Conserved Mechanism of Negative Gene Regulation by Extracellular Calcium
Parathyroid Hormone Gene versus Atrial Natriuretic Polypeptide Gene

Tomoki Okazaki,* Katsuyuki Ando, Tetsuya Igarashi, Etsuro Ogata, and Toshio Fujita
4th Department of Internal Medicine, University of Tokyo School of Medicine, Tokyo 112, Japan

Abstract

We found that a negative calcium-responsive element (nCaRE) originally reported in the human parathyroid hormone gene is conserved among several genes. The results of the present study show that expression of one of the genes, the rat atrial natriuretic polypeptide gene, was negatively regulated in the heart by extracellular calcium by using an in vivo infusion system. Moreover, transfection of the cultured cells revealed that this DNA element conferred negative regulation by extracellular calcium on the reporter gene. It is suggested that there is a gene family whose expression is negatively regulated by extracellular calcium through this conserved DNA motif, nCaRE. (J. Clin. Invest. 1992. 89:1268–1273.) Key words: negative calcium-responsive element • in vivo infusion system • transfection • gene family • nuclear protein

Introduction

Recently, the mechanisms of gene regulation have been extensively clarified. For example, steroid hormone receptors, AP-1 and cyclic AMP-response element binding protein have been identified (1–3). Almost all of the mechanisms stimulate gene transcription via formation of the specific DNA–protein complexes. On the other hand, the mechanisms of negative gene regulation or suppression of gene expression are not so well understood, with a few exceptions such as the glucocorticoid hormone receptor, which has been recently reported to suppress expression of some of its target genes through binding AP-1 (4).

We previously reported that expression of the parathyroid hormone (PTH) gene is negatively regulated by extracellular calcium (5) as well as by 1,25-dihydroxyvitamin D₃ (6). We found that the 684-bp upstream region of the PTH gene contains DNA elements that are necessary to mediate negative regulation by 1,25-dihydroxyvitamin D₃ (6). In this region, however, there is no DNA sequence homologous to the recently identified vitamin D–responsive element (7, 8) or the AP-1 binding site. On the other hand, we recently identified a DNA element that is required for negative regulation of the PTH gene expression by extracellular calcium (9). This element, negative calcium-responsive element (nCaRE), which is located at the −3.5 kbp upstream region, consists of a 15-bp palindrome with thymidine clusters ahead of it (10).

Because we found that a nuclear protein that specifically binds the nCaRE in the human PTH gene (nCaRE–PTH) exists in a wide variety of cell types (9), we assumed that there are other genes whose expressions are negatively regulated by extracellular calcium through the analogous sequence to the nCaRE. Then, we searched for the DNA sequence homologous to the nCaRE–PTH by using the EMBL gene bank. As a result, we found that five genes have DNA sequences that are identical or very homologous to the nCaRE–PTH in their 5′-flanking regions.

In the present study, moreover, we examined the effect of extracellular calcium on expression of one of these genes, the rat atrial natriuretic polypeptide (rANP) gene, in the heart. As expected, the present study showed that expression of the rANP gene is clearly negatively regulated by extracellular calcium. Transfection of the cultured cells with a reporter gene containing an rANP's DNA element that is homologous to the nCaRE (nCaRE–ANP) suggested that this DNA also functions as a negative calcium-responsive DNA element. Then, we propose here that there is a gene family whose expression is negatively regulated by extracellular calcium through binding of the nCaRE to a common putative nuclear protein(s).

Methods

Homology search by computer analysis. Using the core of the human parathyroid hormone (hPTH) gene's nCaRE, GGAGAGGGTCTCA (9, 10), we searched for the DNA sequences that have ≥14 identical bases out of 15 bases of the nCaRE in the EMBL gene bank. In Fig. 1, only the genes that share such sequences in the 5′-flanking region are listed.

Animals and in vivo infusion experiments. Male Wistar rats (7–9 wk old) were used. The femoral vein was cannulated for continuous infusion for 48 h. The contents of infused solutions are as follows (5): calcium-free solution, 20 mM NaCl, 5 mM MgCl₂, 2.5 mM KCl, and 222 mM glucose; calcium-containing solution, the same composition as in calcium-free solution but containing 40 mM CaCl₂. The infusions were carried out at a speed of 2.4 ml/h (calcium load, 96 μmol/h). By using a similar condition, we previously showed that expression of the rat PTH gene was negatively regulated by extracellular calcium (5). 48 h after infusion, blood samples; heart, including both atrium and ventricle; and kidneys were obtained from an individual rat of each condition for the subsequent experiments. Plasma volume of the individual rat was calculated by the method described in reference 10. The

---

1. Abbreviations used in this paper: BHK, baby hamster kidney; CAT, chloramphenicol acetyltransferase; (h)PTH, human parathyroid hormone; nCaRE, negative calcium-responsive element; (r)ANP, rat atrial natriuretic polypeptide; Tk, thymidine kinase.

method for measuring heart rates or blood pressure is described in reference 11.

