The testicular isofrom of hormone-sensitive lipase (HSLtes) is encoded by a testis-specific exon and 9 exons common to the testis and adipocyte isoforms. In mouse, HSLtes mRNA appeared during spermiogenesis in round spermatids. Two constructs containing 1.4 and 0.5 kilobase pairs (kb) of the human HSLtes gene 5′-flanking region cloned upstream of the chloramphenicol acetyltransferase gene were microinjected into mouse oocytes. Analyses of enzyme activity in male and female cells concomitantly with protamine 1 mRNA. We show that 0.5 kb of the HSLtes promoter was sufficient to drive testis-specific expression in transgenic mice. In cell transfection experiments, transactivation of the HSLtes promoter was independent of the cAMP signaling pathway. Four regions bound testicular nuclear proteins essential for the testis specificity of HSL.

Hormone-sensitive lipase (HSL) is a triacylglycerol lipase and a cholesteryl esterase expressed at high levels in adipocytes, testes, and adrenals (1–3). In adipocytes, HSL catalyzes the rate-limiting step in the hydrolysis of triglycerides into fatty acids and glycerol (4). HSL activation is mediated through phosphorylation by the cAMP-dependent protein kinase (5). In rat testis, HSL mRNA and protein are expressed in the seminiferous tubuli and not in interstitial cells with a stage-dependent pattern corresponding to the appearance of haploid germ cells (3, 6). Several isoforms of HSL produced by a single gene have been characterized (2, 7, 8). Human adipose tissue expresses a 2.8-kb mRNA that encodes an 88-kDa protein (7, 9).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AJ132272.

§ Present address: Laboratoire de Nutrition, Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l’Alimentation, Dijon, France.
¶ To whom correspondence should be addressed: INSERM U317, Institut Louis Bugnard, Bâtiment L3, CHU Rangueil, F-31403 Toulouse Cedex 4, France. Tel.: (33) 5 62172950; Fax: (33) 5 61331721; E-mail: langin@rangueil.inserm.fr.

The abbreviations used are: HSL, hormone-sensitive lipase; HSLtes, testicular hormone-sensitive lipase; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer binding protein; CRE, cAMP-responsive element; CREB, CAMP-responsive element binding protein; CREM, CREM product of the CREM gene that acts as a transcriptional activator responsive to the cAMP signaling pathway (12). Several target genes for CREM-mediated activation have been identified only for a few of them. CREM binds to the CREM sequence in the 5′-flanking region suggesting that the expression of different forms of HSL is controlled by several tissue-specific promoters.

During spermatogenesis, specialized transcriptional mechanisms ensure stage-specific gene expression in the germ cells. The factors controlling gene expression in post-meiotic germ cells are beginning to be elucidated. Several germ cell-specific putative transcription factors have been cloned, but target genes have been identified only for a few of them. CREM binds to the CREM sequence in the 5′-flanking region suggesting that the expression of different forms of HSL is controlled by several tissue-specific promoters.
present in testis and not in liver. One of the region bound Sox

EXPERIMENTAL PROCEDURES

Northern Blot Analyses—A mouse HSL DNA probe (477 bp) was generated by PCR on mouse genomic DNA with primers located in the first adiocyte coding exon 5-AGAT TTA TGA CGC AGT AGA CCA CAG-3' and 5'-TAG CGT AAC ATA TCT TTA GAA CAA-3'. Pro- nekaphelin (227 bp) and protamine 2 (207 bp) cDNA probes were generated by reverse transcription-PCR on total RNA using 5'-GAC AGC AGC AAA CAG GAT GA-3' and 5'-TTC AGA AGC TCG GAG TT-3', and 5'-AGC AAA AGC AGC AGA TG-3' and 5'-AGA TGT GGC GATG TCT TT-3' primers, respectively. PCR reactions were performed using the proofreading pfu DNA polymerase (Stratagene). PCR products were cloned into pBluescript (Stratagene) using the TA cloning procedure (17). Identity of the amplicons to published sequences was checked by automatic DNA sequencing (Applied Biosystems).

