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Genetic Variations in Human Testosterone–Estradiol Binding Globulin

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The human testosterone–estradiol-binding globulin (hTeBG) is a plasma heterogeneous glycoprotein with high affinity for a number of circulating steroid hormones. The heterogeneity originates from differential glycosylation of a common protein precursor. Analysis of desialylated hTeBG by isoelectric focusing (IEF) has revealed that microheterogeneity could be partly attributed to variability in sialic acid content or rearrangement of amino acid composition. We have studied this possibility by the analysis of desialylated serum hTeBG by Western blotting of proteins previously separated on IEF-gels. Two distinct well-defined IEF patterns were identified. The most frequent consisted of two major IEF-bands of equal color intensity. The other pattern consisting of four IEF-bands was present in only 5.55% of the total serum samples analyzed. Family studies showed that these phenotypes were autosomally inherited with a simple Mendelian transmission and allele frequencies had an excellent agreement between the observed and expected phenotypes. Androgen affinity constants and serum concentrations of hTeBG variant were similar to those of normal hTeBG. Molecular analyses of each of the exons of hTeBG gene by denaturing gradient gel electrophoresis revealed the presence of a point mutation in exon 8. The studies presented herein confirm and extend previous reports on the existence of structural variants of hTeBG. In addition, the mutation reported in this study is probably the same as that recently identified within numerous ethnic groups throughout the world, thus further supporting the concept of a two allele gene worldwide concoding hTeBG.

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INTRODUCTION

Human testosterone–estradiol-binding globulin (hTeBG) is a homodimeric glycoprotein [1, 2] with a single binding site for testosterone and estradiol [3, 4]. The human and rabbit protein has been extensively characterized [5–7] and their amino acid sequences determined [8]. Human TeBG is synthesized in the liver [9] and recent physicochemical and molecular studies indicate that this protein functions as a homodimer of a 373 amino acid polypeptide. As in the case of many other glycoproteins, glycosylation of hTeBG may play a crucial role in determining its biological half-life and survival in the circulation. It is also important for the presence of microheterogeneity which in part is attributed to variability in sialic acid content or rearrangement in amino acid composition [10–13]. Although neuraminidase treatment of sera or purified TeBG reduced the number of bands detectable by isoelectric focusing [6, 10, 13], residual microheterogeneity after desialylation has been previously reported [11–14]. Genetic polymorphism of hTeBG has been previously detected in individual serum samples [15–19]. This trait seems to be inherited in an autosomal manner which is consistent with recent evidence that TeBG is encoded by a single gene on the short arm of chromosome 17 [20].

The present report summarizes the results of a variety of studies from our and other laboratories aimed at investigating the presence and nature of genetically determined polymorphism of hTeBG.

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EXPERIMENTAL

Materials

Nonradioactive steroids were purchased from Steraloids Inc. (Wilton, NH) and their chemical purity established by paper chromatography and recrystallization. [1,2-^{3}H]dihydrotestosterone ([^{3}H]DHT; 40–60 Ci/mmol) and [1,2-^{3}H]testosterone ([^{3}H]T; 40–40 Ci/mmol) were obtained from Du Pont NEN Research Products (Boston, MA). Acrylamide, *N,N'*-dallyltartardiamide (DADT), *N,N'*-methylenebisacrylamide (bis), *N,N,N',N'*-tetramethylethylenediamine (TEMED), nitrocellulose paper, isoelectric focusing standards, empholytes and other electrophoretic reagents were obtained from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin, Tris, glycine, glutamic acid, Tween-20, glycerol, neuroaminidase from clostridium perfringes Type X and trypsin inhibitor Type I-S were obtained from Sigma Chemical Co. (St Louis, MO). Rabbit anti-human TeBG antiserum was a gift from Dr C. W. Bardin (The Population Council Center for Biomedical Research, New York, NY). The radiolabeled [^{32}P]dCTP was purchased from Amersham International Plc. (Bucks, England). The human TeBG cDNA was kindly provided by Dr G. L. Hammond (University of Western Ontario, London, Ontario, Canada). The cDNA was amplified as 5′ (0.64 kb) and 3′ (0.55 kb) EcoRI fragments in pBR322, and labeled with [^{32}P]dCTP using an oligolabeling kit from Pharmacia LKB (Uppsala, Sweden). All other chemical reagents and solvents were of analytical grade.

