Functional consensus for mammalian osmotic response elements

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Ferraris, Joan D., Chester K. Williams, Akihiko Ohtaka, and Arlyn García-Pérez. Functional consensus for mammalian osmotic response elements. Am. J. Physiol. 276 (Cell Physiol. 45): C667–C673, 1999.—The molecular mechanisms underlying adaptation to hyperosmotic stress through the accumulation of organic osmolytes are largely unknown. Yet, among organisms, this is an almost universal phenomenon. In mammals, the cells of the renal medulla are uniquely exposed to high and variable salt concentrations; in response, renal cells accumulate the osmolyte sorbitol through increased transcription of the aldose reductase (AR) gene. In cloning the rabbit AR gene, we found the first evidence of an osmotic response region in a eukaryotic gene. More recently, we functionally defined a minimal essential osmotic response element (ORE) having the sequence CGGAAAATCAC(C) (bp 1092 to 1105). In the present study, we systematically replaced each base with every other possible nucleotide and tested the resulting sequences individually in reporter gene constructs. Additionally, we categorized hyperosmotic response by electrophoretic mobility shift assays of a 17-bp sequence (∼108 to ∼1092) containing the native ORE as a probe against which the test constructs would compete for binding. In this manner, binding activity was assessed for the full range of osmotic responses obtained. Thus we have arrived at a functional consensus for the mammalian ORE, NGGAAAWDHMC(N). This finding should accelerate the discovery of genes previously unrecognized as being osmotically regulated.

osmoregulation; osmotic stress; organic osmolytes; gene regulation; gene expression

EVOLUTIONARILY, ADAPTATION to hyperosmotic stress through the accumulation of osmotically active organic solutes (organic osmolytes) is a highly conserved mechanism. In organisms other than halophilic bacteria, cells accumulate high concentrations of organic osmolytes instead of inorganic ions. In cells that respond with an initial short-term adaptive accumulation of inorganic ions, inorganic ions are replaced by organic osmolytes in the long term. This occurs because elevated concentrations of organic osmolytes apparently do not perturb the macromolecular function as would equivalent concentrations of inorganic ions (28). Although the cellular responses to hyperosmotic stress are among the most profound, the molecular mechanisms involved have only begun to be addressed. Thus, in contrast to what is known about another environmen-

tal perturbation, heat shock, relatively little is known about signal transduction between the initial extracellular stimulus (hyperosmolality) and the ultimate adaptive response (13, 23, 27).

Hyperosmotic accumulation of organic osmolytes is transcriptionally regulated. Organic osmolytes include betaine in bacteria (e.g., Escherichia coli) (17), glycerol in yeast (e.g., Saccharomyces cerevisiae) (1, 14), and sorbitol and betaine in cells of the mammalian renal medulla, which, among mammalian cells, are uniquely exposed to hyperosmotic stress in normal physiological conditions (9). In bacteria, transport of betaine is encoded by ProU (17). Osmotic control of proU operon transcription is exerted for the most part by a negative-acting “downstream regulatory element” (reviewed in Refs. 11, 17, and 20). There additionally may be minimal involvement of an “upstream activating region” extending ∼200 bp in length (17). Although deletion analysis of this region has been performed, no discrete cis element that confers osmotic response has been identified (16). In S. cerevisiae, GPD1 encodes glycerol-3-phosphate dehydrogenase, which catalyzes the synthesis of glycerol. Osmotic stress increases glycerol-3-phosphate dehydrogenase activity and mRNA levels (1), but osmotically responsive cis elements in the GPD1 gene have not been identified. In the yeast high-osmolality glycerol (HOG) response, most is known about the cascade of signals that immediately follows an increase in osmolality (reviewed in Ref. 14). HOG1 (a mitogen-activated protein kinase gene)-dependent osmotic induction of genes other than GPD1 has been shown to act via yeast stress response elements (STREs) (22). Yeast STREs function under various insult conditions, not only osmotic stress, and there is no homology between the osmotic response element (ORE) and the STREs.

