Cloning and Characterization of Two Promoters for the Human Calcium-sensing Receptor (CaSR) and Changes of CaSR Expression in Parathyroid Adenomas*

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Histological analyses showed that expression of the parathyroid calcium-sensing receptor (CaSR) is decreased in parathyroid adenomas. Because reduced expression of CaSR may result in insufficient suppression of parathyroid hormone secretion, the elucidation of regulatory mechanisms of CaSR expression is indispensable for understanding the pathogenesis of parathyroid adenomas. Two cDNA clones for human CaSR with different 5'-untranslated regions have been isolated. However, the structure of the promoter region of human CaSR and the mechanisms of production of multiple CaSR mRNAs are unknown. We have cloned promoter regions of human CaSR by screening a genomic library. The human CaSR gene has two promoters and two 5'-untranslated exons (exons 1A and 1B), and alternative usage of these exons leads to production of multiple CaSR mRNAs. The upstream promoter has TATA and CAAT boxes, and the downstream promoter is GC-rich. Northern blot analysis showed that expression levels of exon 1A in parathyroid adenomas are significantly less than those in normal glands. However, expression of exon 1B was not different between adenomas and normal glands. Thus, specific reduction of the transcript driven by the upstream promoter was observed in parathyroid adenomas. Further analyses of factors that modulate the activity of the upstream promoter are necessary to clarify the pathogenesis of parathyroid adenomas.

The parathyroid calcium-sensing receptor (CaSR) plays pivotal roles in the regulation of parathyroid hormone (PTH) secretion and hence serum calcium levels. Binding of calcium to CaSR induces activation of phospholipase C and inhibition of PTH secretion (1, 2). Derangements of this negative feedback system between serum calcium and PTH secretion change the set point of PTH secretion and serum calcium levels. In some hypercalcemic diseases, the elevation of the set point of PTH secretion has been reported. For example, inactivating mutations of CaSR result in inadequate suppression of PTH secretion and inappropriately high serum levels of PTH in patients with familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism (3–7). In contrast, no mutation in the CaSR gene has been reported in parathyroid adenomas from patients with primary hyperparathyroidism, the most common cause of hypercalcemia (8). However, it has been shown by immunohistochemistry and in situ hybridization that the expression of CaSR is decreased in parathyroid adenomas (9–11). Because reduced expression of CaSR may also result in insufficient suppression of PTH secretion, the elucidation of the regulatory mechanisms of CaSR expression is indispensable for understanding the pathogenesis of parathyroid adenomas.

The coding region of human CaSR is coded by six exons. In addition, two cDNA clones for human CaSR with different 5'-untranslated regions have been isolated from parathyroid adenoma (2). Therefore, it is suggested that the translation start site of CaSR is in exon 2, and there is at least one 5'-untranslated exon in the human CaSR gene (6). However, the structure of the promoter region of human CaSR and the mechanism of the production of multiple CaSR mRNAs are unknown. In this study, we have identified and characterized the promoter regions of human CaSR by screening a genomic library. The expression levels of multiple mRNAs in normal and adenomatous parathyroid glands were also analyzed. The results indicate that the human CaSR gene has at least two promoters and that alternative usage of 5'-untranslated exons leads to the production of multiple CaSR mRNAs. In addition, the expression of CaSR mRNA produced by one of the two promoters of the CaSR gene is specifically reduced in parathyroid adenomas.

EXPERIMENTAL PROCEDURES

Materials—Parathyroid adenoma tissues were obtained from patients who underwent surgical treatment for primary hyperparathyroidism after informed consents were obtained. All these patients had grossly abnormal single adenomas. Normal parathyroid glands were also obtained from some of these patients because of associated thyroid diseases. The tissues were frozen in liquid nitrogen as soon as possible and stored at −80 °C until use. The Megaprime DNA labeling system, [α-32P]dCTP, and Hybond-N were obtained from Amersham Pharmacia Biotech (Tokyo). The human genomic library was provided by the Japanese Cancer Research Resources Bank. Taq and LA Taq polymerases were purchased from TaKaRa (Otsu, Japan). The TA cloning kit and pCDNA3 were from Invitrogen (Carlsbad, CA). pBluescript II SK was from Stratagene (La Jolla, CA). The dual luciferase reporter assay system and the PGL3-basic vector were from Promega (Madison, WI).

