Original Article

Molecular Analysis of the Sex Hormone-Binding Globulin Gene in the Rat Hypodactylous Mutation (*Hd)*

(rat hypodactyly / male infertility / limb malformation / sex hormone-binding globulin / androgen-binding protein / linkage mapping / gene expression / sequence analysis)

F. LIŠKA[1], C. GÖSELE[2,3], V. KŘEN[1,4], N. HÜBNER[2], D. KŘENOVÁ[1]

[1]Institute of Biology and Medical Genetics, Charles University, Prague, Czech Republic
[2]Max Delbrück Centre for Molecular Medicine, Berlin, Germany
[3]Max Planck Institute for Molecular Genetics, Berlin, Germany
[4]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Abstract. Sex hormone-binding globulin or ABP/SHBG is an extracellular androgen and oestrogen carrier. In the rat, ABP/SHBG is secreted by Sertoli cells of the testis and is thought to regulate androgen bioavailability in the male reproductive tract. During ontogenesis, ABP/SHBG is expressed in many mesoderm-derived tissues, including interdigital mesenchyme of the developing autopodium. *Shbg* is thus a candidate for *Hd*, comprising autopodium (hand and foot) reduction and male sterility resulting from spermatogenesis impairment. Moreover, linkage mapping of *Hd* revealed that an intragenic marker for *Shbg*, D10Wox12, was non-recombinant with *Hd*. Sequencing of the entire coding sequence of *Shbg* failed to identify any variation in hypodactylous animals, distinct from two control strains. However, RT-PCR analysis revealed a significantly higher level of the *Shbg* transcript in hypodactylous rats compared to SHR controls. Whether *Shbg* expression is upregulated due to a *cis*-acting mutation in regulatory elements of the *Shbg* gene or it is a secondary result of spermatogenesis failure remains to be determined.

Sex hormone-binding globulin, or alternatively androgen-binding protein (ABP/SHBG) is an extracellular carrier glycoprotein that binds androgens and oestrogens (more particularly testosterone, dihydrotestosterone and oestradiol) with high affinity (Westphal, 1986). Besides the proposed regulation of free androgen or oestrogen concentration and thus the steroid hormone response in various tissues, ABP/SHBG also functions as part of a novel steroid-signalling pathway, independent of the intracellular receptors, acting through the cell surface G-protein-coupled SHBG receptor (Nakhla et al., 1999). The major ABP/SHBG transcript is coded by eight exons (Joseph et al., 1988a, b). There are several alternative transcripts differing in the 5' sequence and/or internal splicing. In the rat, two alternative first exons were identified (Wang et al., 1990; Sullivan et al., 1993). These exons are localized in the genome 5' to the main variant exon 1, and replace this exon in alternative transcripts. Alternative transcripts containing these exons were first identified in the brain and foetal liver (Wang et al., 1990; Sullivan et al., 1993). A different alternative isoform was isolated from human testes, with a unique 5' end (alternative exon 1) and lacking exon 7 (Hammond and Bocchinfuso, 1996). The functional significance of the alternative transcripts is unknown. Furthermore, the rat alternative exons are different from the human exon and our unpublished data indicate that these alternative first exons are not conserved among mammalian genomes.

The expression pattern of ABP/SHBG is also species-dependent. In rodents, ABP/SHBG is produced by Sertoli cells of the testis (Hagenas et al., 1975), secreted into the lumen of seminiferous tubules, transported with tubular fluid to the epididymis, where it is internalized by caput epithelium (French and Ritzen, 1973). The testicular ABP/SHBG can also be detected in the serum of adult rats, but in contrast to humans, there is no liver production of SHBG in the adult rat. ABP/SHBG was also detected in the adult brain (Wang et al., 1990), foetal liver (Sullivan et al., 1991) and embryo, the latter suggesting possible developmental

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Corresponding author: František Liška, Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University Prague, Albertov 4, 128 00 Praha, Czech Republic.

