Introduction

THE mechanism of gene regulation by signal transduction molecules such as the cyclic AMP (cAMP)-A kinase or the phorbol ester-C kinase systems has been extensively studied [1, 2]. Among them, nuclear transcription factors CREB (cAMP response element binding protein) and AP1 were cloned and their molecular characteristics including structure, dimerization, subcellular localization, phosphorylation and protein-protein interactions have been vigorously clarified [1-3].

On the other hand, the mechanism of gene regulation by another second messenger, calcium (Ca), remains only partially understood. One underlying reason is that there are very few instances where external stimuli elicit transcriptional regulation only by way of activation of the intracellular Ca system. It is believed that wherever Ca plays an important role, it is almost always accompanied by substantial activation of the A kinase and/or the C kinase systems [4]. In fact, one of the recently-identified Ca-responsive DNA elements in the c-fos gene is the CRE itself [5]. Furthermore, whereas this CRE senses cytoplasmic Ca oscillation, another Ca responsive DNA element in the same c-fos gene, SRE (serum response element), is reported to handle the oscillation of nuclear Ca [5]. Therefore, the difference both in the temporal and spatial patterns of intracellular calcium signaling further complicates the mechanism of Ca-mediated gene regulation [5, 6].

The second reason is that changes in extracellular Ca (Ca_e) do not perturb the intracellular Ca level in general [7], thereby leaving the true intracellular or intranuclear messenger molecule(s) unknown. In this regard, expression of the parathyroid hormone (PTH) gene is one of a few exceptions in that Ca_e affects its transcription [8]. Parathyroid cells in which the PTH gene is exclusively expressed is one of the few cells known to transmit the signal of increased Ca_e into the cell, resulting in an increase in intracellular Ca. Such a rise in intracellular Ca is believed to be responsible for subsequent suppression of PTH secretion [7]. In this process, the recently discovered extracellular Ca-sensing receptor (CaSR) [9], is closely involved, though its relationship to transcriptional event is still not clear.

The molecular basis of transcriptional regulation of the PTH gene by Ca_e has not been reported until recently, when we found that suppression of gene expression by Ca_e was, at least in part, mediated by an interaction between two nCaREs (negative calcium responsive DNA elements) and their binding nuclear protein nCaREBs (nCaRE binding proteins). We succeeded in identifying these cis- and trans-acting molecules and analyzed the molecular mechanisms in detail [10-13]. Intriguingly, we found that this mechanism of gene regulation was not confined to regulation of the PTH gene, but was rather widely employed among many cell types and genes. Furthermore, during these studies, we also noticed that not only Ca_e, but also other extracellular stimuli such as changes in osmolarity utilized similar, if not identical,
nuclear machineries [14]. Lastly, we obtained several lines of evidence strongly suggesting that one of the nCaREBs identified here also plays an important role in another distinct, but linked, transcriptional event, i.e., negative gene regulation by vitamin D [15].

The Structure of the PTH Gene

So far, human, bovine, rat and chick PTH genes have been cloned [16, 17]. The chick PTH might also regulate egg shell formation. Amphibia, but probably not fish, are thought to possess a functional PTH gene, suggesting that the calcitropic action of PTH is necessary for vertebrates living in a water-free environment. All the PTH genes show signs of shared structures, three exons and two introns. The human PTH gene is located on chromosome 11p15. Its first exon contains no protein-coding sequences. Followed by an about 2-kbp first intron, the second exon encoding the first 29 amino acids of preproPTH molecule ensues. After the short second intron, there is the third exon encoding the remaining 86 (pro) PTH residues and 3'-non-coding region. Unlike the PTHrP (PTH-related polypeptide) gene, no alternate splicing pattern is known in the PTH gene, although both genes are supposed to be derived from a common ancestor [17]. In fact, eight out of the first thirteen amino acids between the two are identical and both utilize the same transmembrane G protein-coupled receptor, PTH/PTHrP receptor [18]. So far, in the human PTH gene, some 4-kbp 5'-flanking sequences including the promoter regions with two functional TATA boxes have been cloned. In this region, several important regulatory DNA elements are found as described below.

