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Mapping of the calcium-sensing receptor gene (CASR) to human Chromosome 3q13.3-21 by fluorescence in situ hybridization, and localization to rat Chromosome 11 and mouse Chromosome 16

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Abstract. The calcium-sensing receptor (CASR), a member of the G-protein coupled receptor family, is expressed in both parathyroid and kidney, and aids these organs in sensing extracellular calcium levels. Inactivating mutations in the CASR gene have been described in familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT). Activating mutations in the CASR gene have been described in autosomal dominant hypoparathyroidism and familial hypocalcemia. The human CASR gene was mapped to Chromosome (Chr) 3q13.3-21 by fluorescence in situ hybridization (FISH). By somatic cell hybrid analysis, the gene was localized to human Chr 3 (hybridization to other chromosomes was not observed) and rat Chr 11. By interspecific backcross analysis, the *Casr* gene segregated with *D16Mit4* on mouse Chr 16. These findings extend our knowledge of the synteny conservation of human Chr 3, rat Chr 11, and mouse Chr 16.

Introduction

The calcium-sensing receptor (CASR), a member of the G-protein coupled receptor family, is expressed in parathyroid (Brown et al. 1993; Garrett et al. 1995), kidney (Riccardi et al. 1995), and brain (Ruat et al. 1995), and aids these organs in "sensing" extracellular calcium levels. Several studies have now documented that individuals with familial hypocalciuric hypercalcemia (FHH) are heterogeneous, and individuals in these families with neonatal severe hyperparathyroidism (NSHPT) are homozygous for CASR genes having inactivating mutations (Pollack et al. 1993; Pearce et al. 1994; Heath et al. 1994; Janicic et al. 1995). Chou and associates (1992) first mapped the FHH disease locus to 3q21-24 by linkage analysis. Heath and colleagues (1993) also mapped the locus to 3q by linkage analysis, but determined it to be more centromeric in region 3q11.1-12. In another study (Janicic et al. 1995), by haplotype analysis with 3q markers in two families with FHH/NSHPT and assessment of recombination events, the locus could be assigned to 3q13-21. Finegold and coworkers (1994) mapped the disease locus in a family with autosomal dominant hypoparathyroidism to 3q13 by linkage analysis. This group later showed that affected family members had one copy of the CASR gene having a missense 'activating' mutation (Perry et al. 1994). Pollak and associates (1994) reported an activating mutation in CASR segregating with the affected status in a family with autosomal dominant hypocalcemia.

In addition, the FHH trait demonstrates genetic heterogeneity. Heath and colleagues (1993) reported one family in which the disease locus segregated with 19p markers rather than those for 3q, and Trump and coworkers (1995) described another family in which the affected status segregated with neither 3q, 19p, nor 11 markers. This raises the possibility of the existence of other related CASR genes at loci other than 3q in the human genome.

We report here the mapping of the human CASR gene to 3q by an independent method, that of fluorescence in situ hybridization (FISH), and the chromosomal localization of the *Casr* in the rat and mouse genomes.

Materials and methods

Probe preparation. Human CASR cDNAs corresponding to exons IV and VII were prepared by polymerase chain reaction (PCR) amplification of genomic DNA with the following primer pairs. Exon IV: forward primer IVF, 5′-ACTCATTCACCATGTTCTTGGTTCT-3′, and reverse primer IVR, 5′-CCCAACTCTGCTTTATTATACAGCA-3′; the product size was 0.95 kb; Exon VII: forward primer VIIF, 5′-AAGTGCCCAGATGACTTCTGGTCCA-3′, and reverse primer VIIR, 5′-CCATGGCGTTCTTCTGAGGCTCATC-3′; the product size was 1.2 kb. The products were cloned into the pCRII TA vector (Invitrogen).

