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Mammalian expression of the human sex steroid-binding protein of plasma (SBP or SHBG) and testis (ABP)

Characterization of the recombinant protein

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A full-length 1,209 bp cDNA encoding the human sex steroid-binding protein of plasma (SBP or SHBG) and testis (ABP) was constructed and expressed in BHK-21 cells. The sequence agrees with the published gene and protein sequences. The cells were found to secrete SBP following transfection and G418^r selection. The recombinant protein binds 5α-dihydrotestosterone with a K_d of 0.28 nM. It also binds testosterone and 17β-estradiol but not progesterone, estrone or cortisol revealing a steroid-binding specificity identical to that of human SBP. SDS-PAGE patterns are less complex than human SBP and show a monomeric molecular weight of about 43 kDa.

Sex steroid-binding protein; SBP; SHBG; Mammalian expression

1. INTRODUCTION

The sex steroid-binding protein, SBP (or SHBG, sex hormone binding globulin), of human plasma specifically binds testosterone (T) and 17β-estradiol (E_2) with high affinity (see [1] for most recent comprehensive review). Human SBP was extensively characterized and sequenced by protein sequencing methods [2]. The human SBP gene was cloned [3,4] and the coding sequence is identical to that of a cDNA clone coding for the human testis androgen-binding protein (ABP). A number of partial human SBP cDNAs have been cloned [5-7] and their deduced amino acid sequences agree with the sequence obtained by direct protein sequencing methods, except for Leu^{259} which is reported to be Phe^{259} by Hammond et al. [4,6]. This laboratory is interested in describing the molecular structure of SBP/ABP for understanding both the specific and general features of steroid–protein interaction and the role of the protein in the transport of sex steroid hormones. In order to apply site-directed mutagenesis to the understanding of SBP structure and function, we have constructed a full-length SBP cDNA and expressed it in mammalian cells, and have begun to characterize the recombinant protein.

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2. MATERIALS AND METHODS

DH10B *E. coli* was from Gibco-BRL, pBluescript II and SURE *E. coli* cells were from Stratagene, and pcDNA/Neo and MC1061/p3 *E. coli* cells were from Invitrogen. Golden hamster kidney cells (BHK-21, #F-7697) and Hep G2 cells (#8065) were from ATCC. Hep G2 cells were grown in MEM and 5% FBS (Gibco-BRL), 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. BHK-21 cells were grown in 50 ml flasks containing Dulbecco's MEM with 10% FBS. poly(A)$^+$ RNA was prepared from Hep G2 cells [8] and first-strand cDNA was synthesized with random and oligo dT primers. SBP cDNA was amplified using the GeneAmp kit (Perkin Elmer Cetus) with 1 ng each of random-primed and oligo dT primed cDNA template. Amplification was done in the Perkin Elmer Cetus Thermocycler for 25 cycles at 94°C for 2 min and 72°C for 2 min. PCR products were cloned into pDVEG' or pVEGT' and sequenced [9] using ^{35}S-dATP from DuPont/NEN [10] with the protocol of Chen and Seeburg [11] using T7 DNA polymerase (U.S.B.) [12] and were primed with a universal primer or with oligonucleotides complementary to PCR-generated sequences. Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer. Probes for Southern blots were fragments 1, 2 and 3 labeled with biotin-14-dATP using random oligonucleotide-primed synthesis [13] according to published protocols [14]. Agarose gels were blotted onto nylon membranes (S & S Nytran, Schleicher & Schuell) according to Southern [15]. The blots were probed with a mixture of the three biotinylated fragments and developed with streptavidin and biotin-conjugated alkaline phosphatase. Partial digestion with *Eco*RI was with 0.5 U, 37°C, 10 min; all other digestions and DNA manipulations were carried out as described [14, 16]. DNA fragments were isolated from gels using NA-45 DEAE-filter (Schleicher & Schuell). Transformations were done using 2 μl of ligation mix and 40-60 μl of competent cells in ice-chilled cuvettes (2 mm electrode gap) and electroporated at 2.0 kV, 25 μF, and 400 Ω with the Gene pulser (Bio-Rad). Transfection of BHK-21 cells was done with 10 μg pcDNAI/Neo/SBP using the calcium phosphate procedure [17] as described [16].