In mammalian renal medullary cells, including cultured PAP-HT25 cells, which are derived from the rabbit inner medulla, hyperosmotic stress results in the accumulation of sorbitol as a predominant osmolyte (2, 3). Sorbitol accumulates because of a rise in the rate of synthesis of aldose reductase (AR) (19), the enzyme that catalyzes the conversion of glucose to sorbitol. Using PAP-HT25 cells, we originally demonstrated that hyperosmotic stress increases the transcription of the AR gene (24), which leads to a rise in AR mRNA levels (10). In cloning and characterizing the rabbit AR gene, we found the first evidence of a eukaryotic ORE (8).

Since then, there have been several discoveries of mammalian OREs, including the definition of the minimal essential ORE of the rabbit AR gene (7). A sequence

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containing an ORE was identified for the canine Na\textsuperscript{+}- and Cl\textsuperscript{-}-coupled betaine transporter gene (25), within which it is possible to find homology with the rabbit AR gene minimal essential ORE. Another functional ORE, which is now designated ORE-X, was originally thought to be upstream of the human AR gene (21). ORE-X is strongly homologous (9 of 11 bp are identical) to the rabbit AR gene ORE, but the gene to which it belongs is as yet unknown (12). Putative OREs of the AR gene in the human (12) and mouse (4) now have been found. This constitutes a total count of five OREs, three of which are in the same gene (the AR gene), although the genes are from different species. The scarcity of demonstrated active OREs makes it unlikely that an accurate, strong consensus for mammalian minimal essential OREs can be derived merely by sequence comparison. Here, we present the systematic derivation by functional assessment of the mammalian minimal essential ORE.

**METHODS**

Reporter gene expression analysis of transient transfectants. Expression vector ARL has been described previously (7). ARL contains the rabbit AR gene promoter (bp –208 to +27) in unique XhoI--Hind III sites immediately upstream of the firefly luciferase gene. ARL was previously demonstrated
Sequences were verified by primer-directed, double-stranded synthesis. Oligonucleotides containing the appropriate restriction enzyme sites at their 5' and 3' ends were created by annealing synthesized sense and antisense oligonucleotides (model 392 DNA/RNA synthesizer; Applied Biosystems), as previously described (7). Oligonucleotides were directionally doped into the Xho I–Hind III sites of multiple cloning regions immediately upstream of the Renilla luciferase gene in the promoterless pRL-null vector (Promega).

Oligonucleotides were prepared by the method of Dignam et al. (5, 6) with buffer C containing 0.42 M KCl. 32P-end-labeled double-stranded oligonucleotides (bp −1108 to −1092 in the rabbit AR gene containing the minimal essential ORE, −1105 to −1094; 100 fmol) were incubated with 6 µg nuclear protein extract, 0.5 µg poly(dI-dC), and 0–5 pmol (0- to 50-fold molar excess) unlabeled specific (AR gene bp −1092 or −1088) or mutant (AR gene bp −1108 to −1092) firefly luciferase construct (3 µg) and phRL19 (0.03 µg) by using a CellPhect transfection kit (Pharmacia). From each transfected 150-mm-diameter dish, cells were seeded into six 35-mm-diameter dishes and left overnight. The medium in three of the dishes was changed to fresh isosmotic medium (300 mosmol/kgH2O); the medium in the other three dishes was changed to the same medium made hyperosmotic (500 mosmol/kgH2O) with NaCl. Twenty-four hours after changing the medium, cells were harvested by adding 100 µl of passive lysis buffer (Dual-Luciferase reporter assay system; Promega).

Cell lysates were analyzed for total protein and firefly and Renilla luciferase activities in relative light units (RLUs) by using the following kits in accordance with the manufacturer's instructions. Total protein was determined by using the Bio-Rad protein assay kit with bovine serum albumin as the standard.

Renilla luciferase activity in the experimentally active promoter (bp −1088 to −1094) was used as the maximum binding capacity. This value was typically three to four times that of the equivalent band(s) shifted in the presence of isosmotic extract.

