

Presence of a bi-directional S phase-specific transcription regulatory element in the promoter shared by testis-specific TH2A and TH2B histone genes

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ABSTRACT

During mammalian spermatogenesis, somatic histones are replaced by testis-specific variants. The synthesis of the variants occurs primarily in the germ cells undergoing meiosis in the absence of DNA replication. We have cloned the genes encoding rat somatic and testis-specific H2A (TH2A) histones. The two genes share 300 bp of 5' upstream region with respective H2B genes: somatic H2A with somatic H2B and testis-specific TH2A with testis-specific TH2B gene. The deduced amino acid sequences show that H2A and TH2A histones have eight amino acid differences in the first half of the molecules and three consecutive changes in the C-terminal region. TH2A gene is expressed only in testis. Although synthesis of TH2A and TH2B histones is independent of DNA replication and insensitive to inhibitors of DNA synthesis in testis, the regulatory region shared by the two genes contain a bi-directional S phase-specific transcription regulatory element. In addition, TH2A gene, like TH2B gene, contains the consensus sequence element in the 3' non-coding region which is involved in the S phase-specific stabilization of histone mRNA.

INTRODUCTION

During mammalian spermatogenesis, somatic histones are replaced with corresponding testis-specific histone variants (1-3). Unlike somatic histones, synthesis of the testis-specific histone variants is not coupled with DNA replication and unaffected by inhibitors of DNA synthesis (3-5). In the rats, the synthesis of testis-specific histones occurs primarily in pachytene spermatocytes during meiotic prophase I (2,3). It is not clear what is the biological significance of the replacement of somatic histones by the testis-specific types. The pachytene chromatin is more relaxed than chromatin of somatic cells (6,7), and the relaxed chromatin structure may enhance the extensive DNA recombinations occurring in the synaptonemal complexes

(8-10). In a previous study we reported molecular cloning of the cDNA of testis-specific H2B variant (TH2B) and a somatic H2B gene from a rat genomic library (11). *In situ* cytohybridization showed that the expression of TH2B gene is most extensive in pachytene spermatocytes and that of H2B gene occurs only in dividing cells such as spermatogonia and Sertoli cells in rat testis (11). Comparison of the amino acid sequences of H2B and TH2B histones showed that there are extensive differences in amino acid sequences in the N-terminal regions of the two histones (11). The notable difference is the substitution of a nine amino acid stretch starting at the third amino acid of TH2B. This causes a significant reduction in the hydrophilicity of the N-terminal region of TH2B histone. The changes in the primary structure may be responsible for the higher sensitivity of the H2B-binding site in pachytene nucleosome to DNase I (7). The testis-specific H1 gene was also cloned and the deduced amino acid sequence shows extensive variations in the N- and C-terminal regions from somatic H1 histone (12).

We found that a testis-specific H2A (TH2A) gene is located upstream a testis-specific H2B (TH2B) gene in a rat genomic clone. The two genes are transcribed in opposite directions and share about 300 bp 5' flanking sequence. We have also cloned a rat genomic DNA containing somatic H2A and H2B genes which have the same structural organization as the TH2A and TH2B gene pair. Although the expression of TH2A and TH2B genes is testis-specific and independent of DNA replication in testis, the promoter region shared by the two genes contains a bi-directional S phase-specific transcription regulatory element.

MATERIALS AND METHODS

Molecular Cloning and Sequencing of Rat Genomic DNAs Containing Somatic and Testis-Specific H2A and H2B Genes

DNA fragments containing somatic H2B and testis-specific TH2B were cloned from a rat genomic library established in Charon 4A (13) using oligonucleotide probes specific for each gene (11). The regions containing histone genes were subcloned into

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M13mp11 phage DNA. The DNAs were sequentially deleted from one end of the inserts according to the method of Dale et al. (14), and about 2 kb DNA regions were sequenced by Sanger's dideoxy chain termination method (15).

