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Tissue Specific Expression of Testis Angiotensin Converting Enzyme Is Not Determined by the −32 Nonconsensus TATA Motif

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Testis ACE is an isozyme of angiotensin-converting enzyme (ACE) made by male germ cells. These cells recognize a small intragenic promoter which contains a positive regulatory element, TCTTAT, at position −32. A probe containing this element was gel shifted to an identical location by rat testis and rat liver nuclear extracts; formation of the complex was blocked by anti-TATA binding protein antibody. Recombinant TATA binding protein recognized the testis ACE TCTTAT motif as well as a probe containing the consensus TATA motif. Mice transgenic for testis ACE promoter constructs containing either the wild type testis ACE motif or a consensus TATA sequence expressed a reporter gene at high levels only within the testis. These data suggest that the testis ACE motif TCTTAT is a non-consensus TATA, but is not responsible for the highly restricted pattern of testis ACE gene expression.

As male germ cells develop, they express a number of unique proteins including germ cell-specific isoforms of somatic enzymes.[1,2] One such example is the abundant production of testis angiotensin-converting enzyme (ACE).[3] Two isozymes of ACE (EC 3.4.15.1) have been found in mammals.[4] Somatic ACE is produced by several somatic tissues and plays an important role in the regulation of blood pressure through the conversion of angiotensin I into the vasoconstrictor angiotensin II. Testis ACE is only produced by round and elongating spermatids and is approximately half as large as somatic ACE. The study of ACE knockout mice has shown that testis ACE is important for male fertility.[5]

Both isozymes are encoded by the same ACE gene.[6] Previous studies showed that a 91 base pair sequence within the 12th intron of the mouse somatic ACE gene functions as a tissue specific testis ACE promoter.[7] In transgenic mice, this promoter is sufficient to target high level expression of a reporter gene to round and elongating spermatids. Two positive regulatory DNA motifs have been identified within the 91 base pair promoter by in vitro analysis.[8] One element, located at positions −55 to −48, has been shown to be the binding site for cyclic AMP response element modulator (CREM) proteins in vitro.[9] The other element, TCTTAT, is found at positions −32 to −27, a location often occupied by a TATA box. However, the sequence of this motif is quite different from TATAAA, the consensus for the TATA sequence.[10] Here we present data showing that despite its unusual sequence, the promoter motif is recognized by TATA binding protein (TBP). Transgenic mice studies show that this unusual TATA box is not the determinant for germ cell specific expression of testis ACE.

METHODS

Gel retardation assay. Double stranded fragments containing either the TCTTAT element (CAGGCTTGGCTCTTATTGGCCGGTGA) or the consensus TATA (CAGGCTTGGCTATAAATGGCCGGTGA) were generated from oligonucleotides.[8] Two complementary single stranded oligonucleotides were annealed and purified from an agarose gel, followed by phenol extraction and ethanol precipitation. These fragments were end labeled using T_7 DNA polymerase and [α-^{32}P] dCTP.

For gel retardation assay in the presence of antibody, 12 μg of nuclear proteins (testis or liver) were incubated with 0,

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Abbreviations: ACE, Angiotensin converting enzyme; TBP, TATA binding protein.

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6 or 13.5 μl anti-TFIIDτ antibody (0.1 mg/ml, Santa Cruz), in a 20 μl volume containing 20 mM HEPES pH 7.6, 32 mM KCl, 0.8 mM EDTA, 8% glycerol, 0.8 mM DTT, 1 μg double stranded poly(dI.dC) (Pharmacia), and 3.0×10^4 cpm probe at room temperature for 30 minutes. The reaction mix was then loaded onto a 5% native polyacrylamide gel. The gel was run in 45 mM Tris-borate, and 1 mM EDTA buffer (0.5 × TBE) at 4°C as described.[8] The gel was dried and exposed to X-ray film.

