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CHICKEN HISTONE GENES

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A STUDY OF THE REGULATION OF CHICKEN HISTONE GENES

By

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ABSTRACT

A STUDY OF THE REGULATION OF CHICKEN HISTONE GENES

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It has been well established that histone gene expression and DNA replication are temporally and functionally coupled. We have studied the regulated expression of several chicken histone genes by transfecting them into Rat 3 cells. The transfected cells were grown to confluence, serum starved, and stimulated to divide synchronously with serum. Cellular RNA was then isolated at different stages of cell cycle. Levels of exogenous chicken histone mRNAs were low in resting cells, but increased by 8-10 fold during S phase. This suggests that the transfected chicken histone genes exhibited their normal pattern of cell cycle regulation in the synchronized rat cells. This proved to be true for a variety of subcloned chicken histone genes flanked by different lengths of chicken DNA. In particular, one H3 histone gene showed normal cell cycle regulation with only 130 bp of its 5' flanking region DNA. Hybrid genes were made using this H3.2 gene and a replication independent chicken H3.3 histone gene, and examined in this manner. Our results indicate that this 130 bp portion of the H3.2 promoter region is responsible for about half of the usual increase in H3 histone mRNA in S phase relative to G_0/G_1 phase, and that the H3.2 histone gene 3' end

(containing the stem-loop region) is independently responsible for the other half. Detailed kinetic analysis indicates that the promoter effect begins earlier in the cell cycle, presumably before S phase, and the 3' end effect (post-transcriptional) is established more slowly, peaking late in S phase. Experiments with a DNA synthesis inhibitor (aphidicolin) showed that the 3' end of the H3.2 gene confers increased mRNA stability only in the presence of DNA synthesis. We also tested the effect of introns in the H3.3 gene and found that the introns were not responsible for constitutive expression of this gene. Our results also suggest that there is little or no effect of internal coding sequence on cell cycle regulation of histone gene expression. In particular, a hybrid chicken-yeast-chicken H2B histone gene was used to show that the ambiguous nucleotides (wobble sites) of codons in the chicken gene sequence are not required for cell cycle-regulated expression.

To Mom and Dad

To My Wife and Children

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Introduction

The histone proteins are highly conserved in evolution. The five classes of histone proteins are encoded by multiple copies of genes in all species examined. In higher eucaryotes, there are a number of nonallelic variants of each histone protein which are encoded by distinct histone genes organized in clusters. Most histone genes and mRNAs from various organisms have common structural features, including the absence of intervening sequences and the presence of a 3'-terminal stem-loop structure in place of the usual mRNA polyadenylated terminus. The expression of these replication dependent histone genes is coupled to DNA synthesis and is coordinately regulated at both the transcriptional and post-transcriptional levels. In contrast to replication dependent histone genes that are expressed mainly during S phase of the cell cycle, there are replication independent histone genes that are expressed constitutively at a low level throughout the cell cycle. One of the replication independent variants is the chicken H3.3 histone gene which has introns and encodes polyadenylated mRNA.

In order to analyze the contribution of various DNA elements to cell cycle regulation of the chicken histone genes, we carried out DNA-mediated gene transfer experiments with cloned genes. DNA fragments containing chicken histone

genes with various amounts of 5' and 3' flanking regions, as well as hybrid genes, were introduced into Rat 3 cells, and the level of mRNA produced from the transfected genes at different stages of the cell cycle was compared. In Chapter 3 the expression of various chicken histone genes transfected on either phage or plasmid DNA is described. The phage and plasmid constructs used in the transfection experiments differ in the extent of 5' and 3' genomic chicken histone sequences. DNA sequences of 5' flanking regions of two chicken H3.2 histone genes are also described. Hybrid genes were constructed using a chicken H3.2 (replication dependent) and a H3.3 (replication independent) histone gene, (or using a chicken H2B and a yeast H2B histone gene) in order to investigate the role of various regions of the histone genes in their cell cycle-regulated expression. Chapter 4 describes the result of these experiments.

Chapter 1

Literature Review

HISTONES

Histones are a group of small basic proteins that interact with DNA within the nucleus to form the elemental subunit of chromatin structure, the nucleosome (1). There are five major classes of histones based on their electrophoretic mobility: the core histones (H2A, H2B, H3, and H4), and the linker histone, H1 (2). Representatives of each of these five classes of histone have been found in every eukaryotic species examined with the exception of S. cerevisiae, which lacks H1 (2). A sixth type of histone, H5, is also recognized. The H5 histones are unique in that they are limited to species of birds, fish, reptiles, and amphibians (2). It is thought that H5 represents a highly divergent member of the H1 histone class.

As a result of technical improvements in protein fractionation and the fact that they are relatively abundant proteins, the isolation and subsequent amino acid sequencing of histones from many different species have been accomplished (2). Upon analyzing the sequence data from several species it was noted that the histones are very stable evolutionarily . The arginine rich H3 and H4 histones show the most sequence

conservation. In fact, only two amino acid changes distinguish a pea H4 histone from a calf thymus H4 (3) and only four amino acid changes separate a pea H3 histone from a calf thymus H3 (4). The lysine rich H2A and H2B histones exhibit sequence conservation of roughly 80% and 70%, repectively, when genera as divergent as Saccharomyces (yeast) (5), Parechinus (sea urchin) (6,7), Gallus (chicken) (8) and Bos (cow) (9) are compared. In addition, these four core histones, as they are known, display a common primary sequence organization. The amino-terminal third of each protein contains most of the molecule's charged amino acids and the carboxyl two thirds is composed mostly of hydrophobic residues (2). This may indicate that the core histones share a common ancestral gene (10). The changes that do occur in the core histones are usually seen in the hydrophilic amino-terminus rather than in the hydrophobic carboxyl-terminus (2). This is probably due to the function of the core histones within the nucleosome. McGhee and Felsenfeld (1) reported that the amino-terminal portion of each core histone is external to the core particle, based on nuclear magnetic resonance, sensitivity to trypsin digestion, and antibody binding results. Through in vivo reconstitution experiments the ability of trypsinized core histones to assemble into complexes resembling nucleosomes has been demonstrated (11). These results suggest that the basic amino-terminal portion of the core histones are of peripheral importance to the

assembly of the nucleosome core.

H1 histones (including H5) are very lysine rich and show the most variation of all the histones. Even so, H1 histones are well conserved evolutionarily. H1 histones have charged amino acids in both the amino- and carboxyl-terminal ends and have an apolar central region. Most changes in the protein sequence occur in one of the two hydrophilic termini rather than in the central region (2).

The histones were known to complex with DNA but the exact structural role of the histones was unclear until the discovery of the nucleosome (1, 12), the basic subunit of chromatin structure. Reconstitution experiments (13), cross linking data (14), and X-ray crystallographic data (15) taken together suggest that the nucleosome consists of a histone octamer core, containing an $(H3:H4)_2$ tetramer and two H2A:H2B dimers (Figure 1.). The basic amino-terminal ends of the core histones form the external surface of the nucleosome while the apolar carboxyl-terminal ends interact each other inside the core (1). Wrapped twice around this histone core are 140 base pairs of DNA. Between two adjoining nucleosomes is somewhere from twenty to eighty base pairs of DNA.

It has been argued that the H1 (and H5) histones are under less selective pressure than the core histones because

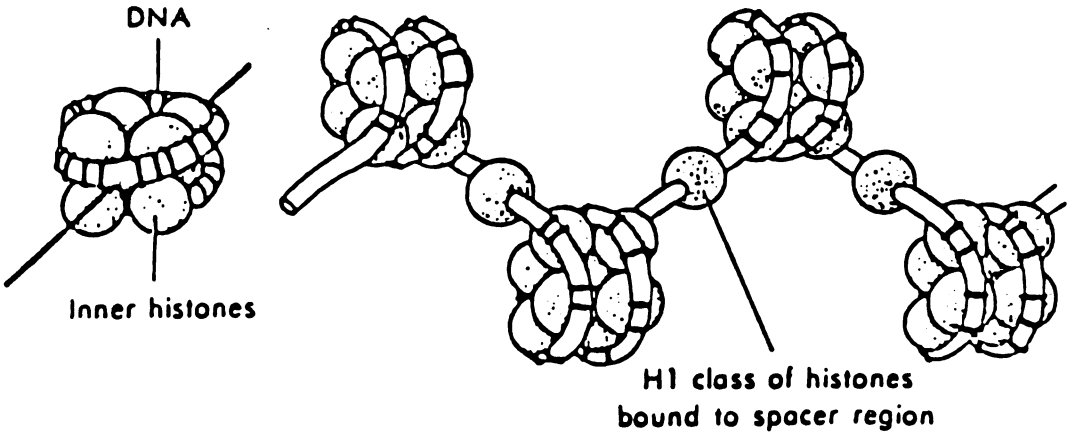


Figure 1. Structure of the nucleosome

The histone octamer consists of an H3:H4 tetramer and two H2A:H2B dimers. 140 base pairs of DNA is wrapped twice around this core. The H1 class of histones bind to the linker region between two adjoining nucleosomes(figure from ref. 179).

they are located on the outside of the nucleosome, in association with the DNA found between nucleosomes, the linker DNA (16, 17). In this position their function presumably depends on fewer critical protein-protein interactions than are required of the core histones, thereby permitting a greater degree of coding freedom. One of the functions proposed for the two basic ends of H1 histone is the 'sealing' of the DNA on the nucleosome where it enters and leaves. A second function assigned to the H1 histones is to function in the formation of higher order chromatin structure. The H1 histone protein appears to be essential for forming the 30 nM fiber, which consists of six nucleosomes arranged in a coil (2). This fiber itself can be further compacted to form even more complex structures whose molecular details are as yet unclear. The H5 histone also is responsible for the assembly of higher order chromatin structure. The replacement of H1 histone with the H5 histone protein within the erythroid chromatin condenses the chromatin, and may play a role in rendering the mature erythrocyte transcriptionally inactive (18).

Though the histones are usually grouped within the five major classes (H1, H2A, H2B, H3, and H4), when individual species are examined the data reveals that a particular class of histone may be composed of a number of variants or subtypes. This finding has led to the development of the

concept of histone families. Each family is composed of individual members that display a significant degree of similarity but differ in a few to many amino acid residues.

The histone variants may be grouped according to timing of their synthesis (19, 20). The major class is that of the replication-dependent histones. The transcripts coding for these histones are most abundant in rapidly dividing tissues. These histone gene transcripts are induced during S phase of the cell cycle and synthesized coordinately with DNA replication. But when DNA synthesis is halted, they are degraded rapidly. These are called replication variants. A second group of histone variants is that of the partially replication-dependent histones. These were discovered during studies on mouse liver regeneration. These histones are induced at the beginning of S phase but unlike the first group of histones that are repressed at the end of S phase, these continue to be synthesized after DNA replication has ceased. A third group of histone variants is that of the replication-independent histones or replacement variants. These histones are first found in the early stages of embryogenesis and continue to be expressed through adulthood. Their synthesis is not affected by inhibitors of DNA replication. The ratio of replacement to replication variants is thought to be determined, to some extent, by the cell's mitotic activity. The experiments performed on regenerating liver (20) and mouse

erythroleukemia cells (21) have confirmed this prediction. The rapidly dividing cells contained a large proportion of replication-dependent variants in their chromatin and replacement variants dominated in less mitotically active, mature cells. The best known examples of the latter group are the H3.3 variants (22, 23), histone H5, $H2A_F$ (24), and $H1^0$ from mammals (25). Messenger RNA coding for these histones is synthesized constitutively at a low level regardless of the replication state of the cell.

Some histone variants only appear at certain stages of an organism's development or maturation. The best characterized processes during which histone variants are expressed include embryogenesis, spermatogenesis and erythropoesis in sea urchin, chicken and mouse species. The developing sea urchin embryo undergoes many rapid cleavage divisions following fertilization. The need for an adequate supply of histone during these very early cell divisions is met by the translation of stored maternal histone mRNAs (26, 27). After the first few cleavage divisions, the main supply of histone protein is translated from mRNA actively synthsized from the early histone genes (28, 29). These variant histones are then replaced by another set of histones known as the late or larval type histones (30, 31). The synthesis of late histones begins at the conclusion of blastulation (32) and they continue to be synthesized, replacing all other variants

entirely in the adult echinoderm's chromatin.

The maturation of nucleated erythrocytes in species of birds, fish, reptiles and amphibians represents an unusual example of tissue-specific histone expression (2, 18). In these animals H5 synthesis is uncoupled from DNA replication and becomes the only histone synthesized in these cells after cell division stops. Although H5 histone exists only in non-mammalian vertebrates, it does share substantial amino acid sequence homology with the mammalian H1 variant $H1^0$ (2, 33).

One role for variant histones is suggested by evidence that indicates newly synthesized DNA becomes associated with newly formed core particles (34, 35, 36) in a conservative (strand-specific) manner. These core particles could be composed of histone variants which might affect the functioning of the nucleosome in such a way as to alter the chromatin structure, for example affecting the accessibility of its DNA to transcription. Using nucleases, such as DNase I, Weintraub and others (16) demonstrated that the enzymes' ability to nick nuclear DNA is a function of the state of its chromatin. Genes which are actively transcribed are more susceptible to enzymatic attack, and domains of sensitivity surround such genes for roughly several kilobase pairs. The nature of this nuclease sensitivity has been found to be partly attributable to the nonhistone proteins HMG 14 and 17. These proteins recognize some structural feature in the

nucleosome particle associated with transcriptionally active DNA (16). One hypothesis suggests that the HMG proteins recognize changes in the core histones, perhaps particular variants.

All histone types can undergo secondary (post-translational) chemical modifications. One such modification is the methylation of certain lysine groups on both H3 and H4 histones, which appears to be reversible (2). Increased levels of methylation may be detected during late S and G_2 phases of the cell cycle (37). The function of this modification is unknown.

Acetylation may occur on the amino-terminal serine residue of H1, H2A and H4 as well as specific N-terminal lysine residues of H3, H4, H2A and H2B. The terminal N-acetyl-serine group appears to be a stable, irreversible modification but its function is not clear. In contrast, the internal acetylations are reversible through the action of the enzyme deacetylase (38). H2B, H3 and H4 have four modification sites while H2A has only one site (2). The core histones are acetylated soon after they are synthesized but not all possible sites are modified. This type of acetylation is transitory, appearing during S phase and lost in G_2 phase. There is evidence that hyperacetylated histones congregate in certain discrete regions of the genome (39) and a number of

these regions are DNase I sensitive (40, 41). This is of interest since DNase I sensitivity has been shown to occur in those areas of the genome that are transcriptionally active or potentially active (42, 43). It is thought that the hyperacetylation of the histones affects their interactions with the DNA and allows the chromatin to relax, but this needs to be confirmed (44).

Phosphorylation is another common secondary modification of histone proteins. The phosphate moiety is covalently attached to specific threonine, serine, and occasionally histidine (H4) and lysine (H1) residues during S phase, mitosis and in response to hormonal stimulation. The phosphorylation is usually lost after the anaphase stage (2). Gurly et al. (45) have suggested that the various organizational levels of chromatin can be correlated with different forms of phosphorylation. As an example, if phosphorylation involves H1 then a change in the extent of chromatin compaction (coiling) might be the result. In addition, the phosphorylation of H2A has been shown to accompany heterochromatin condensation.

The most unusual secondary modification of a histone is the linkage of ubiquitin to the terminal amino group of lysine #119 of certain H2A proteins (46). This complex is known as A24 (47). Ubiquitination of a small number of H2B histones

has also been demonstrated (48). The cellular levels of A24 complex are thought to be related to the cell's mitotic activity such that non-dividing cells contain more A24 than dividing ones (10). Finally, addition of a poly(ADP-ribose) moiety to the glutamate or aspartate residues of H2A, H2B, H3, and H1 is thought to affect the interactions between the histones but no conclusive evidence is yet available (2).

ORGANIZATION OF HISTONE GENES

During the early stages of sea urchin embryogenesis a series of rapidly occuring cell divisions take place and the embryo's requirement for histone proteins is at a maximum. It was known that about 60% of the embryo's translational activity at this time of development is devoted to the synthesis of nuclear proteins (49, 50). Therefore, the sea urchin embryo represents an enriched source of histone mRNA. By using various techniques, several investigators were able to demonstrate that the genes coding for 9S mRNA, which was associated with polysomes engaged in histone synthesis in S phase (51, 52), are repeated several hundred times in the sea urchin genome and these genes were in actuality histone genes (53, 54, 55). When individual histone mRNAs were used to probe cloned and high molecular weight genomic DNA, the remarkable finding was made that all five histone genes are organized in clusters 5-7 kb (kilobase pairs) in size (54) that are tandemly repeated 300-600 times in the genome (56). It was originally believed that most other organisms would share this arrangement or something similar. As more species have been studied however, it has become apparent that the organizational pattern of the sea urchin genes is unique. It reflects their need for a large amount of histone synthesis in a short period of development rather than a standard motif.

The five major early histone genes of sea urchins are

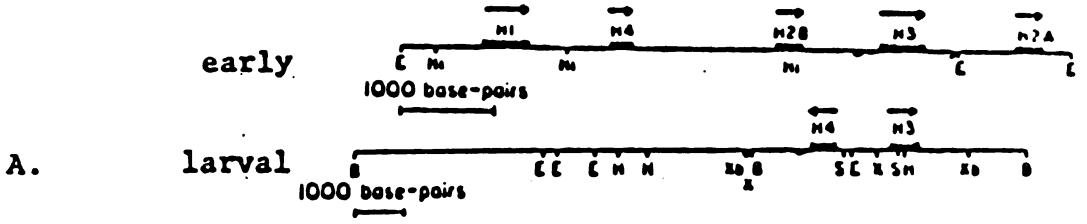
organized into a quintet that is tandemly repeated several hundred times (54). The order of the histone genes within the quintet (5' to 3' relative to transcription) is H4-H2B-H3-H2A-H1. Each early gene is separated from its neighbors by intergenic spacer DNA of varing length that is AT-rich, and which appears to contain the needed regulatory elements (57). The genes in a cluster are all transcribed from the same strand of DNA but not as a polycistronic message, and none of the histone genes contain intervening sequences. The sea urchin late histone genes have been found to be present in only 5-12 copies per haploid genome and are organized in a very different way than their early gene counterparts. Furthermore, DNA sequence analysis has confirmed that these genes have diverged considerably with respect to the early histone gene sequences. It was also discovered that a third class of sea urchin histone gene exists. These genes, called 'orphons', appear to be solitary genes unlinked to any of the other histone genes. It is uncertain if some of these are transcriptionally active or if they are all pseudogenes (58).

The quintet pattern of organization has also been found in Drosophila and amphibians. The organizational pattern of these genes show similarities to that of the sea urchin but with distinct differences as well (Figure 2.). The order of the Drosophila histone genes is H1-H3-H4-H2A-H2B, and the genes are not all transcribed from the same DNA strand. In

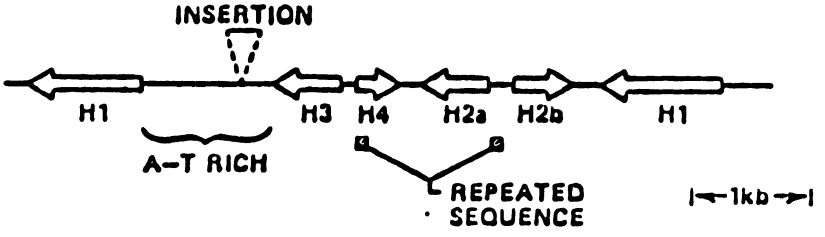
Figure 2. Comparison of the genomic organization of histone genes.

A. The major early histone gene repeat and larval stage of H3 and H4 histone genes of the sea urchin, L. pictus (from ref. 180).
B. The major D. melanogaster histone gene repeat. The site of the 240 bp insertion element is indicated (from ref. 181).
C. The major X. laevis histone gene repeat (from ref. 182).
D. The chicken histone genes (from ref. 183).

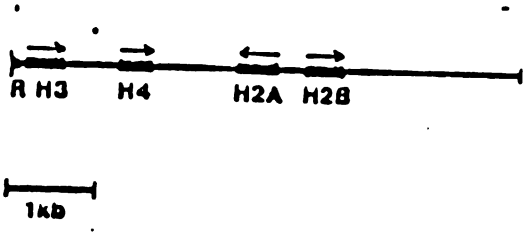
A.


early
1000 base-pairs
larval
1000 base-pairs

B.


INSERTION
H1
H3
H4
H2a
H2b
H1
A-T RICH
REPEATED SEQUENCE
1kb

C.


R H3
H4
H2A
H2B
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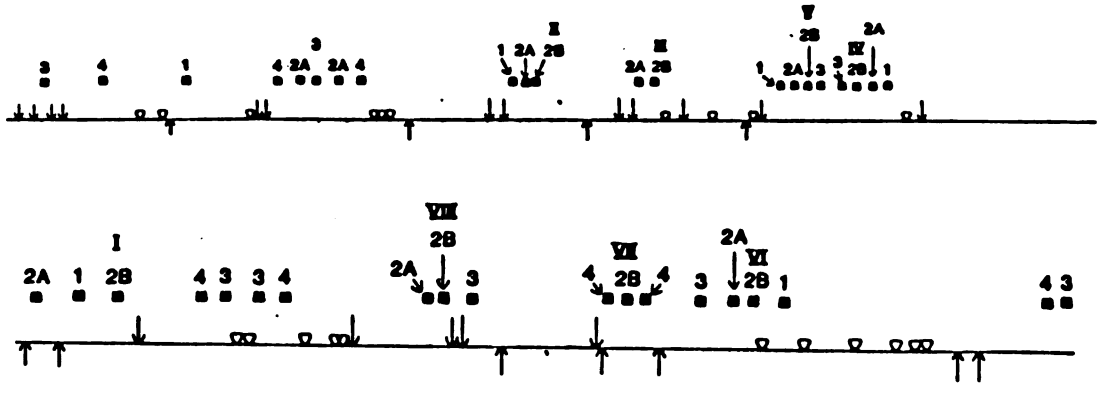
D.



Drosophila there are about 100 quintets, present in two sizes, which differ by an insertion of 240 bp (base pairs) of spacer (59). Flies have been bred that lack one or the other repeat unit with no apparent deleterious effects (60, 61). It appears as though either type of repeat unit is sufficient to maintain the required level of histones. About 60% of the genes in Xenopus borealis are organized in a highly conserved quintet. In contrast, the closely related frog Xenopus laevis has at least three cluster orders, each order showing high heterogeneity but still principally organized in the quintet fashion. Both frogs have minor gene rearrangements (62).

