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Localization of a Site for Interaction with Hepatic Male-Specific Proteins in Two Rat Estrogen Sulfotransferase Genes

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Abstract—Using electromobility shift assay the interaction of fragments of two paralogous rat estrogen sulfotransferase (*Ste*) genes with proteins of nuclear extracts from male and female rat liver was studied. Male-specific DNA–protein complexes were revealed with labeled oligonucleotides corresponding to fragments +1150/+1449, +1358/+1449, +1397/+1449, and +1417/+1449 of intron 1 of the *Ste1* gene. The removal of a 20 bp region corresponding to the sequence +1430/+1449, or even either 5′- or 3′-terminal 5 bp of this region abolished the selective interaction of the oligonucleotides with the male-specific protein(s). According to the results of the experiments on mutual competition of the oligonucleotides, the fragment of the *Ste2* gene corresponding to the sequence +1397/+1449 of the *Ste1* gene formed complexes with the same male-specific protein(s) as the fragment of the *Ste1* gene did. The data suggest the mapped element to participate in gender differentiation of the expression of the *Ste1* and *Ste2* genes.

Key words: estrogen sulfotransferase, sex differentiation, gene expression, regulatory elements, DNA–protein interaction, liver

Estrogen sulfotransferases (STE) (EC 2.8.2.4), enzymes catalyzing estrogen sulfation, are members of a large superfamily of sulfotransferases (ST) [1, 2]. The reaction products, sulfated estrogens, are not able to bind to cognate receptors, and therefore lack the hormonal activity. In addition, the sulfation increases the steroid reabsorption limit in the kidney, thus promoting their excretion from the organism. Studies of STE have shown that these enzymes catalyze the sulfation of natural estrogens (estradiol, estrone, and estriol) with K_m in a low nanomolar range [3-5]. Thus, STE can actually be involved in maintenance of active estrogen balance in the tissues *in vivo* and affect the expression of genes controlled by these hormones. The expression of estrogen sulfotransferase genes is tissue-specific and subject to complex hormonal control [4, 6-10]. Until now two closely related STE cDNA encoded presumably by paralogous genes have been cloned from the rat liver [4, 6]. The expression of these genes shows high sexual dimorphism. They are activated in male liver at puberty but remain lowly active in female liver throughout the life span. There are evidences that *Ste* expression in rat liver is regulated by sex steroids and growth hormone [6, 11], but the fine molecular mechanisms of such regulation remain unknown.

By screening of the genomic library and PCR on rat genomic DNA we have previously isolated the fragments encompassing entirely two estrogen sulfotransferase genes, *Ste1* and *Ste2*, and established their exon–intron organization [12]. The search for the potential regulatory elements within the promoter region of *Ste1* by electromobility shift assay and footprinting did not revealed any elements with sexual specificity of binding of rat hepatic nuclear extract proteins [13, 14]. In the present work the region of intron 1 of the rat *Ste1* gene was mapped whose binding to nuclear extract proteins from male and female liver shows remarkable differences. A similar region was shown to exist within the rat *Ste2* gene as well.

MATERIALS AND METHODS

Nuclear extracts were prepared from liver of adult male and female mongrel white rats. Nuclei were isolated by sucrose gradient centrifugation as described by Gorski et al. [15], and nuclear extracts were obtained according to Parker and Topol [16] with minor modifications [13].

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Electromobility shift assay. Double-stranded DNA fragments used as probes were prepared by annealing of complementary single-stranded oligonucleotides or by cleavage of plasmids bearing corresponding fragments with restriction endonucleases.

Fragment I (+1150/+1449) of *Ste1* intron 1 (Fig. 1) was obtained by PCR with specific primers complementary to regions +1150/+1177 (5′-GTTGTCAAAAGAATGGAAACTTATTTCT-3′) and +1417/+1449 (5′-AAGACTGGTGAAATGCTGCTGCTGACCTGCTA-3′) of *Ste1* gene using plasmid p5/8 as template. The plasmid p5/8 carries an insert containing 2 kb of 5′-flanking region, 5′-untranslated exons 1a and 1b, intron 1 and exon 2 of *Ste1* [14]. PCR-product of 300 bp was cloned in T-vector prepared from the plasmid pBluescript II KS (–). The vector was obtained by cleavage of the plasmid with restriction endonuclease *Eco* 32I (isoschizomer of *Eco* RV; MBI Fermentas, Lithuania) and treatment with *Taq*-DNA polymerase (Dialat, Russia) in the presence of 2 mM dTTP for 2 h at 70°C.

