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 )

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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 (Nakhaia 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
function (Joseph, 1994). Becchis et al. (1996) analysed
ABP/SHBG expression during embryonic development of
the rat. They demonstrated, using different antibod-
ies against ABP/SHBG, the presence of ABP/SHBG in
many tissues of mesodermal origin, including interdig-
tal 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 signifi-
cantly higher frequency in patients with oestrogen-
dependent breast cancer than in a control group
(Becchis et al., 1996). Becchis et al. (1999). Low plasma SHBG levels were
associated to several components of the metabolic syn-
ystem, especially abdominal obesity, hyperinsuli-
naemia and insulin resistance (Hajamor et al., 2003).

Hd is an autosomal recessive mutation leading in
 homozygous condition to defective autopodium develop-
ment (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 develop-
ment, 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 mat-
ing. WHD females were mated to BN/Cub or
SHR/OlaIpcv males, respectively. BN-Hd is in 9th
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 ma-
le)) progeny (further referred to as BC), 2) intercross
F1(F1 female (WHD female xBN/Cub male) x F1 male (WHD
female xBN/Cub male)) progeny (referred to as F2).

The limb phenotype was scored postnatally accord-
ing 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 haemocy-
tometer and immediately evaluated. The sample was
counted normal if there was high density of spermato-
zoa, most of the spermatozoa were motile, and any mor-
phological 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 F2 were
resolved by further backcrossing (to WHD females) or
intercrossing (to F1 hybrids WHDXBN) with subse-
quent geno- and phenotyping of the resulting advanced
BC or F2.
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) and the band intensity was determined by ImageJ (http://rsb.info.nih.gov/ij/). Hercules, CA) and the band intensity was determined

ethidium bromide were photographed using an 8-bit quantification, the 1.5% agarose gels stained with (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 (Kfenová 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 F2 animals and in total 320 backcross animals (including 89 backcross animals from Kfenová 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 D10Mit60. The centromeric border was defined by a normodactyly and fertile backcross male, homozygous for WHD alleles of D10Mit8 and upstream markers. The telomeric border was defined by a F2 female, whose two F3 sons bearing the recombinant chromosome were hypodactyly and infertile, but heterozygous for D10Mit60 and downstream markers. The interval D10Mit8-D10Mit60 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 reference strains. Splice sites were evaluated by NNSPLICE 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.

**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 difference 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).
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 regulatory 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 region 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. 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, F₂ 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.

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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 BoïÊ), no recombination occurred 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.

References


