Communication

ORE, a Eukaryotic Minimal Essential Osmotic Response Element

THE ALDOSE REDUCTASE GENE IN HYPEROSMOTIC STRESS*

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Joan D. Ferrarist, Chester K. Williams, Kyu-Yong J ung, Jennifer J. Bedford, Maurice B. Burg, and Arlyn García-Pérez

From the Laboratory of Kidney and Electrolyte Metabolism, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-1598

Organisms, almost universally, adapt to hyperosmotic stress through increased accumulation of organic osmolytes but the molecular mechanisms have only begun to be addressed. Among mammalian tissues, renal medullary cells are uniquely exposed to extreme hyperosmotic stress. Sorbitol, synthesized through aldose reductase, is a predominant osmolyte induced under hyperosmotic conditions in renal cells. Using a rabbit renal cell line, we originally demonstrated that hyperosmotic stress induces transcription of the aldose reductase gene. Recently, we cloned the rabbit aldose reductase gene, characterized its structure, and found the first evidence of an osmotic response region in a eukaryotic gene. Now, we have progressively subdivided this 3221-base pair (bp) region into discrete fragments in reporter gene constructs. Thereby, we have functionally defined the smallest sequence able to confer hyperosmotic response on a downstream gene independent of other putative cis-elements, that is, a minimal essential osmotic response element (ORE). The sequence of the ORE is CGGAAAATCAC(C) (bp –1105/-1094). A 17-bp fragment (–1108/–1092) containing the ORE used as a probe in electrophoretic mobility shift assays suggests hyperosmotic induction of a slowly migrating band. Isolation of trans-acting factor(s) and characterization of their interaction with the ORE should elucidate the basic mechanisms for regulation of gene expression by hyperosmotic stress.

In nature, one of the most prevalent types of stress is that caused by prolonged exposure of an organism to a hyperosmotic environment. Although the cellular responses to hyperosmotic stress are among the most profound, the molecular mechanisms involved have only recently begun to be addressed. Thus, contrary to the earnest study of heat shock at the molecular level, relatively little is known about the cascade of signals between the initial extracellular stimulus (hyperosmolality) and the ultimate adaptive response. It is recognized that, across the evolutionary spectrum, organisms have developed a universal adaptation process to cope with hyperosmotic stress; that is, increased accumulation of osmotically active organic solutes (organic osmolytes) (1). Cells accumulate high concentrations of organic osmolytes in place of inorganic ions. This is because, unlike equivalent concentrations of inorganic ions, the organic osmolytes apparently are not perturbing to cellular macromolecules (1).

Some of the predominant organic osmolytes are: betaine in bacteria (e.g. Escherichia coli) (2), glycerol in yeast (e.g. Saccharomyces cerevisiae) (3, 4), and sorbitol and betaine in cells of the mammalian renal medulla, the only mammalian tissue routinely exposed to extreme hyperosmotic stress in normal physiological conditions (5). In bacteria, where the molecular mechanisms for accumulation of organic osmolytes have been studied most extensively, hyperosmotic stress induces transcription of the proU operon. proU encodes the transport system involved in accumulation of betaine (2). Osmotic control of proU transcription is exerted, at least in part, by an “upstream activating region” that is currently being characterized by several groups (reviewed in Ref. 2). In S. cerevisiae, studies of the high-osmolality glycerol (HOG) response have concentrated on the signal transduction pathway immediately following hyperosmotic stress and preceding induction of target genes that directly control accumulation of glycerol (reviewed in Ref. 4). Thus, only one ultimate gene target in the HOG pathway has been identified. That is GDP1, which encodes glycerol-3-phosphate dehydrogenase, one of the two enzymes that catalyze the synthesis of glycerol. Most recently, osmotic stress was shown to increase glycerol-3-phosphate dehydrogenase activity and mRNA levels (3). The identification of GDP1 gene regions that control the osmotic response has not been reported.