S1 Mapping of Transcription Initiation Sites

The transcription initiation sites of H2A and TH2A genes were mapped by the procedure of Weaver and Weismann (16). RNA was isolated from various rat organs by direct homogenization in 6 M guanidine isothiocyanate and subsequent centrifugation through a cushion of 5.7 M CsCl (17). 5' end-labeled probes were prepared by digesting H2A or TH2A genes with XmaIII. The 5' ends were dephosphorylated and labeled with ³²P in the presence of T4 polynucleotide kinase and ³²P-γ-ATP (NEN, 6000 Ci/mmol). The labeled DNA for H2A was cleaved with NcoI and the one for TH2A with SmaI, respectively. The restriction fragments were then resolved on a low melting temperature agarose gel, and a 638 bp H2A and 645 bp TH2A fragments were excised. The DNA probes were eluted from the gel, and 15,000 cpm of each probe was annealed with 20 μg of total RNA in 10 μl of hybridization buffer at 43°C overnight. The hybrids were treated with S1 nuclease, and the S1-resistant fragments were subjected to electrophoresis on a 43 cm-long polyacrylamide gel containing 8 M urea. The gels were exposed to Kodak X-ray film at -75°C.

Other Methods

Growth of mouse embryo fibroblast C3H 10T½ cells, DNA transfection, cell synchronization, assay for β-galactosidase and chloramphenicol acetyltransferase, preparation of nuclear extracts, and DNase I footprint assays were described in previous reports (18,19). Construction of recombinant DNAs, site-directed mutagenesis, and DNA sequencing were carried out by using standard protocols (20).

RESULTS

Molecular Cloning of a Rat Somatic H2A Gene and a Testis-Specific H2A Variant Gene

In a previous study, we cloned the cDNA for rat testis-specific H2B (TH2B) mRNA (11). Using the regions unique for the TH2B and somatic H2B histone genes as probes, we cloned DNAs containing testis-specific TH2B and somatic H2B histone genes from a rat genomic library established in lambda phage Charon 4A (13). The sequences of TH2B cDNA and the rat somatic H2B gene were reported in a previous report (11). The sequences of the genomic DNAs revealed that there are H2A histone genes in the upstream of H2B and TH2B genes. The DNA sequences of the two H2A genes with the 5' flanking sequences are shown in Fig. 1. The H2A gene present in the upstream of TH2B gene will be referred as TH2A gene in this report. Both the H2A and TH2A genes share 300 bp 5' non-translated flanking region with respective H2B genes, and the H2A and H2B genes are transcribed in opposite directions. The 300 bp flanking region shared by TH2A and TH2B genes contain many elements which are also found in the somatic H2A-H2B genes. For example, the octamer ATTTGTCAT element found in many H2B genes (21 and references therein) and CCAAT elements are found in both gene clusters. The role of some of these elements in the S phase-specific transcription of TH2A and TH2B genes will be discussed later. The non-translated 3'-flanking regions of H2A and TH2A genes also contain a palindromic sequence element (GGCTC-