Somatic cell hybrids. Mapping of the human and rat genes was carried out with somatic cell hybrids. Two panels of somatic cell hybrids were used: a panel of human × mouse (HA and HB series) or human × rat (HR and JV series) hybrids that segregate human chromosomes, and a panel of rat × mouse (LB) hybrids that segregate rat chromosomes. Both panels were described previously and have been used to localize several human and rat genes respectively (see Pausova et al. 1994, and references therein). DNA was extracted and analyzed by the Southern blot method (Southern 1975) after blotting to nylon (Nytran) membranes. Blots were hybridized separately with both the CASR exon IV and exon VII probes after the plasmid inserts had been labeled with [^{32}P] by the random primer method.

FISH detection system and image analysis. The Exon VII probe was prepared by PCR amplification of genomic DNA with the following primer pairs: forward primer, VIIF described above, and reverse primer FISH R, 5′-TCTTCCTCAGAGGAAAGGAGTCTGG-3′; the product size was 1.5 kb. Mapping of the probe was performed by fluorescence in situ hybridization (FISH; Lichter et al. 1990) to normal human lymphocyte chromosomes counterstained with propidium iodide and 4′,6-diamidin-2-phenylindol-dihydrochloride (DAPI). The biotinylated probe was detected with avidin-fluorescein isothiocyanate (FITC). Images of metaphase preparations were captured by a thermoelectrically cooled charge coupled cam-

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era (Photometrics, Tucson, Ariz.). Separate images of DAPI-banded chromosomes (Heng and Tsui 1993) and of FITC targeted chromosomes were obtained. Hybridization signals were acquired and merged by use of image analysis software and pseudo-colored blue (DAPI) and yellow (FITC) as described (Boyle et al. 1992) and overlaid electronically.

Interspecific backcross mice. C3H/HeJ-*gld* and *Mus spretus* (Spain) mice and [(C3H/HeJ-*gld* × *Mus spretus*)F_1 × C3H/HeJ-*gld*] interspecific backcross mice were bred and maintained as previously described (Seldin et al. 1988). *Mus spretus* was chosen as the second parent in this cross because of the relative ease of detection of informative restriction fragment length variants (RFLV) in comparison with crosses using conventional laboratory strains.

DNA isolated from mouse organs by standard techniques was digested with restriction endonucleases, and 10-μg samples were electrophoresed in 0.9% agarose gels. DNA was transferred to Nytran membranes (Schleicher & Schull, Inc., Keene, N.H.), hybridized at 65°C, and washed under stringent conditions, all as described previously (Watson and Seldin 1994). Gene linkage was determined by segregation analysis (Green 1981). Gene order was determined by analyzing all haplotypes and minimizing crossover frequency between all genes that were determined to be within a linkage group. This method resulted in determination of the most likely gene order (Bishop 1985).

Results

Somatic cell hybrids. The human CASR gene. In *Pvu*II-digested human DNA, the exon IV probe detected a 3.1-kb fragment, and the exon VII probe detected a ~25-kb fragment that could be distinguished from the homologous mouse or rat fragments (data not shown). In mouse × human or rat × human cell hybrids, the 3.1-kb and ~25-kb human fragments segregated clearly with human Chr 3. No discordancy was found with either probe for Chr 3, while at least two (of 14), for the exon IV probe, and four (of 13), for the exon VII probe, discordant clones were counted for each of the other chromosomes (Table 1).

The rat Casr gene. The exon IV probe detected a 7.2-kb *Pvu*II fragment in rat DNA, distinct from the mouse restriction fragment (data not shown). This 7.2-kb rat fragment was present in all rat × mouse hybrids containing rat Chr 11. At least four (of 13) discordant clones were counted for each of the other chromosomes (Table 2). These data show that the *Casr* gene resides on rat Chr 11. Confirmatory data were obtained with the exon VII probe (not shown).

