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Histone H1t: A Tissue-Specific Model Used to Study Transcriptional Control and Nuclear Function During Cellular Differentiation

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Abstract One of the most prominent and best studied family of genes is the histone gene family. In recent years, histone gene regulation during the cell cycle of somatic cells has been studied extensively. This paper is intended to highlight and emphasize recent data concerning the tissue-specific expression of histone H1t using spermatogenesis as a model system. In this article we describe a unique DNA element within the proximal promoter of the histone H1t gene. This element has been shown to bind exclusively to nuclear proteins from pachytene spermatocytes and early spermatids. Thus, there is a strong temporal correlation between the appearance of the testis-specific DNA-binding protein and the onset of transcription of the testis-specific histone H1t gene. © 1993 Wiley-Liss, Inc.*

Key words: spermatogenesis, testis-specific, chromatin, transcriptional regulation, DNA-binding protein

The family members of H1 linker histones including the nucleated erythrocyte H5 [Yaguchi et al., 1979; Briand et al., 1980] are components of chromatin associated with the transition of a 10 nm nucleosome containing DNA fiber into a higher ordered structure, the 30 nm solenoid [Thoma et al., 1979]. This transition results in sequestering of genes into heterochromatin. Histone H1 binds to linker DNA between nucleosomes in all eukaryotes except some fungi [Finch and Klug, 1976].

Members of the H1 family of proteins exhibit the common characteristics of having a short, randomly coiled basic amino-terminal arm and a long, randomly coiled, basic carboxy-terminal arm which flank a central, trypsin-resistant, globular domain [Hartman et al., 1977; Aviles et al., 1978]. The basic N-terminal arm and the C-terminal arm are thought to interact with linker DNA while the globular domain is essential for histone H1 interaction with the nucleosome [Clore et al., 1987; Turnell et al., 1988; Segers et al., 1991; Ramakrishnan et al., 1993]. Each cell contains a complement of H1 family members which can vary during development and differentiation. Some H1 subtypes are present in only a specific cell lineage such as the H1t subtype found in the germinal cells of the mammalian testis [Branson et al., Bhatnagar et al., 1985; Owen et al., 1986; 1975; Meistrich, 1989; Kistler, 1989] and the H5 subtype found in nucleated erythrocytes [Appels and Wells, 1972].

It is widely accepted that two general classes of histone H1 genes are present in most eukaryotes: 1) replication-dependent and 2) replication-independent. Replication-dependent histones are expressed during the S-phase of the cell cycle. The replication-dependent class is further characterized by genes that do not have introns and have corresponding mRNAs that lack polyadenylation [Osley, 1991]. Histone genes of the second class can have introns and can have corresponding mRNAs that are polyadenylated [Osley, 1991]. The replication-independent genes are expressed at a low but constant level throughout the cell cycle [Wu and Bonner, 1981]. A third class of histones and the focus of this paper is the testis histone subtype expressed in the germinal cells during spermatogenesis.

NUCLEAR FUNCTION OF HISTONE H1

Clearly, during the progression of spermatogenesis, chromosomal structure undergoes dra-

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matic conformational changes which ultimately results in compaction into a sperm head. Once compacted there are no significant processes involving DNA until fertilization. Little is known about histone contribution, if any, to the processes of homologous pairing, molecular recombination, and meiotic division.

Very little work has been conducted on the average spacing between nucleosomes during the spermatocyte and early spermatid stage of spermatogenesis where H1t is expressed. Several researchers have recently demonstrated the importance of nucleosome core positioning and displacement on transcriptional initiation and elongation [for reviews see Adams and Workman, 1993; Zlatanova, 1990]. Histone H1 participates in transcriptional regulation by stabilization of nucleosomes and conversion of DNA into a higher ordered structure. Recently, histone H1 has been shown to compete with transcriptional activators as measured by in vitro competition for binding to specific sites on DNA [Croston et al., 1991; Laybourn and Kadonaga, 1991]. Histone H1 added to naked DNA was shown to inhibit transcription of a test gene flanked by binding sites for Sp1, GAL4-VP16, or the GAGA factor [Croston et al., 1991]. Addition of the appropriate factor, was shown to counteract the H1 mediated repression [Croston et al., 1991]. Addition of histone H1 to DNA templates previously reconstituted with core histones yielded similar results to experiments with naked DNA [Laybourn and Kadonaga, 1991].