Analysis of RNA. Total RNA was extracted from the heart or the kidneys of the rat of the condition using guanidinium–cesium chloride method (12). In Northern blot analysis, 15 μg of total RNA of the heart or the kidneys from the individual rat was loaded on a 1.5% agarose, 2.2 M formaldehyde denaturing gel and then transferred onto a nylon membrane filter. Prehybridization and hybridization were performed at 42°C under the standard condition (6). The filter was washed at 65°C three times with 1× SSC for a total of 2 h. The nick-translated probe (5 × 10⁶ cpm/μg) used in the experiments was derived from a 698-bp KpnI fragment of the rat renin cDNA (13) or a 368-bp HindII–Stul fragment of the rANP cDNA (14). The actin probe was described in reference 6.

Synthetic oligonucleotides and plasmid constructions. Oligonucleotides corresponding to the nCaRE in the hPTH gene and the rANP gene were made by a DNA synthesizer (type 8770, Biosearch, San Rafael, CA). Synthetic complementary oligonucleotides were subsequently annealed and used for ligation to construct chimeric plasmids (1). Composition of each oligonucleotide is as follows.

Oligo nCaRE–PTH (GATCG)TTTTGACAGCCTCTCTG(T) (G)AAAACTCTGTCTCCAGAAGTGAGAC(AGATC)

Oligo nCaRE–ANP (GATCG)TTTTGACAGCCTCTCTG(T) (G)AAAACTCTGTCTCCAGAAGTGAGAC(AGATC)

Oligo nCaRE–ANPmut (GATCG)TTTTGACAGCCTCTCTG(T) (G)AAAACTCGGCTCAGTATATC(AGATC)

Bases in the parentheses are BamHI and XbaI cohesive ends to facilitate subsequent ligation to a BamHI–XbaI larger fragment of PUTKAT1 (10, 15). The mutated sequences in oligo nCaRE–ANPmut are underlined.

Transfection and chloramphenicol acetyltransferase (CAT) assay. Baby hamster kidney (BHK) cells were grown in DME supplemented with 10% FCS. 2 μg of each CAT plasmid was introduced into BHK cells by the DEAE–dextran method (16). After transfection, cells were equally split into three dishes to avoid difference in transfection efficiency among dishes. 6 h later, cells were attached to the plate, and media were changed to DME with three different concentrations of extracellular calcium by adding EGTA. 40 h later, cells were harvested and the subsequent CAT assays were performed with 300 μg of cellular protein (9, 10). Average CAT activities were calculated and represented as mean±SEM after five separate transfections using ¹⁴C scintillation counting (9). A CAT activity driven by PUTKAT1 (15) in the presence of 2.0 mM extracellular calcium is arbitrarily represented as 100.

Gel retardation assay. The methods for obtaining crude nuclear extracts (from BHK cells or from the rat heart) or for the gel retardation assay was described in reference 9. An oligonucleotide nCaRE–PTH or nCaRE–ANP was used as a probe after end-labeling by [γ-³²P]ATP and T4 polynucleotide kinase. 6 μg of nuclear protein as well as 2 μg of poly (dl)–(dc) were used in each assay. BHK nuclear extracts were prepared from the cells grown in 10% FCS-supplemented calcium-free DME (Gibco Laboratories, Grand Island, NY) in which CaCl₂ was added to maintain the cells with three different extracellular calcium concentrations (0.2, 1.5, and 3.0 mM) for 48 h and each of the extracts was incubated with the ³²P-end-labeled nCaRE–ANP (see Fig. 5, lanes 10–12).

Results

Fig. 1 shows the genes that, in the 5'-flanking regions, have 14 identical DNA bases out of 15 bases of the palindromic DNA sequence of nCaRE² in the human PTH gene (TGAGACAGGGGTCTCA). We have found that three genes have identical DNA sequences and two genes have very homologous (14/15) DNA ones in their 5'-flanking regions. Very interestingly, not only the palindromic portion but also thymidine clusters or thymidine-rich sequences ahead of it are very well conserved among them, suggesting that these thymidines also play an important regulatory role.² So far, expression of these genes has not been reported to be negatively regulated by extracellular calcium as is seen in the PTH gene. However, some investigators demonstrated that secretion of rANP was suppressed by extracellular calcium (17), though the effect of extracellular calcium on its transcription as well as on its secretion is still controversial (17–19).

