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# Linkage Mapping of the *Bra, Brb* and *Brg* Genes for Rat Protein Phosphatase 2A 55 kDa B-Regulatory Subunit Isotypes

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**We previously identified the rat *Bra, Brb* and *Brg* genes, which encode α, β and γ isotypes of the 55 kDa B-regulatory subunit of protein phosphatase 2A. Polymerase chain reaction-single strand conformation polymorphism analysis in the present study identified polymorphisms in *Bra, Brb* and *Brg* between the ACI and BUF, ZI and TM, and BN and WTC strains, respectively. Linkage analysis using mapping panels composed of F2 or back-crosses of these strains allowed *Bra, Brb* and *Brg* to be assigned to chromosomes 15, 18 and 14, respectively. Furthermore, it was revealed that *Bra* is located close to the *Rb1* locus. Using polymorphism in *Bra*, loss of heterozygosity (LOH) was analyzed for rat mammary tumors induced in (SD×F344) F1 female rats by a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, and a typical mammary carcinogen, 7,12-dimethylbenz[*a*]anthracene. No LOH was detected at the *Bra* locus.**

Key words: B-regulatory subunit — Rat — Protein phosphatase 2A — Linkage mapping — Mammary tumor

Protein phosphatase 2A (PP2A), a major serine/threonine phosphatase present mainly in the cytosol fraction of most cells, plays various roles in cell physiology.[1, 2] It exists in various forms. One type which has been frequently isolated is a heterotrimeric form, consisting of the 37 kDa catalytic subunit (C), the 65 kDa A regulatory subunit (AR) and several kinds of B regulatory subunit (BR).[3, 4] The core component is the C-AR complex to which a BR binds through the AR amino-terminal region, thereby determining the substrate specificity[5] and specific activity of PP2AC.[6] Several kinds of BR, including 54 kDa, 55 kDa, 56 kDa, 61 kDa, 72 kDa (with a 130 kDa splicing variant) and 74 kDa forms, have been isolated from different tissues and organisms.[3, 4]

*In vivo* studies on *Drosophila* and yeast having mutations in the 55 kDa BR genes, *DPR55* and *CDC55*, have suggested that BR is involved in cell-cycle regulation, especially in mitosis, cellular morphogenesis, specification of tissue development and neurogenesis.[7–10] The 55 kDa species has therefore received the most attention.[11–16] It has been demonstrated by cDNA cloning that three isotypes, BRα, BRβ and BRγ, exist for rat 55 kDa BR encoded by the *Bra*,[12] *Brb*[14] and *Brg*[16] genes, respectively. *Bra* mRNA is expressed ubiquitously, whereas *Brb* mRNA is abundant in the brain and testes, but undetectable in other organs.[14] *Brg* mRNA is abundant in the brain and the spinal cord, but absent in the testes, and thus is considered to be an isotype specific for the central nervous system.[15] In addition, as hyper-phosphorylation of cellular proteins could be causative molecular events during malignant transformation of cells, mutations or deletions in these regulatory subunits of protein phosphatase could be implicated in tumorigenesis.

Laboratory rats have been used for experimental animal models of many human diseases, including cancers. In the study of alterations in genomic organization in rat models, information about polymorphisms of relevant genes should be crucially important. However, genetic information about rats is still limited when compared to the human and mouse.

In this study, we determined the chromosomal localizations of the rat *Bra*, *Brb* and *Brg* genes by linkage analysis, and performed an LOH analysis of *Bra* to clarify whether it might play a role in mammary carcinogenesis induced by the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and a typical mammary carcinogen, 7,12-dimethylbenz[*a*]anthracene (DMBA).

## MATERIALS AND METHODS

**Rat genomic DNA** DNA samples were obtained from the Sprague-Dawley (SD) outbred strain and the following

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB000808.

7 inbred rat strains: ACI, BN, BUF, F344, TM, WTC and ZI. The ACI and BUF DNAs were isolated as previously reported by Canzian *et al*.[17] The sources of the SD and F344, and BN, TM, WTC and ZI DNAs were reported by Toyota *et al*.[18] and Yokoi *et al*.,[19] respectively.

