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DIFFERENTIAL REGULATION OF

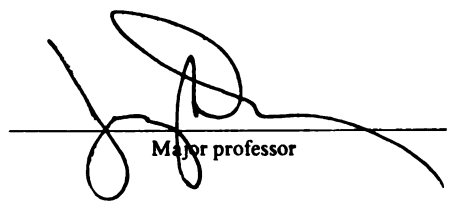
CHICKEN HISTONE H1 GENES

presented by

HUEI-MIN LIN

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology



Major professor

Date 12/18/96

DIFFERENTIAL REGULATION OF CHICKEN H1 HISTONE GENES

By

Huei-Min Lin

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1996

ABSTRACT

THE DIFFERENTIAL REGULATION OF CHICKEN

H1 HISTONE GENES

By

Huei-Min Lin

The chicken contains six closely related histone H1 genes which express distinct H1 proteins at different levels in various chicken tissues and cell lines. The four conserved promoter elements are very similar in sequence and location in all chicken H1 genes, which led us to investigate how the differential expression is regulated.

Transient transfections of transcriptional fusions indicate that approximately 200 base pairs of each promoter is sufficient to drive the observed spectrum of H1 promoter activity. The differential activity of chicken H1 promoters is mainly regulated by the Sp1 binding site, G box, and a newly identified element found between CAAT and TATA that we have termed Dus (differential upstream sequence). Gel mobility shift assays suggest that the primary nuclear binding protein to the G box is one or more avian homologs of the Sp1 transcription factor. The Dus region binds multiple nuclear proteins, one of which is the recently described IBR/IBF factor. The differential affinities of the G1 box and Dus sequences of the H1

promoters for their respective nuclear binding factors correlate well with their relative promoter activities.

Coding region of some histone genes have been reported to control gene expression either at transcriptional level or translational level. Chicken histone H1 transcriptional and translational fusions were compared in their reporter gene activities and steady-state mRNA levels. The protein codon 1-30 region of H1 genes significantly enhances the reporter gene expression and this is partially resulted from increasing the mRNA level. Further analysis suggests that the protein coding region may contribute to differential expression of H1 genes by controlling both the mRNA level and translational efficiency.

Overall, our study indicates the differential expression of chicken H1 genes are regulated at transcriptional and translational levels.

Dedication

To my parents, family and my two lovely children, Tiffany and Dustin.

Thanks for their supports and love.

ACKNOWLEDGMENTS

I want to thank my mentor, Dr. Jerry Dodgson for his support, patience, wisdom and guidance through the years. I also like to thank my committee, Dr. Don Salter, Dr. Richard Schwartz, Dr. Sue Conrad and Dr. Zachary Burton, for their generous advice. I would like to give special thanks to my lab mates Natalie Moore, Kyoung-Eun Kim, Yi Li, William Payne, Wynne Lewis, Ron Okimoto and Steve Suchyta for their friendship and companionship. Good friends in this old building are especially appreciated, for their generosity in sharing materials and equipments. Finally, the love, inspiration and lovely trouble from my children, Tiffany and Dustin, have once taught me to learn the value of life.

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CHAPTER 1

Literature Review

Functions of Histone H1

Eucaryotic chromosomes consist primarily of DNA, histones and other chromosomal proteins. The DNA is tightly bound to an equal mass of histone proteins, which serve to form a repeating unit called the nucleosome (1). The nucleosome particle contains about 200 bp of DNA and histones. Five types of histones are found in the nucleosome, and they are classified into two groups: the core histones and the linker histone, histone H1. The core histones, composed of two molecules of H2A, H2B, H3 and H4, are responsible for coiling about 165 bp of DNA into the nucleosomal core particle (1,2). Adjacent nucleosomal particles are connected by a 40-60 bp linker DNA to which histone H1 binds. Approximately, 0.7-1.0 molecule of histone H1 per core particle binds at the entry and exit sites of the linker DNA (3,4).

Various structural roles have been ascribed to histone H1, including formation of nucleosomal particles, determination of average nucleosomal spacing and facilitation and maintenance of higher-order chromatin structures (5-8). Histone H1 is thought to participate in chromatin organization through electrostatic interaction between positively charged lysine residues and negative phosphate groups of linker DNA (4). Up to now, most of our understanding of the role of histone H1 was based on *in*

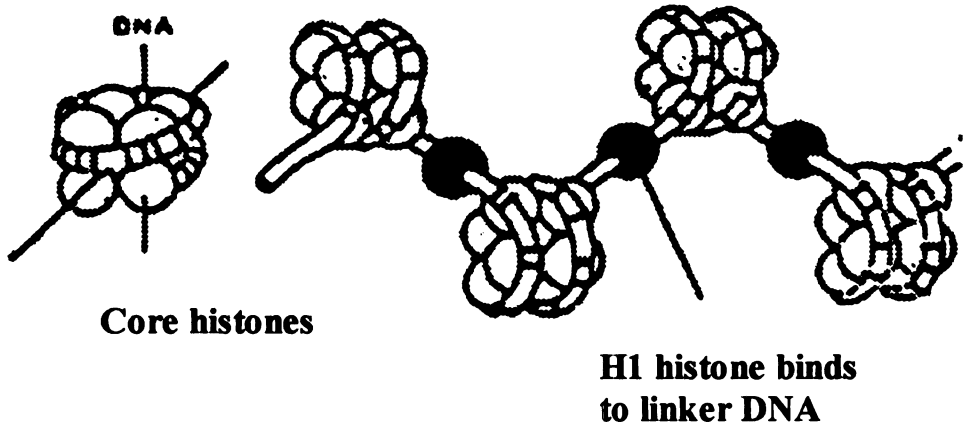

FIG. 1: Nucleosome Structure.
(Olins, D., T. Koller, and A. Klug. 1979. J. Cell Biol.83:403.)

vitro studies.

Recently, histone H1 gene disruption in Tetrahymena has demonstrated that the linker histones are not essential for cell growth in this organism (9). However, cells lacking the H1 gene showed enlarged nuclei, suggesting that linker histones are involved in chromatin packing and condensation *in vivo*. Meanwhile, a low level of expression of a sea urchin histone H1 gene in yeast, which normally lacks linker histones, resulted in no obvious phenotype, while overexpression resulted in dramatic condensation of nuclei, inhibition of growth and low cell viability (10,11). These data provide evidence that histone H1 proteins have a direct role in organizing chromatin structure.

Histone H1 not only serves architectural roles in chromatin structure but also is functionally important in gene expression. Numerous studies have suggested that the H1 histones act as general repressors of DNA-dependent reactions, such as transcription, replication, recombination and repair. Transcription of active genes has repeatedly been found in association with chromatin fractions that are depleted of H1 molecules (12-14). Transcription initiation by RNA polymerase II and III has been shown to be greatly reduced when histone H1 molecules are added to a nucleosomal or DNA template *in vitro* (14-16). In addition , initiation and elongation of *in vitro* transcription by T7 RNA

polymerase was also greatly inhibited by histone H1 (17). The inhibition of elongation has been shown to result from an increase in premature termination. It is generally proposed that the H1-mediated repression results from blocking the binding of basal and regulatory transcription factors to their target sequences on DNA. However, the repression of transcription factor binding by histone H1 has been shown to be differential (18). For example, binding of histone H1 to nucleosomes *in vitro* significantly repressed the binding of USF, but only slightly inhibited GAL4-AH binding. When activation of a specific gene occurs, this H1-mediated repression is thought to counteracted by specific transcription factors (19-21). It is suggested that sequence-specific transcription factors disrupt the interactions of histone H1 and DNA and facilitate the access of the basal transcription factors to the DNA template. In contrast to the accumulated data on the influence of histone H1 on transcription, very limited studies have focused on the effect of H1 on the replication of chromosomal DNA. Recently, the influence of histone H1 on DNA replication was examined using an SV40 minichromosome reconstituted with H1 as a template (22).

In contrast to the inhibition of transcription, H1 molecules, when present at up to one molecule per nucleosome, did not affect DNA replication *in vitro*.

However, ratios higher than one decreased the replication of the reconstituted SV40 template. It has also been suggested that cell cycle-dependent phosphorylation of H1 modulates the status of chromatin condensation (23). Histone H1 is one of the substrates of the cdc-kinase family and is differentially phosphorylated during the cell cycle. Phosphorylation of histone H1 is low in the G1 phase and increases continuously as cells progress to S phase (24). During mitosis, phosphorylation of H1 reaches its highest level at metaphase and decreases thereafter (25). When salt-treated SV40 minichromosomes were reconstituted with H1 proteins isolated from G0-, S-, and M-phase monkey CV-1 cells, the S-phase histone H1 induced a more open chromatin structure than G0- or M-phase histone H1 (26). Meanwhile, the replication of the reconstituted template with S-phase histone H1 was more efficient than those reconstituted with G0 and M phase histone H1. These data suggest that phosphorylation prior to the onset of DNA replication might create a chromatin structure which is favorable for DNA replication to proceed. This result agrees with a previous study that showed temperature-sensitive mutants, defective in H1 phosphorylation, exhibited incomplete DNA replication and a defect in chromosome condensation (27).

It has also been suggested that histone H1 molecules bind to DNA in a sequence-specific manner. H1 proteins bind

more specifically to eucaryotic DNA than to prokaryotic DNA (review, 28). The general preference for binding sequences includes A/T rich regions (29), the 5' end of genes, the first half of protein-coding regions (30) and scaffold attachment sites (31). Whether the histone H1 molecule possesses certain specific structural features that make a specific interaction possible is not yet clearly defined. However, it has been shown that the globular domain of the H1 molecule displays some similarity with the nucleotide-binding domain of adenine nucleotide binding proteins (32). In addition, the crystal structure of the chicken H1 histone globular domain has similarities to the homeodomain of *Drosophila* Antp protein (33) and that of the histone H5 globular domain shows similarity to the DNA-binding domain of the *E. coli* CAP protein (34), thus suggesting that the histone H1 molecule could potentially interact with DNA in a sequence-specific manner. In fact, H1 molecules have been demonstrated to bind specifically to a CTF/NF-I recognition sequence 5′-TTGGCAnnnTGCCAA-3′ in the mouse α2(I) collagen promoter (35) and to albumin (30) and globin promoters (36). These observations further support the possibility that histone H1 may act as a sequence-specific DNA binding protein to regulate the expression of individual genes, in addition to acting as a general repressor by assembly of chromatin into higher order structures.

H1 variants and differential expression

Separation of histone proteins by size and charge on polyacrylamide gels has revealed the presence of multiple histone variants or subtypes in most organisms (37,42,44) . These variants may differ either in only a few amino acid residues or more considerably, both in sequence and length. The synthesis of a specific variant occurs in either all tissues or only in specific cells. Some variants appear throughout the whole developmental process while others are synthesized at specific stages of differentiation (37-43). The structural roles of histone H1 in chromatin are well examined but the functional roles of individual variants remain to be elucidated.

Histone H1 is the most diverse histone gene subfamily, Most veterbrates contain 5-7 histone H1 variants, which are products of independent genes (37,39,42,43). They can be grouped into 4 classes according to their time of expression and cell-type specificity (see review 44). 1. Embryonic histone H1, described only in amphibians, is expressed specifically during early embryogenesis and during oogenesis. 2. Somatic histone H1 variants are commonly found in most or all somatic cells and named according to their mobilities on gels as H1a, H1b, H1c etc. 3. Testis-specific histone, H1t, is a tissue-specific histone H1 and only found in male germ cells. 4. The differentiation-associated H1

histones, $H1^{0}$ and H5, are expressed primarily in cells as they become terminally differentiated. Histone $H1^{0}$ is found in all vertebrates examined. The accumulation of $H1^{0}$ was shown to be absent in dividing cells and to increase along with a decreased rate of cell proliferation (38,45). During the maturation of avian erythrocytes, the condensation of chromatin is closely associated with the increased expression of tissue-specific histone H5 (46). In mammals, the H5 gene is no longer present, and the $H1^{0}$ represents the only differentiation-specific histone H1. It is suggested that histone $H1^{0}$ may play a role, similar to that proposed for histone H5 in avian red blood cells, in silencing gene transcription in terminally differentiated cells.

Changes of the relative levels of H1 variants have also been observed in normal as well as in neoplastic cells (38,39,42). For example, alteration in the distribution of histone H1 variants has been demonstrated during the differentiation of murine erythroleukaemic (MEL) cells which are virus-transformed erythroid precursor cells (42). When dimethyl sulphoxide was added to induce the MEL cells to differentiate, the relative amount of each variant changed with a strong increase in histone $H1^{\circ}$ and H1c, and a decrease in H1a, H1b and H1d. Overall, that the expression of H1 variants is sometimes tissue-specifically or developmentally regulated suggests a possible functional

role for them in modulating chromatin structure and, perhaps, gene expression.

It is thought that the differential distribution of H1 variants may be involved in generating chromatin structures of differential stability, which could thereby play a role in regulating the accessibility of genes to transcription factors. This idea is supported by the finding that histone H1 variants possess different abilities to bind DNA templates and to condense chromatin fragments *in vitro* (47,48). Meanwhile, analysis of mammalian H1 proteins indicate a nonrandom distribution of variants between active and inactive chromatin (49,50).

To study the functional role of H1 variants in living cells, ectopic expression of H1 has been examined in different systems. The expression of an inducible transfected H5 gene in rat sarcoma cells arrested cell proliferation and selectively suppressed transcription of cellular genes (46,51). The H5 protein accumulated in nuclei and partially replaced H1 proteins in chromatin. However, the nucleosome repeat length of chromatin was not altered even when the replacement of H1 by H5 achieved levels similar to those in mature chicken erythrocytes (51). This suggests that H5 alone is not sufficient to determine the nucleosome spacing in maturing erythroid cells. A retroviral vector expressing the H5 gene in primary and transformed

avian cells produced very different results (52). The levels of the expressed H5 protein were comparable to those observed in normal chicken erythrocytes. Overexpression of the H5 histone in transformed chicken embryo fibroblast (CEF) and quail QT6 cells had only slight effects on growth rate and DNA replication. However, expression of the H5 gene in primary CEF and quail cells severely affected cell growth and condensed the nuclei to more compact structures. Further analysis showed the expressed H5 protein was phosphorylated in the transformed cells but not in the primary cells. This suggests that phosphorylation may inhibit the chromatin condensation capability of H5 histone and lead to normal cell growth. This finding is consistent with a previous observation which suggested histone H5 dephosphorylation is a crucial factor for gene inactivation and chromatin condensation during the maturation of erythroid cells (53).

Saccharomyces cerevisiae, which is believed to lack an endogenous H1 histone, provides a useful tool to study the functional roles of H1. Low level expression of a sea urchin early H1 histone in yeast was tolerated, while overexpression (in amounts similar to those of core histones) affected cell growth, transcription and plasmid stability but did not change nucleosomal repeat length (10). Subsequently, overexpression of two mouse H1 variants was carried out in mouse 3T3 cells (54). Overexpression of the

H1c variant had no visible effect on the cell cycle and cell growth rates but increased the expression of some genes. However, cells that expressed fairly high levels of the $H1^{0}$ histone exhibited transient inhibitions of G1 and S phase progression and a significant reduction in the transcription of many genes, including c-fos, c-myc, cdc2 and cyclin D2. Also, when the $H1^{0}$ gene was disrupted in transgenic mice, no significant changes in development were observed in mice harboring a homozygous null mutation in the $H1^{0}$ gene (55). These studies strongly suggest that functional differences exist among H1 variants. However, how the differential expression of H1 variants is regulated in cells is still poorly understood. Cloning of H1 gene family members and identification of generic and specific regulatory elements may facilitate the understanding of the differential regulation of H1 histone genes.

Gene organization

The arrangement of histone genes in the lower eucaryotes tends to be more organized while that in higher eucaryotes is less ordered (review, 56). It has been suggested that the evolutionary transition of histone gene organization from an ordered to a dispersed arrangement is an indication of the declining frequency of recombinational events as the gene copy number becomes low.