Abbreviations: ABP/SHBG – androgen-binding protein/sex hormone-binding globulin, *Actb* – beta actin gene, BC – backcross, BN = BN/Cub – Brown Norway, CDS – coding sequence, F_2 – intercross, second filial generation; *Hd* – rat hypodactyly, *Shbg* – sex hormone-binding globulin gene, SHR = SHR/OlaIpcv – spontaneously hypertensive rat, WHD – Wistar hypodactylous rat.

function (Joseph, 1994). Becchis et al. (1996) analysed ABP/SHBG expression during embryonic development of the rat. They demonstrated, using different antibodies against ABP/SHBG, the presence of ABP/SHBG in many tissues of mesodermal origin, including interdigital mesenchyme of the developing autopodium. Some insight into the role of ABP/SHBG in the reproductive system was gained from studies of mice transgenic for rat *Shbg*. Overexpression of rat ABP/SHBG in the testis led to apoptosis-driven depletion of germ cells and eventually male infertility (Jeyaraj et al., 2003).

In humans, the major site of ABP/SHBG production and the only source of serum SHBG is the liver (Khan et al, 1981). The testicular ABP/SHBG in humans is produced by germ cells instead of Sertoli cells, using the alternative promoter and exon 1. This isoform is not secreted into the lumen, but accumulates in acrosomes, as inferred from mice transgenic for the 11-kb human genomic fragment containing the full *Shbg* gene plus 6 kb upstream sequence. In sharp contrast with mice transgenic for rat *Shbg*, the overexpression of human protein has no pathological outcome (Jänne et al., 1998, 1999; Selva et al., 2002).

On the other hand, several human studies suggest a possible role for ABP/SHBG in three diverse traits. Human mutation P156L in SHBG was associated with hyperandrogenism and ovarian dysfunction (Hogeveen et al., 2002). Human variant D327N, introducing an additional N-glycosylation site, was found in a significantly higher frequency in patients with oestrogen-dependent breast cancer than in a control group (Becchis et al., 1999). Low plasma SHBG levels were associated to several components of the metabolic syndrome, especially abdominal obesity, hyperinsulinaemia and insulin resistance (Hajamor et al., 2003).

Hd is an autosomal recessive mutation leading in homozygous condition to defective autopodium development (reductive changes of digital arch of both fore- and hind limbs in both sexes), and male infertility caused by impairment of spermatogenesis (Sabourdy and Božić, 1960; Moutier et al., 1973; Křenová et al., 1999a).

In the presented work, we use linkage mapping to establish *Shbg* as a positional candidate for *Hd.* Considering additional functional information, e.g. ABP/SHBG function in the male reproductive tract, namely infertility in rat *Shbg*-transgenic mice and expression of ABP/SHBG in interdigital mesenchyme, which is potentially important for autopodium development, *Shbg* is also a promising functional candidate for *Hd*. We therefore proceed to sequence and expression analysis of the gene.

Material and Methods

Animals

We obtained a breeding nucleus of an outbred Wistar *Hd* (WHD) strain from Germany (prof. Schleiermacher's colony) and maintained the strain by backcross mating of homozygous (*Hd/Hd*) females with heterozygous (*+/Hd*) males. The breeding strategy was changed to inbred – homozygous *Hd*/*Hd* females were mated with their +/*Hd* brothers for more than 15 generations. However, WHD cannot be an ordinary inbred strain because of a small segment around the *Hd* locus, bearing the normal counterpart of the *Hd* allele, that is necessary for fertility of the +/*Hd* males.

Congenic strains BN-*Hd* and SHR-*Hd* were derived by cross-intercross or marker-assisted backcross mating. WHD females were mated to BN/Cub or SHR/OlaIpcv males, respectively. BN-*Hd* is in 9^{th} equivalent backcross (NE) generation whereas SHR-*Hd* is in NE11.

Both BC and intercross mapping strategies were accommodated for the purpose of *Hd* positional cloning. Two segregating progenies were derived: 1) backcross BC(WHD female xF1 male (WHD female xBN/Cub male)) progeny (further referred to as BC), 2) intercross F_2(F1 female (WHD female xBN/Cub male) x F1 male (WHD female xBN/Cub male)) progeny (referred to as F_2).

The limb phenotype was scored postnatally according to Moutier et al., 1973.