In mammalian renal medullary cells under hyperosmotic stress, the synthesis of sorbitol, catalyzed by aldose reductase (AR), is increased (6). PAP-HTZ5 cells are a line of rabbit inner medullary cells (7) that accumulate large amounts of sorbitol (8) and other organic osmolytes (9) under hyperosmotic conditions. Using this line, we originally demonstrated that hyperosmotic stress induces transcription of the AR gene (10), resulting in increased AR mRNA levels (11), followed by a rise in AR protein synthesis rate (12) and, ultimately, increased sorbitol accumulation (13). Recently, we cloned the rabbit AR gene, characterized its structure, and found the first evidence of an osmotic response element (ORE) within a eukaryotic gene (14). A 3221 base pair (bp) fragment of the 5’-flanking region of the aldose reductase gene was shown to confer osmotic response to a downstream luciferase gene with the AR promoter as well as with a heterologous, B19, promoter (14). Here, we present the identification of the minimal essential ORE that controls induction of AR transcription by hyperosmotic stress.

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† To whom correspondence should be addressed: National Institutes of Health, Bldg. 10, Rm. 6N307, 10 Center Dr., MSC 1598, Bethesda, MD 20892-1598. Tel.: 301-496-1559; Fax: 301-402-1443; E-mail: jdf@helix.nih.gov.
‡ On sabbatical leave from Department of Physiology, University of Otago, Dunedin, New Zealand. Dr. Jennifer J. Bedford’s sabbatical leave was supported by the Health Research Council of New Zealand and the New Zealand Lottery Grants Board.

1 The abbreviations used are: AR, aldose reductase; ORE, osmotic response element; CAT, chloramphenicol acetyltransferase; bp, base pair(s); nt, nucleotide(s); TK, thymidine kinase; PCR, polymerase chain reaction.

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Experimental Procedures

Reporter Gene Expression Analysis of Transient Transfectants—Expression vectors, 007Luc, ARLuc, and B19CAT, were as described (14). 007Luc is promoterless and contains the luciferase gene, whereas ARLuc contains the rabbit AR promoter (bp −208 to +27) in unique XhoI-KpnI sites immediately upstream of the luciferase gene. ARLuc was previously demonstrated to exhibit basal promoter activity (as construct ARLuc6 in Ref. 14). B19CAT contains the B19 promoter immediately upstream of the luciferase gene. Luciferase activity in relative light units/μg of cell protein was normalized by CAT protein in picograms/μg cell protein (Luc/CAT). **HYPER/ISO is the ratio of Luc/CAT in hyperosmotic medium divided by Luc/CAT in isosmotic medium (mean ± standard error).

