Hyperosmolarity-induced Gene Stimulation Is Mediated by the Negative Calcium Responsive Element*

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The negative calcium responsive elements of the parathyroid hormone gene bind to a specific set of nuclear proteins in an extracellular calcium (Ca\textsuperscript{2+})-dependent manner. We have found that one of the negative calcium responsive elements, named oligo B, is found in the 5′-flanking region of such vasoactive genes as the vasopressin and atrial natriuretic polypeptide genes. Furthermore, the oligo B-like sequence 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 examined whether oligo B is involved in gene regulation by hyperosmolarity. Here, we demonstrate that the binding between oligo B and its binding nuclear proteins including a redox factor 1 was reduced by hyperosmolarity generated by sodium chloride but not by urea. Such attenuated binding was reversed by dephosphorylating nuclear proteins by a potato acid phosphatase, suggesting that NaCl treatment elicited phosphorylation of these nuclear proteins to weaken their binding activity to oligo B. Furthermore, these nuclear events led to hyperosmolarity-mediated transcripational stimulation of the genes bearing this DNA element in the cultured cells.

The negative calcium responsive elements of the parathyroid hormone gene bind to a specific set of nuclear proteins in an extracellular calcium (Ca\textsuperscript{2+})-dependent manner. We have found that one of the negative calcium responsive elements, named oligo B, is found in the 5′-flanking region of such vasoactive genes as the vasopressin and atrial natriuretic polypeptide genes. Furthermore, the oligo B-like sequence 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 examined whether oligo B is involved in gene regulation by hyperosmolarity. Here, we demonstrate that the binding between oligo B and its binding nuclear proteins including a redox factor 1 was reduced by hyperosmolarity generated by sodium chloride but not by urea. Such attenuated binding was reversed by dephosphorylating nuclear proteins by a potato acid phosphatase, suggesting that NaCl treatment elicited phosphorylation of these nuclear proteins to weaken their binding activity to oligo B. Furthermore, these nuclear events led to hyperosmolarity-mediated transcripational stimulation of the genes bearing this DNA element in the cultured cells.

To maintain a constant cell volume against extracellular osmotic perturbations, virtually all living cells must be equipped with sensing machineries that transmit information from the cell membrane to the nucleus. The molecular mechanisms by which eukaryotic cells sense the changes in external osmolality and transduce that information into a certain response are partially understood (1–5). For example, cells of the mammalian renal medulla are exposed to markedly elevated osmoticum-mediated transcriptional stimulation of the vasoactive genes such as the vasopressin and atrial natriuretic polypeptide genes. Furthermore, the oligo B-like sequences in the rat atrial natriuretic polypeptide (ANP) and vasopressin genes (11). Expression of these two genes is known to be influenced by external biomechanical stimuli such as osmolality (14–16). Therefore, we examined the possibility that oligo B mediates osmolarity-induced regulation of gene expression in the cell nucleus.

MATERIALS AND METHODS

Homology Search by Computer Analysis—Human PTH’s nCaRE (oligo B)-like sequences were searched in the EMBL GenBank (11), and several matches are shown in Fig. 1. In the case of vasopressin gene, the oligo B-like sequences were well conserved among many species at similar upstream positions. Although PTH genes in species other than human were registered, the reported sequences might be too short to reach the possible nCaRE-like sequence in the far upstream region. This might be the case with other genes such as ANP or endothelin.

Synthetic Oligonucleotides and Plasmid Constructions—All the oligonucleotides used in this report were made by a DNA synthesizer (Biosearch type 8700). Oligo B, oligo B-like sequence in the rat ANP gene (oligo B\textsubscript{RANP}), oligo B1, and oligo CRE described in Refs. 10–13 and oligo B4 (4 tandem oligo Bs) were synthesized as follows: oligo B, 5′-TTTTTGTGACAGGTTCTCCTCCTCCTG 3′ and 3′-AAAAACTCTGTCACAAGGTTGAG 5′; oligo B\textsubscript{ANP}, 5′-TTTTGAGACAGGTCTCCTCCTG 3′ and 3′-AAAAACTCTGTCACAAGGTTGAG 5′; oligo B1, 5′-TTTTTGTGACAGGTTCTCCTCCTG 3′ and 3′-AAAAACTCTGTCACAAGGTTGAG 5′; oligo CRE, 5′-ATTCCCTGACCTTGGTTGGCCTG 3′ and 3′-ATTCCCTGACCTTGGTTGGCCTG 3′; oligo CRE 1, 5′-ATTCCCTGACCTTGGTTGGCCTG 3′ and 3′-ATTCCCTGACCTTGGTTGGCCTG 3′.

