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Binding Sites for Transcription Factor SF-1 in Promoter Regions of Genes Encoding Mouse Steroidogenesis Enzymes 3βHSDI and P450c17

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Abstract—Using gel retardation of DNA samples and specific antibodies, binding sites for the transcription factor SF-1 were found in positions −53/−44 and −285/−270 in the promoter region of the mouse Cyp17 gene and in position −117/−108 of the promoter region of the mouse 3βHSDI gene.

Key words: SF-1 (steroidogenic factor 1), steroidogenesis enzymes, Cyp17, 3βHSD, binding sites for SF-1, gel retardation of DNA

The steroid hormone testosterone plays an extremely important role in the regulation of the male reproductive system in vertebrates. In the male organism, testosterone is mainly produced by interstitial Leydig cells of the testicles. Biosynthesis of testosterone occurs with involvement of four key enzymes: cytochrome P450 cleaving the side chain (P450scc) of cholesterol and converting it into pregnenolone, 3β-hydroxysteroid dehydrogenase (3βHSD) catalyzing progesterone production from pregnenolone, 17α-hydroxylase/C17-20-lyase (P450c17) responsible for successive transformation of progesterone into 17α-hydroxyprogesterone and further to androstenedione, and, finally, 17-ketosteroid reductase (17KSR) involved in production of testosterone from androstenedione [1]. The enzyme P450scc is located on the inner mitochondrial membrane, and the three other enzymes (3βHSD, P450c17, and 17KSR) are microsomal and located in the endoplasmic reticulum membrane. Cholesterol is transported from the outer mitochondrial membrane to the inner membrane by the steroidogenic acute regulatory protein (StAR). This process limits the rate of biosynthesis of steroid hormones in gonads and adrenals [2].

We studied genetic control of steroidogenesis in primary culture of Leydig cells from testicles of six inbred mouse strains (A/He, CBA/Lac, C57BL/6J, YT, DD, PT), and a correlative inheritable variability was found in activities of three microsomal enzymes of testicular steroidogenesis (3βHSD, P450c17, 17KSR) [4, 5]. In particular, the activities of all enzymes studied were increased in PT males with a relatively high steroidogenic function of the gonads, and, on the contrary, activities of these enzymes were decreased in the strains A/He, and CBA/Lac with a low androgenic function of testicles. Recently we also revealed a coordinated variability in the mRNA level of all the above-mentioned enzymes of testicular steroidogenesis in mice of seven strains (BALB/c, A/He, CBA/Lac, C57BL/6J, YT, DD, PT) [6]. We suggested that this correlative genetic variability in activities of the enzymes under study and expression of their mRNAs should be caused by coordinated transcription of the genes encoding these enzymes, and the transcription factor SF-1 (steroidogenic factor 1) most likely coordinates their expression in the testicles.

SF-1 is a nuclear receptor that plays an important role in the regulation of steroidogenesis in gonads and adrenals and is necessary for development and functioning of all levels of the hypothalamus–hypophysis–gonadal and adrenocortical complexes [7-11]. So far, binding sites for SF-1 have been found in regulatory regions of many genes constituting these complexes in various vertebrate species [7-11]. As to the genes encoding enzymes and other proteins of mouse testicular steroidogenesis, binding sites for SF-1 were found in the promoter region of the Cyp11A gene encoding the enzyme P450scc and of the gene StAR [12-16]. There are still no data on the binding sites for this transcription fac-

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tor in regulatory regions of the mouse genes Cyp17, 3βHSDI, and 17KSR. But such sites have been found in the human [17], bovine [18, 19], and rat gene Cyp17 [20, 21] and also in the human gene 3βHSDII [22].

Based on hypothesis that SF-1 could be a factor responsible for the coordinated inheritable variability found by us [4, 5] in androgenic activity of Leydig cells of mouse testicles and also on the detection of binding sites for SF-1 in promoters of the gene orthologs Cyp17 and 3βHSD, we searched for potential binding sites for this factor in the mouse genes Cyp17 and 3βHSDI. We did not look for potential sites for SF-1 in the promoter region of the mouse gene 17KSR because the sequence of the 5′-flanking region of this gene was absent in the nucleotide sequence bases EMBL/GeneBank and the available literature. Based on the sample of 42 binding sites for SF-1 of the Transcription Regulatory Region Database (TRRD) section created by us for genes responsible for biosynthesis of steroid hormones, we designed the more precise consensus of the SF-1 site, which appeared as GTCAAGGTCA [23, 24]. By homology with the resulting consensus, we found, respectively, five and six putative sites for this factor in the 5′-flanking regions of the mouse genes 3βHSDI and Cyp17.