The binding capacity after a single-base substitution was determined by the ability of an excess (10 or 50 times the mass of the probe) of these mutants to compete effectively, that is, to significantly reduce the maximum binding capacity of the probe in the presence of hyperosmotic extract, as described by Ruepp et al. (21). Significant reduction was equated with the ability of a 50-fold excess of a mutant to reduce binding capacity at least to the level found in the presence of isosmotic extract.
RESULTS AND DISCUSSION

The original demonstration of a putative ORE was in a 3,221-bp fragment of the 5' flanking region (−3429 to −209) of the rabbit AR gene (8). Subsequently, we functionally defined, within that fragment, the smallest sequence capable of conferring osmotic response on a downstream gene, a minimal essential ORE (7). The ability of this ORE to confer osmotic response was independent of that of other putative cis elements that may potentiate the response (7).

We derived the putative consensus for a mammalian ORE (Fig. 1) from the minimal essential rabbit AR gene ORE (7), putative AR gene ORES from humans (12) and mice (4), ORE-X from an unknown gene (12, 21), and the ORE of the canine Na⁺- and Cl⁻-coupled betaine transporter gene (TonE) (25). Three of these are in the same gene, and a set of only three different genes is sufficient to clearly define a true consensus. Therefore, we have now functionally defined a consensus for mammalian ORES. Starting with the rabbit AR gene minimal essential ORE (−1105 to −1094) (Fig. 1), we systematically replaced each base individually with every other possible nucleotide and tested each construct. This allowed us to examine the nucleotide requirement at each position within the minimal essential ORE for the ability to confer osmotic response to a firefly luciferase gene driven by the AR promoter (−208 to +27) (7, 8) in transient transfection assays (Fig. 1). Relative to the osmotic response of the negative control (ARL containing the promoter alone), we defined the ability to confer an osmotic response as a hyperosmotic-to-isomotic ratio of >−1.4 based on statistical analysis (square root transformation of ratio data followed by one-way ANOVA with separation of significant means by Dunnett's multiple-comparison test). Additionally, to assess binding activity, we used EMSAs. In these, a 17-bp sequence (−1108 to −1092) containing the native ORE (−1105 to −1094) was used as a probe against which the test constructs would compete for binding (Fig. 2). We initially determined the binding profiles of the majority (8 of 9) of constructs that were clearly positive in osmotic response (ratio ≥ 1.7) and those that were clearly negative (14 of 24) in osmotic response (ratio ≤ 1.3). The binding activity of constructs with ratios ≥ 1.7 was positive; the binding of constructs with ratios ≤ 1.3 was negative. All other constructs having intermediate response ratios (1.4–1.6) were then examined. In this manner, binding activity was assessed for the full range of osmotic response ratios obtained (2.7–0.8; Fig. 1). EMSA was repeated at least twice for each construct examined. Constructs that did not effectively compete (i.e., binding capacity reduced to the level found in the presence of isosmotic extract or lower), even at a 50-fold molar excess, with the native

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probe were considered osmotically inactive (Fig. 2). This also defined construct hyperosmotic-to-isosmotic ratios of 1.4 as active (Fig. 1). Accordingly, the nucleotide requirements for each position within the minimal essential ORE were defined as follows. Position \(-1105\) can be filled by any of the four nucleotides. However, position \(-1105\) cannot be eliminated and the osmotic response retained (7). Positions \(-1104\) through \(-1100\) must be GGAAA, respectively. Positions \(-1099\) through \(-1096\) can vary; \(-1099\) can be A or T; \(-1098\) can be T, A, or G; \(-1097\) can be C, A, or T; and \(-1096\) can be A or C. Position \(-1095\) must be C, and \(-1094\) can be any of the four nucleotides. These results define the functional consensus for the mammalian ORE as NGGAAA(A/T)/(T/A/G)(C/A/T)(A/C)(N) or, in single-letter nomenclature, as NGGAAWDHMC(N). The further dispensability of G nucleotides in each strand has been addressed by Miyakawa et al. (18) by using an in vivo footprinting technique in Madin-Darby canine kidney (MDCK) cells exposed to hypertonic medium. They concluded that G residues in the canine betaine transporter gene ORE corresponding to positions \(-1104\), \(-1103\), and \(-1095\) (in the rabbit AR gene ORE) are indispensable, whereas a G at position \(-1098\) is not. These results are consistent with the functional consensus ORE. However, because this functional consensus was derived from the known mammalian OREs, which have strong homology, the possibility of the future discovery of mammalian OREs that bear no resemblance to this consensus cannot be discounted.