FISH analysis. The regional assignment of the 1.5-kb biotinylated cDNA probe was determined by the analysis of approximately 50 well-spread human metaphases. Hybridization signals were visualized by CCD camera, and scoring for the number of positive cells was 20%. Positive cells demonstrated doublet hybridization signals at 3q13.3-21 on one or both chromosome homologs (Fig. 1). The band assignment was determined by measuring the fractional chromosome length and by analyzing the banding pattern generated by the DAPI counterstained image. No hybridization signals distinct from background were visualized on any other chromosome including 19.

Mouse chromosomal localization and fine mapping. To determine the chromosomal location of the *Casr* gene, we analyzed a panel of DNA samples from an interspecific cross that has been characterized for over 800 genetic markers throughout the genome. The genetic markers included in this map span between 50 and 80 centiMorgans on each mouse autosome and the X Chr (for example, see Saunders and Seldin 1990 and Watson et al. 1992). Initially, DNA from the two parental mice [C3H/HeJ-*gld* and (C3H/HeJ-*gld* × *Mus spretus*)F_1] were digested with various restriction endonucleases and hybridized with the HCAR4 cDNA probe to determine restriction fragment length variants (RFLVs) to allow haplotype analyses. An informative *Pvu*II RFLV was detected: C3H/HeJ-*gld*, 10.0 kb; *Mus spretus*, 6.8 kb, 3.5 kb.

Comparison of the haplotype distribution of the *Casr* RFLV indicated that this gene cosegregated in 113 of 114 meiotic events with the microsatellite marker *D16Mit4* locus on mouse Chr 16 (Fig. 2). The haplotype distribution among other genes localized to mouse Chr 16 indicated that the best gene order (Bishop 1985) ± the standard deviation (Green 1981) is (centromere) *D16Mit4*–0.9 cM ± 0.9 cM–*Casr*–9.6 cM ± 2.8 cM–*Gap43*.

Discussion

It was reported previously that by Southern blot the CASR cDNA hybridized with restriction fragments present in digests of hamster–human hybrid cell DNA containing only human Chr 3, but not cell hybrid DNA containing only Chr 12 (Pollack et al. 1993). We

Table 1. Segregation of human CASR gene.

Hybrids	Human CASR gene[a]	Human chromosomes[b]																						
		X	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Y
HA13	−	+	+	−	−	+	+	+	−	+	−	−	+	+	−	−	−	+	−	−	−	+	+	−	−
HA212	+	−	+	−	+	−	−	−	−	−	−	−	+	−	−	−	+	−	+	−	−	−	+	−	−
HA221	−	+	−	−	−	+	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−	+	+	−	−
HA232	+	−	−	−	+	+	−	+	−	−	−	−	+	−	−	(+)	+	+	−	−	−	−	+	+	−
HB26	+	+	−	+	+	−	−	+	+	−	−	+	(+)	−	+	+	+	+	+	+	(+)	+	+	+	−
HB29	+	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
HB33	+	−	−	−	(+)	(+)	−	+	−	+	−	−	(+)	−	−	+	−	−	(+)	−	−	−	−	+	−
HB43	+	+	+	+	+	+	−	+	+	+	−	−	+	+	−	+	+	+	+	+	+	+	+	+	−
HB111	+	−	−	−	+	(+)	−	−	−	(−)	−	−	+	+	−	+	−	−	+	−	−	−	+	−	−
HB112	+	−	−	(+)	+	−	−	−	−	(+)	−	−	+	(+)	−	+	−	−	+	−	−	−	+	−	−
HB142-2	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	+	−	−	−
HB181	+	−	−	+	+	−	−	+	+	+	−	−	+	+	+	+	+	−	+	+	+	+	+	+	−
JV211	+	−	(+)	+	+	(−)	+	+	+	+	−	+	+	+	(+)	+	−	+	+	−	−	+	−	+	−
HR40C8	−	+	−	−	−	−	−	−	+	−	(−)	+	−	+	+	+	+	(−)	+	+	+	(+)	+	(+)	−
Independent discordant clones[c]:		9	6	4	0	7	7	4	5	3	7	6	2	5	5	3	5	5	3	5	5	6	5	3	7

[a] A + or − indicates the presence or absence of the human gene, respectively.