An element of sea urchin organization is thus found in amphibians. However, in mammals and birds the histone genes are far more disordered. They have irregular clusters of histone genes without tandem repeat units (20, 63-68). In the chicken there are two such clusters which vary in both content and in organization (64, 65). There is no long range repeat, but there are preferred associations, such as H1 genes with paired, divergently transcribed H2A-H2B gene pairs and H3-H4 associations. However, there are exceptions, and even when associations such as H1-H2A-H2B are maintained, the order of these genes within a cluster may not have been. Also there are unrelated clusters in which genes are symmetrically ordered around central H3 genes, and in one such cluster the boundaries of a duplicated H2A-H4 gene pair contained related

repeat sequences (69). The genes for the tissue-specific H5 histone and other variant histones are not linked to any other histone genes. This may be related to the differences in the way they are expressed.

STRUCTURE OF HISTONE GENES

The histone genes in the tandemly repeated quintets of sea urchin are notable for their lack of introns. Histone genes from a wide variety of organisms studied, including Drosophila, yeast, Xenopus, mouse, human and chicken, subsequently have also been found to lack introns. But there are important exceptions. The first exception discovered was an H3.3 gene of the chicken (23). To this can be added the $H2A_f$ gene of the chicken (24), which is similar to the basal mammalian histone $H2A_z$. It has been found that the H3 and H4 genes of Neurospora also contain introns (86).

Brush et al. (22) characterized two nonallelic H3.3 replacement variant histone genes. The two H3.3 variants share a variety of properties which differ from the replication variant gene class. One contains 3 or more introns and the other has 4 introns. But the location of the two introns within the coding regions of the two genes have been exactly conserved, whereas the intron positions in their respective 5' flanking regions differ. Both genes have a 5' untranslated leader segment spliced to the coding body of the mRNA. Both contain long 3'and 5' untranslated regions and are not linked to any other histone genes. Although both H3.3 genes predict the identical histone polypeptide sequence, they are as different from one another as each of them is from a more common replication variant H3.2 gene in silent base

substitutions within the coding sequences. The H3.3 gene mRNA are post-transcriptionally polyadenylated. The gene which codes for the tissue-specific H5 histone, which in some ways resembles a replacement variant of H1 histone, does not contain introns (92). It is, however, unlinked to other histone genes and its transcript is polyadenylated (122).

Except for the TATA box and CCAAT box related sequences, no other principal homologies 5' to the coding sequences of all histone genes are found. But a few histone gene families have subtype-specific sequences of their own. One example is the H1-box (121). The H1 gene-specific element (5'-AAACACA-3') is located about 100 bp upstream from the cap site and is considered to play an important role in cell cycle regulation of the gene. Another example is an octamer sequence (ATTTGCAT) which is an H2B subtype-specific consensus sequence (95). This element is also important in cell cycle regulation of the H2B gene by interacting with a regulatory protein (94). In sea urchin, the spacers that separate all five histone genes by similar lengths are AT-rich and highly conserved (57). Such 'prelude' sequences are a common feature of all sea urchin histone genes and hence are inferred to have specific functions in gene expression.

In many cell types, including cultured mammalian cells, certain histone mRNAs are the only mRNAs known to belong

exclusively to the nonpolyadenylated fraction. However, in the unicellular eukaryotes, yeast and tetrahymena, histone mRNAs are polyadenylated (62). In higher eukaryotes, H5 mRNA is polyadenylated, but the chicken H5 gene lacks the usual polyadenylation signal AATAAA. Similarly, the chicken H3.3 and $H2A_F$ transcripts appear in the poly(A)$^+$ mRNA fraction, although neither gene contains the AATAAA sequence except for one of the two chicken H3.3 genes and this sequence is far upstream of the actual poly(A) addition site (22). It thus appears possible that these transcripts are polyadenylated by a mechanism other than that applying to most other mRNAs.

The replication dependent histone mRNAs which are nonpolyadenylated usually end in a stem-loop structure with 6-base pair stem and a 4-base pair loop (57). This general structure has been highly conserved during evolution and is found in histone genes from many species, including those from sea urchins, Drosophila, Xenopus, chickens and mammals (75. Table 1.). The stem-loop structure is considered to be partially responsible for the cell cycle regulation of histone gene expression by affecting the half-life of the replication variant histone mRNAs.

Gene	Hyphenated Dyad Symmetry
H4	
h22	AACGGCTCTTTTCAGAGCCACCAaatattCAAGAAAGA
h19	AACGGCTCTTTTCAGAGCCACCAaataatCAAGAAAGA
Sp17/2	AACGGCTCTTTTCAGAGCCACCAaataatCAAGAAAGA
pXlch4	ATAAGCCCTTTTAAGGGCCACAA-PolyA
pXlH4W1	AACGGCCCTTTTAAGGGCCACCA-PolyA
pXbH4W1	AACGGCCCTTTTAAGGGCCACAA-PolyA
mus-hi-1	AACGCCCCTTTTTAGCGCCACCA-10b -CAGGAGACC
H3	
h22	AACGGCTCTTTTCAGAGCCACCAcaccccCAAGAAAGA
h19	AACGGCTCTTTTCAGACCCACCAcaaccccAAGAAAGA
Sp17/2	AACGGCTCTTTTCAGAGCCACCAcaaccccAAGAAAGA
Ph70	AACGGCTCTTTTCAGAGCCACCAcaaccccAAGAAAGA
Dm500	ATCGGTCCTTTTTCAGGACCACAA- 8b -CAATGAGAT
Xl-hi-1	AAAGGCTCTTTTCAGAGCCACAAcattccCAGTCAAAT
H2A	
h22	AACGGCCCTTATCAGGGCCACCAaatattCAAGAAAGA
h19	AACGGCCCTTATCAGGGCCACCAattactCACGAAAGA
Sp17/s	AACGGCCCTTATCAGGGCCACCAattactCACGAAAGA
CHO1	AA GCTCTTTTCAGAGCCACCA- 8b -CAGGAGACT
H2B	
h22	AACGGCCCTTTTCAGGGCCACCAaacatcCAAGAAAGA
h19	AACGGCCCTTTTCAGGGCCACCAaataatCAAGAAAGA
Sp17/2	AACGG--- ---CAaataatCAAGAAAGA
CHO2	AAAGGCTCTTTTCAGAGCCACCAtttgttCTAATAAAA
H1	
h22	AACGGCTCTTTTCAGAGCCACCAcatttcCACGAAAGA
h19	AACGGCTCTTTTCAGAGCCACCAaataatCAAGAAAGA
Dm500	ACAAGTCCTTTTCAGGGCTACAA- 8b -CAAGAGAAA
CONSENSUS	AACGGC[C/T]CTTTTCAG[G/A]GCCACCA 3' CAAGAAAGA

Table 1. Histone gene 3' homology blocks.

Genes h22, h19, Sp17/2 and Ph70 are from sea urcins; Dm500 is from Drosophila; pXlch4, pXlH4W1, pXbH4W1 and Xl-hi-1 are from Xenopus; mus-hi-1 is from mouse; CHO1 and CHO2 are from chicken (from ref. 57).

EXPRESSION OF HISTONES IN THE CELL CYCLE

As mentioned in the earlier section, the major class of histones is the replication-dependent histones. To understand the regulation of histone genes, one must understand a typical animal cell cycle. The observation that animal cells duplicate their DNA during a discrete interval in interphase allowed the cell cycle to be divided into four classical phases: G_1, S (DNA synthesis period), G_2, and M (mitosis). G_1 is the gap period between M and S. For most growing cell lines in tissue culture, the interval between divisions is 10-30 hours (Figure 3.).

Variation in cell cycle times among different cell types is mainly due to variation in the length of G_1, with the duration of S (6-8 hr) + G_2 (2-6 hr) + M (1 hr) being relatively constant. In addition, there is considerable variability in the length of G_1 among individual cells in a single population. This variability has been explained by phenotypic variation in cells at birth. An alternative model proposes that the nature of the cell cycle is such that cells switch from G_1 to S with a constant probability per unit time, thus creating an inherent G_1 variability. Differences in generation times among populations would be accounted for by differences in the magnitude of this probability (70). Animal cells can also exist in a nongrowing quiescent state during which they do not divide for long periods. Under most

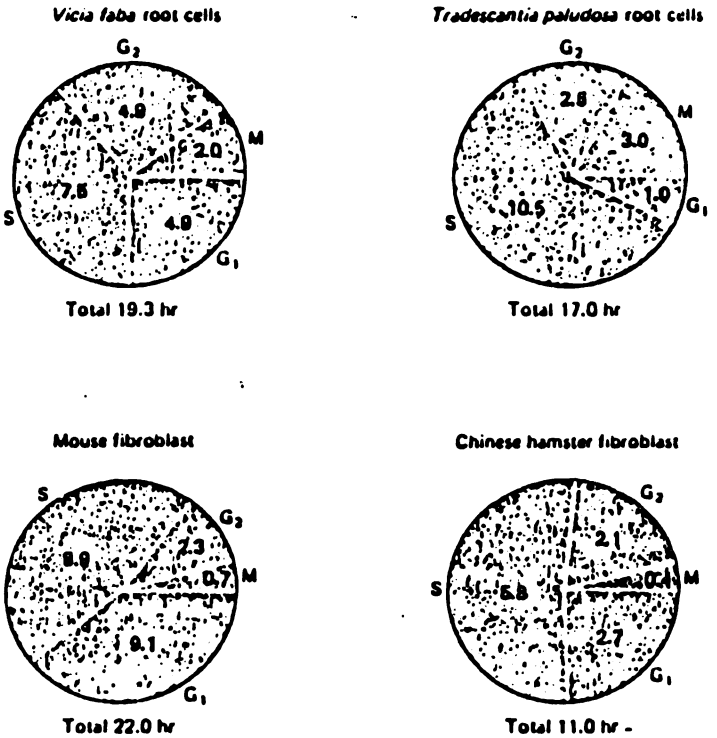


Figure 3. The cell cycle in various cell types (from ref. 184).

circumstances, normal cells that have ceased to grow have the G_1 content of DNA. Whether these cells have left the cell cycle to enter a qualitatively distinct G_0 state or are arrested in a prolonged G_1 is a subject of debate (71).

The crucial control events for the regulation of growth seem to reside in G_1. Evidence has accumulated for the existence of a restriction or commitment point in mid- to late G_1, at which time a cell decides whether to initiate DNA synthesis and undergo division or to cease proliferation. Environmental conditions influence this decision; suboptimal growth conditions shift normal cells into quiescence. Transformed cells can lose this restriction point control in whole or in part (72). After the restriction point, a cell proceeds through the rest of the cell cycle even after serum has been removed. The commitment point has been determined to be about 2 hr before the G_1/S boundary in 3T3 cells (131, 132, 133, 134).

In order to perform biochemical studies on the cell cycle, it is generally necessary to obtain a population of cells that is synchronous with respect to the cell cycle. This can be done by selectively detaching mitotic cells from the growth surface and replating them, by blocking cells at a specific point in the cycle with a drug and then releasing them, by using serum or amino acid limitation or growth to

confluence to shift cells into quiescence and then stimulating them to grow by addition of complete medium, by cell elutriation by size or by various combinations of these methods. When quiescent cells are stimulated to divide, an array of biochemical changes occur at various times before the initiation of DNA synthesis. It has been a difficult task to distinguish which of many observed changes during the G_0 to S or M to S transition are either necessary or sufficient for entry into S. Two approaches to the study of causal relationships in this process are the isolation of temperature-sensitive mutants that are blocked at a specific point in the cell cycle, and the study of the effects of drugs that inhibit specific biochemical processes. The study of differences between normal and transformed cells also suggests which biochemical parameters are important in growth regulation, as does the study of the factors that stimulate quiescent cells to proliferate (72).

Since the original observation that histone protein synthesis and DNA replication are tightly coupled (73), an extensive literature describing the regulation of histone expression during the mammalian cell cycle has been generated. Histone mRNAs coding for the replication-dependent histone proteins that are synthesized coordinately with DNA synthesis are encoded by a multigene family in animal cells. These histone mRNAs are formed by an endonucleolytic cleavage via

a mechanism which has an essential RNA component. Galli et al. (76) showed that the 12S nuclear fraction containing small RNAs of about 60 nucleotides in length could enhance the generation of 3' ends of sea urchin histones when they are injected into the frog oocyte functional nucleus. Other investigators later demonstrated that the 60 nucleotide RNA is a component of a SnRNP (small nuclear ribonucleo-protein), and both this SnRNP and the presence of a histone-specific dyad symmetry element (stem-loop) are required to yield genuine 3' ends of histone mRNAs (77, 78). Transcription must extend at least a few nucleotides past the 3' end of the gene, as it does in other genes transcribed by RNA polymerase II. The actual size of the transcription unit and whether there are precise termination sites remain to be evaluated (77, 97, 98).

The periodic fluctuations in synthesis of these replication-dependent histones in proliferating cells are paralleled by similar fluctuations in the levels of the corresponding mRNAs (79). This regulation must involve both transcriptional and post-transcriptional factors, since during the S phase there is a 15-fold increase in the levels of histone mRNAs in HeLa cells and this results from both an increased rate of RNA synthesis and a lengthening of the half-life of histone mRNAs. To determine the degree to which transcription and post-transcriptional processes are

responsible for histone mRNA accumulation, Heintz et al. (80) measured both the rates of the synthesis and the half-life of histone mRNA during or in the absence of DNA synthesis. Quantitation of nascent histone mRNA synthesized during a 5-minute pulse-labeling in vivo suggested that the rate of histone mRNA synthesis is 3 to 4 fold higher in cells at the point of maximal rate of accumulation of histone mRNA (2.5 hr into S phase) than cells blocked at the G_1/S boundary. Their studies of the half-life of the histone mRNA after a block in DNA synthesis (8 minutes) or during S phase (40 minutes) suggested that the stability of histone mRNA might be increased as much as 5 fold during DNA synthesis. Transcription rate measurements indicated that the triggering of histone mRNA synthesis occured in late G_1 at a point prior to initiation of DNA replication, and that this mRNA was synthesized at its maximal rate 3 to 5 hours before its peak of accumulation (118, 119).

Early work on yeast histone genes suggested that the periodic transcription of an H2A-H2B gene pair during the cell cycle required an ARS (autonomous replicating sequence) down stream from the H2B gene (74). More recent results, however, indicate that the ARS is dispensable for cell cycle regulation. Osley et al. (81) have localized the promoter sequences required for periodic expression by deletion analysis and further analyzed these isolated elements by

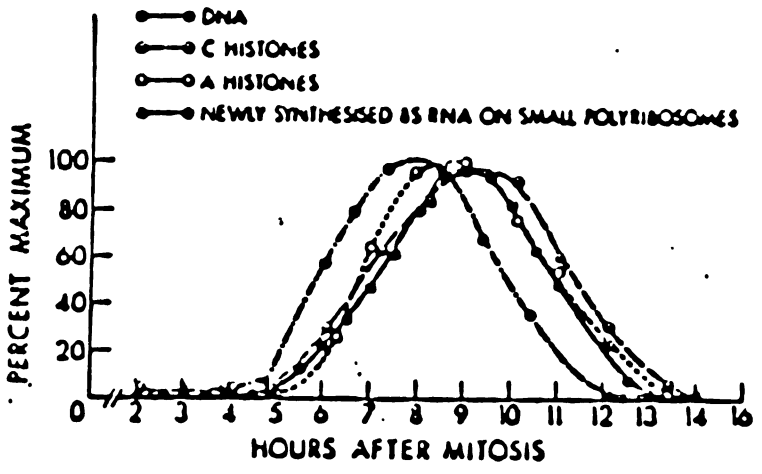


Figure 4. Rates of DNA replication, histone synthesis and the amount of newly synthesized 7-8S RNA (called 8S in the figure) associated with polysomes throughout the HeLa cell life cycle. This 7-8S RNA is histone RNA (from Borunet al. Biochemistry 58:1977-1983 (1967).

inserting them into a heterologous promoter. The cell-cycle-specific regulatory sequences are located in the 5' promoter region separating the divergently transcribed H2A-H2B genes at the yeast TRT1 locus. The TRT1 promoter contains a bifunctional upstream region of approximately 150 bp with two separable timing functions. Gene activation is achieved by a repetitive 16 bp sequence (UAS; upstream activating sites) that may constitute a histone specific UAS because it appears in two additional yeast histone promoters. Although the TRT1 UAS appears sufficient to account for the periodic pattern of histone gene expression, a second function related to cell cycle control is also found in the upstream promoter. This function (CCR; cell cycle regulation) contains negative regulatory sequences that periodically repress transcription during the cell cycle. A 17 bp sequence of dyad symmetry in the CCR element is responsible for cell cycle specific repression because deletion of the dyad from the TRT1 promoter results in elevated level of transcripts.

The human H4 histone gene is transcribed 3 to 10 fold more efficiently in nuclear extracts from S phase HeLa cells than in extracts from non-S phase cells (82). In contrast, transcription of other non-cell cycle regulated genes is equally efficient in S and non-S phase extracts. Competition studies suggest that the H4-specific transcription activity can be sequestered by preincubation with the H4 template DNA.

Mutational analysis indicates that maximal transcription of the human H4 gene requires, in addition to the TATA box and cap site, promoter elements between 70 and 110 nucleotides upstream from the transcription initiation site. These distal promoter elements are recognized preferentially in extracts from synchronized S phase HeLa cells (83). These results indicate the involvement of both an H4-specific transcription factor and distal promoter elements in the in vitro transcription of this gene and suggest that these components may be important for cell cycle regulation of this gene in vivo. When they introduced a human H4 gene into mouse L cells, Capasso and Heintz (84) have found that the 5' flanking region of the human H4 histone gene can function as a promoter in vivo and apparently contains enough DNA sequence information for its specific recognition by putative trans-acting factors of murine origin.

Sequence-specific DNA binding proteins acting on cis-regulatory control elements have been considered to be key elements in eukaryotic gene transcription. But DNA binding proteins with affinity for the 5' regulatory regions of cell cycle-dependent histone genes had not been identified until Dailey et al. (85) identified two proteins in an S phase HeLa cell nuclear extract that bound to separate regions of the human H4 histone gene promoter. Competition experiments with H4 promoter mutants and DNase protection assays demonstrated

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that these factors bound to regions of the H4 promoter that are essential for maximal expression in vitro. One of these factors (H4TF-1) binds to the sequences between 80 to 110 nucleotides upstream of the H4 cap site, whereas the other (H4TF-2) binds to the H4 subtype-specific sequence immediately upstream from the TATA box. They concluded that H4TF-1 and H4TF-2 are required for expression of the H4 histone gene and that they are necessary, if not sufficient, for cell cycle regulation.

A series of deletion, linker-substitution and point mutation studies of a human H2B histone gene promoter have identified a number of discrete functional elements, each of which is required for maximal levels of accurate transcription in nuclear extracts derived from HeLa cells (93). These elements are localized between 118 and 21 nucleotides 5' to the transcription initiation site. Elements recognized include (from 5' to 3') a series of direct repeats, a CCAAT homology, a hexamer sequence conserved among all human histone genes, an H2B-specific consensus sequence and a TATA box. The H2B-specific consensus sequence extends from -53 to -39 and is conserved among H2B promoters in sea urchin, frog , chicken and human (95). An essential part of this consensus sequence is the octamer element (ATTTGCAT). Mutations in the octamer element had no effect on transcription in cells arrested at the G_1/S phase boundary, but completely eliminated induction

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of H2B transcription as cells entered S phase. Conversely, mutations in the other promoter elements lowered transcription both in G_1/S arrested cells and S phase cells, but did not block induction upon entry into S phase.

Fletcher et al. (94) purified a 90Kd protein that binds specifically to this octamer element through the use of DNA affinity chromatography, and the factor was identified by renaturation of activity following sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified factor retained the ability to efficiently stimulate H2B transcription in a reconstituted in vitro system. This effect was dependent upon an intact octamer element and was observed in the absence of the other H2B promoter elements except the TATA box. Furthermore, this activity was not detected in nuclear extracts prepared from cells synchronized in G_2 phase. Therefore, this octamer-binding protein (OTF-1) might be the S phase specific, H2B transcriptional regulatory factor.

Dalton and Wells (91) investigated the role of the H1 gene-specific element (H1-box, 5'-AAACACA-3'), located about 100 bp upstream from the cap site, in H1 histone gene expression. When HeLa cell lines are transfected with chicken H1 histone genes, they exhibit S phase regulation of those genes. But deletion or base-substitution of the H1 element results in a 15 to 30 fold decrease in the level of H1 steady-

state mRNA and eliminates cell cycle control of transcription in synchronized cells. Transfection of multiple copies of H1-box elements into cells drastically decreases the chicken H1 mRNA levels. In contrast, introduction of mutated H1 elements into these cells has no detectable effect. These results imply that an interaction between the H1-box and a sequence-specific trans-acting factor modulates transcriptional control of H1 genes *in vivo*. Gallinari et al. (96) identified a second H1 subtype-specific element which is highly conserved among H1 genes for 18 bp and includes a CCAAT motif. This second element also acts positively to increase transcription *in vivo* and *in vitro*. They identified two distinct proteins in HeLa cell nuclear extracts, H1TF1 and H1TF2, which bind to the H1-box and the second H1 proximal subtype-specific consensus element, respectively. It seems likely that H1TF1 is related to the H1-SF complex reported by Dalton and Wells (91) in their analysis of proteins binding to the chicken H1 histone promoter *in vitro*. Although H1TF2 depends on an intact CCAAT sequence for binding, it is distinct from CCAAT-binding proteins in both molecular size and binding properties. Whether both of these two subtype-specific elements and their binding proteins are needed or one is sufficient for cell cycle regulation of H1 histone gene expression is not known at this time.

Not much is known about H2A and H3 histone genes as far

as promoter elements and specific binding proteins are concerned. Earlier studies by Artishevsky et al. (89) have shown that a DNA fragment derived from a hamster H3 histone gene, which contains about 1.1 kb of 5' flanking sequences as well as sequences encoding the first 20 amino acids of the gene product, confers cell cycle regulation to the coding sequence of the bacterial neomycin resistance gene. Later, they found that a 32 nucleotide region, located about 150 nucleotides upstream of the TATA sequence, contains crucial control signals for cell cycle regulation and that the coding region is not required (90).