There are several histone gene families within the sea urchin genome which are expressed at different stages of embryogenesis or in adult tissues (56). For example, the early histone gene family is composed of 300-500 tandem repeats. Each repeat contains one copy of each of the five histone classes, with a non-transcribed spacer sequence between each gene. In contrast, yeast has eight genes per haploid genome encoding the four core histone proteins. The eight genes are arranged into four divergently transcribed gene pairs which consist of either one H2A and one H2B gene, or one H3 and one H4 gene. The four loci are not linked on physical the map (57,58).

Histone genes in birds and mammals are fewer in number and are not organized in tandem repeats. In chicken, 43 core histone and H1 genes are located within four non-overlapping regions that span about 180 Kb in the chicken genome (59,60,61). 40 of these histone genes are located in 2 major clusters and the other 3 genes are in two other clusters. We and others have isolated and sequenced many of the chicken histone genes. It is surprising that none of them shows characteristics of a pseudogene although some histone pseudogenes have been found in mammals and *Xenopus* (56). This suggests that all these histone genes code for functional proteins in one or more cell types. Six chicken H1 genes are dispersed in the two major gene clusters

(61,63). The erythrocyte-specific H5 is a solitary gene without any nearby H1 or core histone genes (62). The histone gene distribution in mammals is even more complicated due to higher numbers of H1 histone and core histone genes. So far, cloning of mouse (64) and human histone genes (65) shows that they are clustered but are not organized in a regular arrangement. Recently, the cloning of six human H1 genes, including the testis specific variant H1t, has allowed them all to be mapped to the short arm of chromosome 6 (66). However, the $H1^0$ gene is located on chromosome 22 and is not associated with any core histone genes.

Histone H1 protein structure

The histone H1 family is a set of lysine-rich and small basic proteins. The binding of histone H1 to DNA is associated with the formation of nucleosomes and facilitation of the 10 nm nucleosome-containing chromatin fiber into higher order structures. Approximately one molecule of H1 binds per core nucleosome (30). While core histones are generally well conserved among species, histone H1 molecules are more diverse both in DNA and protein sequences. Despite this divergence, linker histones have maintained a tripartite structure, a central globular domain region, flanked by a short N-terminal tail and a long C-

terminal basic tail. The globular domain is the most highly conserved portion of the H1 protein among species. It has been suggested that the globular domain is responsible for sealing the region of entry and exit of DNA from the core particle(67). The NH_2-terminal tail is not required for the formation of the nucleosome core but is involved in positioning the globular domain of histone H1 in nucleosomes. The positively-charged C-terminal tail is thought to interact with linker DNA through charge neutralization which thus could permit further condensation of the chromatin fiber.

The $H1^0$ and H5 histone proteins are very similar in amino acid sequence. However, they differ from somatic H1 proteins in several aspects. The H1/H5 proteins are generally shorter (about 190 amino acids) than somatic H1 molecules (about 215 amino acids). H5 is enriched in arginine residues, and most of these sites are occupied by lysine residues in $H1^0$ and somatic H1 proteins (68). The increased basic nature of the H5 protein may be associated with its role in the repression of transcription and condensation of DNA in erythrocyte nuclei. Moreover, the H5 C-terminal domain, which is highly conserved in most H1 molecules, shows only partial homology with somatic H1 histones.

Histone H1 gene structure

There are two types of histone genes found in most eukaryotes. The most common type is the replication-dependent histone gene, whose expression is regulated during the cell cycle, while the second type, occurring less frequently, is the replication-independent or replacement histone gene, whose expression is maintained at a basal level through the cell cycle (69). The role of these replication-independent histones may be to allow production of histone proteins in the absence of DNA synthesis, thereby allowing the modification of chromatin structure to support the expression of certain tissue-specific phenotypes post-proliferatively.

So far, most histone H1 genes identified are replication-dependent. Generally, replication-dependent histone H1 genes display typical characteristics of eucaryotic histone genes, including lack of introns and poly-A addition signals at the 3' end (70). The non-polyadenylated mRNA contains a short leader sequence and a characteristic 3′ stem-loop motif which participates in RNA processing (71,72).

Generally, the mRNAs of replication-independent histone genes are polyadenylated and the typical stem-loop structure involved in RNA processing is not present at the 3' end. The gene structure and expression of the H5 gene are typical of

replication-independent histones. However, the polyadenylation of H5 mRNA is not mediated by the conventional AAUAAA sequence; instead, two palindrome sequence elements found at the 3' end of H5 mRNA may be involved in poly-A addition (80). $H1^0$ mRNA is polyadenylated, as mediated by a AAUAAA signal present at its 3' end, but its expression is not strictly replication-independent. Studies of $H1^0$ expression in synchronized MEL cells showed that $H1^0$ mRNA accumulated during S phase and the inducibility of $H1^0$ was optimal during S phase (76). In contrast to animal H1 histone genes, the first few histone H1 genes isolated from higher plants all contain introns and poly-A signals at their 3' ends (77,78). Based on the limited data, it seems that plants have retained their introns and poly-A signals whereas animals have generally lost them from their histone H1 genes during evolution.

The promoters of replication-dependent histone H1 genes generally contain 4 regulatory elements: a H1 gene-specific element (H1 box), GC box, CAAT box, and TATA box (Figure 2) (70,79). The four elements have been shown to be highly conserved in somatic histone H1 genes among all vertebrates; therefore it is likely that each element plays a similar regulatory role in all histone H1 genes. Histone H5, a replication-independent H1 variant, displays quite different characteristics in gene structure from other histone H1

genes (Figure 2). The H5 gene contains a poorly conserved TATA box (CTTAAAT), a GC box and an UPE site which is similar to the histone H4-specific site II elements. The H1 box found in all H1 genes studied to date is absent in the H5 gene (80). A UCN element involved in the down-regulation of the H5 gene has been identified upstream of the UPE site(81,82).

Expression of the $H1^0$ gene has been found to be associated with cell differentiation. Several cis-acting elements have been identified as involved in the transcriptional regulation of the $H1^0$ gene (83-85). Comparison of the $H1^0$ promoters and other replication-dependent H1 promoters has showed the conservation of the TATA box, G box and H1 box in analogous locations (Figure 2). However, the CAAT box is absent in $H1^0$ and its site is replaced by a histone H4-specific site II element. A $H1^0$-specific element described as the UCE (upstream conserved element) is found at -435 (85), and it is highly conserved in sequence between mouse(87), human (68) and Xenopus (84). Studies of $H1^0$ in mouse and Xenopus have revealed that the H1 box, H4-specific element and UCE are required for transcriptional regulation of the $H1^0$ gene (84,87). Upstream of the UCE, a retinoic acid response element (RARE), which is composed of a direct repeat of a GGTGACC sequence separated by 7 bp, is found in the mouse $H1^0$ promoter (86).

This DNA motif has been shown to bind retinoic acid receptors in response to treatment with retinoic acid (88).

Regulation of histone H1 gene expression

Histone genes are the primary example of a gene family which regulates its expression coordinately with the cell cycle (review, 79). Multiple levels of control are involved in confining the synthesis of histone mRNA and protein to the S-phase of the cell cycle, including those of transcription (89,90,93,94), RNA processing (92), mRNA stability (91,93) and translation (95,96). Both temporal and maximal expressions of histone genes rely on specific interactions between cis-acting elements and trans-acting factors. In yeast, 8 core histone genes are regulated by the same set of trans-acting factors and the same set of regulatory elements that are present in each of the histone genes (79). In contrast, vertebrate histone genes are controlled by subtype-specific consensus elements that are not shared between different histone genes.

The four distinct sequence elements described previously have been found in the promoters of all vertebrate H1 histone genes examined to date. The importance of each element in H1 histone transcription regulation has been demonstrated by analysis either of deletion mutants or site-specific mutations (97,99,102). Progressive deletion of

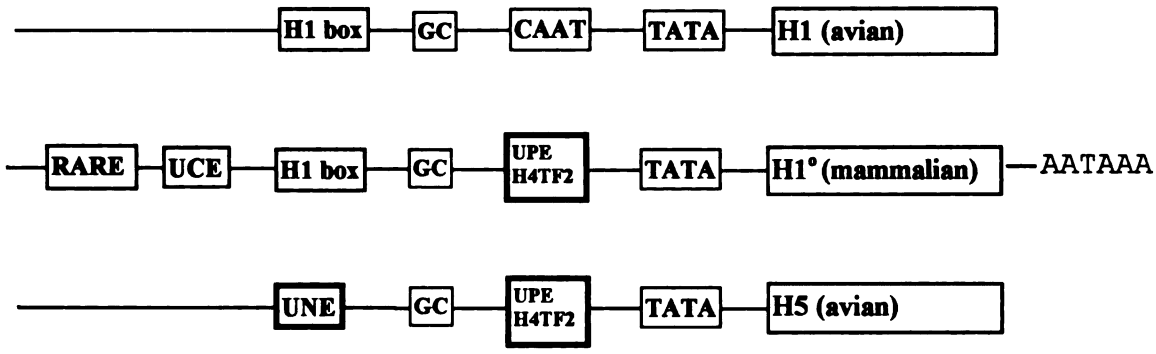

FIG. 2: Organization of H1 gene promoters. The conserved elements TATA, CAAT, GC box and H1 box are indicated. The H1 box is present in all vertebrate H1 genes but is absent in the avian H5 gene (80). A negative regulatory element (UNE) is found in the H5 promoter (82). A UCE (upstream conserved element) is found in the mammalian H1° promoter(85). RARE is a retinoic acid receptor binding element(86). The UPE element harbors a sequence similar to that of the H4-specific element which is bound by H4TF2 transcription factor (84). This element is also present in the same location in the H5 gene with a similar sequence.

H1 promoters results in incremental loss in transcriptional activity, suggesting a positive and additive role is played by each element in H1 gene expression (102).

Attention has been focused primarily on the roles of the H1 box and CAAT box for their contribution to the regulation of S phase-dependent transcription of histone H1 genes. The H1 box, a H1-specific element with a consensus sequence of 5' AAACACA 3', was shown to be required for optimal expression of histone H1 in S-phase (97). Deletion of, or four base substitutions in the element caused a 15-30 fold decrease in H1 mRNA and abolished cell cycle control of transcription in stably transfected HeLa cells (97). In transient assays, transfection of multiple copies of H1-elements into cells significantly decreases the level of H1 mRNA. In contrast, Younghusband and others showed that the deletion of the H1 box in a chicken H1 promoter shows no decrease in transcriptional activity both in microinjected Xenopus oocytes and transiently transfected HeLa cells, arguing against the significance of the H1 box on H1 gene expression (98,99). This discrepancy may arise from the differential dosage effect of the transfected H1 gene in stable and transient cells. The significance of the H1 box on H1 transcriptional regulation was further demonstrated by the observation that a sequence-specific DNA-binding protein

(H1-SF1 in chicken and H1TF1 in mammals) interacts with this element (97,101). In chicken, the level of H1-SF1 binding activity increases 12-fold from G1 to S-phase in synchronized cells and decreases in G2 phase during the cell cycle (100). Overall, these results imply that the interaction between the H1 box and a trans-acting factor modulates transcriptional control of chicken H1 genes.

In studies of human H1 gene regulation, two distinct binding factors have been identified in nuclear extracts from HeLa cells. H1TF1 was found to interact with the H1 box and is required for maximal expression of H1 genes (101). However, no increase in the binding activity of H1TF1 to the H1 box was observed during the cell cycle (102). H1TF2, interacting with the CAAT box, has been shown to be a heterodimer composed of two polypeptides, 33Kd and 43Kd in size, which are not antigenically related (103). This factor is different from H1TF1 and other CAAT box-binding proteins in molecular weight and binding activity. The binding activity of H1TF2 to the CAAT element increased significantly in S-phase nuclear extracts prepared from synchronized cells (102,103). Meanwhile, partially purified H1TF2 was shown to activate histone H1 transcription *in vitro* specifically through the CAAT element. These data suggest that two H1-specific elements and two H1-specific

transcription factors are required for S-phase regulation of human H1 histone genes.

The expression of the H5 histone gene is mainly controlled at the transcriptional level (81). The gene is not transcribed until the preerythroblast-to-erythroblast transition, and this is accompanied by a decreased potential for cellular proliferation (104). The high activity of the H5 gene is maintained until the cells approach maturation. Like other erythrocyte-specific proteins, the expression of H5 histone declines during the latter stages of maturation because of the decreased rate of transcription, and little transcriptional activity remains in mature erythrocytes. The accumulation of histone H5 in erythrocytes seems to meet the requirement of inactivation of nuclei upon the maturation of these cells (46). The transcription of the H5 gene is regulated by several ubiquitous and blood cell-specific factors interacting with enhancers located at the 5' and 3' ends of the gene (81,82). Cooperation among these enhancers is responsible for the increased transcription of the H5 gene in cells which are induced to differentiate. The binding of the GATA-1 factor is required for the function of the 3' enhancer (82). However, the cellular concentration of GATA-1 decreases during cell differentiation, and overexpression of this factor had little effect on H5 transcription (82). Therefore, this suggests the regulation

of the enhancers during differentiation is not dependent on changes in the cellular concentration of GATA-1.

Initiation binding factor (IBR) was isolated from immature and mature adult erythrocytes but was not found in early erythroid cells and HD3 cells which actively transcribe the H5 gene (105). The appearance of IBR seems to parallel the decrease in H5 gene activity. Indeed, it was found that IBR repressed H5 gene transcription *in vitro* (106). IBR, a glycosylated protein, binds to the GC-rich sequences around the transcription start site of the H5 gene. Evidence showed that IBR repressed H5 transcription by interfering with the binding of general transcription factors other than TFIID, possibly TFIIB (106). The unglycosylated form of IBR protein isolated from early erythroid cells and HD3 cells was named IBF which recognizes the same sequences as IBR (106). The glycosylation modification of IBR/IBF seems not to be required for its DNA binding activity or its repressive effect on H5 gene transcription *in vitro*. However, one can't exclude the possibility that the steric effect of the sugar groups or other post-translational modifications could affect the interactions of IBR/IBF with the H5 promoter *in vivo*.

Proposal

In chicken, six different histone H1 variants (63,70) have been cloned and sequenced besides the erythroid specific variant, H5 histone. They express six highly conserved but distinct proteins with sizes ranging from 217 to 224 amino acids (Fig. 3). These proteins are highly homologous to mammalian somatic H1 subtypes and are distinct from H5. The distribution of the chicken H1 variants has also been shown to vary from tissue to tissue (39,107,108) and during differentiation of certain cell types (40,110). Shannon and Wells, using a modified gel system, separated six H1 proteins from chicken erythrocytes and named them according to their mobility on acid/urea polyacrylamide gels as H1-a, a', b, c, c' and d, meanwhile, they also identified the corresponding H1 genes which express each individual variant (109). Quantitative analysis of the expression levels of H1 variants demonstrated that histone H5 made up 60-65% of the total lysine-rich histones (H1 plus H5) in erythrocytes. H1-a/a' together comprised 36-38% of the total H1 in erythrocytes, however H1-a' only made up a very small portion of the H1-a group. H1-b represented 20% of the total H1, H1-c/c' 23% and H1-d 18-19%.

How the balance of H1 variant expression is maintained and modulated in cells is not yet understood. Such an understanding of the transcriptional regulation of the H1 histone family at the molecular level would provide insight

into the control mechanisms operating on the differential expression of a set of closely related genes. This thesis describes research designed to help elucidate the regulation of the differential expression of chicken H1 histone genes. The analysis mainly focuses on the transcriptional regulation of histone H1 genes in avian cells. Sequence comparison has shown that the four conserved regulatory elements are very similar in sequence and position in the six chicken H1 promoters (Fig. 4). We hypothesize that new cis-acting elements (and possibly trans-acting factors) besides these four conserved elements and their binding factors are necessary for the differential expression of chicken H1 genes.

