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Cloning, genomic organization, and osmotic response of the aldose reductase gene

(osmoregulation/osmotic stress/organic osmolytes/gene regulation/gene expression)

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ABSTRACT **Diverse organisms accumulate organic osmolytes to adapt to hyperosmotic stress. The molecular basis of eukaryotic gene osmoregulation remains obscure. Aldose reductase [AR; alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21], which catalyzes the conversion of glucose to sorbitol (an organic osmolyte), is induced in renal medullary cells under hyperosmotic conditions. Elevated extracellular NaCl increases AR mRNA transcription in PAP-HT25 cells, a cell line derived from the rabbit renal papilla. We have cloned and characterized the rabbit AR gene to determine how it is regulated by hyperosmolality. The length of the gene, not including 5′ or 3′ flanking regions, is approximately 14.7 kilobases (kb) organized into 10 exons and 9 introns. The transcription start site is 36 base pairs upstream of the initiator methionine codon. A 5-kb fragment containing approximately 3.5 kb of 5′ flanking region was isolated. The 3.5-kb sequence was examined for basal promoter activity and hyperosmotic response in luciferase reporter gene constructs. A 235-base-pair fragment (base pairs −208 to +27) was able to drive the downstream reporter gene in transfected PAP-HT25 cells under isoosmotic conditions (300 mosmol/kg of H_2O). When this fragment plus the remaining upstream sequence (from approximately base pair −3429 to base pair +27) was used, cells in hyperosmotic medium (500 mosmol/kg of H_2O) showed about 40-fold induction of luciferase expression compared with cells in isoosmotic medium. The upstream fragment (from approximately base pair −3429 to base pair −192) also conferred osmotic response to a heterologous promoter (B19). This finding evidences putative osmotic response element(s) (OREs) within a specific DNA fragment in a eukaryotic genome. Identification and characterization of OREs within this fragment and their associated trans-acting factors should reveal the molecular mechanisms of gene regulation in osmotic stress.**

Diverse organisms, including bacteria, yeast, plants, and animals, adapt to hyperosmotic stress. A common mechanism used in this adaptation is the accumulation of organic osmolytes (1). Although osmotic regulation of genes involved in osmolyte accumulation is best understood for the *proU* operon of *Escherichia coli*, only recently have some putative cis-acting elements been proposed (2, 3).

Renal medullary cells are the only ones routinely exposed to a greatly hyperosmotic milieu in mammals. A principal osmolyte accumulated by these cells during adaptation to hyperosmotic stress is sorbitol (4), a sugar alcohol synthesized from glucose in a reaction catalyzed by aldose reductase (AR; alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21) (5–9). PAP-HT25 is a line of rabbit inner medullary cells (10) that accumulate large amounts of sorbitol (7) and other organic osmolytes under hyperosmotic conditions (11). Using this cell line, we originally demonstrated that extracellular hyperosmolality induces transcription of the AR gene (12), resulting in an increase in AR mRNA (13), followed by increased AR protein synthesis rate (14) and, ultimately, increased sorbitol accumulation (15). To understand the molecular mechanisms involved in the osmotic regulation of the AR gene, we cloned the rabbit AR (rAR) gene and characterized its structure, which we now report.

Recently, the human AR (hAR) and ratAR genes were cloned (16, 17), and the hAR promoter region was carefully characterized (18). This last study also briefly examined the promoter region and a DNA fragment encompassing about 4.2 kilobases (kb) upstream for the presence of osmotic response. The authors were unable to detect osmotic response in their system (18). Herein we report the discovery of sequences upstream of the rAR promoter that confer hyperosmotic response capability. This finding evidences putative osmotic response element(s) (OREs) within a specific DNA fragment in a eukaryotic genome.

MATERIALS AND METHODS

Genomic Cloning. Rabbit spleen genomic DNA was isolated (19) and used to prepare a custom Lambda FIX II library (Stratagene). Forty clones were identified by screening with a 1287-base-pair (bp) AR cDNA insert released from pAR10 (13) by *Eco*RI digestion and subsequently radiolabeled with [α-^{32}P]dCTP (random primed DNA labeling kit, Boehringer Mannheim). Plaque purifications were performed with two probes derived from pAR10, a *Pst* I restriction fragment containing 137 base pairs of a 5′ translated region and a 70-base-pair *Pvu* II restriction fragment containing a 3′ untranslated region. A single clone, λgAR1 (Fig. 1), hybridized to both probes.