The purpose of the present work was to experimentally test the binding of the transcription factor SF-1 with a number of these sites by means of the gel retardation approach and specific antibodies.

MATERIALS AND METHODS

Animals. Ten-day-old male Wistar rats from the Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences (ICG SD RAS) were used.

Preparation of extracts from nuclei of testicular cells. Extracts from nuclei of testicular cells were prepared as described in [25] using the modification [26].

Oligonucleotide retardation in polyacrylamide gel with proteins of nuclear extracts. Single-chain oligonucleotides Cyp17 −59/−35 (5′-cagtCACGTCTTCAAGGTGACAATCAGAA-3′ and 5′-cagtTTCTGATTGTCACCTTGAAGACGTG-3′), Cyp17 −290/−265 (5′-cagtCTCTGAAACCTTGATCTTAATCTGAT-3′ and 5′-cagtATCAGATTAAGATCAAGGTTTCAGAG-3′), 3βHSDI −89/−65 (5′-cagtGGCAGGAATAAAGGACATAAGGTTT-3′ and 5′-cagtAAACCTTATGTCCTTTATTCCTGCC-3′), 3βHSDI −126/−102 (5′-cagtATCACAGTGTAACCTTGAAGCTGGC-3′ and 5′-cagtGCCAGCTTCAAGGTTACACTGTGAT-3′) were synthesized by V. F. Kobzev (ICG SD RAS) [27]. After annealing, the resulting double-chain oligonucleotides were labeled with [α-^{32}P]dATP as described in [28].

The binding of oligonucleotides with the nuclear extract proteins was analyzed by a partly modified routine method: 50 μl of the nuclear extract from testicles was incubated on ice with ultrasonicated salmon sperm DNA (1 μg DNA per 7 μg total protein) for 10 min. In experiments with antibodies to the DNA-binding domain of SF-1 (Upstate Biotechnology Inc., USA) the nuclear extract was concurrently incubated with salmon sperm DNA and antibodies (1 μl antibodies per 2-3 μg protein). Afterwards, 3 μg extract was added to samples containing 1 ng DNA-probe labeled with [α-^{32}P]dATP, and the total volume of the reaction mixture was adjusted to 16 μl with buffer (25 mM Hepes (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.2 mM EGTA, 10% glycerol). The binding reaction was performed at room temperature for 10 min. Then the samples were separated in 4.5% nondenaturing polyacrylamide gel. Electrophoresis was performed in 0.5× TBE buffer (89 mM Tris, 89 mM H_3BO_3, 2 mM EDTA) at the electric field strength of 10 V/cm for 45-50 min at 4°C, and then the gel was dried and exposed with to an X-ray film for 3-5 days.

The oligonucleotide Con (5′-cagtGGCCACAGTTCAAGGTCAAGGAGAA-3′ and 5′-cagtTCTCCTTGACCTTGAACTGTGGCCA-3′) was used as the control; in work [29] its binding to the transcription factor SF-1 was shown. As the negative control the mutant oligonucleotide Mut (5′-cagtGGCCACAGTGTAATATCAAGGAGAA-3′ and 5′-cagtTCTCCTTGATATTACACTGTGGCCA-3′) was used.

RESULTS AND DISCUSSION

The table presents potential binding sites for the transcription factor SF-1 in the regulatory regions of the mouse genes Cyp17 and 3βHSDI which were detected earlier based on homology with the consensus [23, 24]. We have chosen for experimental study the potential site for SF-1 in the −53/−44 position from the gene Cyp17 promoter region and two overlapping potential sites for SF-1 in the positions −285/−276 and −279/−270. The site location in the −53/−44 position is the most like locations of functional SF-1 sites in the positions of −53/−44 and −67/−58 in the proximal region of the promoters of the bovine [18, 19] and rat [20, 21] Cyp17 gene, respectively. The potential site for SF-1 in −53/−44 (5′-tTCAAGGTgA-3′) of the mouse Cyp17 gene and the SF-1 site in −67/−58 (5′-GTCAAGGTgA-3′) of the rat gene are different only in one nucleotide. The positions −285/−276 and −279/−270 of the potential SF-1 sites in the mouse Cyp17 gene are similar to the position −289/−274 of the SF-1 site in the human Cyp17 gene which in complex with SF-1 is involved in regulation of transcription of this gene [17].