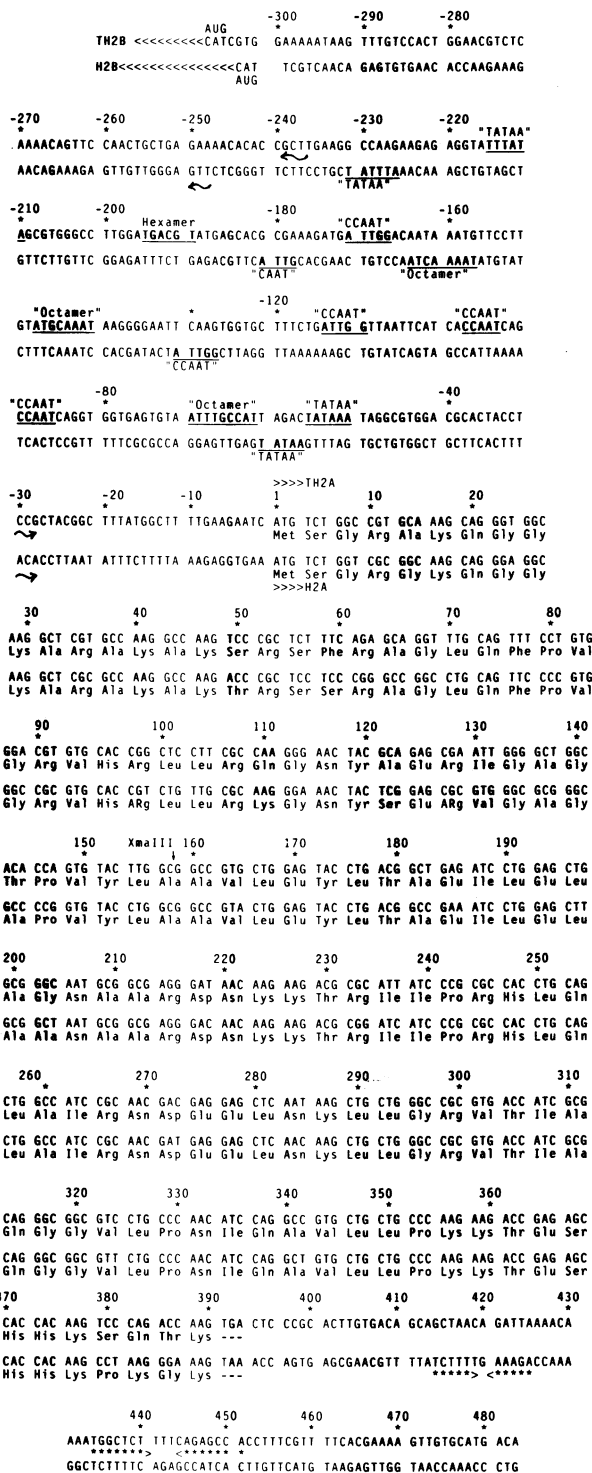


Fig. 1. The DNA sequences of rat testis-specific TH2A and somatic H2A histone genes. The amino acids which are different in the two histones are shown by bold letters. The palindromic sequences present in the 3' nontranslated regions are indicated by *. The 5' flanking regions were numbered from the translation start codon of the two genes. The sequence elements of interest are indicated by bold letters and names. Note that TH2B and H2B histone genes are located about 300 bp upstream of TH2A and H2A genes, respectively. Wavy arrows indicate approximate positions of transcription initiation sites.

T...AGAGCC). The element plays an important role in the processing as well as in the S phase-specific stabilization of histone mRNA (for review, see 22). The same sequence is also found in H2B and TH2B genes (11).

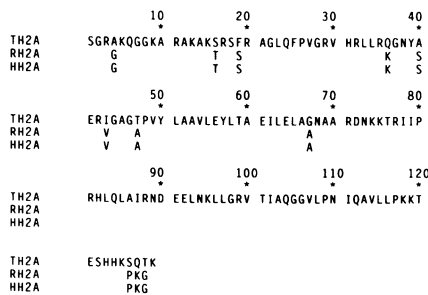


Fig. 2. Comparison of the deduced amino acid sequences of rat TH2A and rat somatic H2A with the amino acid sequence of human H2A histone.

Deduced Amino Acid Sequences

The deduced amino acid sequences show that the two H2A histones are very similar except for the eight amino acid differences in the first half of the molecules and the three consecutive changes in the C-terminal regions. The rat somatic H2A histone sequence is identical to the sequence of human H2A histones (23) as shown in Fig. 2. Thus TH2A appears to be a variant of somatic H2A histones. Hydropathic plots of the two amino acid sequences show that there is no significant difference in the arrangement of hydrophilic and hydrophobic regions between H2A and TH2A histones (data not shown).

Testis-Specific Transcription of the Variant H2A Gene

Due to the extensive sequence similarity of the two histone genes, it was difficult to prepare DNA probes specific for each gene. Therefore, the transcription pattern of H2A and TH2A histone genes was investigated by S1 mapping analysis. RNA was prepared from different tissues as well as from testis of different ages of rats. The transcription initiation sites for H2A, H2B, TH2A and TH2B genes are indicated in Fig. 1. As shown in Fig. 3, transcription of TH2A occurs only in testis in an age-dependent manner. In testes of 7 days old rats which are enriched with rapidly dividing spermatogonia, there is a low but significant level of TH2A mRNA. The level increases dramatically in testis of 14 and 21 days old rats as the proportion of primary spermatocytes increase. In the case of somatic H2A mRNA, there are two S1-resistant RNAs, one protected at about 5 bases (Fig. 3, band b) and another at 30 bases (Fig. 3, band a) upstream of the translation initiation site. It is possible that the protection at about 5 bases upstream of AUG codon could have resulted from an H2A mRNA species derived from a different somatic H2A gene which has a similar sequence as the cloned H2A gene in the protein coding region. The protection site could correspond to the AUG codon of the different H2A gene. This possibility is being investigated. The shorter S1-resistant RNA is more abundant than the RNA initiated at -30 bases. Both species are transcribed at a higher level in the rapidly dividing spleen and thymus cells than in testis. The level of both H2A mRNA appears to be about the same in testis of different ages of rats. The absence of H2A mRNA in liver is most likely due to the relatively small number of dividing cells in liver.

