complexes described in Fig. 5-2 and DNaseI footprint activities.

On the antisense-strand we observed a DNaseI footprint between nt -197 and -178 (van Wijnen et al., 1991d) and on the sense-strand

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Figure 5-4. <u>Competition for HiNF-D binding by multiple distinct H4 and</u>

<u>H3 histone genes.</u> A) Competition analysis of the HiNF-D3:H3-Site II

(left panel) and HiNF-D:H4-SiteII (right panel) protein/DNA interactions

using a 400-fold molar excess of oligonucleotides spanning,

respectively, binding sites of HiNF-D (DS-II) and HiNF-M (DD-1), short

consensus elements within H4-Site II (PD-2), the H3-Site II CCAAT-box,

and a MYB binding site (28,64); the first lane does not contain specific

competitor DNA (C). The probes used for detection span promoter

sequences of, respectively, H3-ST519 (nt -139/-20) and H4-FO108 (nt -

97/-38). Assay conditions were as described in van Wijnen et al., 1991b.

Arrowheads indicate protein/DNA complexes mediated by HiNF-D3, HiNF-D,

HiNF-B3 and HiNF-M, respectively abbreviated as D3, D, B3 and M.
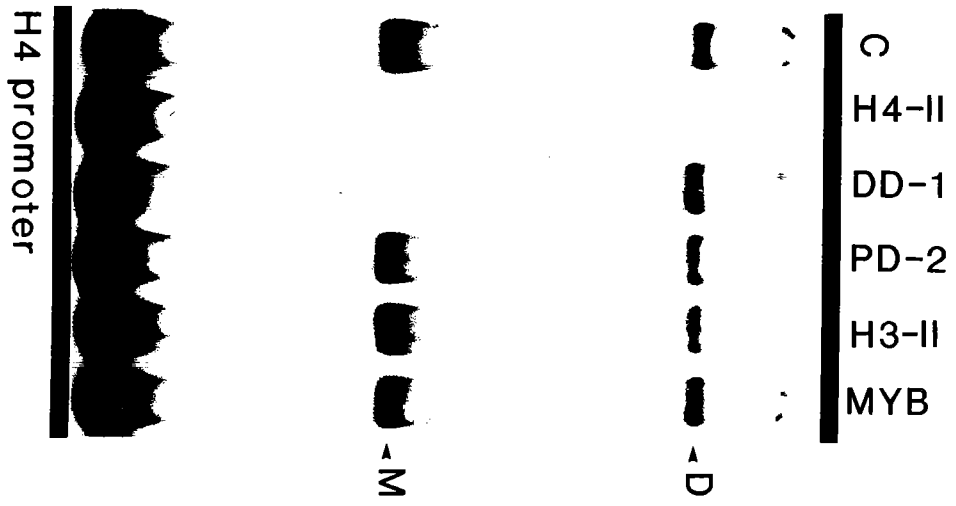
Competition analysis

160
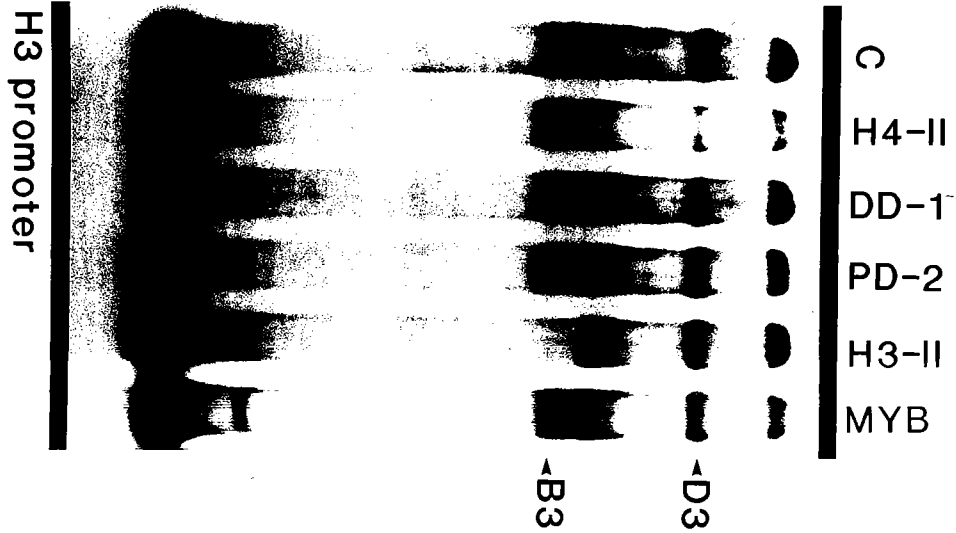
Figure 5-4 (continued). Panel B) Competition analysis of HiNF-D and HiNF-D3 with DNA fragments spanning human H4 histone genes ST523 and ST512. Competition was performed by the inclusion of restriction fragments spanning the 3′ region of ST523 (C; left four lanes), or the 5′ regions of the human H4 histone genes ST523 (middle section) and ST512 (left four lanes). Lanes in each case contain, respectively, 0, 5-, 10-, 20-fold molar excess of each fragment. Binding reactions each contain 2 μg nuclear protein and 2 μg poly I/C DNA. DNA fragments spanning the promoters of H3-ST519 (nt -200/-20; top) and H4-F0108 (nt -130/-38) were used as probes.