PCR for the 5′-Region of CaSR Using Genomic DNA as a Template—Genomic DNA was extracted from peripheral leukocytes as described...
(12). PCR using genomic DNA and LA Taq polymerase was conducted to clarify the relationship between exons in the 5'-region of the CaSR gene. As shown in Fig. 1, the forward primers 5'-GCTGCAGCCGGAGACCAGGCGAGCTG-3' and 5'-CTCCTGCTGGCCGACGCGAAG-3' and the reverse primers 5'-AGGTTAATGATGGGAGTGCTGAGT-3' and 5'-TCTTTAACGATGAGGACGTGAGT-3', corresponding to clones A and B, respectively, were used. PCR conditions using LA Taq polymerase were as follows: initial denaturation at 98 °C for 1 min, 30 cycles of 98 °C for 20 s and 70 °C for 1 min, and final elongation at 72 °C for 5 min.

Screening of the Genomic Library for the Untranslated Region of the CaSR Gene—The PCR product generated by the forward primer in clone A and the reverse primer in clone B was cloned by TA cloning and sequenced as described below. The insert was cut out by EcoRI and used a probe for screening the genomic library. The library screen was conducted by standard plaque hybridization method. A positive clone was isolated and purified using the polyethylene glycol precipitation method. The phage insert was mapped by restriction digestion and Southern blot analysis. A fragment of 4 kb containing the 5'-region of the CaSR gene was subcloned into the BamHI site of pBluescript II SK and sequenced. Sequencing indicated that the 5'-regions of the two different CaSR clones (clones A and B) are derived from two untranslated exons (exons 1A and 1B). In addition, to clarify the DNA sequence of the upstream region of a coding exon containing the translation start site (exon 1A), PCR using a phage library was employed (13). A reverse primer in an exon containing the translation start site (5'-CCAAATGATGAGAAGAGGCCCCCCAAG-3') (see Fig. 2) and a primer corresponding to the left arm of the phage (5'-TTATGC-GCGAGAAGATGGTTGAGGCAAT-3') were used. The conditions for PCR were one cycle at 94 °C for 2 min, 30 cycles at 98 °C for 20 s and 70 °C for 5 min, and one cycle at 72 °C for 7 min. The PCR product was subcloned by TA cloning and sequence by the same primer.

DNA Sequencing—The samples were sequenced using the PRISM Ready- Reaction dye terminator cycle sequencing kit and an ABI Model 373S-36 autosampler (Perkin-Elmer, Chiba, Japan) according to the manufacturer's instructions.

RNA Extraction—Total RNA was prepared from each tissue by the acid guanidinium thiocyanate-phenol-chloroform method described previously (14). 5'-Rapid Amplification of cDNA Ends (5'-RACE) —5'-RACE was carried out using a 5'-RACE system (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, total RNA (0.9 μg), isolated from human parathyroid, was reverse-transcribed, and the first-strand cDNA was purified using GlassMAX DNA isolation spin cartridges. The tailed cDNA was then diluted with 950 μl of serum-free medium and added to Transfection membrane containing the same samples were prepared. Initial hybridization was conducted using a DNA probe for the full-length coding region of CaSR generated by the Megaprime DNA labeling system (12). To determine the expression levels of exons 1A and 1B separately, PCR products corresponding to exons 1A and 1B were cloned by TA cloning. The primers used were 5'-GCTGCAGCCGGAGAGATGAGCTG-3' and 5'-AGGTTAATGATGGGAGTGCTGAGT-3', but their 5'-flanking region and in exon 1A, respectively (see Fig. 2). Therefore, to test the promoter activity of the CaSR gene, pBluescript II containing the same sample was prepared. Initial hybridization was conducted using a DNA probe for the full-length coding region of CaSR generated by the Megaprime DNA labeling system (12). To determine the expression levels of exons 1A and 1B separately, PCR products corresponding to exons 1A and 1B were cloned by TA cloning. The primers used were primer-driven [α-32P]UTP-labeled antisense riboprobes were prepared using a Maxiscript kit (Ambion Inc., Austin, TX). Each membrane was hybridized with a probe for either exon 1A or 1B, respectively. The filters were washed to the high stringency of 0.1× SSC at 65 °C and exposed to X-Omat AR (Eastman Kodak Co.). The filters were then stripped and rehybridized with a cDNA probe for GAPDH. The amount of mRNA in each lane was quantified using a densitometer and normalized by the expression level of GAPDH.