**Cloning and sequencing of the rat *Bra* 3′ non-coding region** *Bra* clones were isolated from a rat testis cDNA library constructed in λZapII (Stratagene, Cambridge, MA) using rat *Brb* cDNA as a probe.[14] The insert was sequenced using Sequenase version 2 (United States Biochemical, Cleveland, OH) according to the manufacturer's protocol.

**Identification of polymorphisms** Genetic polymorphisms in the *BR* gene were assessed using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. PCR was performed in a 5 μl reaction mixture containing 100 ng of rat genomic DNA, 2.5 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 50 μ*M* of dATP, dCTP, dGTP and dTTP each, 2 μCi [α-$^{32}$P]dCTP, 400 n*M* primers and 0.5 U *Taq* polymerase (TaKaRa Biomedicals, Otsu) for 30 cycles at 94°C for 30 s and 60°C for 2 min. The primers used, as shown in Table I, were designed from rat *Bra*, *Brb* and *Brg* cDNA sequences.[12, 14, 16] Where necessary, 1 μl aliquots of PCR products were digested with appropriate restriction enzymes in a 10 μl reaction mixture containing 1× reaction buffer at 37°C for 1 h, and SSCP analysis was performed as described earlier[20] in the presence or absence of 5% glycerol at room temperature. Gels were then dried and exposed to X-ray films.

**Linkage analyses for *Bra, Brb* and *Brg*** Linkage analyses for *Bra*, *Brb* and *Brg* loci were carried out using linkage panels, 1, 2 and 3, respectively: 1, the (ACI×BUF) F2 intercross panel, *n*=105, 245 markers[21] and *Rb1*[22] have been typed: 2, the (ZI×TM)×ZI backcross panel, *n*=48, 74 markers have been typed: 3, the (BN×WTC)×WTC backcross panel, *n*=48, 121 markers have been typed.[19] Genotyping of *Bra*, *Brb* and *Brg* was carried out by PCR-SSCP analysis as described above. Multipoint linkage analyses were performed using MAPMAKER/EXP ver. 3.0 for the *Bra* locus and GENELINK for the *Brb* and *Brg* loci.

**LOH analysis of *Bra* for mammary tumors induced by PhIP and DMBA** Female (SD×F344) F1 hybrid rats were placed on 5% or 23.5% corn oil AIN-76A diets (B.S. Reddy modification, Diet 11274; Dyets, Inc., Bethlehem, PA) at the age of 43 days. After 1 week, the animals in the PhIP-treated group were fed AIN-76A diet containing 150 ppm of PhIP-HCl (Nard Institute, Osaka) for 11 weeks and subsequently switched to a diet containing 100 ppm of PhIP-HCl. In the DMBA-treated group, the animals were given a single dose of 5 mg of DMBA by gavage. Pairs of DNA samples obtained from mammary tumors and normal portions of liver from each rat, the same as reported previously,[18] were used for analysis. PCR-SSCP analysis was performed for *Bra*, using BRa-N1 and BRa-6 primers (Table I) under the same conditions as described in the "Identification of polymorphisms" section.

## RESULTS

**Polymorphism of *Bra* in the 3′ non-coding region** Since no polymorphisms could be detected in the coding regions of *Bra*, *Brb* or *Brg* between SD and F344 rats (data not shown), we next examined polymorphisms in the 3′ non-coding regions of these three genes. A *Bra* clone was isolated from a rat testis cDNA library constructed in λZapII using the rat *Brb* cDNA as a probe,[13] and the insert was sequenced. Although the downstream nucleotide sequence from nucleotide position of 1729 was completely different from the one reported previously,[12] genomic PCR with a primer set across the 1729 nucleotide position, 5′-CTGTGTCAGCATTGATGTAC-3′ (BRa-4) and 5′-GGAGTGATTACAGCCAACTG-3′ (BRa-6 in Fig. 1), gave a single product with an expected size of 366 bp. As the novel sequence at the 3′ half of this PCR product was demonstrated to exist in both genomic and cDNA fragments, we concluded that the clone isolated is a part of the *Bra* gene. PCR-SSCP analysis was