Gel retardation experiments with recombinant TBPs were performed as follows: 1, 2, 5 μl yeast TBP (kindly provided by Dr. Danny Reines), or 1 μl human TBP (1 fpu/μl, Promega) were incubated with 3.0×10^4 cpm probe in a 10 μl reaction volume containing 20 mM Tris (pH 8.0), 2 mM DTT, 80 mM KCl, 10 mM $MgCl_2$, and 10% glycerol at room temperature for 15 minutes. The reaction mix was loaded onto a 6% polyacrylamide gel. The gel was prepared with a running buffer consisting of 0.5 × TBE, 2 mM $MgCl_2$ and 0.01% NP-40. After running at 4°C, the gel was soaked in 1% glycerol solution for 15 minutes before drying.

For competitive gel retardation assay, 5, 10, 50 and 250 ng of unlabeled double stranded oligonucleotides were incubated with 10 μg testis nuclear extract in a 15 μl volume containing 20 mM HEPES pH 7.6, 32 mM KCl, 0.8 mM EDTA, 8% glycerol, 0.8 mM DTT, 1 μg double stranded poly(dI.dC) (Pharmacia) at room temperature for 5 min. 3.0×10^4 cpm probe was then added and incubated at room temperature for 10 minutes. The reaction mix was loaded onto a 5% native polyacrylamide gel. The gel was run in 0.5 × TBE at 4°C, followed by drying and exposing to X-ray film.

Transgenic mice. After site directed mutagenesis, the plasmid tACE. TA-pLacI was created by cloning a 698 bp Nco I fragment (−682 to +17) containing the mouse testis ACE promoter with the consensus TATA sequence into the NcoI site of the β-galactosidase expression vector pLacI.[11] A KpnI-Hind III restriction fragment containing the testis ACE promoter, the *E. coli* LacZ gene, an intron and a poly(A) addition site from the mouse metallothionein II gene was released from vector DNA and purified. This fragment was injected into the male pronuclei of one-cell stage FVB/N embryos to create transgenic mice. Integration of the transgenic DNA fragment into the genome was examined by PCR analysis of tail DNA.

Fresh tissues were removed from transgenic and non-transgenic mice and kept on ice. To quantitate enzymatic activity, the tissues were homogenized in 10 ml/g of PM2 buffer (20 mM NaH_2PO_4, 80 mM Na_2HPO_4, 0.1 mM $MnCl_2$, 2 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.3) with 0.1% Triton X-100, 0.2 mM PMSF, and 5 mg/l leupeptin. After centrifugation at 12,500 × g for 10 minutes, the supernatant was removed and tested for β-galactosidase activity using the substrate O-nitrophenyl-β-D-galactopyranoside (ONPG).[12] Protein concentration of the tissue extract was measured as described by Bradford, using reagents from Bio-Rad.[13] One unit of enzyme activity is equivalent to the conversion of 1 nmol ONPG per min at 37°C.

RESULTS

Gel shift analysis was used to study the binding of the mouse testis ACE TCTTAT motif to proteins present in a rat testis or a rat liver nuclear extract. A 23-base pair double-stranded oligonucleotide from positions −40 to −18 of the testis ACE promoter was end-labeled and used as a probe. Both the testis and liver nuclear extracts retarded the probe and gave a single band at an identical location (Fig. 1). To investigate whether TBP is part of the protein-DNA complex, anti-TBP antibody was added to the binding reaction. This antibody greatly reduced formation of the protein-DNA complex, suggesting that TBP, present in both nuclear extracts, recognized the DNA fragment containing the testis ACE TCTTAT motif.

To further assess the interaction of TBP with the testis ACE TCTTAT motif, we used recombinant yeast and human TBP in a gel shift assay (Fig. 2). These experiments were performed in the absence of any other nuclear proteins. The probe used in Fig. 2, lanes 1 to 5 was the testis ACE motif oligonucleotide. Lanes 6 to 10 are the gel shift pattern of the consensus TATA oligonucleotide. All lanes contained an equal amount of probe. As expected, in the absence of exogenous TBP, no gel retardation was observed (Lanes 1 and 6). Yeast TBP induced gel retardation of both oligonucleotide probes. However, the consensus TATA probe appeared to be more efficiently recognized and retarded. Lanes 5 and 10 are the gel migration pattern of the testis ACE TCTTAT motif and the consensus TATA oligonucleotides in the presence of recombinant human TBP. Recombinant human TBP (38 kDa) is larger than recombinant yeast TBP (27 kDa), and this is reflected in a slower migrating band.[14] Recombinant human TBP was similar to yeast TBP in that it bound the consensus TATA oligonucleotide (TATAAA) more efficiently than it bound the testis ACE TCTTAT motif probe. To verify that the gel shift reaction was not the result of the nonspecific recognition of DNA, we created a DNA fragment with an A to G point mutation at position −28 of the testis ACE promoter. In all other ways this oligonucleotide was identical to that