FIG. 3: Comparison of protein coding sequences of chicken H1 genes. Derived amino acids are presented in one-letter code. Protein coding sequences are compared to the H1-c' sequence. Where protein sequences differ from the H1-c' sequence, an asterisk is shown. Gaps(-) are inserted into sequences for alignment. Boundaries of domains are separated by spaces. Total amino acid numbers of each H1 protein are indicated at the end of the sequence. (109)

	H1-Box	G Box	CAAT	TATA
	-118	-74	-55	-32
H1-c'	AAGAAACACAAA...32....GCGGGGCGGGCT....7....GCACCAATCA...15....CTAAAAATA			
	-120			
H1-d	AAGAAACACAAA...32....GCGGGGCGGGCT....7....GCACCAATCA...15....CTATAAATA			
	-118			
H1-c	AAGAAACACAGA...32....GTTAGGCGGGCT....7....GCACCAATCA...15....CTATAAATT			
	-105			
H1-a	AAGAAACACAAG...27....GCGGGGAGGGCT....7....GCACCAATCA...15....CTATAAATA			
	-113			
H1-a'	AAGAAACACAAG...31....GCGGGGCGGGGC....5....GCACCAATCA...14....CTATAAATG			
	-89			
H1-b	AAGAAACACAAC...17....GCGGGGCGA----------ACCAATCA...15....CTATAAAGG			

FIG. 4: Conserved promoter elements of six chicken H1 genes. The numbers on the top of each conserved element indicate the position relative to the transcription start site of each gene. Distances between conserved elements are in base pairs. Dashed lines are introduced for alignment.(109)

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CHAPTER 2

Elements regulating the differential activity of chicken histone H1 gene promoters

ABSTRACT

The chicken genome contains 6 H1 histone genes, each of which encodes a different H1 protein sequence. Sequence comparisons and functional studies have identified 4 common elements in H1 histone promoters, which are very similar in sequence and location in all 6 chicken H1 genes. Here we study the mechanisms by which the 6 H1 genes are expressed at significantly different levels. Transient transfections of reporter gene fusions in QT6 quail cells indicate that approximately 200 base pairs of each promoter is sufficient to generate the characteristic spectrum of H1 promoter activity. Our study shows that the difference between one of the most active and the least active H1 promoters can almost totally be explained by relative activity of the previously characterized G box region, a putative Sp1 binding site, and that of a region between CAAT and TATA that we have termed Dus (differential upstream sequence). Gel shift analysis suggests that the primary nuclear binding protein to the G box is one or more avian homologues of the Sp1 transcription factor. The recently described IBR/IBF factor is also identified as one of the nuclear proteins which bind to the Dus region. The differential affinities of the G box and Dus sequences of the H1 promoters for the nuclear binding factors correlate well with their relative promoter activities in reporter gene fusions.

INTRODUCTION

H1 histones constitute a family of lysine-rich chromosomal proteins that participate in the packaging of eucaryotic DNA into compact structures (4). H1 proteins bind to the entry and exit sites of the linker DNA which connects the adjacent nucleosomal particles (2,3). Histone H1 is believed to facilitate folding of the nucleosome chains into 10 nm chromatin fibers and high order structures (9).

Histone H1 genes comprise the most diverse of the histone gene subfamilies. Most vertebrates express at least 5-7 non-allelic histone H1 variants (35,36). In different species, the synthesis of histone H1 variants has been shown to be tissue-specific or developmentally regulated (14-16, 28). For example, the accumulation of histone H1° in non-proliferative and terminally differentiated cells, but not in dividing cells, has suggested that H1° may be involved in transcriptional silencing (37). During the maturation of avian erythrocytes, the condensation of chromatin is closely associated with the increased expression of the tissue-specific histone H5 (43,44). Furthermore, changes in the relative levels of H1 subtypes are observed in normal as well as in neoplastic cells (14,25,38). In addition, expression of different H1 variants in vivo results in differential effects on the cell cycle and gene expression

(7,33) further suggesting a functional significance for each H1 variant.

It is well established that histone gene expression is regulated by both transcriptional (21,41) and post-transcriptional mechanisms (22,46). To date, studies of H1 gene promoters have focused on generic sequence motifs present in all replication variant H1 genes (11,24,29). Four such motifs are generally observed: a histone H1 gene-specific element (H1 box), a putative Sp1 binding site (G box), a CAAT box, and a TATA box. These four elements are highly conserved among the replication-dependent H1 histone of all vertebrates. Sequence-specific DNA-binding factors have been identified that bind the H1 box and CAAT box (12,18). The four conserved elements and their interaction with their trans-acting factors have been shown to be required for maximal and cell cycle regulated expression of H1 histone gene (12,31,32).

Chickens have one of the smallest and most thoroughly studied histone gene complements. All six chicken replication histone H1 variants have been cloned and sequenced (8,13,42) in addition to the gene encoding the erythroid specific variant, H5 histone (39). These genes express six highly conserved but distinct proteins (40) at different levels in various chicken tissues and cell lines (5,35,46). All six chicken H1 histone gene promoters

contain the four conserved regulatory elements described previously . The sequences and relative locations of all four regulatory motifs are very similar among the six H1 promoters, whereas the rest of these promoter sequences are highly divergent. We have examined the sources of the differential expression of members of the H1 gene subfamily, and our results indicate that two major components contribute to differences in chicken H1 promoter activity. First, transcription levels are extremely dependent on the sequences in and around the G box, and this correlates with affinity for the presumed avian homologue of the Sp1 transcription factor. Second, a novel cis-acting element, Dus, has been identified which differs substantially in sequence and binding affinity among different H1 variant promoters.

MATERIALS AND METHODS

H1-*lacZ* reporter constructs. Transcriptional fusions were constructed according to the strategy shown in Figure 1. The promoter region of each histone H1 variant was cloned by PCR from the corresponding genomic subclone (8,42). Specific primers annealing to the 3′ end of each of the H1 variant promoters were designed which in each case converted the sequence containing the translation initiation ATG into a *Hind*III site. PCR was performed using the specific 3′ primers in combination with the 5′ T3 plasmid vector primer to amplify fragments containing the promoter sequences of each H1 variant. Primers used are shown in Table 1. The amplified fragments were digested with *Hind*III, isolated and then subcloned upstream of a *lacZ* reporter gene at the *Hind*III site of the p*lacZ* plasmid to give H1-a-p, H1-a′-p, H1-b-p, H1-c-p, H1-c′-p and H1-d-p constructs. Individual chicken H1 histone genes and their corresponding proteins are identified according to the nomenclature of Shannon and Wells (40). The p*lacZ* plasmid was constructed by subcloning the *lacZ* gene excised at the *Hind*III and *Bam*HI sites from pCH110 (Pharmacia) into the pBluscriptII vector (Stratagene) . H1-c-p2, harboring 1.3 kilobase pairs (kb) of the H1-c-p promoter sequence, was produced by cloning a 1kb *Hind*III fragment from its corresponding genomic clone (8)

Fig 1. Strategy used in H1 promoter-LacZ reporter construction. Specific H1 primers used in PCR reactions are listed in Table 1. The H1 promoter regions are indicated as filled boxes and protein coding region are indicated as open boxes. The thin line represents the sequences of pBS vector. Restriction sites and vectors used are indicated.

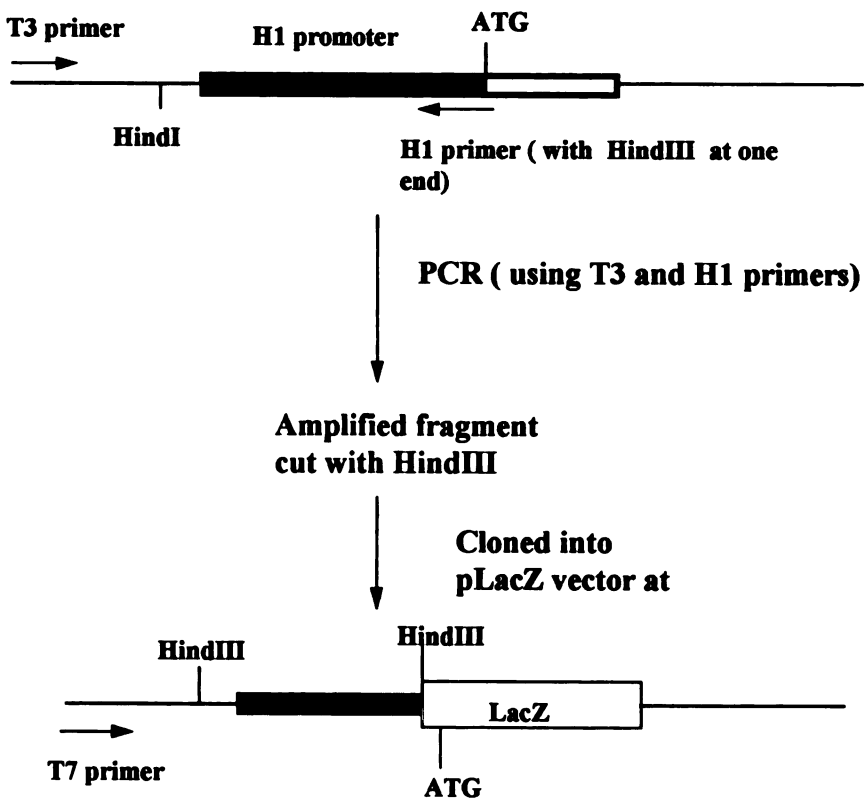

T3 primer
H1 promoter
ATG
HindI
H1 primer (with HindIII at one end)
PCR (using T3 and H1 primers)
Amplified fragment cut with HindIII
Cloned into pLacZ vector at
HindIII
HindIII
LacZ
T7 primer
ATG

Table 1: Oligomers used in PCR reaction and mobility shift analysis

Purpose and oligomer	sequence (5' to 3')	Amplified fragment [d]
A. H1 promoter-lacZ		
JD37	CTCAAGCTTCGCGGCGCAGT [a,b]	H1-c'-p (3')
JD38	CTCAAGCTTGGCCGCGCTG	H1-a'-p (3')
JD39	CTCAAGCTTGGTGACGGACG	H1-d-p (3')
JD40	CTCAAGCTTGGCGCCTCCT	H1-b-p (3')
JD41	CTCAAGCTTCGCGGCAGAGA	H1-c-p (3')
HM-H1-a5'	GGGCTGCAGATGACTCGGAATTAC	H1-a-p (5')
JD47	TCTCAAGCTTGTCGGAGCTGCGCG	H1-a-p (3')
B. Deletion mutants		
JD86	ATCGGGCACTCCTTTAAT	H1-d-2Ru (5')
JD85	TCTCTATGAGCCTGTGTTA	H1-d-2Rd (5')
JD91	GGAAAGCTTCGTGTTGGCGGAA	H1-d-210 (5')
JD166	GTGATTTCGAGCCCGGCATT	H1-c-210 (5')
C. Fusion constructs		
JD141	GCGGCGGGCGGGCTCTGCACC	Fus-1 (5')
JD143	TTTATAGAGCGGAGCCGC	Fus-3 (3')
JD144	CTCCGCTCTATAAATTCAGGC	Fus-3 (5')
D. Site-specific mutants		
JD150	CTGAATTTATAGAGCGGAGCCGCGCGGTGATTGGTGC	H1-c-D (3')
JD144	CTCCGCTCTATAAATTCAGGC	H1-c-D (5')
JD99	CCCGCCGTTAGCCAGGAAGAG	H1-c-G* (3')
JD101	TTCCTGGCTAACGGCGGGGCGGGCTCT	H1-c-G* (5')
JD127	AGGGAGCTCTGCGCCGTGCGGTTAGGCGGGCTCTG	H1-d-G2 (5')
JD163	CAGAGCCCGCCTAACCGCA	H1-d-G2 (3')
JD125	GCGCTGCAGAGCCGGATCCG	H1-d-G4 (3')
JD126	GGATCCGGCTCTGCAGCA	H1-d-G4 (5')
JD157	GCGCACCAATCACAGATCACCGCTTCGCTATAAA-TACGAGGCCGCCGACTTGCTCCGGGCC	H1-d-D3
E. Mobility shift primers		
H1-d-G	TGCGCCGTGCGGCGGGGCGGGCTTCTG [c]	
H1-c-G	CCTGGCTAACAGTTAGGCGGGCTCTG	
SP1	ATTCGATCGGGGCGGGGCGAGC	
PAL	GTAGACTGCGCATGCGCATCT	
H1-d-Dus	CAATCACCGCGCGGCTCCGCTCTATA	
H1-c-Dus	CAATCACAGATCACCGCTTCGCTAT	

a. Restriction sites created in the primer are underlined.
b. The positions of mutations in each primer are indicated as bold letters.
c. The sequence of the upper strand of gel-shift primers is listed.
d. Primer used to amplify the designated promoter fragment from the 5' or 3' end (with respect to H1 histone gene transcription) is shown.

and inserting it directly upstream of the 5′ *Hind*III site of the H1-c-p construct.

Deletion mutants. Promoter deletion mutants were generated by restriction enzyme digestion and, in some cases, by PCR techniques. All H1-d 5′ deletion clones were based on the H1-d-p construct. H1-d-SH was constructed by inserting the 0.14 kb *Hind*III-*Sac*I fragment from H1-d-p into the p*lacZ*120 vector. H1-d-105 was produced by digestion of H1-d-p with *Pst*I, followed by self-ligation. Three separate PCR primers (Table 1) which anneal to positions -650, -560 and -210 in the H1-d promoter were used to create H1-d-650, H1-d-560 and H1-d-210, respectively. The amplified fragments were cut with *Hind*III and inserted into the p*lacZ* vector. A primer with a *Hind*III site at its 5′ end was used to create H1-c-210 which contains sequences from -210 to +1 of the H1-c promoter. A similar strategy was used to produce the H1-c-105 construct containing sequences from -105 to +1.

Fusion constructs and site-specific mutants. Various portions of the 5′ promoter region of H1-d-210 were fused to the 3′ portion of the H1-c promoter using PCR techniques (26). The primer JD141 (Table 1) corresponding to the -121 to -97 sequence of H1-d-210 was used as the upstream primer with JD41 as the downstream primer to amplify a fragment from the H1-c-p template to produce the Fus-1 construct

which contains the G box region of the H1-d promoter fused to the remainder of the promoter in the H1-c-p construct. Fus-2 was generated by cloning the 1.5 kb *Pst*I-*Eco*RV fragment of the Fus-1 construct into the H1-d-210 construct at its *Pst*I and *Eco*RV sites. Fus-3 was created by amplifying the region upstream of the CAAT to TATA sequences of H1-d-210 with primers T7 and JD143, and amplifying the downstream region of H1-c-p using primers JD41 and JD144, separately. The amplified fragments were fused at the overlapping ends in a subsequent PCR reaction using just the outside primers, T7 and JD41.

Site-specific mutations in the G box and Dus regions of the H1-c and H1-d promoters were generated by the same double amplification strategy (26) as described for Fus3 above, in which the mutation was designed within the overlapping region of the central PCR primers. The mutagenic primers used to create individual mutants are listed in Table 1. All clones generated by PCR amplification were verified for correct amplification and ligation by manual sequencing with the dideoxy chain method termination method (45) or by automated DNA sequence analysis (ABI, Model 373A).

Cell culture and transfection. Quail fibroblast QT6 cells were cultured in Dulbecco's Modified Eagle's Medium at

37°C with additions of 5% fetal bovine serum, 1% chicken serum, 2% tryptose phosphate broth and 10 mg/ml gentamicin. LMH cells, a chemically-induced chicken liver cell line, and primary chicken embryonic fibroblast 15B1 cells (CEF) were grown in DMEM supplemented with 10% serum and 1% chicken serum.

For transient transfection, 6 μg of pH1-*lacZ* DNA and 1 μg of the luciferase containing plasmid pGL2 (Promega) as control were co-transfected into $1x10^6$ cells by the calcium phosphate precipitation method (34). After overnight incubation, the transfection solution was removed and cells were placed in fresh medium. Cells were washed with cold phosphate buffer 2 times and lysed with 0.5 ml of diluted reporter lysis buffer (Promega) 48 hr after transfection. Cell debris was removed by centrifugation at 14,000 rpm in a microfuge (Eppendorf) for 10 min at 4°C. Total protein was measured with the BCA protein assay reagent (Pierce) using bovine serum albumin as a standard, and β-galactosidase activity was quantified as previously described (34). Ten microliter aliquots of each cell lysate were assayed for luciferase activity according to the recommended protocol described by Promega. β-galactosidase activity was normalized to total protein and luciferase activity to correct for changes in transfection efficiency. Each assay reported represents the mean of 5 to 10 trials from

independent transfections using at least 2 different batches of column-purified plasmid DNA. Standard deviations generally fall between 10 to 15% of the mean value reported.