Serum samples and treatment

Blood samples were taken from adult individuals and sera kept frozen at −80°C until assayed. Samples were treated with Affi-gel Blue and neuraminidase as previously described [21]. Desialylation of hTeBG was evaluated by isoelectric focusing before and after treatment with neuraminidase as detailed below.

Isoelectric focusing

Isoelectric focusing was performed in polyacrylamide gels (IEF–PAG) in Gelamide-coated 5 mm tubes with a final acrylamide concentration of 5% T, 15% DATD and 2% Ampholine in a pI range of 3–10 and 5–8. The anolyte and catholyte electrode solutions consisted of 0.05 M Tris-base and 0.05 M glutamic acid, respectively [22]. Electrofocusing was also performed on plastic-backed IEF–PAG (11 × 12 × 0.9 cm) using a LKB Multiphor II Electrophoresis unit. The desialylated serum samples were diluted with distilled water and focused at 4°C at a constant current. The pI of the IEF–PAG standards and serum proteins was calculated by interpolation.

Protein blotting and immunostaining

Transfer of proteins from the polyacrylamide gels to nitrocellulose sheets (NTC) was performed as follows. After IEF, plastic backed IEF–PAG were incubated in transfer solution (0.7% acetic acid) for 30 min at room temperature. The blot consisted of three sheets of filter paper, two sheets of NTC, all of them pre-wetted in transfer solution, the plastic backed IEF–PAG (gel side down) and a piece of glass with 5 kg weight on the top. After blotting, the NTC membrane was peeled away from the gel and washed in TSB (0.5 M Tris, pH 7.4, 0.15 M NaCl) twice at room temperature. After blocking the unreacted NTC binding sites for 8 h at 4°C with 5% BSA in TSB, the NTC was incubated overnight with a 1/100 dilution of anti-hTeBG. Unbound antibodies were removed by several TBS washes and NTC membranes incubated with peroxidase-labeled antiserum and stained for peroxidase in TBS containing 0.3 mg/ml 3,3′-diaminobenzidine and 0.0005% hydrogen peroxide. In the case of gel tubes, IEF–PAG tubes were frozen and sliced into 2 mm segments. PAG slices were eluted with continuous shaking in TG buffer (0.02 M Tris, pH 7.4, 10% glycerol) for 18 h at 4°C. After centrifugation at 3000 ***g*** supernatants were saved at −80°C until assayed.

Analytical steady-state polyacrylamide gel electrophoresis

Desialylated serum samples were analyzed by steady-state polyacrylamide gel electrophoresis (SS–PAGE) as described by Ritzen *et al.* [23] and modified by Musto *et al.* [24]. SS–PAGE was performed at 0°C in gels of 5, 6, 7.5, 8.5 and 10% acrylamide concentration with multiphasic buffer system 2860.0.X (pH 9.63) as described previously [25] Stacking gels of 3.125% T and 15% C_{bis} were used. Tritium labeled DHT ([^{3}H]DHT) and nonlabeled steroids were evaporated and dissolved in the gel solutions before polymerization. Stacking and resolving gels contained 5×10^{-9} and 1.5×10^{-9} M [^{3}H]DHT, respectively. After electrophoresis gels were frozen and sliced into 2 mm segments and placed into counting vials containing scintillation fluid.

Binding parameters of hTeBG

The equilibrium binding characteristics of hTeBG were determined by saturation analysis using the dextran-coated charcoal (DCC) assay as described previously [26]. The affinity constants and the number of binding sites were calculated according to the method of Scatchard [27]. The dissociation rate and half-time of dissociation of the hTeBG–testosterone complex were studied either at 4 or 28°C on 10-fold diluted charcoal-stripped serum samples containing saturation amounts of [^{3}H]testosterone (1×10^{-9} M) as previously described [26]. After 1 h incubation, an excess of unlabeled testosterone (100 ×) was added and aliquots were withdrawn at various periods of time. The dissociation half-time and dissociation rate constants were calculated from the linear regression line. Radioactivity

was measured in a Packard Tri-Carb spectrometer with a counting efficiency of 52%.