Total testis RNA was prepared from prepuberal and sexually mature mice by a single-step guanidinium thiocyanate phenol/chloroform extraction (18). RNA samples (25 μg) were separated on a 1% agarose, 2.2 M formaldehyde, 1× TBE gel and stained with ethidium bromide. The gel and hybridization with a rat β-actin probe. Membranes were pre-hybridized for 1 h in hybridization buffer (500 mM Na2HPO4, 1 mM EDTA, 7% SDS, 1% bovine serum albumin) and then hybridized overnight in 10 ml of the same buffer containing 1.5×106 cpm/ml HSL and pronekaphelin cDNA probes and 106 cpm/ml protamine cDNA probe. After hybridization, membranes were washed twice with 0.3 M NaCl, 30 mM tri-sodium citrate, 0.1% SDS 20 min at room temperature and once with 30 mM NaCl, 3 mM tri-sodium citrate, 0.1% SDS for 30 min at 65 °C. Membranes were subjected to digital imaging (Molecular Dynamics).

Plasmid Constructs—A 1.6-kb HindIII/BglII human DNA genomic restriction fragment was isolated from a cosm id clone containing the entire human HSL gene (3). The fragment was subcloned into the HindIII and BamHI sites of pBluescript and sequenced by automatic DNA sequencing (Applied Biosystems). It contained 1.4 kb of the 5'-flanking region upstream of the testis-specific exon. The construct was digested with HindIII and XbaI, and the 1.6-kb fragment was ligated upstream of the chloramphenicol acetyltransferase (CAT) gene into the pCAT basic vector. The 1.6-kb HindIII/BglII DNA genomic restriction fragment was also cloned upstream of the luciferase gene into the promoterless pG3-basic vector (Promega) (p1.4HSLlesLUC). About 900 bp of p1.4HSLlesLUC 5'-flanking sequence was deleted by digestion with SmalI to produce p0.5HSLlesLUC. The SmalI site used to generate p0.5HSLlesLUC and the AvaI site used to generate the microinjected fragment 0.5HSLles- CAT gene above are overlapping. The complete 1-kb CREM-cDNA (12) was subcloned into the expression vector pSVSport (Life Technologies, Inc.).

Transgenic Mice—The two transgenes were prepared by digesting p1.4HSLlesCAT with HindIII and BamHI or with AvaI and BamHI to give, respectively, 1.4HSLlesCAT and 0.5HSLlesCAT. These two fragments were isolated on agarose gel by electrophoresis and purified using an elutip-d column (Schleicher & Schuell). Transgenic mouse were produced by microinjection of the transgenes into the pronuclei of fertilized B6D2F1 mouse eggs (19). Microinjected embryos were transferred to pseudo-pregnant B6-CBA/F1 female mice and carried to term. Screening of the positive transgenic animals was performed with DNA prepared from tail samples using Southern blot or PCR using as sense primer an oligonucleotide located in the human HSLex5 5'-flanking sequence and as antisense primer an oligonucleotide located in the CAT gene. Subsequent generation of heterozygous mice were produced by mating transgenic mice with wild type B6-CBA/F1 mice. The transmission of the transgene was ~50% in the progeny of all founders indicating Mendelian transmission. Protein extracts for CAT assays were prepared from hemizygous transgenic mice. Briefly, tissues were rapidly frozen and homogenized in 0.14 M NaCl, 8.8 M MgCl2, 25% glycerol, 0.5 mM spermidine and 0.14 M NaCl) using an all-glass Dounce homogenizer (pestle B). An aliquot (10 μl containing 100 μg of protein) was added to 0.7 M NaCl) was added dropwise, and the extract was gently shaken for 10 min at 4 °C. After incubation 45 min on ice, the precipitated proteins were centrifuged in an SW28 rotor at 37,000 rpm for 30 min at 4 °C. The pellets were resuspended in 200 μl of dialysis buffer (200 mM HEPES, pH 8, 100 mM EDTA, 8.8 mM MgCl2, 25% glycerol, 0.5 mM spermidine, 0.15 mM spermine, 0.14 M NaCl) using an all-glass Dounce homogenizer (pestle B). An appropriate volume of the extract was added to 1 ml of gel fractionation buffer (8.8 mM MgCl2, 25% glycerol, 0.5 mM spermidine, 0.15 mM spermine, 0.7 M NaCl) was added dropwise, and the extract was gently shaken for 45 min. The viscous lysate was then centrifuged at 35,000 rpm for 1.5 h at 4 °C to pellet the chromatin. Solid (NH4)2SO4 was progressively added and the absorbance was monitored at 260 nm was measured. The nuclear suspension was diluted at 40×, and the absorbance was measured at 40×. Proteins were separated by electrophoresis through a 7.5% SDS–PAGE gel. After electrophoresis, the gel was stained with 0.1% Coomassie blue (Sigma) when specified. Cells were harvested 48 h post-transfection for Firefly and Renilla luciferase activity determinations according to the manufacturer’s instructions (Promega).