Control transfections were carried out with 10 μg pcDNAI/Neo and 10 μg salmon sperm DNA. Cells were incubated for 16 h and shocked with medium containing 15% glycerol. After 48 h, cells were split and grown for 24 h before starting G418 selection at 500 μg/ml. G418^r clones appeared after 10 days. Clones synthesizing recombinant SBP were detected with an immunofilter lift assay using polyclonal SBP antibodies. Human SBP was purified as previously described [18]. Recombinant SBP was partially-purified on Con A agarose pre-equilibrated at 4°C with 300 ml of 50 mM TRIS–Cl, 100 mM NaCl, 1 mM $CaCl_2$, $MnCl_2$ and $MgCl_2$, pH 8.0. After elution of the breakthrough peak, which did not contain steroid-binding activity, the column was washed, and recombinant SBP was eluted with the same buffer containing 200 mM methyl α-D-mannopyranoside. Recombinant SBP, detected with ELISA [19] and filter [20] assays, was concentrated and dialysed in the ProDiCon (Biomolecular Dynamics). Samples (1–5 μg SBP) were taken for SDS-PAGE [21] and transferred to nitrocellulose membranes (cat# HAHY 304FO, Millipore) in the Transblot apparatus (Bio-Rad). The Western blots were probed with goat anti-SBP and rabbit anti-goat IgG coupled to horseradish peroxidase. Scatchard analyses were carried out with the filter assay [20]. Competition studies with other steroids were carried out by incubating 150 μl of 20 nM recombinant SBP with 20 nM [^{3}H]5α-DHT (5α-dihydrotestosterone) and 10-fold molar excess of radio-inert DHT, T, E_2, E_1 (estrone), P (progesterone), and F (cortisol). The extent of competition was expressed in percent relative to DHT.

3. RESULTS

3.1. *Preparation and cloning of fragments 1 (5' end fragment) and 2 (middle fragment) into pBluescript II SK +/–*

Four PCR primers were made to amplify and clone these two fragments: sense primer 1, 5'AGGGAGACCGGAATTGGATCCACCATGGAGAGCAGAGGCCCACTGGCT3'; antisense primer 2, 5'GACAGAGCACAGAATTGCCGCGGTCCAGCACCCACCGTAAGCTG3'; sense primer 3, 5'AGGGAGACCGGAATTGCCGCGGCTGGATGATGGGAGATGGCAC3'; and antisense primer 4, 5'GACAGAGCACAGAATTGATGAGGCTGGGGAATGTCTCGGAG 3'. Primers 1 and 2 were used to amplify fragment 1 encompassing adenine^{+1} to cytosine^{370} (Fig. 1). The underlined sequence contains an *Nco*I site adjacent to the translation start codon, a *Bam*HI site, and a 'Prime' element for efficient cloning of PCR-amplified DNA into 'Prime' vectors [22, 23]. The underlined sequence of primer 2 contains the complementary prime cloning sequence for pDVEG' and a cytosine (underlined) for site-directed mutagenesis at position 366. This silent mutation produces a new and unique *Sst*II site in the cDNA for facilitating the cloning of fragments in later steps. Primers 3 and 4 were used to amplify fragment 2 from cytosine^{364} to thymine^{732}, including the mutagenic nucleotide at position 366 for introducing the *Sst*II site, and the natural *Eco*RI site at position 701. The underlined region contains 'Prime' sequences for cloning in pVEGT' [23]. The relative position of all four PCR primers is shown in Fig. 1. Following amplification, fragments 1 and 2 were each cut back with T4 DNA polymerase, gel purified, and cloned into pDVEG' and pVEGT', respectively. Restriction analyses of purified plasmids confirmed the presence of both fragments (data not shown). The DNA sequences of the fragments agreed with the corresponding exons of the published gene sequence [3,4]. Recombinant pDVEG' and pVEGT' were each digested with *Bam*HI and *Sst*II, and *Sst*II and *Eco*RI, respectively. A three-part ligation consisting of fragments 1, 2, and *Bam*HI and *Eco*RI-digested pBluescript II SK +/– was carried out followed by electroporation into DH10B cels. The predicted 709 bp *Bam*HI–*Eco*RI, 376 bp *Bam*HI–*Sst*II, and 333 bp *Sst*II–*Eco*RI fragments were all obtained (data not shown).

