The Tonicity-sensitive Element That Mediates Increased Transcription of the Betaine Transporter Gene in Response to Hypertonic Stress*

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BGT1, the Na⁺- and Cl⁻-coupled betaine transporter, is responsible for the accumulation of high concentrations of the non-perturbing osmolyte betaine in hypertonic Madin-Darby canine kidney (MDCK) cells and presumably in the hypertonic renal medulla. In MDCK cells, the increase in activity of the betaine transporter is preceded by an increase in transcription of BGT1 and in the abundance of BGT1 mRNA. To investigate the molecular mechanism of transcriptional regulation by hypertonicity, we have characterized the 5'-flanking region of the gene. Transient transfection assays in MDCK cells cultured in isotonic or hypertonic medium using luciferase reporter constructs containing various fragments of the 5'-flanking region revealed that the region spanning base pairs -69 to -50 5' to the transcription initiation site (-69/-50) has hypertonicity-responsive enhancer activity. A double-stranded -69/-50 concatemer cloned 5' to an SV40 basal promoter and luciferase reporter gene in hypertonic cells exhibited more than 11-fold the activity in isotonic cells. Expression assays and electrophoretic mobility shift assays of mutants of -69/-50 identified a smaller region that is required for hypertonicity to induce increased expression and a slowly migrating band on mobility shift assays.

In response to exposure to a hypertonic environment, prokaryotes, plants, and animal cells accumulate small organic solutes that protect them from the adverse effects of hypertonicity (1). In contrast to high concentrations of intracellular electrolytes, the small organic solutes do not perturb the function of macromolecules. Glycine betaine (betaine) is one of the major non-perturbing small organic solutes (osmolytes) (1) accumulated by prokaryotes (2), plants (1), and animal (3) cells exposed to hypertonicity. In prokaryotes, hypertonicity induces a betaine/proline transport system by relieving repression of the proU operon (4). In animals, the renal medulla is the only tissue that normally, as part of the urinary concentrating mechanism, becomes hypertonic. In Madin-Darby canine kidney (MDCK) cells, betaine is accumulated to concentrations 1000 times the extracellular concentration (5) as the result of an increase in the \( V_{\text{m}} \) of a sodium- and chloride-coupled transporter whose cDNA has been named BGT1. The cotransporter is a member of the sodium- and chloride-coupled neurotransmitter transporter gene family (6). The increase in BGT1 transporter activity in response to hypertonicity is preceded by an increase in transcription of BGT1 and in the abundance of BGT1 mRNA (7). Hypertonicity also leads to the accumulation of sorbitol and myo-inositol in kidney cells by increasing the transcription of the genes for aldose reductase (8) and for the sodium-coupled myo-inositol transporter (9–11), respectively. The molecular mechanism of regulation of transcription of these genes in response to hypertonicity is not understood. We have characterized the 5'-flanking region of the BGT1 gene and have found a hypertonic stress-responsive element. Hypertonicity induces the formation of two complexes in MDCK cells that bind to the element.

EXPERIMENTAL PROCEDURES

Cell Culture, Transient Transfection, Luciferase Assays, and Chloramphenicol Acetyltransferase Assays—MDCK cells were maintained in a defined medium (12). The day before transfection, MDCK cells were seeded onto 60-mm tissue culture dishes (approximately 5 x 10⁴ cells/dish). They were transfected using DEAE-dextran (13) with plasmid DNA (10 μg) of the luciferase reporter gene constructs containing various fragments of the 5'-flanking region of the BGT1 gene or double-stranded oligonucleotides, and, for assessing transfection efficiency, 5 μg of CMV-CAT, a plasmid containing the chloramphenicol acetyltransferase gene under the control of the cytomegalovirus promoter). Transfected cells were maintained in isotonic medium for 24 h, then switched to hypertonic medium or maintained in isotonic medium for another 24 h. Forty-eight hours after the transfection, the cells were harvested and assayed for luciferase (14) and chloramphenicol acetyltransferase (15) activity. Each transfection was performed at least three times. Medium was made hypertonic by adding raffinose to 500 mosm/kg H₂O.

Electrophoretic Mobility Shift Assays—Nuclei were isolated from MDCK cells cultured in isotonic or hypertonic medium (16), and nuclear extracts prepared (17). Nuclear extracts (4 μg) were incubated with a 32P-end-labeled oligonucleotide corresponding to -69/-35 of the BGT1 gene (see Fig. 1), in 30 μl of binding reaction mixture (20 mM HEPES (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1.5 μg of poly(dA-dT), 5 mM MgCl₂). After a 30-min incubation at room temperature, the mixture was electrophoresed on a 4% polyacrylamide gel (7.5:1, acrylamide/ bisacrylamide) in TAE buffer (40 mM Tris acetate, pH 8.5, 2 mM EDTA) at 4 °C. The oligonucleotides used were as follows.