Animals were fed standard chow and tap water *ad libitum*. All animal experiments were approved by The Charles University Animal Care Committee.

Sperm analysis

Animals were killed by anaesthetic overdosing and/or cervical dislocation. Cauda epididymidis was isolated, placed into 2 ml of PBS, cut several times with scissors and mixed. The appropriate volume of the sperm suspension was applied to a Bürker haemocytometer and immediately evaluated. The sample was counted normal if there was high density of spermatozoa, most of the spermatozoa were motile, and any morphological abnormalities were rare. On the other hand, in mutant samples the sperm number was at least 10-fold decreased, spermatozoa were almost immotile, exhibited substantial tail fragility and had a bulky appearance of the head (probably due to persistence of the cytoplasmic droplet).

Linkage mapping

Both backcross and intercross were used for linkage mapping. The uninformative recombinants in F_2 were resolved by further backcrossing (to WHD females) or intercrossing (to F1 hybrids WHDxBN) with subsequent geno- and phenotyping of the resulting advanced BC or F_2.

DNA was isolated from tail biopsy by phenol extraction. Polymorphic microsatellite loci were amplified by PCR. Polymorphic markers were selected from public databases (Rat Genome Database, http://rgd.mcw.edu/sslps/, The Wellcome Trust Centre for Human Genetics, http://www.well.ox.ac.uk/rat_mapping_resources/markers_info/primers_chr10.TXT, or Whitehead Institute/MIT Center for Genome Research, http://www-genome.wi.mit.edu/cgi-bin/rat/gmap_search). To develop additional polymorphic markers, rat genomic DNA was searched for simple tandem repeats by POMPOUS (Fondon et al., 1998) included in the PANORAMA bioinformatic tool (Pertsemlidis et al., 2000, http://atlas.swmed.edu/panorama_form.shtml), Primer3 (see later) was employed for primer design. The markers were dubbed D10Cub1–D10Cub14, where Cub is for Charles University, Institute of Biology. Primer sequences and information about polymorphism are available on request.

Map Manager QTX (Manly et al., 2001, http://mapmgr.roswellpark.org/mapmgr.html) was used for integrated linkage map construction.

RNA isolation and RT-PCR

Animals were killed as mentioned above. Two hundred mg of testicular tissue were extracted with 1 ml of Trizol reagent (Gibco BRL, Gaithersburg, MD). One µg of total RNA was reverse-transcribed using MMLV reverse transcriptase (Gibco BRL) and oligo dT primer in 25 µl reaction mix according to manufacturer's instructions. The product of the reverse transcription was 4 x diluted with 5 mM Tris·Cl, pH 8.5 (to the volume 100 µl). Two µl of the reaction product were used for subsequent PCR amplification with primers Shbg_1F (ATATTCTGAGCCACTGGGTG) and Shbg_1309R (AGGTCCCAATTCACCTCTCC). PCR products were excised from agarose gel, purified by Qiagen's QIAquick Gel Extraction Kit and both strands were sequenced. For semi-quantitative RT-PCR, 2 µl of diluted cDNA were amplified using 400 nM primers GACCTGCAACCTGGACTGTT (Shbg_694F) and TAAAGCCCCAAGGGAGAGAT (Shbg_1092R) and 50 nM control primers ATGGTGGGTATGGGTCAGAA (ß-actin_130F) and GCTGTGGTGGTGAAGCTGTA (ß-actin_611R), with 1.7 mM $MgCl_2$, for 22 cycles with annealing temperature 60˚C. For quantification, the 1.5 % agarose gels stained with ethidium bromide were photographed using an 8-bit (grey scale) digital camera (BioRad Laboratories, Hercules, CA) and the band intensity was determined by ImageJ (http://rsb.info.nih.gov/ij/).