Statistical Analyses—Statistical significance was analyzed either by one-way analysis of variance followed by Bonferroni's method for comparison of multiple means or by Student's t test. An unadjusted p value less than 0.05 was considered to be significant.

RESULTS

Cloning and Characterization of the 5'-Region of the Human CaSR Gene—Two previously reported CaSR cDNA clones (clones A and B) have the same sequence back to −242 bp from the translation start site (boldface), but their 5'-upstream regions have completely different sequences (12). The primers used for PCR using genomic DNA as a template and for exon-specific riboprobes are underlined. The translation start sites are boxed.

Dual Luciferase Reporter Assay for the Promoter Activity—Promoter activity was measured using the dual luciferase reporter assay system and a MiniLumat LB9506 luminometer (Berthold, Osaka, Japan). Cells were transfected with 0.25 μg of each reporter recombinant along with 12.5 ng of Renilla luciferase vector (pRL-SV) as an internal control. Each luciferase activity was normalized for transfection efficiency by the Renilla luciferase assay.

Northern Blot Analysis—6 μg of total RNA from normal parathyroid glands and 15 μg from adenoma tissues were electrophoresed on a formaldehyde-containing 1.5% agarose gel and transferred to Hybond-N. Each sample was electrophoresed twice, and two Hybond-N membranes containing the same samples were prepared. Initial hybridization was conducted using a DNA probe for the full-length coding region of CaSR generated by the Megaprime DNA labeling system (12). To determine the expression levels of exons 1A and 1B separately, PCR products corresponding to exons 1A and 1B were cloned by TA cloning. The primers used were primer-driven [α-32P]UTP-labeled antisense riboprobes were prepared using a Maxiscript kit (Ambion Inc., Austin, TX). Each membrane was hybridized with a probe for either exon 1A or 1B, respectively. The filters were washed to the high stringency of 0.1× SSC at 65 °C and exposed to X-Omat AR (Eastman Kodak Co.). The filters were then stripped and rehybridized with a cDNA probe for GAPDH. The amount of mRNA in each lane was quantified using a densitometer and normalized by the expression level of GAPDH.
5'-region of human CaSR, we conducted PCR using genomic DNA as a template. PCR using the forward primer in clone A and the reverse primer in clone B produced a PCR product of ~800 bp (data not shown). However, no PCR product was observed by PCR using the forward primer in clone B and the reverse primer in clone A (data not shown). These results indicate that the exon forming the 5'-region of clone A is in the 5'-position to the exon forming the 5'-region of clone B. To isolate the promoter region of human CaSR, we labeled the 800-bp PCR product described above and screened a human genomic library. A phage clone containing the 5'-flanking region of the human CaSR gene was cloned and purified using the polyethylene glycol precipitation method. A fragment of 4 kb was subcloned into pBluescript II SK. DNA sequencing of this plasmid showed that the 5'-regions of clones A and B are derived from two different untranslated exons (exons 1A and 1B) (Fig. 2). In addition, exon 1B was not connected to the exon containing the translation start site (exon 2). However, because exon 2 was not included in the phage clone, the length of the intron between exons 1B and 2 was not evident. The DNA sequence of the upstream region of exon 2 was determined by PCR using the phage library as a template as described under “Experimental Procedures.” Sequencing indicated that the intron between exons 1B and 2 is a U2-type GT-AG intron (15). There are TATA and CAAT boxes in the upstream region of exon 1A (Fig. 2). In contrast, the region between exons 1A and 1B is GC-rich, but has no TATA box (Fig. 2). Therefore, the human CaSR gene has at least two 5'-untranslated exons, and alternate usage of these exons produces multiple CaSR mRNAs.

5'-RACE—The DNA sequences of exons 1A and 1B in Fig. 2 are the longest ones identified by 5'-RACE. 5'-RACE for clone B using mRNA from parathyroid tissue showed that clone B is at least 50 bp longer in its 5'-end than originally described (2). 5'-RACE for clone A also showed that clone A is at least 430 bp longer than the originally described clone (2) (Fig. 2).