The site for SF-1 in the −117/−108 position was chosen for experimental studies from five potential sites in the 3βHSDI gene as the closest to the consensus (table). Another site located in position −82/−73 was

Potential binding sites for the transcription factor SF-1 in the regulatory regions of mouse Cyp17 and 3βHSDI genes detected by homology with the consensus

Gene	Consensus GTCAAGGTCA	Consensus TGACCTTGAC
Cyp17	<u>−53/−44 tTCAAGGTgA</u>	−505/−496 gGcCCTTGAg −435/−426 TGACCTTatg −337/−328 TGACaTTaAt <u>−285/−276 aaACCTTGAt</u> <u>−279/−270 TGAtCTTaAt</u>
3βHSDI	<u>−82/−73 aTaAAGGaCA</u> −54/−45 GggAAGGaCA −3/+7 GTCAAGaTat	<u>−117/−108 TaACCTTGAa</u> +86/+95 TGACtTTtAa

Note: To the right and to the left the alignment is presented in the direct and inverse orientation, respectively, relative to consensus of the binding site for SF-1. Three potential sites are shown which differ from the consensus in no more than three nucleotides. Nucleotides coinciding with the consensus sequence are presented in capital letters. The sites for SF-1 experimentally tested by gel retardation of DNA sample are underlined.

chosen based on its coincidence with the consensus with GG nucleotides in the 6 and 7 positions of the consensus, which are the most significant for binding with the SF-1 protein molecule [23, 24].

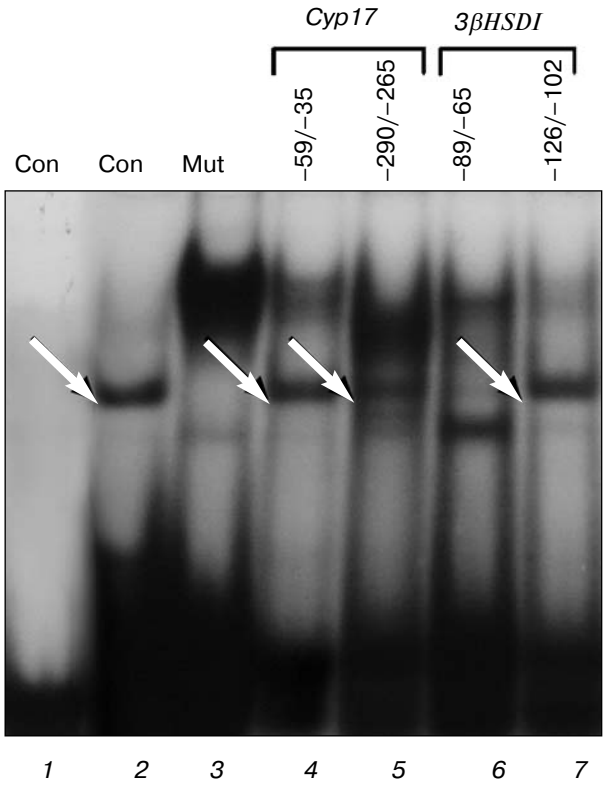

Fig. 1. Binding of proteins of nuclear extract from cells of 10-day-old rat testicles with oligonucleotides Con, Mut, −59/−35 and −290/−265 of Cyp17 and −89/−65 and −126/−102 of 3βHSDI: *1*) free probe; *2-6*) binding with extract from cells of rat testicles (3 μg protein). The arrows show retardation bands corresponding to transcription factor SF-1.

Moreover, the SF-1 sites −53/−44 and −285/−270 in the Cyp17 gene and the SF-1 site −117/−108 in the 3βHSDI gene were revealed using the SITECON computerized approach [30]; the SF-1 site −117/−108 in 3βHSDI was also detected using the SiteGA computerized method for site recognition [30].

In the present work the transcription factor SF-1 was established to bind to oligonucleotides which corresponded to potential sites in the positions −53/−44 and −285/−270 (lanes *4* and *5*, respectively) in the 5′-regulatory region of the Cyp17 gene and an oligonucleotide corresponding to the potential SF-1 site in position −117/−108 (lane *7*) in the promoter region of the 3βHSDI gene (Fig. 1). SF-1 did not bind to the oligonucleotide, which corresponded to the potential site for SF-1 in position −82/−73 of the promoter region of the 3βHSDI gene (Fig. 1, lane *6*). The experiment with antibodies to transcription factor SF-1 (Fig. 2) confirms that retardation bands of DNA–protein complexes (shown with the arrow in Fig. 1) really contain this factor. In particular, the retardation band corresponding to the SF-1 complex with the oligonucleotide −59/−35 of the Cyp17 gene was attenuated (lane *3*). Antibodies to SF-1 inhibited SF-1 complexing with oligonucleotide −126/−102 of the 3βHSDI gene (lane *9*) and oligonucleotide −290/−265 (lane *6*) of the Cyp17 gene.