| Construct | +HYPER/ISO (n) | **HYPER/ISO relative to promoter |
|-----------|---------------|---------------------------------|
| ARLuc (−208/+27) | 3.5 ± 0.64 (8) | 1.0 |
| −3429/+209 (3221 bp) | 3.31 ± 3.83 (8) | 9.4 ± 1.09 |
| −3429/−2686 | 1.9 ± 0.18 (2) | |
| −2705/−1152 | 1.4 ± 0.36 (2) | |
| −1170/−209 (962 bp) | 2.11 ± 0.16 (2) | 6.0 ± 0.04 |
| −510/−209 | 1.7 (1) | |
| −1170/−492 (679 bp) | 20.2 ± 4.16 (3) | 5.7 ± 1.18 |
| −1170/−894 (277 bp) | 9.2 ± 1.52 (3) | 2.64 ± 0.43 |
| −993/−697 | 3.0 ± 1.05 (3) | |
| −770/−492 | 2.9 ± 0.63 (3) | |
| ARLuc (−208/+27) | 2.4 ± 0.09 (27) | |
| −1170/−492 (679 bp) | 11.5 ± 1.30 (7) | 4.8 ± 0.54 |
| −1170/−894 (277 bp) | 12.7 ± 2.46 (2) | 5.3 ± 1.26 |
| −1202/−1057 (146 bp) | 8.1 ± 0.54 (15) | 3.4 ± 0.22 |
| −1100/−957 (154 bp) | 4.1 ± 0.66 (6) | 1.7 ± 0.28 |
| −1026/−870 | 1.7 ± 0.37 (3) | |
| −1170/−1118 | 1.8 ± 0.20 (3) | |
| −1117/−1071 (47 bp) | 5.3 ± 1.08 (3) | 2.2 ± 0.45 |
| −1070/−1026 | 1.7 ± 0.02 (3) | |
| −1144/−1101 | 1.5 ± 0.12 (3) | |
| −1100/−1051 | 2.5 ± 0.18 (3) | |
| −1120/−1096 | 2.3 ± 0.47 (4) | |
| −1095/−1071 | 2.3 ± 0.10 (3) | |
| −1108/−1083 (26 bp) | 4.5 ± 0.51 (3) | 1.9 ± 0.17 |
| −1108/−1083 2X (26 bp 2X) | 15.1 ± 0.55 (3) | 6.3 ± 0.23 |
| −1108/−1092 (17 bp) | 4.8 ± 0.22 (16) | 2.0 ± 0.09 |
| −1108/−1092 3X (17 bp 3X) | 28.6 ± 2.51 (3) | 11.9 ± 1.05 |
| −1104/−1091 | 2.7 ± 0.08 (4) | |
| −1104/−1091 3X | 2.9 ± 0.12 (3) | |
| −1108/−1092 | 2.3 ± 0.12 (6) | |
| 007Luc (promoterless) | 5.6 ± 0.83 (4) | 2.5 ± 0.36 |
| | 2.0 ± 0.28 (8) | |
Fig. 1. The aldose reductase minimal essential osmotic response element (ORE). Transfected PAP-HT25 cells were maintained in isoosmotic medium (Iso) (300 mosm/kg H2O) or exposed to hyperosmotic medium (Hyper) (500 mosm/kg H2O) for 18–24 h. Values are expressed as the ratio of Hyper divided by Iso. ARL contains the rabbit AR promoter (bp −208/+27) upstream of the luciferase gene. DNA fragments from the rabbit AR gene were inserted upstream of the AR promoter in ARL. Positions of nucleotides that define the AR gene DNA fragments are numbered with the first nucleotide of exon 1 as +1. Negative numbers are nucleotides upstream of +1. As indicated by an underline, bp −1102 was mutated from an A to a G and bp −1094 was mutated from a C to an A. Cells were co-transfected with a given luciferase construct and B19CAT. B19CAT contains the B19 promoter upstream of the CAT gene. Luciferase activity in relative light units/μg of cell protein was normalized by CAT protein in picograms/μg of cell protein (Luc/CAT). *Hyper/Iso is the ratio of Luc/CAT in hyperosmotic medium divided by Luc/CAT in isoosmotic medium (mean ± standard error). **Hyper/Iso is the ratio of Luc/CAT in hyperosmotic medium divided by Luc/CAT in isoosmotic medium expressed relative to ARL (Hyper/Iso = 2.4 ± 0.09 and Table I) (mean ± standard error); these data are shown only for constructs demonstrating hyperosmic response. [n] is the number of independent transfections.

Kit (Bio-Rad, using γ-globulin as standard). Luciferase activity was determined using the Enhanced Luciferase Assay Kit and a Monolith 2010 Luminometer (Analytical Luminescence Laboratory). The amount of CAT was determined using CAT Elisa Kits (5 Prime—3 Prime, Inc. or Boehringer Mannheim) and a TiterTek Multiskan plate reader with a 405-nm filter.

Transfection Data Analyses—Luciferase activity in relative light units/μg of cell protein was normalized by CAT protein in picograms (from the co-transfected B19CAT construct)/μg of cell protein.