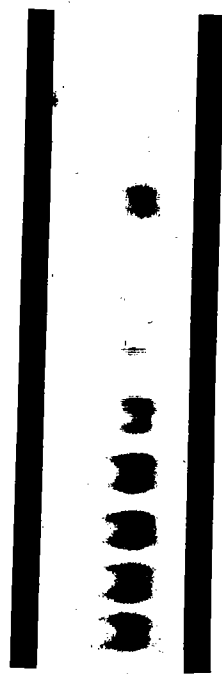between nt -200 to -174 (van Wijnen et al., 1991d). This DNaseI footprint activity coelutes with the factor mediating complex I, but not with gel retardation activities mediating the other H3-Site I complexes (Ia and Ib)(data not shown). Using DMS protection analysis (van Wijnen et al., 1991d) we observed protection of two guanines (nt -190 and -187) located in the ATF element. The vitro DNaseI footprints (van Wijnen et al., 1991d) coincide with the proximal part of the in vivo genomic DNaseI footprint H3-Site I (nt -223/-174)(Pauli et al., 1989), and the guanines protected in vitro (van Wijnen et al., 1991d) are identical to the in vivo DMS fingerprint (Pauli et al., 1989). Comparison of these in vitro and in vivo results indicate that the proximal part of H3-Site II interacts with an ATF-related DNA binding activity.

In vivo protein/DNA interaction domain H4-Site I of the H4-F0108 histone gene (Pauli et al., 1987) also contains an ATF element that interacts with a factor (HiNF-E/ATF-84) related to the ATF family of DNA binding proteins (Wright et al., 1991b). The in vitro and in vivo DMS fingerprints of the H4-Site I element are very similar to those of the ATF sequence in H3-Site I (Fig. 5-3). To directly address whether each site interacts with the same binding activity, we performed competition analysis of the H3-Site I protein/DNA complexes (Fig. 5-5). Using total nuclear protein we observed that protein/DNA complexes I and Ia competed specifically with an oligonucleotide (DS-I) containing the H4-Site I ATF element (Fig. 5-5). Competition occurred, albeit to a lesser degree, with a short DNA fragment designated NMP-1. The NMP-1 oligonucleotide (S.Dworetzky, JS & GS; unpublished data) contains another ATF-like

sequence located within a sequence context different from the DS-I oligonucleotide. Taken together, the results of in vivo and in vitro approaches are in agreement with the ATF-element of H3-Site I and H4-Site I interacting with a DNA binding protein that is a member of the ATF family of transcription factors.

5.3.4: Protein/DNA interactions involving the CCAAT-box of H3-Site II

Deletion analysis of the multiple complexes mediated by the H3-ST519 histone promoter in vitro (Fig. 5-2) suggests a partition between distal and proximal binding events, thereby reflecting the bimodular structure of this promoter as established by in vivo analysis of protein/DNA interactions. In vitro DNaseI protection analysis using nuclear protein shows that the same area (nt -139 to -112) that is protected in vivo is also protected in vitro (data not shown, see van Wijnen et al., 1991). To relate this DNaseI footprint activity to gel retardation complexes, we investigated the interactions of H3-Site II in closer detail using a DNA fragment spanning nt -139 to -20 (Fig. 5-6). With these short DNA fragments, we can clearly observe the HiNF-D3 (="II") complex, and two other complexes designated B3 and F3 (mediated by HiNF-B3 and HiNF-F3). Deletion of nt -139 to -109 abolishes detection of complexes B3 and D3, whereas detection of F3 requires nt -109 to -83 (Fig. 5-6). Hence, this analysis indicates that these complexes require different sequences (F3 versus B3 and D3), or require DNA binding activities eluting in separate chromatography fractions (D3 versus B3 and F3). These results suggest that these complexes are mediated by distinct entities.

Figure 5-5. <u>Characterization of the protein/DNA interactions at the ATF-</u><u>element of H3-Site I: cross-competition with H4-Site I.</u> Competition analysis of H3-Site I protein/DNA complexes (I and Ia) by gel retardation analysis using a 200-fold molar excess of oligonucleotides spanning the ATF binding site of H4-FO108 Site I (DS-I), a palindromic ATF binding site (NMP-1), and a MYB binding site (Biedenkapp et al., 1988). The probe spans nt -200 to -143 of the H3-ST519 promoter. In vitro DNaseI footprinting and DMS fingerprinting data of the ATF-element in the proximal portion of H3-Site I have been presented in van Wijnen et al., 1991d.