DNA amplification

Genomic DNA was isolated from peripheral blood lymphocytes by the method of Singer *et al.* [28]. This DNA served as a template to amplify the coding regions (exons 1–8) of the hTeBG gene by polymerase chain reaction using specific oligonucleotide primers for each exon. At the 5′ end, these primers contained an attached GC clamp of 40 bp in order to increase detectability of possible mutations [29]. PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) [30].

RESULTS AND DISCUSSION

Screening test and population survey

Isoelectric focusing analysis of serum samples incubated in the presence or absence of neurominidase revealed the presence of two well-defined IEF-binding peaks. The first peak focused in a broad pH area between 5.4 and 5.85, and the second peak of binding activity focused in a more well-defined pH area of 6.9. These areas of binding activity corresponded to native and desialylated serum samples, respectively, and were specific to hTeBG since incubations containing an excess of unlabeled DHT displaced the binding of radioactive DHT. IEF-patterns were indistinguishable when desialylation was performed in the absence or presence of protease inhibitors (Fig. 1). These studies were important since they demonstrated the effectiveness of neuroaminidase treatment of serum samples to change the isoelectric point of native hTeBG and suggested that neither desialylation nor IEF conditions altered the DHT-binding properties of hTeBG. Furthermore it demonstrated that pre-treatment of serum samples with neuraminidase greatly simplifies the isoelectric focusing data making feasible the comparison of patterns among different samples. We have performed desialylation of serum samples under a variety of experimental conditions, including long incubation times in the presence of different neuraminidase concentrations. We have also carried out repeated blood sampling of the same individual, conditions which always resulted in identical focusing patterns of desialylated hTeBG. With this methodological approach, we have investigated more than 500 serum samples and so far we have failed to demonstrate a single case of genetic variation of the pI of hTeBG. For this reason, we decided to submit desialylated serum samples to isoelectric focusing in polyacrylamide slab gels followed by immunoblotting with specific hTeBG antiserum. With the use of this methodology, 110 serum samples of unrelated individuals, corresponding to 55 married couples, were investigated. Two main patterns were observed that will be further referred to as the 1–1 and the 1–2 pattern. The 1–1 pattern with a frequency of 0.9773 consisted of two well-defined bands with equal color intensity focusing at pHs of 6.5 and 6.63, respectively. The 1–2 pattern with a frequency of 0.0227 consisted of the bands seen in the 1–1 pattern plus two additional cathodic bands with a pI of 6.70 and 6.76, respectively (Fig. 2). In all cases the color intensity of desialylated hTeBG was

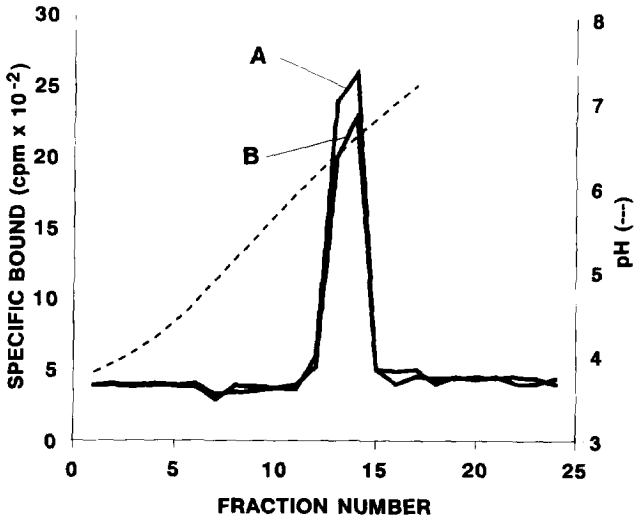

Fig. 1. Isoelectric focusing profiles of serum desialylated hTeBG. Serum samples were incubated with neuraminidase in the absence (A) or presence (B) of a protease inhibitor and submitted to IEF in polyacrylamide gel tubes. After focusing gels were frozen, sliced and eluted. Human TeBG was identified in the eluates by [^{3}H]DHT binding as described in experimental.

