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Chromosomal Mapping of Human Angiotensinogen Gene and Human Renin Gene by Fluorescence In Situ Hybridization (FISH) in Transgenic Mice

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Abstract: We attempted to apply FISH to chromosomal mapping of the human angiotensinogen (hAG) and human renin (hRN) transgenes which are carried by the parental strains of the Tsukuba hypertensive mouse. We report here that the hAG gene is mapped in the C2 region of Chr 19 and the hRN gene in the A1 region of Chr 6.

Key words: FISH, human angiotensinogen gene, human renin gene, mapping, PCR, Tsukuba hypertensive mouse

Fukamizu *et al.* [1] reported that the Tsukuba hypertensive mouse produced by mating between two transgenic lines carrying human angiotensinogen (AGT assigned on human Chr 1q42–q43) and human renin (REN assigned on human Chr 1q32) genes is a remarkable model for human hypertension.

So far, isolated DNA clones have been assigned on chromosomes by hybridization *in situ* (HIS) with radioisotopes. Recently, *in situ* hybridization with fluorescence such as FITC, namely FISH (fluorescence *in situ* hybridization), has been developed and applied to chromosomal mapping of DNA clones in humans and laboratory animals. In this study, we attempted to apply FISH to chromosomal mapping of the hAG and hRN transgenes which are carried by the parental strains of the Tsukuba hypertensive mouse.

Mating experiment: Male mice of C57BL/6J-

TgN(hAG)2-5 and C57BL/6J-TgN(hRN)8-12 strains were used in this study. A mating experiment was performed to confirm the location of the hAG transgene on the chromosome. One hundred and thirty-nine backcross mice were obtained by crossing (C57BL/6J-TgN(hAG) × DBA/2Jcl) F1 and DBA/2Jcl.

Genomic DNA preparation: Livers obtained from backcross mice were treated overnight with mincing buffer (500 μ l) containing Proteinase K at 37°C. Phenol/chloroform (500 μ l) was added and reacted for 5 min with shaking, and the mixture was then centrifuged for 10 min at 15,000 rpm. The upper layer was mixed for 5 min with chloroform with shaking. After centrifugation for 10 min at 15,000 rpm, the upper layer was mixed with 2.5 fold volumes of 100% ethanol. Precipitated DNA was washed in 70% ethanol and then dried. DNA dissolved in the TE buffer (pH 8.0) was

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used as the template for PCR.

PCR: The nucleotide sequences of the primers for hAG were as follows: 5'-AGAGAGCCCACAGAGTCTAC-3' and 5'-TTCTACTGCTCACTCCATGC-3'. The primers were purchased from KURABO, Japan. The primers of three microsatellite DNA markers of Chr 19, D19Mit28, D19Mit40 and D19Mit13, were purchased from Research Genetics Inc. (Huntsville, AL, USA). The solutions were mixed in microcentrifuge tubes in the following order: dNTP, 10 × buffer, 50 mM MgCl₂, distilled water, primers, *Taq* DNA Polymerase (Life Technologies Inc., NY, USA) and DNA template. Mineral oil (Sigma, USA) was dripped onto the mixture. The PCR conditions were as follows: 15 sec at 94°C for the denaturing steps, 60 sec at 55°C for the annealing steps, and 30 sec at 72°C for the extension steps, with 35 cycles. The PCR products were electrophoresed for 40 min with 3% NuSieve™ agarose (FMC, Rockland, ME, USA) prepared in TBE buffer containing ethidium bromide. Band patterns of the PCR products were photographed under a transilluminator (FUNAKOSHI, Japan).

Chromosome preparation: Chromosome preparation was basically carried out according to the method of Matsuda *et al.* [3]. The procedure was as follows. Spleen cells treated in the Gey's solution were cultured in RPMI1640 supplemented with 20% FCS in a CO₂ incubator. Con A (3 µg/ml) and LPS (10 µg/ml) were used for stimulation of lymphocytes. Incubation was continued for 44 hr with 50 µl of thymidine (final concentration of 300 µg/ml), and then for 14 hr with BrdU (final concentration of 200 µg/ml). After 4.5 hr (62.5 hr from the beginning of the culture), colcemide was added to a final concentration of 0.02 µg/ml. The cells were harvested after 30 min. Cells harvested were treated with hypotonic solution (0.75% KCl), incubated at 37°C for 15 min, and then fixed with a fixative containing ethanol and acetic acid at a ratio of 3:1. Metaphase spreads were prepared according to the standard protocol. After chromosome preparations were soaked in Hoechst 33258 prepared in phosphate buffer (1 µg/ml), UV irradiation was carried out with the Stratalinker® UV Crosslinker (Stratagene, USA). Chromosome preparations were then stored at -80°C until use.