To examine the possibility that expression of the rANP gene is suppressed by extracellular calcium in heart, we used the in vivo rat infusion system (5). Using this system (5), we previously showed that expression of the rat PTH gene is negatively regulated by extracellular calcium in the parathyroid gland. By Northern blot analyses, we compared the level of ANP mRNA obtained from the heart infused with calcium-free solution to that obtained from the rat heart infused with calcium-containing solution. These solutions were infused for 48 h. During this period, plasma volume of the Ca-free rat (4.5±0.4 ml/100 g body wt) was not significantly different from that of the Ca-plus rat (4.6±0.1 ml/100 g body wt) (10). Mean blood pressure or heart rate in conscious rats measured by the method described in reference 11 did not significantly differ between these two groups either (data not shown). These observations potentiate the validity of our analyses, because we can attribute the difference in the levels of rANP mRNA, if any, to the difference in the level of extracellular calcium (see below). As shown in Fig. 2, the level of rANP mRNA from the heart of the calcium-infused rats (serum calcium level was 12.5–12.6 mg/dl) was markedly lower compared with that from the rats without calcium (serum calcium level was 9.6–9.8 mg/dl). On the other hand, the level of actin mRNA did not significantly differ between the two with and without calcium.

Because secretion of renin is also known to be suppressed by extracellular calcium (20), we also studied the effect of calcium on the level of renin mRNA in the rat kidney. As shown in Fig. 3, the increase in extracellular calcium did not affect the level of renin mRNA in the rat kidney. In a computer search, we found that the rat renin gene does not have DNA sequences > 80% homologous to the nCaRE, including thymidine clusters (data not shown).

We next synthesized a short DNA sequence corresponding to the putative nCaRE in the rANP gene (nCaRE–ANP) as well as a DNA sequence where five bases are mutated (nCaRE–ANPmut). The nCaRE–ANP has one base different from the palindromic portion of the hPTH gene's nCaRE (T to G; see Fig. 1) and similar thymidine clusters ahead of it. Each of these DNA sequences was ligated to the upstream portion of the herpes viral thymidine kinase (Tk) gene promoter–CAT plasmid (15)² and introduced into BHK cells. As shown in Fig. 4, reduction of extracellular calcium concentrations from 2.0 to 1.0 mM significantly elevated CAT activity driven by the plasmid containing nCaRE–ANP as much as threefold to ~ 30% of the CAT activity elicited by the parental PUTKAT1. Similar results were obtained using the hPTH gene's nCaRE (nCaRE–PTH) Tk promoter–CAT plasmid.³ On the other hand, CAT activities driven by the parental PUTKAT1 that has mutated or by no nCaRE–ANP were not affected by the same procedure. Although we have used a rather higher amount of the cellular protein (300 μg) to clearly show the difference in CAT
nCaRE

TGAGACAGGGTGCTCTCA

-3.5kb

TUTTTTTTTTTTTTTTTTTTGAGACAGGGTGCTCTCA

-748

TTTCTTTTTTTTTTTTTTTTTATGCAGGGTGCTCTCA

-1985

TAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATGCAGGGTGCTCTCA

-590

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Figure 2. Negative regulation of the rANP gene by extracellular calcium. The results of two separate experiments are shown. 15 μg of total RNA obtained from the heart of the individual rat infused with calcium-free solution [left lanes of (1) and (2)] and with calcium-containing solution (right lanes) are compared by Northern blot analysis. The levels of ANP and actin mRNA are separately shown. At the bottom, serum total calcium concentrations of the rats of each condition are indicated.