The results from these experiments provide evidence that histone H1 can act as a general repressor that can be selectively removed or depleted by specific transcriptional factors. The repressor function of histone H1 may not be a generalized mechanism whereby the whole chromosome is covered nonspecifically by H1, but occurs in specific localized regions [Parseghian and Hamkalo, 1993; Schulze et al., 1993]. This role of histone H1 may reflect the specific histone subtype as well as specific post-translational modifications such as phosphorylation [Roth and Allis, 1992]. In any case, the presence of the histone H1t subtype correlates with significant changes in transcription of a large number of genes involved in spermatogenesis.

TESTIS-SPECIFIC HISTONE H1t

Histone H1t, the testis-specific H1 variant, is synthesized only in pachytene primary spermatocytes. Synthesis of H1t is dependent upon the steady-state level of H1t mRNA. Human and rat histone H1t genes are transcribed only in testis as determined from steady-state levels of H1t mRNA by Northern blot analysis [Cole et al., 1986; Grimes et al., 1987, 1990; Drabent et al., 1991]. Total cellular RNA from 7-day-old rats is devoid of H1t mRNA as analyzed by Northern blot analysis. Therefore, we conclude that H1t mRNA does not accumulate in the testis before the spermatocyte stage. Additionally, there is no detectable H1t mRNA in early spermatids. Transcription of the H1t gene must decrease dramatically or H1t mRNA turnover must increase or both during or immediately following the first meiotic division. Total cellular RNA isolated from enriched populations of pachytene primary spermatocytes exhibits high steady state levels of H1t mRNA indicating a high level of transcriptional activity probably combined with a low turnover rate in this germinal cell type. Nongerminal testis cells such as Leydig cells are devoid of histone H1t mRNA.

Data generated by Northern blot analysis may indicate a low rate of transcription coupled with a high H1t mRNA turnover rate in somatic cells and germline cells excluding the pachytene spermatocytes. Examination of H1t transcription by nuclear transcription assays resulted in no detectable transcription in liver cells, but a significant level of transcription in rat testis [Wolfe and Grimes, 1991]. Upon further examination of enriched populations of the various cell types from testis, we were surprised to find transcriptional initiation in both the pachytene spermatocyte enriched fraction as well as the early spermatid enriched fraction. Since there is no detectable full-length or mature histone H1t mRNA in early spermatids, transcription may be terminated prematurely or mRNA transcripts may be degraded at a rapid rate rendering H1t mRNA undetectable by Northern analysis.

Comparison of sequences of the known mammalian histone H1 promoters revealed four sequence elements that are highly conserved [Kistler, 1989; Grimes et al., 1990]. These elements are the H1/AC box, the H1/GC box the H1/CCAAT box, and the TATA box, respectively [Dalton and Wells, 1988; van Wijnen et al., 1988; Osley, 1991]. These elements are present in the H1t gene and are shown in Figure 1. These four elements are conserved in most H1 promoters, but the spacing between the elements can vary. The H1/GC box appears to be

H1t PROMOTER

H1/AC BOX BINDING FACTOR | SP1-LIKE BINDING FACTOR | H1t/CCTAGG BINDING FACTOR | H1/CCAAT BOX BINDING FACTOR | TFIID BINDING FACTOR | RNA POLYMERASE II + GENERAL TRANSCRIPTION FACTORS

-113 TGGGGGAAAAGAAACACAAATGCCCCTTCCCCAGGGGCGGGGGAGGCGCCTAGGGATGCACCAATCACAGCGCGCCCTGCTCTATATAAGGCCCCCCCCCGGACCCCCTCTCTACTCCAGCGCTG 12