-69/-35: 5'-TACCTTGCTGCAAGGCTCAGCAGTTACCCCT ATTGACCACCTTTTACGTGCAACCCATGCGGGA-3'

-69/-50: 5’-TGATACATTGTTGGAAGAATTCCAG AGTAAAACCTTTCTGACCTGACCCATGCGGGA-3’

-80/-56: 5’-GACCCAGAGGCCGACCTGTTCGATGGA-3’

API consensus oligonucleotide was obtained from Promega.

RESULTS

The BGT1 gene has three independent transcription initiation sites under control of three independent promoters. Only the 5'-flanking region of the most 5' exon, 1A, displays hyper-
tonicity-responsive enhancer activity,\(^3\) so we further characterized only this region. We inserted various lengths of the 5' flanking region of exon 1A of the BGT1 gene\(^5\) to a luciferase reporter gene and assayed luciferase expression in isotonic and hypertonic cells (Table I). All constructs demonstrated promoter activity as seen previously.\(^2\) Initial studies found that there was no difference in luciferase activity in response to hypertonicity in cells transfected with a construct containing the 2,400 bp upstream of the transcription initiation site compared to cells transfected with a construct containing 500 bp (construct -501/+53 of Table I; data not shown). Further deletions from -501 to -373, to -185, to -69, manifested essentially the same enhancer activity. In contrast, a construct beginning 49 bp 5' to the start site of transcription was not responsive to hypertonicity (Table I), suggesting that the segment -69 to -50 contains the hypertonicity-responsive element. To confirm the location of the hypertonicity-responsive region, we tested promoter activity in the correct orientation and in the reverse orientation. Hypertonicity induced luciferase activity independent of the orientation (Table II, part A). A concatemer of the 20-bp region was dramatically responsive. These results establish that the 20-bp region contains a TonE.

To evaluate the -69/-50 region further, we cloned that 20-bp oligonucleotide upstream of an SV40 promoter and the luciferase reporter gene in the correct orientation and in the reverse orientation. Hypertonicity induced luciferase activity independent of the orientation (Table II, part A). A concatenator of the 20-bp region was dramatically responsive. These results establish that the 20-bp region contains a TonE.

To test for interaction of TonE with transcription factors, we performed electrophoretic mobility shift assays using a double-stranded oligonucleotide corresponding to -69/-35 as a probe. Nuclear extracts from isotonic MDCK cells contained at least one binding factor that resulted in a broad band (Fig. 1, lane 3).

A similar band was also formed by nuclear extracts from hypertonic cells (lane 4). Binding to this band is markedly competed by an excess of AP1 sequence (lanes 6 and 7). The band that remains in the presence of excess AP1 sequence may represent incomplete competition or binding to another sequence. There is an AP1 sequence (18) at -45/-39 in the probe. Extracts from hypertonic cells led to two additional bands, a broad band migrating faster than the AP1-like band that is designated α, and a narrow band migrating more slowly, designated β. The 20-bp TonE element competed for binding with both of the hypertonicity-induced bands (lanes 8 and 9), whereas the sequence -80/-56 competed for β but not for α (lanes 10 and 11), suggesting that bands α and β are independent and represent binding at the 3' and 5' portions of the TonE element, respectively.

To characterize the element further, we synthesized three sets of oligonucleotides that contained contiguous 5 bp mutations in the sequence spanning -69/-50 (Fig. 2A) and used them as cold competitors in the electrophoretic shift assays (Fig. 2B). Mut67/63 competed for complex α but not complex β (lanes 4 and 5) and, in expression assays increased luciferase activity in hypertonic cells (Table II, part B). In contrast, Mut60/56 did not compete for complexes α or β (Fig. 2B, lane 7) and, did not lead to increased luciferase activity in the expres-

\(^3\) M. Takenaka, A. S. Preston, H. M. Kwon, and J. S. Handler, unpublished results.
that the MAP kinase pathway is involved in regulation of the activation of transcription of the glycerol synthetic pathway through a two component signal transduction pathway (25). Protein kinase C-dependent activation of the MAP kinase cascade by hypertonicity has been reported in MDCK cells (26). Since osmolyte transporter mRNA accumulation is stimulated by hypertonicity in protein kinase C-depleted MDCK cells, it is unclear whether MAP kinase is involved in regulation of osmolyte transporter genes in higher eukaryotes. Mammalian cells use three mechanisms for organic osmolyte accumulation: decreased degradation (glycerophosphorylcholine), induction of increased synthesis (aldose reductase), and increased uptake by induction of specific cotransporters (betaine, myo-inositol, and taurine) (5,27). Thus, a number of mechanisms appear to be involved in this important function.

This is the first TonE identified. The data we present indicate that hypertonic stress induced the formation of complexes bound to the TonE in MDCK cells. It is not clear, at this point, whether other tonicity-regulated genes, namely aldose reductase (8) and the myo-inositol cotransporter (11), use common cis- and trans-acting elements responsive to hypertonicity. Further characterization of trans-acting factors that interact with TonE, as well as identification of cis- and trans-acting elements responsive to hypertonic stress in the other tonicity-regulated genes, are needed.

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