Figure 3. The level of the rat renin mRNA in kidneys. 15 μg of total RNA from the kidneys of the individual rat was analyzed. The experimental condition was the same as the one shown in the left part of Fig. 2.
protein were increased when extracellular calcium concentration was decreased. However, it did not reach the level elicited by a parental plasmid where no nCaRE was included (PUTKAT1). Such incomplete recovery of the CAT activity was also recognized when we used the nCaRE–PTH–PUTKAT1 previously. Even when we used 0.2 mM extracellular calcium adjusted by adding CaCl₂ to the Ca-free DME, CAT activity driven by these plasmids remained at the same level as that in the presence of 1.0 mM extracellular calcium (data not shown). The reasons for these observations might be twofold. First, in our plasmid, the distance between the nCaRE and the promoter region necessary for basal transcription might be too close to elicit physiological extracellular calcium–mediated transcriptional regulation. The nCaRE–PTH and the nCaRE–ANP normally exist in a far upstream region from their own transcriptional start site, respectively (Fig. 1). The plasmid in our previous report containing the nCaRE–PTH linked to the hPTH promoter- (but not the Tk promoter-) CAT gene produced the CAT activity almost equal to that generated by the parental PTH–CAT plasmid containing no nCaRE–PTH in 1.0 mM extracellular calcium concentration. In that case, the distance between nCaRE and the hPTH promoter is ~650 bp, in contrast to a distance of ~50 bp between nCaRE–ANP and Tk promoter in our present construct. Presumably, a smaller distance between the nCaRE and the promoter region would yield stronger suppression of the CAT activity by extracellular calcium. Second, the basic transcriptional apparatus functioning at the Tk promoter region might interact more tightly with the nCaRE-binding protein than the apparatus functioning at the PTH promoter region, leading to weaker reactivation of the CAT activity even in the presence of lower extracellular calcium. Both possibilities remain to be elucidated. Further, we cannot rule out the possibility that not only the nCaRE–ANP but also another DNA element(s) is required for full suppression of the rANP gene expression by extracellular calcium. This issue will be clarified more extensively by using the rANP promoter instead of the Tk promoter, which is currently under investigation in our laboratory.

![Figure 4. CAT assays in BHK cells transfected with the reporter genes containing the nCaRE–ANP, nCaRE–ANPmut, or no nCaRE. After transfection of the cells by the DEAE-dextran method, cells were split into three dishes to avoid differences in transfection efficiency among the dishes. The concentrations of extracellular calcium were manipulated by adding three different concentrations of EGTA to DME containing 10% FCS. The concentrations of extracellular calcium in each condition are shown. Average CAT activities after five independent transfections are shown in each condition as mean±SEM. The mean CAT activity driven by the parental PUTKAT1 is arbitrarily represented as 100. In this figure, one of the typical results is shown.](image)

![Figure 5. Gel retardation assays using nuclear extract from BHK cells and the rat heart. The nCaRE–PTH (lane 1) or the nCaRE–ANP (lanes 2–12) was used as a probe. A 5-fold (lanes 5 and 7), 20-fold (lanes 6 and 8), and 80-fold (lane 3) molar excess of the nonradiolabeled nCaRE–ANP was added as a competitor. In lanes 10–12, BHK nuclear extracts were obtained from the cells grown in DME containing 0.2 mM (lane 10), 1.5 mM (lane 11), and 3.0 mM (lane 12) extracellular calcium. In lanes 1 and 4–10, extracellular calcium concentration was 2.0 mM.](image)

We have not examined the possibility that the homologous sequence to the nCaRE is seen in the PTH or ANP gene of other species. Although DNA sequences of many genes have been completely identified, including their upstream portions, such identification may not cover the far upstream region where the nCaRE may usually be located (Fig. 1).

The membrane machineries that sense the level of extracellular calcium or intracellular signals followed by changes of extracellular calcium leading to transcriptional suppression by extracellular calcium have not been identified in this study. In many cells, including atrial and ventricular, changes in the level of extracellular calcium are never supposed to alter the level of intracellular calcium, which is in good agreement with the observations that the level of intracellular calcium is by far less abundant compared with the level of extracellular calcium (25). In this regard, parathyroid cells are one of few exceptions, in that changes of the level of extracellular calcium easily alter the level of intracellular calcium (26). This might suggest that the intracellular signal for extracellular calcium–mediated transcriptional suppression is intracellular calcium, because the first example of this kind of regulation was seen in the PTH gene, which is exclusively expressed in the parathyroid cells. However, the results of this study contradict this notion because negative regulation of the rANP gene was seen in the heart cells. We previously showed that an nCaRE-binding protein(s) is actually distributed among various types of cells (9, 10). As shown in Fig. 5, the rat heart cells also possess this protein, which binds to the nCaRE–ANP in a sequence-specific and extracellular calcium–dependent manner as the nCaRE–PTH–binding nuclear protein does (9). Presumably, the signal transduction machinery activated by changes of extracellular calcium is also widely distributed among many cell types.
Acknowledgments

We thank Drs. K. Nakao and A. Fukamizu for the generous gifts of the rANP and renin cDNA, respectively.

This work was supported by Grants-In-Aid for Encouragement of Young Scientists (T. Okazaki) and Grants-In-Aid for Scientific Research (E. Ogata), both from the Ministry of Education, Science and Culture of Japan and by a grant from Toyo Jozo Co.Ltd.

References