S Phase-Dependent Transcription Elements for TH2A Gene

The testis-specific TH2A and TH2B histones are produced mostly in primary spermatocytes in the absence of DNA replication, and the synthesis is unaffected by inhibitors of DNA synthesis (3-5). On the other hand, synthesis of somatic histones is tightly coupled

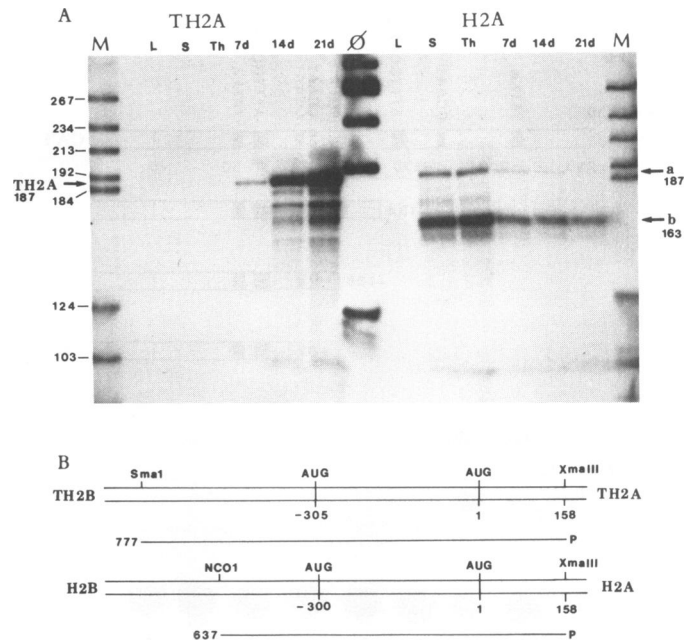


Fig. 3. Testis-specific transcription of TH2A gene. The transcription pattern of TH2A and H2A genes were investigated by S1 mapping. RNAs were extracted from various tissues and hybridized with an end-labeled DNA probe and the duplexes were digested with S1 nuclease. The size of the protected DNA was analyzed by high-resolution polyacrylamide gel electrophoresis as described in Methods. (A). Transcription of TH2A and H2A genes. Lanes: L, liver; S, spleen; Th, thymus, 7d, testis of 7 days-old rat; 14d, testis of 14 days-old rat; 21d, testis of 21 days-old rat. Markers: lanes M, pBR322 digested with HaeIII; lane h, ϕ X174 digested with HaeIII. (B). Diagram for the regions corresponding to the probes used for S1 mapping.

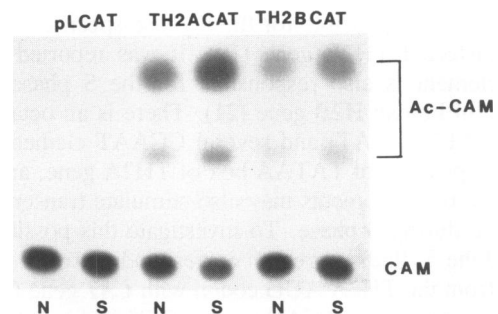


Fig. 4. S phase-dependent synthesis of CAT under the control of TH2A and TH2B promoters. TH2B (485 bp) and TH2A (253 bp) promoters were fused with CAT gene and the fused genes (6 μ g each) and pRSV β gal DNA (5 μ g) were co-transfected into mouse embryo fibroblast C3H 10T $\frac{1}{2}$ cells, and the cells were treated with aphidicolin for 24 h for cell synchrony as described in previous reports (18,19). The cells released from the drug for 3 h (S phase) or incubated with aphidicolin for additional 3 h (non-S phase) were harvested and lysed by repeated freezing and thawing. After centrifugation of the lysed cells, the extracts were assayed for β -galactosidase (18,19). The cell extracts containing the same level of β -galactosidase activity were assayed for CAT activity as described before (18,19). Shown here are the thin-layer chromatogram of labeled chloramphenicol (CAM) and acetoxy-CAM (Ac-CAM). S, S phase; N, non-S phase. pLCAT represents promoterless CAT gene.