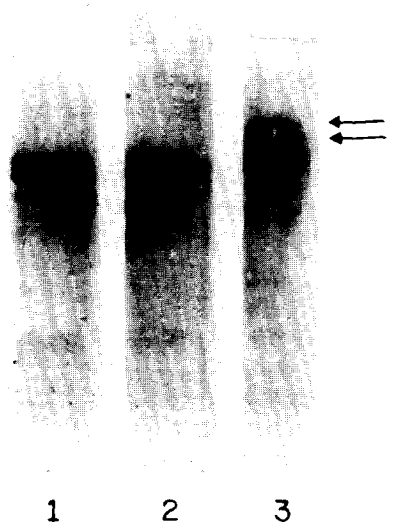

Fig. 2. Isoelectric focusing patterns of desialylated hTeBG. After focusing, proteins were transferred to nitrocellulose and immunostained with polyclonal anti-hTeBG antibodies. Lanes 1 and 3 show the 1–1 and 1–2 patterns, respectively. Lanes 2 and 3 represent the results of serum samples incubated in the presence of 10 times more neuraminidase than the one used in Lane 1. Cathode is at the top of the figure. A minor non-specific band was always observed at the anode.

stronger in individuals having the 1–1 pattern than those showing the 1–2 pattern. In the population studied, it was not possible to detect a single-band pattern nor a sex predilection for the presence of a given isoelectric focusing pattern of desialylated hTeBG. Unfortunately, in the course of this study we have not found a single subject showing the 2–2 pattern characterized only by the presence of the two extra cathodic bands seen in subjects with the 1–2 pattern.

Although initial evidence that the 1–2 pattern was not the result of protein denaturation was derived from the observation that the antiserum maintained its specificity for both IEF-phenotypes, it could be argued that residual microheterogeneity might have been due to incomplete desialylation. However, these two IEF-patterns remained the same independently of the focusing time conditions and the amount of neuraminidase present during desialylation of serum samples.

Furthermore, the heterogeneity of these IEF-patterns in the original sample was the same in repeated analyses of serum samples drawn at different time intervals from the same individual. In addition to these observations, the two extra bands in the 1–2 pattern always focused at the cathodic side of the 1–1 pattern, thus suggesting that these bands did not correspond to incomplete removal of sialic acid.

Further support for the presence of charge differences between both hTeBGs was derived from Ferguson's plots on native polyacrylamide gel electrophoresis (log RF vs acrylamide concentration) where similar slopes but different Y_0 intercepts of 1–1 and 1–2 hTeBGs could be demonstrated.

Family studies

In order to obtain further evidence for genetic inheritance of 1–1 and 1–2 IEF-patterns, we studied family members of individuals with these two phenotypes. The results of segregation analyses in two families demonstrated that the 1–2 pattern was genetically transmitted (Fig. 3). The study of three families with parents showing a 1–1 pattern resulted, in all cases, in offspring ($n = 12$) with this phenotype. According to these observations, it was clear that segregation of hTeBG phenotypes was consistent with a simple Mendelian inheritance of an autosomal gene and appeared to fit in a polymorphic bi-allelic (hTeBG-1 and hTeBG-2) codominant autosomal system. On these bases the results were mathematically analyzed using the Hardy–Weinberg law; $p^2 + 2pq + q^2 = 1$, where p and q are the allele frequencies of the hTeBG-1 and the hTeBG-2 allele, respectively. The results, as judged by the χ^2 value, demonstrated an excellent agreement between the observed and expected phenotypes (Table 1).