To obtain upstream sequences, a Lambda DASH II rabbit genomic library (Stratagene) was screened with two probes—a λgAR1, intron 1-specific probe amplified by PCR (GeneAmp, Perkin–Elmer) [primers: base pairs 547–566 (5′-ACAGGACGGGGACGTTTCCG) and 922–903 (5′-AGGGTCTGCACACCTGGAGC) of the rAR gene][§] and a 465-base-pair *Bam*HI restriction fragment, extending from exon 1 to exon 5 of the 1287-base-pair pAR10 cDNA insert. Plaque purifications were performed with both probes; a single clone, λgAR56, was characterized further.

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Abbreviations: AR, aldose reductase; hAR, human AR; rAR, rabbit AR; CAT, chloramphenicol acetyltransferase; ORE, osmotic response element; RLV, relative light unit(s).

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§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U12316 (rabbit AR mRNA), U13688–U13694 (genomic rabbit AR DNA gene fragments), and U12317 (genomic rabbit AR DNA composite sequence)].

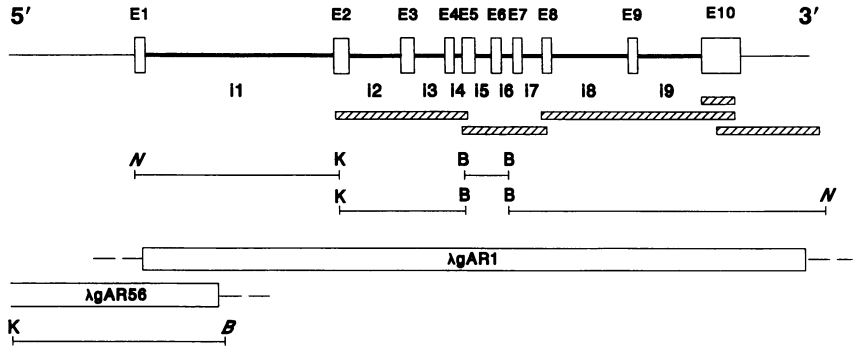

FIG. 1. Schematic diagram of the rAR gene (14.7 kb plus 3.5 kb of 5′ flanking region and 2 kb of 3′ flanking region). Lengths of exons E1–E10 (open boxes) in base pairs are as follows: E1, 102; E2, 168; E3, 117; E4, 78; E5, 123; E6, 107; E7, 82; E8, 84; E9, 83; and E10, 367. Lengths of introns i1–i9 (bold lines) in base pairs are as follow: i1, ≈5383; i2, ≈1500; i3, ≈800; i4, 213; i5, 473; i6, 310; i7, 556; i8, ≈2335; and i9, ≈1816. Genomic clones λgAR1 and λgAR56 (open bars are AR gene regions, and broken lines are λ arms) are shown in relation to the gene. Other representations are as follows: PCR fragments, hatched bars; restriction fragments, bracketed lines; B, *Bam*HI; K, *Kpn* I; N, *Not* I. Restriction sites in vector sequences are italicized.

Structure and Characterization. The transcription initiation start site and sequence of the 5′-most 14 amino acids, which are not contained in pAR10, were determined by two methods. In both methods the template was poly(A)$^+$ RNA isolated from PAP-HT25 cells exposed for 24 hr to medium (10) made hyperosmotic (500 mosmol/kg of H_2O) with NaCl. Primer extension analysis was performed by direct mRNA dideoxynucleotide sequencing (20). Primers used are numbered 5′ to 3′, with +1 being the transcription initiation start site in the rAR mRNA.[§] Antisense primers used were nucleotides 67–43, 111–87, and 299–280 (Fig. 2*A*). A PCR-enhanced method to determine the 5′-end sequence (21) was also used; the primers were nucleotides 111–87 (antisense), 87–111 (sense), and 67–43 (antisense).