Post-transcriptional regulation during the cell cycle is as important as transcriptional regulation because the rate of histone gene transcription changes to a much smaller extent than the steady-state level of histone (80, 99, 100). Further, the histone mRNA levels are rapidly reduced after treatment of cultured cells with DNA synthesis inhibitors like hydroxyurea or cytosine arabinoside (80, 100-105). In the absence of DNA synthesis, the half-life of histone mRNAs becomes much shorter than the normal histone mRNA half-life. This destabilization can be counteracted by treating cells with protein synthesis inhibitors. In fact, protein synthesis inhibitors can superinduce histone mRNAs by increasing histone mRNA stability both in the presence and absence of DNA synthesis (100, 105-108). These results suggest that histone

	GENE	UPSTREAM SEQUENCE ELEMENT III	UPSTREAM SEQUENCE ELEMENT II	UPSTREAM SEQUENCE ELEMENT I
H1	*X. laevis* X1h1	(-305)CTGGAGCTGTTCCGG-CCAATCTCTGTAACC	(-165)TTTTTGAGACTCTGGAAACACAGACCG	(-105)ATGGGCGGGGT
	X. laevis X1h3	(-305)TCAGGGCTGTTTTGGGACAATCCCTCCAACC	(-170)TTATTGAGAGCCTGGAAGCACAGAATG	(-110)AATGGCGTGAC
	X. laevis XLH#2	insufficient sequence	(-155)TTTTCCAGACTCTAAAAACACAGACTC	(-100)ATGGGCGGGGT
	X. laevis XLH#3	insufficient sequence	(-155)TTTTCCAGACTCTAAAAACACAGACTC	(-100)ATGGGCGGGGT
	Human	(-100)TTGGAGCTTCAAACTGCCAAATTCTGTA-CC	(-135)ATTTTGAGGACTAACAAACACAATTTG	(-100)AUCGGCGGGCC
	Chicken	insufficient sequence	(-125)TTTTCGCCTGTTAAGAAACACAAAATA	(-105)AATAGCGGGGA
	Consensus	TTGGAGCTGTTTYGGGCCAATCTCTGTAACC	TTTTYGAGACTCTAGAAACACAGACTG	ATGGGCGGGGT
	Protein Factor	H1TF2(ref. 96)	H1-SF, H1TF1(ref. 91 and 96)	
H2A	*X. laevis* X1h1		(-180)CACTGACGAATTTATG	(-80)ATTGGCTGC
	X. laevis X1h3		(-185)CACTGGGACATTTTCG	(-95)GTTGGCTGC
	X. laevis X1h11		insufficient sequence	(-90)GTTGGCTGG
	Human		(-180)AAATAGAGTATTTCTC	(-85)CTTGGCGAA
	Chicken		(-165)CACTCTTTCATTGGTC	(-95)ATTGGCTGC
	P. miliaris		(-145)CACCA-TTCACTAGTG	(-85)ATTGGACAA
	Consensus		CACTkRYNCATTTNTG	ATTGGCTGC
H2B	*X. laevis* X1h1		(-85)GTCATGTGACAAAA	(-45)CTGCCTCGTTTGCATGGG
	X. laevis X1h3		(-90)GTCATGCCACAGAG	(-50)GCCAGTGCTTTACATGGG
	X. laevis X1h11		(-90)ACCATGTGACAAAA	(-60)CTGCCTTATTTGCATGGG
	Human		not found	(-35)TCACCTTATTTGCATAAG
	Chicken		not found	(-45)GCTTCTCATTTGCATAGA
	P. miliaris		not found	(-75)GAGCCTCATTTGCATACG
	Consensus		GTCATGTGACAAAA	GYGCCTCATTTGCATRGG
	Protein Factor			OTF-1(ref. 94)
H3	*X. laevis* X1h1			(-100)AACCAATAAGA
	X. laevis X1h3			(-90) AACCAATCAAA
	X. laevis X1h11			(-120)GAGCAACATCC
	Human			(-100)GGCCAATCCAA
	P. miliaris			(-90) GACCAATCAAG
	Consensus			GACCAATCAAA
H4	*X. laevis* X1h1-c	(-185)ATACCATATAATTGACACATCGTTG	(-100)ATCTAGAAGGAAGC	(-60)GTTCCCTATCAG
	X. laevis X1h1-d	(-290)ATACCATATAATTGGCACTTCATTG	(-100)AACTACACGGAAGC	(-60)GTTCCCTATCAG
	X. laevis X1h3	(-290)ATAACATACTTTGTGCGCTTTACAG	(-100)AACT-CGTAGAAGC	(-60)GTTCCCTATCAG
	X. laevis X1h11	insufficient sequence	(-95) AAC-ACACTGATAT	(-50)GTTCTCTGTCAG
	Human	not found	not found	(-60)GTTCTCAGTTCG
	Chicken	insufficient sequence	not found	(-60)GTTT-CAATCAG
	P. miliaris	not found	not found	(-45)GTCCGCAATGGT
	Consensus	ATACCATATAATTGGCACTTCATTG	AACTACACGGAAGC	GTTCCCTATCAG
	Protein Factor		H4TF1(ref. 85)	H4TF2(ref. 85)

Table 2. Histone gene class-specific upstream sequence elements and binding protein factors

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mRNA stability is sensitive to DNA synthesis or deoxyribonucleotide metabolism and that a short-lived protein may be involved in histone mRNA degradation. However it has not yet been shown that the regulatory mechanisms detected in these inhibitor experiments are the same ones controlling histone mRNA levels during a normal cell cycle.

Luscher et al. (109) showed that fusion of the SV40 early promoter to a DNA fragment containing the 3'-terminal half of the mouse H4 histone gene, including 230 bp of spacer sequences, led to the regulated expression of SV40/H4 fusion RNA. They suggested that the sequences in the 3' terminal part of the mouse H4 histone gene can regulate gene expression during the cell cycle, so long as they are not positioned further away from the terminus than normal. Various fragments from the 3' end of this gene were introduced into transcription units controlled by the SV40 early promoter. Mutational analyses of the H4 gene 3' flanking region indicate that the minimal sequences necessary for this regulation are contained within an 80 bp fragment which contains two histone-specific, highly conserved sequence elements (110). These are located at the 3' end of histone mRNA (stem-loop) and in the adjacent spacer region.

Several groups have investigated the importance of the stem-loop structure in post-transcriptional regulation of

histone genes (111, 112, 113). Pandey and Marzluff (113) made chimeric genes by fusing mouse histone genes with the human globin gene. The genes were introduced into mouse L cells and the stability of the chimeric mRNA was measured when DNA synthesis was inhibited. An mRNA containing all the globin coding sequences and the last 30 nucleotides of the histone mRNA was degraded at the same rate as histone mRNA. They concluded that the stem-loop structure is necessary and sufficient for the regulation of histone mRNA stability. However, histone mRNAs engineered to have a longer 3' untranslated region than normal or to be polyadenylated are not regulated, even though they contain the normal 3' stem-loop structure (116).

Ross et al. (114) suggested that histone mRNA is degraded in a 3' to 5' direction when they detected two sets of short-lived histone mRNA decay products. Luscher and Schumperli (115) showed that a heat-labile component of the mRNA processing apparatus, identified in HeLa cell nuclear extracts, was limiting in extracts from G_1 arrested cells. They also found that nuclear histone mRNA precursors accumulated in G_1 arrested cells, and that this activity was in excess in extracts from exponentially dividing cells. These findings have led them to suggest that these fluctuations in heat-labile activity may contribute to cell cycle dependent histone gene expression.

In summary, replication-dependent histones are regulated both at the transcriptional level and at the post-transcriptional level (117). At the transcriptional level, trans-acting protein factors and histone subtype-specific sequences in the 5' flanking region of the gene are usually essential for proper regulation. At the post-transcriptional level, RNA processing factor(s) and the stem-loop structure in the 3' untranslated region of the histone mRNA are important.

The H5 histone gene is a divergent member of the H1 histone gene family which lacks an H1-box but contains a remnant of the 3' stem-loop structure. However, H5 histone mRNA is polyadenylated at a site further downstream (122). These two differences may affect the transcriptional and the post-transcriptional regulation of H5 gene expression in comparison to that of typical H1 histone genes (91, 121). During the cell cycle, the steady-state level of the tissue-specific chicken H5 histone mRNA remains relatively constant. According to <u>in vitro</u> pulse-labeling experiments (120), transcription of the H5 histone gene is not initiated at any particular stage of the cell cycle but is constitutive.

Replication-independent histone genes, such as H3.3, contain intervening sequences and their mRNA is polyadenylated (123, 124, 125). The steady-state level of H3.3 mRNA is

nearly the same in all tissues and ages of animals examined (124) and it is not affected by inhibitors of DNA or protein synthesis in cultured cells (103, 126). Thus, factors which induce cell cycle-specific regulation of the H3.2 gene appear not to act to regulate H3.3 mRNA.

It has been suggested that histone biosynthesis is subject to some form of autoregulation (127, 128, 129). Peltz and Ross (130) showed that human H4 histone mRNA was degraded 4 to 6 fold faster in reaction mixtures containing core histones and a cytoplasmic S130 fraction than reaction mixtures lacking these components and suggested that accelerated histone mRNA degradation occured as a result of an autogenous negative regulatory circuit triggered by the accumulation of free histone proteins in the cytoplasm. At the end of S phase, when histones are no longer required for nucleosome formation, newly synthesized histones may accumulate in the cytoplasm until they reach a critical concentration at which they induce accelerated histone mRNA degradation. Similarly, DNA synthesis inhibitors may reduce histone mRNA stability by increasing pools of free histone proteins which have no available DNA to which to bind. This would correlate with the fact that concurrent inhibition of protein synthesis (presumably blocking histone protein production) reverses the decrease in histone mRNA stability induced by the DNA synthesis inhibitors. However, a specific

molecular mechanism by which free histone protein may alter histone mRNA half-life has not yet been elucidated.

OTHER CELL CYCLE REGULATED GENES

It has been known for some time that the activities of many enzymes involved in DNA replication increase as cells enter S phase and decrease after the completion of DNA synthesis. The S phase-specific enzymes, such as thymidine kinase (135), dihydrofolate reductase (136), thymidylate synthetase (137), ribonucleotide reductase (138), and DNA polymerases (139), follow a similar pattern of increasing activity through S phase with a maximum near the S/G_2 boundary.

Because dihydrofolate reductase (DHFR) and its mRNA are present at low levels in normal mouse fibroblast 3T6 cells, most DHFR studies have been conducted with a methotrexate-resistant derivative of mouse 3T6 fibroblasts that overproduces the enzyme and its mRNA by a factor of 300 but regulates the level of the enzyme during the cell cycle in the same manner as normal 3T6 cells (140). Kaufman and Sharp (141) reported that the level of DHFR in growing cells is approximately 10 times that in stationary cells. More specifically, commencing within two hours into S phase and continuing throughout the duration of S phase, there is a 90% increase in DHFR specific activity. This results from a 2.5 fold increase in the level of DHFR, while total soluble protein increases 50% during the same period. This increase is the result of new synthesis of DHFR molecules initiated

after the cell is physiologically commited to DNA replication, and the maximum peak of DHFR activity is coincident with the maximum rate of DNA synthesis within the last 6-7 hours of S phase (142). When resting cells were serum stimulated in the presence of DNA synthesis inhibitors, the increase in DHFR synthesis was the same as in control stimulated cells (140, 143) indicating that there is no tight coupling between DNA synthesis and DHFR gene expression. Actinomycin D inhibits the increase in DHFR accumulation if added 8 hr after stimulation but has no effect if added 16 hr after stimulation. This is consistent with the idea that the increase in DHFR gene expression requires increased transcription of the gene, and that DHFR mRNA synthesis begins at about the time the cell initiates DNA replication. Using mouse 3T6 fibroblasts containing amplified DHFR genes, Johnson and colleagues (144, 145, 146) found that DHFR mRNA production is controlled by regulating the rate of transcription in cells undergoing a serum-induced transition from the resting to growing state. However, Kellem and co-workers have come to the opposite conclusion (147, 148). They examined growth-stimulated, DHFR amplified mouse S180 cells and concluded that the appearance of cytoplasmic DHFR mRNA depends on the relative stability of nuclear DHFR RNA and is not dependent on the rate of transcription. The discrepancy between these two reports could be due to the differences in the experimental protocols used to measure transcription rate or

to differences in the physiological states of cells in which synchrony was induced. Farnham and Schimke (149) also showed that the transcription rate of DHFR gene is low in G_1, increases 7 fold at the beginning of S phase, decreases almost immediately thereafter, and remains low throughout the remainder of S and into G_2. This cell cycle regulation seen in the G_1 to S phase transition is achieved by increasing the rate of transcription from a single promoter region which is similar to promoter regions of other housekeeping genes.

Thymidine kinase (TK) has two mammalian isozymes: a minor mitochondrial isozyme which does not fluctuate during the cell cycle and the cellular isozyme which reaches a high level in cycling cells (150). The activity of TK increases by 10-20 fold at or near the G_1/S border (151, 152). Like DHFR, the increase in TK level is not blocked when cells are stimulated in the presence of inhibitors of DNA synthesis, indicating that there is no tight coupling between these two processes (153). But when cells are treated with cycloheximide, the level of TK decreases with a half-life of 4-5 hours, indicating that the enzyme is relatively unstable. Increases in TK enzyme levels seen after stimulation are paralleled by an equivalent increase in the steady-state levels of TK mRNA (154). Furthermore, this induction of TK is inhibited by actinomycin D suggesting that induction may primarily be at the level of transcription initiation (152). When the

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promoter of the chicken TK gene is replaced by the promoter of the herpesvirus TK gene, which is not regulated during the cell cycle, the regulated pattern of expression is retained (155). Hofbauer et al. (156) showed similar results when the cDNA for mouse TK was linked to the herpesvirus TK promoter. Using human TK cDNA, Stewart et al. (157) also suggested that the body of the TK cDNA is sufficient to insure cell cycle-regulated expression, regardless of the promoter or polyadenylation signal used. While a few groups (158, 159) suggested that the principal control of TK gene expression is post-transcriptional, other groups (157, 160) showed that TK gene expression is controlled at both the transcriptional and post-transcriptional level during the mammalian cell cycle. They showed that the increase in transcription rates in growth-stimulated cells is at most 2-4 fold, but that the level of TK mRNA increases more than 20 fold. The half-life of TK mRNA is 8-12 hours in the S and M phases and decreases as cells enter quiescence. Based on these results, they concluded that the appearance of TK mRNA at the beginning of the S phase in serum-stimulated cells is controlled not only by the rate of gene transcription but also by the decreased rate of mRNA degradation.

When resting mouse 3T6 fibroblasts are serum-stimulated to re-enter the cell cycle, thymidylate synthetase (TS) activity remains at the level found in resting cells until 12

hours following stimulation. It then increases sharply as the cells enter S phase (137). Enzyme activity increases about 20 fold over that of resting cells by 30 hours following stimulation and continues to increase linearly for at least another 30 hours. The increase is blocked by inhibitors of protein or RNA synthesis, suggesting that the increase in TS activity is the result of de novo synthesis of the enzyme and its mRNA. However the increase in TS activity is not affected by the presence of DNA synthesis inhibitors, indicating there is no tight coupling between the increase in TS gene expression and DNA synthesis itself. Since TS and its mRNA represent only a tiny fraction of total cellular protein and mRNA, Jenh et al. (161) isolated a 3T6 cell line that is resistant to 5-fluorodeoxyuridine (FdUrd) and that overproduces TS and its mRNA about 50 fold. This cell line still regulates the expression of the TS gene in the same manner as the parental cells. Using a pulse-labeling experiment, they showed that the rate of synthesis of TS protein increased 8-9 fold by 25 hours after serum-stimulation and the half-life of TS in growing cells was greater than 24 hours. TS mRNA increased 20-40 fold as cells progress from resting to late S phase. The increase in TS mRNA was the result of an 8 fold increase in the rate of production. The half-life of TS mRNA was similar in resting and growing cells and the rate of transcription of the TS gene, as determined in isolated nuclei, increased only by a factor of three to

four during the S phase (162). Since the content of the message increased to a much greater extent than the rate of transcription of the gene, post-transcriptional controls must also play a role in regulating the content of TS mRNA under these conditions. From these results, they suggested that the cell may regulate the distribution of thymidylate synthetase mRNA between a relatively stable poly(A)$^+$ RNA species and a labile poly(A)$^-$ RNA species.

Ribonucleotide reductase is the enzyme responsible for conversion of ribonucleotides to deoxyribonucleotides. This enzyme has always been an obvious candidate for cell cycle dependency, but the degree of cell cycle regulation is unclear. It has been documented by some as having a strict correlation with S phase (163), while others found ribonucleotide reductase activity in the S, G_2 and M phases (138). When G_1 arrested cells were allowed to progress to S phase, ribonucleotide reductase activity increased in parallel with [^{3}H]-thymidine incorporation into DNA. The cell cycle pattern of ribonucleotide reductase activity involves negligible levels in G_1 phase, a progressive increase of activity upon entry into S phase (paralleling overall DNA synthesis), continued retention of significant ribonucleotide reductase activity well into the metaphase period of mitosis, and a very rapid decline in activity during the later phases of mitosis (164). The enzyme is composed of two dissociable

subunits, proteins M1 and M2, which are inactive alone, but are fully active when combined. Cells in G_1 phase have decreased ribonucleotide reductase activities and decreased protein M2 activity, but the levels of protein M1 activity were almost constant (165). A 3-7 fold increase in the concentration of active protein M2 was observed when cells passed from the G_1 to S phase of the cell cycle. Pulse-chase experiments showed that the half-life of protein M1 was 15 hours and that of protein M2 was 3 hours (166). Therefore, ribonucleotide reductase appears to be primarily regulated during the cell cycle by the level of protein M2.

Not all cell cycle regulated genes are specific to the S phase of the cell cycle. Examples of some G_1 phase dependent genes include the c-fos and c-myc genes, both of which are transiently activated within minutes after quiescent fibroblasts or lymphoid cells are stimulated to enter the cell cycle (167-171). Specifically, c-fos transcription transiently increases more than 15 fold very soon after stimulation, returning to its initial levels within 30min. No further changes in c-fos transcription are observed as the 3T3 cells continue to progress through the cell cycle from G_1 to S phase. On the other hand, expression of c-myc is induced more than 20 fold 1 hr after stimulation with serum followed by a slow decrease until reaching the basal level of quiescent cells after about 18 hours. Induction of c-fos clearly

preceded the activation of c-myc mRNA expression and was detectable as early as 5 min after stimulation, while c-myc transcripts appeared at significantly elevated levels after 30 min of stimulation and reached the maximum after 1 hr (170). This induction of c-fos and c-myc mRNA occurs in the presence of cycloheximide and, therefore, does not require the synthesis of new protein species (168, 172). These proto-oncogenes encode post-translationally modified nuclear proteins (173). The study of fusion genes by Treisman (174) showed that in addition to the 5' activating element, transient accumulation of human c-fos RNA following serum stimulation requires sequences at the 3' end of the human c-fos gene. These findings may be related to recent observations that exposure of resting monocytes to inhibitors of protein synthesis induced a rapid and marked (300 fold) increase in c-fos mRNA levels, despite only a 9 fold increase in c-fos transcription, and that such exposure prolonged the half-life of c-fos mRNA (175). Thus, while post-transcriptional control is responsible for the limiting c-fos mRNA levels in both resting and activated cells, transcriptional mechanisms are responsible for the transient increase in c-fos expression after stimulation.

The c-myc gene was found to be transcribed at a high rate in resting cells, although the level of mature c-myc mRNA level is barely detectable (176). When these resting cells

are stimulated by growth factors, the early and dramatic increase in c-myc mRNA levels that occurs is not accompanied by any appreciable changes in the transcription rate of the c-myc gene. These findings suggest the post-transcriptional regulation of c-myc expression at the level of mRNA degradation. It should be mentioned that Thompson et al. (177) insisted that the transient increase in c-myc mRNA levels following the activation of quiescent cells was not due to cell cycle-dependent regulation. They showed that although c-myc mRNA does undergo a transient increase within 2 hours of serum stimulation of quiescent cells, the level of c-myc mRNA is constant throughout the cell cycle and does not diminish in density-arrested cells maintained in the presence of serum growth factors. Before cells can proliferate, they must be activated by growth factors to a state where they are competent to enter the cell cycle. They argued that the transient increase in c-myc mRNA levels could be the result of this activation process, rather than the result of the regulation during the proliferative cell cycle. They also showed that the synthesis, half-life and modification of c-myc proteins are constant throughout the cell cycle of normal and transformed cells (178). This discrepancy could also be due to the differences in the experimental protocols used to measure the amount of c-myc mRNA, to differences in effects of different mitogens in stimulating resting cells, or to degree of synchrony of cells.

Histone genes have been shown to be regulated during cell cycle. We have chicken histone genes isolated in our lab and one of them is H3.3 which is a replacement variant gene. These isolated chicken histone genes along with Rat 3 cells which have a selective marker open the possibility of an in depth study of cell cycle regulation of histone genes.

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CHAPTER 2

Materials and Methods

Materials

Restriction enzymes, calf alkaline phosphatase, T4 DNA ligase, T4 polynucleotide kinase, RNase-Free DNase I, S1 nuclease, RNasin, RNase A and RNase T1 were obtained from following sources: Bethesda Research Laboratories, USB (United States Biochemical Corporation), IBI (International Biotechnologies, Inc.), Promega Biotec, New England Biolabs or Boehringer Mannheim. Aphidicolin was obtained from Sigma and T3 RNA polymerase was obtained from Stratagene. The plasmid containing human thymidine kinase (TK) cDNA, the plasmid containing rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and Rat 3 cells were gifts from Dr. Susan E. Conrad.

Methods

Most of the cloning procedures listed below follow the protocols outlined by Maniatis, Fritsch and Sambrook (1).

Cell Culture

Rat 3 cells (2), which lack cytoplasmic thymidine kinase, were maintained in Dulbecco Modified Eagle's Medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10%

calf serum. These cells grow well in normal media but can be easily characterized by their inability to grow in a selective medium containing HAT. This medium contains 110 μM hypoxanthine, 20 μM thymidine and 2 μM aminopterin. Aminopterin inhibits dihydrofolate reductase thereby causing a block in the main pathway of thymidine phosphate and purine nucleotide synthesis (3). In the presence of an exogenous source of thymidine kinase, Rat 3 cells can grow normally in HAT medium.

For synchronization, the medium was removed after the cells reached confluence, and it was replaced by medium containing 0.1% calf serum. Cells were allowed to incubate for 48 hours to obtain synchrony in G_0/G_1. For serum stimulations, fresh medium containing 10% calf serum was added. At various times after the stimulation with serum, cells were harvested for RNA analysis.