Genomic DNA sequencing

DNA was isolated from three hypodactylous strains (WHD, BN-*Hd* and SHR-*Hd*) and two control strains (BN/Cub and SHR/OlaIpcv) as described above. Primer3 (Rozen and Skaletsky, 2000, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was employed for design of exon-flanking primers. Primer sequences are available on request. In addition to the eight exons of the main *Shbg* gene product (GenBank M19993), two alternative first exons (GenBank accession numbers U85959 and M62613) were sequenced. The genomic DNA sequence flanking the alternative exons was identified by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search of the rat genome (version 2). PCR products were purified by Qiagen kits and sequenced according to the standard protocols. BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) sequence alignment editor was employed for comparison of the sequences from different strains. Splice sites were evaluated by NNSPLICE 0.9 (Reese et al., 1997) athttp://www.fruitfly.org/seq_tools/splice.html.

Results and Discussion

Hd maps to a 0.33 cM segment of RNO10

In our previous work (Křenová et al., 1999a, b), *Hd* was assigned to RNO10 (Rattus norvegicus chromosome 10), to a 6.8 cM interval between markers D10Rat30 and D10Rat31.

For fine linkage mapping, 447 F_2 animals and in total 320 backcross animals (including 89 backcross animals from Křenová et al., 1999a) were bred. Both limb and reproduction (in males) phenotypes were evaluated. *Hd* position was thus refined to 0.33 cM between D10Mit8 and D10Rat60. The centromeric border was defined by a normodactylous and fertile backcross male, homozygous for WHD alleles of D10Mit8 and upstream markers. The telomeric border was defined by a F_2 female, whose two F_3 sons bearing the recombinant chromosome were hypodactylous and infertile, but heterozygous for D10Rat60 and downstream markers. The interval D10Mit8-D10Rat60 spans 1,324 kb in the rat genome – version 3.1 (Fig. 1). The region contains the gene for sex-hormone-binding protein (*Shbg).* In intron 7 of *Shbg* there is a SSLP marker D10Wox12, polymorphic in our cross and non-recombinant with *Hd*. That supports *Shbg* as a good positional candidate, besides being a functional candidate (Becchis et al., 1996; Jeyaraj et al., 2003).

Shbg contains no mutation in the coding sequence

Eight exons and two alternative exons of *Shbg* were sequenced in three different strains homozygous for the *Hd* allele (WHD and two *Hd*-congenic strains BN-*Hd* and SHR-*Hd*) and two control strains (BN/Cub and SHR/OlaIpcv). The only diference identified was an A to G transition of the third base at the intronic side of the splice site of the brain alternative exon 1 (exon sequence M62613, Wang et al., 1990) in all *Hd/Hd* strains plus in the control SHR strain. Thus, this variation cannot have any causal relationship to the *Hd* phenotype. Moreover, *in silico* splice site analysis suggested only a negligible impact of the transition on splice efficiency (Fig. 2).