C
DS-I
NMP-1
MYB

ATF

◄ I

◄ Ia
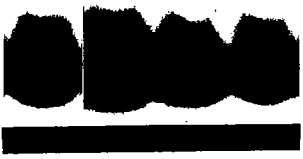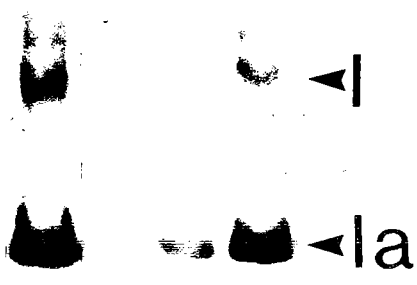
H3-I

To examine in vitro protein/DNA interactions mediated by the H3-Site II sequences that correspond to the in vivo genomic DNaseI footprint, we assessed binding of total nuclear protein to an oligonucleotide (nt -139 to -112) spanning H3-Site II and the region required for complexes B3 and D3 (Fig. 5-6). Competition analysis shows that this DNA fragment mediates formation of complex B3, but not the HiNF-D3 complex. These results are consistent with HiNF-D3 interacting with a more extended DNA sequence (see Fig. 5-2), and are similar to results for the analogous H4-Site II:HiNF-D interaction that also requires an elongated sequence (van Wijnen et al., 1991b). These results also demonstrate that nt -139 to -112 are sufficient for sequence-specific binding of HiNF-B3.

The H3-Site II footprint (nt -138/-112) contains a CCAAT-motif that resembles the consensus binding site of the CP1/NF-Y class of transcription factors (Hooft van Huijsduijnen et al., 1990; and references therein) suggesting that HiNF-B3 is a CCAAT-box binding protein. The protein/DNA contacts of complex B3 were established by methylation interference (Fig. 5-6), and we observed that that methylation of guanines nt -126 and -125 is inhibitory for binding. These nucleotides coincide with the H3-CCAAT element (nt -126 to -122) and are equivalent to protein/DNA contacts of the heteromeric CCAAT-box factor HiNF-B (van Wijnen et al., 1988a & 1988b). Therefore, it is possible that the DNA binding activities interacting with the H3- and H1-CCAAT boxes are the same.

Figure 5-6. <u>Characterization of protein/DNA interactions with the CCAAT-</u>
<u>element of H3-Site II.</u> Panel A) Stairway assay of the H3-Site II region
using unfractionated (NE) and fractionated nuclear proteins (PO-100 and
P100-400)(28) were performed with fragments labelled at nt -20 (HindIII
site) and which span sequences up to the following sites: BstNI/-139 (ln
1,5,9), ThaI/-109 (ln 2,6,10), AvaII/-83 (ln 3,7,11) and HinfI/-63 (ln
4,8,12). Indicated by arrowheads are complexes mediated by HiNF-B3, -D3
and -F3.

Deletion analysis

NE   P0-100 P100-400

D3▸

F3▸
B3▸

BstNI→
ThaI →
AvaII →
HinfI→

H3-Site II

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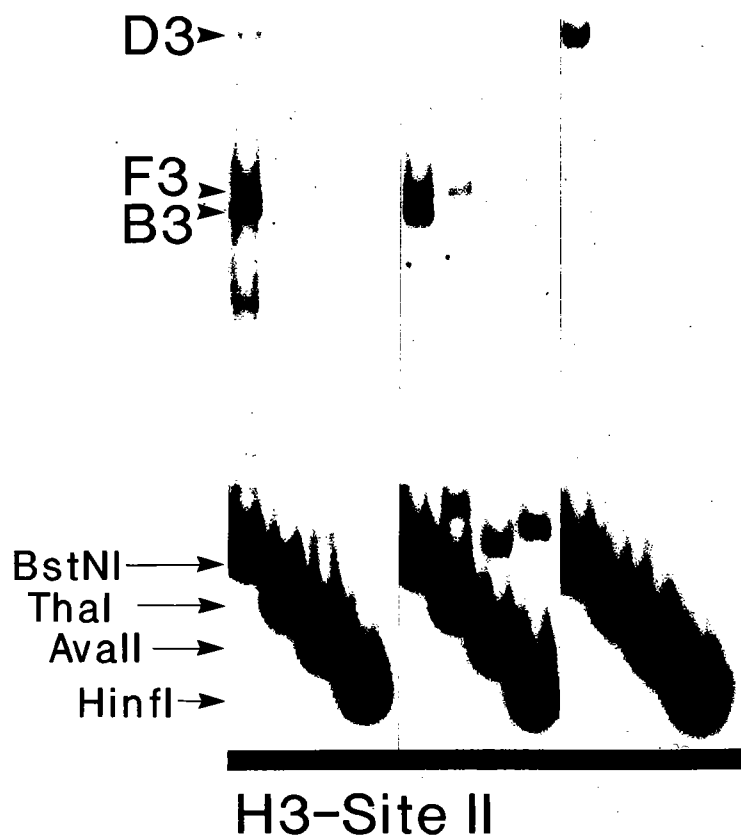
Figure 5-6 (continued). Panel B) Competition analysis of HiNF-B3 binding to a radio-labelled oligonucleotide (H3-II; 0.5 ng) spanning the in vivo and in vitro footprint of H3-Site II. Binding mixture were performed with 10 μg nuclear protein and a 200-fold exces of the indicated oligonucleotides (see Fig. 5-2 for the sequences of these DNA fragments).