Nuclear extract preparation. Crude nuclear extracts were prepared as described (1) with slight modification. Briefly, cells were washed with cold phosphate buffer before resuspension in 5 volumes of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF). The cells were allowed to swell on ice and lysed by rapid extrusion through a 25G needle. Samples were centrifuged and the pelleted nuclei were extracted in cold buffer C (20 mM HEPES, pH7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5 mM DTT, 5μg/ml leupeptin). Nuclear extracts were aliquoted and stored at -70°C until use.

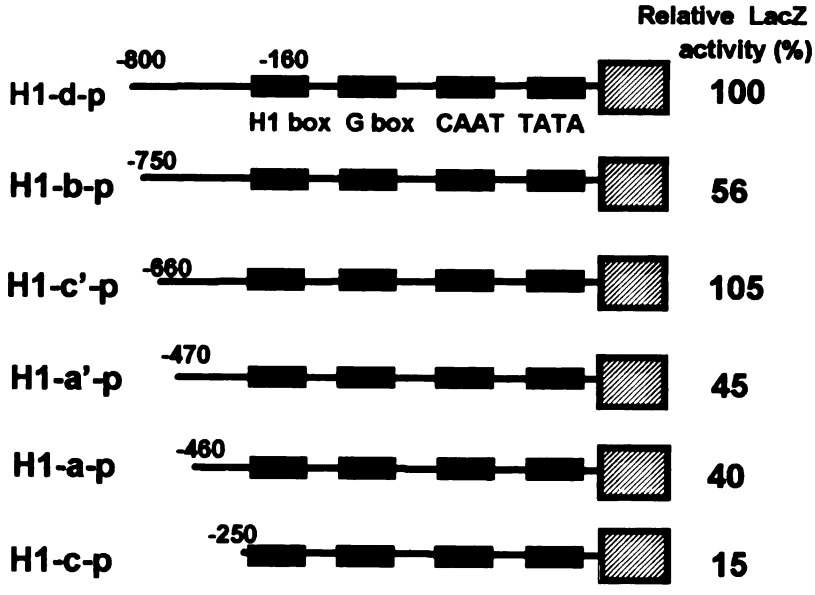
Mobility shift assays. Double-stranded oligonucleotides used for mobility shift assays were prepared by annealing two synthetic complementary oligomers in 0.5M NaCl, 0.5M Na_2HPO_4 at 90°C for 5 min, 65°C for 5 min and 4°C for 4 hr followed by purification on an HPLC ion-exchange column. The double-stranded oligomers were labeled by filling in the 5' overhang with Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. Binding reactions were carried out at room temperature in 10 mM HEPES pH 7.9, 50 mM

KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll, with 5-10 μg of nuclear proteins and 1μg of non-specific competitor, either poly(dI)poly(dC), poly(dI-dC), or poly(dA-dT), as noted in figure legends. About 0.2 ng of probe (3-5 x 10^4 cpm) was added followed by incubation for 20 min at room temperature. For competition assays, a 10-200 molar excess of the specific unlabeled competitor was added to the reaction 10 min prior to addition of the probe. Complexes were resolved on 6% non-denaturing polyacrylamide gels (acrylamide:bisacrylamide 19:1) electrophoresed in 0.5X TBE (45 mM Tris-HCl, 45 mM Boric Acid, 0.5 mM EDTA) at 150V for 2.5 hr at room temperature. The gel was dried and autoradiographed. In the case of supershift experiments, non-immune or anti-SP1 (SC-59X, Santa Cruz Biotech) antisera were incubated with purified human Sp1 protein (Promega) or nuclear proteins on ice for 30 min before adding to the binding reaction. The samples were separated on 5.5% polyacrylamide gels as described above. The rabbit anti-IBR antibody was generously provided by Dr. A. Ruiz-Carrillo (18). The IgG fraction was purified by protein A-Sepharose chromatography and dialysed against PBS buffer.

RESULTS

Differential activity of histone H1 gene promoters in QT6 cells. In order to examine whether the differential expression of H1 histone variants is correlated with the transcriptional strength of their respective promoters, reporter gene fusions were constructed between each of the 6 chicken H1 promoters and the β-galactosidase gene. The promoter regions were fused at their respective ATG start codons to the same site upstream of *lacZ*, and the structure of the constructs was confirmed by DNA sequencing. Although the original six transcriptional fusion constructs contained different lengths of their respective promoter regions, they all included all four previously defined regulatory elements (Figure 2). The activity of the *lacZ* reporter gene was used to directly evaluate the transcriptional strength of each promoter. The H1-d and H1-c′ promoters are the most active of the 6 H1 histone promoters, and their activities are about 7-fold higher than that of the weakest promoter, H1-c. The H1-a, H1-a′, and H1-b promoters show intermediate expression levels, ranging from 40% to 55% of H1-d in QT6 cells. The relative promoter activities of the chicken H1 genes have also been examined in other transfected avian cells. In chicken LMH cells a similar expression spectrum was observed except that the H1-c'-p decreased to about 50%

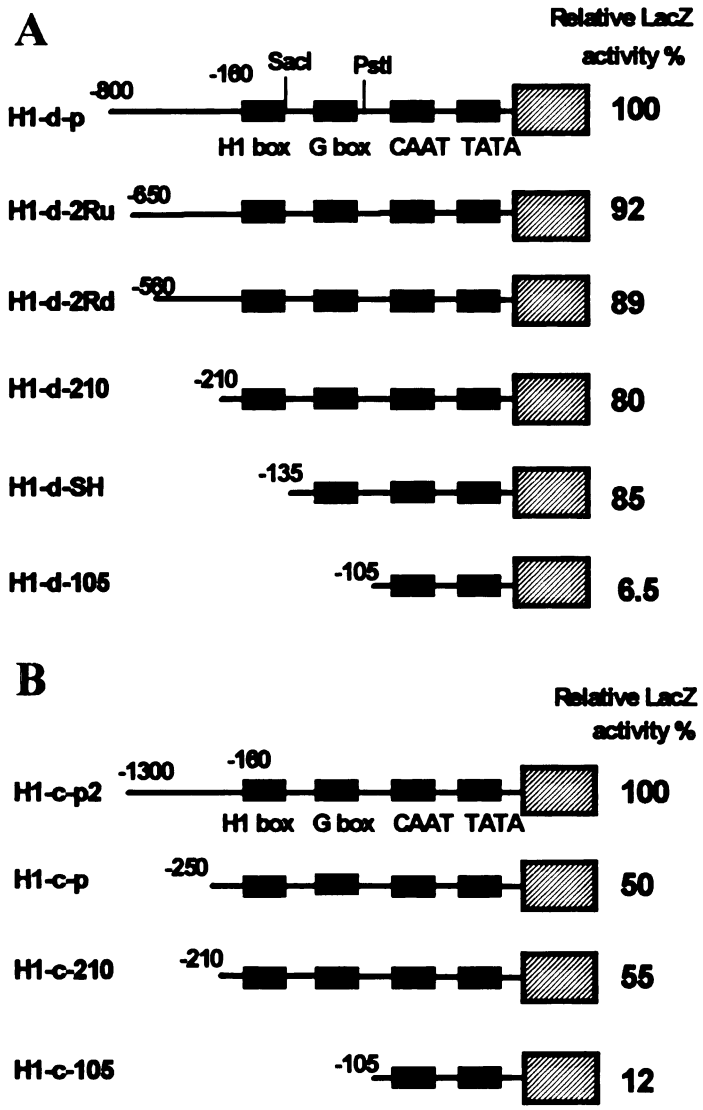
FIG. 2. Activities of six chicken H1 promoters in QT6 cells. The positions of TATA, CAAT, G box and H1 boxes are as indicated. The 5' end of each promoter is numbered with respect to the A in the H1 histone ATG site as +1. H1 promoter-*lacZ* activity was assayed as described in Materials and Methods. The promoter activity of each reporter construct is calculated as a percentage relative to the activity of H1-d. Each value represents the mean of 6-12 independent transfections. Standard deviations range from 3 to 9% for different H1 constructs.


Relative LacZ activity (%)
-800
-160
H1-d-p
H1 box G box CAAT TATA
100
-750
H1-b-p
56
-660
H1-c'-p
105
-470
H1-a'-p
45
-460
H1-a-p
40
-250
H1-c-p
15

of its level in QT6 cells relative to a control chicken β-actin promoter (results not shown). However, the promoter activities of all 6 H1 genes in primary CEF cells were at least 100-fold lower (relative to that of chicken β-actin) than those in QT6 and LMH cells. Although H1-d and H1-c' remained the most active promoters and H1-c the weakest promoter, the relative promoter activities of H1-a, H1-a' and H1-b were only 20 to 35% of the H1-d (data not shown). While it is difficult to compare these promoter activities to *in vivo* H1 protein levels due to the difficulty in resolving H1-a and H1-a′ and H1-c and H1-c′ in protein gels, H1-c and H1-a' appear to be the least expressed of the H1 proteins in chicken erythrocytes with H1-a, H1-b and H1-d being those most highly expressed (35,40). By this measure, the H1-a′ and H1-c′ promoters appear to be more active than would be expected on the basis of their protein levels in erythrocytes, which may relate to the variety of post-transcriptional influences known to be exerted on histone gene expression (22,41). Although the H1-a′ and H1-c constructs used for this comparison contain shorter promoter sequences than the other four, analogous constructs with longer promoter regions (up to 1.3 kb) have been tested which result in no more than a 2-fold increase in activity (data not shown).

Upstream promoter sequences have minimal effect on H1 promoter activity. Further analysis was performed of regions controlling the transcriptional levels of individual H1 histone promoters. Promoter deletion mutants of H1-d, one of the most active promoters and H1-c, the least active promoter, were prepared. Deletion of the sequences from -800 to -210 of H1-d only reduces the relative *lacZ* activity by 20% (Fig. 3A). Further deletion of the highly conserved H1 box (H1-d-SH) resulted in a slight increase in promoter activity. This result is similar to observations of Wells and colleagues of the H1-c' promoter in transiently transfected Hela cells and injected Xenopus oocytes (24,47). However, H1-d-105, in which the G box is also deleted, exhibits a dramatic decrease in promoter activity. A similar analysis was performed on the H1-c variant (Fig. 3B) which showed that deletion of the -1300 to -210 fragment in the promoter only resulted in a reduction in transcriptional activity of about 50%. Further deletion of both the H1 box and G box led to nearly a 90% drop in expression (H1-c-105). This suggests that the primary sequences responsible for the expression of the H1-d and H1-c variants are located within about 200bp of the ATG initiation codon, a region which covers the 4 conserved elements previously defined. Similar results were obtained with deletion mutants of the H1-a' and H1-c' promoters. In agreement with Wells et al(47), the H1

FIG 3. Analysis of H1-d and H1-c histone deletion mutant promoter activity. Deletion mutants were derived from the H1-d (A) or H1-c (B) constructs. The positions of four conserved elements and the restriction sites are as indicated. QT6 cells were co-transfected and *lacZ* activity was determined as described in Materials and Methods. The activity of H1-d (A) or H1-c-p2 (B) was set as 100%.


A
Relative LacZ activity %
-800
-160
SacI
PstI
H1-d-p
H1 box
G box
CAAT
TATA
100
H1-d-2Ru
-650
92
H1-d-2Rd
-580
89
H1-d-210
-210
80
H1-d-SH
-135
85
H1-d-105
-105
6.5
B
Relative LacZ activity %
-1300
-160
H1-c-p2
H1 box
G box
CAAT
TATA
100
H1-c-p
-250
50
H1-c-210
-210
55
H1-c-105
-105
12

box appears not to be required to maintain full activity and, indeed, may slightly repress promoter activity in transiently transfected cells. The G box, however, plays a major a role in maintaining full H1 histone promoter activity.

Sequence comparison of the minimal promoters of the H1-d and H1-c variants. In order to analyze the differential expression of the various chicken H1 histone gene promoters, their sequences from -210 to +1 were aligned and compared (Fig. 4). About 50% of the promoter sequences of the highly active promoter, H1-d, and the least active promoter, H1-c, are identical with about 70% similarity in the region from the H1 box to the TATA box. There is very little sequence similarity between these two promoters outside of the four conserved elements. However, three of the four conserved elements, the H1, CAAT and TATA boxes, are virtually identical and therefore unlikely to contribute to the differences in promoter activities. There is, however, a 3 bp difference within the G box, with the H1-d promoter having a G box of CGGGGCGGGCT and the H1-c promoter containing the sequences of TTAGGCGGGCT.

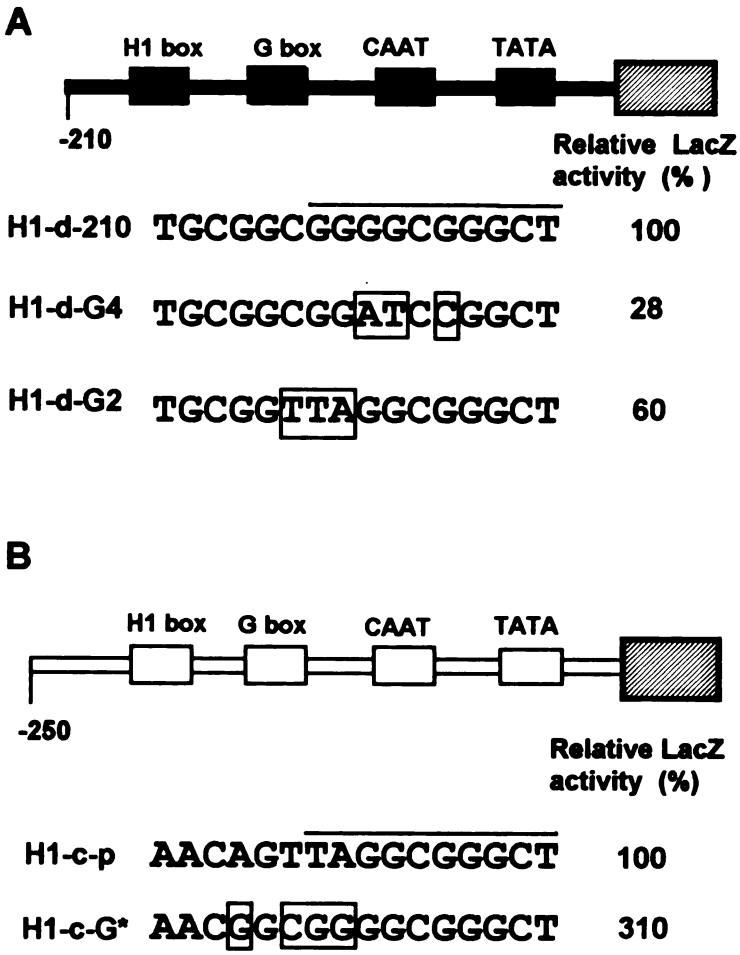
Sp1 binding to the G box is critical for H1 histone promoter activity. The core consensus sequence GGGCGG

FIG 4. DNA sequence comparison of minimal promoters of H1 variants. H1 promoter sequences are aligned in parallel with the A of their ATG initiation codons defined as +1. The numbering shown is for H1-d. The locations of the four conserved elements are underlined. The Dus element of the H1-d promoter is indicated by a line over the sequence in the -73 to -88 region. Asterisks (*) indicate sequence identify with H1-d and dashed (-) indicate deletions proposed to maximize alignment. Two unique restriction sites, *Pst*I and *Sac*I, only present in the H1-d promoter are underlined.