It should be noted that in the presence of antibodies to SF-1 the retardation bands of less mobile complexes of the oligonucleotides −59/−35 and −290/−265 with proteins of the nuclear extract from the testicle cells were attenuated and disappeared. Thus, these complexes were suggested to also contain SF-1. The absence of a supershift of the retardation bands of DNA–protein complexes on addition of antibodies is explained by using the antibodies directed to the DNA-binding domain of SF-1. Other authors obtained similar results with such antibodies [31].

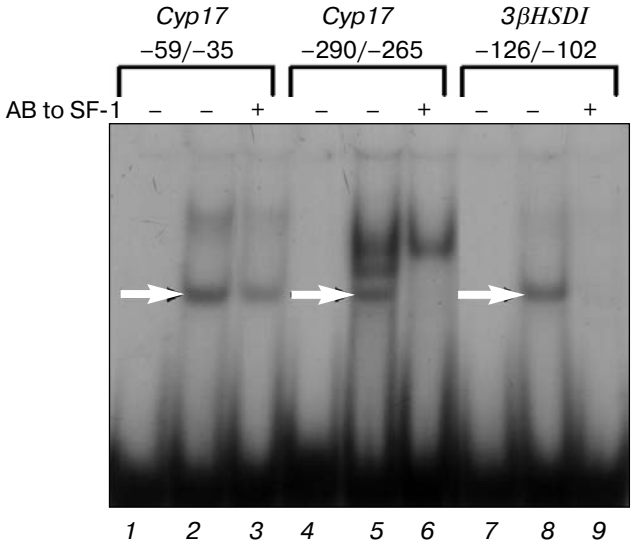

Fig. 2. Binding of proteins of the nuclear extract from cells of 10-day-old rat testicles with oligonucleotides −59/−35 and −290/−265 of Cyp17 and −126/−102 of 3βHSDI in the presence of antibodies (AB) to the transcription factor SF-1: *1*, *4*, *7*) free probe; *2*, *5*, *8*) binding with nuclear extract from cells of rat testicles; *3*, *6*, *9*) binding with nuclear extract preincubated with antibodies to SF-1 ("+" indicates 1 μl of the antibodies). The arrows show retardation bands corresponding to SF-1.

Thus, by gel retardation of DNA sample, we have established the presence of binding sites for SF-1 in the mouse genes Cyp17 and 3βHSDI. The literature data show the presence of functional binding sites for this transcription factor in the regulatory regions of the mouse genes StAR and Cyp11A [12-16]. These results taken together support the hypothesis that SF-1 can be a factor responsible for the coordinated inheritable variability of the androgenic activity of testicles [4, 5] because it regulates the gene transcription of key enzymes necessary for biosynthesis of testosterone in testicles.

Our diallele analysis of the cAMP- and substrate-dependent production of testosterone by Leydig cells of six inbred mouse strains and their reciprocal first generation hybrids showed that these parameters are under coordinated and polygenic control. Based on these data, we developed a four-locus segregation model of the coordinated inheritable variability of the hormonal activity of Leydig cells [32-34]. Based on current ideas on the key role of the SF-1 factor in the regulation of expression of many genes of the hypothalamus–hypophysis–gonadal system [11] and our and literature data on the presence of binding sites for SF-1 in the promoter regions of the genes encoding the enzymes of steroidogenesis in mouse testicles, the gene encoding this factor may be considered as a candidate in the four-locus model of inheritance of the coordinated hormonal activity of Leydig cells.

Data on SF-1 polymorphism in mice appeared recently [35, 36]. Two SF-1 forms different in the presence of alanine or serine in position 172 of its molecule were revealed. Mouse strains C57BL/6J and C57BL/10J carrying the A172 allele (alanine in position 172) are characterized by high steroidogenic activity of Leydig cells, whereas interstitial cells of testicles of the mouse strains C3H/HeJ and DBA/2J carrying the S172 allele (serine in position 172) produce twofold less testosterone on stimulation with gonadotropins. These data also favor SF-1 as a possible factor regulating the coordinated genetic variability of the androgenic function in mice detected by us, and make it reasonable to search for polymorphism of this factor in the inbred strains under study (BALB/c, A/He, CBA/Lac, C57BL/6J, YT, DD, PT) [4, 5, 32-34].

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