Intron positions in λgAR1 were determined by using PCR with overlapping primer sets (22). The following mRNA sense and antisense primer sets were used: nucleotides 130–154 (sense) and 549–530 (antisense), 534–558 (sense) and 819–800 (antisense), 798–822 (sense) and 1284–1258 (antisense), and 1162–1183 (sense) and T7 primer (Lambda FIX II). To confirm intron positions, restriction analysis of λgAR1 with *Bam*HI, *Kpn* I, and *Not* I (Fig. 1) followed by hybridization to specific end-labeled oligonucleotide probes was performed. λgAR56 was also mapped with *Bam*HI, *Kpn* I, and *Not* I (Figs. 1 and 3). Restriction fragments (Figs. 1 and 3) were subcloned into pBluescript SK(+) II (Stratagene) and sequenced by primer-directed, double-stranded plasmid sequencing (13) (Sequenase DNA sequencing kit, United States

A

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+40 GCGACCCACCTTGTGCTCTACAACGGCGCCAAGATGCCGATCCTGGGGCTGGGCACCTGG  +99 ARmRNA
+100 AAGTCACCACCGGGCCAGGTGACCGAGGCCGTGAAGACAGCCATCGACCTCGGGTACCGC +159 ARmRNA
+160 CACATCGACTGCGCCCACGTGTACCAGAACGAGAACGAGGTCGGGGTGGCCCTGCAGGAG +219 ARmRNA
+220 AAGCTCAAGGAGCAGGTGGTGAAACGTGAGGAGCTCTTCATCGTCAGCAAGCTGTGGTGC +279 ARmRNA
+280 ACGTCCCACGACAAGAGCCT                                          +299 ARmRNA
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B

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-208                                  CAGCGGTAGGAGAGGACGGAATCCGGTGC -180 rAR
                                       |||| | || | ||| ||||||| |||
-197                                  GAGCGTTGGGGGCGGAAAGAATCCGCTGC -169 hAR

-179 CGCTAGGACCTGGCGGAAGCTCCACCCGGGCGGGGTCGCCGGGAAAGCCCCCGGGGCAAC -120 rAR
     | ||||||||| ||||||||  || ||  || |   |  |||||  ||| ||| |||| |
-168 CACTAGGACCAGGCGGAAGAAGCATCCCCGCCGACCCTTGGGGAAGGCCGCCGCGGCACC -109 hAR

-119 GCCCACCTCGGGGAGTGCGGCCAATCAGATGGCGCTTTCGCACATTCGCACGCCAATCGC  -60 rAR
      ||          || ||  |||||||||| ||| | |||||  ||   || ||||||  |||
-108 CCC----------AGCGCAACCAATCAGAAGGCTCCTTCGCGCAGCGGCGCGCCAACCGC  -59 hAR

 -59 GGGCGCCGATTCTGCAG-CCGCACGGGCTATTTAAAGGTGCGCGGCGCGGCGCCGAAGCT   -1 rAR
      ||||||  ||||||  | ||  |||||||||||||  ||||  |||||  ||  | ||
 -58 AGGCGCCCTTTCTGCCGACCTCACGGGCTATTTAAAGGTACGCGCCGCGG--CCAAGGCC   -1 hAR

  +1 ACAACGGTTCTGGGCCTGGGTCTCTG-AGGG--GCGGCCATG                    +39 rAR
      || | || ||||||  ||||||  | || |  || ||||||
  +1 GCA-CCGTACTGGGCGGGGGTCTGGGGAGCGCAGCAGCCATG                    +41 hAR
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FIG. 2. (*A*) Nucleotide sequence of renal rAR mRNA from nucleotide +40 to nucleotide +299. Primers used in primer extension analysis and PCR-enhanced determination of the 5′-end sequence are overlined. In the gene, introns 1 and 2 are positioned between nucleotides 102 and 103 and between nucleotides 270 and 271, respectively. (*B*) Nucleotide sequence of the 5′ upstream region of the rAR gene. Numbering is positive downstream beginning with +1 as the transcription initiation start site. The sequence is aligned with hAR (18) through the first codon (ATG). cis elements previously shown to affect basal promoter activity (18) (TATA box, CAAT box, and GA element) and the first codon (ATG) are doubly underlined. Primers used to PCR-amplify gene fragments for reporter gene expression analysis are overlined.