Preparation of Liver and Testis Nuclear Extracts—Total nuclei extracts were performed as described by Howard et al. (22) with modifications. Four adult rat testis and 10–15 mg of adult rat liver (perfused with 0.9% NaCl to wash out blood) were washed in ice-cold saline containing 0.1 mM PMFS, decapsulated, and minced with scissors in 40 mM of homogenization buffer (10 mM HEPES, pH 8, 1 mM EDTA, 25 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 10% glycerol, 0.5 mM DTT, 0.5 mM PMFS, 0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, and sucrose (1.5× for testis, 2× for liver)). Tissues were then homogenized in a glass tissue grinder with a motor-driven Teflon pestle until cells were broken. The homogenate was then completed to 80% (testis) and 100% (liver) with homogenization buffer, and 28-ml aliquots were layered over 10-ml cushions of the same buffer in SW28 tubes. The tubes were centrifuged at 27,000 rpm for 1 h at 4 °C. The supernatants were carefully removed, and the tube walls were washed with water and dried. Nuclei pellets were resuspended in 3× (testis) or 5× (liver) of buffer A (20 mM HEPES, pH 8, 100 mM EDTA, 8.8 mM MgCl2, 25% glycerol, 0.5 mM spermidine, 0.15 mM spermine, 0.14 M NaCl) using an all-glass Dounce homogenizer (pestle B). An aliquot (0.5 ml) of the extracts was added to 1 ml of gel fractionation buffer and digested digestion for 45 min. The products were separated by electrophoresis through a 7.5% SDS–PAGE gel. After electrophoresis, the gel was stained with 0.1% Coomassie blue (Sigma) when specified. Cells were harvested 48 h post-transfection for Firefly and Renilla luciferase activity determinations according to the manufacturer’s instructions (Promega).
RESULTS

HSL*es Expression in Germ Cells—The developmental expression of HSL*es mRNA was examined by Northern blot analysis of testis total RNA from mice at different ages. In rodents, the time at which a transcript appears during the first wave of spermatogenesis in prepuberal animals can be used to identify the spermatogenic cell type in which transcription initiates (31). The levels of proenkephalin and protamine 1 mRNA were therefore determined. In rodents, somatic and spermatogenic cells expressed a 1.4- and a 1.7-kb proenkephalin mRNA, respectively. The testis-specific proenkephalin mRNA is expressed at high levels in late pachytene spermatocytes, and protamine 1 mRNA expression appears in round spermatids (32–34). The proenkephalin germ cell form was also detected in the collected fraction indicating the expression of HSL in sperm after spermiogenesis (data not shown). No apparent variation in CAT activity was observed in the offspring of the founders and subsequent generations (data not shown). These data provided evidence that 0.5 kb of the 5'-flanking region are sufficient to drive expression of the CAT gene in testis. Next, we sought to determine if the 5'-flanking regions conferred tissue-specific expression. In males, CAT activity levels were very low in all non-gonadal tissues. In females, the low level of CAT activity seen in all tissues was comparable to the level detected in tissues of non-transgenic male and female mice (data not shown). Therefore, the sequences present in the first 0.5 kb of the human HSL*es promoter are critical for specific expression in testis. CAT activity was also determined in testis of 25- and 60-day-old mice from lines A and D. Four animals were analyzed per line at both ages. In young mice, the levels of CAT activity were 21 ± 3 cpm/min/mg protein for line A and 18 ± 1 cpm/min/mg protein for line D. In older mice, CAT activity levels were 818 ± 56 and 862 ± 43 cpm/min/mg protein, respectively. The marked increase in CAT activity showed that the transgenes were expressed in post-meiotic germ cells.