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The methods to prepare double-stranded DNAs and to construct the PTH promoter-based CAT reporter plasmid containing the 684-bp 5′-flanking region of the human PTH gene (pc301, Ref. 10) or the Tk promoter-based CAT reporter plasmid (12) were described.

Transfection and CAT Assay—HeLa or HEK (human embryonal kidney) 293 cells were grown in Ca\textsuperscript{2+}-free Dulbecco’s modifed Eagle’s Ham F12 media (Sigma) supplemented with 10% fetal calf serum and 1.8 mM CaCl\textsubscript{2} unless otherwise mentioned. The CAT plasmids were introduced into these cells by the DEAE dextran method (10). After transfection, cells were equally split into 2–4 dishes to avoid differences in transfectional efficiency among dishes. Final amounts of transfected plasmids per each dish were 5 μg. Three to five h later when the cells

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1 The abbreviations used are: Ca\textsuperscript{2+}, extracellular calcium; nCaRE(B), negative calcium responsive element (binding protein); PTH, parathyroid hormone; CAT, chloramphenicol acetyltransferase; Ref1, redox factor protein; EMSA, electrophoretic mobility shift assay; Tk, thymidine kinase; HEK cells, human embryonal kidney cells; ANP, atrial natriuretic polypeptide; CRE, cyclic AMP response element.
were attached to the plate, the medium was changed into Ca\(^{2+}\)-free Dulbecco’s modified Eagle’s/Ham F12 media containing 10% fetal calf serum with several different concentrations of CaCl\(_2\) (2.5 M stock solution), NaCl (2.5 M stock solution), or urea (5 M stock solution). The final concentration of total Ca\(^{2+}\) in the medium was measured by atomic absorption spectrophotometry. Calculated osmolarity in the media was shown in each figure. In each case, 40 h after transfection unless otherwise mentioned, cells were harvested, and CAT assays were performed. Where indicated, average CAT activity was calculated after three separate transfections using 

\[ ^{14}C \text{ scintillation counting, and typical results were shown in the figures with a given CAT activity as 100 in each case. HeLa cells stably transfected with a Ref1 expression vector in the antisense orientation or an empty vector alone were described (12).} \]

Preparation of Nuclear Extracts and EMSA—Nuclear extracts were prepared by the method of Schreiber et al. (17) from HeLa or HEK 293 cells 40 h (or 20 h where indicated) after maintaining the cells in the media as indicated. The synthetic oligonucleotides were end-labeled with [\(^{32}\)P]ATP by T\(_4\) polynucleotide kinase. 10\(^6\) cpm of the probe (10\(^8\) cpm/\(\mu\)g) was incubated with 10 \(\mu\)g of the nuclear protein along with 1 \(\mu\)g of poly(dI-dC) in each reaction for 30 min at room temperature. Final KCl concentration was adjusted to 80 mM by the binding buffer containing no KCl (10). The total reaction volume was 25 \(\mu\)l. DNA-protein complexes were resolved on 4% non-denaturing acrylamide gels, dried, and visualized by autoradiography as described (10–13). To explore the complexes were resolved on 4% non-denaturing acrylamide gels, dried, and visualized by autoradiography as described (10–13). To explore the effects of NaCl and/or urea on the protein-DNA interaction, proteins were obtained from the cells treated in the same manner as described in the transfection method. Where indicated, 0.1 unit of potato acid phosphatase (Sigma) was directly included in the reaction mixture at 37 °C for the indicated period. Protein concentrations were determined by the Bio-Rad assay kit (10).

**RESULTS**

Sequence Similarity among Various nCaRE-like DNA Sequences—We found by computer search that one of the nCaREs, oligo B, in the human PTH gene (10) shared DNA sequences similar to those located in the 5’-flanking regions of several other genes shown in Fig. 1. Some of these genes encode vasoactive substances. Interestingly, the portions of similarity among these genes overlap a recently proposed cis-acting DNA shear stress response element (18). Furthermore, in the ANP and vasopressin genes, sequence similarity extends to the 5’ portion of oligo B; all of them contain AT-rich clusters ahead of the palindromic sequence. We previously reported that expression of the ANP gene was repressed by a rise in Ca\(^{2+}\) concentrations in the heart, as was that of the PTH gene in parathyroid cells (11). Moreover, we noticed that oligo B-like elements in the vasopressin gene are well conserved at the upstream positions among various species as shown in Fig. 1. The reason why the positions of oligo B-like sequences in the vasopressin gene are not similar between these three species is currently unknown.