C DS-I DS-II H3-II MYB

B3►
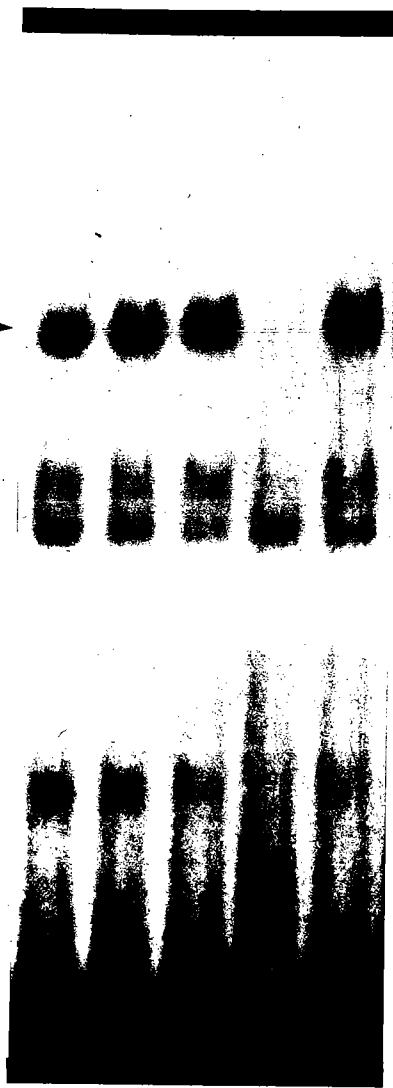
H3-II

Figure 5-6 (continued). Panel C) Methylation interference analysis of
HiNF-B3. Results were obtained by isolating the HiNF-B3 complex from the
gel after DMS treament of binding reactions containing PO-100 protein
and a probe labelled at the anti-sense strand (HindIII/BstNI fragment,
nt -142 to -13). Ln 1-2: DNA cleaved at purines (G>A); ln 1: unbound DNA
(U), ln 2: bound DNA (HiNF-B3 complex)(B), ln 3: DNA cleaved at
pyrimidines (C+T).

U B CT

CCAAT
H3-Site II

−142 ►
OO
GGTTA ►

−110 ►

−67 ►

1 2 3

173
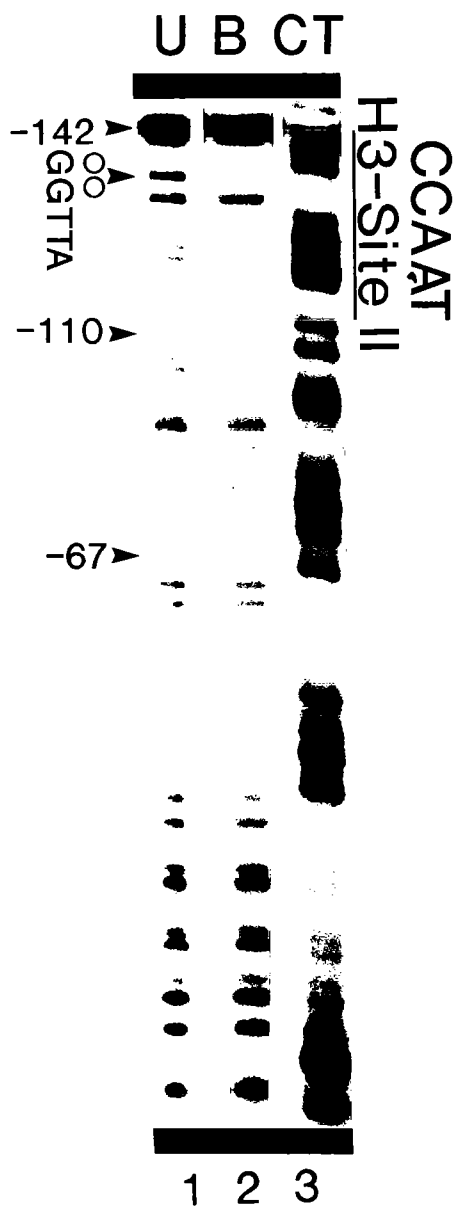
Figure 5-7: **CCAAT-box binding protein HiNF-B interacts with both the H3-ST519 and H1-FNC16 histone genes.** Competition analysis of protein/DNA complexes interacting with the H3-ST519 (nt -139/-20) and H1-FNC16 (nt -213/-78) promoters using the oligonucleotides indicated above the lanes. Binding reactions were performed using nuclear protein from human HeLaS3 cells (left panel, H3: ln 1-4; and right panel, H1: ln 1-4), mouse fetal liver (H3: ln 5-8) or fetal brain (H3: ln 9-12). The complexes with similar migration rates and competition behavior are indicated by arrowheads and equivalent letters.