Tissue-Specific Expression of the PTH Gene

Except for very rare cases of ectopic production in malignant cells, expression of the PTH gene is strictly confined within the parathyroid cells [19]. This phenomenon is ascribed to the interaction either between parathyroid-specific nuclear proteins and PTH gene enhancer, or between non-parathyroid nuclear proteins and PTH gene silencer, but none of these cis-acting DNA elements have been identified, partly because no permanent authentic parathyroid cultured cell lines are yet available. Of interest, however, is one case report describing ectopic PTH production in ovarian cancer which demonstrates that translocation of the 5'-regulatory sequence of the human PTH creates a new fusion gene in which the PTH coding sequence is linked to an unknown DNA sequence [19]. Another report suggests that, in parathyroid adenoma cells, the regulatory region of the human PTH gene is fused to the cyclin D1 gene leading to overproduction of this gene in the parathyroid cells, thus triggering adenoma formation [20].

The Mechanism of Transcriptional Regulation of the PTH Gene

In PTH regulation, there is no involvement of the hypothalamic-pituitary axis. Instead, the chief player is the level of Ca_2+, which tightly regulates the secretion as well as the biosynthesis of PTH. Responses to both hypocalcemia and hypercalcemia should be closely interwoven. Nonetheless, the notion that the actual blood calcium level is maintained at a level slightly higher than the so-called set-point [7] suggests that hypocalcemia rather than hypercalcemia might be a more efficient stimulus of the PTH level and that constitutional suppression of PTH is operating in ordinary settings. In the physiological environment, this condition is achieved by negative regulation of PTH by both Ca_2+ and vitamin D. To meet rapid changes in the blood calcium level, secretion of PTH is acutely regulated. To back up this acute regulation, the regulation of the PTH gene at the transcriptional level in the same direction is absolutely necessary. As shown below, we have identified key players in the negative regulation of the PTH gene by Ca_2+ [10–13].

Our initial observation that the 4700 bp of the upstream region of the human PTH gene conferred suppressed CAT (chloramphenicol acetyltransferase) activity on the minimal PTH promoter-CAT construction in the transfected cultured cells led us to define two DNA elements responsible for transcriptional repression of the PTH gene [10]. By means of deletion analysis and protein-DNA interaction experiments, we found
that these elements were located at \(-3.5\) kbp and 
\(-2.4\) kbp upstream of the gene, respectively. The 
distal one consisted of a 15 bp palindrome sequence, 
TGAGACAGGGTCTCA (oligo B) and the proximal 
one contained an AT-rich sequence of 
ATTTGATATGCAGAA (oligo A), which could be 
considered an inverted repeat of the octamer 
(ATTTGATCA)-like sequence [21]. Fusion of each 
oligonucleotide (nCaRE) with the neutral TK 
thymidine kinase)-promoter CAT plasmid as well 
as with the PTH promoter-CAT plasmid created 
CAT activities clearly inhibited by a rise in the Ca\textsubscript{e} 
concentration after transfection into various types 
of cultured cells. Moreover, electrophoretic 
mobility shift assay (EMSA) revealed that the 
binding of each of the nCaREs to the nuclear protein 
obtained from these cultured cells was sequence-
specific and was augmented several-fold when the 
nuclear protein was obtained from the cells grown 
in the higher Ca\textsubscript{e} concentration. By competition 
assay in EMSA, we found that both nCaREs, oligo 
A and oligo B, bound a common set of the nuclear 
proteins [10-12]. Because this putative nuclear 
protein (s) is widely distributed among various 
types of cells including the parathyroid cells, we 
assumed that the core sequence of nCaRE, oligo B, 
is also conserved among many genes whose 
expression is inhibited by a rise in the Ca\textsubscript{e} 
concentration through a common mechanism. By computer search, we found that several genes other 
than the PTH gene had identical or very 
homologous nCaRE (oligo B) sequences (Fig. 1). 
Interestingly, not only the 15 bp palindromic 
sequence of oligo B but also AT-rich sequences 
ahead of it were also conserved among them [11]. 
Recently one group reported that both oligo A and 
oligo B are the DNA sequences contained in the 
Alu family [22]. There have been several reports 
indicating that some DNA sequences within the 
Alu repeat play an important role in transcriptional 
regulation as cis-acting DNA regulatory elements 
[23]. Further, not only in human but also in the 
rat and mouse 5'-flanking region of several genes, 
these oligo B-like elements are evolutionally 
conserved, underscoring the functional importance 
of the oligo B-like DNA elements. It is of note that 
we also found similar oligo B-like sequences in the 
upstream regions of the recently-identified 25- 
vitamin D\textsubscript{3}-1\alpha hydroxylase genes of human [24] as 
well as mouse origin [25]. Although the expression 
of this gene is presumed to be inhibited by a rise 
in the Ca\textsubscript{e} concentration [26], whether this 
regulation is also mediated through these DNA 
elements remains to be defined. On the other hand, 
we have yet not found DNA sequences homologous 
to oligo A. Presumably, exact matching to oligo A 
might not be required for the nCaRE-like activity. 
Detailed analyses of the base requirements in the 
oligo A-sequence are now in progress.

