NOTE ***Physiology***

Fine Mapping of the Region Including Hypogonadism (*hgn*) Locus on Rat Chromosome10

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ABSTRACT. The hypogonadic rat (*hgn/hgn*) shows male sterility, reduced female fertility, and renal hypoplasia, controlled by a single recessive gene located on rat chromosome 10. We developed a fine map around the *hgn* locus using 565 rat backcross progeny and a Rat/Hamster radiation hybrid panel. The *hgn* locus was linked to *Aldoc* (aldolase c) and *whn* (winged helix of nude), and located in a 0.34-cM region between *D10Rat30* and *D10Rat68*. The distance of the region was approximately 840-kb on rat physical map. Neither loci responsible for male sterility nor renal hypoplasia has been mapped on the homologous regions of mouse chromosome 11 and human chromosome 17. Identification of the gene responsible for the *hgn* mutation would provide important information on urogenital development.

KEY WORDS: hypogonadism, linkage map, radiation hybrid map.

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Male hypogonadic rats (*hgn*/*hgn*) show severe hypogonadism that inherited as an autosomal single recessive manner. The weight of an adult *hgn*/*hgn* testis is almost equal to that of a normal female ovary and about 1% that of a normal rat testis. These animals have small male reproductive accessory organs but no female reproductive organs [18]. Plasma testosterone is low and levels of gonadotropins are high, indicating that the cause of hypogonadism lies within the testis itself [5]. Testicular pathogenesis begins in the fetal period and progresses during postnatal testicular development [20]. Most germ cells degenerate in the testes before entering meiosis because of Sertoli cell dysfunction [13, 17]. *hgn*/*hgn* males show not only hypogonadism but also bilateral hypoplastic kidney (HPK) [19]. The HPK of *hgn*/*hgn* rat contains only one-quarter the number of nephrons that are found in the normal kidney [12]. Therefore, the HPK causes chronic progressive renal failure [14]. The phenotypes of this disorder resemble those of oligomeganephronia, a congenital renal hypoplasia reported in humans [2], in respect of the reduced number of nephrons with hypertrophy of individual nephrons [12]. This combination of HPK and hypogonadism makes it possible to distinguish *hgn*/*hgn* females from phenotypically normal ones in the HGN strain [19]. *hgn*/*hgn* females identified by the presence of HPK have hypoplastic ovaries at birth, reduced reproductive performance, and early reproductive senescence [11]. Therefore, the female *hgn*/*hgn* rat is a congenital animal model of premature ovarian failure (POF) in women [9, 11]. No other mutant animal showing a phenotype similar to that of the *hgn*/*hgn* rat has been reported. Therefore, the *hgn*/*hgn* rat would be a useful model for investigating the development of the mammalian reproductive and urinary organs. Our recent report revealed that the *hgn* locus was located in the region close to the *D10Mit2* locus on rat chromosome 10 [15]; in the previous study we presented only a rough linkage map around the *hgn* locus, using 48 backcross progeny and 11 rat microsatellite markers. In the present study, we performed further linkage analysis using 565 rat backcross progeny to make a fine linkage map around the *hgn* locus. Radiation Hybrid (RH) mapping was also performed to determine the order of the markers mapped at the same positions in the linkage analysis.

All rats used in this study fed a certified commercial diet (CR-LPF: Oriental Yeast Co., Ltd., Tokyo, Japan) and were kept in a clean conventional animal room under controlled light (14L: 10D), temperature (22 ± 1°C), and humidity (55 ± 5%) [17]. The experimental procedure and care of animals were in accordance with the guideline of the Animal Care and Use Committee of Nippon Veterinary and Animal Science University. One hundred and twenty-six *hgn*/*hgn* females were selected from a population of the HGN inbred strain by laparotomy for the detection of HPK at weaning [19]. They were mated with the F_1 (+/*hgn*) males obtained by mating between a BN (Brown Norway strain) male (+/+) and a *hgn*/*hgn* female (HGN strain) [15]. As a result, 510 male backcross progeny were obtained. They were sacrificed at 21 days of age. Their phenotypes were determined by measuring their testicular weights with an electric balance [19, 20]. Their livers were excised and frozen at –20°C until DNA extraction. In a total of 388 out of the 510 male progeny, kidneys, adrenal glands, spleen, thymus, heart, lung, brain, and pituitary gland were also weighed. Student's *t*-test was used for statistical analysis of the data. The testes were fixed in 4% neutral formalin, dehydrated with graded alcohol, embedded in paraffin, and sectioned at 3 μm. The sections were stained with hematoxylin and eosin and examined under a light microscope [20].