Although in our study population the expected 2–2 IEF-pattern was not observed, the estimated gene frequency for hTeBG-2 allele ($q = 0.0227$) gives the probability to find homozygous for hTeBG 2–2

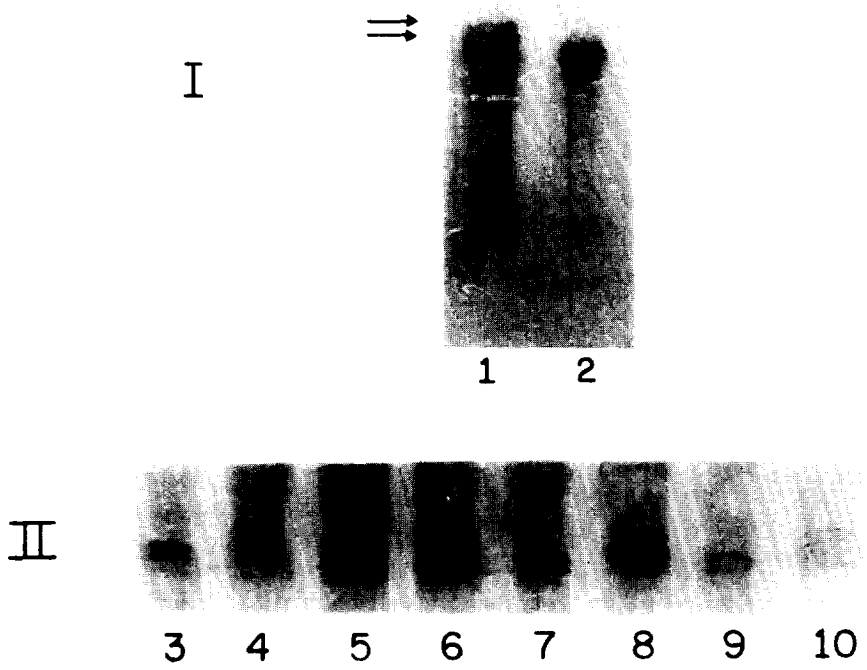

Fig. 3. Isoelectric focusing of serum hTeBG from family members of one representative individual with the 1–2 pattern. The arrows indicate the two additional cathodic bands present in one parent that were transmitted to the offspring.

Table 1. Distribution of phenotypes and gene frequencies among the population studied

System	Phenotypes	No.	Gene	Frequency	χ^{2}*	P
hTeBG	hTeBG 1–1	105	hTeBG 1	0.9773		
	hTeBG 2–1	5	hTeBG 2	0.0227		
	hTeBG 2–2	0				
					0.053	
						<0.95

*χ^{2}-value for Hardy–Weinberg equilibrium test.

Table 2. Association constants and serum concentrations of normal and variant hTeBG

hTeBG pattern		4°C		28°C	
		10^{9} M^{-1}	pmol/ml	10^{9} M^{-1}	pmol/ml
1–1					
1	M*	2.4	22	1.2	15
2	F	1.0	43	1.5	48
3	F	2.0	40	2.0	38
4	M	1.0	16	1.0	26
1–2					
1	F	1.1	50	1.5	54
2	F	2.0	38	1.7	42
3	M	2.0	14	1.4	16
4	M	1.0	9	1.3	10
5	M	1.7	17	1.5	15

M, male; F, female.

phenotype ($q^{2} = 0.0005$) of 1/2000 individuals. These observations are in line with those reported recently by Van Baelen *et al.* [19] where several racially different population samples were studied. In this survey, the phenotype distribution and allele frequencies of hTeBG corresponded in part to those reported by our laboratory [18], particularly in those cases when a similar number of screened individuals are compared.