with DNA replication and blocked by inhibitors of DNA synthesis (22, 24-26). However, the cloned TH2B gene with upto 140 bp of 5' flanking sequence (from the transcription initiation site) is transcribed in parallel with DNA replication when the DNA is introduced into somatic cells (18). We found that two elements,

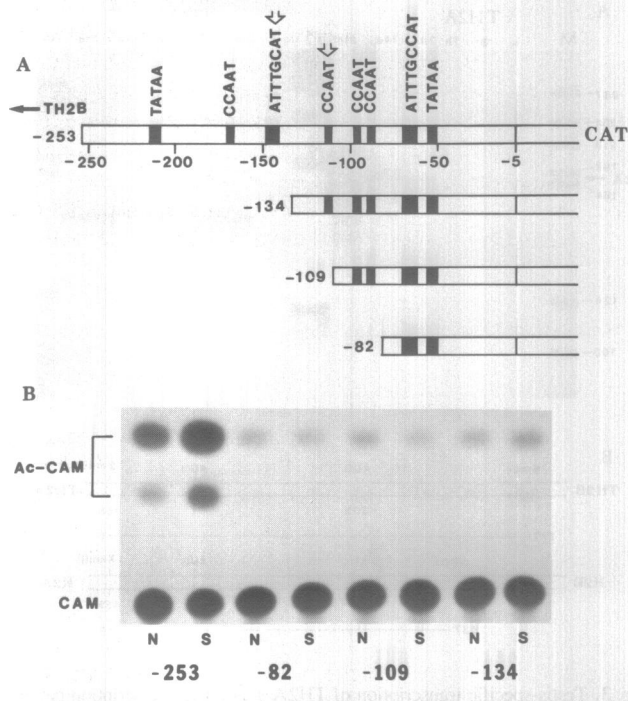


Fig. 5. Effect of deletions on the promoter activity of TH2A gene. TH2A 5' flanking region in TH2ACAT gene was deleted sequentially and production of CAT from the deletion mutants were assayed as described in Fig. 4. S, S phase; N, non-S phase. (A). Diagram of the deletion mutants. The elements indicated by open arrows are involved in the S phase-dependent expression of TH2B gene (19). (B). Results of CAT assay. Lanes N, non-S; S, S phase; CAM, chloramphenicol; Ac-CAM, acetylated CAM.

the CCAAT at -110 bp and octamer ATTTGCAT at -141 bp (see Fig. 1) are responsible for the S phase-specific expression of the transfected TH2B gene (19). It was reported that the octamer element is also responsible for the S phase-specific expression of human H2B gene (21). There is an octamer-like element (ATTTGCCAT) and several CCAAT elements in the immediate upstream of TATAA box of TH2A gene, and it was possible that these elements may also stimulate transcription of TH2A gene during S phase. To investigate this possibility we have fused the 5' flanking region corresponding from -5 bp to -253 bp from the TH2A AUG codon with CAT gene (pTH2A-CAT; Fig. 4). pTH2ACAT and pRSV β GAL (27) were cotransfected into mouse embryo fibroblast C3H 10T $\frac{1}{2}$ cells, and the cells were arrested at the border of G1/S in the presence of aphidicolin as described before (18). After release from the block for three hours (S phase cells), the cells were harvested and the production of CAT was determined. The amount of cell extracts containing the same amount of β -galactosidase activity was used for CAT assay to normalize the differences in transfection efficiency among different culture dishes as described before (18). Fig. 4 shows that the production of CAT from pTH2ACAT was increased 6 fold in S phase cells compared to non-S phase cells (the cells maintained in aphidicolin containing medium). Similar result was obtained with pTH2BCAT as reported earlier (18,19). The promoter-minus CAT (pLCAT, ref. 18) produced no detectable level of CAT. The degree of stimulation of CAT production from pTH2ACAT and pTH2BCAT in S phase is about the same. The TH2A promoter appears to be more active than that of TH2B since the amount of CAT produced from TH2ACAT in non-S and S phase cells