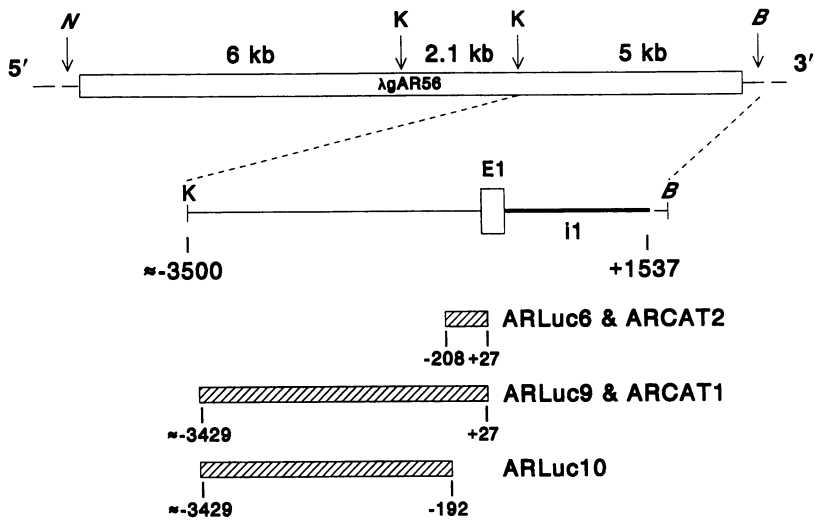

FIG. 3. Schematic diagram of λgAR56 showing positions of *Kpn* I restriction sites and an enlargement of the 5-kb restriction fragment (gAR56_5). Hatched bars represent PCR-amplified gene fragments used in the primary reporter gene constructs. ARLuc6 and ARCAT2 contain the promoter alone as defined (18), ARLuc9 and ARCAT1 contain this promoter plus the upstream sequence, and ARLuc10 contains the upstream sequence alone. B, *Bam*HI; K, *Kpn* I; N, *Not* I. Restriction sites in vector sequences are italicized.

Biochemical). PCR fragments (Fig. 1) were sequenced directly (23).

Reporter Gene Expression Analysis of Transient Transfectants. Expression vectors 007Luc and B19CAT were obtained from K. McDonagh (Hematology Branch, National Heart, Lung and Blood Institute). Both 007Luc and B19CAT are expression vectors derived from pUC8. 007Luc is promoterless and contains the luciferase gene, whereas B19CAT contains the B19 promoter in unique *Xho* I–*Hin*dIII sites immediately upstream of the chloramphenicol acetyltransferase (CAT) gene (24). LKEM-B19Luc was constructed by insertion of the B19 promoter from B19CAT into unique *Xho* I–*Hin*dIII sites in 007Luc immediately upstream of the luciferase gene. 007CAT was constructed by removal of the B19 promoter, then blunting and ligating the ends.

All AR fragments to be subcloned into expression vectors were PCR-amplified by using primers synthesized with appropriate restriction enzyme sites toward their 5′ end. All construct sequences were verified.

ARLuc6. To examine basal promoter activity as previously characterized (18), a PCR-amplified fragment from base −208 to base +27 (Figs. 2*B* and 3) was subcloned directionally into the *Xho* I–*Hin*dIII sites in 007Luc. The primers used were nucleotides −208 to −192 (sense) and +27 to +10 (antisense) (Fig. 2*B*). To control for spurious effects due to mere disruption of the vector by insertion of a DNA fragment that might produce artifactual promoter activity, we constructed ARLuc1. ARLuc1 contains a *Hha* I fragment of λgAR1, extending from intron 3 to intron 7, subcloned into the *Xho* I–*Hin*dIII site of 007Luc.

ARLuc9. To determine promoter plus putative osmotically regulated activity, a PCR-amplified fragment extending from estimated base −3429 (sense primer: 5′-TGGACCTGCAGCTTGATGT) to base +27 (antisense primer, nucleotides +27 to +10) (Figs. 2*B* and 3) was subcloned directionally into the *Xho* I–*Hin*dIII sites in 007Luc.

ARLuc10. To determine putative osmotic regulation of a heterologous promoter, a PCR-amplified fragment extending from estimated base −3429 to base −192 (antisense primer, nucleotides −192 to −208) (Figs. 2*B* and 3) was subcloned in the forward direction into the *Xho* I site of LKEM-B19Luc immediately upstream of the B19 promoter.

ARCAT2. The same fragment as described for ARLuc6 was PCR-amplified and subcloned directionally into the *Xho* I–*Hin*dIII sites of 007CAT immediately upstream of the CAT gene.

ARCAT1. The same fragment as described for ARLuc9 was PCR-amplified and subcloned directionally into the *Xho* I–*Hin*dIII sites of 007CAT immediately upstream of the CAT gene.