Table I. Primer Sequences and Restriction Enzymes for Genotyping of *Bra*, *Brb* and *Brg* Loci

| Primer pair | Sequence | Amplified region[b] | Restriction enzyme |
|---|---|---|---|
| BRa-N1[a] | 5′-TACAGCTCATTCTTACTGTGGC-3′ | *Bra* exon VII | (–) |
| BRa-6[a] | 5′-GGAGTGATTACAGCCAACTG-3′ | | |
| BRb-2 | 5′-CACTGGAGAGTTACTAGCGA-3′ | *Brb* intron V | *Nla*III |
| BRb-3 | 5′-GGCTCTGGAATGTGCTGTAA-3′ | | |
| BRg-3 | 5′-GGGATTACCTCACTGTCAAG-3′ | *Brg* intron VI | *Alu*I |
| BRg-8 | 5′-TACTCCGCAGGTAGTCATGG-3′ | | |

*a*) The BRa-N1 and BRa-6 primers were also used for the LOH analysis.
*b*) Amplified regions were estimated from the *Drosophila PR55* gene structure.

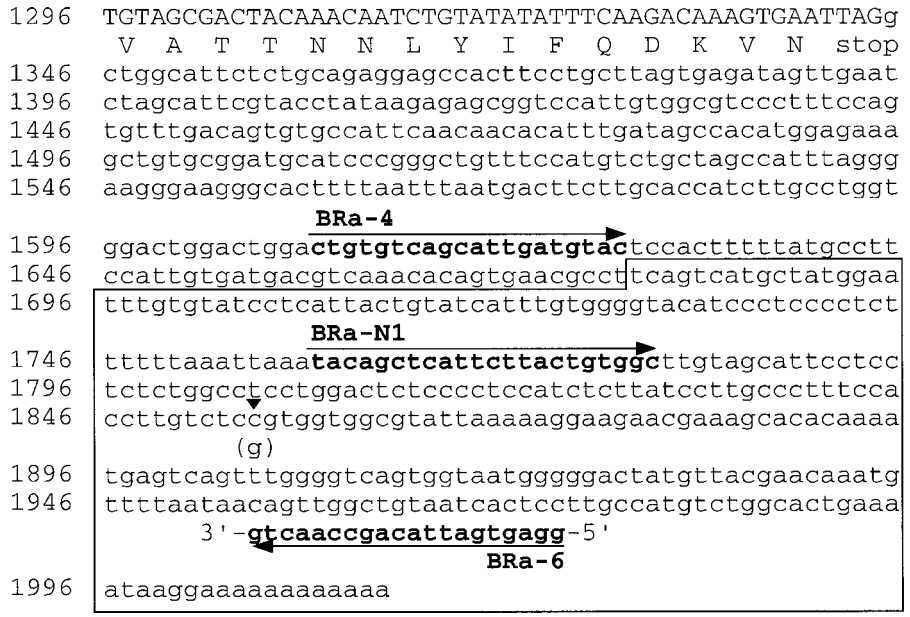


Fig. 1. Nucleotide sequence of rat *Bra* cDNA 3′ region. BRa-N1 and BRa-6 indicate primer sequences used for genotyping and LOH analysis of *Bra*. BRa-4 was also used to amplify genomic DNA fragments. The triangle indicates C/G polymorphism at nucleotide position 1855. The boxed region did not coincide with the previously reported sequence.