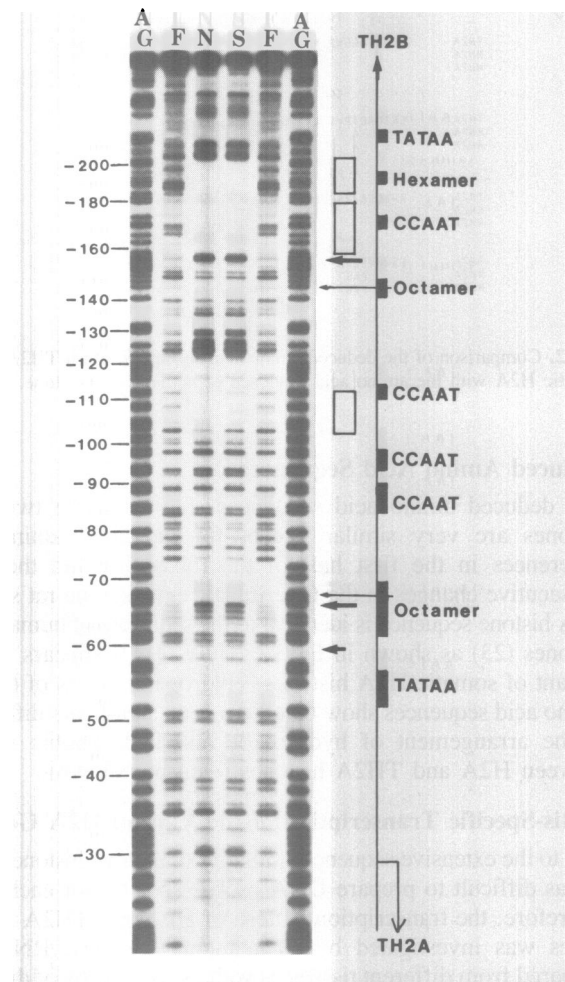


Fig. 6. Protein binding sites on TH2A promoter. The nuclear proteins extracted from non-S and S phase cells were incubated with a DNA fragment derived from -5 bp and -253 bp from the translation initiation site of TH2A. The DNA was labeled with 32 P- γ -ATP and T4 polynucleotide kinase at the -5 bp position. The mixture was digested with a minimal amount of DNase I, and the DNA was resolved by high-resolution polyacrylamide gel electrophoresis. Shown here is an autoradiogram. Lanes F, protein-free DNA; N, non-S phase; S, S phase; A-G, the DNA probe cleaved at adenines and guanines. The regions resistant to DNase-digestion are shown on the side as open boxes, DNase-sensitive sites by bold arrows; resistant sequence by a long thin arrows. The position of various elements are shown by filled boxes. The numbers on the left side indicate positions from AUG codon.

was higher than that from TH2BCAT. To determine the regions responsible for the S phase-dependent production of CAT, a series of deletions was made from the 5' end of TH2A promoter, and effect of these deletions was investigated. As shown in Fig. 5, deletion from -253 bp to -134 bp abolished most of the basal as well as the S phase-dependent production of CAT. The 134 bp 5' flanking region contains the octamer-like element as well as three upstream CCAAT elements. Apparently these elements are not involved in transcription of TH2A gene. Rather the region between -253 and -134 bp could be important for the expression of TH2A gene. This region includes the octamer element which is required for the S phase-dependent expression of TH2B gene (19). The importance of the region upstream of -134 is also reflected from the protein-binding studies shown in Fig. 6. The protein-binding sites on TH2A promoter sequences were mapped by DNase footprinting assay. Nuclear proteins extracted from S and non-S phase cells were incubated with a