To address this question more directly, we electrophoresed probes spanning the H3-ST519 nt (-139/-20) and H1-FNC16 (-213/-78) histone promoters in parallel in gel retardation assays (Fig. 5-7). The complexes of HiNF-B3 and HiNF-B have similar relative mobilities and indistinguishable competition behavior. Specifically, the HiNF-B:H1-CCAAT box interaction competes with the H3-Site II CCAAT-box oligonucleotide that competes for HiNF-B3 binding to the H3 histone gene. Thus, we have observed similarities in electrophoretic mobility, size and sequences of the DNA binding domain, equivalent methylation interfence contacts, and cross-competition. In addition, both the H3 and H1 CCAAT-box binding activities are regulated in parallel, and are constitutively present during tissue development from fetus to adult (van Wijnen et al., 1991c). We postulate that the CCAAT-boxes of the H3-ST519 and H1-FNC16 histone genes interact with the same binding activity.

Interestingly, the H3 promoter (nt -139/-20) also mediates formation of complex F3, and this complex has an apparent counterpart of similar migration in the H1 promoter (complex F1)(Fig. 5-7). The intensities of complexes F3 and F1 relative to complexes B3 and B1 respectively, are dependent on the non-specific competitor DNA present in the binding reaction (compare Figs. 5-6 and 5-7). The results of specific competition analysis using a panel of oligonucleotides show that these complexes compete specifically with a DNA fragment spanning sequences of the H4 histone promoter. However, HiNF-F3 and HiNF-F1 can not be detected in nuclear protein preparations from fetal mouse tissues

(Fig. 5-7) that actively transcribe histone genes (van Wijnen et al., 1991a). Thus, the significance of these protein/DNA complexes is unclear at present.

5.4: Discussion

5.4.1: Involvement of ATF/AP1 related proteins in transcriptional regulation of several histone gene classes

In this work, we have established the binding site of an TGACG-box binding protein that binds to a portion of genomic protein/DNA interaction domain H3-Site I of the H3-ST519 histone gene. This activity competes with the binding site of factor HiNF-E/ATF-84 located in the analogous domain (H4-Site I) of the H4-FO108 histone gene. Deletion of the HiNF-E/ATF-84 binding site in H4-Site I results in a decrease, but not abolishment, of H4 histone gene transcription both in vitro (Wright et al., 1991b) and in vivo (Ramsey et al., 1991). This indicates that HiNF-E/ATF-84 is a positively acting, auxiliary transcription factor, consistent with the postulated roles of other members of the ATF-family (Ziff, 1990). Based on comparison of DNaseI and DMS protection patterns in vivo (Pauli et al., 1987 & 1989) and in vitro, as well as cross-competition, we suggest that H4-Site I and H3-Site-I interact with similar DNA binding activities. Hence, HiNF-E/ATF-84 may be involved in a mechanism that coordinately stimulates transcription of both H4 and H3 histone genes.

This conclusion does not exclude a role for ATF-sites in other histone genes. For instance, Sive et al. have shown that mutation of

upstream sequences (nt -115 to -100) of a human H2B histone gene reduces in vitro and in vivo transcription (Sive et al., 1986). This region appears to coincide with an ATF element (Fig. 5-8), suggesting that ATF factors may also influence transcription of H2B histone genes. Interestingly, Tabata et al. (Tabata et al., 1989) have shown that a similar ATF-element is functionally involved in the transcription of a wheat H3 histone gene. This suggest that ATF-sites may represent an ancient component of a highly conserved, histone gene regulatory transcription mechanism that is operative in all eukaryotic species.

Sharma et al. have shown that a hamster H3 histone gene interacts with a factor that is immunologically related to the JUN oncoprotein (with JUN representing a component of the AP1 transcription factor)(Sharma et al., 1989). Recently, Hai and Curran (Hai & Curran, 1991) have shown that certain members of the ATF family (ATF-1, ATF-2, etc.)(Ziff, 1990) may form selective cross-family heterodimers with members of the AP1 family (JUN, FOS, FRA-1, etc.)(Mitchel & Tjian, 1989), suggesting that the various ATF and AP1 members represent a superfamily of related transcription factors (Hai & Curran, 1991). Consistent with the close sequence similarity between AP-1 (5′dTGACTCA) and ATF (5′dTGACGTCA) elements, which differ only by the spacing between two half-sites, cross-family heterodimers may recognize both elements, albeit with different affinities (Hai & Curran, 1991). Thus, it is clear that at least one member of the ATF/AP1 superfamily may be involved in regulation of several histone gene classes. However, the subunit composition of the specific ATF/AP1 species that is physiologically most

relevant to histone gene expression remains to be established.

5.4.2: Interactions of heteromeric CCAAT-box binding proteins with several different histone gene classes

We have also defined the binding site of a CCAAT-box binding protein that corresponds to the in vivo DNaseI footprint of H3-Site II, and presented evidence here that this site interacts with the heteromeric H1 histone CCAAT-box binding protein HiNF-B (van Wijnen et al., 1988a & 1988b). The H3 and H1 histone CCAAT-boxes display extensive sequence similarities with elements in these and other histone subtypes. These elements resemble the binding sites of heteromeric CCAAT-box proteins of the CP1/NF-Y class that have been evolutionarily conserved from yeast to man (Hooft van Huijsduijnen et al., 1990). Gallinari et al. (Gallinari et al., 1989) and Sive et al. (Sive et al., 1986) have shown that mutation of identical CCAAT-elements (i.e., conforming to an extended consensus sequence) in H1 and H2B histone genes decreases transcription, and similar "CP1/NF-Y type" CCAAT-boxes have been shown to influence transcription of many other genes (reviewed by Mitchel & Tjian). We deduce that the CCAAT-boxes of human H3 and H1 histone promoters are conserved transcriptional elements interacting with equivalent DNA binding activities that may collectively enhance transcription of H3, H2B and H1 histone genes.