Of particular interest, expression of the two genes, ANP and vasopressin, is known to be altered by changes of osmolarity (14–16). In addition, secretion of PTH as well as exocytosis of myeloperoxidase were reported to be influenced by osmolarity (19, 20). Both of the latter two “non-vasoactive” genes also contain oligo B motifs, although the effect of osmolarity on the expression of these two genes is unknown. Based on this information, we examined whether the oligo B motif plays a role in transcriptional regulation in response to changes of osmolarity.

**Hyperosmolarity Impairs the Binding between Oligo B and Its Binding Protein(s)—**To test the possibility that the nCaRE

is involved in gene regulation by osmolarity, we first examined whether changes of osmolarity affect the interaction between oligo B from the human PTH gene and its binding protein (nCaREB) using the electrophoretic mobility shift assay (EMSA). When nuclear proteins were prepared from HeLa cells grown in higher sodium chloride (NaCl) concentration (50 mM NaCl was added to the Dulbecco’s modified Eagle’s/F12 media), binding to radiolabeled oligo B (Fig. 2A) or oligo B-like sequence in the rat ANP gene (not shown) was suppressed in both high (2.0 mM) and low (0.2 mM) Ca\(^{2+}\) conditions. Binding of a control oligonucleotide, the CRE site, and its binding protein(s) was not affected by the same procedures (Fig. 2A). A 3–4-fold inhibition on the oligo B binding by NaCl was consistently seen in the higher Ca\(^{2+}\) level. On the other hand, its effect in lower Ca\(^{2+}\) was also suppressive although the degree of suppression varied considerably among the repeated experiments presumably due to the combined toxic effects of low Ca\(^{2+}\) and high NaCl (see “Discussion”). Hereafter, we examined the effect of NaCl on DNA-protein binding in higher Ca\(^{2+}\) (2.0 mM), that is in a more physiological condition. Dose responses in HeLa and HEK 293 cells revealed a clear inverse relationship between the binding activity and NaCl concentration until the osmolarity went to 450 mM Osm/kg (by adding 76 mM NaCl), whereas no changes of the binding activity between the CRE site and its binding protein(s) were observed (Fig. 2B). Time course experiments using HeLa cells revealed that the effect of NaCl on the oligo B binding was already seen 20 h after treatment and lasted up to 40 h. Interestingly, a similar osmotic rise driven by urea did not exert an effect (Fig. 2C). Sequence-specific binding of oligo B was confirmed in Fig. 2C, as reported previously (10). Taken together, these results suggest that an osmotic increase...
An Osmotic Rise Enhances CAT Activity Driven by the Oligo B-containing Plasmid in HeLa and HEK Cells—We previously showed that, after a rise in Ca\(^{2+}\) concentration, increased binding of oligo B to its binding protein(s) led to transcriptional repression (10–13). By analogy with this observation, we speculated that the reduced binding between oligo B and its binding protein(s) due to hyperosmolarity (Fig. 2) would lead to transcriptional activation of a gene bearing the oligo B motif. HeLa cells were transfected with a reporter CAT plasmid containing no (TkCAT (11)) or one copy of oligo B (oligo B-CAT). We examined the effect of changing osmolarity on the CAT activities 40 h after transfection. As shown in Fig. 3A, CAT activity driven by oligo B-TkCAT, but not by the parental TkCAT plasmid, was increased in a NaCl concentration-dependent manner in these cells in high Ca\(^{2+}\) condition. On the other hand, urea which created a comparable osmotic rise to 37 kDa in size was Ref1 protein by immunodepletion with a specific antibody raised against the carboxyl-terminal portion of Ref1 protein (12). As shown in Fig. 4A, the binding activity of Ref1 was diminished by 75 mM NaCl but not by 150 mM urea treatment, although the calculated osmolarity was the same between the two conditions. Interestingly, several bands other than Ref1 protein behaved similarly by NaCl treatment although these proteins were not always visible probably due to subtle differences in washing conditions (Ref. 12 and see Fig. 6), and they were not characterized further in this study. Nonetheless, in each experiment, there always existed some bands other than Ref1, whose binding activity to oligo B4 was not affected at all by osmotic changes. On the other hand, NaCl treatment always attenuated the binding activity of Ref1, suggesting the specific effect of NaCl, but not of urea, on Ref1 protein. The amount of Ref1 was not changed by NaCl treatment as shown in the Western blotting (Fig. 4B). We also observed dose-dependent and time-dependent decreases in the oligo B4-binding activity of Ref1 protein (Fig. 4C), which coincided with the EMSA pattern shown in Fig. 2.