To prepare radioactive probes, the cloned fragment I was cut out by *Eco* RI and *Hind* III, labeled with [α-^{32}P]dATP (5000 Ci/mmol) by Klenow fragment of DNA polymerase I (MBI Fermentas) and isolated from 5% polyacrylamide gel. Probes corresponding to fragments +1150/+1303 (fragment II, Fig. 1) and +1358/+1449 (fragment III, Fig. 1) of the *Ste1* gene were obtained by cleavage of labeled fragment I with restriction endonuclease *Alu* I and separating in 5% polyacrylamide gel.

To obtain the labeled fragment of *Ste2* gene (fragment VI, Fig. 1), corresponding to the region +1397/+1449 of *Ste1*, oligonucleotides complementary to region +1397/+1429 of both *Ste* genes (fragment V, Fig. 1) (5′-AAAGGATGCTTTTACCCTGTGTAGCAGGTCAGC-3′) and to region +1417/+1449 of *Ste2* gene (5′-AAGACTGGTTAGATGCTGCTGCTGACCTGCTAC-3′) were annealed and extended by Klenow fragment of DNA polymerase I, using [α-^{32}P]dATP (5000 Ci/mmol) and unlabeled dGTP, dCTP, and dTTP.

Double-stranded oligonucleotides corresponding to fragments IV and V of *Ste1* gene, oligonucleotides partially overlapping 3′-terminal region of fragment IV (+1420/+1444, +1415/+1439, +1435/+1459), oligonucleotide corresponding to regulatory region of rat tyrosine aminotransferase gene (TAT) and containing steroid-responsive element [17] (5′-GATCCTCTGC**TGTACA**GGA**TGTTCT**AGCTAGA-3′, half-sites of the element shown in bold), and oligonucleotide with a random sequence (5′-AATTTAATACGACTCACTATAGGGATATC-3′), used as unlabeled competitors were also obtained by annealing of respective single-stranded oligonucleotides. All oligonucleotides were synthesized by Litekh (Russia).

Binding of DNA fragments to nuclear proteins was done as follows. Nuclear extracts (1-3 μg protein) were preincubated for 5 min at room temperature in 13 μl of buffer (25 mM HEPES, pH 7.6, 40-150 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1.25 mM DTT, 10% glycerol), containing 2-4 μg poly[(dI-dC)·(dI-dC)] or 1 μg poly[(dA-dT)·(dA-dT)] as a carrier, and in some cases unlabeled double-stranded oligonucleotides as competitors. Then, the labeled probe was added and incubation proceeded for 20 min at room temperature. In a separate experiment, after addition of the probe, the incubation with rabbit polyclonal antibodies reacting with rat, mouse, and human STAT5 proteins (Santa-Cruz Biotechnology, USA, sc-1081 and sc-835) was done for 10 min.

DNA–protein complexes were separated in 5-7.5% polyacrylamide gel (30 : 1 or 80 : 1) containing 2.5% glycerol. Electrophoresis was run in 0.5× Tris-borate buffer (50 mM Tris, 41.5 mM boric acid, 0.5 mM EDTA, pH 8.3) under 10 V/cm. Gels were fixed in 10% acetic acid, dried and exposed to X-ray film (Retina, Germany) with an intensifying screen.

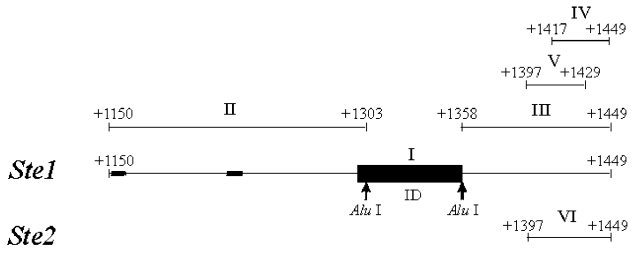

Fig. 1. Scheme of *Ste1* and *Ste2* intron 1 fragments (I-VI) used as probes for binding of rat liver nuclear extract proteins. Numbering is given in relation to the first transcribed nucleotide of the *Ste1* gene [14]. The repeated ID element of the *Ste1* absent in the *Ste2* gene is shown as a black box. Arrows indicate the *Alu* I cleavage sites used for preparing fragments II and III from fragment I of *Ste1*. Thicker lines mark clusters of potential STAT5 binding sites.