5.4.3: Histone promoters contain unique permutations of shared promoter elements

Parallel studies on transcriptional regulation or protein/DNA interactions in distinct human H1, H2B, H3 and H4 histone genes by

distinct laboratories have resulted in the definition of several

protein/DNA interaction elements. The list of nuclear factors from human

HeLa S3 cells characterized to date include OTF-1 (Fletcher et al.,

1987), CP1/NF-Y related CCAAT-box factors (HiNF-B/H1-TF2)(van Wijnen et

al., 1988b,; Gallinari et al., 1989), H1-SF/H1-TF1 (Dalton & wells,

1988a & 1988b; Gallinari et al., 1989), Sp1/H4-TF1 (van Wijnen et al.,

1989; Dailey et al., 1988), and ATF-84/HiNF-E (Wright et al., 1991b),

and we have shown for the H4-F0108 and H3-ST519 histone gene that

several of these elements represent protein/DNA interaction sites in

vivo (Pauli et al., 1987 & 1989).

We aligned these binding sites with the immediate 5'regions of

recently characterized mammalian histone genes for which only sequence

information is available (Wells & McBride, 1989). We observed in each

individual gene a limited number of extended sequence similarities that

overlap with putative core consensus sequences for the aforementioned

factors. The most striking observations are the following: (I) the H2B

subtype specific element (OTF-1 binding sites)(LaBella et al., 1988) is

associated with both H2B and H2A histone genes; (II) an H1 histone

consensus sequence (H1-SF binding site)(Dalton & Wells, 1988a & 1988b)

in one case is also found associated with an H4 histone gene; (III)

extensive CCAAT-box similarities (of the "CP1/NF-Y" type)(van Wijnen et

al., 1988b), and referred to as a secondary "H1 subtype specific

element" (Gallinari et al., 1989), are present in H1, H2a, H2b, and H3

histone genes, but are usually not associated with H4 histone genes;

(IV) the Sp1 consensus sequence (Kadonaga et al., 1986) is frequently

associated with H1, H3 and H4 histone genes, but Sp1 consensus sequences with the same high degree of similarity are not found in H2a and H2b histone genes; (V) ATF-elements are usually present in H3 and H4 histone promoters, and less frequently in other histone genes. Based on the apparent occurrence of putative H1-subtype and H2B-subtype specific consensus elements in more than one subtype, the function of these elements in mediating binding of histone gene transcription factors is most likely not confined to specific classes of histone genes.

We conclude from this analysis that individual histone genes are associated with a limited number of protein/DNA interaction elements. These elements represent a permutated subset of the full complement of histone promoter binding sites. Although the subset of putative factor binding sites is unique for each individual gene, these 5′ regions frequently have one or more lements in common. Consequently, the modular organization of the prototypical histone promoter is reflected by a mosaic of cis-acting elements that is to various degrees similar to that of another histone gene. These heterogeneous permutations may govern the cellular competency to transcribe histone genes by increasing the likelihood that stimulatory factors interact with these genes. Shared protein/DNA interaction elements may reflect regulatory coupling at the transcriptional level of multiple distinct histone genes by a relatively small number of transcription factors.

5.4.4: Constitutive and proliferation-specific protein/DNA interactions

In vivo H4-Site I protein/DNA interactions involving ATF and Sp1 factors are not downregulated during differentiation of HL60 cells at

the cessation of histone gene transcription (Stein et al., 1989a). Moreover, we have shown that histone CCAAT-box binding activity (HiNF-B) remains constitutive during hepatic development (reflecting the onset of in vivo quiescence and differentiation in the intact animal)(van Wijnen et al., 1991c), although downregulation of histone gene transcription occurs during this process as shown using transgenic mice (van Wijnen et al., 1991b). Thus, permutated sets from a fixed complement of shared promoter elements including ATF, Sp1 and CCAAT-box factors may be a constitutive component of a mechanism by which histone genes selectively stimulate groups of histone genes. Another component is represented by a set of analogous proliferation-specific protein/DNA interactions in the promoters of the H4-FO108, H3-ST519 and H1-FNC16 histone genes involving HiNF-D and related factors (van Wijnen et al., 1991c). This set is coordinately controlled during the cell cycle, differentiation and development, and is collectively deregulated during tumorigenesis. Together, these findings suggest that the interplay of constitutive and cell cycle regulated components may mediate coordinate control of five histone multi-gene families.

CHAPTER 6

GENERAL DISCUSSION & CONCLUSIONS

6.1: Relevance of in vitro protein/DNA interactions.