The Involvement of Ref1 Protein—Several lines of evidence in our previous paper (12) suggested that nuclear proteins that specifically bound to oligo B contained a redox factor, Ref1 protein. We showed in EMSA that HeLa nuclear proteins gave rise to a protein-oligo B complex(es), which was partially supershifted by the anti-Ref1 antibody (12). To test whether Ref1 protein is also involved in oligo B-mediated gene regulation by osmotic changes, we first examined the binding of Ref1 to radiolabeled oligo B4 (4 tandem oligo Be) by the Southwestern assay (12). As shown in Fig. 4A, the radiolabeled oligo B4 bound to several nuclear proteins in HeLa and HEK 293 cells. We previously identified that one of these bands corresponding to approximately one-fifth as much Ref1 protein as wild-type HeLa cells (12). As shown in Fig. 5, in high Ca\(^{2+}\) conditions, elevated osmolarity by NaCl did not raise CAT activity driven by oligo B-TkCAT in these cells, whereas cells stably transfected with an empty vector (12) yielded an osmolarity-induced rise in CAT activity by the same plasmid. Similar results were obtained when we used the TkCAT plasmid linked to the oligo B-like sequence in the rat ANP gene (Fig. 5 and Ref. 11). These treatments were not toxic to the cells because the CAT activity by the parental TkCAT was not altered by the same procedure. These results suggest that these cells utilize common, at least in part, nuclear pathways to fulfill gene regulation by both Ca\(^{2+}\) and osmotic changes created by NaCl.
nCaRE Mediates Transcriptional Stimulation by Hyperosmolarity

Fig. 3. CAT assay in HeLa and HEK 293 cells. A, oligo B-TkCAT (right, see Ref. 12) or the parental TkCAT (left, see Ref. 12) was introduced into HeLa cells grown in 2.0 mM Ca\(^{2+}\) condition. Cells were equally split into three conditions after transfection. Forty h after addition of 0, 50, and 75 mM NaCl, cells were harvested and CAT assays were performed. The most representative results were shown. Average CAT activity after arbitrarily represented as 100.

Three different transfections was indicated below each condition. Here, average CAT activity by TkCAT in normo-osmolar conditions was indicated statistically significant differences (*asterisks*) raised against Ref1 protein (12) was shown. Molecular mass markers among the experiments probably due to the subtle differences in wash-

Fig. 4. Changes of the binding between oligo B and its binding proteins including Ref1 protein by NaCl treatment. Southwestern assay with a \(5\)-\(\text{dP}-\text{end-radiolabeled oligo B4 as a probe (A and C). B.}

Immunoblotting of HeLa nuclear proteins with the specific antibody raised against Ref1 protein (12) was shown. Molecular mass markers are shown to the left. The 37-kDa band indicated by a solid arrow had been identified as Ref1 protein by immunodepletion (12). Although several bands other than Ref1 sometimes seemed similarly regulated in the Southwestern assay, their intensities were considerably different among the experiments probably due to the subtle differences in washing conditions (12), and they were not characterized further in this study. A. Southwestern assay. HeLa (left) or HEK (right) nuclear proteins were prepared 40 h after maintaining the cells in the conditions indicated below each lane. Densitometric scanning revealed that the degree of inhibition on the binding by NaCl was 55 ± 10% in HeLa cells and 68 ± 7% in HEK cells, respectively. The numbers were represented as mean ± S.E. after three separate experiments, and one of the typical results was shown in the figure. B. Immunoblotting of HeLa nuclear proteins treated identically to A. Ref1 protein was indicated by a solid arrow. One band corresponding to about 70 kDa in size was a nonspecific band. C, Southwestern assay. Dose- and time-dependent decreases of binding activity of Ref1 protein to oligo B4 in HeLa cells after NaCl treatment.