Transfection, Luciferase, and CAT Assays. For each transfection, PAP-HT25 cells (passages 65–68) were grown in isoosmotic medium (300 mosmol/kg of H_2O) (10, 13) to 70–80% confluency on a 150-mm dish (Corning). Cells were then cotransfected with a given luciferase construct (3 μg) and B19CAT (12 μg) by using Cell-Phect (Pharmacia). Alternatively, cells were cotransfected with a given CAT construct (12 μg) and LKEM-B19Luc (3 μg). From each 150-mm dish, transfected cells were seeded into six 35-mm dishes (Corning) containing an equal volume of 0.2 mM chloroquine in isoosmotic medium for 2.5 hr. Cells were then treated with medium containing 20% dimethyl sulfoxide for 5 min, washed twice with medium, and left overnight. Then, the medium in three of the dishes was changed to fresh isoosmotic medium (300 mosmol/kg of H_2O); the medium in the three other dishes was changed to the same medium made hyperosmotic with NaCl (500 mosmol/kg of H_2O). Twenty-four hours after changing the medium, cells were harvested by adding 150 μl of lysis buffer (enhanced luciferase assay kit, Analytical Luminescence Laboratory, San Diego). Routinely, transfections were performed in groups that included appropriate positive and negative controls—e.g., a transfection of ARLuc6 was performed concomitantly with transfections of LKEM-B19Luc, 007Luc, and transfection components only (no DNA).

Cell lysates were analyzed for total protein, luciferase activity, and CAT protein as described by the manufacturers of the following kits. Total protein was determined by the Bio-Rad protein assay kit (Bio-Rad) with γ-globulin as the standard. Luciferase activity expressed as relative light units (RLU) per μg of total cell protein was determined with the enhanced luciferase assay kit and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego). The amount (pg/μg of total cell protein) of CAT was determined with the CAT ELISA kit (5 Prime → 3 Prime, Inc.) and a Titertek Multiskan plate reader with a 405-nm filter.

Transfection Data Analyses. In transfections where the primary construct carried the luciferase gene, luciferase activity in RLU per μg of total cell protein was normalized by

dividing by CAT protein in pg per μg of total cell protein (from the cotransfected B19CAT construct) (luc RLU/CAT pg). In transfections where the primary construct carried the CAT gene, CAT protein in pg per μg of total cell protein was normalized by dividing by the luciferase activity in RLU per μg of total cell protein (from the cotransfected LKEM-B19Luc) (CAT pg/luc RLU). All values represent the mean of a minimum of three independent transfections.

RESULTS AND DISCUSSION

Structure of the rAR Gene. We had previously cloned a partial cDNA from a PAP-HT25 cell library containing all of the coding region excluding the first 14 amino acids (beginning at nucleotide 80, Fig. 2*A*) and the complete 3′ untranslated region (13). Two independent methods were used to determine the missing 5′ sequence. Poly(A)$^+$ mRNA from PAP-HT25 cells that had been exposed for 24 hr to medium made hyperosmotic with NaCl to increase rAR mRNA abundance was used as the template (12, 13). Primer extension analysis was performed by direct mRNA dideoxynucleotide sequencing with three different antisense primers (Fig. 2*A*) (20). All three reactions stopped at the same position, but the precise identity of the transcription start site nucleotide (+1) was unclear. We then used a PCR-enhanced method to identify the nucleotide (21) (Fig. 2*A*). The total mRNA sequence excluding the poly(A) tail is 1311 nucleotides long. The coding region comprises nucleotides 37–987.

The complete genomic structure was derived from two overlapping clones, λgAR1 (insert size, 16.6 kb) (Fig. 1) and λgAR56 (13.1 kb) (Fig. 3). Initially, intron/exon boundaries were identified by PCR (22) in λgAR1. Because we were unable to amplify one of the regions of λgAR1 (between exons 1 and 2) we also characterized the clone by restriction analysis. The combination of *Bam*HI, *Kpn* I, and *Not* I yielded four fragments (Fig. 1), the relative positions of which were determined by hybridization to specific end-labeled oligonucleotide probes. Sequencing the PCR fragments and the restriction enzyme fragments and additional characterization of λgAR56 (below) resulted in the following data. The total length of the gene is approximately 14.7 kb, excluding 5′ and 3′ flanking regions. Exon sequences are identical to the rAR mRNA (ref. 13 and primer-extension analysis above) except for a conservative substitution at nucleotide 699 in the coding region of the rAR mRNA (GCA in the mRNA and GCC in the gene, both of which code for alanine). All exon/intron boundaries conform to the GT–AG rule. Positions of the introns in reference to the mRNA sequence are as follows: intron 1, between nucleotides 102 and 103; intron 2, between nucleotides 270 and 271; intron 3, between nucleotides 387 and 388; intron 4, between nucleotides 465 and 466; intron 5, between nucleotides 588 and 589; intron 6, between nucleotides 695 and 696; intron 7, between nucleotides 777 and 778; intron 8, between nucleotides 861 and 862; and intron 9, between nucleotides 944 and 945. The relative lengths of exons and introns are shown in Fig. 1. All introns in the rAR gene occurred in the same position relative to the mRNA sequence, as those of hAR and rat AR genes (16, 17).