RESULTS AND DISCUSSION

Analysis of the rat *Ste1* gene promoter region by footprinting and electromobility shift assay did not reveal any regions that differentially interact with nuclear extract proteins from female and male liver [13, 14]. One could propose that *cis*-elements located in other regions of the gene might be involved in sex-dependent regulation of transcription. Recently, sexual dimorphism of hepatic expression of some other genes, for example some cytochrome P450, was shown to be mediated by interaction of transcription factors of STAT family, STAT5a and STAT5b, with specific elements of nucleotide sequence [18-20]. We have searched the potential STAT5-binding sites (consensus sequence, 5′-TTCNNNGAA-3′) in the

sequenced regions of the *Ste1* gene using computer program MatInspector ([21], http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl). Two clusters of "weak" potential binding sites were found in the 5′-portion of intron 1 fragment I of the *Ste1* gene (EMBL database AC: AJ131835) (Fig. 1).

To test the possible involvement of these sites in sex-dependent expression of the gene we studied the binding of 300 bp fragment I of intron 1 with nuclear extract proteins from female and male rat liver. Incubation of fragment I with the proteins in the presence of nonspecific carrier poly[(dI-dC)·(dI-dC)] (200 μg/ml) results in formation of DNA–protein complexes whose electrophoretic mobility differs drastically in the case of female and male liver extracts (Fig. 2, lanes *2* and *3*, respectively).

To confirm the involvement of potential STAT-binding sites in observed gender differences in DNA–protein interaction, similar experiments were done with fragments II and III corresponding to the first 154 bp and the last 92 bp of fragment I (Fig. 1). Since the potential STAT-binding sites reside in fragment II (within the region +1150/+1230 relative to the 1st transcribed nucleotide), while fragment III does not contain such sites, the results of these experiments (Fig. 3) were quite unexpected: sex-differentiated DNA–protein complexes not observed with fragment II (Fig. 3a) were clearly visible with fragment III (Fig. 3b). One slowly migrating DNA–protein complex was formed with the female liver extract while the male liver extract produced three complexes with higher mobility (**a**, **b**, **c**). These data allow suggest that sex-specific DNA–protein complexes lack STAT proteins. This was confirmed by incubating of fragment I with nuclear extract in the presence of anti-STAT5a and anti-STAT5b antibodies. Electrophoretic mobility of DNA–protein complexes did not changed after incubation with antibodies at 60 or 150 mM KCl (data not shown). Thus, sex-differentiated interaction of fragment I with rat liver proteins distinct from STAT5 is mediated by the region corresponding to fragment III. Analysis of the 3′-terminal sequence of this fragment (+1397/+1449, Fig. 4) shows that it contains several potential binding sites for a number of transcription factors: those for nuclear receptors (ROR, COUP-TF; +1417/+1427), cAMP-responsive elements (CREs), which might interact with the following factors—CREB, CREM, ATF-1, ATF-2, ATF-3, c-Jun, TTF-1, AP-1, AP-4 (+1420/+1428), as well as binding sites for C/EBP and NF-IL6 factors (+1436/+1444). Note the presence in this fragment the sequences that are nearly perfect mirror copies of the nuclear receptor binding sites (+1406/+1416, +1436/+1446). As a necessary step in identification of factors present in sex-differentiated DNA–protein complexes, their binding sites within the fragment III of rat *Ste1* gene were more precisely mapped. The competitive displacement of the

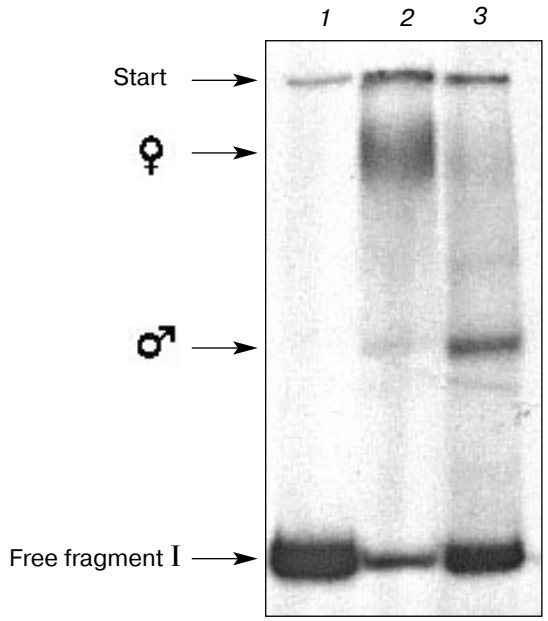

Fig. 2. Electromobility shift assay of the *Ste1* fragment I. End-labeled with [α-^{32}P]dATP fragment +1150/+1449 (0.02 pmol) was incubated without (*1*) or with 2 μg of nuclear extracts from female (*2*) or male (*3*) rat liver in the presence of poly[(dI-dC)·(dI-dC)] (200 μg/ml). Complexes formed were separated in 4% (30 : 1) polyacrylamide gel.