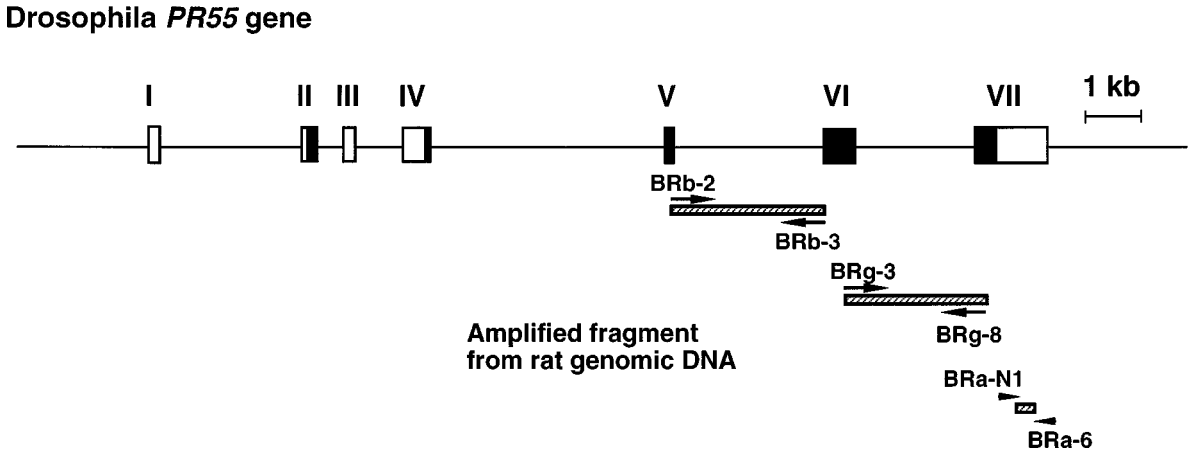


Fig. 2. Primers used for genotypings were designed from rat cDNA sequences referring to the *Drosophila PR55* gene genomic structure. Exons are depicted as boxes, open and closed for non-coding and coding sequences, respectively. Primer pair positions corresponding to the *Drosophila PR55* gene are indicated by short arrows. Amplified regions of rat *Bra*, *Brb* and *Brg* were estimated to be a part of the non-coding region of exon VII, the entire intron V and the entire intron VI, respectively.

then performed as described[22] on eight rat strains using the primer set BRaN-1 and BRa-6 (Fig. 1). PCR-SSCP analysis demonstrated shifts of the mobility of bands for ACI, WTC and SD rats from those of BUF, BN, ZI, TM and F344 strains (Fig. 3). Nucleotide sequencing of the PCR product from an SD rat revealed a C→G substitution at nucleotide position 1855 (Fig. 1). We were not able to detect any shifted bands in the 3′ non-coding exons of *Brb* or *Brg* by SSCP analysis (data not shown).

**Polymorphism of *Brb* in putative intron V** A *Brb* genomic fragment containing an intron sequence was able to be amplified by PCR with a primer set, BRb-2 and BRb-

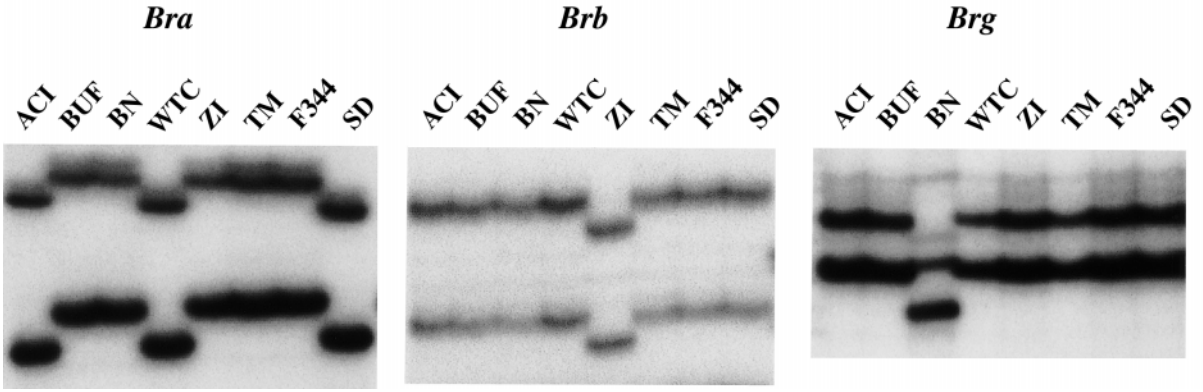


Fig. 3. Polymorphisms detected in *Bra*, *Brb* and *Brg* among eight rat strains by PCR-SSCP analysis. ACI, SD and WTC for *Bra*, ZI for *Brb* and BN for *Brg* are segregated from other strains. Using these polymorphisms, genotyping was performed.