λgAR1 contained approximately 2000 base pairs of 3′ flanking region but only the last 26 base pairs of exon 1. λgAR56 (insert size 13.1 kb) was selected by using an intron 1-specific probe. A restriction analysis of λgAR56 yielded three fragments (Fig. 3). Hybridization to an upstream, exon 1-specific oligonucleotide identified the 5-kb *Kpn* I–*Bam*HI fragment (gAR56_5). Sequencing of gAR56_5 showed that it contained the first 1435 base pairs of intron 1, all of exon 1, and approximately 3500 base pairs of the 5′ flanking region. gAR56_5 was used as the template for all PCR amplifications for the principal reporter gene constructs.

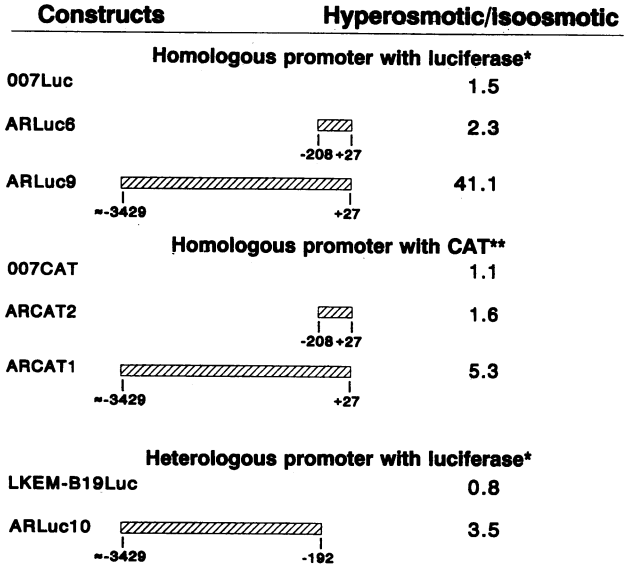

FIG. 4. Effect of hyperosmolality on reporter gene expression in transient transfectants. Transfected PAP-HT25 cells were maintained in isoosmotic medium (300 mosmol/kg of H_2O) or exposed to hyperosmotic medium (500 mosmol/kg of H_2O) for 24 hr. Values shown are the hyperosmotic/isoosmotic ratio of the luc/CAT or CAT/luc measurements (see text) in the two conditions. ARLuc6 and ARCAT2 contain the promoter alone (base pairs −208 to +27) (Figs. 2*B* and 3) as defined (18), ARLuc9 and ARCAT1 contain this promoter plus the upstream sequence (from estimated base pair −3429 to base pair +27), and ARLuc10 contains the upstream sequence alone (from estimated base pair −3429 to base pair −192). All values represent the mean of three or more independent transfections. *Cells were cotransfected with a given luciferase construct and B19CAT. Luciferase activity in RLU per μg of cell protein was normalized by dividing by CAT protein in pg per μg of cell protein; calculation of the hyperosmotic/isoosmotic ratio cancels residual units. **Cells were cotransfected with a given CAT construct and LKEM-B19Luc. CAT protein in pg per μg of cell protein was normalized by luciferase activity in RLU per μg of cell protein; calculation of the hyperosmotic/isoosmotic ratio cancels residual units.