Binding studies

In order to estimate the binding properties and dissociation rates of hTeBG phenotypes, representative serum samples from individuals with the 1–1 and 1–2 IEF-patterns were studied by saturation analyses as described previously in this manuscript. Affinity constants and serum concentrations of hTeBG 1–1 and hTeBG 1–2 measured at two different temperatures were similar and did not reveal any statistically significant differences between them (Table 2). In addition, we examined the dissociation rate and the half-time of dissociation for both hTeBGs. Neither the dissociation rate constants nor the half-time of dissociation measured at 4°C and at 28°C were statistically different for these two forms (Table 3). These data suggest that the potential mutation point responsible for the generation of the 1–2 pattern did not involve the steroid binding site of the molecule. Although our study did not allow us to identify the homozygous variant (2–2 IEF-pattern) and therefore its steroid-binding affinity properties, a more recent study by Van Baelen *et al.* [19] demonstrated, for a similar variant as described herein, identical binding characteristics for [^{3}H]DHT amongst the different phenotypes encountered, including the homozygous state. Traditionally, steroid carrier proteins have been characterized by their abilities to bind sex-steroid hormones. More recently, it has been shown that hTeBG not only binds and transports hormonal steroids but also may function as a carrier of steroids to specific cells by its interactions with plasma membrane receptors [31, 32]. In this study, androgen binding affinities and the number of binding sites of hTeBG 1–2 were identical to those of hTeBG 1–1 suggesting that the mutation identified herein does not affect the binding domain. However, these data do not eliminate the possibility that other metabolic actions attributable to hTeBG could be affected.

Inasmuch as neither a specific domain for hormone-binding nor alterations of this function have been identified so far suggests that for this specific functional property of the protein a higher order molecular structure rather than a simple binding region in the polypeptide is required.

Analysis of genomic DNA-encoding hTeBG IEF variants

The molecular basis for the cathodal IEF shift of this hTeBG variant was determined by the amplification of

Table 3. Dissociation rate constants (K-1) and dissociation half-times ($t_{1/2}$) for normal and for isoelectric focusing variant of hTeBG

hTeBG pattern	4°C		28°C	
	K-1 (min^{-1})	$t_{1/2}$ (min)	K-1 (min^{-1})	$t_{1/2}$ (min)
1–1	0.034 ± 0.001	20.13 ± 1.029	0.25 ± 0.024	2.60 ± 0.26
1–2	0.032 ± 0.004*	20.58 ± 0.56*	0.25 ± 0.04*	2.90 ± 0.058*

*Nonsignificant K-1 and $t_{1/2}$ for normal vs variant at 4°C and 28°C, respectively.

each exon of the hTeBG gene by the polymerase chain reaction (PCR) using as a template genomic DNA isolated from individuals expressing the 1–1 and the 1–2 phenotypes. A portion of each PCR-amplified genomic DNA sample was analyzed by denaturing gradient gel electrophoresis [30]. The gels were stained with ethidium bromide and examined by UV-transillumination. The results showed that when amplification products were electrophoresed on non-denaturing gels, no separation of the fragments was observed eliminating gross deletion or insertion as a source of the variation. However, four bands were observed by DGGE when DNA from a heterozygous individual was amplified with intron-specific oligonucleotide primers for exon 8. These bands corresponded to the homoduplex DNA fragments of the normal and variant alleles, respectively, and to the two heteroduplex DNA fragments. These results demonstrate the presence of sequence variation in exon 8, the origin of which will be determined by DNA sequencing analysis. Power *et al.* [33] identified a single nucleotide substitution in exon 8 at residue 327 which resulted in replacement of the normal aspartic acid by asparagine in the hTeBG polypeptide in homozygous subjects. An identical mutation in the heterozygous individuals in this study would explain in part the cathodal shift on IEF induced by the loss of the negative charge provided normally by the aspartic acid residue. Further support for this concept is derived from other mutations that involve aspartic acid residues at different locations in a protein molecule result in similar mobility shifts toward the cathode when analyzed by isoelectric focusing [34]. In conclusion, this study established that hTeBG is encoded by two autosomal codominant alleles confirming, for the first time in heterozygous individuals, the prevalence of the variant allele (hTeBG-2) in the world population. Isoelectric focusing patterns were the result of combinations of two codominant alleles with gene frequencies in genetic equilibrium and family studies were consistent with a simple Mendelian inheritance of an autosomal gene. Finally, the mutation in this study is probably the same as that recently identified with numerous ethnic groups throughout the world [19], thus confirming a two allele gene worldwide encoding hTeBG.