Miyakawa et al. (18) recently reported deriving a consensus ORE (Fig. 3). Their consensus differs from ours. A principal difference is due to the fact that Miyakawa et al. did not derive their consensus entirely empirically. For example, none of the bases were individually replaced by all other possible nucleotides. Therefore, many of the conclusions are derived from

\[
\text{Putative Consensus} \quad Y \ G \ G \ A \ A \ A \ A \ T / O \ Y \ C / C^C / A
\]

\[
\text{Consensus ORE} \quad N \ G \ G \ A \ A \ A \ A \ / T \ / O \ / C^C / A^T / C \ N
\]
assumptions; e.g., pyrimidines (C or T) and purines (A or G) were considered equal without consideration of differences in hydrogen bonding (A and T form two bonds; C and G form three bonds). Specific examples follow (Fig. 3). 1) The base corresponding to position −1099 of the rabbit AR gene ORE was concluded to be N, whereas we conclude that it can be an A or T. It is an A in all known active OREs, and Miyakawa et al. replaced it only with a T and found a positive response. They concluded that this base could be an N simply because a pyrimidine (T) and a purine (A) had been tested. This conclusion did not take into account the fact that both A and T form two hydrogen bonds. We demonstrated empirically that neither C nor G can effectively substitute at this position. 2) The base corresponding to position −1095 of the rabbit AR gene ORE was concluded to be a C or T, whereas we conclude that it can only be a C. Miyakawa et al. based their conclusion on the presence of a T in OreA and OreB from the human AR gene “osmotic response region” (12) and did not test it empirically. Neither OreA nor OreB has been shown to be independently active. Independent osmotic activity is the underlying assumption in the experimental approach of Miyakawa et al. (and of ours). However, two notable discrepancies in osmotic response were found when individual bases were substituted at AR gene ORE positions −1105 and −1100. We found osmotic response with a G at −1105, whereas Miyakawa et al. did not. Miyakawa et al. found osmotic response with a T at −1100 and concluded that −1100 could be an N. We substituted at −1100 with C, G, and T and lost osmotic response, concluding that the base at −1100 could only be an A. The reasons for these discrepancies are not apparent; there is, however, at least one important experimental difference. Miyakawa et al. used a tandem repeat of the OREs being tested, whereas we used a single copy. We do not believe these discrepancies to be a result of a species difference (dog vs. rabbit cell lines) because we obtained osmotic response with the AR gene ORE in MDCK cells (as Miyakawa et al. used) and with TonE in PAP-HT25 cells (unpublished results).

On the basis of our data, we can conclude that native, active OREs fit this functional consensus. However, we then asked whether the converse was true, that is, is any sequence that fits the functional consensus ORE necessarily osmotically responsive? The functional consensus allows more latitude per position than the predicted putative consensus derived merely by maximizing the homology of presently known native OREs (Figs. 1 and 4). We asked how many substitutions that fit the functional consensus but deviate from maximum homology among known native OREs can one make and still obtain osmotic response. Figure 4 illustrates one example of this test. When the test construct contained either four or three positions that deviated from maximum homology, osmotic response was lost, although the position changes still fit the functional consensus (Fig. 4). Two or fewer position changes that deviated from maximum homology resulted in the retention of osmotic response. These data are consistent with previous observations that most functional elements must be related to the consensus by no more than one or two substitutions (15). We conclude that, although a functional ORE fits the functional consensus, the converse is not true. Any gene sequence identified as a possible ORE by homology scanning with the consensus sequence must ultimately be assessed by the expression of osmotic response. For example, a putative osmotic response region in the bovine Na+/inositol cotransporter gene (SMIT) was recently identified (29). This region contains a sequence that shares partial similarity with that of the ORE (7 of 11 bp are identical); however, the sequence has G at corresponding base −1099, and this does not fit the functional consensus. Whether the 11-bp SMIT ORE is osmotically responsive remains to be examined. Our determination of a functional consensus for mammalian OREs should accelerate the recognition of genes that are osmotically regulated.

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