FISH: Analyses were carried out according to the method of Takahashi *et al.* [7]. The procedure was as follows. Probes used for detection of the hAG and hRN

genes were phAGTM14 (14 kb) and phRNTM15 (15 kb) which were packaged in pUC19. Restriction enzyme maps of the probes are shown in Fig. 1. The 400–600 ng/µl probe DNA was labeled with biotin-16-dUTP by nick translation. After the probe was mixed with sperm DNA and transfer RNA, precipitation was carried out with 100% ethanol. Denaturing was performed for 10 min at 75°C and the probe was stored at 4°C until use. The chromosome preparation was hardened for 3 hr at 65°C. Treatment of the preparation with 70% formamide/2 × SSC for 2 min at 70°C was followed by dehydration with 70% ethanol and 100% ethanol. Ten microliters of the hybridization medium (a mixture of 50 µl of 10 × SSC, 25 µl of BSA (20 mg/ml) and 50 µl of 50% dextran sulfate) was mixed with 10 µl of the denatured probe. Twenty microliters of this mixture was dripped on the denatured chromosome preparation, and the preparation was covered with parafilm. Hybridization was performed for 15–24 hr at 37°C in a humidity box containing 2 × SSC. After hybridization, the preparation was treated for 20 min at 37°C with 50% formamide/2 × SSC, and washed three times in 4 × SSC for 15 min at room temperature. Seventy microliters of a mixture of 1.5 µl of goat anti-biotin antibody and 500 µl of 4 × SSC/1% BSA was dripped onto the chromosome preparation, which was then covered with parafilm, and incubated for 1 hr at 37°C in a humidity box. Thereafter, the preparation was washed in 4 × SSC, 4 × SSC/0.05% NP40 and 4 × SSC for 5 min each time. To amplify the signals, the chromosome preparation was treated with 70 µl of a mixture of 1.5 µl of FITC labeled anti-goat IgG antibody and 500 µl of 4 × SSC/1% BSA. After incubation for 1 hr at 37°C, the preparation was washed in 4 × SSC, 4 × SSC/0.05% NP40 and 4 × SSC for 10 min with shaking in a dark box, followed by treatment with 2 × SSC for 5 min without shaking. The chromosome preparation was stained for 30 min with propidium iodide in a dark box. Observation of chromosomes was performed with a Nikon Microphoto FXA. Chromosomes were observed in orange color by PI and signals in yellow color by FITC. Metaphase chromosomes with signal(s) were photographed with Kodak Ektachrome film (ASA 100). Chromosomes were identified according to the R banding standards [6].

Chromosomal mapping of the hAG transgene in the C57BL/6J-TgN(hAG): Of 27 metaphase chromosomes with signals, five (19%) showed twin-spot signals in the

phAGTM14 **phRNTM15**

1kb

Fig. 1. Structure of phAGTM14 and phRNTM15. The size of phAGTM14 used to detect hRN is 14 kb and that of phRNTM15 used to detect hAG is 15 kb.

(a)

(b)

Fig. 2. Chromosomal localization of hAG. (a) Metaphase spread after FISH with hAG probe, phAGTM14. Arrows indicate hybridization signals with twin dots. (b) The left picture is G-band standard, the center is R-band standard and the right is a R-banded Chr 19 with a fluorescent signal. The signal is observed in the C2 region.

Table 1. Recombination values between hAG and three microsatellite markers on Chr 19

Markers	Total No. of tested	hAG+		hAG-		χ^2	Recombination Values
		Mit ^{B6}	Mit ^{D2}	Mit ^{B6}	Mit ^{D2}		
D19Mit28 (9 cM)*	139	61	8	8	62	82.4	11.51 ± 2.65
D19Mit40 (16 cM)	139	63	6	3	67	105.7	6.47 ± 2.08
D19Mit13 (20 cM)	139	64	5	5	65	101.9	7.19 ± 2.12

*: Positions of microsatellite markers on Chr 19 are shown in parentheses.