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REFERENCES

1. Hammond G. L.: Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocrine Rev.* 11 (1990) 65–79.
2. Rosner W.: The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocrine Rev.* 11 (1990) 80–91.
3. Corvol P., Chrambach A., Rodbard D. and Bardin C. W.: Physical properties and binding capacity of testosterone–estradiol-binding globulin in human plasma, determined by polyacrylamide gel electrophoresis. *J. Biol. Chem.* 246 (1971) 3435–3443.
4. Rosenbaum W., Christy N. P. and Kelly W. G.: Electrophoretic evidence for the presence of an estrogen-binding globulin in human plasma. *J. Clin. Endocr. Metab.* 26 (1966) 1399–1403.
5. Mickelson K. E., Teller D. C. and Petra P. H.: Characterization of the sex steroid binding protein of human pregnancy serum. Improvements in the purification procedure. *Biochemistry* 17 (1978) 1409–1415.
6. Cheng C. Y., Musto N. A., Gunsalus G. L. and Bardin C. W.: Demonstration of heavy and light protomers of human testosterone-estradiol binding globulin. *J. Steroid Biochem.* 19 (1983) 1379–1389.
7. Musto N. A., Larrea F., Cheng S-L., Kotite N., Gunsalus G. and Bardin C. W.: Extracellular androgen-binding proteins: species comparison and structure–function relationships. *Ann. N.Y. Acad. Sci.* 383 (1982) 343–359.
8. Walash K. A., Titani K., Takio K., Kumar S., Hayes R. and Petra P. H.: Amino acid sequence of the sex steroid binding protein of human blood plasma. *Biochemistry* 25 (1986) 7584–7590.
9. Khan M. S., Knowles B. B., Aden D. P. and Rosner W.: Secretion of testosterone–estradiol-binding globulin by a human hepatoma-derived cell line. *J. Clin. Endocr. Metab.* 53 (1981) 448–449.
10. Van Baelen H., Heyns W. and DeMoor P.: Microheterogeneity of the testosterone-binding globulin of human pregnancy serum demonstrated by isoelectric focusing. *Ann. Endocr.* 30 (1969) 199.
11. Cheng C. Y., Musto N. A., Gunsalus G. L. and Bardin C. W.: The role of the carbohydrate moiety on the size heterogeneity and immunological determinants of human testosterone–estradiol-binding globulin. *J. Steroid. Biochem.* 22 (1985) 127–134.
12. Hammond G. L., Robinson P. A., Sugino H., Ward D. N. and Finne J.: Physicochemical characteristics of human sex hormone binding globulin: evidence for two identical subunits. *J. Steroid Biochem.* 24 (1986) 815–824.
13. Mischke W., Weise H. C., Graesslin D., Rusch R. and Tamm J.: Isolation of highly purified sex hormone binding globulin (SHBG): evidence for microheterogeneity. *Acta Endocr. (Copenh.)* 90 (1979) 737–742.
14. Petra P. H., Stanczyk F. Z., Senear D. F., Namkung P. C., Novey M. J., Alexander Ross J. B., Turner E. and Brown J.A.: Current status of the molecular structure and function of the plasma sex steroid binding protein (SBP). *J. Steroid Biochem.* 19 (1983) 699–706.
15. Gershagen S., Henningsson K. and Fernlund P.: Subunits of human sex hormone binding globulin. Interindividual variation in size. *J. Biol. Chem.* 262 (1987) 8430–8437.
16. Khan M. S., Ehrlich P., Birken S. and Rosner W.: Size isomers of testosterone–estradiol binding globulin exist in the plasma of individual men and women. *Steroids* 45 (1985) 463–472.
17. Luckock A. and Cavalli-Sforza L. L.: Detection of genetic variation with radioactive ligands. V. Genetic variants of testosterone-binding globulin in human serum. *Am. J. Hum. Genet.* 35 (1983) 49–57.
18. Larrea F., Oliart R. M., Granados J., Mutchinick O., Díaz-Sánchez V. and Musto N. A.: Genetic polymorphism of the human sex hormone-binding globulin: evidence of an isolectric focusing variant with normal androgen-binding affinities. *J. Steroid. Biochem.* 36 (1990) 541–548.
19. Van Baelen H., Convents R., Cailleau J. and Heyns W.: Genetic variation of human sex hormone-binding globulin: evidence for a worldwide bi-allelic gene. *J. Clin. Endocr. Metab.* 75 (1992) 135–139.
20. Bérubé D., Séraline G. E., Gagné R. and Hammond G. L.: Localization of the human sex hormone-binding globulin gene (SHBG) to the short arm of chromosome 17 ($17_{p12\text{-}p13}$). Cytogenet. *Cell Genet.* 54 (1990) 65–67.