DNA Transfection and HAT Selection

The transfection protocol is that of Wigler et al. (4). Twenty four hours before transformation, Rat 3 cells were plated to a density of $5x10^5$ cells per 100 mm tissue culture plate. Approximately 1 microgram of a plasmid containing human TK cDNA and 10 micrograms of histone plasmid were ethanol precipitated along with 10-20 micrograms of high molecular weight carrier DNA (Rat 3 DNA). The DNA was resuspended in 0.45 ml of sterile double distilled water (dd

H_2O), and adjusted to a final concentration of 250 mM $CaCl_2$ by addition of 0.05 ml of 2.5 M $CaCl_2$. The DNA/$CaCl_2$ mixture was rapidly added to an equal volume of 2X HBS (Hepes-buffered saline; 280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.05-7.15). The DNA-calcium phosphate precipitate was allowed to form for 20-30 minutes at room temperature. One ml of this mixture was added to one 100 mm plate containing 10 ml of medium. After about 16 hours this mixture was removed and replaced with fresh medium without HAT. After an additional 24 hours the medium was removed and replaced by HAT containing medium. This was changed to fresh HAT-containing medium every 3-4 days until HAT resistant colonies were clear (about 2 weeks).

RNA Isolation

Total RNA was prepared from tissue culture cells as follows. Cells were washed twice with phosphate buffered saline (PBS) without calcium and magnesium. One ml of lysis buffer (100 mM Tris-HCl, pH 7.5; 12 mM EDTA; 150 mM NaCl; 1% sodium dodecyl sulfate) containing 200 micrograms per ml of proteinase K was added to each plate. DNA in the cell lysate was sheared by passage through a 22-gauge needle and the lysate was transferred to a tube. This solution was incubated at 37°C for 45 minutes and then extracted with 50:50 v/v phenol: chloroform. Sodium acetate was then added to 0.3 M and the solution was ethanol precipitated. Samples were spun

in a Sorvall RC-2 centrifuge at 10,000 RPM for 20 minutes, the ethanol poured off and pellets allowed to air dry. Pellets were resuspended in 400 microliters of RNase-free TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and transferred to Eppendorf tubes. Four microliters of 1 M $MgCl_2$, 100 units of RNasin and one microliter of a 1 mg/ml solution of RNase-free DNase I was added, and the tubes were incubated at 37°C for 30 minutes. Next, 16 microliters of 0.5 M EDTA and 20 microliters of 20% sodium acetate was added, this mixture was extracted twice with 50:50 v/v phenol:chloroform, and the aqueous phase was ethanol precipitated at -70°C. RNA was then pelleted in a microcentrifuge at 4°C for 15 minutes and pellets were dried in a vacuum pump dessicator. Pellets were resuspended in 150 microliters of 20% sodium acetate and spun for 10 minutes in a microcentrifuge at 4°C. Supernatants were discarded and the remaining pellets were resuspended in 100 microliters of TE and then ethanol precipitated after the addition of 10 microliters of 20% sodium acetate. For determination of optical density, samples were spun down at 4°C in a microcentrifuge for 15 minutes, drained, dried and resuspended in 200 microliters of RNase free dd H_2O. 5 microliters of each sample was diluted into 500 microliters of dd H_2O and optical density was read at 260 nm. One O.D. is equivalent to 50 μg/ml of RNA.

S1 Nuclease Analysis

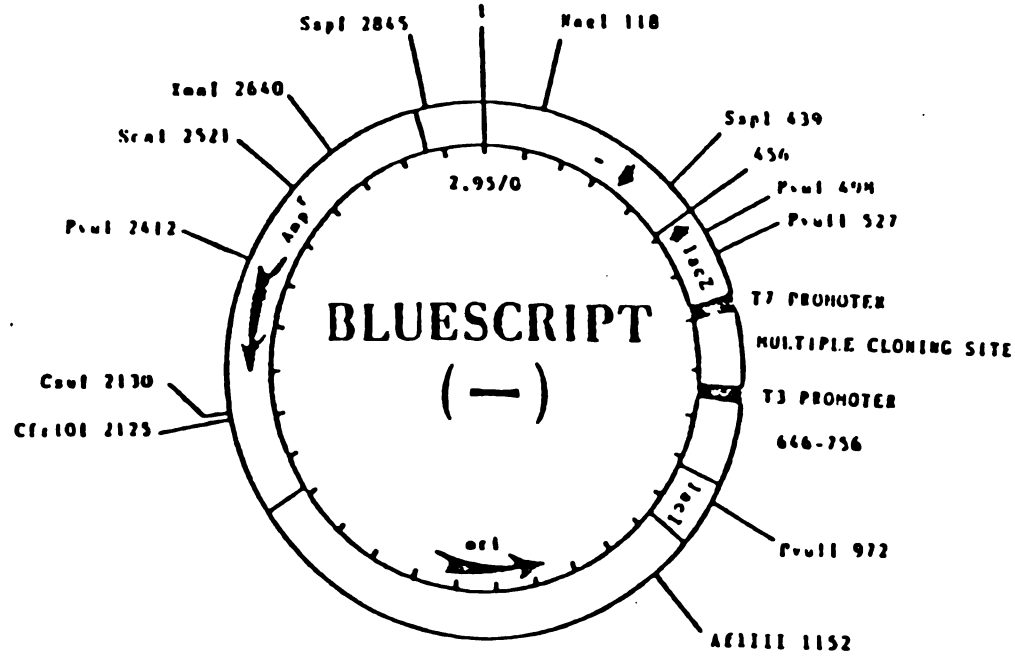
Various DNA probes for different histone genes were used to analyze the RNA samples obtained. For example, pCH1a-SH4 was used to analyze the RNA transcribed from one of the H3.2 histone genes. In this case, the SalI restriction enzyme site present at +63 was used. Twenty micrograms of the plasmid pSH4 was digested with 20 units of SalI for 4 hours at 37°C, then the terminal phosphates were removed by incubation with 2 units of calf alkaline phosphatase at 37°C for 1 hour. This DNA was radioactively labeled by treatment with [γ-^{32}P]ATP and 3 units of T4 polynucleotide kinase at 37°C for 1 hour. The DNA was then digested with 20 units of the restriction enzyme HindIII to remove the unwanted labeled end. The DNA was separated on a 1.2% agarose gel, and the desired fragment was isolated. The labeled DNA fragment was mixed with the RNA being studied and both were ethanol precipitated. The pellet was resuspended in 20 microliters of hybridization buffer (80% formamide; 0.4 M NaCl; 0.04 M Pipes, pH 7.25). The sample was heated at 90°C for 5 minutes to denature both the RNA and the DNA probe. The sample was allowed to hybridize at 55°C for 12 hours. After the hybridization was completed, 300 microliters of S1 buffer (0.03 M sodium acetate, pH 4.5; 0.25 M NaCl; 4 mM $ZnSO_4$; 50 micrograms/ml denatured, sheared salmon sperm DNA) was added to stop the reaction. The sample was then split into two tubes and 100 and 200 units of S1 nuclease were added to each tube. The reaction was allowed to proceed for

15 minutes at 37°C, then was stopped by extraction with an equal volume of 50:50 v/v phenol:chloroform mixture. The supernatant was ethanol precipitated. The reaction products were analyzed on a 6% denaturing polyacrylamide gel.

RNase Protection Assay

Various RNA probes were used in the RNase protection assay as described in the Figure legends. Usually, a DNA fragment containing the 5' portion of a histone gene and some of its flanking region was cloned into a Bluescript vector (obtained from Stratagene, La Jolla, CA.) which has multiple cloning sites in between T3 and T7 promoters (Figure 5). The RNA probe was made by *in vitro* transcription using T3 or T7 RNA polymerases. The *in vitro* transcription was done according to the manufacturer's recommendation. The reaction mixture includes transcription buffer (40 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 2 mM spermidine; 50 mM NaCl), 1 microgram of restricted, proteinase K-treated DNA template, 0.4 mM rATP, 0.4 mM rCTP, 0.4 mM rGTP, 30 mM DTT (Dithiothreitol), 25 units of RNasin, 5 microliters of 800 Ci/mM, 10 mCi/ml [α-^{32}P]rUTP, and 10 units of T3 or T7 RNA polymerase in a final volume of 25 microliters. The reaction mixture was incubated at 37°C for 30 minutes. Following the RNA synthesis reaction, 1 microliter of 1 mg/ml RNase-free DNase I was added to remove the DNA template followed by incubation at 37°C for 15 minutes. Extraction with an equal volume of a 50:50 v/v

phenol:chloroform mixture and ethanol precipitation followed. The pellet was then resuspended in 100 microliters of 0.15 M sodium acetate, precipitated with ethanol again, and resuspended in 50 microliters of DEPC-treated dd H_2O. The labeled RNA transcript was mixed with the RNA isolated from Rat 3 transformants and both were ethanol precipitated. The pellet was resuspended in 30 microliters of hybridization buffer (80% formamide; 0.4 M NaCl; 0.04 M Pipes, pH 7.25). The sample was heated at 90°C for 5 minutes to denature both RNAs. The sample was allowed to hybridize at 55°C for 12-16 hours. Following the hybridization, 300 microliters of RNase buffer (0.3 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA) containing RNase A (40 micrograms per ml) and RNase T1 (2 micrograms per ml) were added, and the reaction was incubated at 37°C for 1 hour (5). The RNase digestion was terminated by the addition of 20 microliters of 10% SDS and 50 microliters of 10mg/ml proteinase K and followed by an additional incubation at 37°C for 15 minutes. The reaction mixture was extracted with an equal volume of phenol:chloroform (1:1) and the ^{32}P-labeled RNA was precipitated with ethanol (sometimes with the addition of carrier tRNA). The pellet was washed with 70% ethanol, dissolved in a loading buffer containing 90% formamide and analyzed by denaturing polyacrylamide gel electrophoresis (6).



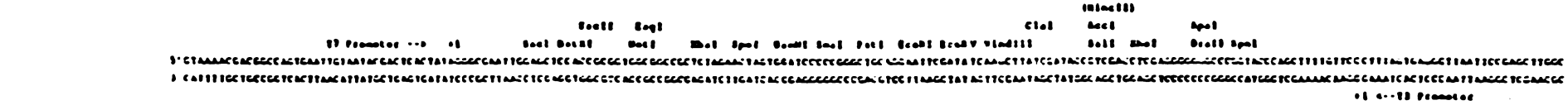


Figure 5. KS Polylinker

DNA Sequencing

pCH1a-SH4 and pCH1a-SH12 were sequenced by the chemical degradation method of Maxam and Gilbert (6) as modified by Smith and Calvo (7). Both plasmids were digested with EcoRI to linearize the circular DNA and the DNA was labeled as described above for S1 probe preparation. The labeled DNA was digested with SalI, and the desired fragment was gel isolated.The end-labeled DNA fragment was treated as described (6, 7), and the reaction product was run on an 8% denaturing polyacrylamide gel followed by autoradiography of the gel.

Cell-Cycle Analysis

Rat 3 cells stably transfected with foreign DNA fragments were grown to confluence before being incubated in a medium containing 0.1% calf serum for 48 hours. Cells were harvested by trypsinization and centifugation at 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 hours after the serum stimulation. Cells (up to $2x10^6$) were resuspended in 200 microliters of medium. 400 microliters of buffer A (0.1% v/v Triton X-100; 190 mM sucrose; 0.1 mM EDTA; 40 mM citric acid; 20 mM sodium phosphate, dibasic) was added and cells were allowed to equilibrate overnight at 4°C. 400 microliters of freshly made buffer B (100 mM NaCl; 9 mM citric acid; 10 mM sodium phosphate, dibasic; 0.002% acridine orange) were added 30-45 minutes before analysis. Cells were then analyzed for their DNA content in a cell sorter (8).

Densitometric Analysis

Since it is critical to compare the level of mRNA of interest in different stages of cell cycle, an LKB 2222-010 UltraScan XL Laser Densitometer (Bromma, Sweden) was used to measure the level of mRNA quantitatively. Protected bands of expected size in a radiogram were monitored along the lanes and peaks were compared.

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CHAPTER 3

Results

In order to analyze the contribution of various DNA elements to cell cycle regulation of the chicken histone genes, we needed to identify a system which had the following properties: 1. Cells must grow efficiently in culture (preferably as an established line) and be readily transfected with exogenous DNA. 2. Transfected cells must be able to be synchronized and induced to go through at least one round of the cell cycle in a reasonably coordinate fashion. 3. Expression of exogenous chicken histone genes must be measurable without interference from endogenous histone gene expression. 4. Exogenous wild type chicken histone genes must show their normal pattern of expression after transfection.

Rat 3 cells, which lack cytoplasmic thymidine kinase (TK), were cotransfected with a DNA fragment of interest and a human TK cDNA plasmid. When transfected cells were selected in HAT medium, they were allowed to grow together in the same plate for a mass culture or individual colonies were transferred into other plates. For synchronization, they were allowed to grow to confluence before changing medium to 0.1% serum containing medium. Cells were incubated for 48 hours

and then subjected to serum stimulation.

Expression of Chicken H4 Histone Gene in λCH1a DNA

λCH1a was isolated previously in our lab from a chicken DNA library (1). The restriction map of λCH1a is shown in Figure 6. This phage DNA was introduced into Rat 3 cells with a plasmid containing human TK cDNA. It was shown previously that (from left to right) one H4, two H3.2 and the H2B histone genes were appropriately regulated during the cell cycle (Figures 19 and 20). As an example, the test for cell cycle regulation properties of the second (from left) H4 histone gene will be discussed. The H4 histone gene in pCH1a-RH4.6 was tested for expression with a cell line called CH1a-1 which is a Rat 3 cell line transfected with λCH1a DNA and human TK cDNA. The cells were grown to confluence and then incubated in a medium containing 0.1% calf serum for 2 days. Because the Rat 3 cells start DNA synthesis 6-8 hours after the serum stimulation and the S phase lasts almost 10 hours (see Chapter 4), total RNA was prepared from cells 12 hours after the serum stimulation and from unstimulated cells. S1 nuclease analysis was performed using these samples. The probe used in this experiment was made as follows (Figure 7). Since a NcoI restriction site was found in a conserved region 278 bp downstream from the cap site of another chicken H4 histone gene (2) and NcoI sites were also found in apparently analogous locations in both H4 genes in λCH1a, the plasmid

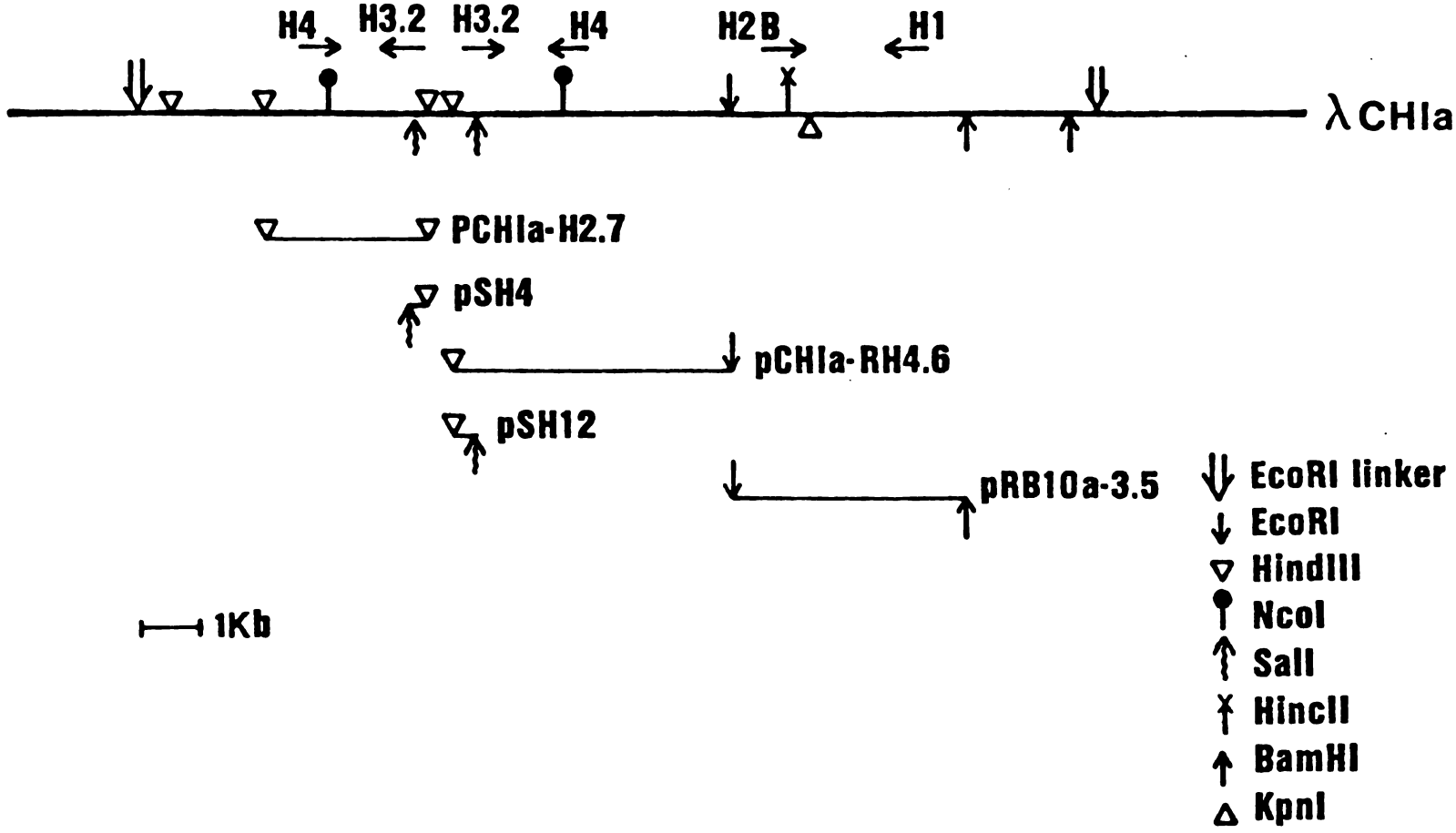


Figure 6. Restriction map of λCH1a and its subclones. The direction of transcription is shown by the horizontal arrows.

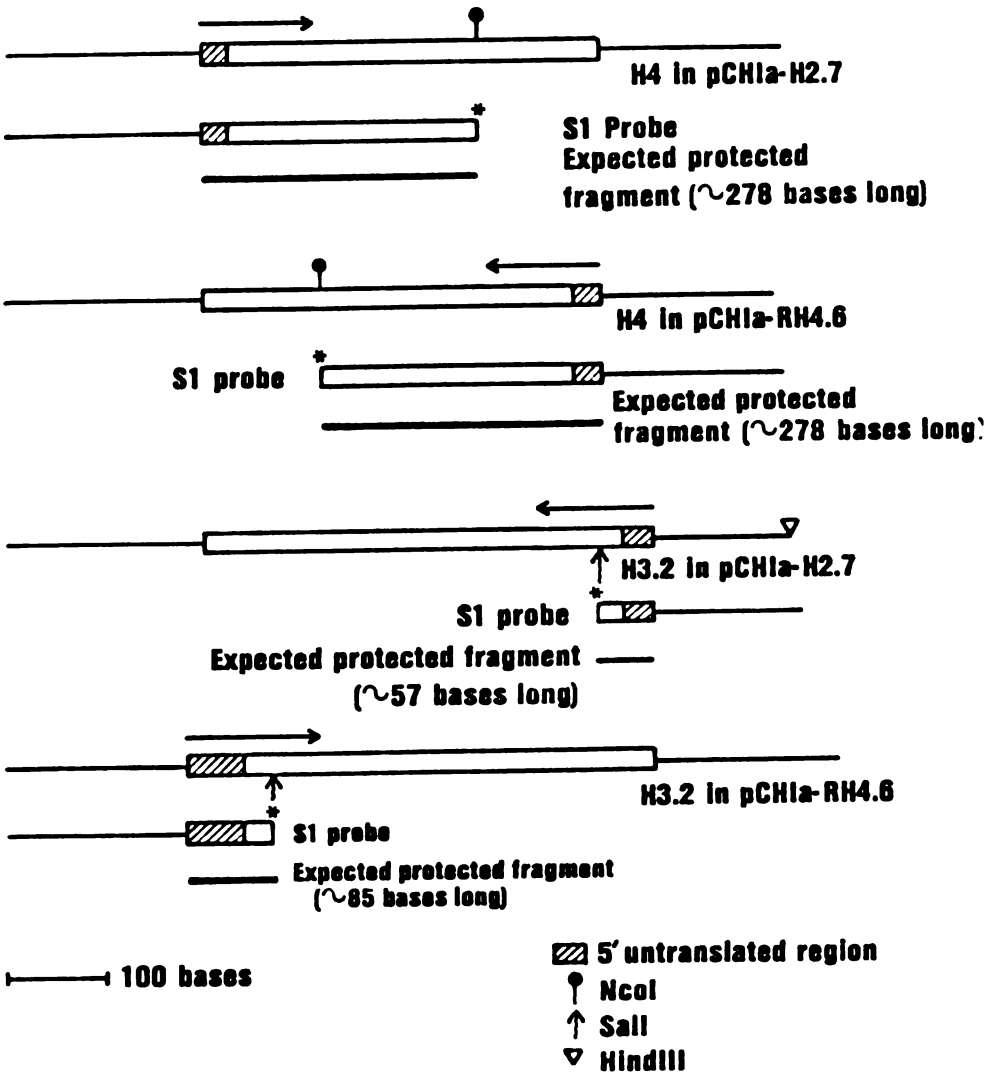


Figure 7. S1 probes for various chicken histone genes. The direction of transcription is shown by the horizontal arrows.