[b] A + indicates that the human chromosome is present in more than 55% of the metaphases; (+) indicates that the human chromosome is present in 25–55% of the metaphases; (−) indicates that the human chromosome is present in less than 25% of the metaphases; − indicates that the human chromosome is absent.

[c] Independent hybrid clones derived from distinct fusion events. They are identified by unrelated numbers (non-independent clones are: HB26 and 29, HA212, 221 and 232, HB111 and 112). When a chromosome was present in less than 25% of the metaphases (− in parentheses), the hybrid in question was not taken into account to establish the number of discordances for that particular chromosome.

Table 2. Segregation of rat *Casr* gene.

Hybrids	Rat *Casr* gene[a]	Rat chromosomes[b] X	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
LB20	–	+	–	(+)	(+)	–	–	(–)	+	–	–	–	–	+	+	–	–	+	(+)	+	+	–
LB150-1	+	+	–	–	+	+	–	–	+	–	+	(+)	+	+	+	–	–	(+)	(+)	+	+	–
LB161	–	+	–	+	+	+	+	+	+	–	+	+	–	(+)	+	+	+	+	+	+	+	(+)
LB210-I	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	–
LB251	–	+	+	+	–	+	–	(+)	+	–	–	+	–	+	+	–	–	–	+	–	+	–
LB330	–	+	–	+	+	+	–	+	–	–	–	+	–	+	–	–	–	–	+	–	–	–
LB510-6	–	+	–	+	+	+	–	–	+	–	–	–	–	+	+	+	+	+	+	+	–	–
LB600	+	+	+	+	+	+	+	(+)	+	–	(–)	+	+	+	+	+	+	+	–	+	+	–
LB630	+	+	(–)	–	+	+	(+)	+	+	–	+	–	+	+	+	(+)	+	+	–	+	+	(–)
LB780-8	+	+	–	+	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	+	–	–
LB810	+	+	–	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	+	–	+	(+)
LB860	+	+	–	+	+	+	–	–	+	–	+	–	+	+	+	–	+	+	+	+	–	(+)
LB1040TG1	+	–	–	–	–	+	–	+	–	–	–	–	+	(+)	–	–	+	+	–	+	–	–
Independent discordant clones[c]:		7	6	8	6	5	6	6	5	6	4	6	0	6	7	7	4	4	9	5	6	5

[a] A + or – indicates the presence of absence of the rat gene respectively.
[b] A + indicates that the rat chromosome is present in more than 55% of the metaphases; (+) indicates that the rat chromosome is present in 25–55% of the metaphases; (–) indicates that the rat chromosome is present in less than 25% of the metaphases; – indicates that the rat chromosome is absent.
[c] Independent hybrid clones derived from distinct fusion events. In this table, all clones are independent. When a chromosome was present in less than 25% of the metaphases (– in parentheses), the hybrid in question was not taken into account to establish the number of discordances for that particular chromosome.