In this study, we have delineated multiple protein/DNA interaction sites in the proximal promoters of H4, H3 and H1 histone genes. For example, the H4 histone promoter interacts with at least six different DNA binding proteins (HiNF-A, HiNF-C, HiNF-D, HiNF-E, HiNF-M and HiNF-P)(Chapters 2 & 4); the H3 histone promoter interacts with DNA binding activities indistinguishable from, respectively, HiNF-A, HiNF-B, HiNF-D and HiNF-E (Chapters 2 & 5); the H1 histone promoter contains binding sites for HiNF-A and HiNF-B, as well as a HiNF-D related factor (HiNF-D1)(Chapters 2 & 3). After establishing regional sequence-specificity of these factors using stairway assays, the binding sites were in most cases determined at single nucleotide resolution by a variety of approaches. The sequence-specificity of these factors (with the possible exception of HiNF-A) is consistent with a promoter-selective role in the regulation of gene-expression.

Interestingly, the binding sites coincide with (I) in vivo protein/DNA interaction domains (Pauli et al., 1987 & 1989), and functional cis-acting elements defined both (II) in vivo (Ramsey et al., 1991) and (III) in vitro (Wright et al., 1991b). Moreover, (IV) several DNA binding activities (HiNF-A, HiNF-B, HiNF-D and HiNF-E) interact with more than one histone promoter (Chapters 2 & 4), and (V) some factors (HiNF-B, HiNF-C and HiNF-E, respectively, Sp1-, ATF-, and CCAAT-box

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factors) are similar to generally established trans-activating proteins identified by others during the course of this study (reviewed by Mitchel & Tjian, 1989). Hence, the relevance of these in vitro protein/DNA interactions is firmly indicated by compatibility of the results obtained with parallel experimental approaches in this and other laboratories.

6.2: The complement of factors interacting with H4, H3 and H1 histone promoters.

Do the protein/DNA interaction sites described for the H4-FO108, H3-ST519 and H1-FNC16 genes (over-)represent the full complement of DNA binding activities associated with each of these genes? The primary experimental approach was based on the gel retardation assay. The high sensitivity intrinsic to this assay could conceivably lead to the establishment of too many protein/DNA interactions, thereby resulting in over-representation of the complement of bona fide histone gene regulatory factors. However, because this assay monitors electrophoretically stable protein/DNA complexes that are mediated by proteins remaining stable during both the isolation procedure and the binding reaction, this places several constraints on the ability to detect DNA binding activities.

The detection of six different DNA binding activities with DNA fragments spanning the H4-FO108 histone promoter required variation of multiple experimental parameters. Several lines of evidence now indicate that HiNF-C and HiNF-E are the primary activities binding to in vivo

protein/DNA interaction domain H4-Site I (Wright et al., 1991b).

However, unlike HiNF-C, HiNF-E activity could not be detected in earlier

studies (Chapter 2), although the presence of a HiNF-E binding site in

the H4-F0108 histone gene was inferred from studies with the H3-ST519

gene (Chapter 5). Mutational analysis of the evolutionarily conserved

H4-Site II sequences (Ramsey-Ewing et al., 1991) indicates that apart

from HiNF-D (Chapter 2) at least two other factors should interact with

this domain (Chapter 3) to account for in vivo transcription data

(Ramsey et al., 1991). Modification of experimental conditions was

required to identify two novel factors (HiNF-M and HiNF-P) each with a

DNA binding activity complementary to that of HiNF-D (Chapter 4). At

present, and with the possible exception of HiNF-A, the H4 histone

promoter factors described here may very well represent the functional

complement of proteins selectively involved in transcriptional

regulation of the H4-F0108 histone gene.

The H3-ST519 and H1-FNC16 histone genes have not been studied in

the same detail as the H4-F0108 histone gene. Based on transcriptional

studies using other histone genes (Dalton & Wells, 1998a & 1988b;

LaBella et al., 1988 & 1989; Wright et al., 1991b) and the presence in

the H3-ST519 and H1-FNC16 promoters of extensive sequence similarities

with established cis-acting elements, it is possible to make reasonable

estimates of the complement of factors interacting with these two genes.

The HiNF-E binding site in the H3-ST519 histone gene is located in the

proximal part of in vivo domain H3-Site I (Chapter 5). However, the

distal part H3-Site I contains palindromic sequences and several groups

of in vivo protein/DNA contacts. We speculate that this region may
interact with a novel dimeric protein, possibly in cooperation with
HiNF-E. H3-Site II interacts with HiNF-B and HiNF-D, but considering the
presence of an Sp1 consensus element, this domain is also likely to
interact with HiNF-C. However, to date we have not been able to detect
in vivo or in vitro binding of HiNF-C to the H3-ST519 promoter. This
suggests that this Sp1 element is perhaps a low affinity HiNF-C binding
site. We postulate that the H3-ST519 promoter interacts with
approximately five distinct DNA binding activities. Following the same
reasoning, we estimate that the number of factors interacting with the
H1-FNC16 is similar to that of the H3-ST519 and H4-F0108 genes. Thus,
the number of in vitro protein/DNA interactions detected for the H3-
ST519 and H1-FNC16 genes in this study most likely reflect an under-
representation of the binding events occurring in vivo.