-200
-180
-160 H1 Box
H1-d GATTCGTGTTGGCGGAATTGTAGAAAAAACGCGCTTTTTCGCCTGTTAAGAAACACAAA
H1-c TGA*TTC*AGCC***C***T*CC*******A**AA***ATAA**CCG**********G*
H1-c' CGA*TTG**G*CA*A****CCGAGG****TA*A*****GTTAG*CCA**********
H1-a' CGA*TTC**GACA*AT*AA***T*******A*A****A*TCT*C*CG**********G
H1-a *TGC**C*ACCA*CAAT**T**CC****T*A*A******T*AGCAGG**********G
H1-b TGCG**G*CG*T*AATT*CTGTAGG****G*A*A*****G*A*C*AC**********C
-140
-120
G Box
H1-d ATAGCGGGGAGAAGGGAGCTCTGC-GCCGTGCG---GCGGGGCGGGCTCTGCAGCG-----
H1-c C*C*GA**AC*G*--A******T*CTGGC*AACA--*TTA**************AA*-----
H1-c' -*C*A*CAC*CCGAA*G****CC*G*******A---************TA***A**-----
H1-a' ----*C*A*CCGGCC****G*C*A-**A*CAGCGGA**********GCGGCGT*-------
H1-a ----------CCGA*AT**AGC**-****G***---**********GAGG**TC**CAACG
H1-b ----------C*****GGC*GC----------*GCC******GCG----------
CAAT Box
-80
TATA Box
+1
H1-d CACCAATCACCGCGCGGCTCCGCTCTATAAATACGAGGCCGCCGACTTG...48..ATG
H1-c **********A*AT*AC*G*TT*G********TCAG*CATCGG*GT*AC...46..ATG
H1-c' **********************T*****A*********CATCTGAC*CGC...45..ATG
H1-a' *********G***C**CTG*GCTG********GG***CG*CG**G*C*C...28..ATG
H1-a **********C**CTG*GCTG*********A***CG*CT**G*CGC...30..ATG
H1-b A********G*A****CGG*GCTG*******GGG*GC*G**G**G*GAC...35..ATG

usually specifies a high affinity Sp1 binding site. However, Kadonaga et al. (27) have shown that flanking sequences can have a major effect on binding of the mammalian Sp1 factor. Both the sequence comparison of the H1-d and H1-c promoters and the results with deletion mutants (Fig. 3) point to the G box as being a critical component of H1 promoter activity. To confirm this, several promoter constructs were prepared with altered G box sequences. When the core sequence of the G box was altered from GGGCGG to GATCCG in H1-d-210, the transcriptional activity was reduced to 24% of the wild type (Fig. 5A). This is a substantial decrease but has less effect than deletion of the entire G box region and upstream sequence (H1-d-SH, Fig. 3). This indicates that there is an additive effect of upstream elements on H1 histone promoter activity. However, when 3 bp of the flanking sequence were altered (H1-d-G2) , such that the H1-d G box becomes virtually identical to that of H1-c, the promoter activity was decreased to 60% of the wild type. Conversely, changing the H1-c G box so that it becomes identical to that of H1-d results in a 3-fold enhancement of its expression (Fig. 5B). These experiments suggest that some, but not all, of the transcriptional activity difference between these two H1 promoters relates to differences in the presumed Sp1 binding site or the G box.

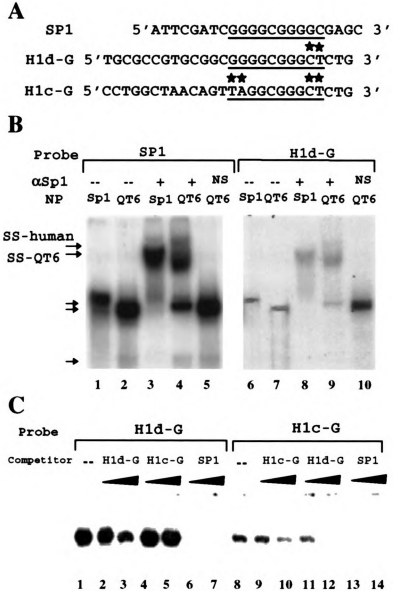
FIG 5. Effect of site-directed mutagenesis of G box elements on promoter activity. The putative consensus sequence for Sp1 binding in the G box of H1-d-210 (A) and H1-c-p (B) are indicated by a line above them. Constructs were made and assayed as described in Materials and Methods. The positions of altered nucleotides in mutant constructs are boxed. The activities of all mutants were calculated relative to that of H1-d-210 (A) or H1-c (B), respectively.


A
H1 box
G box
CAAT
TATA
-210
Relative LacZ activity (%)
H1-d-210 TGCGGCGGGGCGGGCT 100
H1-d-G4 TGCGGCGGATCCGGCT 28
H1-d-G2 TGCGGTTAGGCGGGCT 60
B
H1 box
G box
CAAT
TATA
-250
Relative LacZ activity (%)
H1-c-p AACAGTTAGGCGGGCT 100
H1-c-G* AACGGCGGGGCGGGCT 310

To confirm that Sp1 binding is involved in G box-regulated expression, we wished to demonstrate a correlation between Sp1 binding and transcriptional activity. Since the avian Sp1 gene is not presently available, we used recombinant Sp1 protein of human origin (hSp1) and the antibody against human Sp1 to test this possibility. First the binding of recombinant hSp1 to the H1-d G box sequence was compared to that of a control SP1 oligonucleotide containing the Sp1 decanucleotide consensus sequence, GGGGCGGGGC, which has been shown to bind Sp1 with high affinity (27) (Fig. 6A). As expected, a single complex was detected between the hSp1 protein and both SP1 and H1-d-G probes. However, the affinity of hSp1 protein for the consensus SP1 oligonucleotide was at least 10-fold higher than that for the H1-d-G probe (Fig. 6B). When a crude QT6 nuclear extract was tested under the same assay conditions, two closely migrating complexes and a weaker, faster migrating complex were observed with both the SP1 and H1-d-G probes. The formation of these complexes was abolished by competition with a 100-fold molar excess of homologous competitor, indicating that these shifted complexes are specific. One of the two intense QT6 complexes is clearly bound by anti-hSp1 antibody, suggesting that an avian analogue of mammalian Sp1 factor exists in QT6 nuclei and it is capable of binding the H1-d G box sequence, although with

FIG 6. Comparison of Sp1 binding activity to sequences from the H1-d and H1-c promoters. (A) Oligonucleotide probes used for mobility shift assays are listed. Putative Sp1 binding sites are underlined. Differences between the putative Sp1 binding sites in H1 promoters and the SP1 oligonucleotide containing the GGGGCGGGC Sp1 consensus binding site found in the SV40 promoter (6) are indicated by asterisks. The sequences of only one strand of the double-stranded probes are shown. (B) SP1 and H1-d-G probes were incubated with either purified recombinant human Sp1 protein or crude QT6 nuclear extract using poly(dI-dC) as non-specific competitor as described in Material and Methods. Monoclonal antibody to hSp1 (αSp1) and non-immune rabbit serum (NS) were included in the reactions as indicated. Three complexes shifted by QT6 nuclear extract are indicated by arrows. Two different antibody-shifted bands are indicated as SS-human and SS-QT6. Films were exposed for 8 hr and 2 d, respectively, for the SP1 probe and the H1-d-G probe. (C) H1-d-G or H1-c-G probes were competed with excess unlabeled H1-d-G, H1-c-G, or SP1 oligonucleotides as indicated, in binding to QT6 nuclear extract (-: indicates no competitor added). Filled triangles indicate increasing competitor levels with the lesser amount being a 25-fold molar excess and the larger being a 100-fold molar excess, except for the SP1 competition in which the

lower amount is a 10-fold molar excess and the larger amount a 40-fold molar excess.


A
SP1 5'ATTCGATCGGGGCGGGGCGAGC 3'
H1d-G 5'TGCGCCGTGCGGCGGGGCGGGCTCTG 3'
H1c-G 5'CCTGGCTAACAGTTAGGCGGGCTCTG 3'
B
Probe
SP1
H1d-G
αSp1 -- -- + + NS -- -- + + NS
NP Sp1 QT6 Sp1 QT6 QT6 Sp1 QT6 Sp1 QT6 QT6
SS-human
SS-QT6
1 2 3 4 5 6 7 8 9 10
C
Probe
H1d-G
H1c-G
Competitor -- H1d-G H1c-G SP1 -- H1c-G H1d-G SP1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

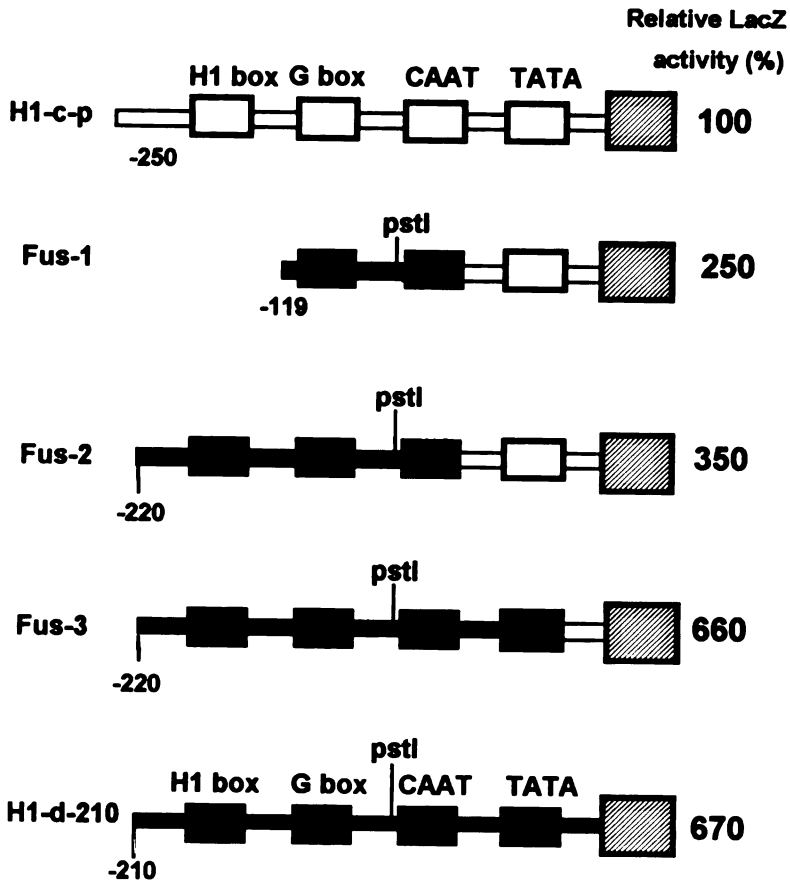
less affinity than a consensus Sp1 sequence. It appears that the other slowly migrating complex and the faint, more rapidly migrating complex are not recognized by anti-hSp1. The formation of these complexes may be due to the binding of avian homologues of the Sp1-related proteins, Sp2, Sp3, and Sp4, which have been shown to recognize an identical binding motif in mammals (23,30) or to other avian nuclear binding factors unrelated to the Sp1 family. A competitive gel shift analysis also shows that the G box of the H1-c promoter is bound by the same nuclear proteins but with an approximately 2.5-fold lower affinity than the G box of the H1-d variant (Fig. 6C). The difference in affinity for nuclear binding proteins, at least one of which appears to be an avian Sp1 homologue, between the H1-d and H1-c G box sequences correlates well with their relative promoter activities demonstrated previously (Fig. 2).

Identification of a second element controlling the differential H1 promoter activity. The altered G box of the H1-c promoter only partially accounts for its lower activity. To further identify other regulatory elements responsible for the difference, promoter hybrids were constructed between H1-d-210 and H1-c-p (Fig. 7). First, two base pairs in the H1-c-p promoter (-100,-101) were altered to create a *Pst*I site for convenience in hybrid

promoter construction. This change makes promoter sequences of H1-d-210 and H1-c-p identical downstream of the G box through the CAAT box and, by itself, has little effect on H1-c-p promoter activity (data not shown). Therefore, differences in subsequent fusion gene expression levels from either H1-d-210 or H1-c-p promoters must lie upstream of position -114 (using the H1-d numbering, Fig. 4) or downstream of -87. As expected, both Fus1 and Fus2 (Fig. 7) which fuse the G box alone or the G box and H1 box, respectively, of H1-d-210 to the rest of the H1-c-p promoter increase H1-c-210 promoter activity (2.5 to 3.5-fold) in agreement with the site mutagenesis results (Fig. 5B). However, neither fusion generates equivalent activity to the full H1-d-210 promoter. As shown with Fus3, full H1-d-210 promoter activity is achieved by additionally replacing the 16 bp of sequence between the CAAT and TATA elements in the H1-c-p promoter with the corresponding sequence of the H1-d-210 promoter. This region is very different in sequence between the H1-d-210 and H1-c-p promoters (Fig. 4). We have named this region the Dus element (Differential upstream sequence).

The G box and Dus element together account for differences in H1 histone promoter activity. To further examine the effects of the G box and the Dus element on the

FIG 7. Activity of fusion constructs of H1-d and H1-c promoters. Construction of fusion promoters and *lacZ* activity analysis are as described in Materials and Methods. The regulatory elements of H1-d-210 are shown as filled boxes, while those of H1-c-p are shown as open boxes. Two nucleotides in H1-c-p were altered to create a *Pst*I site as shown for convenience in constructions. Activities were calculated relative to that of H1-c, set at 100%.


Relative LacZ
activity (%)
H1 box G box CAAT TATA
H1-c-p
-250
100
pstI
Fus-1
-119
250
pstI
Fus-2
-220
350
pstI
Fus-3
-220
660
pstI
H1 box G box CAAT TATA
H1-d-210
-210
670

promoter activity of H1 variants, corresponding elements in the H1-d-210 and the H1-c-p promoters were exchanged (Fig. 8). The H1-c-D construct containing the Dus element of H1-d-210 showed an increase in activity of about 2.3-fold (Fig. 8B). As noted previously (Fig. 5B), the H1-c-G* construct with the G box of H1-d-210 showed a 3-fold increase. When both elements in H1-c-p were altered to their sequences in H1-d-210, promoter activity increased by 6.2-fold, nearly approaching the full H1-d-210 promoter activity (Fig. 8B). The converse series of mutations in the H1-d-210 promoter have effects which are in general agreement (Fig. 8C), although the G box conversion has a less dramatic effect in this direction, probably due to sequences just upstream of the G box (for example, nucleotide -121 in the H1-d sequence remains a G nucleotide in the H1-d-G2 construct which probably contributes to a greater activity than would be expected were this nucleotide also converted to A as in the H1-c promoter). These results support a model in which the effect of the G box and the Dus element on the promoter activity of the H1-d and H1-c are of similar magnitude and are additive. Together, their influence can account for almost all of the differential activity observed between the most and least active histone H1 promoters.

FIG 8. Effect of exchanging G box and Dus element regions on H1 gene expression. (A) Sequences of the G box and Dus element of the H1-d and H1-c promoters which are exchanged in the constructs tested. (B) and (C) Mutants are described graphically with the four conserved regulatory elements shown as rectangles and the Dus elements as ovals which are open for the H1-c-p promoter sequences and filled for H1-d-210. Constructs were prepared and activities were measured as described in Materials and Methods. Promoter activities are given as percents in comparison to that of H1-c (B) or H1-d (C), respectively.