H1/AC BOX | H1/GC BOX | H1t/CCTAGG | H1/CCAAT BOX | TATA BOX

Fig. 1. Model of the testis-specific histone H1t promoter. A hypothetical model of the histone H1t promoter shows DNA-protein binding sites based upon published data from other H1 genes and the four highly conserved H1 consensus sequence elements (H1/AC box, H1/GC box, H1/CCAAT box, and TATA box). This model emphasizes the newly discovered testis-specific H1t/CCTAGG element found in mammalian H1t promoters. The protein factors (cross-hatched except for the H1t/CCTAGG binding protein which is solid) are drawn showing hypothetical interactions with DNA, with each other, and with RNA Polymerase II to form a transcription preinitiation complex. This linear depiction of the H1t proximal promoter and cognate binding factors does not include accessory transcription factors which may be essential for formation of a transcription preinitiation complex. Accessory factors do not bind directly to DNA.

the least conserved of the four motifs and is totally absent in some H1 promoters.

The H1/AC box (AAACACA) is a highly conserved H1 promoter element that has been shown to be essential for optimal expression of histone H1 genes in S-phase [Dalton and Wells, 1988]. As mentioned previously, the histone H1t gene contains this element, but there is no replication in pachytene primary spermatocytes where the H1t gene is transcribed. The H1/CCAAT box and the adjacent TATA box have also been shown to be essential for the optimal transcription of H1 genes during S-phase [Gallinari et al., 1989; LaBella et al., 1989]. The H1/CCAAT box and TATA box region is the most highly conserved portion of the promoter. Again, both of these elements are present in the H1t gene (Fig. 1). However, a study of these elements has not provided insight into mechanisms involved in regulating tissue-specific transcription of the H1t gene.

THE UNIQUE TESTIS-SPECIFIC HISTONE H1t/CCTAGG PROMOTER ELEMENT

Examination of the H1t promoter revealed a sequence element located between the H1/GC box and the H1/CCAAT box homologous to an element that directs the testis specific expression of β2-tubulin in *Drosophila* [Michiels et al., 1989; Grimes et al., 1990]. The central portion of the element contains the palindromic sequence CCTAGG (Fig. 1). Additionally, this palindrome is found in the human, monkey, and rabbit H1t promoters in the same location [Koppel et al., 1992]. Further examination revealed that the H1t/CCTAGG palindrome is actually the center of a larger 18 to 19 bp element. The nucleotide sequence within this element is highly conserved with only two base differences being noted upon comparison of five species of mammals. Thus far, this element has been found only in histone H1t promoters. Since a portion of the *Drosophila* element is homologous to a sequence element found in H1t promoters, we decided to determine whether the element could have a similar role in mammalian H1t gene expression. It should be mentioned that there is evidence that a sequence upstream from the proximal promoter modulates transcription of the rat H1t gene in somatic cells in transient transfection assays [Kremer and Kistler, 1992], but it is not clear that this sequence modulates transcription in testis germinal cells.

The H1t/CCTAGG containing sequence element currently appears to be an excellent candidate for interaction with a transcriptional regulatory protein. Since this element is highly conserved in mammalian H1t promoters [Koppel et al., 1992], it potentially represents a binding site for a transcriptional factor. We have drawn a model in Figure 1 showing the position of the element in the H1t promoter and the binding of testis-specific protein to the element to form a transcription preinitiation complex. In this model the testis-specific DNA-binding protein could interact directly with the DNA-

binding proteins that bind to the H1/GC box and the H1/CCAAT box. It could possibly interact directly or indirectly (through an accessory factor) with the other factors in the transcription complex.

BINDING OF TESTIS-SPECIFIC NUCLEAR PROTEINS TO THE H1t/CCTAGG ELEMENT

Proteins from crude nuclear extracts from rat testis bind specifically to the 18 bp element from the rat H1t promoter. Activity is specifically competed with a 10-fold excess of homologous DNA. On the other hand, activity is retained in the presence of 1,000-fold excess of heterologous DNA. We have tested nuclear extracts from several other rat tissues and all fail to bind to the 18 bp element. Further testing revealed that only nuclear extracts from sexually mature rats bind to the element. Nuclear extracts from sexually immature rats (7 day old male) fail to bind whereas extracts from enriched populations of pachytene spermatocytes and early spermatids bind [Grimes et al., 1992a,b].