Analysis of Promoter Activities of the Human CaSR Gene—Before examining the promoter activity of the human CaSR gene, we searched for cell lines that express CaSR. We found by Northern and Western blot analyses that MCF-7 cells express CaSR (data not shown). Therefore, we used this cell line for analyses of promoter activities. When transiently expressed in CaSR (data not shown). Therefore, we used this cell line for analyses of promoter activities. When transiently expressed in

Northern Blot Analysis of the Expression of CaSR mRNAs in Parathyroid Tissues—Hybridization with a probe for the full-coding region of CaSR identified main transcripts of ~5.4 and ~4.4–4.2 kb and a minor band of ~10 kb in both normal and adenoma tissues (Fig. 4) as already reported (2). We used riboprobes to distinguish the expression levels of exons 1A and 1B. Analyses by the exon 1A-specific riboprobe identified 5.4-, ~4–4.2-, and 10-kb transcripts, suggesting the existence of alternative splicing in 3'-untranslated exons (Fig. 4). In contrast, the exon 1B-specific riboprobe showed only a ~4–4.2-kb band (Fig. 4). Although detailed mechanisms of the production of multiple transcripts from the human CaSR gene are not clear at the moment, these results indicate that 5.4- and 10-kb transcripts are specific for exon 1A.

Expression Levels of Exons 1A and 1B in Normal Parathyroid Glands and Parathyroid Adenomas—The expression levels of exon 1A were evaluated by the exon 1A-specific riboprobe, and those of the 5.4-kb band were quantified and normalized by GAPDH expression. The expression levels of this transcript in adenomas were ~60% of and significantly less than those in normal parathyroid glands (p < 0.01) (Fig. 5A). In contrast, the expression levels of exon 1B were not different between nine adenomas and three normal glands (Fig. 5B). These results indicate that the reduction of the expression of CaSR in parathyroid adenomas is specific for mRNA containing exon 1A.

DISCUSSION

We have shown that the human CaSR gene has at least two promoters and 5'-untranslated exons and produces multiple mRNAs. The coding region of human CaSR is 3234 bp, and the 5'-untranslated region that is common to both exons A and B is 242 bp long (2). Two 3'-untranslated sequences of ~180 bp and 1300 bp have also been reported (2). We have shown here that exon 1A for human CaSR is ~560 bp. Therefore, using two 3'-untranslated regions, CaSR mRNAs containing exon 1A seem to be ~4.2 and 5.4 kb. The mechanism of the production
of 10-kb CaSR mRNA is not clear. However, mRNA of a similar size has also been reported in a human medullary thyroid carcinoma cell line and in other species including mouse (16–18). On the other hand, exon 1B is ~250 bp long. If the short 3’-untranslated region is used, mRNA containing exon 1B is calculated to be ~3.9 kb. The absence of larger transcripts by the exon 1B-specific probe indicates that only the short 3’-untranslated sequence is used. Although the mechanism of selective usage of the 3’-untranslated exon is unclear at the moment, probes specific for these two 3’-untranslated regions may be useful to further characterize multiple CaSR mRNAs.

Human CaSR has at least two promoters. The upstream promoter has TATA and CAAT boxes, and the downstream promoter is a GC-rich promoter without a TATA box. The promoter has TATA and CAAT boxes, and the downstream promoter regions of human CaSR is different, it is likely that the regulation of promoter activity is promoter-specific. It is also likely that these two promoters are regulated in a tissue-dependent manner. Further study employing other tissues is necessary to address these issues. Although these two promoters are active in both normal human parathyroid glands and parathyroid adenomas, the expression of the transcript driven by the upstream promoter and containing exon 1A is decreased in adenomas, whereas that driven by the downstream promoter is not altered. These results suggest that the upstream promoter activity is reduced in parathyroid adenomas. Although normal parathyroid glands were obtained from patients with primary hyperparathyroidism in our study, the expression levels of CaSR in normal parathyroid glands were similar to those from eucalcemic normal controls (11). Previous study using immunohistochemistry or in situ hybridization indicated that the expression of CaSR in parathyroid adenomas was reduced to ~60% of that in normal glands (10, 11). Our results show that the expression level of CaSR mRNA containing exon 1A in adenomas is ~60% of that in normal parathyroid glands. It is difficult to precisely analyze the relative amount of CaSR mRNAs containing exons 1A and 1B because the specific activities of probes for exons 1A and 1B are not the same. However, since both transcripts containing exons 1A and 1B are evident by Northern blot analysis, our results indicate that the difference in the expression level of CaSR between adenomas and