In order to check if the transcriptional start site of the chimeric genes expressed in transgenic mice and of the endogenous human HSL*es gene were identical, we performed RNase H mapping analyses with human-specific oligonucleotides on RNAs from human and transgenic mouse testis (Fig. 2). In both tissues, a band of ~ 175 nucleotides was detected. The data show that the human HSL*es promoter in transgenic mice used the same initiation site as the endogenous human promoter. Moreover, the length of the 5'-noncoding region deduced from RNase H mapping corresponded to the size (277 nucleotides) found using 5'-rapid amplification of cDNA ends PCR (3). HSL*es Promoter Activation Is cAMP- and CREM* independent—It has been shown that CREM* binds to cAMP-responsive elements (CREs) and stimulates transcription of several germ cell-specific genes (12, 13). CREM* functions as a transcriptional activator after phosphorylation by cAMP-dependent protein kinase. Computer-based (35) and visual analyses did not reveal apparent consensus sequences for CREs in the HSL*es promoter. Since functional CRE-like sites can substantially diverge from the palindromic sequence TGCAGTCA (36), we wished to determine whether CREM* and cAMP had an effect on HSL*es transcriptional activity. Because of the lack of haploid germ cell lines, cotransfection experiments were performed in JEG3, a human choriocarcinoma cell line bearing an efficient cAMP-dependent transduction pathway (13). To ensure that CAT activity were detected in testis and epididymis from sexually mature mice (between 60 and 90 days old) for the two transgenes (Table 1). The activity in epididymis was ascribed to sperm, since, when mature sperm is washed from the epididymis, CAT activity was between 200 and 800 cpm/min/mg protein in the collected fluid. HSL enzymatic activity and protein was also detected in the collected fraction indicating the expression of HSL in sperm after spermiogenesis (data not shown).
mature transgenic mouse testis and bridged with $^32$P-labeled DNA probe located 5'-phoresed on a polyacrylamide-urea gel. The resulting blot was hybridized with nuclear proteins present in testis, a series of present in nuclear extracts prepared either from rat testis or nucleotides was used to map interaction sites for factors tides spanning the entire region (Fig. 4). Each of the 20 oligonucleotide on human HSL tes cDNA. RNA size markers were pro-
duced by in vitro transcription and labeled by incorporating $[^32]$PUTP into the reaction mixture.

our transfection system was valid to study CAMP-dependent transactivation, we used a control CRE-LUC vector containing four copies of CREs upstream of a minimal promoter. This reporter construct was strongly CAMP-inducible whether the cells were treated with dibutyryl CAMP, a stable and permeable analogue of CAMP, or cotransfected with an expression vector for the catalytic subunit of the CAMP-dependent protein kinase. This result was predictable because JEG3 cells contain endogenous CREB. CREM transactivation resulted in a further increase of CAMP-induced luciferase activity. The results obtained with 0.5HSLtesLUC were strikingly different. No significant increase in luciferase activity was observed showing that the HSLtes promoter does not represent a cellular target of CAMP/CREB transregulatory function.