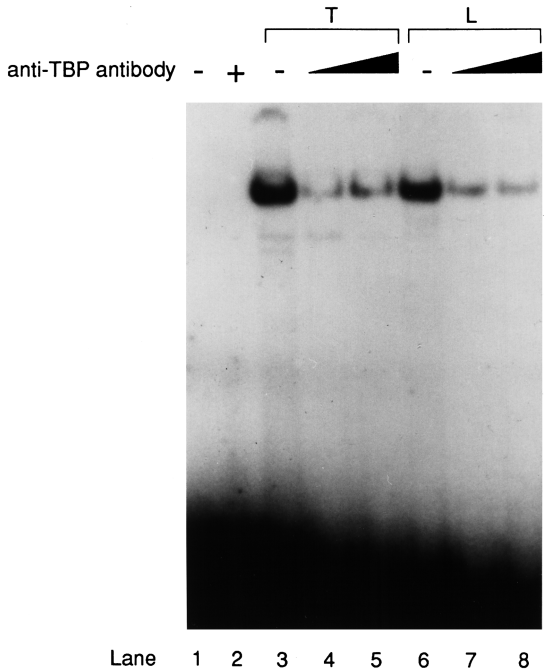

FIG. 1. Gel retardation. A 23 bp DNA fragment containing the testis ACE TCTTAT motif was used as a probe. Lane 1 shows the migration of the free probe. In lane 2 the probe was mixed with 1.35 μg of anti-TBP antibody in the absence of nuclear proteins. In lane 3 the probe was mixed with 12 μg of rat testis nuclear proteins. Lanes 4 and 5 are similar to lane 3 but the reaction mix also contained 0.6 and 1.35 μg of anti-TBP antibody. In lanes 6 to 8 the probe was reacted with 12 μg of liver nuclear proteins in place of the testis nuclear proteins. Lanes 7 and 8 contain 0.6 and 1.35 μg of anti-TBP antibody.

containing the wild type testis ACE motif. The modified oligonucleotide did not gel shift with either yeast or human TBP (data not shown).

Competitive gel retardation was also performed (Fig. 3A and B). In this protocol, the gel retardation observed with a testis nuclear extract was competed by adding increasing amounts of unlabeled (cold) oligonucleotide. The testis ACE TCTTAT oligonucleotide and the consensus TATA oligonucleotide were used separately, both as probes and as cold competitors resulting in four separate experimental protocols. In Fig. 3A the labeled probe was the testis ACE motif oligonucleotide while Fig. 3B used the consensus TATA oligonucleotide. The gel shifted bands were quantitated using a PhosphorImager and data is reported such that the shifted band in the absence of cold competitor was assigned a value of 100 percent. This analysis demonstrated that each oligonucleotide probe was most effective in competing with itself. We also observed that the testis ACE TCTTAT oligonucleotide competed very poorly for the protein factors that recognized the consensus TATA probe.

We have created transgenic mice containing either the wild type testis ACE promoter or a testis ACE promoter in which the TCTTAT motif has been mutated to a consensus TATA sequence. The wild type promoter construct consisted of a 698-base pair DNA fragment containing the testis ACE promoter and translation start site from −682 to +17 linked to the *E. coli* LacZ gene. The DNA construct also contained an intron and a poly (A) addition site from the mouse metallothionein II gene.[11] A second construct was identical to this first construct with the exception that the mouse testis ACE TCTTAT motif was converted to the sequence TATAAA by site directed mutagenesis. Both constructs were isolated free of plasmid DNA and were microinjected into one cell stage embryos. Transgenic animals were identified by PCR screening of tail DNA. In order to quantitate