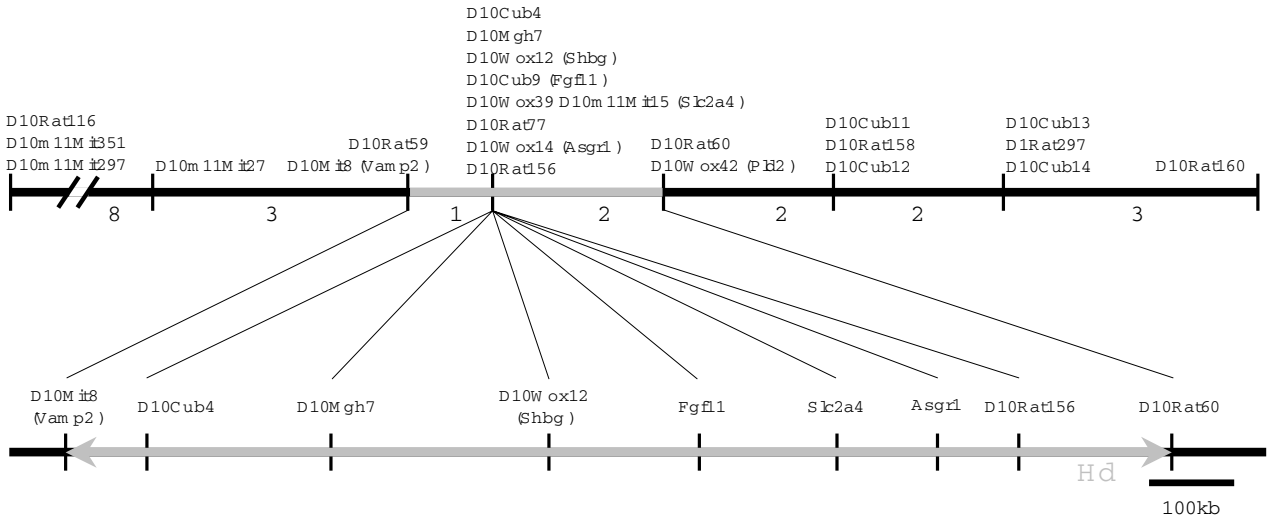

Fig. 1. Linkage and physical map of the *Hd* locus
Upper track – integrated linkage map. The number of recombination events is indicated below the track (1 recombination = 0.08 cM, F_2 n = 447, BC n=320). **Lower track** – physical map – rat genome release, version 3.1, NCBI supercontig NW_047334. Critical interval for *Hd* (grey) spans 1324 kb between D10Mit8 and D10Rat60, 42689–44013 kb in the supercontig. Only positions of the linkage-mapped markers are shown on the physical map. Cub markers (Charles University, Inst. of Biology) are microsatellites or insertion/deletion polymorphisms identified during the project (see Methods).

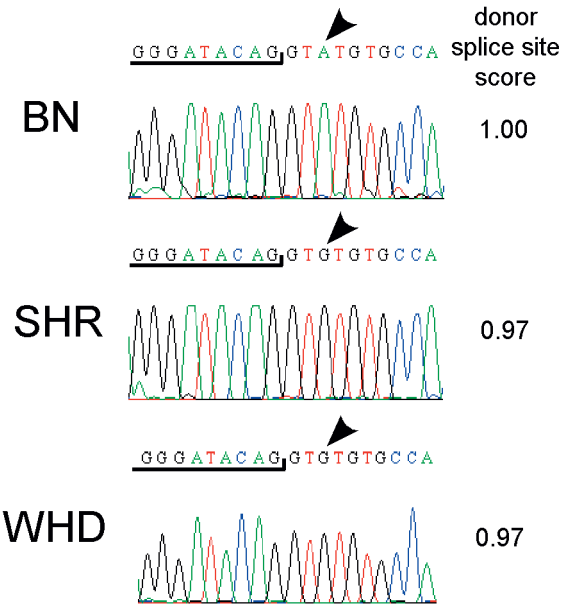

Fig. 2. A to G transition in the donor splice site adjacent to brain alternative exon 1 of *Shbg* (GenBank M62613)
The BN/Cub sequence (top) is identical with the rat genome sequence (strain BN/SsNHsdMCW, NCBI supercontig NW_047334). Donor splice site prediction by NNSPLICE 0.9 gives a maximum score – i. e. 1.00. In SHR and WHD, the third intron base is changed to G, thus lowering the donor splice site prediction score to 0.97.
The exon sequence is underlined, the splice site is indicated by a short vertical bar. The site of the A to G transition is marked by arrowheads.

Shbg expression in hypodactylous testes

RT-PCR analysis of *Shbg* expression in adult testicular tissue revealed that *Shbg* mRNA is expressed in mutant WHD as well as in control SHR males. We therefore amplified a cDNA fragment containing full *Shbg* CDS from WHD and SHR testicular cDNA and sequenced the PCR product – sequences of both WHD and SHR were identical. Therefore, we can infer from the genomic sequence of *Shbg* as well as from the cDNA sequence that *Shbg* does not contain any mutation in the coding sequence nor any mutation causing aberrant splicing of the major transcript.

Semi-quantitative RT-PCR analysis was thus performed to determine the expression level of *Shbg* in testes. We used duplex design with beta-actin amplification as an internal control. The *Shbg* transcript was more than 2-fold more abundant in mutant (WHD) than in control (SHR) testes (Fig. 3).

The observed difference in *Shbg* expression may reflect a mutation in a hypothetical *cis*-acting regularory element of the *Shbg* gene. The observed infertility of *Hd/Hd* males would therefore result from depletion of free testosterone due to overexpression of ABP/SHBG, in a way similar to observed infertility in male mice overexpressing rat ABP/SHBG (Jeyaraj et al., 2003).