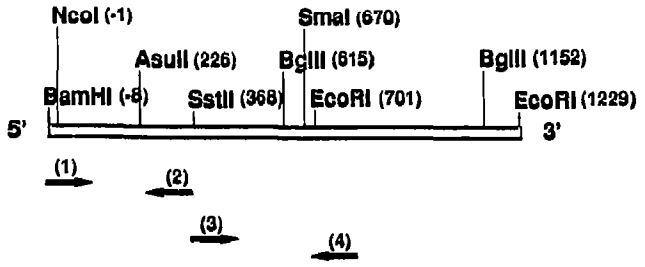

Fig. 1. Partial restriction map of SBP cDNA showing the newly created *Sst*II, the two *Bgl*II, and *Eco*RI sites. Arrows indicate positions for the four PCR primers used in amplifying the 5' end of the cDNA. Nucleotide numbering, shown in parentheses, starts at adenine^{-24}.

3.2. *Subcloning of fragment 3 (the 3' end fragment)*

Fragment 3 was obtained from a λgt11 clone previously isolated form a Hep G2 cDNA library by an immuno-screen (B.G. Que, F.S. Hagen and P.H. Petra, unpublished result). The *Eco*RI fragment of this clone was subcloned into lambda HG3 which contains pGEMT, pGEM-1 with a T7 RNA polymerase terminator [23]. A clone containing the SBP sequence was identified with a SBP-specific oligonucleotide probe. DNA was purified [24], and pGEMT contained in lambda HG3 was removed by the lambda-pop procedure [22,23]. This procedure liberated pGEMT having *Not*I termini and containing the SBP insert. After electroporation, the plasmid was amplified in DH10B and fragment 3 was excized with *Eco*RI digestion. The DNA sequence revealed that fragment 3 contained the expected sequence from the internal *Eco*RI site (adenine^{701}) to the terminal 3'end *Eco*RI site (Fig. 1).

3.3. *Cloning of full-length SBP cDNA into pBluescript II SK +/–*

pBluescript II SK +/– containing fragments 1 and 2 was linearized with *Eco*RI, dephosphorylated, and fragment 3 was cloned into the site. Fig. 2A and B show the presence of the predicted full-length cDNA of 1,267 bp released by *Bam*HI and *Xho*I (lane 11) which includes 34 bp from the cloning site of pBluescript, and 1,233 bp by partial *Eco*RI digestion of the *Bam*HI linearized vector (lane 12). The predicted 709 bp and 532 bp