A

H1d-G GGGGGCGGGC H1d-Dus CCGCGCGGCTCCGCTC

H1c-G TTAGGCGGGC H1c-Dus CAGATCACCGCTTCGC

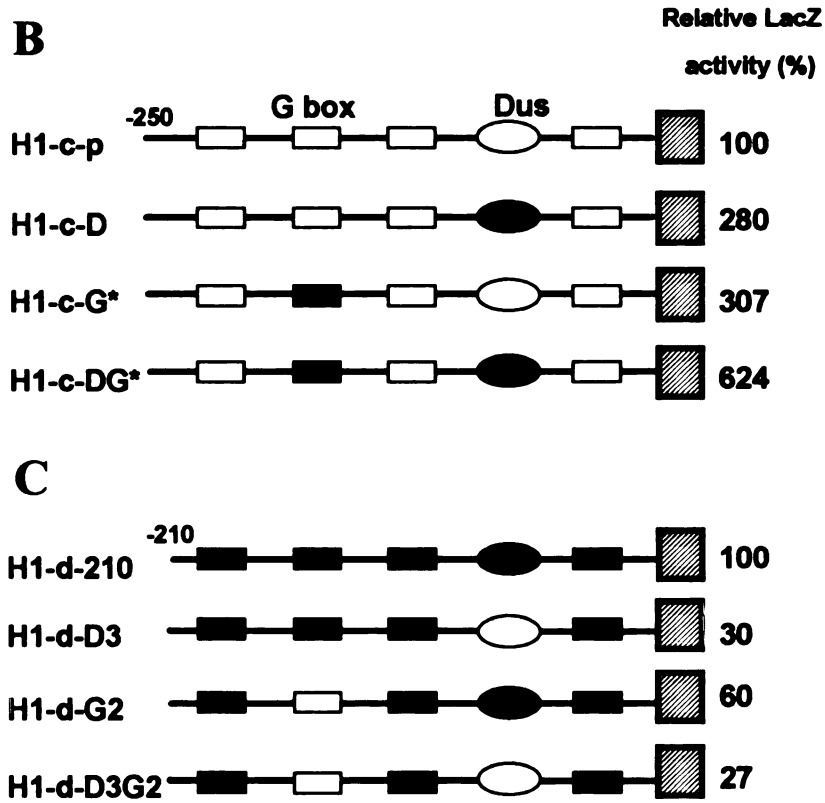
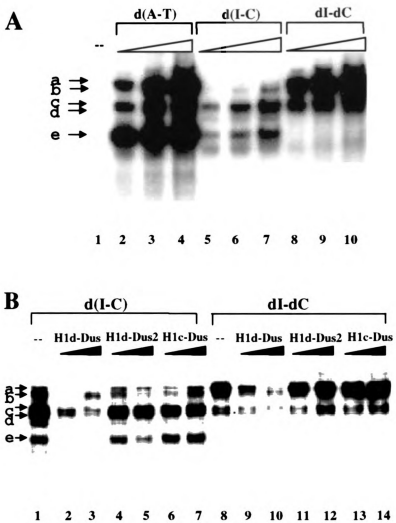


FIG. 9. QT-6 nuclear factor binding to the Dus element. (A) 0.2 ng of the ^{32}P-labeled H1-d-Dus probe (nucleotides -95 to -70, Fig. 4) was incubated with increasing amounts (5, 10, and 15 μg) of QT6 crude nuclear extract, as indicated by open triangles in the presence of 1 μg of poly(dA-dT), poly(dI-dC) and poly(dI)poly(dC) as indicated. The lane labeled as - indicates no added extract. Arrows a, b, c, d and e indicate the positions of specific gel-retarded complexes. (B) Binding of the H1-d-Dus probe in the presence of a 50-fold or 200-fold molar excess (as indicated by filled triangles) of unlabeled H1-d-Dus, H1-d-Dus2 and H1-c-Dus. The H1-d-Dus2 oligonucleotide contains AATC in place of the CGCG at -85 to -82 of the H1-d sequence (Fig. 4). The H1-c-Dus oligonucleotide corresponds to sequences -93 to -69 of the H1-c promoter (Fig. 4). Reactions included 1 μg of poly(dI-dC) or poly(dI)poly(dC) as indicated. Specific complexes are indicated by arrows.


A
d(A-T)
d(I-C)
dI-dC
--
a
b
c
d
e
1 2 3 4 5 6 7 8 9 10
B
d(I-C)
dI-dC
-- H1d-Dus H1d-Dus2 H1c-Dus -- H1d-Dus H1d-Dus2 H1c-Dus
a
b
c
d
e
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Multiple nuclear factors bind to the Dus region. The Dus region was tested for binding to proteins in QT6 nuclear extracts in gel shift experiments. Using three different non-specific competitors, poly(dI)poly(dC), poly(dI-dC), poly(dA-dT), 5 complexes labeled as a, b, c, d and e were observed on 6% polyacrylamide gels (Fig. 9A). (Complexes a and b and c and d require long gel runs or 5.5% polyacrylamide gels to be fully resolved, see Fig. 9B). All five complexes

bind to the Dus sequence most strongly in the presence of poly(dA-dT), which indicates that the G-C rich nature of the Dus element is a factor in binding. The Dus region of the H1-d promoter contains a CGCGCG sequence, binding to which is most effectively competed by poly(dI-dC), as seen for complexes a and b. It also contains a strong strand bias for C in one strand and G in the other which may be reflected in the competition of poly(dI)poly(dC) for complexes c, d and e. As expected, all binding to the 5 complexes was effectively competed by excess cold Dus oligonucleotide (Fig. 9B). Neither DNA containing the H1-c promoter Dus region nor a mutated H1-d Dus sequence, in which CCGCGCGGCTCCGCTC has been changed to CCGAATCGCTCCGCTC, were effective in such a competition assay (Fig. 9B). Complex formation to the H1-d probe is observed to increase by up to 40% when 5 mM $MgCl_2$ is included in the reaction

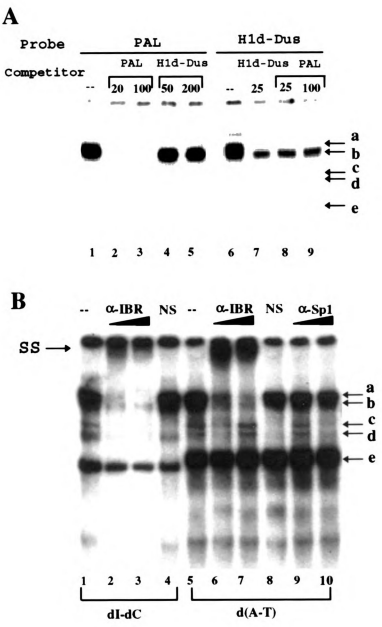
(data not shown). When 5 mM $MgCl_2$ conditions are used, it is also possible to observe complexes a, d and e with the H1-c Dus region probe. However, the binding activity observed appeared to be quite weak, and the complexes can be competed away with 40-fold molar excess of the H1-d Dus oligonucleotide (data not shown). These results suggest that more than one nuclear factor may bind to the H1-d Dus element in a sequence-specific manner, but that they bind poorly to the analogous region of the weaker H1-c promoter.

Identification of IBR as a Dus binding protein. No strong match to the 16 bp of H1-d Dus region sequence was found in a search of the Transcription Factor Database maintained at GenBank. However, the recent cloning and analysis of the chicken IBR, initiation binding receptor, demonstrated that its consensus binding sequence showed some similarity to the H1-d Dus sequence. IBR is involved in the repression of histone H5 transcription in mature erythrocytes (20). IBR was found only in immature and mature erythrocytes. Isolation of the IBR gene revealed that it is encoded by the same gene as IBF which is found in erythroid precursor HD3 cells and other cell types (19). IBR protein differs from IBF in its glycosylation (20). Both factors bind to GC-rich sequences spanning the transcription initiation site of the H5 histone gene. The optimal binding

site for IBR/IBF is identical to that of human α-Pal factor which activates the transcription of translation initiation factor IF-2α (17). Both factors bind with highest affinity to the sequence TGCGCATGCGCA. The 16 bp H1-d Dus region (CCGCGCGGCTCCGCTC) contains half of this binding site and is very similar to the IBR/IBF binding site found in the H5 histone gene (CGCGCGGCCGCA). As expected, a PAL oligonucleotide (19), which contains the optimal IBR/IBF binding site, is bound by crude QT6 nuclear extract proteins (Fig. 10A). Competition with the H1-d Dus region sequence only partially decreases the intensity of the shifted PAL complex indicating that the H1-d Dus sequence has a lower affinity for IBR/IBF than PAL. Conversely, one of the H1-d-Dus gel shifted bands (complex a, Fig. 10A) is very effectively competed by the PAL oligonucleotide, whereas PAL has little or no effect on complexes b, c, d and e. Addition of antibody against IBR to the H1-d-Dus probe binding reaction (Fig. 10B) confirms that complex a contains IBR/IBF. Non-immune rabbit serum has no effect on any of the complexes. Anti-IBR/IBF can also supershift some of complex b, suggesting that IBR/IBF may also form part of the b complex. However, since the PAL oligonucleotide did not compete for binding to the H1-d-Dus b complex, perhaps the IBR/IBF in the b complex has an altered sequence specificity. IBR/IBF antibody has no effect on the c, d and

e complexes of H1-d-Dus. Since there is some sequence similarity of the H1-d Dus region to the Sp1 consensus sequence, we also examined the Dus binding complexes by adding antiserum against the hSp1 protein (Fig. 9B). None of the H1-d-Dus complexes are supershifted by the anti-hSp1 antiserum, indicating that the cross-reactive avian Sp1-like protein detected in our G box studies does not bind directly to H1-d-Dus, but this does not rule out binding by one or more Sp1 homologues that do not cross-react with the human Sp1 antibody.

Figure 10. Involvement of IBR/IBF in Dus element binding. (A) A PAL oligonucleotide probe (19) (lanes 1-5), containing sequences with high affinity for IBF (19), and H1-d-Dus (lanes 6-9) were incubated with QT6 nuclear extracts as described in Materials and Methods in the presence of unlabeled PAL (lanes 2, 3, 8, and 9), H1-d-Dus (lanes 4, 5, and 7), or no added competitor (lane 1 and 6). Molar excesses of competitors are as indicated above the autoradiogram. All reactions contained 1 μg of poly(dI)poly(dC) as non-specific competitor and were electrophoresed on a 5.5% native polyacrylamide gel. (B) QT6 nuclear extract was preincubated with increasing amounts of protein A-purified rabbit antiserum against IBR (α-IBR; 2 μg and 4 μg, as indicated by the open triangles) or polyclonal antibody against hSp1 (α-Sp1; 1 μg and 2 μg, as indicated by the open triangle) prior to adding the labeled H1-d-Dus probe. NS indicates the addition of 5 μg of non-immune rabbit whole serum and a - indicates no added antiserum. SS indicates the position of antibody-supershifted complexes. Assays were performed in the presence of 1 μg of either poly(dI)poly(dC) or poly(dA-dT) as indicated at the bottom of the figure.


A
Probe
PAL
H1d-Dus
Competitor
PAL
H1d-Dus
H1d-Dus
PAL
-- 20 100 50 200 -- 25 25 100
a
b
c
d
e
1 2 3 4 5 6 7 8 9
B
-- α-IBR NS -- α-IBR NS α-Sp1
SS
a
b
c
d
e
1 2 3 4 5 6 7 8 9 10
dI-dC
d(A-T)

DISCUSSION

Several studies have focused on the generic regulation of histone H1 promoters, demonstrating four critical control elements(10,12,24,47). There has been little attention given to the differential expression of an organism's complete H1 histone gene complement. Such an analysis is possible in the chicken, since all six H1 variants have been cloned and their corresponding proteins been identified (11,40). H1 histone promoter-*lacZ* transcriptional fusions are used to classify the 6 chicken H1 promoters into three sets: two with high activity (H1-d and H1-c'), three with intermediate activity (H1-a, H1-a' and H1-b) and one with lowest activity (H1-c).

Deletion mutagenesis studies of the H1-d and H1-c promoters demonstrated only a limited effect (less than 2-fold, at most) of sequences upstream of the H1 box on transcriptional activity. This confirms that the promoter regions harboring the four conserved regulatory elements (TATA box, CAAT box, G box and H1 box) of H1 promoters are sufficient to generate normal transcription levels in transiently transfected cells. A similar result was observed with deletion mutants of the H1-a' and H1-c' promoters (results not shown).

Detailed site-specific mutagenesis of the promoters of the H1-d and H1-c variants have demonstrated that

differences in their activities result from the additive effects of different flanking sequences adjacent to the G box element and the Dus element which exists considerable sequence differences in a region between CAAT and TATA boxes. The G box of the H1 histone genes contains a GGGCGG core sequence of Sp1 binding site originally identified in SV40 promoter sequences (6). Our gel retardation supershift studies (Fig. 6) confirm that part of the nuclear extract binding activity for the G box cross-reacts with anti-hSp1 antibody. It also has been shown that the relative affinity of both the QT6 nuclear binding activity and hSp1 for the H1-d G box sequence (GGGGCGGGCT) is about 10-fold lower than for the best Sp1 binding motif (CGGGCGGGGC) and about 2.5-fold higher than that for the H1-c G box (Fig. 6B). These results correlate well with mutagenesis studies which demonstrate that the similarity of the H1-d and H1-c G box to the consensus Sp1 binding motif is partially responsible for their differential promoter activities. A similar observation has been made in studies of the expression of sea urchin H1 genes during early embryogenesis in that a single-base difference in the core Sp1 binding sites of early and late histone H1 genes dramatically affects the promoter activities and the temporal accumulation of mRNA (32).

While the question cannot be fully resolved at this point, our observations suggest that G box activity derives from binding of an avian Sp1 homologue. There may also be an additional G box binding activity that could derive from non cross-reacting Sp1 homologues or other avian cell nuclear binding factors.

The Dus region is a 16-bp, G/C rich sequence originally found between the TATA and CAAT boxes of the H1-d variant. Mutagenesis studies demonstrate that this region has a differential effect on H1-d versus H1-c promoter activity similar in magnitude to the differences in their G box regions. Interestingly, the Dus region sequence of the two most active H1 promoters (H1-d and H1-c', Fig. 3) is nearly identical, while the three with intermediate activity (H1-a, H1-a' and H1-b) all share a rather different(but still G/C rich) Dus sequence, and the Dus region of the least active promoter (H1-c) differs most from that of the H1-d and is the most A/T rich. Evidence is provided that multiple QT6 nuclear proteins bind to the Dus element of H1-d. Under three assay conditions, the overall binding patterns are similar, but the intensity of each band depends markedly on the type of non-specific competitor used. This observation suggests that the Dus element may be a composite DNA binding motif containing multiple independent or overlapping binding sites. To date, most of the proteins which bind to the H1-d

Dus region remain unknown, and little hint is provided by sequence comparison to known consensus binding regions. However, some homology was noted with the recently described IBR/IBF binding site (19), which is also noteworthy in that this site was first identified in the promoter of the chicken H5 histone gene, a distantly related H1 variant. Gel shift experiments using antiserum to the IBR/IBF protein confirm that one of the multiple complexes observed between the H1-d-Dus probe and QT6 nuclear extracts contains the IBR/IBF protein. While the exact role of IBR/IBF binding to the Dus element in H1 promoter function remains unclear, the reduced affinity of the H1-c Dus region sequence for nuclear binding proteins correlates well with its lower promoter activity. Our results strongly suggest that IBR/IBF and possibly other nuclear proteins bind to the Dus region (at least of the H1-d and H1-c' genes) and thereby influence the relative activity of different chicken histone H1 promoters.

ACKNOWLEDGMENTS

We thank Dr. Adolf Ruiz-Carrillo (Medical School of Laval University) for providing anti-IBR antibody and discussion of our results and Barbara Okimoto and Dr. Hans Cheng (USDA-ARS Avian Disease and Oncology Laboratory) for assistance in DNA sequence determination.

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Chapter 3

5' Coding sequences are involved in gene expression of chicken H1 histone genes.

ABSTRACT

The chicken contains 6 different histone H1 genes which express distinct H1 proteins at different level in chicken tissues and cell lines. The four common promoter elements and the interaction with their trans-acting factors are required for maximal expression of chicken H1 genes. Transient transfections of transcriptional fusions in several avian cell lines have shown that the differential activity of chicken H1 promoters is mainly regulated by the Sp1 binding site, G box, and a newly identified Dus element. Here, reporter gene activity of translation fusions is compared to that of transcriptional fusions in transient transfections and the results show protein codon 1-30 of chicken H1 genes enhances a heterologous gene expression by 1.5 to 6-fold. Our data suggests that the coding region may contributes to the differential expression of six chicken H1 genes by influencing both mRNA level and translational efficiency.

INTRODUCTION

Histone H1 has been found to participate in the formation of higher order chromatin structures (1,2) and to repress gene transcription in general (3). In chicken, six different histone H1 variants (4,5) have been reported besides the erythroid specific variant, H5 histone. They express six highly conserved but distinct proteins at different levels in various chicken tissues and cell lines (6-8). It is not clear how the differential expression of the six variants is regulated. The role of the different H1 histone gene promoter sequences upstream of the transcription initiation site (TIS) is discussed in chapter 2.

