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Presence of a Repressor Protein for Testis-specific H2B (TH2B) Histone Gene in Early Stages of Spermatogenesis*

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The rat testis-specific TH2B histone gene assumes a hypomethylated chromatin structure at all stages of spermatogenesis. However, the TH2B mRNA level is very low in pre-meiotic spermatogenic cells and rises sharply in meiotic pachytene spermatocytes. The low level of TH2B mRNA in pre-meiotic spermatogenic cells appears to be a result of transcriptional repression of the gene by a pre-meiotic cell-specific protein which binds to a site between the TATA element and the transcription initiation site of TH2B gene.

During mammalian spermatogenesis, chromatin undergoes structural reorganization in meiotic spermatocytes (pachytene spermatocytes) by replacement of somatic histones with germ cell-specific histones (1, 2). Studies on the rat testis-specific H2B (TH2B) histone gene showed that expression of the gene is restricted to pachytene spermatocytes (3), and the gene has conserved the promoter elements involved in the S phase-specific expression of somatic H2B histone genes as well as the S phase-specific stabilization of histone mRNA (3–6). The cloned TH2B gene introduced into somatic cells is expressed in an S phase-dependent manner (4–6). Therefore, the gene has the potential to be expressed during the S phase in all tissues as well as during early stages of spermatogenesis in which spermatogonia undergo rapid cell division (reviewed in Ref. 7). In fact, somatic H2B histones and mRNA, but very little if any TH2B histones and mRNA, are produced in spermatogonia (2, 3, 8). One of the basic questions is how a gene which does not have any cell type-specific regulatory elements can be expressed in a cell type-specific manner during spermatogenesis. In the case of TH2B gene, this appears to be achieved at least at two levels: assembly of germ cell-specific chromatin structure as evidenced by the hypomethylation of the gene from the beginning to the end of spermatogenesis (sperm) (8) and repression of the gene transcription in nonexpressing spermatogenic cells as suggested in this report. Our experiments show that testes of 7-day-old rats which are enriched with spermatogonia (9, 10), but not the testes of older animals, contain a nuclear protein factor which binds to a site between the TATA element and the transcription initiation site of TH2B gene. *In vitro* transcription experiments with nuclear protein extracts prepared from testes of different ages of rat and from liver suggest that the factor inhibits transcription of the TH2B gene.

EXPERIMENTAL PROCEDURES

Animals—Sprague-Dawley rats (Zivic-Miller Laboratories, Zelienople, PA) were used in all studies.

Preparation of Nuclear Extracts, DNase I Footprinting, and DNA Mobility Shift Assays—Nuclear extracts were prepared from pure nuclei (11) as described before (6). DNase footprinting and DNA mobility shift assays were also carried out as described before (6). The composition of the buffer used for these assays (10 μl) was 60 mM KCl, 12 mM HEPES[1] (pH 7.9), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 6% glycerol, 0.3 mM phenylmethylsulfonyl fluoride, 2 μg of poly(dI-dC), labeled DNA, and nuclear proteins.

Construction of the Plasmid Used for in Vitro Transcription—TH2B promoter DNA corresponding to −483 and −2 bp was inserted into the *Sac*I site of p(C2AT)19 plasmid (12) after trimming the *Sac*I-cleaved DNA with T4 DNA polymerase. The circular form of the resulting plasmid pTH2B(C2AT)19 was used for *in vitro* transcription.

In Vitro Transcription Assay—Nuclear extracts were prepared and *in vitro* transcription reactions were carried out essentially as described by Gorski *et al.* (11). The transcription reaction mixture (40 μl) contained 25 mM HEPES (pH 7.6), 50 mM KCl, 6 mM $MgCl_2$, 0.6 mM each of ATP and CTP, 35 μM UTP, 7 μCi of [α-^{32}P]UTP (ICN, 650 Ci/mmol), 0.1 mM 3′-*O*-methyl-GTP, 12% glycerol, and 1 μl of RNasin (300 units), 75 μg of nuclear proteins, and 0.6 μg each of the circular form of pML(C2AT)19 and pTH2B(C2AT)19. Following a 45-min incubation at 30 °C, the mixture was deproteinized and electrophoresed on 4% polyacrylamide gel in 8 M urea.