Promoter Activity and Hyperosmotic Response in the rAR Gene. Recently, the promoter for the hAR gene was characterized (18). Fig. 2*B* shows the alignment of this sequence with that of rAR (72% identity). cis elements previously shown to affect basal promoter activity were the TATA box (in the rAR, base pairs −32 to −27), the CAAT box (base pairs −99 to −95), and a GA element (base pairs −166 to −161). The hAR sequence shown in Fig. 2*B* (base pairs −192 to +31) demonstrated full promoter activity (18). To determine whether the equivalent sequence in the rAR gene would drive a downstream reporter gene, a fragment (rAR, base pairs −208 to +27) (Fig. 3) was cloned directionally immediately upstream of the luciferase gene in 007Luc to produce ARLuc6. Cells cotransfected with ARLuc6 and B19CAT (the latter used for normalization of transfection efficiency) were maintained in isoosmotic medium (300 mosmol/kg of H_2O) until harvested. ARLuc6 showed an 11.3-fold increase in luciferase gene expression compared with 007Luc (mean expressed as luc RLU/CAT pg:ARLuc6, 1455; 007Luc, 129.2). In contrast, ARLuc1, which was constructed to control for artifactual promoter activity due to disruption of the 007Luc vector with a DNA fragment (spanning introns 3–7), showed luciferase gene expression to be less than that of 007Luc. We conclude that the upstream sequence cloned into ARLuc6 can effectively drive the transcription of the downstream gene.

To test for the presence of a region that might respond to extracellular hyperosmolality, we prepared additional constructs—ARLuc9 and ARLuc10 (Figs. 3 and 4). ARLuc9

contains a fragment extending from estimated base pair −3429 to base pair +27 situated upstream of the luciferase gene in 007Luc. This construct is equivalent to ARLuc6 plus approximately 3221 base pairs upstream. When cells cotransfected with ARLuc9/B19CAT were exposed to hyperosmotic (500 mosmol/kg of H_2O) versus isoosmotic medium for 24 hr, luciferase gene expression was >40-fold greater in the cells in hyperosmotic medium (Fig. 4) [means expressed as luc RLU/CAT pg for ARLuc9 were 70,466 (hyperosmotic) and 1714 (isoosmotic)]. In contrast, in 007Luc and ARLuc6 luciferase gene expression in hyperosmotically treated cells was 1.5-fold and 2.3-fold that of isoosmotically treated cells, respectively [means expressed as luc RLU/CAT pg for 007Luc were 188.1 (hyperosmotic) and 129.2 (isoosmotic) and for ARLuc6 were 3343.6 (hyperosmotic) and 1455 (isoosmotic)]. We conclude that the region between estimated base pair −3429 and base pair −208 contains one or more putative OREs.

An equivalent region to that in ARLuc9 had been used in the hAR promoter characterization studies but failed to show hyperosmotic response (18). In these previous studies, the region plus the hAR promoter had been subcloned upstream of the CAT gene. We hypothesized that the difference might lie in the reporter gene used. Therefore, we constructed ARCAT1 (Figs. 3 and 4), containing the same rAR gene fragment as ARLuc9 but subcloned into 007CAT immediately upstream of the CAT gene. When cells cotransfected with ARCAT1/LKEM-B19Luc were exposed to hyperosmotic medium, CAT gene expression increased by 5.3-fold over that of cells in isoosmotic medium (Fig. 4) [means expressed as CAT pg/luc RLU for ARCAT1 were 6.93×10^{-5} (hyperosmotic) and 1.32×10^{-5} (isoosmotic)]. In contrast, CAT gene expression in cells transfected with 007CAT or ARCAT2 (Figs. 3 and 4) and exposed to hyperosmotic medium was 1.1-fold and 1.6-fold that of cells exposed to isoosmotic medium, respectively (Fig. 4) [means expressed as CAT pg/luc RLU for 007CAT were 1.23×10^{-5} (hyperosmotic) and 1.14×10^{-5} (isoosmotic) and for ARCAT2 were 4.34×10^{-5} (hyperosmotic) and 2.71×10^{-5} (isoosmotic)]. Thus, the hyperosmotic response conferred by the region from estimated position −3429 to position −208 is clearly demonstrable in transfected PAP-HT25 cells regardless of the reporter gene used.