Testis Nuclear Protein-binding Sites within the Human HSLtes Promoter—Transgenic analyses demonstrated that the 0.5-kb region located upstream of the transcriptional start site was sufficient to confer testis-specific expression. To assess directly whether sequences within the human HSLtes promoter bound nuclear proteins present in testis, a series of in vitro DNA binding studies was performed. The strategy used consisted in designing 20 overlapping double strand oligonucleotides spanning the entire region (Fig. 4). Each of the 20 oligonucleotides was used to map interaction sites for factors present in nuclear extracts prepared either from rat testis or from rat liver, an organ that does not express HSL. Four probes bound nuclear proteins expressed in testis but not in liver (Fig. 5). Analysis of the sequences of three testis-specific binding regions (TSBR) revealed no binding motifs for known testis transcription factors. TSBR4 contained a sequence AACAAAG regions (TSBR) revealed no binding motifs for known testis transcription factors. TSBR4 contained a sequence AACAAAG.

| TABLE I Chloramphenicol acetyltransferase (CAT) activity of 1.4HSLtesCAT and of 0.5HSLtesCAT in transgenic mice |
|---------------------------------|
| Males | Line | Brain | Heart | Liver | Kidney | Gut | Stomach | Spleen | Lung | Skel. | Adi. | Testis | Epi. |
|-------|------|-------|-------|-------|--------|----|---------|--------|------|-------|------|--------|-----|
| 1.4HSLtesCAT | A | 7 | 11 | 1 | 2 | 2 | 5 | 1 | 10 | 1 | 21 | 750 | 450 |
| B | 12 | 5 | 3 | 7 | 1 | 0 | 4 | 5 | 8 | 15 | 830 | ND |
| C | 1 | 4 | 10 | 3 | 6 | 3 | 8 | 2 | 7 | 6 | 200 | ND |
| 0.5HSLtesCAT | D | 2 | 4 | 0 | 2 | 1 | 5 | 6 | 4 | 0 | 15 | 800 | 550 |
| E | 1 | 2 | 5 | 3 | 8 | 12 | 7 | 5 | 6 | 14 | 700 | ND |

| Females | Line | Brain | Heart | Liver | Kidney | Gut | Stomach | Spleen | Lung | Skel. | Adi. | Testis | Epi. |
|---------|------|-------|-------|-------|--------|----|---------|--------|------|-------|------|--------|-----|
| 1.4HSLtesCAT | A | 8 | 5 | 6 | 12 | 2 | 4 | 2 | 3 | 4 | 7 | 21 | 5 |
| B | 15 | 17 | 7 | 4 | 0 | 0 | 4 | 8 | 0 | 1 | 15 | 2 | 0 |
| 0.5HSLtesCAT | D | 9 | 11 | 0 | 1 | 0 | 6 | 7 | 20 | 6 | 26 | 9 | 0 |

Fig. 2. Analysis of 5'-untranslated region of HSLtes mRNA in human and transgenic mice testes. Total RNA (90 μg) from sexually mature transgenic mouse testis and 1 μg of poly(A)+ RNA from human testis were digested by RNase H in the presence of an antisense oligonucleotide complementary to the 5'-untranslated region of human HSLtes mRNA. The reaction mixture was denatured and electrophoresed on a polyacrylamide-urea gel. The resulting blot was hybridized with a $^32$P-labeled DNA probe located 5' of the antisense oligonucleotide on human HSLtes CDNA. RNA size markers were produced by in vitro transcription and labeled by incorporating $[^32]$PUTP into the reaction mixture.

Fig. 3. Effect of CREM, CAMP-dependent protein kinase, and CAMP on p0.5HSLtesLUC vector in JEG3 cells. The p0.5HSLtesLUC vector contains 0.5 kb of the HSLtes promoter in the promoterless luciferase vector (pG3basic). The pCRE-LUC reporter construct contains four copies of CAMP-responsive elements upstream of a minimal promoter linked to the luciferase gene. The reporter constructs were cotransfected with expression vectors encoding the catalytic subunit of the CAMP-dependent protein kinase (PKA) and CREM. Cells were treated for 4 h with 1 mM dibutyryl CAMP (dbCAMP), a stable and permeable analogue of CAMP, as indicated. Results are expressed relative to the activity of pG3 basic treated under the same conditions. Data represent means ± S.D. of three experiments performed in duplicate.