In the linkage analysis, we typed a total of 565 backcross males, including 55 males obtained previously [15]. The *hgn* locus has been reported to locate in the region close to the *D10Mit2* locus on the rat chromosome 10 [15]. Therefore, the rats were typed for the following rat microsatellite

markers in the close vicinity of *D10Mit2: D10Rat159, D10Rat161, D10Rat69, D10Rat211, D10Arb9* (*Aldoc*: aldolase C), *D10Rat30, D10Rat68, D10Wox24* (*Nos2*: nitric oxide synthase 2), *whn* (winged helix of nude), *D10Rat133, D10Got80, D10Arb27,* and *D10Rat29* [4]. Forward and reverse primers for each locus were purchased from Research Genetics Inc. (Huntsville, AL, USA) or synthesized by an outside supplier (TaKaRa, Kyoto, Japan). The primer sequences for *whn* were 5'-ACCTGACTGCAGGTGTGAATGG-3' and 5'-TTTGATTCCTAGGACCCACATGG-3', which amplify microsatellite DNA of an intron of mouse *whn* genomic DNA [10]. Isolation of genomic DNA, polymerase chain reactions (PCRs), separation of PCR products, and gel staining were done as previously described [16]. Data analysis for linkage was carried out with Map Manager QTb [7]. The order of the loci was determined in a way that minimized the number of recombination events. A Rat/Hamster radiation hybrid (RH) panel was purchased from Research Genetics Inc. (Huntsville, AL, U.S.A.). The PCR of RH mapping was basically similar as genotyping in linkage analysis except for using 25 *n*g genomic DNA of RH clone instead of that extracted from rat liver. The PCR products were separated by non-denatured 10–12.5% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining [16]. The presence or absence of each of 9 microsatellite markers, which located in 1cM-region including *hgn* locus, were determined on 106 RH clones and 4 control samples (consisting of rat FR donor, hamster A23 recipient, HGN rat, and BN rat DNA) in triplicate. Our ability to compare the sizes of the PCR products of RH clones with those of control FR and A23 DNAs excluded the possibility that we would mistake non-specific amplifications as a presence. Data were analyzed using RH option of Map Manager QTb according to Elliot *et al.* [3]. We omitted *whn* from RH mapping analysis, since the product of *whn* from rat FR DNA showed smear. The positions of the makers on rat physical map of chromosome 10 were determined by the similarity search program for the sequence database (BLAST) with their sequences against rat genome [6].

As in our previous report [15], the male rats of the backcross progeny were unambiguously classified into normal and affected groups according to their testicular size at 21 days of age, although it was impossible to identify HPK from normal ones by renal weight. Therefore, we used only the male rats of the progeny for the linkage analysis. Table 1 shows the average values of body weights and relative weights of each organ in 182 *hgn*/*hgn* and 206 +/*hgn* males of the backcross progeny at 21 days of age. The body weight of *hgn*/*hgn* rats was significantly smaller than that of +/*hgn*. The relative weight of testis of *hgn*/*hgn* rats was almost 10% that of +/*hgn* rats at 21 days of age. The histological appearance of the testes in the affected rats of the backcross progeny was basically similar to that of *hgn*/*hgn* rats of the HGN strain. However, compared with the latter, more seminiferous tubules remained in the affected testes of the backcross progeny (data not shown). This suggests that the testicular dysgenesis, with degeneration of seminiferous tubules and their embedding into the interstitial tissue, might result from the interaction of *hgn* and other background genes expressed in testicular development. The relative weights of kidney, thymus, and spleen were significantly smaller in *hgn*/*hgn* rats than in +/*hgn* rats. The relative weights of lung and heart were significantly greater in *hgn*/*hgn* than in +/*hgn*. Similar alterations in body weight and the weights of these organs have been observed in affected rats of the HGN strain [18–20 and unpublished data]. Compared to remarkable reductions in the weights of testis and kidney, those of thymus and spleen were not so obvious in *hgn*/*hgn* rats of the HGN strain. Thus it was meaningful that we detected the significant reduction in the weights of these organs in *hgn*/*hgn* rats under the altered genetic background. In the HGN strain, the weights of *hgn*/*hgn* thymus and spleen were already small at birth compared with those of normal rats. Therefore, the *hgn* gene might be expressed in the fetal thymus and might have an influence on the development of immune organs. An alternative possibility is that the genes close to *hgn* have an influence on thymus and spleen development. The linkage analysis presented here revealed that the *hgn* locus was closely linked to *whn* (*Foxn1* in mouse) locus. Although we have not yet examined the expression of the product of *whn* in the thymus of *hgn*/*hgn* rats, the phenotypes of *hgn*/*hgn* and nude rats were obviously different [10]. The increase in the relative weights of the lung and heart of male *hgn*/*hgn* rats was more obvious with age (18, unpublished data), so it is likely that these effects are complications of endocrine disorders secondary to the hypogonadism and/or the HPK. One of the hypotheses is that the chronic renal failure caused by HPK would lead to alterations in blood circulation system (e.g. hypertension accompanied by the increase of heart weight) in *hgn*/*hgn* rats.