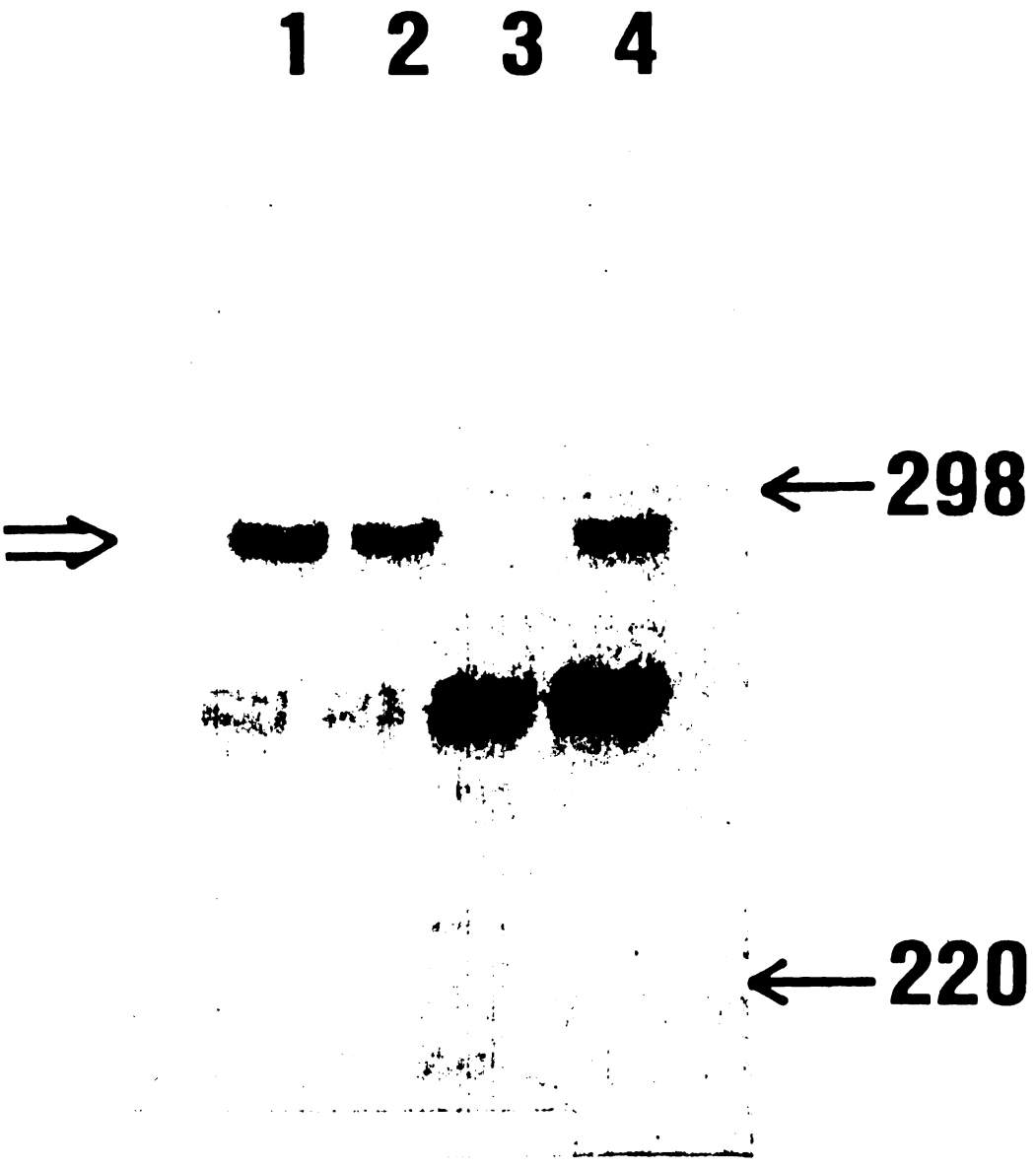


Figure 8. Cell cycle regulation of a chicken H4 histone gene in a cell line (1a-1) transfected with λCH1a. Approximately 0.5 μg of end-labeled DNA was hybridized to 30 μg of total RNA. S1 nuclease analysis was done as described in Chapter 2. The probe used is shown in Figure 7. S1 nuclease level used was 670 u/ml. Lane 1: RNA from stimulated cells (for 12 hours) cotransfected with λCH1a. Lane 2: RNA from unstimulated cells cotransfected with λCH1a. Lane 3: RNA from stimulated Rat 3 cells. Lane 4: RNA from anemic chicken red cells.

pCH1a-RH4.6 was cut with NcoI and end-labeled with ^{32}P at this site. The linearized, labeled plamid DNA was digested with EcoRI, and a 2.6 kb fragment was gel isolated for use as an S1 probe. The protected fragment from S1 digestion was expected to be about 278 nucleotides long. Because the protein coding regions but not the 5' and 3' untranslated regions of histone gene mRNAs are very well conserved, shorter fragments were also expected to arise from hybridization of the probe to endogenous rat H4 histone mRNAs and/or to chicken H4 histone mRNA from the other gene on λCH1a. The difference in length between the band arising from transcription of the H4 gene on pCH1a-RH4.6 and that arising from endogenous rat H4 genes and/or the other λCH1a H4 gene should be the same as the length of the 5' untranslated region of the H4 histone gene in pCH1a-RH4.6, about 25 nucleotides long. As shown in Figure 8, the expression of this H4 histone gene is appropriately regulated during the cell cycle. During the S phase of the cell cycle (lane 1), the level of specific H4 histone mRNA is about 4 times as much as the level of the H4 histone mRNA during resting period (lane 2). Together with previous results, these data show that exogenous chicken histone genes are expressed in an appropriate cell cycle fashion after transfection into cultured Rat 3 cells. Thus the factors which govern such regulation must have been highly conserved throughout evolution. This is in agreement with the results of Capasso and Heintz (3) who showed that a human H4

histone gene was regulated in murine cells. Old et al. (16) also showed that frog histone genes are regulated in mouse cells. This further confirms that this system is an appropriate one in which to test the effects of various DNA elements of chicken histone genes on cell cycle regulation. At this point, we wished to further test whether small subclones of portions of the λCH1a DNA would also show appropriate cell cycle regulation when transfected into Rat 3 cells. Several such subclones were tested after cotransfection into Rat 3 cells.

Expression of the H2B Histone Gene in pRB10a-3.5

The plasmid pRB10a-3.5 contains about 550 base pairs 5' to the cap site of the H2B histone gene (Figure 6). Rat 3 cells were cotransfected with the plasmid pRB10a-3.5 and the human TK cDNA plasmid, and transfected cells were selected with HAT media. This mass culture was grown to confluence before changing the medium to 0.1% calf serum containing medium. Total RNAs were prepared before and 12 hours after serum stimulation, and subjected to an RNase protection assay. Radioactively labeled RNA probe used in this experiment was made as follows (Figure 9). An 0.8 kb EcoRI-HincII DNA fragment from plasmid pRB10a-3.5 was cloned into the multiple cloning site of the vector plasmid pBS(-) at EcoRI and EcoRV sites. This newly constructd plasmid, pBS(-)-H2B, was cut with EcoRI and subjected to *in vitro* transcription using

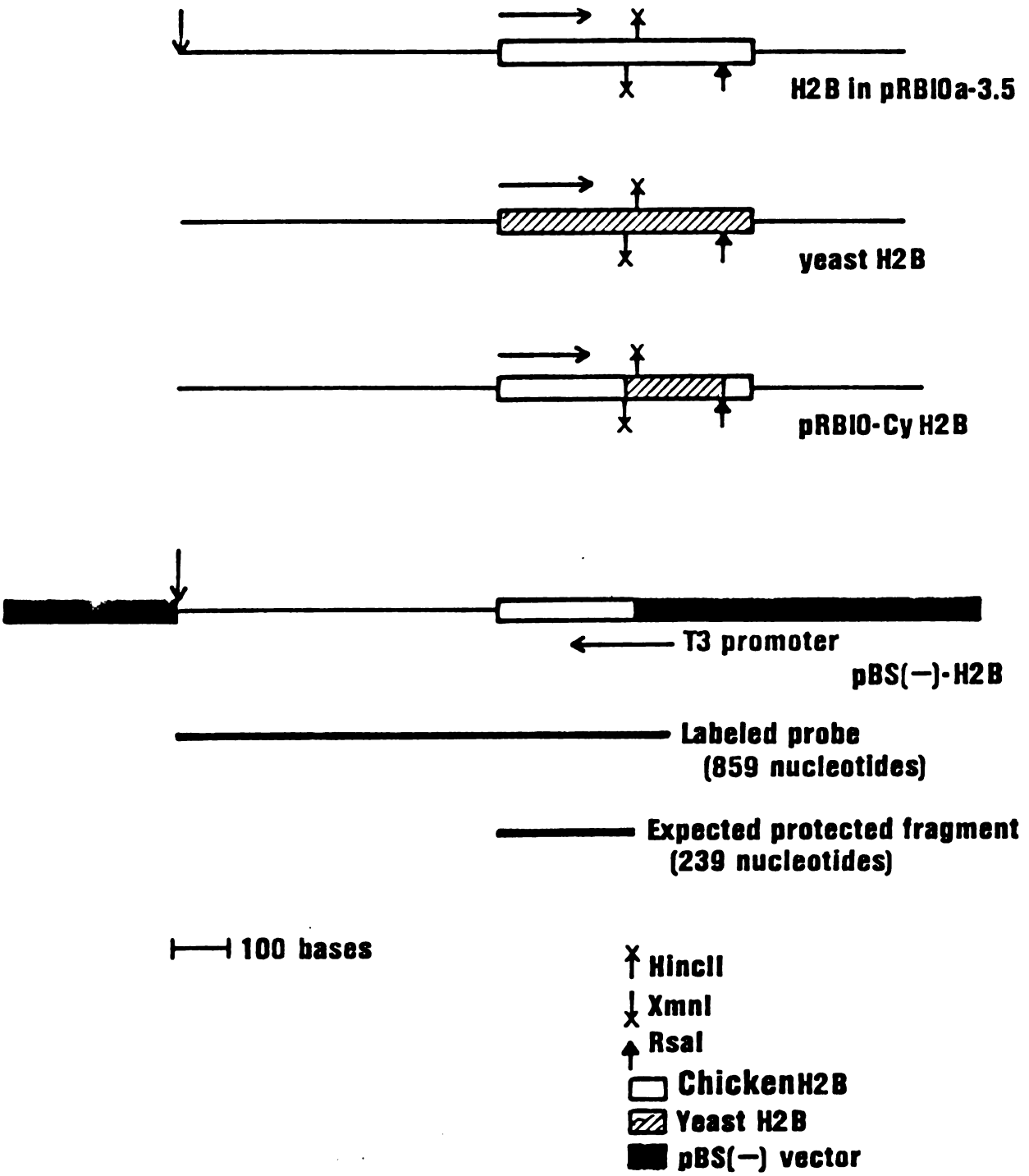


Figure 9. Construction of pRB10-CyH2B and its probe for the RNase protection assay.

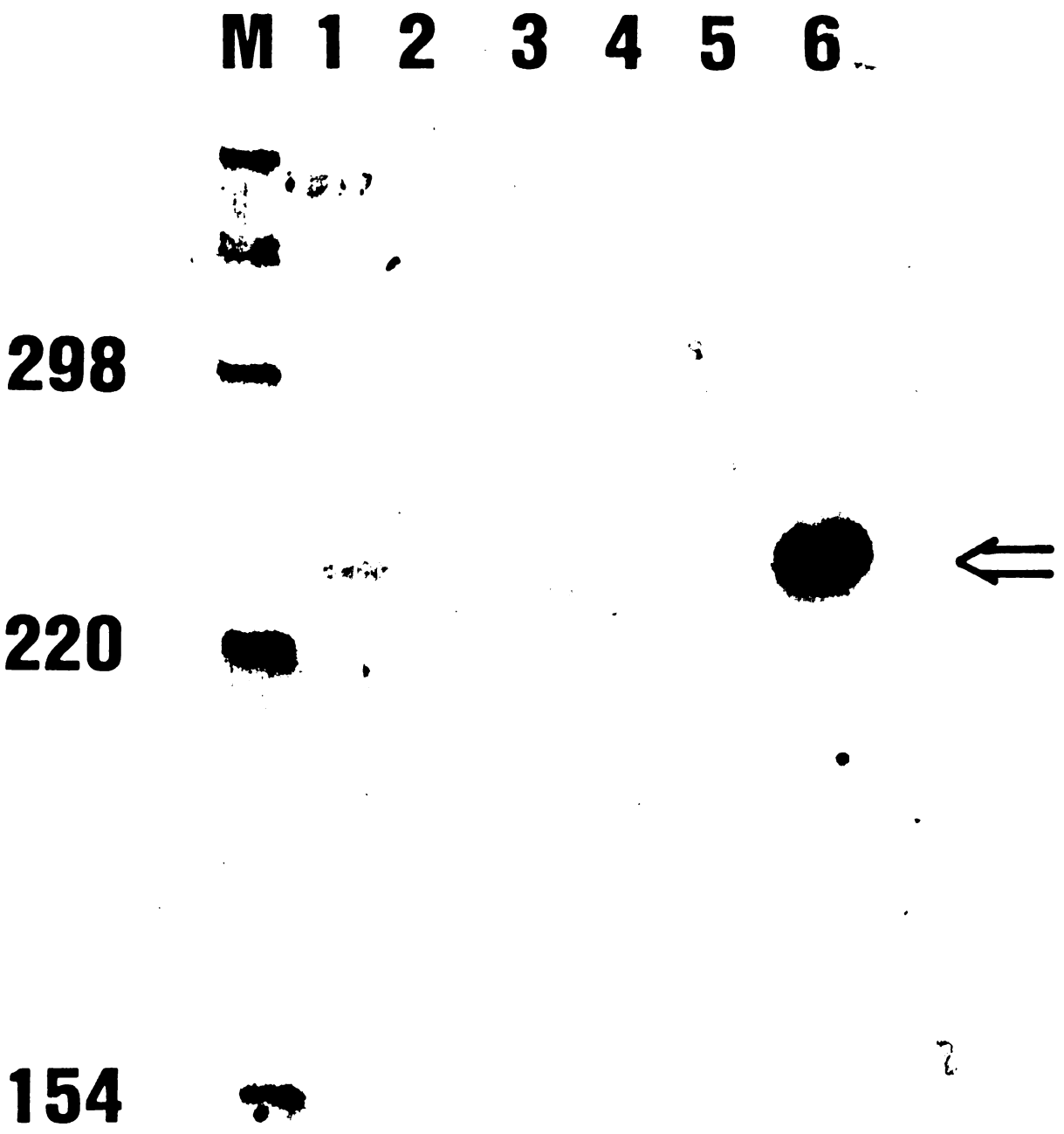


Figure 10. RNase protection assay of a chicken H2B histone gene. Thirty micrograms of total RNA were used in each reaction described in Chapter 2. The probe is made from pBS(-)-H2B, shown in Figure 9. Lane 1: stimulated cells transfected with pRB10a-3.5. Lane 2: unstimulated cells transfected with pRB10a-3.5. Lane 3: stimulated cells transfected with pRB10-CyH2B. Lane 4: unstimulated cells transfected with pRB10-CyH2B. Lane 5: stimulated Rat 3 cells. Lane 6: anemic chicken red cells.

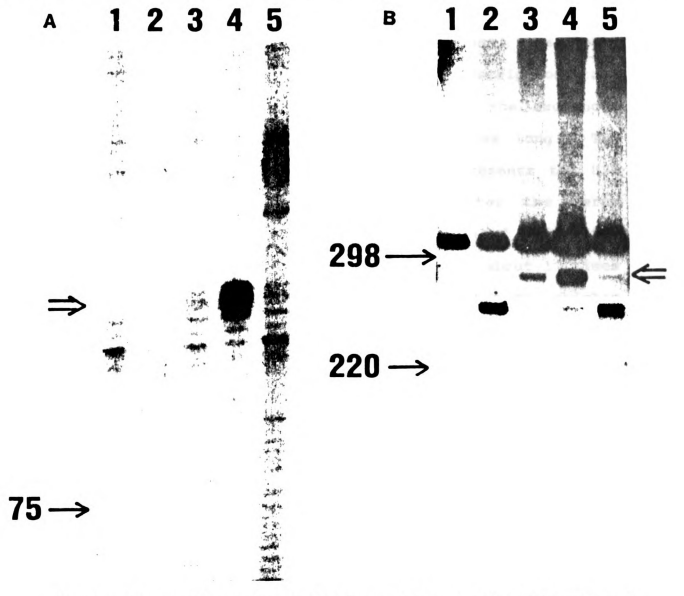


Figure 11. Cell cycle regulation of the chicken H2B (A) and H4 (B) histone genes in a Rat 3 cell line transfected with λCH1a. A: A DNA fragment cut with EcoRI was end-labeled at the BstEII site to be used as a probe. B: A DNA fragment from pCH1a-H2.7 end-labeled at NcoI was used as a probe. Fifty micrograms of RNA and 670 u/ml of S1 nuclease were used for all assays. RNAs tested were: 1. Untreated Rat 3 cells, quiescent (lane 1) or stimulated (12 hr, lane 2). 2. Rat 3 cells cotransfected with λCH1a DNA for lane 3 (unstimulated) and lane 4 (stimulated). 3. Anemic chicken red cells (lane 5).

[α-^{32}P]rUTP and T3 RNA polymerase. The resulting uniformly-labeled RNA probe was about 860 nucleotides long. The HincII site is located 239 bp downstream from the transcription start site, and thus the protected fragment from the exogenous pRB10a-3.5 H2B gene should be 239 nucleotides long. The result is shown in Figure 10. Lane 1 represents the H2B histone mRNA level prepared 12 hours after the serum stimulation and lane 2 represents the mRNA level before the stimulation. The protected band in lane 1 is about 10 times darker than the one in lane 2. This means that the chicken H2B histone gene in pRB10a-3.5 is expressed 10 times more during S phase than during the resting stage at the RNA level. The H2B histone mRNA was increased by 8 fold during S phase when the complete λCH1a phage DNA was transfected (Figure 11-A). These two stimulation levels are not significantly different. The H2B-specific octamer element (ATTTGCAT) which is known to be essential in cell cycle regulation of H2B histone genes (12) was originally found in the λCH1a H2B gene (4). This gene also contains a 3' end stem-loop structure (4), another sequence important in cell cycle regulation of replication-dependent histone genes (13). Thus it is not surprising, given our previous results, that the H2B histone gene on pRB10a-3.5 seems to contain all the sequences necessary for proper cell cycle-regulated expression. Unlike the case with the H4 histone gene, smaller bands expected at 202 nucleotides are not found representing endogenous rat H2B

histone RNA. The coding sequences of this chicken H2B histone gene used as probe are apparently not adequately homologous to those of the rat genes to cross-hybridize in the S1 analysis.

Expression of two H3.2 and a H4 Histone Genes

Since both H3.2 genes were shown to be regulated during the cell cycle when λCH1a phage DNA was transfected into Rat 3 cells, smaller DNA fragments containing these genes separately were tested. The plasmid pCH1a-RH4.6 contains the H3.2 histone gene with about 300 base pairs 5' to the cap site. Several colonies were isolated after cotransfecting with pCH1a-RH4.6 and the human TK cDNA plasmid. When these isolated cell lines were tested for the expression of the chicken H3.2 histone gene by S1 nuclease analysis, two of 12 isolated colonies showed measurable expression. The probe used in this experiment was a 0.4 Kb fragment from pSH12 which was radioactively labeled at the SalI site (Figures 6 and 7). According to the compiled DNA sequence data (5), this SalI site is conserved among most H3 histone genes at 30 nucleotides downstream from the ATG start codon. Our previous experiment showed that this probe protected a fragment of about 85 nucleotides in length (lanes 8-11 in Figure 12). Total RNA isolated from serum stimulated cells (lane 1) showed much higher expression than total RNA from resting cells (lane 2 in Figure 13B) as judged by the 85 nucleotide band

Figure 12. Cell cycle-regulated expression of chicken H3.2 histone genes in λCH1a. Total cellular RNA was isolated from cell line 1a-1 (lanes 1, 2, 8, and 9), cell line 1a-5 (lanes 3, 4, 10, and 11), or untreated Rat 3 cells (lanes 5, 6, 12, and 13). Lanes 7 and 14 result from the assay of chicken red cell cytoplasmic RNA. RNA used for lanes 1, 3, 5, 8, 10, and 12 come from quiescent cells. RNA used for lanes 2, 4, 6, 9, 11, and 13 was isolated from serum stimulated cells (for 12 hours) in S phase. Lanes 1-7 were assayed for the levels of the H3.2 gene present on the pCH1a-H2.7 and lanes 8-14 for levels of mRNA from the other H3.2 gene on λCH1a. Probes used are shown in Figure 7. Thirty micrograms of RNA and 1000 u/ml of S1 nuclease were used for each reaction.