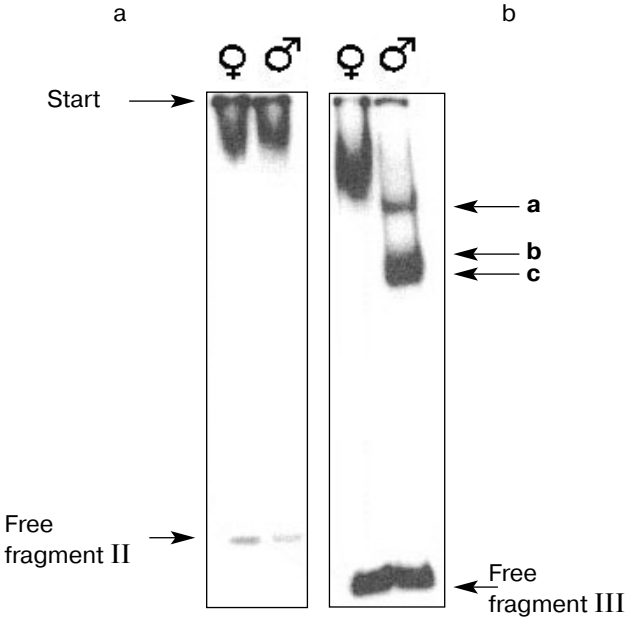

Fig. 3. Electromobility shift assay of fragment II (+1150/+1303) and III (+1358/+1449) of *Ste1*. Fragments II (a) and III (b) (0.02 pmol) end-labeled with [α-^{32}P]dATP were incubated with 2 μg of nuclear extracts from female (a) or male (b) rat liver in the presence of poly[(dI-dC)·(dI-dC)] (200 μg/ml). **a**, **b**, **c**, male-specific complexes. Complexes formed were separated in 6.5% (80 : 1) polyacrylamide gel.

labeled fragment III from the specific complexes with proteins by a shorter partly overlapping double-stranded oligonucleotides IV and V (Fig. 1) was studied. Taking into account the high AT-content (70%) of fragment III, the poly[(dA-dT)·(dA-dT)] instead of poly[(dI-dC)·(dI-dC)] was used in the further experiments as a nonspecific carrier. The pattern of DNA–protein complexes was changed under such conditions. As seen in Fig. 5a (odd lanes), after incubation of fragment III with female liver nuclear extract in the presence of poly[(dA-dT)·(dA-dT)], DNA–protein complexes are virtually not observed. Under the same conditions the male liver proteins form with fragment III only two complexes with similar electrophoretic mobility (Fig. 5a, lane *2*) which are seen as single band **a** in Fig. 3b due to shorter electrophoretic run. The complexes with higher mobility designated in Fig. 3a as **b** and **c** are not formed under such conditions. Adding of 10-fold excess of unlabeled fragment III decreases the intensity of complex with labeled fragment (lane *4*). Labeled fragment III is completely displaced from the complexes with male nuclear protein by 150-fold excess of fragment IV (lane *6*). Fragment V as well as the fragment with the random sequence (nonspecific competitor) added to fragment III in 150-fold excess do not compete for the binding with male liver nuclear proteins (lanes *8* and *10*). The fragment of TAT gene carrying the steroid-responsive element does not compete as well (data not shown).

The results of competitive assay presented in Fig. 5a show that the binding site(s) for male-specific protein(s) resides within fragment IV (+1417/+1449) of the *Ste1* gene. Moreover, the lack of competition for male-specific protein between fragment V and labeled fragment III suggests that the 3′-terminal 20 bp (+1430/+1449) is essential to form the male specific DNA–protein complexes. A similar conclusion follows from the experiments with labeled fragment IV: it formed complexes with male-specific proteins and was displaced from complexes by unlabeled fragment IV but not fragment V (data not shown).