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of recombinant Sox5 HMG box peptide and \textit{in vitro} translated Sox6 protein on TSBR4 was studied using linker scan mutagenesis (Fig. 7). The HMG domain of Sox5 bound the mSRY/Sox probe containing the AACAAT sequence and TSBR4 but not a C/EBP recognition motif (Fig. 7B). Mutation of the AACAAG sequence of TSBR4 strongly decreased the binding. Because Sox6 homodimers do not bind DNA (27), a truncated form of Sox6 deleted of the leucine zipper region was produced by \textit{in vitro} translation. As previously reported (27), incubation of the unprogrammed lysate (data not shown) and of the programmed lysate of the empty vector pRc/CMV with labeled DNA probes of almost identical sizes and base composition gave DNA probes of almost identical sizes and base composition in the retardation assay with recombinant Sox5 HMG box peptide and the different DNA probes. The retarded complexes migrated with a seen mobility that was inversely correlated to the distance between the retardation mapped to the AACAAAG motif of TSBR4. This binding was abolished by mutation of the AACAAAG sequence. These data show that testis Sox proteins as other proteins containing HMG domains bind testis nuclear proteins. HSL\textsubscript{tes} mRNA appears in round spermatids concomitantly to protamine 1 mRNA (Fig. 1). This result is in agreement with \textit{in situ} hybridization data obtained in rat that showed that HSL\textsubscript{tes} mRNA was detected in stages X–XIV of spermatogenesis (6). As shown for many genes expressed during spermatogenesis, the HSL\textsubscript{tes} protein accumulation is delayed to stages XIII–X VIII corresponding to late spermatids (3, 44). The similar stage-specific expression pattern observed for HSL\textsubscript{tes} and protamine 1 mRNAs and other transcripts suggests the presence of common regulatory mechanisms. Since CREM\textsubscript{t} plays an important role during the first steps of spermiogenesis as a transcriptional activator, we checked whether this transcription factor transactivates the HSL\textsubscript{tes} promoter. Cell transfection experiments (Fig. 3) similar to the ones performed with CREM\textsubscript{t}-activated promoters (11, 13, 45) do not support a direct role for CREM\textsubscript{t} and members of the CREB family in HSL\textsubscript{tes} promoter transactivation. An indirect role of CREM\textsubscript{t} that is essential for a complete differentiation of haploid germ cells (15, 16) cannot, however, be ruled out, \textit{e.g.} through the control of expression of a transcription factor activating the HSL\textsubscript{tes} promoter.

The lack of appropriate male haploid germ cell line led us to use transgenic mice to investigate the transcriptional regulation of HSL\textsubscript{tes}. We demonstrate here that 0.5 kb of the region flanking the HSL\textsubscript{tes}-specific exon govern testis expression in transgenic mice (Table 1). Analysis of a large number of tissues in male and female transgenic mice showed the strict testis expression of the transgene. The testis form of HSL that is characterized by larger mRNA and protein species than the other isoforms has only been detected in testis (1–3). Moreover, the 25-day-old transgenic mice showed very little CAT activity other than in testis, compared with the 60-day-old animals. The data in transgenic mice are therefore in agreement with the pattern and timing of expression of HSL\textsubscript{tes}.

In order to determine testis-specific DNA-protein interactions on the HSL\textsubscript{tes} promoter 0.5-kb region, gel retardation assays were performed using overlapping double strand oligonucleotides (Fig. 4). Four regions were shown to bind testis nuclear proteins absent in liver nuclear extracts (Fig. 5). One of them, TSBR4, contained a DNA sequence motif AACAAAG recognized by the HMG domain of SRY/Sox proteins (23, 39). Competition experiments revealed that TSBR4 bound a testis