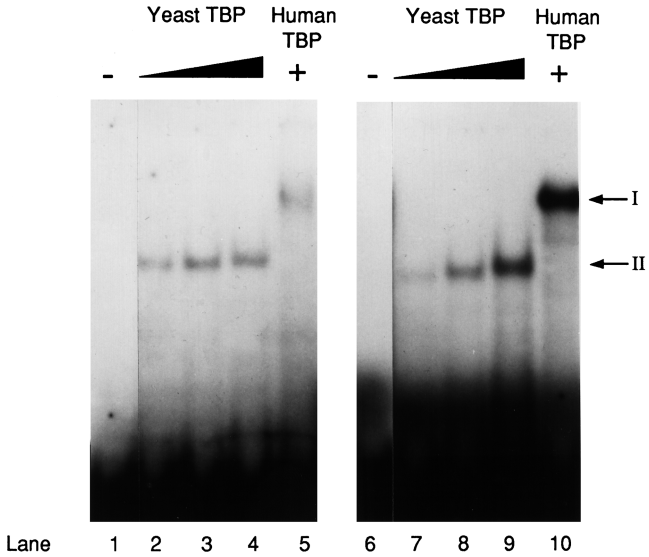

FIG. 2. Gel retardation with recombinant TBP. The probe used in lanes 1 to 5 contained the testis ACE TCTTAT element. Lane 1 is the migration of free probe. Lanes 2 to 4 have an increasing amount of recombinant yeast TBP. Lane 5 has 1 footprinting unit of recombinant human TBP. Lanes 6 to 10 are similar to lanes 1 to 5, except that as probe, we used an oligonucleotide that contained the consensus TATA sequence.

β-galactosidase expression, tissue extracts were prepared from four groups of animals: non-transgenic control mice; mice transgenic for the wild type testis ACE promoter construct; and two separate founder families transgenic for the construct containing the consensus TATA sequence. β-galactosidase activity was determined using the substrate O-nitrophenyl-β-D-galactopyranoside (ONPG).[11] The results of this analysis are shown in Fig. 4 and demonstrate that all of the transgenic animals produce the reporter gene at high levels only within the testis. One family of mice transgenic for the consensus TATA construct (tACE.TA-pLacI(1)), expressed the reporter gene at a level similar to that observed in mice transgenic for the wild type testis ACE promoter (tACE-pLacI). The second family of mice transgenic for the consensus TATA construct (tACE.TA-pLacI(2)) expressed the reporter at approximately 2-fold higher levels within the testis. This difference in level of reporter expression may be the result of the site of integration of the transgenic construct. Thus, these data indicate that in vivo, substitution of the testis ACE TCTTAT element with a consensus TATA sequence does not change the tissue specific expression of the testis ACE promoter.

DISCUSSION

The testis ACE TCTTAT motif is located at a position where most TATA boxes are located.[10] This sequence is identical in the testis ACE promoters of mice, humans, and rabbits.[7] Singer et al. has summarized the criteria to define a TATA element: (1) relative location to the transcription start site in promoters; (2) sequence similarity to the consensus TATAAA sequence; (3) ability to bind TFIID; and (4) the ability to activate transcription in combination with upstream activator proteins.[15] Since the testis ACE TCTTAT motif is found at the right location and acts as a positive transcriptional promoter element, we first studied the binding of the element to transcription factors in nuclear extracts. A critical observation was that nuclear extracts prepared from either the testis or the liver (a non-expressing tissue) retarded migration of the TCTTAT motif to an identical location. These data, plus the inhibition of complex formation by anti-TBP antibody and the