To get more information on the borders of the binding site for male-specific protein(s), the ability of short oligonucleotide fragments (+1420/+1444, +1415/+1439, +1435/+1459) partly overlapping fragment +1430/+1449 to compete with the labeled fragment III for male-specific protein(s) was assayed. None of these fragments was found to possess competitive activity (data not shown). These data suggest that the 10 bp sequence +1435/+1444 and at least one additional nucleotide from both 5′- and 3′-side are required for the binding of male-specific protein(s).

The intron 1 region of the *Ste2* gene corresponding to fragment IV of *Ste1* differs by two nucleotides (Fig. 4), and both substitutions reside in the center of the 3′-terminal 20 bp sequence required as shown above for interaction with male-specific protein(s). Therefore, it was of interest to test the ability of this region of the *Ste2* gene to bind to the same male-liver protein(s) which the *Ste1* region interacts with. Experiments with labeled fragment III of *Ste1* have shown that the *Ste2* fragment corresponding to fragment VI of *Ste1* competes efficiently with the labeled probe for male-specific protein(s) (data not shown). In a direct experiment with the labeled fragment VI of the *Ste2* gene the male liver-specific DNA–protein complexes was found to form (Fig. 5b, lane *2*). Unlabeled fragments VI of *Ste2* and IV of *Ste1* completely displace the labeled probe from the complexes when added in 150-fold excess (lanes *4* and *6*), while fragment V of *Ste1* and oligonucleotide with random sequence do not possess competitive activity (lanes *8* and *10*).

Thus, the differences between *Ste1* and *Ste2* in positions +1438 and +1440 (numbering corresponds to *Ste1*) do not affect significantly the ability of fragments +1417/+1449 to interact with one and the same male-specific protein(s). These nucleotide substitutions in +1136/+1146 of *Ste2* decrease its similarity to CRE and C/EBP and octamer factor binding sites. Thus, the protein(s) interacting with this region of *Ste* genes is likely to be distinct from the mentioned transcription factors. In addition to STAT5, at least four transcription factors whose expression or activity show sexual dimorphism in the rat liver are known: estrogen receptors (ER), androgen receptors (AR), hepatocyte nuclear factor 6 (HNF-6), and constitutively active receptors (CAR) [26-28].

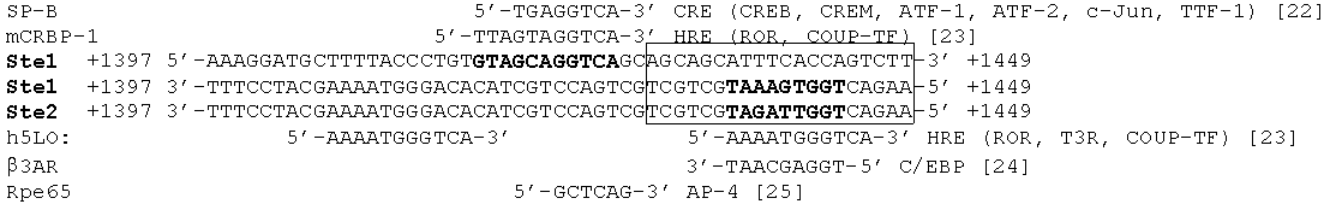

Fig. 4. Nucleotide sequence and potential transcription factor binding sites of fragment +1397/+1449 of *Ste1* and *Ste2* intron 1. The sequences related to hormone-responsive element of nuclear receptors and C/EBP binding site are shown in bold. The 20 bp fragment required for interaction with protein(s) specific for male rat liver is framed. Known functional elements of genes are positioned against compared *Ste* nucleotide sequences.