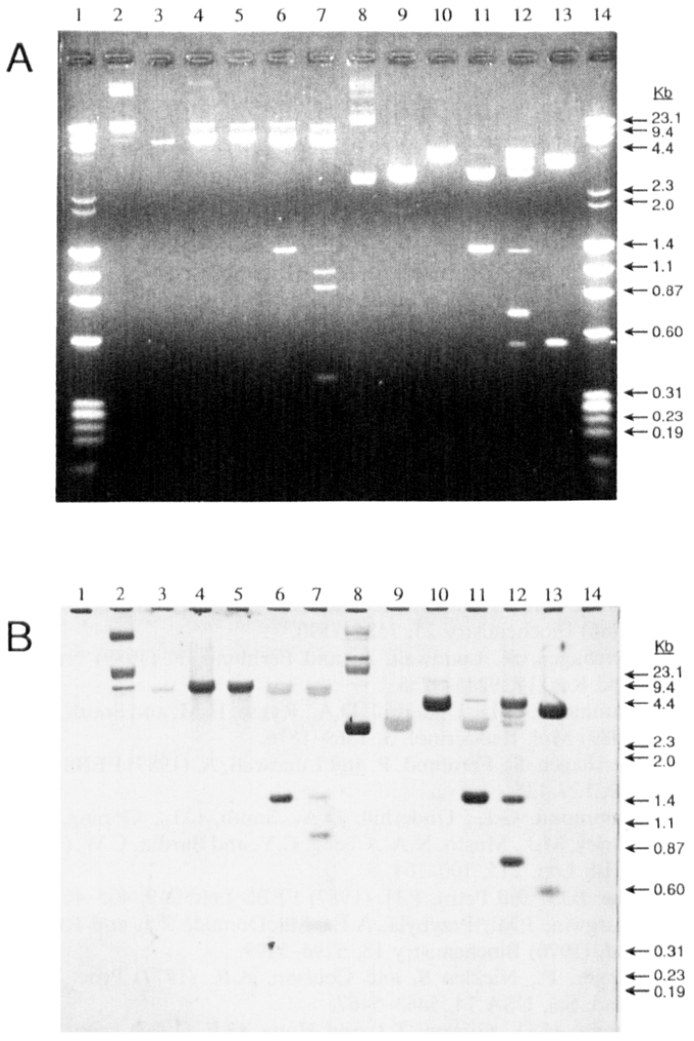

Fig. 2. Restriction analysis of clones containing full-length SBP cDNA. (A) 1.3% agarose gel: (lanes 1 and 14) λHindIII/φX174HaeIII ladder; (lane 2) pcDNAI/Neo/SBP, uncut; (lane 3) pcDNAI/Neo, uncut; (lane 4) *Bam*HI-linearized pcDNAI/Neo/SBP; (lane 5) *Xho*I-linearized pcDNAI/Neo/SBP; (lane 6) *Bam*HI- and *Xho*I-cut pcDNAI/Neo-SBP; (lane 7) *Bam*HI-, *Xho*I- and *Sst*II- cut pcDNAI/Neo/SBP; (lane 8) pBluescript/SBP, uncut; (lane 9) *Bam*HI-linearized pBluescript; (lane 10) *Bam*HI-linearized pBluescript/SBP; (lane 11) *Bam*HI- and *Xho*I-cut pBluescript/SBP; (lane 12) partial *Eco*RI digest of *Bam*HI-linearized pBluescript/SBP; (lane 13) *Bgl*II-cut pBluescript-SBP. (B) Southern blot of agarose gel shown in Fig. 2A. with the same lane assignments. The low background in vector DNA shown in lanes 3 and 9 is due to contamination of the gel-purified SBP fragments with vector DNA during biotinylation (F.S. Hagen, unpublished results). The wells in both figures were marked with black ink.

*Eco*RI fragments are also seen in lane 12, although the latter is barely noticeable in the Southern blot. The presence of a 537 bp *Bgl*II restriction fragment in lane 13 confirms the correct orientation of fragment 3 (see Fig. 1).

3.4. *Cloning of full-length SBP-cDNA into pcDNAI/Neo*

The full-length SBP cDNA was moved into pcDNAI/Neo for mammalian expression. Restriction analyses of the recombinant pcDNAI/Neo/SBP show the presence of the predicted 1,267 bp full-length cDNA released with *Bam*HI and *Xho*I (lanes 6 and 7, Fig. 2A and B). Lane 7 of Fig. 2A and B also shows the predicted 376 bp *Bam*HI/*Sst*II and 895 bp *Sst*II/*Xho*I fragments. There are two vector bands in lane 7 of Fig. 2A that do not hybridize in the Southern blot of Fig. 2B, one located below the linearized vector containing SBP and the other at about 1.1 kb. These arise from digestion at a second *Sst*II site in the vector.

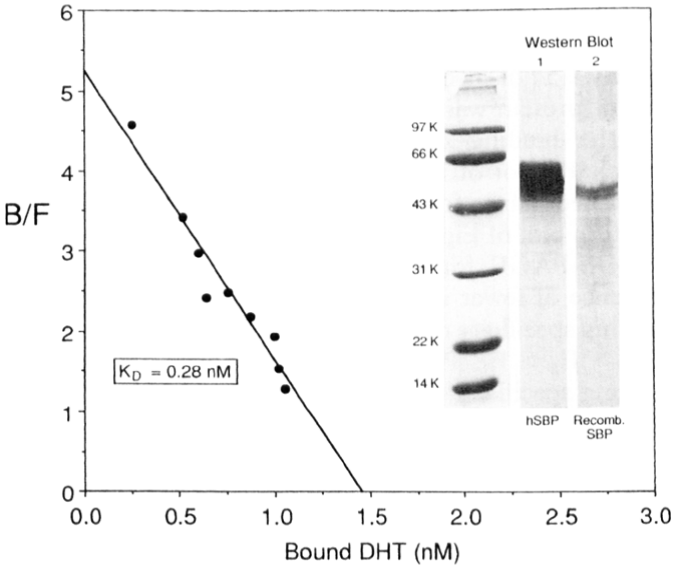

Fig. 3. Scatchard analysis recombinant SBP. The coefficient of determination, $r^2 = 0.95$ for the linear regression, $y = -(3.60\ nM^{-1})x + 1.46$. The inset shows a Western blot of human and recombinant SBP.