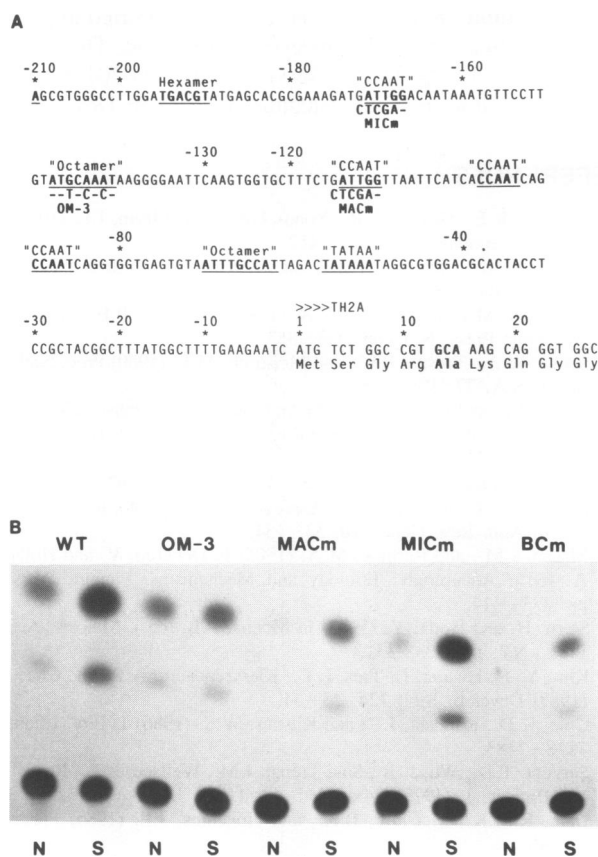


Fig. 7. Effect of mutations of octamer and CCAAT elements on the S phase-dependent expression of TH2ACAT. (A). Shown here are the sequences of the mutated octamer and CCAAT elements. (B). Production of CAT from mutants was determined as described in Fig. 4. S, S phase; N, non-S phase. BCm represents the promoter containing mutations in both CCAAT elements (MACm and MICm). The promoter regions used in both the wild type and the mutants extend up to -253 bp.

DNA fragment labeled at -5 bp position, and the mixture was digested with a minimal amount of DNase I. The sites protected against DNase digestion by the bound-proteins were mapped by high resolution polyacrylamide gel electrophoresis. The result shows that the nuclear proteins mainly bind to the sites upstream of -100 bp. Nuclear proteins bind to the sequences containing the following elements: CCAAT at -110 , octamer at -141 , CCAAT at -167 , TGACGT at -190 bp but not the two CCAAT elements present at -86 and -94 bp. Taken together, the DNA elements present upstream of -110 bp, most likely the octamer near the TH2B gene may be involved in the S phase-specific expression of TH2A gene. It is possible that a protein is bound to the octamer-like element situated immediately upstream of TH2A TATAA element since some of the sequences show enhanced cleavages in the presence of proteins.

Effect of Mutations of CCAAT and Octamer Elements

Previously we introduced mutations into various CCAAT and octamer elements present upstream of -134 bp in the studies on the role of these elements in the S phase-dependent expression of TH2B gene (19). These DNAs were used for construction of mutant TH2ACAT fusion genes as shown in Fig. 7. The results show that the mutation of the octamer at -141 bp (OM-3) abolishes the S phase-specific increase of CAT production and

the mutations in either the CCAAT at -167 bp (MICm) or at -110 bp (MACm) abolish the basal but not S phase-dependent increase of CAT production. Mutations of both CCAAT elements at -167 bp and -110 bp (BCm) decreased the S phase-dependent production of CAT to some extent. Taken together, these results demonstrate that the octamer element present at -141 bp controls the S phase-dependent transcription of both TH2B and TH2A genes. On the other hand, the two CCAAT elements flanking the octamer element control basal level of the promoter activity.

DISCUSSION

In this study, we cloned a testis-specific H2A histone-variant TH2A gene. The gene is located 300 bp upstream of a testis-specific H2B histone variant TH2B gene, and the two genes are transcribed in opposite directions. Transcription of TH2A gene, as TH2B gene, is specific for testis, and the level of TH2A mRNA is much higher in testis of 14 and 21 days old rats than in testis of 7 day old rats. This suggests that either the gene is transcribed at a higher rate or the mRNA is stabilized more in primary spermatocytes than in spermatogonia. The cloned gene encodes a polypeptide which is recognized by H2A antibody and also has the same electrophoretic mobility as testis-specific TH2A histones during acid-urea polyacrylamide gel electrophoresis (data not shown). The deduced amino acid sequences of rat somatic H2A and testis-specific TH2A histones show that there are eight amino acids differences in the first half of histone molecules and three consecutive changes in amino acids at the C-terminal region of the two histones. Hydropathy plots show no significant difference in the hydrophilicity of the two histones. On the other hand, the N-terminal region of TH2B histone is significantly less hydrophilic than that of somatic H2B histones (11). In rat pachytene spermatocytes, greater than 90% of somatic H2B histones is replaced by TH2B but only 15% of somatic H2A is replaced by TH2A (2). The H2B-binding site in pachytene nucleosomes is five fold more sensitive to DNase I than that of somatic nucleosomes (7). The weakening of the interaction of H2B with DNA in pachytene nucleosomes, therefore, could be due to the changes of amino acid sequences in TH2B histone. The relaxed chromatin structure in pachytene spermatocytes could be responsible for the extensive chromosome 'crossing over' during meiosis (8-10). The H2A binding to DNA appears to be not altered in pachytene spermatocytes (7).