Subsequently we showed that expression of one 
of the genes sharing oligo B, the rat atrial natriuretic 
polypeptide (ANP) gene, was dramatically 
repressed by hypercalcemia in the heart by using 
an \textit{in vivo} continuous perfusion system for 48 h. 
On the other hand, expression of the renin gene 
sharing no oligo B-like sequence was unaffected 
by the same procedure in the rat kidney [11].

**Cloning of nCaREB**

We employed two different approaches to clone 
the components of the nCaREB protein. By using 
the protein-DNA binding (Southwestern) assay, we 
found that a redox factor protein, ref-1, was one 
component of nCaREB [12]. Ref-1 was first 
identified as a mammalian homologue of a bacterial 
apurinic endonuclease/repair enzyme [27]. 
Subsequently, it was reported to potentiate DNA 
binding activity of several transcription factors such 
as AP1 and NF \textsuperscript{κB} by modifying the redox state of 
these proteins [28]. In addition to such activities 
of ref-1, we first reported that it also possessed 
nCaRE's sequence-specific transcriptional repressor 
function. Very interestingly, one group reported 
that the promoter region of the human ref-1 gene 
contained both oligo A and oligo B although the 
functional significance of this observation awaits 
further investigation [29], but ref-1 alone could not 
explain all the characteristics of nCaREB activity, 
and we predicted the existence of another nuclear 
protein(s) that functions as nCaREB by cooperating 
with ref-1.

We then employed an oligonucleotide affinity 
column and amino acid microsequencing. We 
succeeded in demonstrating that both subunits of 
the Ku antigen (KuAg) interacted with ref-1 to bind 
to one of the nCaREs (oligo A) and functioned as 
nCaREB [13].

KuAg, which consists of two subunits, p70 and
p80 (p86), plays an important role in double-strand break repair of DNA. In this process, its ability to bind to DNA ends non-specifically is postulated to be related to subsequent actions such as DNA recombination or unwinding. Furthermore, such binding has been reported to be directly coupled with DNA-dependent protein kinase (DNA-PK) activity, which is elicited by the putative catalytic unit of KuAg [30]. On the other hand, sequence-specific binding of KuAg has been demonstrated in some genes, such as the small nuclear RNA [31], T cell receptor [32], transferrin receptor [33], collagen [34], ribosomal RNA [35], heat shock protein genes [36] and mouse mammary tumor virus long terminal repeat (MMTV LTR) [37]. While expression of most of these genes is stimulated by KuAg, transcription of the latter three is repressed by KuAg [35-37], but neither common KuAg-responsive DNA elements nor details of the domain structure of KuAg have been identified in either type of gene regulation. In this regard, it is of note that multimer formation of KuAg with another protein was suggested to be involved in some of the above examples of sequence-specific gene regulation by KuAg [34, 38]. Most importantly, we found that the amino-terminal portion of ref-1 bound to KuAg to function as nCaREB [13]. Furthermore, at the putative leucine zipper motif (residues 395-399) of the p70 subunit of KuAg, there is a sequence, AALCR (residues 395-399), which is similar to the consensus site A-A-K/E/R-C-R, needed for ref-1 interaction [28]. This consensus sequence found in JUN protein has been shown to be important for its binding to ref-1 [28]. Also, a cysteine preceded by a lysine was found in the p80's putative leucine zipper region (residues 156 and 157).

Although functional separation of KuAg into
separate subunits is difficult, our observation raises the possibility that some form of ref-1-KuAg interaction really occurs in vivo. This finding is of particular interest because both ref-1 and KuAg have been reported to be engaged in the basic transcriptional activity coupled with DNA repair/recombination processes [27, 30]. For example, both KuAg (or DNA-PK) [39] and ref-1 proteins [40, 41] were reported to stimulate the function of tumor suppressor p53, although their functional synergism is still an open question. Notably, however, one group recently reported that both KuAg and ref-1 protein bound a certain DNA element to exert hemin-induced transcriptional inhibition [42]. The latter finding also suggests that ref-1-KuAg interaction really occurs in vivo. Gene targeting revealed that homozygous disruption of ref-1 gene caused embryonic death [43] while homozygous deletion of either subunit of KuAg led to a severe defect in the immunoglobulin rearrangement [44, 45]. So far, no reports regarding changes in calcium handling have appeared, although detailed examination of the heterozygous state has not been carried out.