3.5. *Expression of SBP/ABP in BHK-21 cells: preliminary characterization of the recombinant protein*

Stable G418^r BHK-21 clones were grown to estimate the extent of SBP expression. A total of 57 clones were identified by the immunofilter lift assay but only 5 produced detectable steroid-binding activity. The average recombinant SBP levels secreted by these clones

Table I

Relative steroid-binding affinity of recombinant SBP

Steroids	Relative binding (%)	
	Recombinant	Human*
5α-Dihydrotestosterone	100	100
Testosterone	50	33
17β-Estradiol	41	21
Estrone	0	1.3
Progesterone	0	0.8
Cortisol	0	0.7

*Vermeulen and Verdonck [31].

at confluency were in the range of 0.2 μg SBP/ml medium. One such clone was grown to yield approximately 1.5 l of medium from which the secreted recombinant protein was partially-purified. A K_d of 0.28 nM for the binding of DHT was calculated from the Scatchard plot of Fig. 3 which agrees completely with published values for the human protein [20,25,26]. The Western blot of Fig. 3 indicates a slightly faster mobility in SDS-PAGE for the recombinant protein and the presence of fewer isoforms. Table I shows the steroid-binding specificity of recombinant SBP as follows: DHT > T > E_2 > E_1, P, F, which agrees with the steroid-binding specificity of human SBP.

4. DISCUSSION

After several unsuccessful attempts at isolating a full-length SBP cDNA by screening libraries or amplifying the entire sequence by PCR, we decided to construct it from three smaller fragments, two amplified by PCR using Hep G2 poly(A)$^+$ as template, and the other a Hep G2 λgt11 cDNA clone containing the 3′ end sequence. This approach led to the isolation of a full-length cDNA which agrees completely with the protein [2] and gene sequence of Gershagen et al. [3]. It is also consistent with that of Hammond et al. [4] except for guanine^{864} which they report as a cytosine. The presence of guanine^{864} encodes Leu^{259} instead of Phe^{259} as reported by Hammond et al. [4,6]. The Leu^{259} assignment is correct on the basis of the protein sequence [2] and the finding of guanine^{864} by two independent laboratories([3] and this work). Expression of the SBP cDNA in BHK-21 cells, a mammalian cell line that expresses eukaryotic proteins [27,28], yields recombinant SBP with a subunit molecular weight and steroid-binding properties similar to human SBP. The Western blot of Fig. 3 suggests that the extent of glycosylation is less pronounced than human SBP. Our objective was to test whether or not the full-length cDNA could yield active SBP in a mammalian cell line. Although this was achieved, the level of SBP expression was low. We have now expressed SBP cDNA in COS-7 cells and find a level of expression at least 10-times higher which will permit site-directed mutagenesis experiments (L. Sui, W. Zhang, and P.H. Petra, unpublished results).

The findings presented here open the way for studying SBP/ABP with a molecular biology approach. A number of outstanding questions remain with regards to the structure and function of SBP. Which structural parameters are responsible for dimer formation? Is the steroid-binding site located at the interface between the subunits? How many and which kind of amino acid side-chains are involved in steroid binding? Does the monomer bind steroid? Lastly, the recent proposal for the existence of an SBP membrane receptor [29, 30] raises the question as to where on the surface of the SBP molecule does receptor interaction take place? Although recent findings indicate that fully deglycosylated human and rabbit SBPs have the same binding affinity for DHT as native SBP (P.H. Petra, P.R. Griffin, L. Hood. K. Moore and W. Zhang, submitted for publication), the oligosaccharide side-chains could play a role in SBP receptor recognition. Deglycosylated SBP or recombinant SBP having mutated carbohydrate attachment sites may be used to test this proposal.