**DISCUSSION**

In this paper, we show that the proximal 5′-flanking region of the HSL gene functions as a testis-specific promoter and binds testis nuclear proteins. HSL\textsubscript{tes} mRNA appears in round spermatids concomitantly to protamine 1 mRNA (Fig. 1). This result is in agreement with \textit{in situ} hybridization data obtained in rat that showed that HSL\textsubscript{tes} mRNA was detected in stages X–XIV of spermatogenesis (6). As shown for many genes expressed during spermatogenesis, the HSL\textsubscript{tes} protein accumulation is delayed to stages XIII–X VIII corresponding to late spermatids (3, 44). The similar stage-specific expression pattern observed for HSL\textsubscript{tes} and protamine 1 mRNAs and other transcripts suggests the presence of common regulatory mechanisms. Since CREM\textsubscript{t} plays an important role during the first steps of spermiogenesis as a transcriptional activator, we checked whether this transcription factor transactivates the HSL\textsubscript{tes} promoter. Cell transfection experiments (Fig. 3) similar to the ones performed with CREM\textsubscript{t}-activated promoters (11, 13, 45) do not support a direct role for CREM\textsubscript{t} and members of the CREB family in HSL\textsubscript{tes} promoter transactivation. An indirect role of CREM\textsubscript{t} that is essential for a complete differentiation of haploid germ cells (15, 16) cannot, however, be ruled out, \textit{e.g.} through the control of expression of a transcription factor activating the HSL\textsubscript{tes} promoter.

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nuclear protein that shows properties of a Sox protein (Fig. 6). Two members of the family, a short form of Sox5 and Sox6, are expressed in male germ cells at the round spermatid stage (27, 28, 38, 46). The role of these proteins in spermatogenesis has not been documented. The HMG domain of Sox5 and a leucine zipper region-deleted Sox6 was shown to bind TSBR4 (Fig. 7). This observation raises the possibility that the short form Sox5 and/or Sox6 may directly or indirectly participate to the transactivation of the HSL_tes promoter. The HSL gene would therefore represent the first target gene of these proteins.

Cooperation of several Sox proteins and other transcription factors is often necessary to promote target gene expression (41, 46–49). In teratocarcinoma cells, Sox2 and the POU domain transcription factor octamer 3 bind adjacent sites and participate together to the transactivation of the fibroblast growth factor 4 gene through protein-protein interaction (41, 49, 50). Either factor alone is ineffective. Three different Sox proteins, a long form of Sox5, Sox6, and Sox9, are coexpressed in chondrocytes and cooperatively activate the chondrocyte-specific enhancer of the type II collagen gene (46). The activation is facilitated by the dimerization of the long form of Sox5 and Sox6. Sox6 contains a leucine zipper motif that allows dimerization of the protein, and homodimers fail to bind DNA (27). These data suggest that, in testis, Sox6 may bind to another protein as heterodimers to show transactivation properties. The short form of Sox5 expressed in testis does not contain the coiled-coil domain present in the long form. In HeLa cells, expression of the short form Sox5 alone or coexpression of the short form Sox5 and Sox6 did not activate the HSL_tes promoter (data not shown). Other uncharacterized testis Sox proteins might be involved in the activation of HSL_tes. In addition, the cooperation between Sox proteins and other transcription factors might be necessary. The identification of the interacting partners will require extensive investigation since, except Sox binding to TSBR4, the other TSBRs do not activate the HSL_tes promoter. The HSL gene would therefore represent the first target gene of these proteins.

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show significant sequence homology with consensus binding motifs for known testis transcription factors.

Sox proteins could indirectly modulate transcription activity by organizing local chromatin structure. Binding of Sox proteins occurs in the minor groove and results in a bend within the DNA (37). It has been reported that Sox5 HMG box induces a bend to the AACAAT motif with an estimated angle of 74° (28). The nature of the recognition sequence and of the flanking nucleotides influence the angle of the bend (51). Here, we show that the HMG domain of Sox5 induces an estimated flexure of 65–70° through binding to the AACAAAG sequence of TSBR4 (Fig. 8). The data demonstrate that Sox5 can induce a strong bend in DNA in the context of a natural testis-specific promoter. Testis Sox proteins may act through an alteration of local chromatin structure around the AACAAAG site in TSBR4 to facilitate the interaction of distant enhancer nucleoprotein complexes (e.g., on the other TSBRs) with the basal transcription machinery.

To conclude, we have identified a testis-specific promoter that contains four regions binding testicular nuclear proteins. The HSLtes promoter provides a molecular basis to characterize new cis-acting elements and transcription factors responsible for the transactivation of genes in post-meiotic germ cells.

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