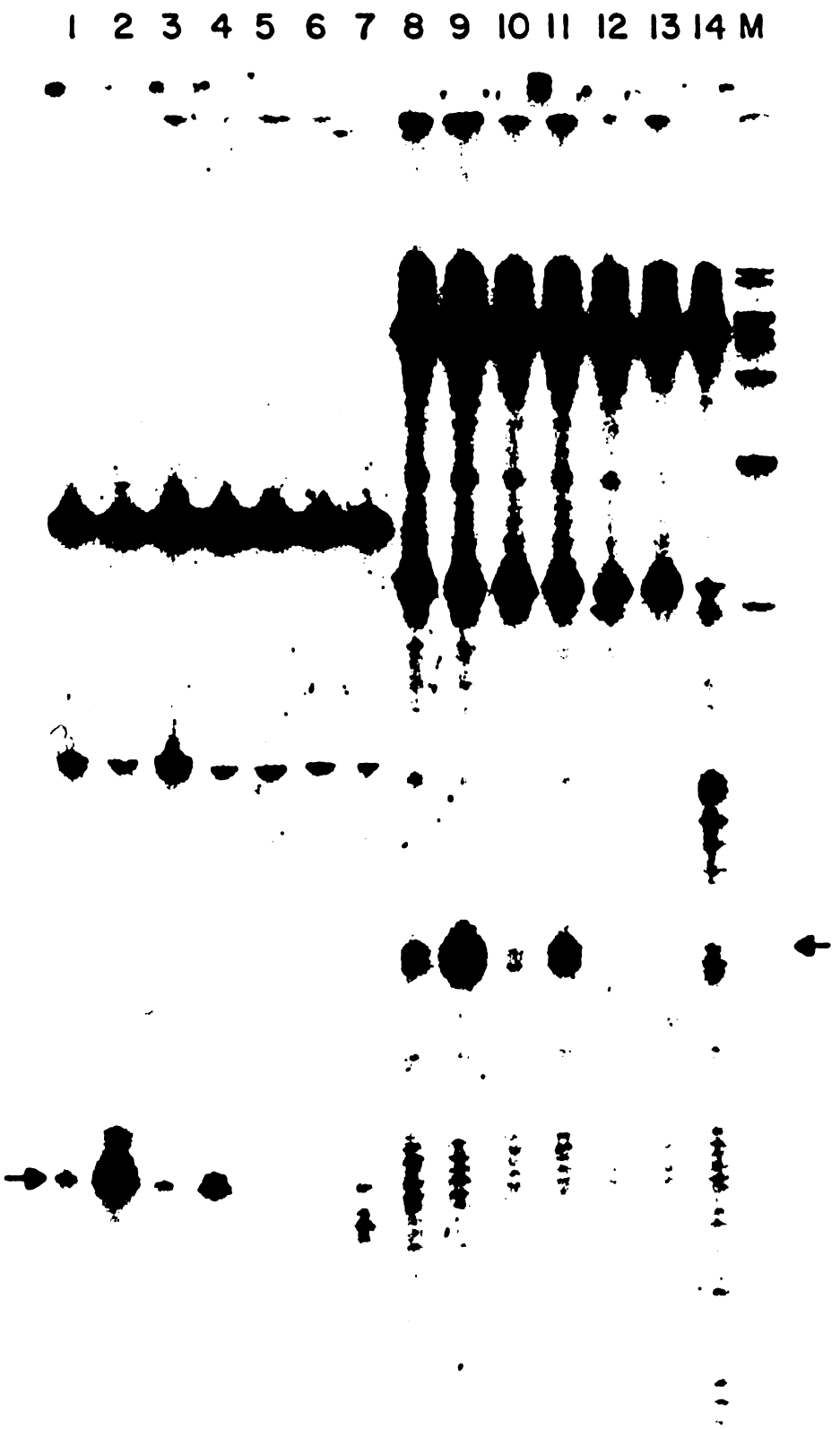
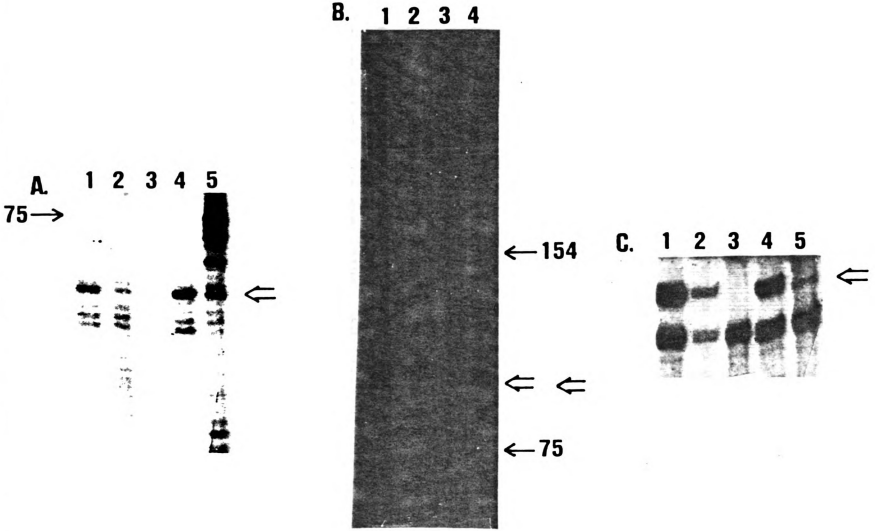

1 2 3 4 5 6 7 8 9 10 11 12 13 14 M

Figure 13. S1 nuclease analysis of chicken H3 and H4 histone genes. Thirty micrograms of RNA and 670 u/ml of S1 nuclease were used in each reaction. A and C shows cells cotransfected with pCH1a-H2.7 and B shows Rat 3 cells cotransfected with pCH1a-RH4.6. RNAs from stimulated (lane 1) and unstimulated (lane 2) cells were tested. Lane 3 represents RNA from stimulated Rat 3 cells and lane 4 represents RNA from stimulated Rat 3 cells transfected with λCH1a DNA. Lane 4 serves as a positive control and so does lane 5 which represents RNA from anemic chicken red cells.


A.
1 2 3 4 5
75
B.
1 2 3 4
154
75
C.
1 2 3 4 5

protected. Thus with only 300 nucleotides of 5' flanking sequences, this chicken H3.2 histone gene appears to be appropriately expressed in transfected Rat 3 cells.

The other chicken H3.2 histone gene on λCH1a contains only 130 nucleotides 5' to its cap site when it is cloned into plasmid pCH1a-H2.7 (Figure 6). This plasmid DNA and the plasmid containing human TK cDNA were transfected and several colonies were isolated by HAT selection. Two of 12 isolated cell lines expressed measurable levels of the H3.2 histone gene mRNA. The probe for the S1 nuclease analysis was a 0.2 Kb fragment from plasmid pSH4 which was end-labeled at the SalI site (Figures 6 and 7). This probe protected a fragment of about 57 nucleotides from cells transfected with λCH1a (Figure 7). Lane 1 of Figure 13A shows a protected band of about 57 nucleotides long which is 8 times darker than the one in lane 2. This means that this H3.2 histone mRNA level was about 8-fold increased in the S phase in comparison to the G_0/G_1 boundary. There was about 10-fold induction when the complete λCH1a phage DNA was transfected. Artishevsky et al. (6) have shown that a 32 nucleotide region, located about 150 nucleotides upstream of the TATA box, contains a crucial control signal for the cell cycle regulation of a hamster H3 histone gene when the promoter region of the hamster H3 histone gene conferred cell cycle regulation on a bacterial neomycin resistance gene. This region is located at 180

nucleotides upstream from the cap site and hence, a total of at least 210 nucleotides 5' to the cap site are needed for the cell cycle regulation of the hamster H3 histone gene. But in our experiment with a chicken H3.2 histone gene, it seems that no more than 130 nucleotides 5' to the cap site are sufficient to confer cell cycle-regulated expression on the gene.

The same cell line that expressed the H3.2 histone gene in pCH1a-H2.7 also expressed the H4 histone gene contained in the same DNA fragment. A 1.1 kb fragment of pCH1a-H2.7 cut with NcoI and HindIII was used as a probe for the S1 nuclease analysis which gave a protected fragment of about 280 nucleotides. This H4 histone gene was also regulated during the cell cycle (Figure 13C). The level of the H4 histone gene mRNA was 8 times higher during S phase than at the G_0/G_1 boundary. This H4 histone gene has about 800 bp 5' to the cap site in this particular subclone. It is not surprising then that all the promoter elements necessary for cell cycle-regulated expression may be located within this 800 bp 5' flanking region. When the complete λCH1a was transfected, the level of this H4 histone gene mRNA was increased by about 6 fold during S phase (Figure 11B), again an increase essentially identical to that seen with the transfected subclone.

DNA Sequencing of 5' Flanking Regions of Two H3.2 Histone Genes

Because the two H3.2 histone genes in pCH1a-H2.7 and in pCH1a-RH4.6 showed cell cycle-regulated expression, their 5' flanking regions were sequenced to see if any regions of significant homology existed. Figure 14 shows the 5' flanking sequences for the two genes. Except for the TATA box and the CCAAT box, no other significant homologies 5' to the coding sequences of these two H3.2 genes were found.

It is interesting that even though the two chicken H3.2 histone genes are less than 1 kb apart, the lengths of their 5' untranslated regions are different by about 28 nucleotides. The H3.2 in pCH1a-H2.7 contains about 27 bp of 5' untranslated region and the H3.2 in pCH1a-RH4.6 contains about 55 bp of 5' untranslated region. This further confirms that even though the protein coding regions of histone genes have been highly conserved throughout evolution, their 5' untranslated regions have not been conserved as well.

Control Genes

We looked for a control gene whose mRNA level is constant throughout the cell cycle. Thompson et al. (14) reported that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was useful as a control gene, because its mRNA level varied little in chicken cells. We obtained a rat GAPDH gene clone from Dr.

1. AAGCTTGTTT TCACTGCTTG CTAGTATCTG GCTTCTTCTC AGGTTAAATG
AGGTGTGTGA AAATGCGATT TATTGCTGAA AGAAGACAAT GAGGGAAGAC
AACTAGATAA AAAGAAGAAA GGCTTTATGA ATCCGTAGCA AACCGAAAAG
AGAAACGCTG GGGTTTAACT ATTAAAGAGC AGCAGTAGGR ACAGCAGGAG
ATTAACGCTG GTTTTTCAAA TTGAACCAAT AATATTCGTC CTTTCTTCAG
CCAATGGCAA TGCAGCGTTC GGTATAAAAG CGAGTCAGGA ACGGCGCCAC
CTCARATGCG GTTTTACGGG TCATTTGTGT AGTTGTGGGA AA

2. AAGCTTCTTT GCAAGGTGGG ACAGGCAGAA GGCTTAGAGT TAGCCAATTA
AATTCATTGA TTTATTGACC AATCAGAGGC GAATGGGCGG GGTTTCATCT
ACTATAAATA AGAGCCGCTG CAACGAGACC GCCTACTTTC GGTTGCAGAG
CAGTTCTGCG AATGGCGCGT ACGAAGCAGA CGRCGYGT

Figure 14. DNA sequences of 5' flanking regions of chicken H3.2 histone genes on pCH1a-RH4.6 (1) and pCH1a-H2.7 (2). Putative transcription initiation sites are marked by arrows. Possible TATA boxes and CCAAT boxes are underlined.

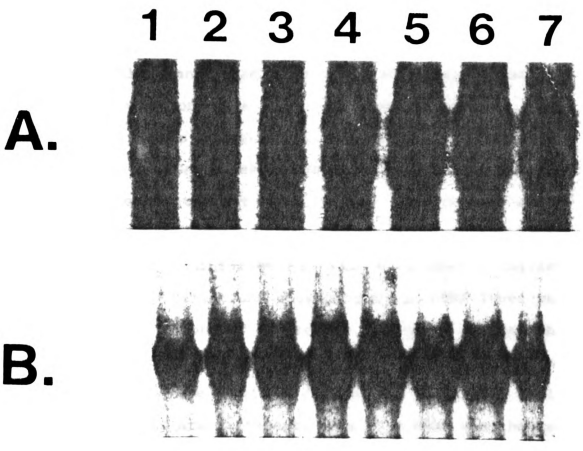


Figure 15. Levels of chicken H3.2 histone mRNA (A) and rat GAPDH mRNA (B) during the cell cycle. RNA samples in both A and B are from Rat 3 cells cotransfected with λCH1a DNA. Thirty micrograms of total RNA isolated at 0, 3, 6, 9, 12, 15, and 18 hr (lanes 1 to 7, respectively) after the stimulation were analyzed by the RNase protection assay. The last lane in B is RNA from untransfected Rat 3 cells.

S. Conrad. By using it to make a RNA probe for an RNase protection assay, we analyzed endogenous GAPDH mRNA levels at different stages of Rat 3 cell cycle. We isolated total RNA from Rat 3 cells transfected with λCH1a at 3 hour intervals after the stimulation. In a parallel experiment, we also analyzed mRNA levels of H3.2 in pCH1a-H2.7. As shown in Figure 15-B, the GAPDH mRNA level was not constant in different stages of the Rat 3 cell cycle. By 12 hour after the stimulation, it increased by more than 2 fold (lane 5), comparing to 0 hr. Linial et al. (15) also used a chicken GAPDH in their experiment and showed that its mRNA level was not constant. Based on our experiment, we could not use the GAPDH gene as a control gene. Although both the H3.2 and the GAPDH mRNA levels are maximum during the S phase, their expression patterns are different. The H3.2 mRNA was induced at 9 hours after the stimulation (lane 4 in Figure 15-A). This was why we chose three different time points at 0, 6, and 12 hours in some of the experiments that will be discussed in Chapter 4. We also tried a human beta tubulin gene to use as a control gene. Since it is not a rat gene, we were able to use Northern blot to determine its regulation. This gene also showed higher mRNA levels during S phase (data not shown).

Discussion

Two chicken H4 histone genes were tested for their expression at the transcription level in relation to the cell cycle. One was transfected in λCH1a DNA and the other was in the plasmid pCH1a-H2.7. We showed that the second H4 histone gene (from left) was not a pseudogene and its expression was stimulated during the S phase of the cell cycle. The other H4 gene also showed cell cycle-regulated expression after its 5' flanking region was cut down to 800 base pairs. According to Dailey et al. (10), two sequence elements are necessary for cell cycle regulation of H4 histone genes because they interact with sequence-specific protein factors. Both chicken H4 histone genes must have at least one of them because it is the H4 subtype-specific sequence element immediately upstream from the TATA box. The other sequence element was found between 80 to 110 nucleotides upstream from the cap site in the human H4 histone gene. A sequence similar to this element may or may not be present in the chicken H4 histone gene because it could be a specific sequence only to the human H4 histone gene. We presently do not have DNA sequence data on these two chicken H4 histone genes. When these genes are sequenced, more detailed studies on the cell cycle regulation of chicken H4 histone genes could be done including mutagenesis of the DNA sequence elements involved, identification and isolation of protein factors, and comparison with H4 histone genes in other species.

The H2B-specific octamer element (ATTTGCAT) has been shown to be essential in stimulating human H2B histone gene transcription upon entering into S phase (12). The chicken H2B histone gene in pRB10a-3.5 has been sequenced previously in our lab (4). Although its location is a little bit different from that in the human H2B gene, this gene contains the octamer element in its 5' flanking region. This H2B histone gene also contains the stem-loop sequence at the end of the transcription unit. These two features may be sufficient to confer cell cycle-regulated expression on the gene. It might be interesting to see if the Rat 3 cells and chicken cells have the same or similar protein factor to that purified by Fletcher et al. (11).

There are no known H3-specific sequence elements in the promoter region of the H3 histone genes which have been shown to be required for cell cycle regulation. In a hamster H3 histone gene, a 32 nucleotide region which is located about 150 nucleotides upstream from the TATA box was proposed to contain crucial control signals for cell cycle regulation (6). We tested two chicken H3.2 histone genes with different lengths of 5' flanking regions. If the chicken H3.2 histone genes were similar in the organization and spacing of regulatory elements to those in the hamster H3 histone gene, the chicken H3.2 gene with 300 nucleotides of 5' flanking sequence in pCH1a-RH4.6 should be regulated and the other H3.2

with 130 nucleotides 5' to the cap site in pCH1a-H2.7 should not be. Both chicken H3.2 genes showed cell cycle regulation. Their mRNA levels increased by 8 fold during S phase. Of course, a region similar to the 32 nucleotide sequence might be located closer to the cap site in at least the latter chicken H3.2 gene or the chicken H3.2 histone genes might require different sequence elements for their regulation. The DNA sequence of the 5' flanking regions of both of the subcloned chicken H3.2 histone genes failed to show any sequence that is similar to the 32 nucleotide element in hamster or to indicate any significantly homologous region between two chicken genes other than the expected CCAAT and TATA boxes. It seems that the H3 histone genes may not be cell cycle-regulated using conserved, subtype-specific sequences 5' to the mRNA cap site. Further studies regarding this point will be described in Chapter 4.

We wanted to include a control gene in our experiment. We tried a rat GAPDH gene and a human beta tubulin gene. Both genes were not expressed constantly in Rat 3 cells during the cell cycle. Although we failed to find an internal control gene, we showed relatively constant expression of an intronless H3.3 gene in Chapter 4 which indicated the reliability of our system.

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CHAPTER 4

Results

A. Does the conservation of coding nucleotide sequence observed among H2B histone genes relate to a functional role in cell cycle regulation?

Construction of a Hybrid Chicken-Yeast-Chicken H2B Histone Gene and Its Expression

Chapter 1 describes studies which suggested the presence of sequence elements that play a role in cell cycle regulation both 5' and 3' to the histone protein coding region. Grandy and Dodgson (4) found in a comparison of 7 of the 8 chicken H2B gene sequences that their internal coding region nucleotide sequence was highly conserved, more so than that level of conservation needed merely to specify the same or similar polypeptide sequences. In other words, nucleotides at ambiguous sites (mostly 3' or wobble sites) still showed a very high level of similarity. These authors proposed that a specific H2B mRNA secondary and/or tertiary structure play an important role in histone gene expression. We wished to test whether such a role, if any, might participate in cell cycle regulation. To do this we took advantage of the fact that while all chicken (and other vertebrate) H2B histone genes examined to date have high levels of G:C base pairs in

ambiguous positions, the yeast H2B genes have high levels of A:T base pairs in these sites.

Yeast H2B histone genes are also regulated during the cell cycle. Even though the protein sequences of chicken and yeast H2B histones are similar, the third nucleotides of their amino acid codons are often different from each other as suggested above. To answer the question whether the third nucleotides of amino acid codons affect the expression pattern of the H2B histone gene, we replaced a part of the chicken H2B histone gene with the analogous portion of a yeast H2B gene, TRT-1 (9). The fact that an XmnI site at codon 68 and an RsaI site at codon 125 are conserved between chicken and yeast H2B genes made this reasonably straightforward (Figure 9). The hybrid chicken-yeast-chicken H2B histone gene thus has a 57 codon (171 bp) yeast insert. The hybrid histone differs from chicken H2B.1 by only 8 of 126 amino acids, but within the 171 bp insert there is only 63.7% nucleotide sequence homology between the yeast H2B gene and the chicken H2B.1 gene (differs in 62 of 171 bp). The hybrid H2B gene in plasmid pRB10-CyH2B was transfected into Rat 3 cells with human TK cDNA. A mass culture was grown after HAT selection. Using the same probe for mRNA from the H2B gene as described previously (Chapter 3), an RNase protection assay was performed. Figure 10 shows the result of this experiment. The protected band in lane 3 (12 hr after serum stimulation) is about 10 times darker than

the one in lane 4 (from unstimulated cells). Lane 5 represents RNA from Rat 3 cells that were used as a negative control and lane 6 represents RNA from anemic chicken red cells that were used as a positive control. This compares well with the 9 fold stimulation observed previously (Chapter 3) for the H2B histone gene in λCH1a transfected cells. Therefore, the ambiguous nucleotides of amino acid codons in the chicken H2B histone gene sequence do not seem to be required for cell-cycle regulated expression.

B. Do the introns that exist in the replication-independent histone genes affect cell cycle regulation?

Constitutive Expression of Transfected H3.3

Our lab previously demonstrated that the replication-independent H3.3 histone genes contain introns (1, 17). This appears to be generally true for replication independent variant histone genes (but not for the H5 histone gene). Furthermore, Seiler-Tuyns and Paterson (10) showed that the presence of artificial introns (e.g., a globin gene intron) could eliminate the cell cycle regulation properties of a replication dependent histone gene. We wished to test whether the presence or absence of introns in the H3.3B gene affected its constitutive manner of expression.

Previously, others in the lab (Dodgson, Masta and Conrad)

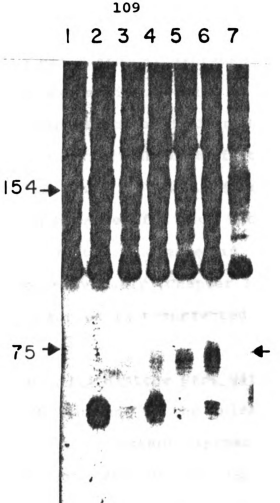


Figure 16. Expression of transfected chicken H3.3 histone gene. Fifty micrograms of RNA and 1000 u/ml S1 nuclease were used in all assays. Samples were assayed with a fragment of the H3.3B gene (Figure 17) labeled at PvuII site. RNA tested were:

1. Untreated Rat 3 cells, quiescent (lane 1) or serum-stimulated (lane 2).
2. Rat 3 cells cotransfected with the chicken H3.3 gene: line 3-5 (quiescent, lane 3; serum-stimulated, lane 4) and line 3-9 (quiescent, lane 5; serum-stimulated, lane 6).

Positive control (lane 7) is anemic chicken red cell total RNA.

showed that the wild type chicken H3.3B gene (1) could be transfected into Rat 3 cells and, if expressed, it would show its usual constitutive expression. This is shown in Figure 16. The difference in expression of H3.3B mRNA at 12 hr after serum stimulation (lane 6) and in absence of stimulation (G_0/G_1 boundary, lane 5) is less than 1.5 fold. (Lanes 3 and 4 were not able to be compared because of a high background in lane 3.) This is less than the increase in the GAPDH gene expression demonstrated previously (Chapter 3). Thus, normal H3.3B expression was observed in transfected cells.

Expression of a Chicken H3.3 Histone Gene Without Introns

To test whether the introns in the chicken H3.3 histone gene are responsible for the constant expression of the gene, a chicken H3.3 histone gene without introns was made from a H3.3 cDNA clone and the genomic H3.3 clone, pBH6b-2.3. Because one EcoRV site each is located in the first (leader) exon and the last exon of the gene, the internal EcoRV fragment of pBH6b-2.3 was cut out and replaced by the analogous cDNA EcoRV fragment. The resulting plasmid, pBH6bΔI (Figure 17), is exactly the same as pBH6b-2.3 except for the absence of all three introns. After the cotransfection and HAT selection, cells were grown to confluence, incubated in 0.1% serum-containing media for 2 days, and stimulated with serum. Total RNAs were prepared from cells at 0, 6, and 12 hours after the stimulation. An RNase protection assay was

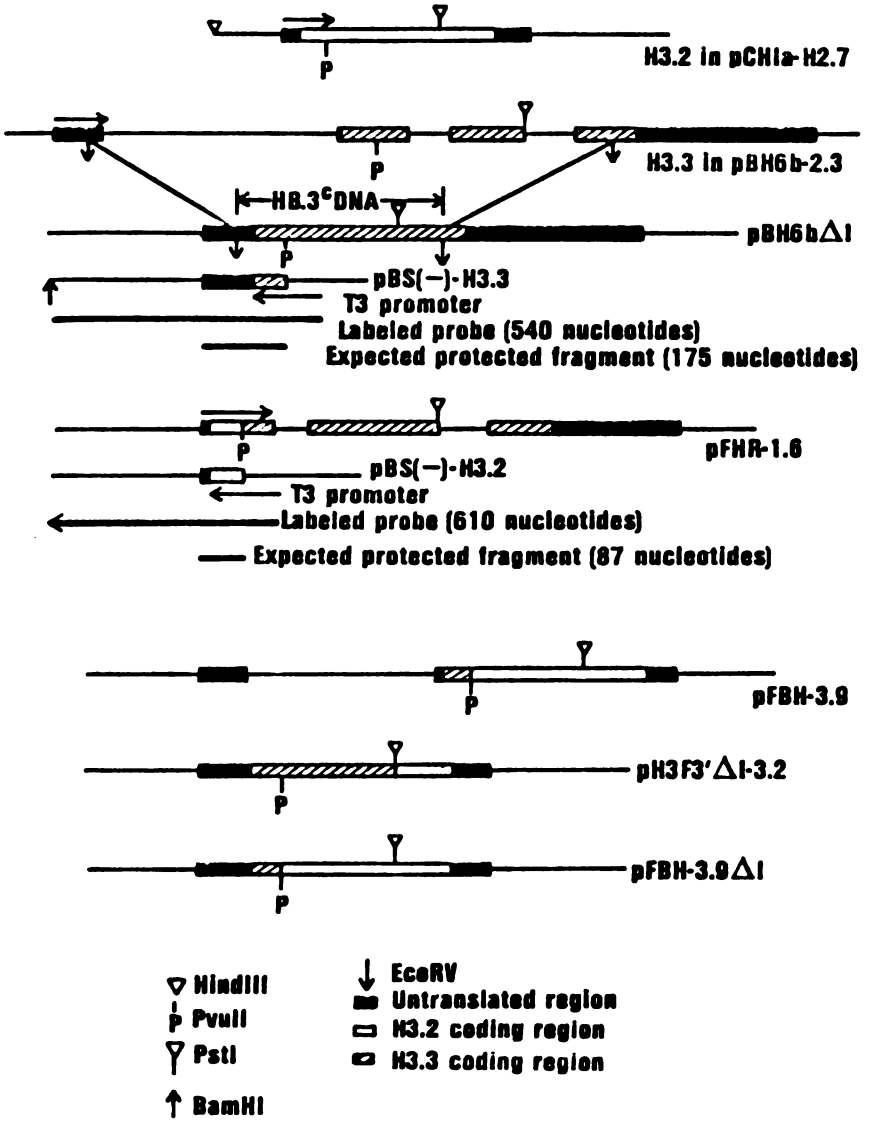


Figure 17. Construction of fusion genes containing chicken H3.2 and H3.3 histone genes. Intronless H3.3 is also shown. Probes for the RNase protection assay were made from pBS(-)-H3.2 or pBS(-)-H3.3.