Fractionation of Sertoli Cells and Pachytene Spermatocytes—Testes of 21-day-old rats were used. Preparation of a single-cell suspension of testicular cells and fractionation of Sertoli cells and pachytene spermatocytes by CelSep apparatus (Eppendorf Co., Fremont, CA) were carried out as described (13).

RESULTS

Pattern of Protein Binding to the Promoter of TH2B Gene—The protein binding sites on the promoter of the TH2B gene were determined by DNase I footprint assays using nuclear protein extracts prepared from rat testes and liver. A site immediately upstream of the translation initiation codon of TH2B gene was labeled with ^{32}P, and the DNA was mixed with nuclear protein extracts of testes and liver. The mixture was digested with a minimal amount of DNase I, and the deproteinized DNA was resolved by electrophoresis on a high resolution polyacrylamide gel. The results show that the previously identified elements important for the S phase-dependent expression of TH2B gene (sites A, B, and C; Ref. 6) and site D are bound by proteins of testes of 7- and 21-day-old and adult (60 days) rats and rat liver (Fig. 1). In addition, site E situated between the TATA element and the transcription initiation site is bound by a nuclear protein in the testis nuclear extract of a 7-day-old rat. The same site was partially protected against DNase when the DNA was incubated with the testis proteins derived from 21-day-old rats but not with the testis proteins of adult rats nor with proteins from rat

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[1] The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s).

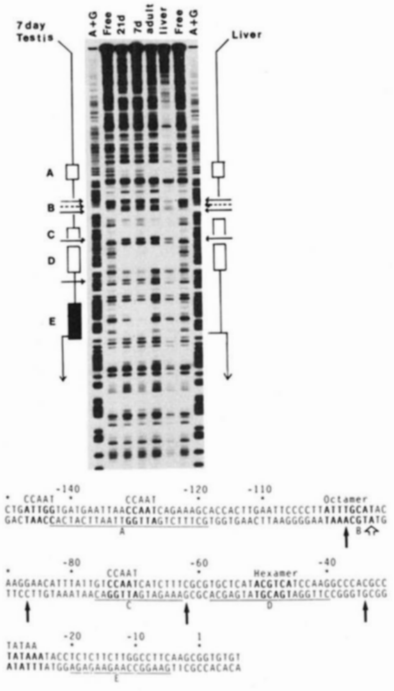

FIG. 1. **Protein-binding sites on the minimal promoter of TH2B gene.** The TH2B DNA fragment corresponding to −485 and +64 bp was labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase at the +64-bp position of the noncoding bottom strand. Nuclear extracts were prepared from pure nuclei (11) as described before (6). The end-labeled DNA was mixed with nuclear extracts and digested with a minimal amount of DNase I, and the deproteinized DNA was resolved by electrophoresis in high resolution polyacrylamide gel as described before (6). *Top*, the *boxes* on the *left* and *right* sides of the figure indicate the nuclease-resistant regions obtained with nuclear extracts of testes of 7-day-old rats and rat liver, respectively. *Arrows* indicate nuclease-sensitive sites. *Lanes A + G* indicate the probe digested at adenine and guanine residues; *lane free*, the probe digested with DNase in the absence of nuclear extracts; *lanes 7d, 21d,* and *adult*, testis nuclear extracts of 7-, 21-, and 60-day-old rats; *lane liver*, nuclear extract of rat liver. *Bottom*, the nuclease-resistant and -sensitive sites on the TH2B promoter sequence are indicated.