21. Van Baelen H., Brepoels R. and DeMoor P.: Transcortin Leuven: a variant of human corticosteroid-binding globulin with decreased cortisol-binding affinity. *J. Biol. Chem.* 257 (1982) 3397–3340.
22. Larrea F., Musto N., Gunsalus G. and Bardin C. W.: The microheterogeneity of rat androgen-binding protein from the testes, rete testis fluid, and epididymis as demonstrated by immunoelectrophoresis and photo-affinity labeling. *Endocrinology* 109 (1981) 1212–1220.
23. Ritzen E. M., French F. S., Weddington S. C., Neyfeh S. N. and Hansson V.: Steroid binding in polyacrylamide gels. Quantitation at steady state conditions. *J. Biol. Chem.* 249 6597–6604.
24. Musto N. A., Gunsalus G. L. and Bardin C. W.: Purification and characterization of androgen binding protein from the rat epididymis. *Biochemistry* 19 (1980) 2853–2860.
25. Chrambach A., Jovin T. M., Svendsen P. J. and Rodbard D.: Analytical and preparative polyacrylamide gel electrophoresis. An objectively defined practical route, apparatus and procedures. In *Methods of Protein Separation* (Edited by M. Castimpoolas). Plenum Press, NY (1976) Vol. 2, pp. 27–44.
26. Larrea F., Musto N. A., Gunsalus G. L., Mather J. P. and Bardin C. W.: Origin of the heavy and light protomers of androgen binding protein from the rat testis. *J. Biol. Chem.* 256 (1981) 12,566–12,573.
27. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51 (1949) 660–672.
28. Singer E., Kuenzle C. C., Thomann P. E. and Hubscher U.: DNA fingerprinting: improved extraction from small blood samples. *Nucl. Acids Res.* 16 (1988) 7738.
29. Abrams E. S., Murdaugh S. E. and Lerman L. S.: Comprehensive detection of single base changes in human genomic DNA using denaturing gradient gel electrophoresis and a GC clamp. *Genomics* 7 (1990) 463–475.
30. Myers R. M., Maniatis T. and Lerman L. S.: Detection and localization of single base changes by denaturing gel electrophoresis. In *Methods and Enzymology* (Edited by R. Wu). Academic Press, San Diego, CA (1987) Vol. 155, pp. 501–527.
31. Rosner W., Hryb D. J., Khan M. S., Nakhla A. M. and Romas N. S.: Sex hormone-binding globulin: anatomy and physiology of a new regulatory system. *J. Steroid Biochem. Molec. Biol.* 40 (1991) 813–820.
32. Larriva-Sahd J., Orozco H., Hernánez-Pando H., Oliart R. M., Musto N. A. and Larrea F.: Immunohistochemical demonstration of androgen-binding protein in the rat prostatic gland. *Biol. Reprod.* 45 (1991) 417–423.
33. Power S. G. A., Bocchinfuso W. P., Pallesen M., Warmels-Rodenhiser S., Van Baelen H. and Hammond G. L.: Molecular analyses of human sex hormone-binding globulin variant: evidence for an additional carbohydrate chain. *J. Clin. Endocr. Metab.* 75 (1992) 1066–1070.
34. Waltz M. R., Pullman T. N., Takeda K., Sobieszczyk P. and Refetoff S.: Molecular basis for the properties of the thyroxine-binding globulin-slow variant in American Blacks. *J. Endocr. Invest.* 13 (1990) 343–349.