Electrophoretic Mobility Shift Assays—Nuclei were isolated (17) from PAP-HT25 cells maintained in isoosmotic medium (300 mosm/kg H2O) and from cells exposed for 18–24 h to the same medium made hyperosmotic (500 mosm/kg H2O) with NaCl (11). Nuclear protein extracts were prepared after the method of Fran (18) except that buffer D contained 650 mM NaCl. 32P-End-labeled double-stranded oligonucleotides (3–5 × 106 cpm containing 10–20 fmol) were incubated with 4 μg of nuclear protein extract, 0.5 μg of poly(dI-dC), and 0–1 pmol (0–100-fold molar excess) unlabeled specific competitor oligonucleotide (AR gene bp −1108−1092) in binding buffer (19) for 30 min at room temperature. Reactions were electrophoresed on a 4% polyacrylamide gel (80:1, acrylamide: bisacylamide) in 1× TAE buffer at 4 °C (19). Autoradiograms were exposed at −80 °C. Oligonucleotides were synthesized directly to produce sequence that corresponds to AR gene bp −1108−1092 (17 bp).

RESULTS AND DISCUSSION

We first identified a 3221-bp region of DNA (−3429/−209) that contained a putative osmotic response element (as construct ARLuc9 in Ref. 14) (Table I). We have now proceeded to functionally identify within that fragment the smallest sequence that could confer osmotic response on a downstream gene independent of other putative cis-elements that may potentiate the response; that is, a minimal essential osmotic response element. Instead of traditional nested deletions, we increasingly subdivided the −3429/−209 region into discrete pieces and tested them individually. This allowed us to examine all fragments for independent osmotic response as opposed to only those fragments that remain after deletion. All fragments were synthesized either by PCR amplification (≥146 bp) or directly on a DNA synthesizer and tested for ability to confer osmotic response to a luciferase gene driven by the rabbit aldose reductase promoter (−208/+27) (14, 20) in transient transfection assays (Table I). As shown in Table I in the ARLuc constructs, the most upstream region (−3429/−2686 and −2705/−1152) of the 3221-bp fragment (−3429/−209) did not generate osmotic response. However, the downstream 962 bp (−1170/−209) retained osmotic response. Within this 962-bp fragment, osmotic response was confined to an upstream 679-bp fragment (−1170/−492).

Initially, for the ARLuc constructs (Table I), the DNA fragments were cloned into the only available unique restriction enzyme (Xhol) site immediately upstream of the AR promoter. After having narrowed down the ORE to 277 bp (−1170/−894), the construct ARL was produced by adding to ARLuc, unique restriction enzyme sites that would allow us to subclone directionally (see “Experimental Procedures”). Since the added bases could affect function, we subcloned into ARL two of the fragments (−1170/−492 and −1170/−894) that had osmotic response in ARLuc. Indeed, these fragments continued to display osmotic response when in the ARL vector (Table I).

Osmotic response was sequentially narrowed down to 47 bp (−1117/−1071), 26 bp (−1108/−1083), and then to 17 bp (−1108/−1092). This 17-bp sequence was also able to confer osmotic response to the TK promoter (Table I, TKL construct −1108/−1092).

Osmotic response had been gradually decreasing with the size of the fragment, as seen in the only other sequence identified as containing a eukaryotic osmotic response element (19). To better evaluate osmotic response, we created tandem repeats of the 26- and 17-bp fragments, hypothesizing that this would magnify the response. As shown in Table I, tandem repeats of sequence containing the ORE (−1108/−1083 two times and −1108/−1092 three times) markedly increased osmotic response. In contrast, tandem repeats (−1104/−1091 three times) of a fragment having no osmotic response (−1104/−1091) were unable to evoke the response. We conclude that the sequence −1108/−1092 alone can confer osmotic response. The gradual drop in magnitude with decreasing fragment size suggests the possibility that other cis-elements may potentiate the osmotic response.
By gradually eliminating base pairs from the 17-bp fragment (−1108/−1092) we continued to functionally define the minimal essential osmotic response element. As shown in Fig. 1, upstream bp −1108 to −1106 are unnecessary for osmotic response. However removal of bp −1105 eliminates osmotic response, thereby defining the upstream end of the ORE to be bp −1105. Relative to the downstream end of the element, construct −1108/−1094 retained osmotic response (Fig. 1). In addition, we had observed previously that when we subdivided the 47-bp fragment (−1117/−1071) into two pieces (Table I, ARL constructs −1120/−1096 and −1095/−1071), thereby splitting between bp −1096 and −1095, all osmotic response was lost. We concluded that bp −1095 was essential. If bp −1094, a cytidine (C), is substituted by an adenosine (A), osmotic response is unaffected. However, ARL construct −1108/−1095 showed a Hyper/Isos response equal to 3.5 ± 0.25 (1.5 ± 0.10 relative to ARL, Fig. 1). We conclude that bp −1094 may be necessary, but it need not be a pyrimidine, and that the minimal essential ORE is defined by bp −1105/−1094.