Calcium-Sensing Receptor

Parathyroid cells in which PTH is exclusively produced are rather unique in that they possess the calcium-sensing membrane G protein-coupled receptor (CaSR) that transmits the Ca$_{\text{c}}$ signal into the cells through mobilization of intracellular calcium via Gq-type G protein [7, 9]. Although relatively numerous varieties of the cell types have been reported to express functional CaSR [46], parathyroid cells are one of a few cell types in which changes of Ca$_{\text{c}}$ level physiologically affect the secretion of endogenously-produced protein. On the other hand, unlike secretion, it is not yet clear, although probable, whether negative regulation of the PTH gene by Ca$_{\text{c}}$ also requires CaSR as a first calcium entry site. Our efforts to link the nCaRE-nCaREB nuclear machinery with the CaSR are now in progress.

Changes in Osmolarity (Fig. 2)

As shown above, we have found that one of the nCaREs, oligo B, is very well conserved among such vasoactive genes as the vasopressin and the ANP genes. Further, the oligo B in the former gene is conserved throughout evolution. Because expression of some of these vasoactive genes is altered by external stimuli which change cell volume, we next examined whether oligo B is involved in gene regulation by hyperosmolarity. Our presumption is that virtually every living cell might be equipped with a common mechanism sensing changes in osmolarity from the cell membrane to the nucleus in order to maintain cell volume. Once a DNA element capable of interacting with such a machinery in the cell nucleus is introduced into the cell along with the reporter gene, the reporter activity would be properly regulated by changes in osmolarity through the interaction between such a nuclear machinery and the introduced DNA element. This may happen even when no endogenous gene is known to be susceptible to osmotic regulation in these cells. We demonstrated that the binding of oligo B to nCaREB including ref-1 was reduced by hyperosmolarity generated by sodium chloride (NaCl) but not by urea. Such attenuated binding was reversed by dephosphorylating some of the nuclear proteins with a potato acid phosphatase, suggesting that NaCl treatment elicited phosphorylation of these nuclear proteins to weaken their activity in binding to oligo B. Furthermore, these nuclear events led to hyperosmolarity-mediated transcriptional stimulation of the genes bearing this DNA element in the cultured cells [13]. This suggests that a certain protein kinase(s) activated by NaCl but not by urea might phosphorylate some of the oligo B-binding proteins including ref-1. These phosphorylated proteins might have weaker activity in binding to oligo B, leading to NaCl-mediated transcriptional stimulation of the gene(s) bearing oligo B. The report implying that ref-1 protein shares consensus amino acid sequences for the substrates of several protein kinases also favors our speculation [28, 47]. The recent identification of p38 MAP kinase as an intracellular mediator of osmotic stimuli [48, 49] might help in understanding the molecular mechanism underlying our observations. Probably the effect of a higher Ca$_{\text{c}}$ concentration and that of hyperosmolarity, that is, a higher NaCl
concentration, is counteracting; one stimulatory and the other inhibitory on the oligo B-protein binding and vice versa on the oligo B-mediated CAT activity. Although precise mechanism remains to be elucidated, we believe that both of these stimuli share, at least in part, a signal transducing mechanism from the cell membrane to the nucleus.

**Negative VDRE (Negative Vitamin D Response Element)**

Lastly and curiously, KuAg was found to play another important role in closely-related gene regulation [14]. We found that the human PTHrP gene contained a DNA element (nVDRE_{PTHrP}) homologous to a negative vitamin D response element in the human PTH gene [50]. It bound to vitamin D receptor (VDR), but not retinoic acid Xα receptor (RXRα) in the human T cell line, MT2 cells. VDR binding to this element was confirmed by Southwestern assay combined with immunodepletion by using anti-VDR monoclonal antibody and this binding activity was repressed by 1,25-dihydroxyvitamin D₃. Such a repression was reversed by the acid phosphatase treatment, suggesting that 1,25-dihydroxyvitamin D₃ phosphorylates VDR to weaken its binding activity to nVDRE_{PTHrP}. To our surprise, we found in EMSA that anti-KuAg antibody specifically supershifted the MT2 nuclear protein-nVDRE_{PTHrP} complex. The nVDRE_{PTHrP} bearing reporter