If *Shbg* upregulation is caused by a regulatory mutation, what would be its nature? Besides a point mutation in a promoter or enhancer/silencer regions (which were to the best of our knowledge not exactly determined at the time of our analysis) one should consider chromosomal rearrangement. The relative recombination “cold

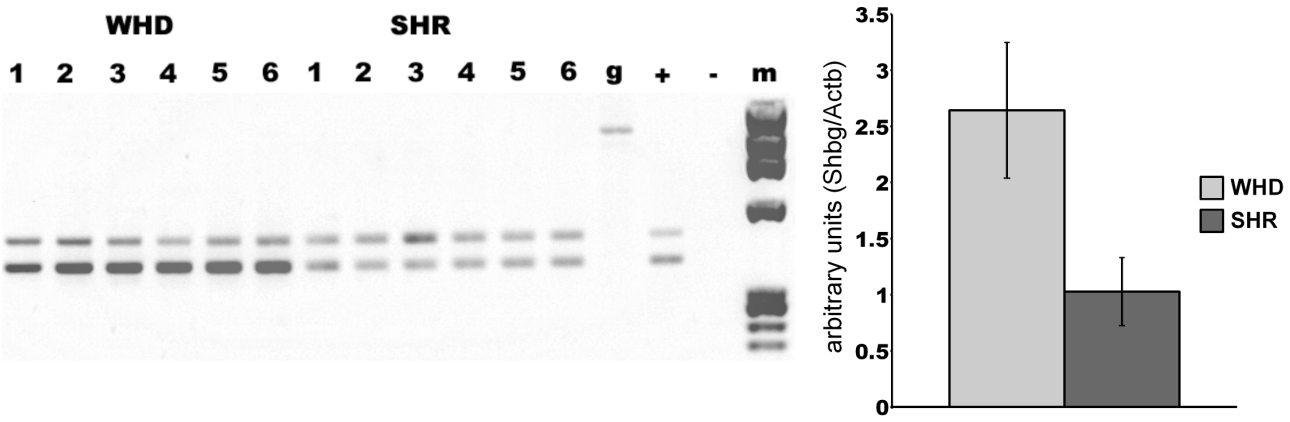

Fig. 3. RT-PCR analysis of *Shbg* expression in the testis
Left panel: representative result of duplex RT-PCR of *Shbg* (399 bp) and β-*actin* (482 bp). Six homozygous WHD (*Hd*/*Hd*) animals and six control SHR were analysed. **g** – amplification of genomic DNA (*Shbg* gene 1086 bp, β-*actin* gene 938 bp), + and **–** – positive and negative control, respectively, **m** – DNA marker ÖX174/HaeIII.
Right panel: Columns indicate the ratio of *Shbg* to control gene (β-*actin*) expression. Data are shown as means ± standard deviation (for each group N = 6). The *Shbg* expression level in the adult testis is significantly higher in mutant (WHD) and control (SHR) males (Student's t-test for independent samples, $P < 0.001$).

spot" around *Hd*, with no recombination in ~1 Mb in ~1200 meioses, raises a possibility of expression dysregulation caused by chromosome micro-rearrangement. This hypothesis is also corroborated by the fact that in the WHD strain, existing since 1960 (Sabourdy and Božić), no recombination occured between the *Hd*-containing maternal chromosomal segment and the homologous paternal chromosomal segment of heterozygous males (necessary for maintaining the strain). If we count only three generations per year, it represents more than 130 *backcross* generations since 1960. This again indicates scarce recombination events between the chromosomal segments in proximity to *Hd*, despite the homogenizing pressure exerted by inbreeding.

In conclusion, the *Hd* position was substantially refined and *Shbg* identified as a positional and functional candidate. Despite the highest functional significance of *Shbg*, our sequence analysis of *Shbg* failed to identify the mutation responsible for the *Hd* phenotype. However, expression analysis revealed a significantly higher level of *Shbg* transcript in testes of the mutant rats. Although the exact determination of the role of elevated *Shbg* expression in the *Hd* phenotype will require further studies, *Shbg* is still a promising candidate gene for *Hd*.

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