**Fig. 3.** Promoter activity of the human CaSR gene in MCF-7 cells. A 2.0-kb fragment of the 5’-upstream region of exon 1A (A-2.0) and a 0.5-kb upstream region of exon 1B (B-0.5) were each subcloned into the luciferase reporter vector. Promoter activity was measured using the dual luciferase reporter assay system. Cells were transfected with 0.25 μg of each reporter recombinant along with 12.5 ng of Renilla luciferase vector. Each luciferase activity was normalized for transfection efficiency by the Renilla luciferase assay. Results are mean ± S.D. from six wells. The same results were obtained in other six independent experiments. * significantly different from control vector pGL3 by one-way analysis of variance followed by Bonferroni’s method.

**Fig. 4.** Northern blot analysis of CaSR mRNA in parathyroid glands. 6 μg of total RNA from normal parathyroid glands and 15 μg from adenoma tissues were electrophoresed on a formaldehyde-containing 1.5% agarose gel. Each sample was electrophoresed twice, and two Hybond-N membranes containing the same samples were prepared. After initial hybridization with a probe for the full-length coding region of CaSR, membranes were stripped, and each membrane was rehybridized with a probe for either the exon 1A- or 1B-specific riboprobe. This figure is a representative result from a normal parathyroid gland. The same expression patterns of these bands were obtained from parathyroid adenomas.

**Fig. 5.** Expression levels of exons 1A and 1B in normal parathyroid glands and parathyroid adenomas. The expression levels of the 5.2-kb band hybridized with the exon 1A-specific riboprobe were quantified by densitometry and normalized by those of GAPDH. The expression levels of exon 1A in parathyroid adenomas were significantly reduced compared with those in normal glands (p < 0.01 by Student’s t test) (A). The expression levels of exon 1B were also evaluated using the exon 1B-specific riboprobe and normalized by those of GAPDH. The expression levels of exon 1B were not different between adenomas and normal glands (B). The results of nine adenomas and three normal glands are shown.
normal parathyroid is less than that in previous reports. This may be due to the difference of methods employed. Because the expression of CaSR is not uniform in parathyroid adenomas (23), Northern blot analysis shows the average expression level of CaSR in whole adenomas. In contrast, immunohistochemistry and \textit{in situ} hybridization would be suitable for investigating the expression pattern of CaSR in different part of adenomas.

The reduced expression of CaSR may contribute to less efficient intracellular signal transduction at a given calcium level. This leads to less suppression of PTH secretion and hence higher PTH levels. Because calcimimetics suppresses proliferation of parathyroid cells (24), it is possible that the reduction of CaSR expression also has stimulatory effects on parathyroid cell growth. Thus, derangement of CaSR expression may explain the two main characteristics of parathyroid adenoma, a higher set point for PTH secretion and enhancement of cell proliferation. However, it is not clear at the moment whether the reduction of CaSR expression can explain all the abnormalities of adenoma cells because the reduction is not prominent. Since no mutation in CaSR was reported in parathyroid adenomas (8), derangement of post-receptor signal transduction is another possibility. Furthermore, it is not known whether the reduction of CaSR expression is a direct cause of parathyroid adenoma or, conversely, the results of tumorigenesis. For example, it is possible that hypercalcemia and/or high levels of 1,25-dihydroxyvitamin D in patients with primary hyperparathyroidism affect expression of CaSR. However, our preliminary experiments indicate that neither hypercalcemia nor 1,25-dihydroxyvitamin D has an effect on the promoter activities of the human CaSR gene (data not shown). Further study is necessary to clarify the effect of systemic factors on CaSR expression because we cannot exclude the possibility that a region farther upstream has some effects on CaSR expression.

In conclusion, the human CaSR gene has two promoters and produces multiple mRNAs. Specific reduction of the expression levels of transcript driven by the upstream promoter underlies the pathogenesis of parathyroid adenomas. Further analyses of the factors that modulate the activity of the upstream promoter of CaSR will be necessary to clarify the mechanisms of reduced expression of CaSR in parathyroid adenomas.

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