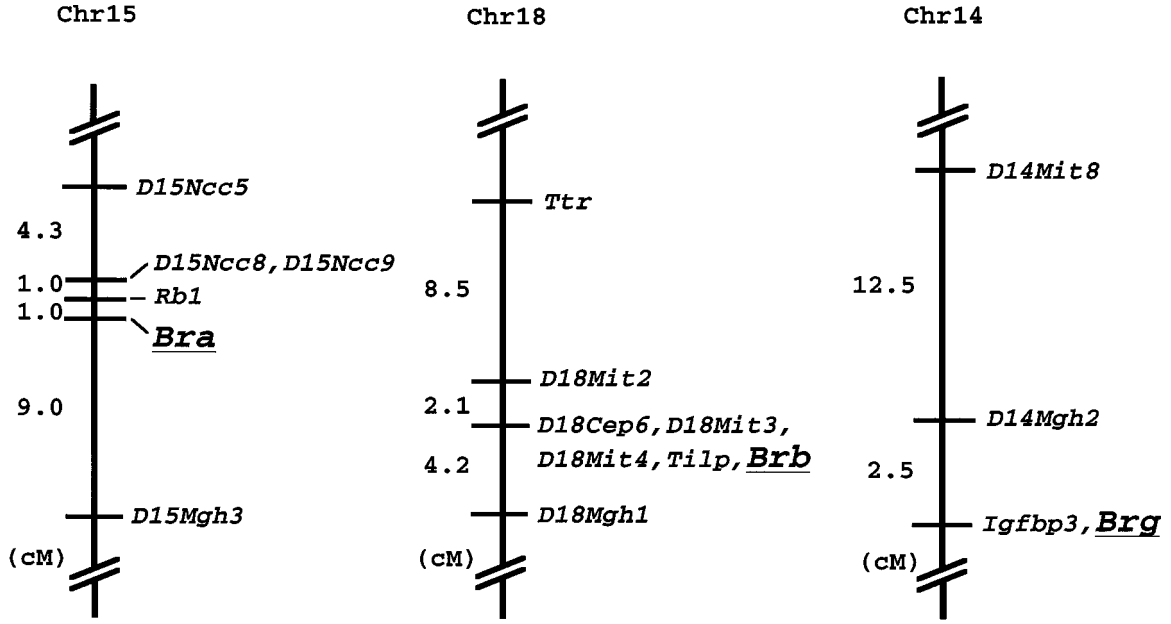


Fig. 4. Locations of rat *Bra*, *Brb* and *Brg* on Chr 15, 18 and 14. The recombinational distances between markers are shown on the left.

3, designed from sequences corresponding to the 3′ end of exon V and the 5′ end of exon VI of the *Drosophila Brb* homologue, *PR55* (Fig. 2 and Table I). PCR products from eight rat strains, which were all about 2.8 kb in length, were digested with *Nla*III and analyzed by SSCP. Only the ZI rat showed a band segregated from those of the other strains (Fig. 3).

**Polymorphism of *Brg* in putative intron VI** A *Brg* genomic fragment containing an intron sequence was also able to be amplified as well as *Brb* using primers BRg-3 and BRg-8, designed from sequences corresponding to the 3′ end of exon VI and the 5′ end of exon VII of the *Drosophila* homologue *PR55*, respectively (Fig. 2 and Table I). Approximately 2.3 kb PCR products from eight rat strains were digested with *Alu*I and analyzed by SSCP. Only the BN case showed a shifted band (Fig. 3).