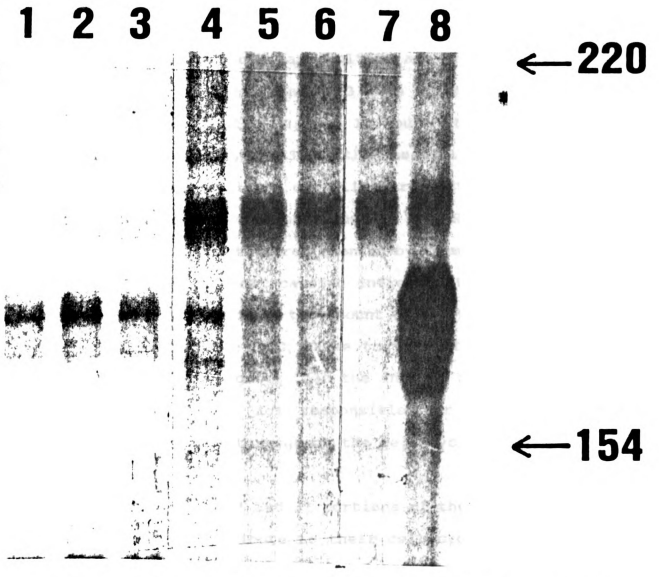


Figure 18. Expression of a chicken H3.3 histone gene without introns. One mass culture (lanes 1-3) and one isolated cell line (lanes 4-6) were grown from Rat 3 cells cotransfected with pBH6bΔI. Total RNAs were isolated at 0 hr (lanes 1 and 4), 6 hr (lanes 2 and 5), and 12 hr (lanes 3 and 6) after the stimulation. Thirty micrograms of RNA were used in each RNase protection assay. The probe used is shown in Figure 17. Lane 7 represents RNA from stimulated Rat 3 cells and lane 8 represents RNA from anemic chicken red cells.

performed by using a uniformly labeled RNA probe that was transcribed *in vitro* from pBS(-)-H3.3 (Figure 17). One isolated cell line and one mass culture are shown in Figure 18. These cells expressed the H3.3 gene without introns at almost the same rate at 0, 6, and 12 hours after the serum stimulation (lanes 1, 2, and 3 for the mass culture and lanes 4, 5, and 6 for the isolated cell line, respectively). Lane 7 is a negative control that contains RNA from Rat 3 cells and lane 8 is a positive control represented by anemic chicken red cell RNA. A densitometer scanning indicated that there was less than 20% fluctuation in the amount of the H3.3 mRNA when the cells went from the G_0/G_1 phase to the later stage of S phase. This result suggests that the introns in the chicken H3.3 histone gene are not responsible for the constant expression of the gene throughout the cell cycle.

C. To what extent do 5' and 3' portions of the chicken H3.2 histone genes contribute to their cell cycle-regulated expression?

The Hybrid H3 Histone Gene Approach

As described above, the chicken H3.2 and H3.3 histone genes, while homologous, show completely different cell cycle regulation properties both in their normal state (1) and as exogenous transfected genes in the Rat 3 cell system (Figures 13 and 16). As outlined in Chapter 1, we expect sequences

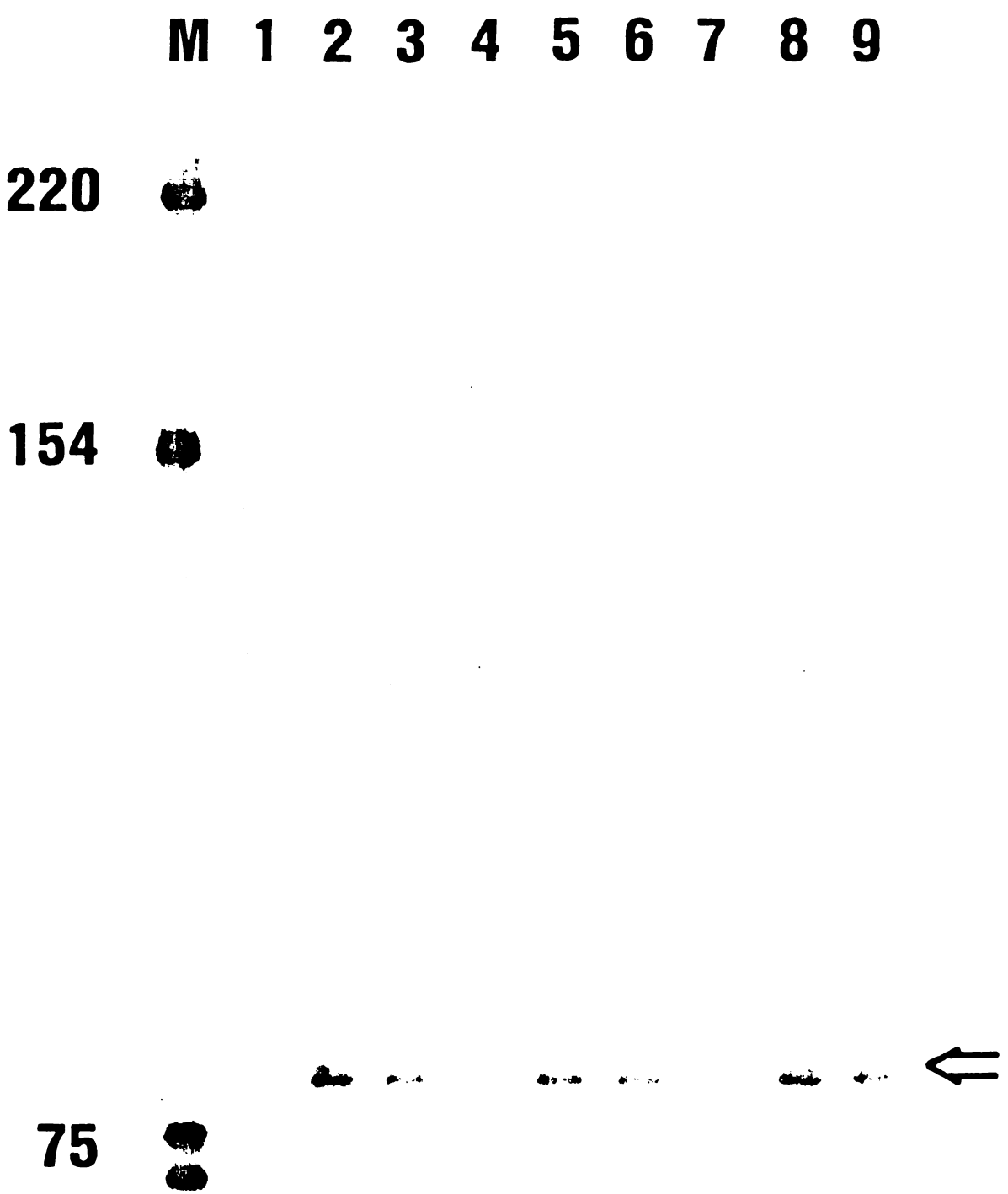
both 5' and 3' to the H3.2 genes to be involved in regulating their expression, presumably involving both transcriptional regulation and post-transcriptional (e.g., mRNA stability) factors. (Experiments in parts A and B of this Chapter suggest that protein coding region and intron sequences are not involved. The former is also suggested by the fact that the overall coding sequence of H3.3B differs from that of another H3.3 variant, H3.3A, by almost as much as it does from a typical H3.2 gene sequence). We chose to construct hybrid H3.2/H3.3 genes to judge the relative contribution of 5' and 3' portions of the H3.2 gene to its cell cycle-regulated expression.

Construction of a Hybrid H3.2-H3.3 Histone Gene and Its Expression

There are a couple of convenient restriction sites that are useful in making chicken H3 histone fusion genes. A PvuII site and a PstI site are located at exactly same sites relative to the coding regions of both genes at the 20^{th} codon and at the 93^{rd} codon, respectively. The H3.3B chicken histone gene on pBH6b-2.3 as described by Brush et al. (1) and the H3.2 gene on pCH1a-H2.7 (Figure 6) were used. A 1.05 kb fragment of pBH6b-2.3 cut with BamHI and PvuII was replaced by a 0.55 kb fragment of pCH1a-H2.7 cut with BamHI and PvuII (Figure 17). (Note that this 0.55 kb fragment contains about 330 bp of plasmid pBR322 vector DNA, 130 bp of 5' flanking

region of the H3.2 gene and 87 bp of 5' untranslated and coding regions of the gene.) The newly constructed plasmid, pFHR-1.6, contains the 5' flanking region and the first 20 amino acid codons from the H3.2 histone gene and the remainder (codon 21 on) of the H3.3B gene. The plasmid pFHR-1.6 was cotransfected with the human TK cDNA into Rat 3 cells and the transfected cells were selected in HAT medium. Three different mass cultures were grown to confluence and kept in HAT medium containing 0.1% calf serum for 48 hours. Then total RNAs were prepared from cells 0, 6, and 12 hours after the serum stimulation. Because Rat 3 cells enter the S phase between 6 to 8 hours after serum stimulation (see below), these three time points should indicate the expression pattern of the gene at the RNA level. The 0.55 kb fragment of pCH1a-H2.7 cut with BamHI and PvuII was inserted into the multiple cloning site of the pBS vector to make a probe for an RNase protection assay. Using pBS(-)-H3.2 cut with BamHI, a uniformly labeled probe of 610 nucleotides was made (Figure 17). (Because the PvuII-cut end of the fragment was ligated to the EcoRV site in the pBS vector which is 60 bp downstream from the transcription initiation site, the probe is 60 nucleotides longer than the 0.55 kb fragment.) This probe should be able to protect RNA from the H3.2 gene transcription initiation site to the PvuII site, which is 87 nucleotides. The result of the RNase protection assay of the three different mass cultures indicated that in all cases the

Figure 19. Cell cycle regulation of a fusion gene in pFHR-1.6. Three mass cultures (C-Ma, C-Mb and C-Mc) were grown after Rat 3 cells were transfected with pFHR-1.6. Total RNAs were isolated at 0 hr (lanes 1, 4, and 7), 6 hr (lanes 2, 5, and 8), and 12 hr (lanes 3, 6, and 9) after the stimulation. Thirty micrograms of RNA from C-Ma (lanes 1, 2, and 3), C-Mb (lanes 4, 5, and 6), or C-Mc (lanes 7, 8, and 9) were used for each RNase protection assay. The probe was made from pBS(-)-H3.2, shown in Figure 17.

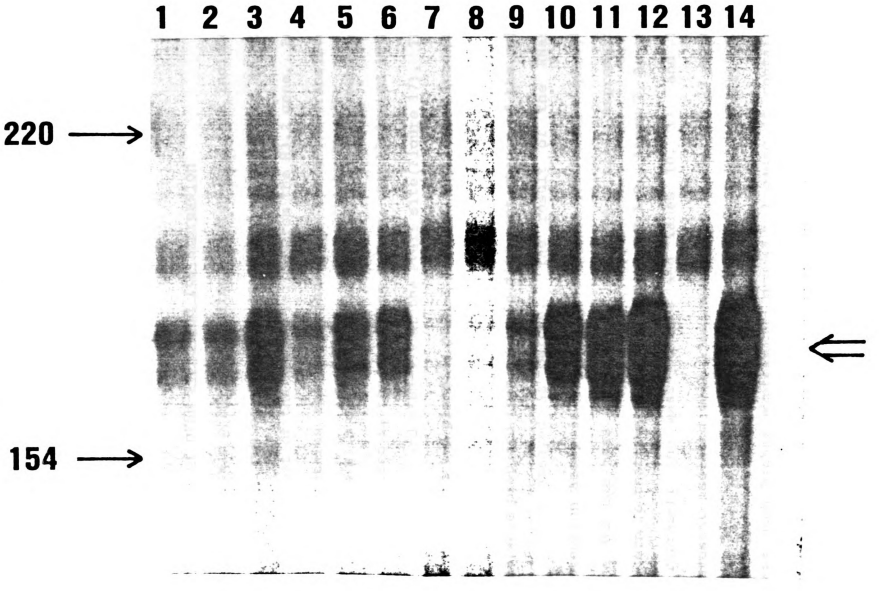

M 1 2 3 4 5 6 7 8 9
220
154
75

expression of this fusion gene was stimulated during S phase of the cell cycle (Figure 19). mRNA levels of the fusion gene at 6 hr (lanes 2, 5, and 8) and at 12 hr (lanes 3, 6, and 9) increased by an average of 4 fold and 3 fold respectively, comparing to 0 hr (lanes 1, 4, and 7). Exogenous fusion gene mRNAs were induced at 6 hr after stimulation or even earlier. This means that the 220 bp H3.2 fragment at the 5' end of the fusion gene is, at least, partially responsible for the cell cycle regulation of the gene, perhaps due to transcriptional regulatory elements in the 130 bp of 5' H3.2 flanking region present in this construct. One thing that is noticeable in this experiment is that the mRNA level at 6 hours after stimulation was a little higher than that at 12 hours after stimulation. It is generally considered that the 5' regions of the cell-cycle dependent histone genes are responsible for regulation at the transcriptional level and the 3' regions are responsible at the post-transcriptional level (Chapter 1). Because this fusion gene contains the 5' region of the H3.2 histone gene and the 3' region of the H3.3 histone gene, the transcriptional stimulation may begin slightly prior to S phase, but normally be ineffective since mRNA with an H3.2 3' end is unstable until sometime further into S phase. This question is addressed further in more detailed experiments later.

Construction of a Hybrid H3.3-H3.2 Gene and Its Expression

In an effort to make a fusion gene that is opposite to pFHR-1.6, the 1.6 kb PvuII fragment of pCH1a-H2.7 was inserted into the PvuII site of pBH6b-2.3. The resulting plasmid, pFBH-3.9, contains the 5' flanking region of the H3.3 histone gene including up to 20th codon and the 3' flanking region of the H3.2 histone gene including most of the coding region and the H3.2 stem-loop 3' end (Figure 17). After cotransfection and HAT selection, the expression of this fusion gene was tested. The probe used in the RNase protection assay was obtained by using part of the intronless H3.3 cDNA clone, pBH6bΔI (Figure 17). A 0.5 kb fragment of pBH6bΔI was inserted into the multiple cloning site of the pBS vector. Using BamHI cut pBS(-)-H3.3, the in vitro transcription using T3 RNA polymerase produced a uniformly labeled probe of 540 nucleotides in length (Figure 17). With this probe a protected fragment of 175 nucleotides in length is expected. The result of this experiment is shown in Figure 20. Lanes 1, 2, and 3 show the mRNA level of the fusion gene in a mass culture and lanes 4, 5, and 6 show the fusion gene mRNA level in a isolated cell line at 0, 6, and 12 hr, respectively. An average increase of 1.3 fold was observed at 6 hr and 2.6 fold at 12 hr. Because this gene contains the 5' region of the H3.3 histone gene, one might expect it to be transcribed in a constitutive fashion. However, since the fusion gene contains the 3' end of the H3.2 gene which is likely to make

Figure 20. Cell cycle regulation of chicken H3.3-H3.2 histone fusion genes. Rat 3 cells were cotransfected with pFBH-3.9 (lanes 1-6) or pFBH-3.9ΔI (lanes 7-12). One mass culture (lanes 1-3 and 7-9) and one isolated cell line (lanes 4-6 and 10-12) for each fusion gene are shown. Thirty micrograms of total RNA were used for each RNase protection assay. The probe used is shown in Figure 17. Total RNAs were isolated at 0 hr (lanes 1, 4, 7, and 10), 6 hr (lanes 2, 5, 8, and 11), and 12 hr (lanes 3, 6, 9, and 12). Lane 13 represents RNA from stimulated Rat 3 cells and lane 14 represents RNA from anemic chicken red cells.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
220
154

its transcripts unstable except during S phase, there is little accumulation of fusion gene mRNA outside of S phase. This experiment indicates that the 3' region of the chicken H3.2 histone gene alone can confer at least partial cell-cycle regulation on expression of the gene.

Construction of pFBH-3.9ΔI and Its Expression

The plasmid pFBH-3.9 contains a fusion gene that includes the promoter region from the H3.3 histone gene and the 3' region and most of the coding region from the H3.2 gene. But it still contains the first intron in the 5' untranslated region of the H3.3 gene. In order to insure that its behavior was not affected by the remaining intron, we eliminated it by fusing pBH6bΔI and pFBH-3.9 at the PvuII site(Figure 17). The newly constructed plasmid, pFBH-3.9ΔI, was introduced into Rat 3 cells with human TK cDNA. After the HAT selection, total RNAs were prepared from cells in different stages of the cell cycle and assayed as before. The result of the RNase protection assay is shown in Figure 20. One isolated cell line and one mass culture both showed a similar expression pattern to that seen previously for pFBH-3.9. Lanes 7, 8, and 9 represent the mass culture and lanes 10, 11, and 12 represent isolated cell line assayed at 0, 6, and 12 hours after serum stimulation, respectively. The isolated cell line shows much higher expression, probably because it contains more copies of pFBH-3.9ΔI than the average of cells in the

mass culture. This experiment again showed that the introns, especially the first intron, appear not to affect the cell cycle-regulated expression of these genes. This also confirms the conclusion from other experiments that the 3' region of the chicken H3.2 histone gene alone can partially confer the cell cycle-regulated pattern of expression.

Construction of pH3F3'ΔI-3.2 and Its Expression

Although it seems likely that the cell cycle-regulated expression of the fusion gene in pFBH-3.9ΔI is mainly due to the 3' flanking region of the H3.2 histone gene, the possible involvement of some of the H3.2 coding region can not be excluded. We tried to replace the H3.2 gene coding region with that of the H3.3 gene as much as possible while retaining the H3.2 gene 3' stem-loop structure. The PstI site, located between the 93rd and the 94th codons, at the same site in the two chicken H3 histone genes, made it possible to construct another fusion gene with much less of the H3.2 coding region. The newly constructed plasmid, pH3F3'ΔI-3.2, contains less than one third of the amino acid coding region of the H3.2 histone gene (Figure 17). After the stable cotransfection with human TK cDNA, the expression of the fusion gene was tested. Figure 21 shows one mass culture (lanes 1, 2, and 3) and an isolated cell line (lanes 4, 5, and 6). The fusion gene mRNA level increased 2 fold by 6 hr (lanes 2 and 5) after stimulation and more than 4 fold by 12 hr (lanes 3 and 6).