To test whether the region containing the putative OREs would have an effect on a heterologous promoter, ARLuc10 was examined. ARLuc10 contains a fragment spanning estimated base pair −3429 to base pair −192 placed upstream of the B19 promoter in LKEM-B19Luc. When cells cotransfected with ARLuc10/B19CAT were exposed to hyperosmotic medium, luciferase expression increased by 3.5-fold compared with cells maintained in isoosmotic medium (Fig. 4) [means expressed as luc RLU/CAT pg for ARLuc10 were 29,486 (hyperosmotic) and 8458 (isoosmotic)]. In comparison, luciferase gene expression in cells transfected with LKEM-B19Luc, containing the B19 promoter alone upstream of the luciferase gene, in hyperosmotic medium was 0.8 of that in isoosmotic medium [means expressed as luc RLU/CAT pg for LKEM-B19Luc were 20,682 (hyperosmotic) and 26,017 (isoosmotic)]. Thus, the region in the rAR between estimated base pair −3429 and base pair −192 contains putative OREs capable of conferring hyperosmotic response to a heterologous promoter. Unfortunately, because that region appears to have a negative effect on basal B19 promoter activity (comparing the means of ARLuc10 and LKEM-B19Luc in cells in isoosmotic medium), this may obscure some of the osmotic response expressed by ARLuc10.

Previously, we had found that transcription of the AR gene was induced by extracellular hyperosmolality (12). We have now cloned the gene and discovered a region of 5′ flanking sequence that confers osmotic response to the gene.

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1. Yancey, P., Clark, M., Hand, S., Bowlus, R. & Somero, G. (1982) *Science* **217**, 1214–1222.
2. Owen-Hughes, T., Pavitt, G., Santos, D., Sidebotham, J., Hulton, C., Hinton, J. & Higgins, C. (1992) *Cell* **71**, 255–265.
3. Mellies, J., Brems, R., Nguyen, A., Ponce, A. & Villarejo, M. (1994) *J. Bacteriol.* **176**, 3638–3645.
4. Garcia-Perez, A. & Burg, M. B. (1991) *Physiol. Rev.* **71**, 1081–1115.
5. Bagnasco, S., Balaban, R., Fales, H., Yang, Y.-M. & Burg, M. (1986) *J. Biol. Chem.* **261**, 5872–5877.
6. Bedford, J. J., Bagnasco, S. M., Kador, P. F., Harris, H. W. & Burg, M. B. (1987) *J. Biol. Chem.* **262**, 14255–14259.
7. Bagnasco, S., Uchida, S., Balaban, R., Kador, P. & Burg, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1718–1720.
8. Cowley, B. D., Jr., Ferraris, J. D., Carper, D. & Burg, M. B. (1990) *Am. J. Physiol.* **258**, F154–F161.
9. Garcia-Perez, A. & Ferraris, J. D. (1994) in *Cellular and Molecular Physiology of Cell Volume Regulation*, ed. Strange, K. (CRC, Boca Raton, FL), pp. 373–381.
10. Uchida, S., Green, N., Coon, H., Triche, T., Mims, S. & Burg, M. (1987) *Am. J. Physiol.* **253**, C230–C242.
11. Moriyama, T., Garcia-Perez, A. & Burg, M. B. (1990) *Am. J. Physiol.* **259**, F847–F858.
12. Smardo, F., Burg, M. & Garcia-Perez, A. (1992) *Am. J. Physiol.* **262**, C776–C782.
13. Garcia-Perez, A., Martin, B., Murphy, H. R., Uchida, S., Murer, H., Cowley, B. D., Handler, J. S. & Burg, M. B. (1989) *J. Biol. Chem.* **264**, 16815–16821.
14. Moriyama, T., Garcia-Perez, A. & Burg, M. B. (1989) *J. Biol. Chem.* **264**, 16810–16814.
15. Uchida, S., Garcia-Perez, A., Murphy, H. & Burg, M. B. (1989) *Am. J. Physiol.* **256**, C614–C620.
16. Graham, A., Brown, L., Hedge, P., Gammack, A. & Markham, A. (1991) *J. Biol. Chem.* **266**, 6872–6877.
17. Graham, C., Szpirer, C., Levan, G. & Carper, D. (1991) *Gene* **107**, 259–267.
18. Wang, K., Bohren, K. & Gabbay, K. (1993) *J. Biol. Chem.* **268**, 16052–16058.
19. Maniatis, T., Fritsch, E. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
20. Geliebter, J. (1987) *Focus* **9**, 5–8.
21. Hofman, M. & Brian, D. (1991) *PCR Methods Appl.* **1**, 44–45.
22. Bruzdzinski, C. & Gelehrter, T. (1989) *DNA* **8**, 691–696.
23. Bachmann, B., Luke, W. & Hunsmann, G. (1990) *Nucleic Acids Res.* **18**, 1309.
24. Liu, J., Fujii, H., Green, S., Komatsu, N., Young, N. & Shimada, T. (1991) *Virology* **182**, 361–364.