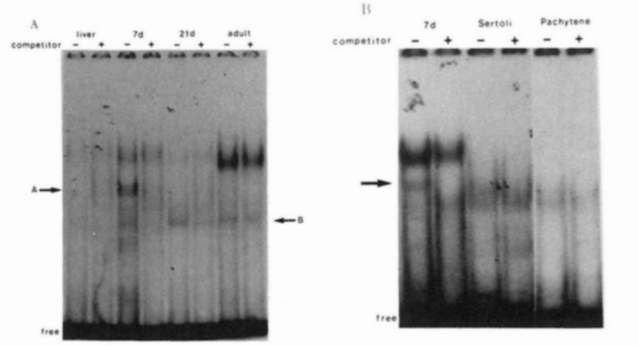

FIG. 2. **Assay for the site E-specific nuclear factors by DNA mobility shift assay.** The double-stranded oligonucleotide corresponding to site E (sequence of upper strand: CTAGACTCTCTTCTTGGCCTTCAAGT) was labeled with ^{32}P and mixed with nuclear extracts (3 μg) in binding buffer described under "Experimental Procedures." Free DNA and protein-DNA complexes were resolved by electrophoresis on a 4% nondenatured polyacrylamide gel as described before (6). *A*: *lanes liver, 7d, 21d,* and *adult* represent nuclear extracts from liver and testes of 7-, 21-, and 60-day-old rats, respectively. − and + indicate absence and presence of 100-fold excess of nonlabeled site E DNA. DNA-protein complex A was present only in the testis nuclear extract of 7-day-old rat and complex B in testis nuclear extracts of 21-day-old and adult rats; *lane free*, free DNA probe. *B*: *lanes 7d, Sertoli,* and *pachytene* represent nuclear extracts from testes of 7-day-old rats, fractionated Sertoli cells, and pachytene spermatocytes, respectively.

liver. Since spermatogenesis proceeds synchronously right after birth (9, 10), the testis of a 7-day-old rat is enriched with the pre-meiotic spermatogenic cells, spermatogonia (9, 10). The proportion of spermatogonia in the testes of older animals is much lower (9, 10). Therefore, the protein which binds to site E could be specific for spermatogonia. This conclusion was further substantiated by DNA mobility shift assay. Double-stranded oligonucleotide corresponding to site E was labeled with ^{32}P, and the labeled DNA was incubated with nuclear protein extracts in the presence and absence of a 100-fold excess of nonlabeled site E oligonucleotide, and the mixture was resolved by electrophoresis on nondenatured polyacrylamide gel (Fig. 2). The results show that testis nuclear proteins of 7-day-old rats form a sequence-specific DNA-protein complex with site E (*complex A* in Fig. 2*A*). Addition of a 100-fold excess of nonlabeled site E DNA prevented formation of the complex with labeled site E. Nuclear extracts obtained from testes of older rats (21-day-old and adult) do not form complex A but form a faster moving complex (*complex B* in Fig. 2*A*). Formation of complex B was partially competed by the nonlabeled site E oligonucleotide. Also, the band intensity of complex B was weaker than that of complex A. Addition of nonrelated sequence (OTF-1 binding sequence) as a competitor did not affect formation of either complex A or B (data not shown). Proteins of Sertoli cells, pachytene spermatocytes, and liver do not form any specific complex with site E. The results suggest that there is a spermatogonia-specific protein which has specificity toward site E of the TH2B gene in the 7-day-old rat testis. It is unlikely that the protein is present in other cell types in testes of 7-day-old rats, since the other cell types such as Sertoli cells are also present in testes of older rats. Nuclear extracts contained varying amounts of proteins which formed nonspecific complexes with site E. Formation of the nonspecific complexes could not be reduced by the presence of an excess amount of nonlabeled site E DNA. There appears to be a discrepancy between the results obtained with the DNase I footprint assay and the DNA mobility shift assay. For example, the site E was partially protected against DNase by the testis proteins of 21-day-old rats in the DNase footprint assay, but complex A was not formed by the testis proteins of 21-day-old rats in the DNA mobility shift assay. The result of the DNase footprint assay is somewhat complicated due to the formation of more than one complex with site E and the changing population of spermatogenic cells of different stages of spermatogenesis with age.