The linkage map we obtained from 565 backcross prog-

Table 1. Body weights and relative weights of organs in backcross/*hgn* and *hgn/hgn* males

	+/*hgn* (206)	*hgn/hgn* (182)
Body weight (g)	36.70 ± 8.99	34.52 ± 8.63*
testis[a)]	2101 ± 243	190 ± 45**
kidney[a)]	5529 ± 493	4864 ± 612**
adorenal gland[a)]	146 ± 40	149 ± 29
spleen	2976 ± 711	2635 ± 621**
thymus	3463 ± 607	2366 ± 547**
heart	5212 ± 621	5766 ± 853##
lung	10427 ± 2435	11178 ± 4031#
brain	39193 ± 7731	39365 ± 8.445
pituitary gland	47 ± 23	49 ± 20

Each value represents the average with the standard deviation. Relative weight of each organ is calculated as organ weight (mg)/ Body weight (kg). a) Average value from bilateral organs. *,**: Signifcantly smaller than that of +/*hgn* as $p<0.05$ and $p<0.01$, respectively. #, ##: Significantly larger than that of +/*hgn* as $p<0.05$ and $p<0.01$, respectively. Number in parenthesis represents the number of animals examined.

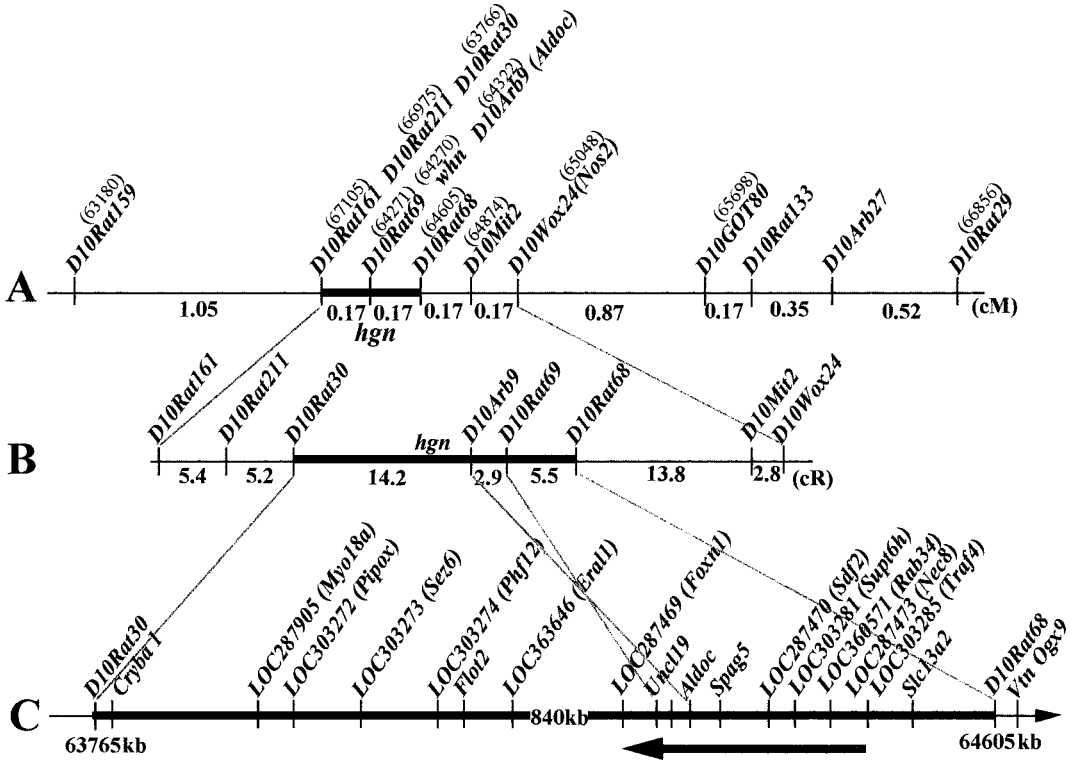

Fig. 1. (A): Fine linkage map of the area around the *hgn* locus on rat chromosome 10, showing the location of *hgn* in relation to other loci. The *hgn* locus is located in the 0.34-cM region between *D10Rat30* and *D10Rat68*. The number in parenthesis attached with locus name represents the position (kbp) of the locus on physical map. (B): Radiation hybrid map showing the order of the markers close to the *hgn* locus. (C): Rat physical map showing the positions of genes with known or inferred functions in the 840-kb region including *hgn* locus. The homologous genes of mouse are shown in the parentheses attached to locus ID number. Thick arrow indicates that the order of the loci in the region is opposite to those shown in mice and human. Thick line shows the distance of the *hgn*-region restricted in each map.

eny, including 271 *hgn*/*hgn* and 294 +/*hgn* males, is shown in Fig. 1A. The segregation ratio of *hgn*/*hgn* and +/*hgn* did not deviate significantly from the expected 1:1 ratio (χ^2=0.94, P>0.20). No recombinant event was observed between *hgn*, *D10Rat69*, *whn*, and *D10Arb9(Aldoc)*. One recombination was observed between *hgn* and *D10Rat68*, and one recombination was observed between *hgn* and *D10Rat30* (in the same position as *D10Rat161* and *D10Rat211*). Therefore, the *hgn* locus was located in a 0.34-cM region between *D10Rat68* and *D10Rat30*. The order and distances of the microsatellite markers revealed in this experiment are basically