The somatic and testis-specific H2A and H2B histone gene pairs have a remarkably similar structural organization suggesting that the testis-specific pair has evolved from the somatic pair. Of particular interest is the presence of similar sequence elements in the promoter regions of the two gene pairs. The TH2A-TH2B pair contains one octamer element (ATTTGCAT) near TH2B gene and an octamer-like element (ATTTGCCAT) near the TH2A gene. In addition, there are four CCAAT elements. The somatic pair also contains one octamer and one CCAAT and one CAAT elements. The octamer element is present in most of H2B histone genes (21 and references therein; 23) and involved in the S phase-specific expression of H2B histone genes (19,21). Also, CCAAT element is involved in the S phase-specific expression of thymidine kinase gene (28). We have previously reported that the octamer at -141 bp and the CCAAT element at -110 bp stimulate expression of TH2B gene in an S phase-dependent manner when the gene was introduced into somatic cells (19). The presence of only one of the two elements reduced the S phase-specific expression to about half (19). Also, the two

elements have to be present on the same surface of DNA helix for maximal expression during S phase suggesting that the proteins bound to these elements interact each other to bring about the maximum S phase-specific stimulation of expression. The CCAAT element at -167 bp has no role for expression of TH2B gene (19). The results provided in this study suggest that the same octamer element activates the TH2A gene about six folds during S phase, but two flanking CCAAT elements at -110 and -167 bp are involved only in the basal expression. The two additional downstream CCAAT elements and the octamer-like element near the TH2A TATAA element apparently have no role in transcription of TH2A gene. This conclusion was also supported by the absence of protein binding to the two CCAAT elements. It is not clear if any protein binds to the octamer-like element near the TH2A TATAA element. As reported earlier (19), the level of the proteins binding to the regulatory elements is unchanged during cell cycle. The reason for the involvement of two CCAAT elements for transcription of TH2A but only one for that of TH2B gene is not clear. It is possible that a neighboring DNA element may affect the function of the protein bound to a CCAAT element. For example, there is a protein-bound hexamer (ACGTCA) element (at -191 bp) in the upstream of the CCAAT at -167 bp. This element is found in many of plant histone genes (29), and the protein which recognizes this element has been characterized (29). A chicken H2A and H2B gene pair is also controlled by an octamer and an adjacent CCAAT elements (30). However, the role of these elements in basal and S phase-specific transcription of the gene pair was not investigated (30).

The presence of S phase-specific transcription elements in the promoters of testis-specific histone gene promoters is surprising. The expression of these genes is not only cell-type specific but also the synthesis of these histones is unaffected by inhibitors of DNA synthesis (4,5). The synthesis of somatic histones is tightly coupled with DNA replication and inhibited by inhibitors of DNA synthesis (22, 24-26). Therefore, the reason for the conservation of the S phase-specific transcription elements and the possible role of these elements in the testis-specific transcription are unclear at the present time. Recent gene transfer experiments with primary cultures of spermatogenic cells showed that the promoter region shared by TH2A and TH2B genes does not contain any germ cell-specific transcription element (Lim, unpublished data). It is possible that the germ cell-specific regulatory element resides outside the TH2A-TH2B genes. The remarkable similarity in the sequence organization of the somatic and testis-specific gene pairs suggests that the testis-specific gene pair was most likely evolved from the somatic histone gene pair. Perhaps a duplicated gene pair was translocated to a DNA domain containing testis-specific genes during evolution and has accumulated changes of DNA sequences which are beneficial for meiosis. The presence of the TH2A-TH2B genes in testis-specific chromatin domain is supported by the methylation of the TH2A-TH2B gene pair in somatic cells including the tubular somatic Sertoli cells but not in germ cells starting spermatogonia to spermatozoa (Choi and Chae, submitted). Investigation of the testis-specific regulation of TH2A and TH2B genes will most likely yield a novel gene regulatory mechanism.

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