The five human histone gene classes represent functionally
expressed multi-gene families of 10 to 30 gene-copies each (Stein et
al., 1984). The 5'regions of these genes display considerable sequence
heterogeneity (Wells & McBride, 1989), despite short stretches of
sequence similarity, suggesting complexity in transcriptional
regulation. Because our findings suggest that each gene interacts with
only 4 to 6 distinct DNA binding activities, and because most of these
factors interact with more than one gene, the combined number of
sequence-specific trans-activating proteins involved in histone gene
expression actually may be limited and could be about an order of
magnitude lower than the total number of histone genes being regulated

by these factors.

6.3: Regulation of histone promoter protein/DNA interactions

The temporal regulation of protein/DNA interactions of H4, H3 and H1 histone promoters was examined during various biological processes. We have shown that HiNF-D binding activity is modulated in conjunction with alterations in histone gene transcription, whereas other DNA binding activities (for example, HiNF-A, HiNF-B, HiNF-C, and HiNF-M) are not directly coupled to histone gene expression (Chapter 3). Thus, these studies not only have defined candidate proteins involved in directing (I) efficient transcription of individual histone mRNA synthesis units, and (II) transcriptional coupling of distinct classes of histone genes, but also (III) in temporally regulating histone gene expression with cell proliferation and DNA replication. Moreover, we have obtained a first indication that (IV) post-translational modifications may influence the DNA binding activities of several histone promoter factors, thereby directly modulating the putative trans-activating potential of these proteins. The most straightforward model that arises from these findings is that coordinate temporal regulation of multiple histone genes is mediated by heterogeneous combinations of constitutive and cell cycle dependent nuclear factors that are subject to regulatory post-translational modifications.

This working model provokes several questions. First, the heterogeneous permutations of transcription factor binding sites implies that each gene interacts with various classes of proteins with different

trans-activating polypeptide domains (reviewed by Mitchell & Tjian, 1989) that operate at various nucleotide-distances from histone mRNA cap-sites. How does the general transcription machinery translate these mixed signals into selective and efficient synthesis of histone mRNAs? This presumably occurs by promotion of protein/protein interactions via adaptor molecules (co-activators)(Pugh & Tjian, 1990) that interface between the TATA-box factor (Cavallini et al., 1988), RNA polymerase II and the various classes of trans-activating protein domains. But because different classes of co-activators may exist, this transfers the same question to a more complex molecular level.

Second, mutational analyses suggest that, with few exceptions, these protein/DNA interactions have auxiliary (and stimulatory) rather than essential functions in histone gene expression. Does this imply that these stimulatory functions are redundant? Assuming that proliferating cells of diverse ontogeny contain widely different levels of these trans-acting factors, the presence of multiple redundant elements would for example promote the likelihood that there is always at least one trans-activator capable of directing histone gene expression at a sub-optimal level. Alternatively, the requirement for a rapid rate of histone protein synthesis during the cell cycle may imply that all stimulatory elements are necessary for efficient synthesis of the histone encoding translational templates (i.e., histone mRNA). Solutions to these questions require a detailed study of multiple kinetic and transcriptional parameters of histone gene expression during mammalian development.

Finally, our studies suggest that HiNF-D is a key regulatory factor in histone gene transcription and this factor is represented by two phosphatase- sensitive electrophoretic species. The ratio of these species fluctuates during the cell cycle in normal diploid cells, and is altered during tumorigenesis. Cell cycle stage specific modifications in the degree of phosphorylation have been observed for several proteins intimately associated with cell growth control (reviewed by Luscher & Eisenman, 1990a & 1990b; Levine, 1990). This provides precedent for the possibility that HiNF-D may also be regulated by cellular phosphorylation mechanisms. The possibility that HiNF-D acts as a specific, cell cycle regulated trans-activating phosphoprotein involved in transcription of at least three distinct histone gene classes can be addressed directly when immunologic reagents and genetic information for this factor are available.

There are many examples of heteromeric transcription factors and it would not be surprising if HiNF-D will turn out to be a composite protein. However, until this is demonstrated, for example, by separation *and subsequent reconstitution of HiNF-D* from its putative components, it is prudent to refer neutrally to the entity as "factor HiNF-D" rather than "the HiNF-D complex".

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BIOGRAPHICAL SKETCH

The author was born in The Hague, Netherlands, on July 16, 1962. He attended Aloysius College, The Hague where he graduated with a High School degree in the spring of 1980. He entered the University of Utrecht (Rijks Universiteit Utrecht) in the fall of 1980, and obtained a B.A. in biochemistry in 1983. In the fall of 1983, he entered the laboratory of Dr. Peter van der Vliet in the Department of Physiological Chemistry. He remained here until the summer of 1984 and his departure to the University of Florida, Gainesville. As a participant in a student exchange program, he visited the laboratory of Drs. Gary and Janet Stein for one year. After his return to the Netherlands in the fall of 1985, he entered the Stein's laboratory again in the beginning of 1986. After official enrollment in the University of Florida graduate program in the fall of 1986, he transferred to the University of Massachusetts, Worcester, in the winter of 1987 to continue his doctoral dissertation project under the supervision of Drs. Gary and Janet Stein.