identical with those of linkage maps reported by others [4, 8]. BLAST search with the sequences of the markers revealed that their positions (parentheses attached to locus names) on rat physical map of chromosome 10 [6]. The positions of *D10Rat161*, *D10Rat211*, and *D10Rat30* mapped on the same position in the linkage map were inconsistent with those presumed from the rat physical map obtained from NCBI [6]. We supposed that the inconsistency might be derived from the incompleteness in the draft sequences of rat genome, since the physical map includes gaps and incorrect segments of chromosome. Our RH map (Fig. 1B) and another fine linkage map around the region reported by our group [1] support our fine linkage map presented here. Consequently, integration of the linkage map with the RH map suggests that the *hgn* locus is located in a 22.6cR-region between *D10Rat68* and *D10Rat30*. Although the region includes unsettled segment of chromosome, in which the order of loci is opposite to that reported in the homologous region of mouse chromosome 11 and human chromosome 17 (Fig. 1C; shown by thick arrow), the distance of the region was estimated as a 840-kb on the physical map (Fig.1C).Comparison of the region with mouse homologous region on chromosome 11 indicates that the region includes 16 genes with known or inferred functions, and 8 segments with unknown function. It has been reported that *Spag5* encoding a protein associated with the mitotic spindle apparatus is abundantly expressed in testis. However recent report revealed that the knockout mice of *Spag5* had normal fertility [21]. At present, neither loci responsible for male sterility nor renal hypoplasia has been mapped on the homologous regions of the mouse chromosome 11 and human chromosome 17 [6]. Therefore, it is strongly postulated that the gene responsible

for *hgn* is one of the genes, of which function have not yet been settled. In order to narrow the distance of the region including *hgn* locus, we will generate new polymorphic markers in the region to type the recombinants in the back-cross progeny. To identify the mutated gene *hgn*, we will examine the expression of the candidate genes in the embryonic organs in which the effects were shown in *hgn*/*hgn* rats. Our efforts to identify the mutated gene *hgn* would lead to discover new information important for development and function of urogenital organs.

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