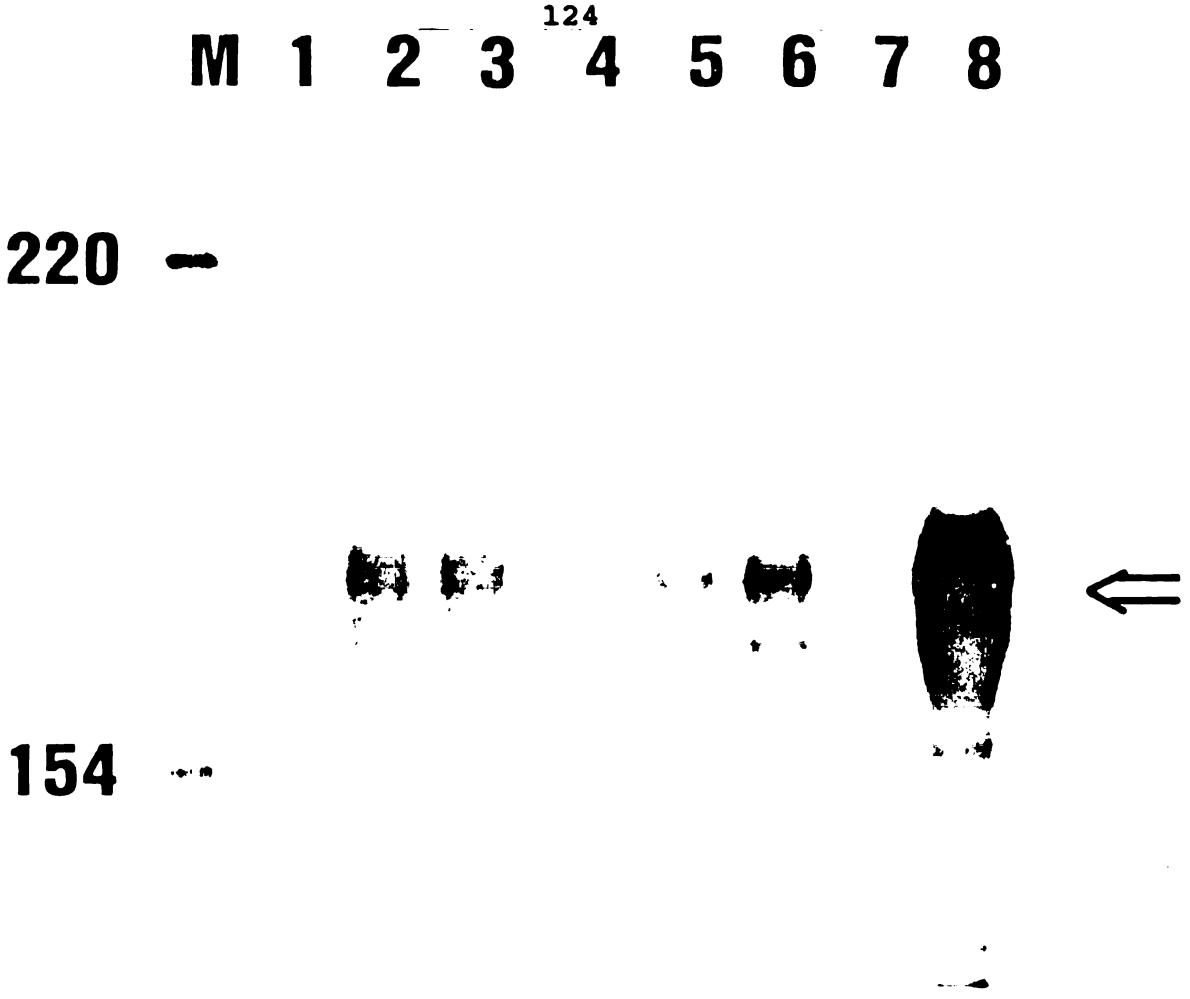
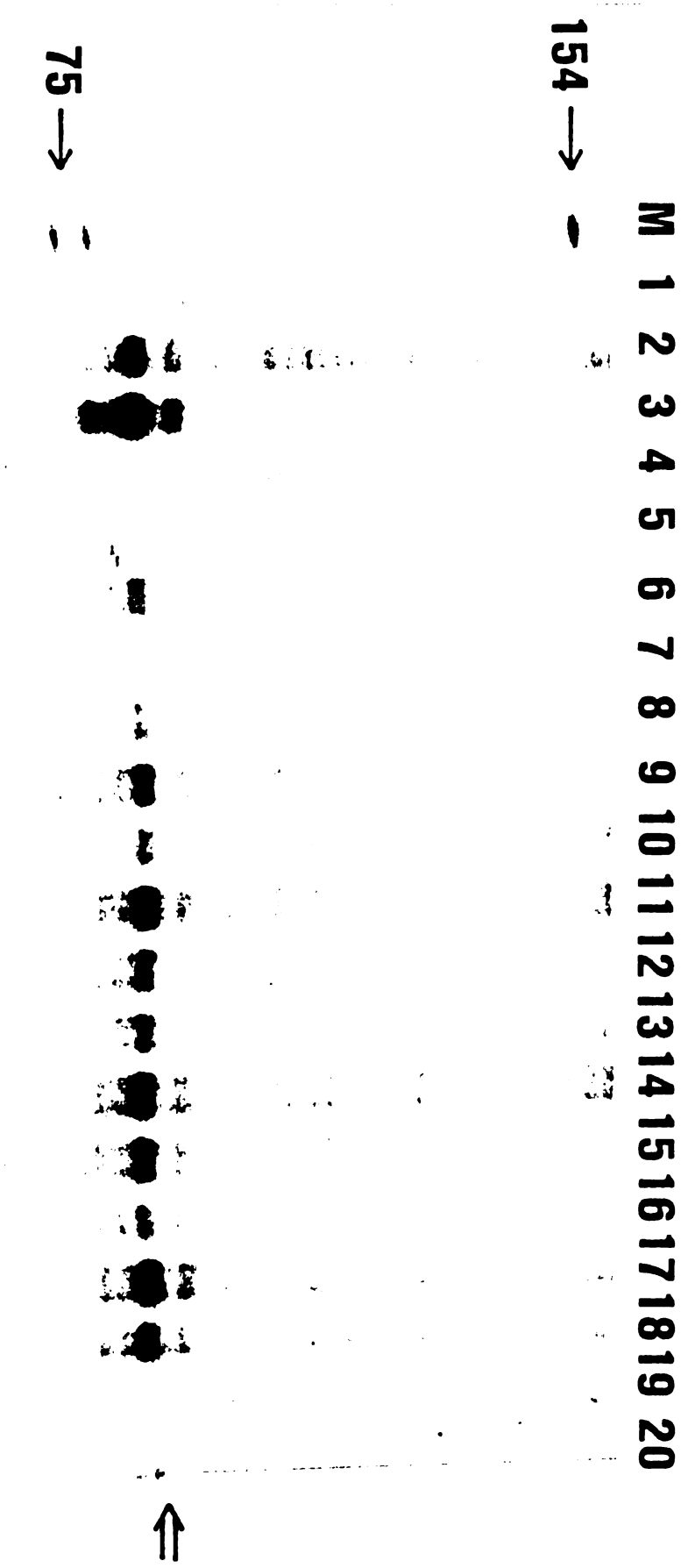


Figure 21. Cell cycle regulation of a fusion gene in pH3F3'ΔI-3.2. One mass culture (lanes 1-3) and an isolated cell line (lanes 4-6) are shown. Thirty micrograms of total RNA were used for each RNase protection assay. The probe used is shown in Figure 17. Total RNAs were isolated at 0 hr (lanes 1 and 4), 6 hr (lanes 2 and 5), and 12 hr (lanes 3 and 6) after the stimulation. Lane M shows a size marker. Lane 7 shows RNA from stimulated Rat 3 cells and lane 8 shows RNA from anemic chicken red cells.

Figure 22. The expression of chicken H3.2 histone and its fusion gene mRNAs. Three mass cultures were grown from Rat 3 cells cotransfected with pCH1a-H2.7 (3-Ma, lanes 1-3, 3-Mb, lanes 4-6, and 3-Mc, lanes 7-9) or with pFHR-1.6 (C-Ma, lanes 10-12, C-Mb, lanes 13-15, and C-Mc, lanes 16-18). Total RNAs were isolated at 0 hr (lanes 1, 4, 7, 10, 13, and 16), 6 hr (lanes 2, 5, 8, 11, 14, and 17), and 12 hr (lanes 3, 6, 9, 12, 15, and 18) after the stimulation. Thirty micrograms of RNA were used in each RNase protection assay with a probe made from pBS(-)-H3.2, shown in Figure 17. Lane 19 represents RNA from stimulated Rat 3 cells and lane 20 represents RNA from anemic chicken red cells.


154
75
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

This result also confirms the previous conclusion that the 3'region of the chicken H3.2 histone gene is at leastpartially responsible for the cell cycle regulation of the fusion gene constructs.

Densitometric Analysis

All radiograms in this chapter were analyzed by a densitometer to measure the expression levels of each fusion gene. An LKB 2222-010 UltraScan XL Laser Densitometer (Bromma, Sweden) was used. To correlate these measurements to the wild type H3.2 gene, a few mass cultures stably transfected with pCH1a-H2.7 were grown. Total RNAs from the mass cultures were tested for the expression of the chicken H3.2 histone gene by the RNase protection assay (Figure 22). The results of densitometric analysis are shown in Table 3. The expression of the H3.2 gene was increased by almost 9 fold at 12 hr after stimulation relative to 0 hr. The chicken H3.3 histone gene without introns showed constant expression as cells go from the G_0/G_1 boundary to late S phase. As mentioned earlier, the fusion gene in pFHR-1.6 was expressed at similar levels at 6 hours and at 12 hours after the stimulation, being increased by a factor of 3-4 fold. The fusion genes that contain the H3.3 5' flanking region and the H3.2 3' flanking region generally showed little or no increase in expression at 6 hr and about a 3-fold increase at 12 hr. The pH3F3'ΔI-3.2 fusion gene showed slightly higher levels of

	0 hr	6 hr	12 hr
H3.2 in pCH1a-H2.7	1	2.6	8.7
pBH6bΔI	1	1.2	1.0
pFHR-1.6	1	3.7	2.7
pFBH-3.9	1	1.3	2.6
pFBH-3.9ΔI	1	0.9	3.4
pH3F3'ΔI-3.2	1	2.1	4.4

Table 3. Densitometric analysis of chicken histone fusion genes. Each number represents an average of at least two mass cultures or isolated cell lines. Numbers at 6 and 12 hr after the stimulation are normalized relative to the numbers at 0 hr.

stimulation at 6 hr and at 12 hr than the other 5'-H3.3-H3.2-3' fusion genes, but it is doubtful if this represents a significant difference. It should be noted that the difference in the histone genes in pFBH-3.9ΔI and pH3F3'ΔI-3.2 are only minor nucleotide changes in the center of the coding region (leading to 4 amino acid changes in the resultant proteins, if they are expressed).

D. How do the kinetics of H3.2 and fusion gene activation correlate with the Rat 3 cell cycle?

Cell Cycle Analysis of Transfected Cells

Although the transfected Rat 3 cell system has been used extensively in other labs, it was necessary to correlate exogenous H3 histone gene expression with the S-phase kinetics of these cells. We also wished to examine fusion gene activation with a more detailed series of time points. In these experiments, Rat 3 cell lines transfected with pCH1a-H2.7, pFHR-1.6, or pFBH-3.9 were tested. Cells were synchronized in G_o/G_1 phase and harvested at 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 hours after serum stimulation. Cells were stained with acridine orange and their DNA contents were analyzed in a cell sorter (11). These three different cell lines showed essentially identical cell cycle kinetics. One pattern is shown in Figure 23. The numbers on the x axis are units of DNA fluorescence and the y axis shows units of cell

number. The cells appear to be well synchronized, and until 4 hours after the stimulation most cells remained in the G_0/G_1 phase. At 6 hr cells had started to move into S phase, and by 12 hr most cells were in S. The peak of DNA synthesis seems to be around 12 hr. From this FACS (Fluorescent Activated Cell Sorter) analysis, it is clear that the transfected Rat 3 cells enter the S phase between 6 and 8 hours after the serum stimulation.

Cell Cycle Analysis of Chicken H3 Histone Genes

Total RNAs from the three cell lines mentioned above were isolated at 2 hour intervals after serum stimulation in the presence or absence of a DNA synthesis inhibitor, aphidicolin (at a concentration of 2.5 μg/ml, 12). The level of wild type chicken H3.2 mRNA in the pCH1a-H2.7-transfected line started to increase at 6 hr and remained high during the S phase (Figure 24-A, lanes 1-10) in agreement with results presented in Chapter 3. However, in the absence of DNA synthesis there was no increase in H3.2 mRNA level (lanes 11-20). Thus the overall level of H3.2 expression seems to correlate with DNA synthesis, as expected for an H3.2 histone gene with both intact 5' and 3' ends.

The fusion gene in pFHR-1.6, which contains the H3.2 promoter region and H3.3 3'region, shows a different pattern of expression (Figure 24-B). The fusion gene mRNA level

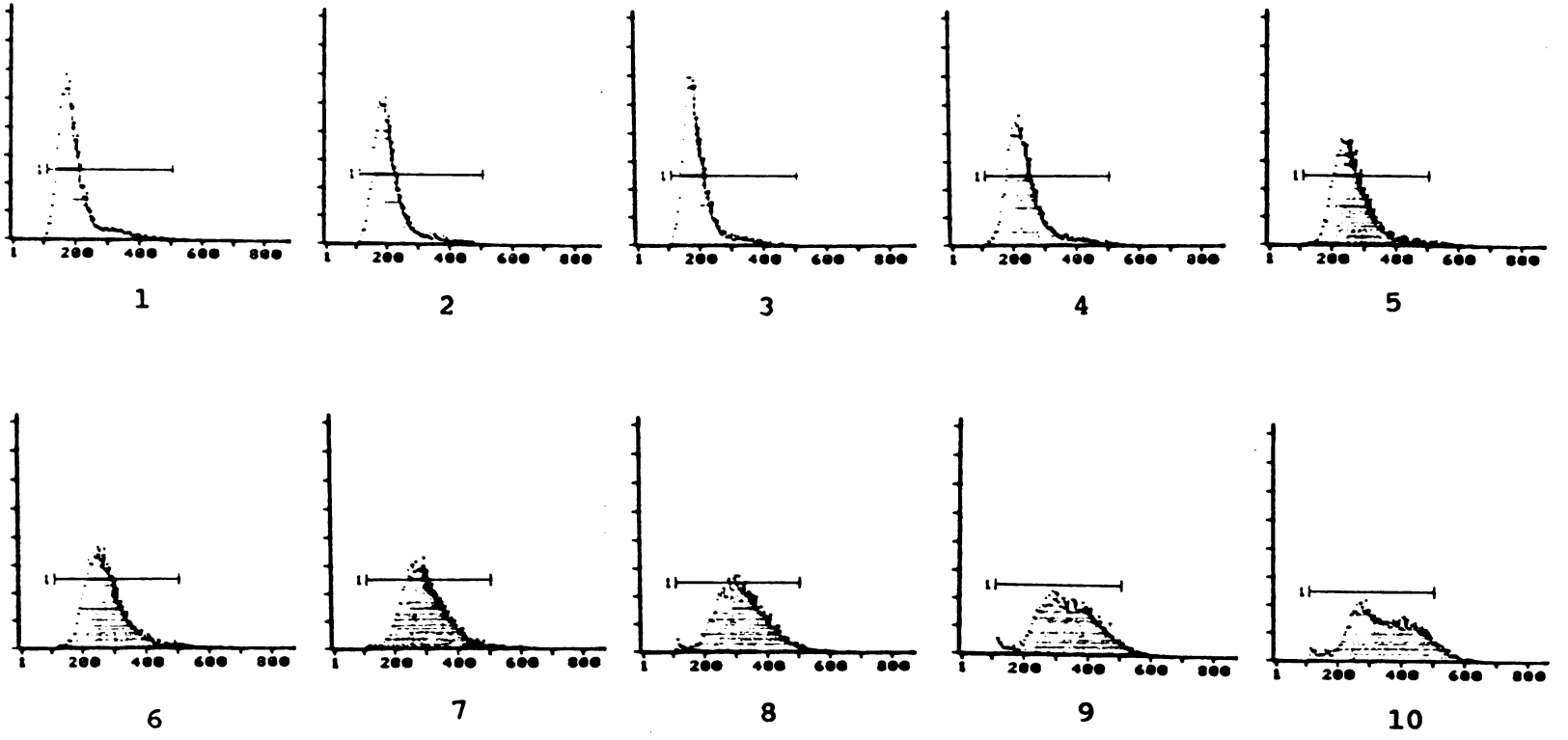
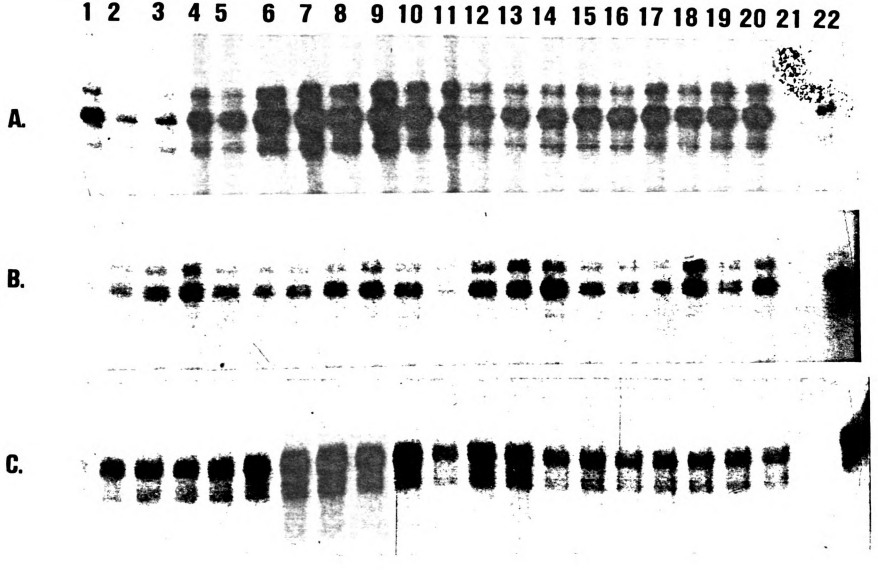


Figure 23. FACS analysis of transfected Rat 3 cell cycle. Cells were harvested at 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 hours (1 to 10, respectively) after serum stimulation. The numbers on the x axis are units of DNA fluorescence and the y axis shows units of cell number.

Figure 24. Effect of aphidicolin in various chicken H3 fusion mRNA expression. A. RNAs from Rat 3 cells cotransfected with pCH1a-H2.7. B. RNAs from Rat 3 cells cotransfected with pFHR-1.6. C. RNAs from Rat 3 cells cotransfected with pFBH-3.9. Total RNAs were isolated from mass cultures at 2 hour intervals from 0 hr to 18 hr after the stimulation in the absence (lanes 1-10) or in the presence (lanes 11-20) of a DNA synthesis inhibitor, aphidicolin. Lane 21 represents stimulated Rat 3 cells and lane 22 represents anemic chicken red cells. Thirty micrograms of RNA were used in each RNase protection assay. The RNA probes used for A and B were made from pBS(-)-H3.2 and for C was made from pBS(-)-H3.3, shown in Figure 17.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
A.
B.
C.

increases rapidly and reaches its peak at 6 hr, followed by a slight decrease after which it remains relatively constant. The expression pattern of this gene was similar in the presence of aphidicolin except for the high level of expression at 14 hr which is unusual, and which could be some sort of artifact of the assay of this one time point. These results tend to confirm our suggestion that the H3.2 promoter may be activated before S phase, probably early in G_1 phase.

The other fusion gene, that in pFBH-3.9, which contains the H3.3 promoter region and the H3.2 3' region, showed an expression pattern different from that of the previous two genes. At 0 hr, the level of the fusion gene mRNA appeared unusually low (lane 1 in Figure 24-C). This should be about the same level as at 0 hr with aphidicolin (lane 11), because there is no difference between these two. If we consider the exceptionally low level of the fusion gene mRNA in lane 1 as a result of an experimental error, the effect of the DNA synthesis inhibitor is evident. In the absence of aphidicolin, the fusion gene mRNA level increased slowly until 12 hr when there was a big increase followed by a relatively steady level. In the presence of aphidicolin, however, there is no big increase in the mRNA level. There was some increase at 2 hr, which is hard to explain. As mentioned earlier, H3.2 histone mRNA is more stable during S phase. The high level of the fusion gene mRNA at 12 hr and 14 hr (in S phase) must

be due to the effect of H3.2 3' region, which presumably stabilizes fusion gene mRNA in an S-phase-specific manner. In contrast, the mRNA stability shouldn't change in the absence of DNA synthesis, and therefore a relatively constant level of fusion gene mRNA from resting stage to the late stage of S phase was observed, as would be expected for a gene with the constitutive H3.3 gene promoter.

Discussion

As outlined in Chapter 1, cell cycle regulation of histone gene mRNA levels involves both transcriptional and post-transcriptional components. For example, DeLisle et al. (3) reported that the level of mouse H3 histone mRNA increased by a factor of 50 during S phase, but the rate of H3 gene transcription increased only 5 fold during this period. Furthermore, Alterman et al. (9) reported that in a mouse H3 histone gene, the difference in transcription rate between S phase cells and resting cells is much smaller in extent than that in the steady-state levels of the mRNA. Most studies of transcriptional regulation have focused on regulatory elements in the 5' flanking regions of histone genes. Most studies on post-transcriptional regulation have focused on histone mRNA stability conferred specifically during S phase by the 3' stem-loop region. The hybrid histone gene approach has allowed us to separate and compare the magnitude of these effects for chicken H3 histone genes.

In the system we have used, a short (ca. 130 bp) portion of the H3.2 promoter region confers about a 3 fold increase in H3 histone mRNA in S phase relative to G_0/G_1 when hooked to the H3.3 histone gene body (Table 3). Similarly, an H3.2 histone gene 3' end containing the stem-loop region confers about a 3 fold increase in S phase mRNA levels (relative to G_0/G_1) when attached to an H3.3 promoter and coding region.

Since the wild type H3.2 gene shows about a 9 fold increase (Table 3) in mRNA from G_0/G_1 to S phase, it appears that the 5' end and 3' end effects are multiplicative and, presumably, independent of one another.

Although the sizes of the 5' and 3' end effects are similar, their kinetics differ considerably. Our results (Figure 19 and Figure 24) suggest that the promoter (transcriptional) effect begins earlier in the cell cycle, at least by 6 hr after serum stimulation and thus slightly before the onset of S phase. This is in agreement with the report of Plumb et al. (2) who suggested that transcriptional regulation is predominant in early S phase and post-transcriptional regulation predominates late in S. Presumably, the early transcriptional activation of replication dependent histone gene promoters such as those of the H3.2 genes normally has little effect, since in absence of DNA synthesis, the resultant H3.2 mRNA is unstable. We observe this activation in the H3.2-H3.3 fusion genes, since these mRNAs contain a normally stable poly(A)$^+$ 3' end. This conclusion is also in agreement with the results of Hereford et al. (13), who reported that the activation of yeast histone mRNA synthesis occured late in G_1, at a point prior to the initiation of DNA replication and of Artishevsky et al. (14), who showed that a critical period necessary for hamster histone mRNA accumulation occured late in G_1 phase. In

addition, the latter authors showed that the maximal rate of histone transcription preceded the peak of DNA synthesis by 4 to 6 hours in a hamster fibroblast cells.

Also in agreement with Plumb et al. (2), our results show that the 3' end effect (S-phase-specific mRNA stability, 6,7) is established more slowly in the cell cycle, not reaching its peak until 12-14 hr after serum stimulation (Figure 24), that is, late in S phase (Figure 23). This can best be seen by examining the RNA accumulation pattern of pFBH-3.9, the H3.3-H3.2 hybrid gene. Our results (Figure 18) and previous results (1) suggest that the H3.3 promoter behaves in a weak, constitutive manner. Thus, the changes in pFBH-3.9 mRNA level during the cell cycle are presumably due to the 3' end effects. The results of Figure 24-C show that the fusion RNA is relatively unstable early in the cell cycle, with increased stability beginning in S phase and peaking in late S. This agrees with the reports by DeLisle et al. (3) who claimed that the half-life of mouse H3 mRNA increased by almost 20 times during S phase and by Heintz et al. (15) who reported a 5 fold increase in stability during S phase.

Finally, our results suggest that there is little or no effect of internal coding sequence on cell cycle regulation of histone gene expression. Furthermore, the natural H3.3 gene introns have no effect on cell cycle-regulated

expression, in contrast with the report of Seiler-Tuyns and Paterson (10) that a globin gene intron blocked cell cycle regulation of histone gene expression.

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