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**Working hypothesis on the mechanism of gene regulation by either extracellular calcium or osmolarity.** Three types of cells are depicted. In parathyroid (left) and heart (right) cells, endogenous production of PTH mRNA or ANP mRNA is inhibited by a rise in the extracellular calcium concentration (white thick arrow). In the cultured cells of various types (middle), introduced DNA containing nCaRE (-like sequence) is also subject to this type of gene regulation, leading to inhibition of the reporter (CAT) activity driven by the neutral promoter. In all the cases, the interaction between nCaRE and nCaREBs containing ref-1 and Ku antigen (represented as dotted and hatched circles) within the nucleus is crucially involved. On the other hand, in these transfected cultured cells, the reporter activity was stimulated by a rise in osmolarity caused by the addition of NaCl (thick black arrow). Here nCaRE-nCaREB interaction is also required. We speculate that in parathyroid or heart cells, endogenous production of PTH mRNA or ANP mRNA might also be affected by changes in osmolarity through a similar mechanism. The first entry site for these extracellular stimuli (filled circles on the cell membrane) has not been identified.
plasmid produced vitamin D-dependent inhibition of the reporter activity in MT2 cells, which was noticeably masked by the introduction of the Ku antigen expression vector in the antisense orientation. On the other hand, such a procedure did not perturb the VDRE [obtained from the mouse osteopontin gene]-mediated gene stimulation by vitamin D. These results indicate that nVDRE<sub>PTH</sub> specifically interacts with KuAg in addition to VDR to mediate gene suppression by vitamin D. It is possible that phosphorylation of VDR by the KuAg, which would have been triggered by the treatment with 1,25-dihydroxyvitamin D<sub>3</sub>, might weaken its activity to bind to nVDRE<sub>PTH</sub> followed by vitamin D-mediated gene repression [14]. These results are particularly reminiscent of the recent report suggesting that protein kinase activity of DNA-PK, the catalytic subunit of KuAg, would modify glucocorticoid receptor activity after the specific binding to DNA [37, 51], but before we confirm that such regulation really occurs in vivo, several issues should be addressed. First, can we demonstrate that this type of gene regulation is seen also in vitamin D-mediated inhibition of PTH or other genes? Second, how can we reconcile these data with Ca<sub>e</sub>-mediated gene inhibition, where KuAg was also shown to be crucially involved? Is ref. 1 protein involved here? Is there any cooperative or synergistic relationship between the action of Ca<sub>e</sub> and vitamin D, or, is it possible that the actions of these agents are mutually exclusive? Once these questions are solved, we will be able to open the way to the new biology regarding the calcitropic hormones.

**Concluding Remarks**

Not only intracellular but also extracellular calcium, whose concentration is maintained ~10,000-fold higher than the intracellular one, has important functions including the clotting of blood, maintenance of skeletal integrity, water handling by the kidney and brain-nerve networks. Only recently has the molecular mechanism whereby the signal of extracellular calcium is transmitted into the cells been investigated. We have identified the nuclear players which deal with the information created by such a mechanism, but there must be other unidentified molecules involved here. One example is an RNA-binding protein(s) mediating Ca<sub>e</sub>-induced suppression of the PTH gene [52]. Furthermore, there have been several reports describing Ca-sensing membrane receptors other than CaSR [46]. The relationship between nCaRE-nCaREB and these receptors including CaSR should be clarified. Finally and probably most importantly, we stress that the most interesting finding described here is that the same molecule(s) might be involved both in Ca<sub>e</sub> and vitamin D-mediated gene regulation, suggesting an unusually close interrelationship in the calcitropic hormone system. The notion that PTH, ANP and probably 1α-hydroxylase are all subject to negative gene regulation by both vitamin D and Ca<sub>e</sub> [10–13, 24, 26, 53] might open the way to understanding the whole story.

**Acknowledgment**

I am very grateful to Dr. Eturo Ogata (Cancer Institute Hospital), Dr. Tetsuya Igarashi (University of Tokyo) and Prof. Toshiro Fujita (University of Tokyo) for their continuous support and encouragement. This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

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