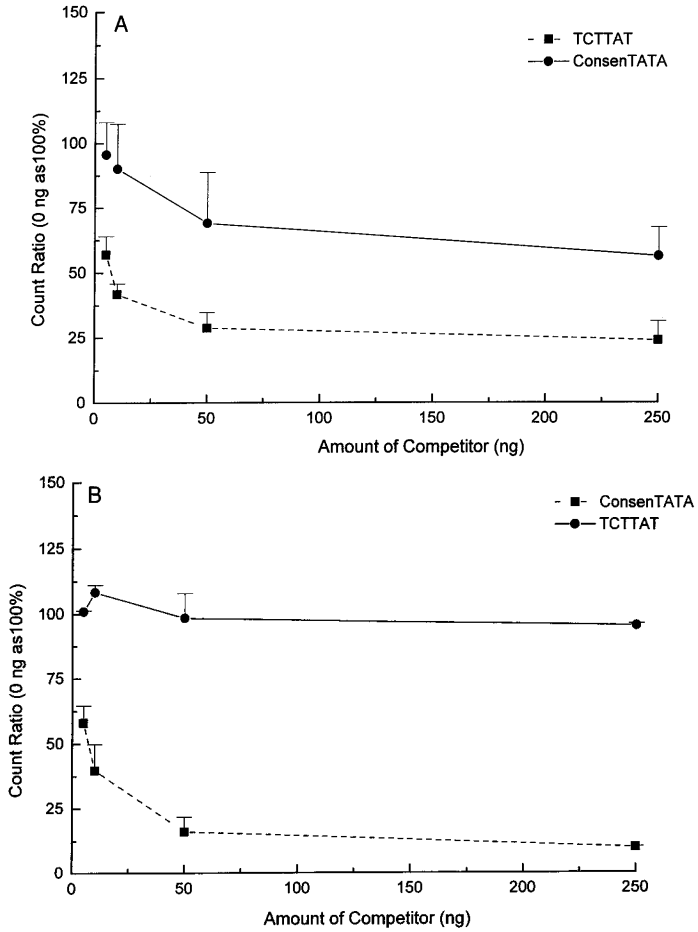

FIG. 3. Competitive gel retardation. (A) The probe used was a 23 bp oligonucleotide containing the testis ACE TCTTAT motif. 12 μg of a rat testis nuclear extract was added in each reaction. Separate reactions also contained 5, 10, 50, and 250 ng of unlabeled competitor. As a competitor, we used either the TCTTAT motif or the consensus TATA oligonucleotides as indicated. The shifted band in the absence of cold competitor was assigned a value of 100%. This graph represents the average and standard error from two independent experiments. (B) The probe used was a 26 bp oligonucleotide containing the consensus TATA sequence. In all other ways this figure is similar to (A).

demonstration that recombinant TBP recognized this motif, strongly suggest that a major role of the testis TCTTAT motif is to act as a nucleation site for TFIID.

To investigate the role of the testis ACE TCTTAT motif in the tissue specific expression of testis ACE, we have created transgenic mice in which this element was mutated to a consensus TATA sequence. Comparison of these animals to animals transgenic for the wild type testis ACE promoter indicates that tissue specificity is not conferred by the unique sequence of the testis ACE TCTTAT element. Specifically, conversion of this element to a consensus TATA sequence did not change the tissue specific pattern of reporter gene expression. These studies suggest that the testis ACE TCTTAT element functions as a non-consensus TATA sequence, but that this element is not responsible for the highly restricted pattern of tissue specific gene expression observed with testis ACE.

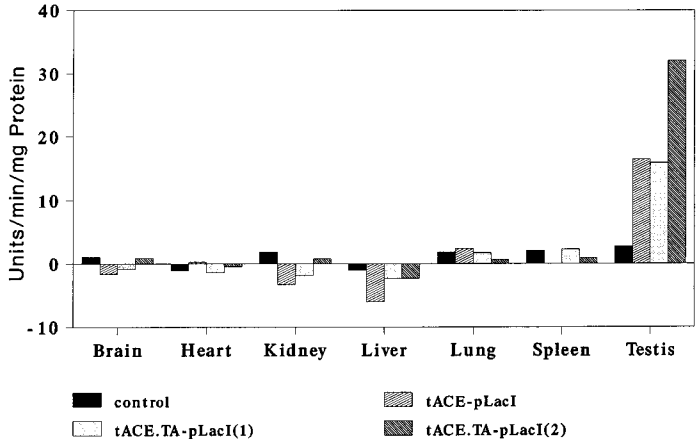

FIG. 4. Reporter in control and transgenic animals. Transgenic animals were created as described in the text. At sacrifice, organs were harvested and assayed for β-galactosidase activity. All animals were male. The data represented are the average of 3 non-transgenic control mice, 2 animals transgenic for tACE-pLacI, 3 animals derived from the tACE.TA-pLacI(1) founder, and 2 animals derived from the tACE.TA-pLacI(2) founder line.

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