Dephosphorylation of nCaREBs Including Ref1 Protein Restores the Binding to Oligo B Once Attenuated by NaCl Treatment—Recently, certain protein kinases such as the mitogen-activated protein kinases have been proposed to be activated in osmotically shocked mammalian cells (2–5). Although molecular events following such kinase activation and nuclear translocation have been clarified for AP1 (21) or serum response factor transcription factors (22), the mechanism by which osmolarity-induced gene regulation through several protein kinase cascades in the cell nucleus has not been clarified. We first examined by EMSA the effect of potato acid phosphatase treatment on the binding between oligo B and HeLa nuclear proteins obtained from high (75 mM) NaCl conditions. As shown in EMSA (Fig. 6A), a time-dependent increase in the binding was observed after the treatment, suggesting that phosphorylation of the protein(s) might have inhibited the interaction with oligo B. By using the same nuclear proteins, we next performed the Southwestern assay to examine which nuclear proteins were affected by this dephosphorylation experiment. As shown in Fig. 6B, Ref1 protein, but not the other protein(s) shown here, was up-regulated in their binding to oligo B4, which also supported our hypothesis indicated above. Here, the clustered bands of 32 kDa frequently seen in Fig. 4 were not visible, suggesting that they were less specific oligo B-binding proteins (12). Experiments to define what kind of protein kinases are involved in these processes are now in progress in our laboratory.

DISCUSSION

We previously proposed a unique palindromic sequence, TGAGAACGGTCTCA, oligo B, is one of the two DNA sequences necessary for negative gene regulation by Ca\(^{2+}\), and speculated that oligo B might mediate osmolarity-induced regulation of gene expression (10, 11). Of note, vasopressin, whose transcription is known to be stimulated by hyperosmolarity (14), contains DNA sequences similar to oligo B, and these sequences are conserved throughout evolution (Fig. 1).

Our hypothesis is that virtually every living cell is equipped with a common sensing machinery against changes of osmolarity that signals from the cell membrane to the nucleus 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 a reporter gene, the reporter activity would be properly regulated by changes of 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. In this respect, a recent report by Shyy et al. (23) encouraged our hypothesis. They postulated that the in-
Fig. 5. Involvement of Ref1 protein in oligo B-mediated CAT activity by NaCl treatment examined by the antisense strategy. Oligo B-TkCAT, oligo BANP-TkCAT, or the parental TkCAT were introduced into HeLa cells stably transfected with a pReCMV empty vector (A, see Ref. 12) or into HeLa cells stably transfected with the same vector encoding Ref1 protein in the antisense orientation (B, Fer cells, see Ref. 12). After transfection, cells were treated as shown in Fig. 3A although only two different NaCl concentrations (0 and 75 mM) were used here. Typical results were shown in the upper panel. In the lower panel, data were expressed as mean ± S.E. of triplicate measurements. The mean CAT activity driven by TkCAT without added NaCl in A was arbitrarily represented as 100.

In this study, we first attempted to explore the hyperosmolar effects on the binding between oligo B and nuclear proteins from HeLa and HEK cells. We previously showed that, in Ca\(^{2+}\)-mediated gene regulation, the binding between oligo B and nCaREB was augmented by a rise in Ca\(^{2+}\) concentration, followed by suppression of expression of the genes bearing oligo B (11, 12). By analogy with this observation, we speculated that hyperosmolarity induces up-regulation of the genes bearing oligo B due to suppression of the oligo B binding to HeLa or HEK nuclear proteins. This hypothesis was verified in several experiments. In EMSA (Fig. 2), we demonstrated that HeLa and HEK nuclear proteins bound to oligo B more weakly as NaCl concentrations were raised. Furthermore, in the Southwestern assay (Fig. 4), similar NaCl treatment of these cells conferred weaker binding activity to oligo B (B4) by the Ref1 protein, which had been shown by us to be one of the nCaRE (oligo B) binding proteins (nCaREBs, Ref. 12). We also demonstrated that these nuclear events correlated with a NaCl-mediated transcriptional stimulation of an introduced reporter CAT gene bearing oligo B in either PTH or Tk promoter context (Fig. 3). Furthermore, our experiments using cells stably transfected with a Ref1 expression vector in the antisense orientation (12) revealed oligo B introduced into such cells no longer mediated gene regulation by hyperosmolarity (Fig. 5). This finding also underscores our hypothesis that oligo B and one of its binding proteins, Ref1, are crucially involved in this type of gene regulation.