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REFERENCES

[1] Petra, P.H. (1991) J. Steroid Biochem. Mol. Biol. 40, 735–753.
[2] Walsh, K.A., Titani, K., Kumar, S., Hayes, R. and Petra, P.H. (1986) Biochemistry 25, 7584–7590.
[3] Gershagen, S., Lundwall, A. and Fernlund, P. (1989) Nucleic Acid Res. 17, 9245–9258.
[4] Hammond, G.L., Underhill, D.A., Rykse, H.M. and Smith, C.L. (1989) Mol. Endocrinol. 3, 1869–1876.
[5] Gershagen, S., Fernlund, P. and Lundwall, A. (1987) FEBS Lett. 220, 129–135.
[6] Hammond, G.L., Underhill, D.A., Smith, C.L., Goping, I.S., Harley, M.J., Musto, N.A., Cheng, C.Y. and Bardin, C.W. (1987) FEBS Lett. 215, 100–104.
[7] Que, B.G. and Petra, P.H. (1987) FEBS Lett. 219, 405–409.
[8] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1970) Biochemistry 18, 5294–5299.
[9] Sanger, F., Nicklen S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[10] Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983) Proc. Natl. Acad. Sci. USA 80, 3963–3965.
[11] Chen, E.Y. and Seeburg, P.H. (1985) DNA 4, 165–170.
[12] Tabor, S. and Richardson, C.C. (1987) Proc. Natl. Acad. Sci. USA 84, 4767–4771.
[13] Feinberg, P. and Vogelstein, S. (1983) Anal. Biochem. 132, 6–13.
[14] Ausubel et al. (eds.) Current Protocols in Molecular Biology, vols. 1 and 2, Wiley, New York.
[15] Southern, E. (1975) J. Mol. Biol. 98, 503–517.
[16] Maniatis, T., Fritsch, E.F. and Stambrook, J. (1982) Molecular Cloning: a laboratory manual, Cold Spring Harbor, New York.
[17] Graham, F.L. and van der Eb, A.J. (1973) Virology 52, 456–460.
[18] Namkung, P.C., Kumar, S., Walsh, K.A. and Petra, P.H. (1990) J. Biol. Chem. 265, 18345–18350.
[19] Bordin, S., Torres, R. and Petra, P.H. (1982) J. Steroid Biochem. 17, 453–457.
[20] Mickelson, K.E. and Petra, P.H. (1974) FEBS Lett. 44, 34–38.
[21] Petra, P.H., Namkung, P.C., Senear, D.F., McCrae, D.A., Rousslang, K.W., Teller, D.C. and Ross, J.B.A. (1986) J. Steroid Biochem. 25, 191–200.
[22] Kuijper, J.L., Wiren, K.M., Mathies, L.D., Gray, C.L. and Hagen, F.S. (1991) Gene (in press).
[23] Hagen, F.S., Grant, F.J., Kuijper, J.L., Slaugher, O.A., Moomaw, C.R., Orth, K., O'Hara, P.J. and Munford, R.S. (1991) Biochemistry 30, 8415–8423.
[24] Helms, C., Graham, M.Y., Dutchik, J.E. and Olson, M.V. (1985) DNA 4, 39–49.
[25] Shanbhag, V.P. and Sodergard, R. (1986) J. Steroid Biochem. 24, 549–555.
[26] Renoir, J.-M., Mercier-Bodard, C. and Baulieu, E.-E. (1980) Proc. Natl. Acad. Sci. USA 77, 4578–4582.

[27] Gronwald, R.G.K., Grant, F.J., Haldeman, B., Hart, C.E., O'Hara, P., Hagen F.S., Ross, R., Bowen-Pope, D.F. and Murray, M.J. (1988) Proc. Natl. Acad. Sci. USA 85, 7307–7311.
[28] Foster, D.C., Rudinski, M.S., Schach, B.G., Berkner, K.L., Kumar, A.A., Hagen, F.S., Sprecher, C.A., Insley, M.Y. and Davie, E.W. (1987) Biochemistry 26, 7003–7011.
[29] Strel'chyonok, O.A. and Avvakumov, G.V. (1990) J. Steroid Biochem. Mol. Biol. 35, 519–534.
[30] Rosner, W. (1990) Endocrinol. Rev. 11, 80–91.
[31] Vermeulen, A.L. and Verdonck, L. (1968) Steroids 11, 609–635.