In recent years, elements that regulate the transcription of genes have also been found 3' with respect to the transcription of the TIS. Most of these intragenic control elements are located in introns (9,10), but some are within the protein coding sequences (11,12). Histone genes generally do not contain introns and their RNAs are usually not polyadenylated. Recently, coding regions of some histone genes have been reported to activate transcription (13,14). Coding region activator sequences (CRAS) were shown to be required for high expression of mouse histone H2A and H3 genes. Deletion of CRAS elements significantly

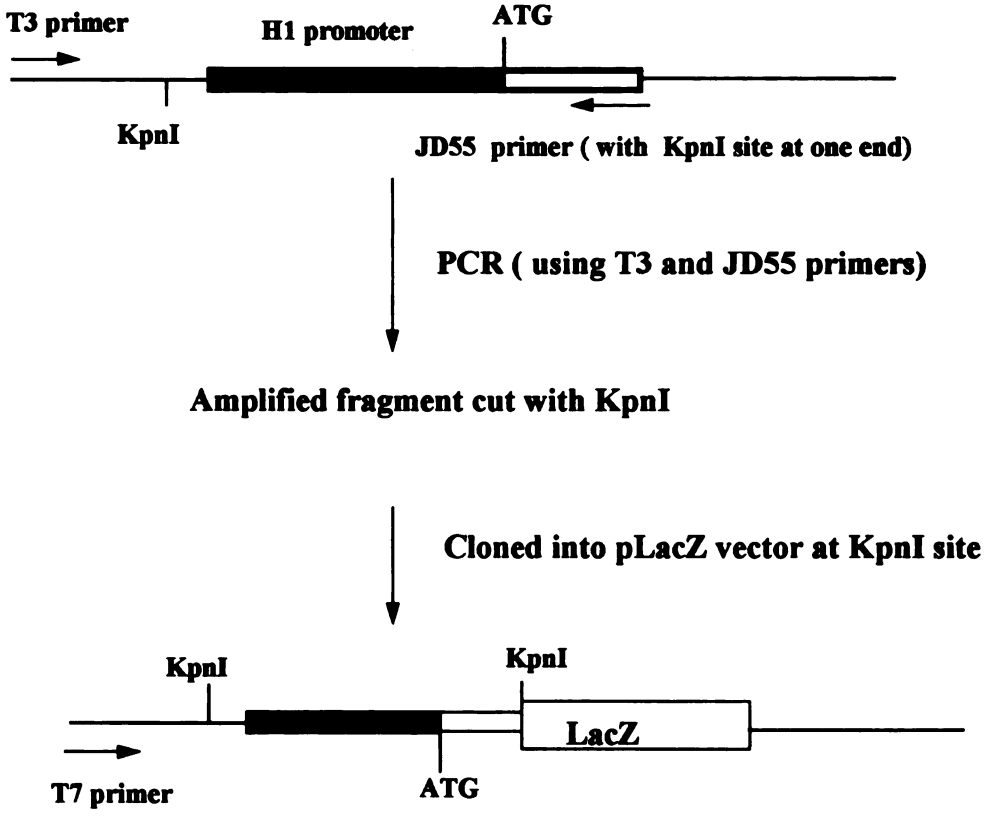
decreases transcription. In addition, CRAS elements have an orientation-dependent and position-independent effect on the transcription of both genes (14). Further analysis led to the identification of two conserved elements α (CATGGCG) and Ω (CGAGATC) within the CRAS elements of both mouse H3 and H2A histone genes(15,16). Both elements interact with distinct nuclear proteins and the binding activities are required for maximal expression. So far, no intragenic element has been reported in any chicken histone gene family. Results reported here suggest the existence of a protein-coding region of chicken H1 histone genes that can activate transcription of a heterologous gene. These data also suggest that 5' protein-coding sequences of chicken H1 histone genes may play a role in translational control.

MATERIALS AND METHODS

Construction of translational fusions. Translational fusions were constructed as shown in Fig 1. The JD55 primer sequence 5' TTTGGGTACCGCCTTCTTCGGCTT 3' contains a *KpnI* site and anneals to the conserved sequence in the NH_2-terminal domain of all chicken histone H1 genes. PCR was performed using this primer in combination with the 5′ T3 primer located in the cloning vector to amplify fragments containing the promoter sequence and about the first 90 bp of the 5' protein coding region from the corresponding genomic clone (17). The amplified fragments were digested with *KpnI*, isolated and then subcloned upstream of the *lacZ* reporter gene at the *KpnI* site of the p*lacZ* plasmid (chapter 2) to give H1-a-c, H1-a'-c, H1-b-c, H1-c-c, H1-c′-c and H1-d-c translational fusions. Individual chicken H1 histone genes and their corresponding proteins are identified according to the nomenclature of Shannon and Wells (5). The p*lacZ* plasmid was constructed by subcloning the *lacZ* gene excised at the *Hind*III and *Bam*HI sites from pCH110 (Pharmacia) into the pBluscriptII vector (Stratagene).

pActin-GL2 which was generated by inserting the 1.3 kb *XhoI-HindIII* chicken actin promoter fragment isolated from the pActin-his plasmid into pGL2-basic (promega) at the same restriction sites. pActin-his was kindly provided by Jean Marie Buerstedde(18). The pRc/H1-a'-p construct used in

FIG. 1: Strategy used in construction of H1 translational fusions. The H1 promoter region is indicated as a black bar and the protein coding region is indicated as an open box. The thin line represents the sequences of the pBS vector. The wide open box designates *lacZ* seque


T3 primer
H1 promoter
ATG
KpnI
JD55 primer (with KpnI site at one end)
PCR (using T3 and JD55 primers)
Amplified fragment cut with KpnI
Cloned into pLacZ vector at KpnI site
KpnI
KpnI
LacZ
T7 primer
ATG

stable transfection was produced by cloning the cassette of the H1 promoter-*LacZ* fusion excised at *ApaI* and *XbaI* sites from the H1-a'-p plasmid into a pRC-4.7 vector, which was produced by removing the CMV promoter (*NruI* to *EcoRV*) from pRC-CMV (Invitrogen). The pRc/H1-a'-c construct was made in the same way.

Deletion mutants. H1-a'-p2 containing 1.1 kb of H1-a' promoter sequence was constructed by inserting the 0.7 kb *Hind*III-*ApaI* fragment from the appropriate genomic λ clone into the H1-a' plasmid at its *ApaI* site. A primer with a *HindIII* site at its 5' end, annealing to position -145, was used to amplify the 145 bp H1-a' promoter fragment. The fragment was cut with *Hind*III and inserted into the p*lacZ* vector to generate the H1-a'-150 construct.

A primer annealing to the 5' coding region of the H1-d gene (+1 to +16) together with JD55 were used to amplify the 5' coding region of H1-d. The amplified 90 bp fragment was cut with *NcoI* and *KpnI* and subcloned into the H1-a'-c plasmid to give H1-a'-cw2. H1-a'-cw1 was generated by replacing the promoter region of H1-b-c with the 0.47 kb *ApaI-NcoI* fragment of H1-a'-c. The same strategy was used to create H1-b-cw which harbors the H1-b promoter and the codon 1-30 region of the H1-a'. All clones generated by PCR amplification were verified for correct amplification and

ligation by manual sequencing with the dideoxy chain method termination method or by automated DNA sequence analysis (ABI, Model 373A).

Cell culture and transfection. Quail fibroblast QT6 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at 37^{0}C with additions of 5% fetal bovine serum, 1% chicken serum, 2% tryptose phosphate broth and 10 mg/ml gentamicin. For transient transfection, 6 μg of pH1-*lacZ* DNA and 0.5 μg of the luciferase-containing plasmid pActin-GL2 as control were co-transfected into 1x10^{6} cells by the calcium phosphate precipitation method (19). After overnight incubation, the transfection solution was removed and cells were placed in fresh medium. Cells were washed with cold phosphate buffer 2 times and lysed with 0.5 ml of diluted reporter lysis buffer (Promega) 48 hr after transfection. Cell lysates were assayed for total protein with BCA protein assay reagent (Pierce) and β-galactosidase activity(19). Ten microliter aliquots of cell lysates were assayed for luciferase activity according to the protocol described by Promega. β-galactosidase activity was normalized to total protein and luciferase activity to correct for changes in transfection efficiency. Each reported value represents the mean of at least 5 independent transfections using different batches of column-purified plasmid DNA. Standard

deviations generally fall between 10 to 15% of the reported mean value.

Stable transfection was carried out by transfecting pRC/H1-a'-p and pRC/H1-a'-c plasmid DNA, separately, into QT6 cells by the calcium phosphate precipitation method. Cells were selected in QT6 media containing 1 mg/ml Geneticin for 12 days. 200-400 Geneticin-resistant colonies were collected and pooled.

Nuclear extract preparation. Crude nuclear extracts were prepared as described (20) with slight modification. Briefly, cells were washed with cold phosphate buffer before resuspension in 5 volumes of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF). The cells were allowed to swell on ice and lysed by rapid extrusion through a 25G needle. Samples were centrifuged and the pelleted nuclei were extracted in cold buffer C (20 mM HEPES, pH7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM, 25% glycerol, 0.5 mM PMSF, 0.5 mM DTT, 5 μg/ml leupeptin). Nuclear extracts were aliquoted and stored at -70^{0}C until use.

Mobility shift assays. Double-stranded oligonucleotides used for mobility shift assays were prepared by annealing two synthetic complementary oligomers in 0.5 M NaCl, 0.5 M Na_2HPO_4 at 95^{0}C for 4 min, 65^{0}C for 4 min and 4^{0}C for 4 hr

followed by purification on an HPLC ion-exchange column. The double-stranded oligomers were labeled by filling in the 5' overhang with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. Binding reactions were carried out at room temperature in 10 mM HEPES pH 7.9, 50 mM KCl, 1mM EDTA, 1mM DTT, 4% Ficoll, with 5-10 µg of nuclear proteins and 1 µg of non-specific competitor, poly(dI)poly(dC). About 0.2 ng of probe (3-5 x 10^4 cpm) was added followed by incubation for 20 min at room temperature. For competition assays, a 10-200 molar excess of the specific unlabeled competitor was added to the reaction 10 min prior to addition of the probe. Complexes were resolved on 6% non-denaturing polyacrylamide gels (acrylamide:bisacrylamide 19:1) electrophoresed in 0.5X TBE buffer (45 mM Tris-Boric Acid, 0.5 mM EDTA) at 150V for 2.5 hr at room temperature. The gel was dried and autoradiographed.

RNA isolation and analysis. Total RNA from stably or transiently transfected cells was extracted using Trizol reagent(BRL) according to the manufacturer's recommendations. RNase protection assays were performed following the recommended protocol in Hybridspeed RNase protection kit (Ambion). Briefly, 30 µg of total harvested RNA for each sample was coprecipitated with yeast tRNA and P^{32}-labeled antisense riboprobes. To each pellet, 10 µl of

HybSpeed hybridization buffer was added and hybridized at 68^{0}C for 15 min. After RNase A/T1 digestion, the reaction was stopped by HybSpeed inactivation/ precipitation mix and precipitated at -20^{0}C for at least 20 min. The protected fragments were analyzed on 5% polyacrylamide-8M urea denaturing gels. The coding region of the *lacZ* gene (*ClaI-EcoRV*) and the Neo gene (*PstI-EcoRV*) were subcloned into pBluescript vector, separately. The plasmids were linearized by restriction enzyme digestion and P^{32}-labeled antisense RNA was transcribed by T3 or T7 RNA polymerase in the presence of P^{32}-UTP.

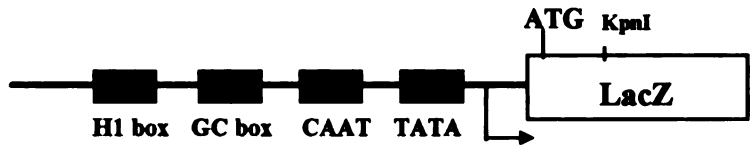
RESULTS

The amino-terminal end (amino acid 1 to 30) of the six chicken H1 genes are the most variable regions of their protein sequences (5). To determine whether this variable region may act as a intragenic element to regulate the expression of chicken H1 genes, transcriptional and translational fusions were compared in their reporter activities. The promoter and protein coding sequences (codon 1-30) were amplified by PCR and cloned upstream of a truncated lacZ reporter gene to produce translational fusions (Fig. 1). The promoter structures of the translational fusions are basically the same as their corresponding transcriptional fusions (as described in chapter 2), harboring the four conserved elements, the same transcriptional start site and the 5' untranslated region (5' UTR) of H1 genes (Fig. 2A). However, the translational fusions include the authentic ATG translational signal of each histone H1 gene, and the transcriptional fusions employ the *lacZ* ATG signal. Moreover, the transcriptional fusions contain an extra 5' UTR of the lacZ gene (85 bp) in addition to the 5' UTR of H1 genes which are included in both transcriptional and their respective translational fusions. However, we have deleted the lacZ 5' UTR from five transcriptional fusions (H1-p) and demonstrated that this 85 bp sequence has no positive or negative effect on reporter

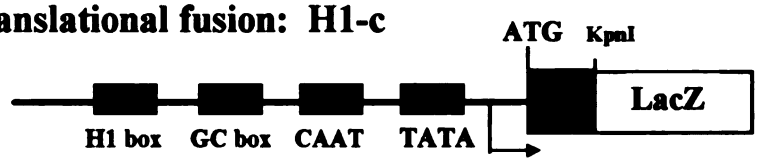
FIG. 2: Reporter activity of transcriptional fusions (H1-p) and translational fusions (H1-c) of histone H1 and *LacZ* reporter gene constructs. (A) Structures of the constructs. Promoter and 5' coding regions of histone H1 genes are indicated in black. The *lacZ* gene is shown in white. Arrows indicate the position of transcriptional start sites which are the same in both constructs. However, the translation signal ATG is located 120 bp upstream of the *KpnI* site in H1-P constructs and about 87 bp upstream of *KpnI* in H1-c constructs. (B) The relative activity of fusion constructs is calculated as a percentage relative to the activity of H1-d-p.

A

Transcriptional fusion: H1-P



Translational fusion: H1-c



B

	Relative LacZ activity (%)		Relative LacZ activity (%)	H1-c/H1-p ratio
H1-d-p	100	H1-d-c	189	1.8
H1-c'-p	105	H1-c'-c	157	1.5
H1-b-p	56	H1-b-c	229	4.1
H1-a'-p	49	H1-a'-c	294	6.0
H1-c-p	15	H1-c-c	37	2.2

gene activity (data not shown), therefore any differences in lacZ activities observed between transcriptional fusions and their translational fusions will result from the addition of H1 gene coding regions.

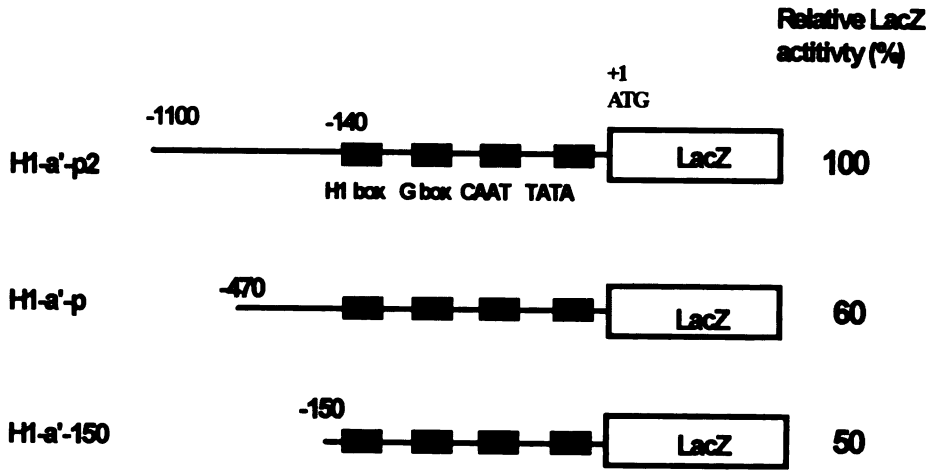
DNA constructs were transiently transfected into QT6 cells and the *lacZ* reporter activity in cell lysates were measured to assess the influence of the sequence in question on H1 gene expression. In transfected QT6 cells, reporter activities of the six H1 translational fusions were 1.5- to 6-fold higher than those of their corresponding transcriptional fusions (Fig. 2B). H1-a'-c and H1-b-c increase dramatically in reporter gene activity compared to their corresponding transcriptional fusions (H1-a'-p and H1-b-p). This result suggests that the first 30 amino acids of H1 protein coding region may potentially play a role in H1 gene expression. However, it can't be determined at present whether the effect of this coding region is exerted at the transcriptional or post-transcriptional level. Since the difference in translational versus transcriptional fusion activity was greatest for the H1-a' gene, further studies focused on this gene.