In Vitro Transcription—Activity of the TH2B promoter was investigated with nuclear extracts prepared from testes of 7-day-old and adult (60-day-old) rats and rat liver. TH2B promoter (−483 and −2 bp) was fused with an artificial template lacking cytosines in the template strand of plasmid p(C2AT)19 (12). The resulting plasmid pTH2B(C2AT)19 was used as a template in a transcription reaction mixture containing ATP, CTP, UTP, but no GTP. The predicted size of the transcript was 370 nucleotides. As a control, the template fused with an adenovirus promoter, pML(C2AT)19, was used. The size of RNA transcribed from this template was 390 nucleotides. When an equal amount of the two templates was added to the same transcription reaction mixture (11), the two templates were transcribed in the reaction mixtures containing either testis nuclear extract of adult rats or nuclear extract of rat liver (Fig. 3*A*). However, very little transcript was produced from pTH2B(C2AT)19 in the reaction mixture

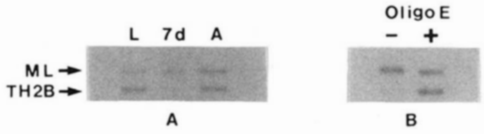

FIG. 3. **Repression of *in vitro* transcription of TH2B template.** *A*, *in vitro* transcription of pML(C2AT)19 and pTH2B(C2AT)19. *Lanes L, 7d,* and *A* represent nuclear extracts of liver and testes of 7-day-old and adult rats, respectively. The transcripts of pML(C2AT)19 and pTH2B(C2AT)19 are indicated as *ML* and *TH2B*, respectively. *B*, effect of oligonucleotide E on *in vitro* transcription in the presence of testis nuclear extract of 7-day-old rats. *Lanes* − and + represent the absence and presence of 1 μg of double-stranded oligonucleotide E (200-fold molar excess). Preparation of nuclear extracts and transcription reaction conditions are described under "Experimental Procedures."

containing the testis nuclear extract of 7-day-old rats (Fig. 3*A*). The transcription of pML(C2AT)19 in the same reaction mixture was comparable with that obtained with the reaction mixtures containing nuclear extracts of either testes of adult rats or liver. Therefore, the results suggest that the transcription of pTH2B(C2AT)19, but not that of pML(C2AT)19, was prevented in the reaction mixture containing the testis nuclear extract of 7-day-old rats. To determine if the lack of transcription of the TH2B template is due to the factor binding to site E, double-stranded oligonucleotide corresponding to site E (200-fold molar excess) was added to the transcription reaction mixture containing the two templates and testis nuclear proteins of 7-day-old rats. As shown in Fig. 3*B*, the addition of site E oligonucleotide relieved the transcription inhibition of the TH2B template presumably by titrating the binding factor away from the TH2B promoter. The site E oligonucleotide had no apparent effect on transcription of pML(C2AT)19. The release of the transcriptional repression of TH2B promoter by oligonucleotide E appears to be sequence-specific. Addition of oligonucleotides of the non-TH2B sequence had no apparent effect on transcription of either pTH2B(C2AT)19 or pML(C2AT)19 (data not shown). Furthermore, there was a plasmid sequence in the same reaction mixture which was at least 300-fold in excess over the site E sequence. pTH2B(C2AT)19 was transcribed by testis nuclear proteins of 21-day- old rats (data not shown).

DISCUSSION

The expression of the TH2B gene, although it contains the same regulatory elements as the somatic H2B gene (4–6), is confined to pachytene spermatocytes during rat spermatogenesis (3). However, the gene appears to be in a transcriptionally competent state from the beginning to the end of spermatogenesis based on the germ cell-specific hypomethylation of the gene in all stages of spermatogenesis (8). The presence of a protein factor which inhibits transcription of the TH2B gene could be responsible for the lack of or very little TH2B mRNA in pre-meiotic spermatogenic cells (3, 8). Molecular cloning of the gene encoding the repressor protein will be of great value for identification of the spermatogenic cell types in which the protein is produced and for elucidation of the role of the repressor factor in regulation of the TH2B gene during spermatogenesis. The expression of the TH2B gene is turned off in spermatids after meiosis. It is unlikely that the nonexpression of the gene in spermatids is also achieved by a similar mechanism, since pTH2B(C2AT)19 was efficiently transcribed in the reaction mixture containing testis nuclear extracts of sexually mature adult rats. The proportion of spermatids in testes of adult animals is high. Again availability of the gene encoding the repressor protein will help to determine if the repressor protein is involved in transcriptional repression of the gene in spermatids.

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