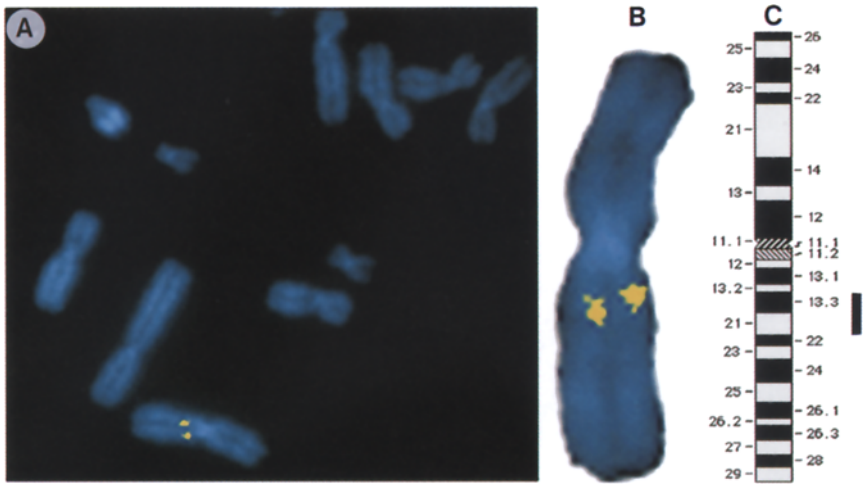

Fig. 1. Regional mapping of the CASR gene by fluorescence in situ hybridization (FISH) to normal human lymphocyte chromosomes counterstained with DAPI. Biotinylated probe was detected with avidin-DCS-fluorescein isothiocyanate (FITC). Separate images of DAPI-counterstained metaphase chromosomes and of hybridization signals were captured and overlaid electronically as described in Methods. Part of a representative metaphase preparation (A) is shown to indicate the position of HCAR(7) probe FISH signals that are visible as yellow fluorescent spots. A DAPI-banded Chr 3 (B), together with schematic ideogram (C) is shown to indicate localization to band 13.3-21.

have confirmed this result here and extend the analysis to show that CASR cDNA did not hybridize to any of the other human chromosomes under the stringent hybridization conditions used. Under relaxed stringency hybridization conditions the probe weakly hybridized to several other restriction fragments (data not shown), but we were not able to demonstrate segregation of these fragments to any particular chromosome. Thus, the CASR gene appears to be a single-copy gene, and it remains to be determined whether related members of a 'CASR gene family' exist in the genome. The most closely structurally related G protein-coupled receptors to the CASR are the metabotropic glutamate receptors which comprise a gene family (Nakanishi 1992). By FISH analysis, we mapped the CASR to Chr region 3q13.3-21. This clarifies the data provided by the linkage analysis studies described above, and more precisely maps the CASR locus.

By Southern blot analysis the *Casr* also appeared to be encoded by a single-copy gene in the rat and mouse. The *Casr* mapped to rat Chr 11 and mouse Chr 16, which are known to be homologous and to show synteny conservation with human Chr 3 (Levan et al., 1991; Nadeau et al., 1991; Yamada et al., 1994; Takada et al., 1995). Human Chr 3 is defined by at least 12 ho-

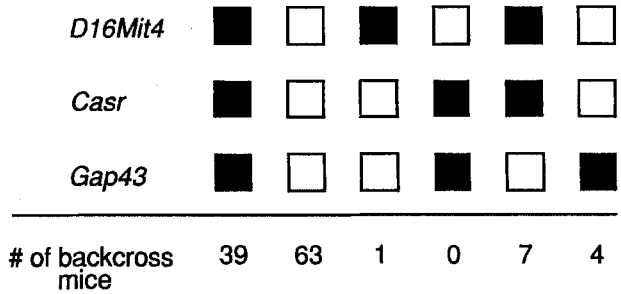

Fig. 2. Segregation of *Casr* on mouse Chr 16 in [(C3H/HeJ-*gld* × *Mus spretus*) F_1 × C3H/HeJ-*gld*] interspecific backcross mice. Filled boxes represent the homozygous C3H pattern, and open boxes the F_1 pattern. The mapping of the reference loci has been described previously (Reeves et al. 1991; Reeves and Citron 1994).

mology clusters located on seven different chromosomes in rat and mouse (Hino et al. 1993). Assignment of CASR to human 3q13.3-21, rat Chr 11, and mouse Chr 16 is consistent with these known homology relationships (Hino et al. 1993). Few loci have been

assigned to rat Chr 11: only 10, relative to 69 for Chr 1 (see Yamada et al. 1994). There are no known mutations involving this region of mouse Chr 16 that would correspond to the FHH/NSHPT phenotype in humans. The nearest mouse mutation on Chr 16 is *aku* (alkaptonuria).

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