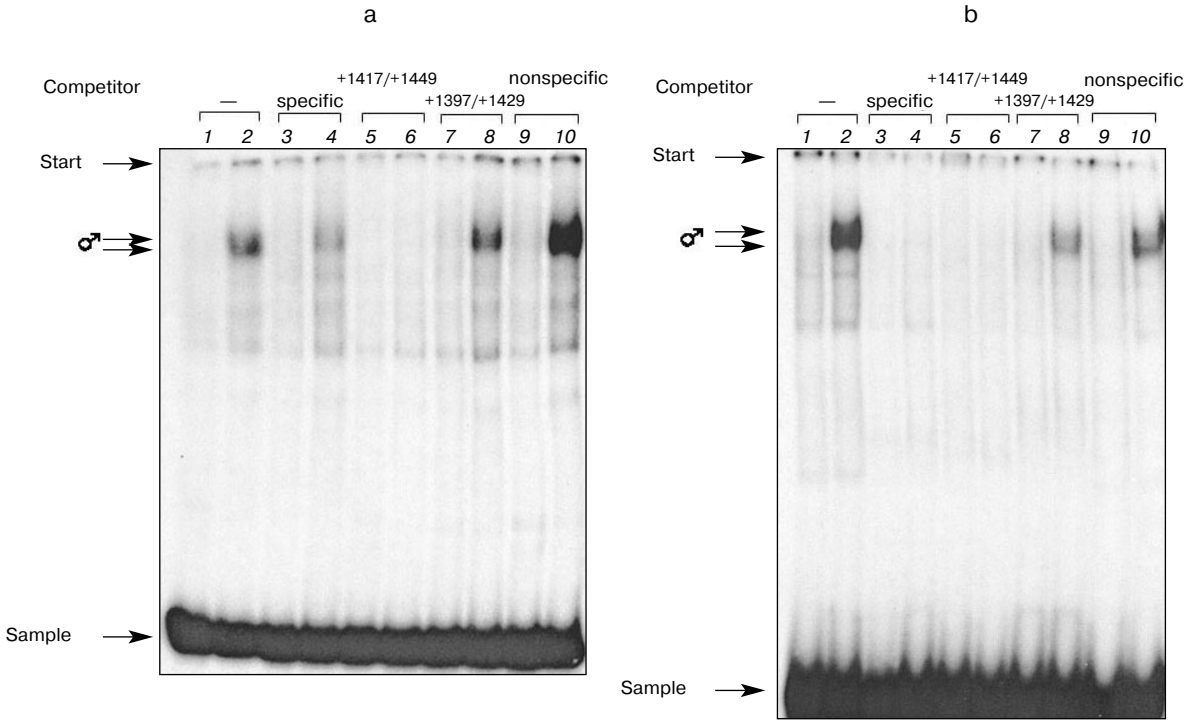

Fig. 5. Electromobility shift assay of the *Ste1* fragment III and *Ste2* fragment VI. a) *Ste1* fragment III (0.02 pmol) end-labeled with [α-^{32}P]dATP was incubated with 2 μg of nuclear extracts from female (*1*, *3*, *5*, *7*, *9*) or male (*2*, *4*, *6*, *8*, *10)* rat liver without competitor (*1*, *2*) or in the presence of 10-fold excess of unlabeled fragment III (*3*, *4*), 150-fold excess of unlabeled fragment IV (*5*, *6*), 150-fold excess of unlabeled fragment V (*7*, *8*) or 150-fold excess of unlabeled oligonucleotide with random sequence (*9*, *10*); b) *Ste2* fragment VI (0.02 pmol) end-labeled with [α-^{32}P]dATP was incubated with 2 μg of nuclear extracts from female (*1*, *3*, *5*, *7*, *9*) or male (*2*, *4*, *6*, *8*, *10*) rat liver without competitor (*1*, *2*) or in the presence of 150-fold excess of *Ste2* unlabeled fragment VI (*3*, *4*), 150-fold excess of *Ste1* unlabeled fragment IV (*5*, *6*), 150-fold excess of *Ste1* unlabeled fragment V (*7*, *8*) or 150-fold excess of unlabeled oligonucleotide with random sequence (*9*, *10*). Double-stranded alternate poly[(dA-dT)·(dA-dT)] was used as a carrier (67 μg/ml). Complexes formed were separated in 6.5% (80 : 1) polyacrylamide gel.

ER and HNF-6 are predominant in female liver and, therefore cannot be responsible for the forming of male-specific complexes. Hormone-responsive element for AR is a palindrome with nucleotide sequence remarkably different from that of elements binding the majority of nuclear receptors [29, 30]. Similar sequences were not found in the studied fragments of *Ste* genes. Moreover, the fragment of rat TAT gene regulatory region containing hormone-responsive element did not compete with the labeled probe for male-specific protein(s). Thus, it seems unlikely that this protein(s) is identical to AR. mRNA CAR is expressed in the liver of rats in gender-independent fashion, but in females of Wistar Kyoto strain (contrary to females of Fischer 344 strain) immunoreactivity, DNA-binding, and functional activities of CAR are significantly lower than in males [28]. Therefore, one cannot exclude that male-specific DNA–protein complexes contain CAR. The data obtained suggest that fragment +1417/+1449 of intron 1 of *Ste1* and *Ste2* is sufficient while its portion, the sequence +1430/+1449, is required for the binding of male liver-specific factor(s), and that these fragments may play an important role in the sexual differentiation of expression of *Ste* genes.

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