We noted, particularly because of the concentration of purines at the 5′ end of the ORE, the similarity between a nucleotide (nt) sequence (nt 5′-CGGAAAATCAC(C)3′), within it and the consensus sequence for the NF-κB element (GGGAATTCAC(C)3′) (21). However, the sequence −1104/−1095 does not fit the NF-κB consensus at nucleotide −1102 where all currently recognized NF-κB elements have a guanosine (G) at the corresponding nucleotide (21). To determine whether the ORE would retain osmotic response if its sequence were modified to include an NF-κB element consensus, we substituted base −1102, an adenosine (A), by a guanosine (G) (underlined in Fig. 1) as in an NF-κB element. Osmotic response was lost (Fig. 1); we conclude that, at least based on cis-element sequence, the ORE does not contain an NF-κB element.

The same experiment provided information relative to a putative ORE consensus. As shown below, the ORE shares six consecutive base pairs (−1104/−1099, GGGAATACTAC) in common with TonE, the only other sequence identified as containing a eukaryotic osmotic response element (19). TonE regulates the hyperosmotic response of the dog renal Na+ - and Cl- -coupled betaine transporter responsible for accumulation of betaine, another organic osmolyte (22–24). In TonE, when five of the 6 bases (GAAAAG) were simultaneously substituted by TCCCC, osmotic response was lost (19).

DogTonE: 5′-TACCTGTTGGAAAAATCTCAC(3′)
Rabbit AR-ORE: 5′-CGGAAAATCAC(C)3′

By defining the minimal essential osmotic response element in the aldose reductase gene, we have shown that these 6 bases, although conserved, do not suffice to confer the response. However, as referred to above, we have also demonstrated that, substitution of only one of the six nucleotides (nt −1102 in the ORE), an adenosine (A), with another purine, a guanosine (G), eliminates osmotic response. Identification of other osmotic response elements should better define a eukaryotic osmotic response consensus, if there is one.

To test for interaction of the ORE with putative osmotically induced transcription factors, we performed electrophoretic mobility shift assays of the ORE in the presence of nuclear protein extracts prepared either from cells maintained in isoosmotic medium or cells exposed to hyperosmotic medium for 18–24 h. We used bp −1108/−1092 as the probe. This fragment had been shown to confer osmotic response to the luciferase gene in transfection analyses (Table I). A slowly migrating but narrow band (arrow) was observed predominantly with extracts from hyperosmotically treated cells in the presence of 0.5 μg of poly(dI-dC) (Fig. 2, lane 6). As shown in Fig. 2, a 50-fold molar excess of unlabelled (cold) probe reduces and a 100-fold molar excess virtually eliminates the narrow slowly migrating band (lanes 7 and 8, respectively). An additional, faster migrating and very broad band also remains in the presence of poly(dI-dC) but is eliminated by a 100-fold molar excess of the specific competitor (bp −1108/−1092). This band is also present in lanes containing extract from isoosmotically treated cells but to a lesser degree than that seen in lanes containing extract from hyperosmotically treated cells. These mobility shift assays represent exploratory evidence that hyperosmotic stress results in the induction of putative trans-acting factor(s) that associate with the aldose reductase ORE. Isolation of these trans-acting factor(s) and characterization of their interaction with the ORE should elucidate the basic mechanisms for regulation of gene expression by hyperosmotic stress.

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