**Linkage mapping of rat *Bra*, *Brb* and *Brg*** One-hundred and five (ACI×BUF) F2 progeny were typed for the

*Bra* locus by PCR-SSCP analysis. Multipoint linkage analysis was performed with 246 genetic markers, and the *Bra* was assigned to chromosome 15, 1 cM away from *Rb1* with a lod score of 35.0. Forty-eight (ZI×TM)×ZI backcross progenies were typed for the *Brb* intron V polymorphism. Multipoint linkage analysis with 74 genetic markers allowed mapping to the same locus as *Tilp* on chromosome 18, which is 10.6 cM away from *Ttr* ($P$<0.001). Forty-eight (BN×WTC)×WTC backcross progenies were typed for the *Brg* intron VI polymorphism. *Brg* was mapped to the same locus as *Igfbp3* on chromosome 14 with 121 genetic markers ($P$<0.001). Chromosomal localizations of *Bra*, *Brb* and *Brg* are depicted in Fig. 4 along with the relative genetic distances between the adjacent markers.

**LOH analysis for *Bra* in mammary tumors induced by PhIP and DMBA** LOH analysis was performed by PCR-SSCP analysis using the *Bra* polymorphism between F344 and SD as described above. Sixteen PhIP-induced mammary tumors and 5 DMBA-induced mammary tumors were analyzed. No LOH was detected in any case (data not shown).

## DISCUSSION

The present study demonstrated the coding regions of *Bra*, *Brb* and *Brg* to be very well conserved among rat strains, with polymorphisms only detected in non-coding exon or intron sites. Further, nucleotide sequencing of PCR products including introns V and VI revealed the exon-intron boundaries of *Drosophila*[9] to be well conserved in rats (data not shown).

In the *Bra* case, we were able to detect polymorphisms between ACI and BUF, and F344 and SD. Since ACI and BUF exhibit different susceptibilities with regard to stomach carcinogenesis,[23] and F344 and SD differ in terms of mammary carcinogenesis,[24] this information may be useful for assessing any involvement of the *Bra* gene. As we noted earlier, deterioration of phosphatase activity brings similar consequences for cells to activation of protein kinases, which is commonly observed in transformed malignant cells. However, the lack of LOH in tumors induced in (SD×F344) F1 rats by administration of a diet containing PhIP or by a single subcutaneous injection of DMBA suggests that *Bra* is not playing a role as a typical tumor suppressor gene in mammary carcinogenesis induced by DMBA and PhIP. At present, however, we can not exclude the possibility that the *Bra* gene is altered in more progressed tumors or cell lines, or that the *Bra* gene is hemizygously mutated, or that *Bra* expression is suppressed without any genetic change. *Rb1* is closely localized to *Bra* on Chr 15,[22] only 1 cM away, and no LOH was detected in any case at the *Rb1* locus either.

The involvement of either *Brb* or *Brg* in mammary tumorigenesis could not be evaluated because of the lack of polymorphism between SD and F344 rats.

*Brb* was assigned to Chr 18, 10.6 cM away from *Ttr*. Chromosomal loci of human *BRB* and mouse *Brb* have yet to be assigned, but both human *TTR* and mouse *Ttr* are present on Chr 18.[25] Therefore, the portion of rat Chr 18 containing the *Brb* and *Ttr* loci may correspond to parts of human and mouse Chr 18. The allele-frequency of polymorphism we detected in *Brb* was 12.5%, and the ZI strain having this allele has not been commonly used in carcinogenesis studies. The allele frequency of the polymorphism we detected in *Brg* was also 12.5% with the BN strain, so the situation is similar to that of *Brb*. Polymorphism with a higher allele frequency would be desirable in analyzing and deducing the biological relevance of segregated alleles in experimental animal models of carcinogenesis.

Rat *Brg* was assigned to chromosome 14, at the same locus as *Igfbp3*. A cDNA clone showing high homology with *BRA* and *BRB* was previously mapped to human chromosome 4.[26] We compared its nucleotide and predicted amino acid sequences with those of our rat *Brg*, and found that it covered 28.2% of the *Brg* coding region, with identities of 90.5% at the nucleotide and 99.2% at the amino acid level. Thus, the gene mapped on Chr 4 appears to be the human *BRG*. It is already established that human Chr 4p and proximal parts of q correspond to rat chromosome 14.[25] Since BRγ is expressed in the central nervous system,[15, 16] it is intriguing to speculate that mutations of this gene may be implicated in the development of neural tumors.

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