In order to define the minimal promoter region required for H1-a' gene transcription, we analyzed its promoter sequence up to -1.1 kb. Our results showed that sequences from -150 to +1 (H1-a'-150) are sufficient to generate 45%

of the presumed full length promoter activity of H1-a'-p2 which carries 1.1 kb of promoter sequence (Fig. 3). This result is in agreement with our previous studies of the H1-d and H1-c promoters which showed that promoter sequences upstream of the 4 conserved H1 elements account for only about half of the maximal expression levels (Chapter 2).

Transient transfections showed that the coding region of the H1-a' histone gene was able to increase the reporter gene activity by 6-fold. In order to see whether the increased activity was related to the high level of gene dosage in transient transfections, we generated stably transfected cells which carry lower copy numbers of transfected plasmids. QT6 cells were separately transfected with plasmids carrying a transcriptional or translational fusion gene. 200-400 neo-resistant colonies were pooled and assayed for *lacZ* reporter activity. Southern blot analysis confirmed that the transfected gene cassette is stably integrated into the QT6 genome without any deletion (data not shown). The *lacZ* activity of cells harboring the translational gene cassette remains about 10 to 12-fold higher than cells harboring transcriptional gene fusion after 10 passages (data not shown). It appears that the effect of the coding region on H1-a' histone gene expression, if anything, is more dramatic at lower gene-dosages.

FIG. 3: Analysis of promoter activities of histone H1-a' deletion mutants. The positions of four conserved elements and the restriction site are as indicated. QT6 cells were transiently transfected and *LacZ* activity was calculated as described in Materials and Methods. The promoter activity of H1-a'-p2 was set as 100%.


Relative LacZ
actitivty (%)
+1
ATG
-1100
-140
H1-a'-p2
H1 box
G box
CAAT
TATA
LacZ
100
-470
H1-a'-p
LacZ
60
-150
H1-a'-150
LacZ
50

The DNA sequence of the NH_2-terminal domains of the 5 chicken H1 genes were aligned and compared (Fig. 4). They are very similar at the nucleotide level; in fact, all are at least 80% identical to the H1-a' sequence. The G+C content of these regions are all greater than 75%, especially in the region from +31 to +57. Fig. 2B shows that the coding regions of H1-a' and H1-b significantly increased reporter activity in translational fusions. To see whether the coding region of different H1 variants is promoter-dependent, coding sequences in the same region were exchanged between different H1 genes. When the codon 1-30 region of H1-a'-c was replaced with that of H1-b (H1-a'-cw1), it slightly reduced the *LacZ* activity (Fig. 5). However, when the same region was replaced with the analogous sequence of H1-d, the *LacZ* activity decreased to 50% of H1-a'-c. Similarly, when the codon 1-30 region of H1-b-c was replaced with that of H1-a', *LacZ* activity increased to 1.5-fold of the H1-b-c. It appears that sequence differences in the codon 1-30 region influence the level of reporter gene activity. It is noteworthy that the effects observed in each case parallel the ratio of translational to transcriptional fusion activity. That is, H1-a' showed the largest ratio of H1-a'-c to H1-a'-p activity and its codon

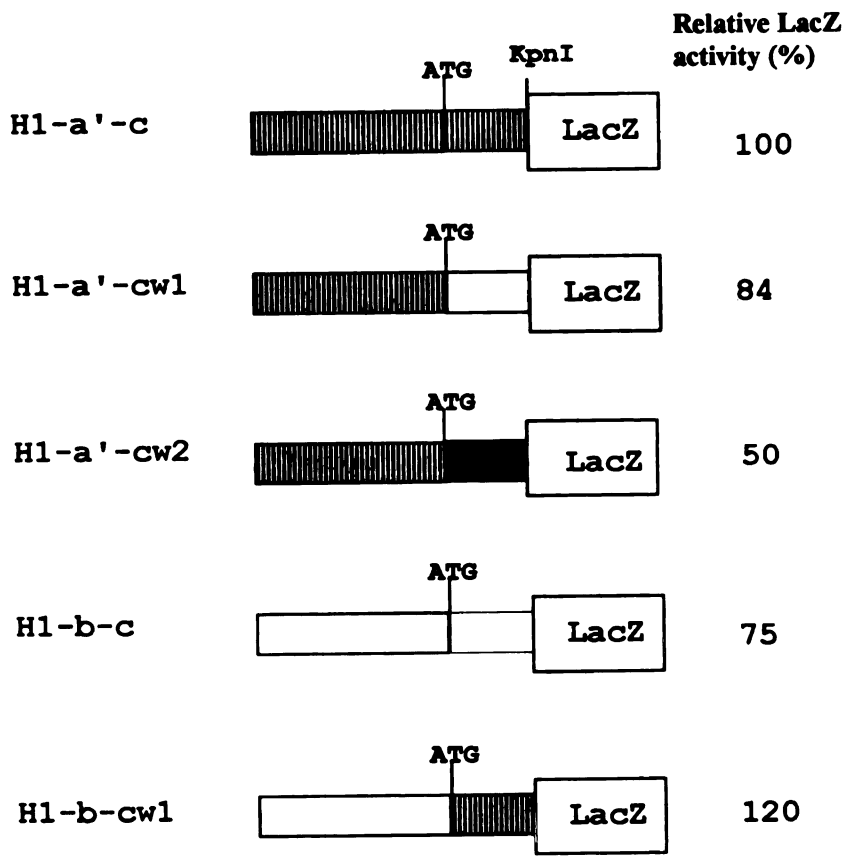
FIG. 4: DNA sequence comparison of amino-terminal protein coding regions of H1 variants. The DNA sequences of H1 genes are aligned and compared to the H1-a' sequence. An asterisk indicates the DNA sequence in the same position is identical to H1-a', but the change is shown where the sequence differs. Dashes are inserted for best alignment. A unique *PstI* site only present in H1-a' is underlined. DNA sequences are numbered according to H1-a' sequence.

```
          +1                                    pstI        +30
H1-a'     ATG GCT GAG ACC GCT CCT GCT GCA GCG CCC
H1-b      *** *** *** *** *** *** *TC **T *** ***
H1-d      *** T*G *** *** **G **C *T* **C *** ***
H1-c'     *** T*C *** *** *** **C **C **C **C ***
H1-c      *** T*G *** *** *** **C **C **C **T ***

          +31                                         +57
H1-a'     GCC GCC --- GCC CCG GCC CCC GCC GCC AAG
H1-b      GAT GTC GCC *** G*C *** A** C*G *** ***
H1-d      **G *TG TCT **G **C *G* --- --- *** ***
H1-c'     *AT **G CCC **G **C *G* --- --- *** ***
H1-c      **T *T* TCC **G **C *** --- --- *** ***

          +58                                         +84
H1-a'     GCC GCC --- GCC AAG AAG CCG AAG AAG GCG
H1-b      **G *** CCC *** *** *** *** *** *** ***
H1-d      *** *** --- *** *** *** *** *** *** ***
H1-c'     *** *** GCC *** *** *** *** *** *** ***
H1-c      *** *** --- *** *** *** *** *** *** ***
```

FIG. 5: Effect of switching the coding region on the expression of translational fusions. The promoter region and codon 1-30 region of the H1-b-c are indicated as white boxes and those of the H1-a'-c as striped boxes. The coding sequence of the H1-d is shown as filled black box. The sequences of the switched coding regions are shown in detail in Fig. 4.

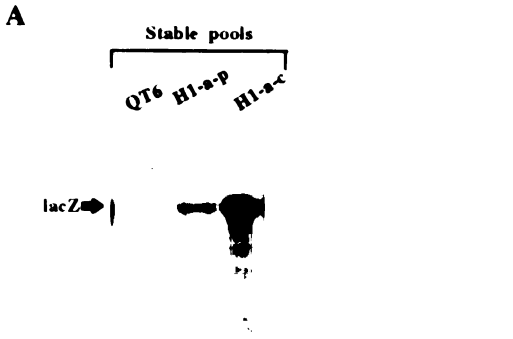

Relative LacZ activity (%)
ATG
KpnI
H1-a'-c
LacZ
100
ATG
H1-a'-cw1
LacZ
84
ATG
H1-a'-cw2
LacZ
50
ATG
H1-b-c
LacZ
75
ATG
H1-b-cw1
LacZ
120

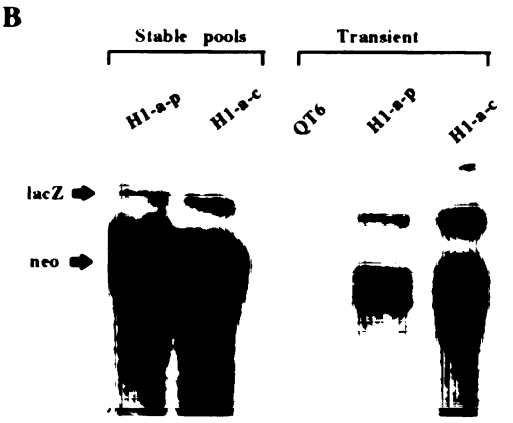
1-30 region appears to generate enhanced activity when inserted into H1-b-c.

In order to distinguish whether the effect of codon 1-30 is exerted at a transcriptional or translational stage, levels of steady state RNA were analyzed in transiently and stably transfected cells. Total RNA was extracted and analyzed by Northern blotting. However, the *lacZ* message was found to co-migrate with the 28S ribosomal band which generated problems in transfer and signal detection. In order to accurately estimate RNA levels, RNase protection assays were performed. Target mRNA was detected with a uniformly labeled anti-sense RNA probe that hybridizes to the 5' coding region of *lacZ* mRNA. The RNA level of stably transfected colonies of translational fusions was about 2-2.5 times higher than that from the corresponding transcriptional fusion transfectants as quantitated by a densitometer (Fig. 6). In transient transfections, *lacZ* mRNA from a translational fusion construct was slightly higher than that from transcriptional fusion. These data suggest that the codon 1-30 region of H1-a' contributes to enhanced expression in part through an increased level of mRNA.

However, the 2-2.5 fold effect is significantly less than the 10-fold increase in total l*acZ* activity observed previously, and no significant RNA effect was observed in the transient transfection. Therefore, it seems likely that

FIG. 6: Codon 1-30 region sequence of the H1-a' histone gene enhances level of mRNA expression. Total RNA samples from stably or transiently transfected QT6 cells were analyzed by RNase protection assays. Transfection and hybridization are described in MATERIALS AND METHODS. (A) 30 μg of RNA from cells stably transfected with pRC/H1-a'-p and pRC/H1-a'-c plasmids were hybridized with a *lacZ* anti-sense P^{32}-labeled riboprobe. QT6 cell RNA is shown as a negative control for lacZ message. The arrow indicates the *lacZ* protected fragment.(B) RNA samples from stably (30 μg) and transiently (15 μg) transfected QT6 cells were analyzed with the *lacZ* riboprobe and an internal control *neo* riboprobe.


A
Stable pools
QT6
H1-a-p
H1-a-c
lacZ


B
Stable pools
Transient
H1-a-p
H1-a-c
QT6
H1-a-p
H1-a-c
lacZ
neo

translational effects also play a role in reporter gene expression. When the translation signals of the six H1 histone genes were compared with the most efficient eucaryotic ATG signal (GCCGCCATGGC)(21), H1-a' and H1-b match perfectly, while H1-d, H1-c, H1-a and H1-c match less well(Table 1). The *lacZ* ATG signal employed by transcriptional fusions matches poorly with only 4 nucleotides identical to the most efficient ATG signal. This result correlates with the expression levels of translational fusions (Fig. 2B) in that H1-a'-c and H1-b-c, with "perfect" ATG signals, have the highest translational to transcriptional fusion expression ratio (H1-c/H1-p) and the others, with less "perfect" ATG signals, show lower ratios. This suggests that differences in the effectiveness of translation may contribute partially to the differential expression of H1 genes.

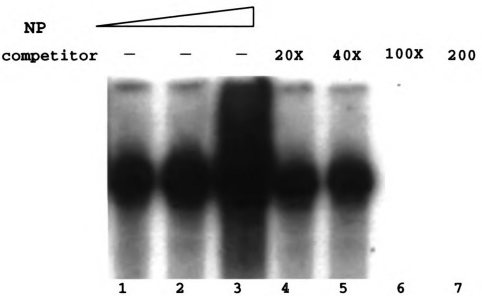
We also tested the nuclear protein binding activity of codon 1-30 region of the H1-a' gene. A complex containing the labeled 87 bp fragment bound to components of QT6 nuclear extract was detected in gel retardation assays. (Fig. 7). The binding activity increases proportionally to an increase in added nuclear extract. In addition, this shifted complex was efficiently competed with 25, 40, 100 and 200-fold molar excesses of the non-labeled homologous competitor, indicating that the binding is specific. The

Table 1: Comparison of the translation initiation signals of histone H1 genes with an optimal eucaryotic ATG signal (21).

Eucaryotic genes	GCCGCCATGGC	Identity
H1-a'	GCCGCCATGGC	10/10
H1-b	GCCGCCATGGC	10/10
H1-d	GTCACCATGTC	7/10
H1-c'	GCCGCGATGTC	8/10
H1-c	GCCGCGATGTC	8/10
H1-a	TCCGACATGTC	7/10
LacZ	CTTCACATGAG	4/10

The mismatched nucleotides are underlined.

FIG. 7: Detection of nuclear binding activity of the H1-a' codon 1-30 region. Lane 1-3, a P^{32} labeled fragment from the H1-a' coding region(+1 to +87) was incubated with increasing amounts of QT6 nuclear extract (5 µg, 10 µg, 15 µg) and 3 µg of poly(dI)poly(dC). Lane 4-7, the labeled probe was competed with X-fold molar excess of non-labeled homologous competitor in the presence of 10 µg of QT6 nuclear extract and 3 µg of poly(dI)poly(dC).


NP
competitor — — — 20X 40X 100X 200
1 2 3 4 5 6 7

probe DNA fragment was cut at its PstI site (Fig. 4) and the binding activity of the larger remaining fragment was tested. One retarded complex was detected with the 60 bp probe, and it was competed effectively with a 100-fold molar excess of cold 87 bp fragment. This implies that a binding site in the codon 1-30 region is located in sequences from +25 to +84.

DISCUSSION

H1 histone transcriptional fusion reporter gene construct expression was compared to that of analogous translational fusions up to codon 30 and the results demonstrated that the H1 codon 1-30 region can enhance the expression of reporter genes by levels ranging from 1.5-fold to 6 fold. Measurement of the steady-state *lacZ* mRNA levels showed that the codon 1-30 sequence of H1-a' enhanced the expression of *lacZ* up to 2.5-fold in stably transfected QT6 cells. In contrast, deletion of the CRAS element (110 bp) in mouse H2A and H3 genes decreased the levels of H2A and H3 mRNA by 20-fold (13,14). It is possible that the less dramatic effect we have observed results in part from the differences in stability between reporter gene *lacZ* mRNA and histone mRNA. Of course, it is equally possible that the codon 1-30 region in the chicken H1 gene has an inherently smaller effect than CRAS elements in mouse H2A and H3 genes. In addition, the predicted effectiveness of H1 histone ATG signals correlates well with the expression levels of their respective translational fusions. Overall, our results suggest that the amino-terminal protein coding region of chicken H1 histone genes influences both mRNA levels and translatability; both of which contribute to the differential expression of the 6 different chicken H1 histone genes.

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