One unexpected finding was that hyperosmolarity generated by NaCl, but not by urea, mediated this type of DNA-protein interaction (Figs. 2 and 4) and gene regulation (Fig. 3). Both of these stimuli are classified into “perturbing” solutes that would disturb many intracellular processes and are supposed to exert similar effects to the cells (1). However, in contrast to the cell-permeant nature of urea, NaCl is non-permeant and known to cause cell shrinkage (26). When we used another cell-permeant osmolyte, glycerol, we found that it did not affect the binding between oligo B and Ref1 nor was the CAT activity driven by oligo B-TkCAT (data not shown). Future experiments using several cell-impermeant osmolytes and/or monitoring of cell volume would clarify whether cell shrinkage rather than cell osmolarity triggers the nuclear events shown here.

Cohen et al. (8) reported that urea, but not NaCl, induced expression of an immediate early gene, egr-1, in renal medullary collecting duct cells, thereby discriminating their effects. They postulated that different types of phosphorylation elicited

interaction between the exogenously added AP1 site and endogenous AP1 protein(s) in HeLa cells mediated gene regulation by fluid shear stress, another stimulus changing cell volume; expression of no endogenous gene is known to be influenced by this stimulus in HeLa cells.

In this study, we first attempted to explore the hyperosmolar interaction between the exogenously added AP1 site and endogenous AP1 protein(s) in HeLa cells mediated gene regulation by fluid shear stress, another stimulus changing cell volume; expression of no endogenous gene is known to be influenced by this stimulus in HeLa cells.

FIG. 6. Effect of phosphatase treatment on oligo B-binding proteins. A, 0.1 units of potato acid phosphatase was added to HeLa nuclear proteins obtained from the high NaCl condition. After the reaction was carried out at 37 °C for the indicated time interval, EMSA were performed with oligo B as a radiolabeled probe. An arrow indicated the protein-DNA complex. Because of the presence of the enzyme in the reaction mixture, the migrating position of the specific oligo B-nCaRE complex differed from those shown in the other experiments (Fig. 2), and only this complex was shown here. B, Southwestern analysis by using the same set of HeLa nuclear proteins as those treated and used in A. Oligo B4 was used as a probe. Molecular mass markers are shown to the left. The arrow indicated the position of a 37-kDa protein, Ref1. The migrating position of Ref1 was not altered by the treatment in this assay. The bands other than Ref1, although not characterized (12), were unaffected by this treatment. The clustered bands of 32 kDa seen in Fig. 4 were not visible, suggesting that, unlike Ref1, they were less specific oligo B-binding proteins (12).
by these stimuli were responsible for their discriminative effects (8). In this regard, it is of particular interest that, in our experiment, dephosphorylation of the oligo B-binding proteins including Ref1 protein by potato acid phosphatase reversed their binding to oligo B once attenuated exclusively by NaCl treatment (Fig. 6). This result suggests that a certain protein kinase(s) activated by NaCl but not by urea might phosphorylate some of the oligo B-binding proteins including Ref1. These phosphorylated proteins might have weaker binding activity to oligo B, leading to NaCl-mediated transcriptional stimulation of the gene(s) bearing oligo B. The recent identification of SGK (Ref. 24, human serum and glucocorticoid-regulated protein kinase) or p38 mitogen-activated protein kinase (3, 4) as an intracellular mediator for osmotic stimuli might help understanding the molecular mechanism underlying our observations. In addition, the report implying that Ref1 protein shares consensus amino acid sequences for the substrates of several protein kinases (25) also favors our speculation.

Recently, several transcription factors and/or specific DNA elements were reported to be involved in osmolarity-mediated gene regulation in renal tubular cells (8, 9). Unlike renal tubular cells that accumulate large amounts of organic osmolytes to compensate for the interstitial hypertonicity, the cultured cells we used in this study do not seem to tolerate such an extraordinary stimulus. Such a difference might explain, at least in part, why oligo B does not share any sequence with the consensus amino acid sequences for the substrates of several protein kinases (25) also favors our speculation.

With regard to our previous studies, we speculate that the effect of higher $\text{Ca}^{2+}$ concentrations and that of hyperosmolarity, that is 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 the precise mechanism remains to be elucidated, we believe that both of these stimuli share, at least in part, signal transducing machinery from the cell membrane to the nuclei.

The results of the EMSA, competition, and Southwestern assays indicate complex interactions of multiple factors including Ref1 with oligo B (or B4) sequence, which appear to differ between the “basal” isosmotic and the hyperosmotic states.

Identification of the upstream components of this pathway in mammalian cells may provide information about intracellular signaling pathways that regulate mammalian cell responses to changing osmotic pressure.

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