Fig. 3. Linkage map of Chr 19. All progeny were obtained from the mating between (C57BL/6J-TgN (hAG) × DBA/2J) F1 and DBA/2J. Gene order from centromere to telomere was D19Mit28 (9 cM) - [11.51 ± 2.65 cM] - hAG - [6.47 ± 2.08 cM] - D19Mit40 (16 cM) - [0.72 ± 0.04 cM] - D19Mit13 (20 cM).

middle of two chromosomes as shown in Fig. 2a, and 22 metaphase 1–3 signals in the same position. Five metaphase chromosomes with twin-spot signals were analyzed for chromosomes according to the R-band standard [6]. As a result, it was demonstrated that the transgene of hAG was integrated in the C2 region of Chr 19 as shown in Fig. 2b. Mating experiments were performed to demonstrate the integrated site of the hAG transgene

on Chr 19 in relation to other markers. All progeny were tested for hAG, D19Mit28, D19Mit40 and D19Mit13, using PCR. As shown in Table 1, recombination values were as follows: 11.51 ± 2.65 cM between hAG and D19Mit28, 6.47 ± 2.08 cM between hAG and D19Mit40 and 7.19 ± 2.12 cM between hAG and D19Mit13. The linkage map of Chr 19 is shown in Fig. 3.

Chromosomal mapping of the hRN transgene in the C57BL/6J-TgN(hRN): Of 15 metaphase chromosomes with signals, three (20%) showed twin-spot signals in the centromere of two chromosomes as shown in Fig. 4a, and 12 metaphase 1–3 signals on the same portion. Three metaphase chromosomes with twin-spot signals were analyzed for chromosomes according to the R-band standard [6] and they were in the A1 region of Chr 6 as shown in Fig. 4b.

It is known that both the angiotensinogen (*AGT*) gene and renin (*REN*) gene are assigned on Chr 1 in humans, and the angiotensinogen (*Agt*) gene is assigned on Chr 8 and renin (*Ren1*) gene on Chr 1 in mice [4]. As shown in Figs. 2a and 4a, we did not observe any signals on mouse chromosomes 8 and 1 in this study. This means that human angiotensinogen and renin DNA probes did not hybridize with mouse homolog genes.

Seo *et al.* [5] reported that the number of copies of the hRN transgene was about 10 and Takahashi *et al.* [8] reported that the number of copies of the hAG transgene was 100 copies. In this study, we observed that the specific signals for hAG were clearly larger than those for hRN. The intensity of FISH signals and that of Southern hybridization bands corresponded.

Linkage analyses were performed to demonstrate the position of the hAG transgene on Chr 19 in relation to other markers. As a result, the hAG transgene was mapped between 9 cM and 20 cM from the centromere. According to the map of Chr 19 [2], the region from 9 cM to 20 cM corresponds to the C1, C2 and C3 bands. In the present study, FISH analyses revealed that the

Fig. 4. Chromosomal localization of hRN. (a) Metaphase spread after FISH with the hRN probe, phRNTM15. Arrows indicate hybridization signals with twin dots. (b) The left picture is the G-band standard, the center is the R-band standard and the right is a R-banded Chr 6 with a fluorescent signal. The signal is observed in the A1 region.

integrated position was C2. Therefore, data from linkage mapping and physical mapping of the hAG transgene by FISH corresponded.

It is said that the resolution ability of FISH may be inferior to that of *in situ* hybridization with isotopes, but this seems to be related to the number of gene copies. Generally, it is known that the number of copies of transgenes integrated on chromosomes in transgenic animals is more than a couple of copies. Although there may be a problem with the resolution ability of FISH, FISH is nevertheless very useful as a method for chromosomal mapping of transgenes because transgenic animals have a large number of copies of transgenes as mentioned above.

Many transgenic mice and rats have recently been produced. Various biological characteristics, not only those caused by the transgene but also the genetic data of the transgene, should be required for transgenic animals to be used as laboratory animals in biomedical research. We think that chromosome assignment of transgenes is an important topic for study.

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