Therefore, the data support the hypothesis that testis-specific transcription of the histone H1t gene is regulated in large part by the newly identified H1t/CCTAGG sequence element and the cognate testis-specific DNA binding protein. The tissue and cell types from which the binding activity can be extracted correlate well with our previous Northern blot and transcriptional initiation data. These data show that H1t transcription occurs in both pachytene primary spermatocytes and early spermatids but that H1t mRNA accumulation occurs only in primary spermatocytes. These data support the hypothesis that H1t mRNA transcription in early spermatids is initiated but mRNA does not accumulate. This may be due in part because of premature termination of transcription and/or specific H1t mRNA degradation.

Concerning the nature of the H1t/CCTAGG binding protein, only one major band is apparent in the electrophoretic mobility shift assays in native gels when testis nuclear extracts bind to the H1t promoter element [Grimes et al., 1992a,b]. The DNA protein complex is stable up to 42°C as determined using the mobility shift assays, but the binding activity is heat labile if the protein is not bound to DNA. UV-crosslinking experiments reveal one major testis-specific band with an apparent molecular weight of 70–80 kDa [Grimes et al., in press]. A representation of the H1t/CCTAGG binding protein is presented in the model in Figure 1.

FUTURE PROSPECTS

The discoveries described in the previous sections raise several important questions. Does the "CCTAGG" sequence element play a role in testis-specific transcription of the histone H1t gene and possibly other testis genes that are expressed in pachytene spermatocytes? Will the purified testis-specific factor enhance transcription when added to in vitro transcription assays? Are histone H1t and the testis-specific factor essential for spermatogenesis?

In order to begin to answer these questions, we plan to continue transcription studies of the H1t promoter and mutant H1t promoters using transient expression assays. Specific bases involved in binding of the testis-specific protein to the H1t/CCTAGG element will be mutated to see the effect on transcription. We have found an 11 bp sequence located between the H1/AC box and H1/GC box of the H1t promoter that is similar to the H1t/CCTAGG sequence. This same 11 bp sequence is also found in the shared promoter of the rat testis-specific histone TH2B-TH2A gene pair. This element or a variant of it may be present in many testis-specific genes.

To better characterize the testis-specific DNA-binding protein, we will attempt to purify the protein. The purified protein or protein-DNA complex can be used to raise polyclonal antibodies. We will test whether the purified protein will stimulate transcription in an in vitro transcription system. The polyclonal antibodies should bind to the factor and block in vitro transcription.

In order to determine whether the 7 kb rat genomic DNA fragment containing the histone H1t and H4t genes can direct testis-specific transcription of the H1t gene, it may be necessary to conduct experiments using transgenic mice. If the 7 kb rat fragment is found to direct faithful testis-specific transcription of the H1t gene, then a second experiment can be conducted with the fragment carrying a mutant H1t/CCTAGG element.

We believe that histone H1t and the testis-specific DNA-binding protein may be essential for spermatogenesis. Histones are responsible in large part for chromatin structure and may be involved in regulating expression of specific genes. The different histone subtypes are not bound to DNA in a random distribution. This

leads to a banding pattern in chromosome spreads when specific H1 variants are assayed. Expression of histone variants (and especially the testis histone variants) may therefore lead to altered chromatin structure and to altered expression of specific genes during spermatogenesis. To test this possibility, it may be necessary to use antisense RNA technology or embryonic stem cell technology to block expression of the genes or "knock out" the genes. It would be informative to delete the coding region of the H1t gene and replace it with the coding region of another H1 variant. This type of experiment should reveal whether other H1 variants can successfully substitute for H1t.

This is an exciting time for the study of the interrelationships between cell structure and gene expression, and in particular the contribution of nuclear architecture to the regulation of tissue-specific structure and function. Existing technology should allow us to answer in the near future most of the questions raised in this section.

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