



Characterization of the Mouse Aldose Reductase Gene and Promoter in a Lens Epithelial Cell Line

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Purpose: To clone and characterize the mouse aldose reductase (AR) gene and evaluate the functional promoter under basal and hypertonic conditions in mouse lens epithelial cells.

Methods: The mouse AR gene structure was determined by DNA sequencing, and its chromosomal localization was determined by fluorescent in situ hybridization. A luciferase reporter gene was utilized to assess promoter activities of mouse, rat, and human AR deletion constructs as well as mouse site-directed mutants containing specific deletions of an aldose reductase enhancer element (AEE) or a tonicity response element (TonE). Electrophoretic mobility shift assays were performed to evaluate binding of trans-acting factors to mouse AEE and TonE.

Results: The mouse AR gene (14.2 Kb) is located on chromosome 6. The basal AR promoter activity was greatest for the rat followed by mouse and human. All 3 species demonstrated increased promoter activity under hypertonic conditions. Deletion of TonE decreased mouse AR basal activity 2.5-fold and substantially reduced the osmotic response. Deletion of AEE had only a slight effect on AR promoter activity. Nevertheless, AEE strongly bound multiple trans-acting factors under nonstressed and stressed conditions, while weaker binding was evident for TonE.

Conclusions: Species-specific differences in AR promoter activities suggest the presence of unique regulatory cis-acting elements. The effects of AEE or TonE on AR transcription appear to involve complex transcriptional regulatory mechanisms.

Aldose reductase (EC 1.1.1.21) is a cytosolic monomeric NADPH-dependent oxidoreductase capable of reducing a sugar such as glucose to its corresponding alcohol, sorbitol. A normal functional role in biological systems attributed to the accumulation of sorbitol is the counter-balancing of osmotic pressure due to excess NaCl and urea in the renal medulla [1]. The hypertonic induction of AR has been demonstrated in different tissue culture systems including rabbit inner renal medulla cells [2,3], rat kidney mesangial cells and Chinese hamster ovary cells [4], dog glomerular endothelial cells [5], dog lens epithelial cells [6], and mouse lens epithelial cells [7]. In addition, osmotic transcriptional control elements in the AR promoters of different species have recently been identified and analyzed [8-13].

The accumulation of sorbitol in diabetes is believed to be a causative factor in diabetic cataract, retinopathy, neuropathy, and nephropathy [14-17]. It has been shown that in hyperglycemic lenses, excess glucose is converted into sorbitol by the action of aldose reductase. Accumulation of sorbitol within the cell causes water influx, cellular swelling, and loss of lens homeostasis leading to opacification [18,19]. AR inhibitors have proven effective in retarding or preventing cataract formation in hyperglycemic or galactosemic animal models [20-23] and yet have met with limited clinical success in humans [24,25]. It has been noted that hyperglycemic mice are resistant to sugar-induced cataract, and levels of AR activity and sorbitol are significantly lower in mice than in rats or humans [26-30]. Strong evidence supporting the correlation

between increased polyol accumulation and diabetic or galactosemic cataract formation is the finding that transgenic mice, which over-express AR in the lens, accumulate higher sorbitol or galactitol levels and develop lens opacities [31].

The mechanism whereby AR gene expression is lower in mice than in rats has not been elucidated. Functional studies of the mouse AR gene, which include basal transcriptional control elements, would be useful in assessing the low AR expression and lack of cataract formation in hyperglycemic mice. Also, analysis of an osmotically inducible mouse AR promoter may elucidate and confirm transcriptional control elements necessary for regulating osmoregulation. To this end, we present the complete mouse AR gene structure and organization, as well as, a comparative functional AR promoter analysis of basal and osmotic-stress induction activities among mouse, rat, and human in a mouse lens epithelial cell line. Also, attention is given to the *cis*-acting elements, AEE and TonE, and their contributions to the transcriptional regulation of mouse AR expression.

METHODS

Genomic Library Screening—Prior to screening a mouse genomic library, an initial PCR of mouse AR intron 2 from mouse genomic DNA (Clontech, Palo Alto, CA) was performed with exon 2 and exon 3 AR-specific primers designed from mouse AR cDNA sequence. The PCR fragment was then sequenced in order to obtain intron 2 specific information for subsequent primer design. An intron-specific primer was used in order to identify a putative AR gene among the many pseudogenes observed in the mouse genomic library. A P1 mouse genomic library [32,33] was screened by PCR amplification (Genome Systems, Inc., St. Louis, MO) utilizing primers designed from mouse AR intron 2 and exon 3 sequences.

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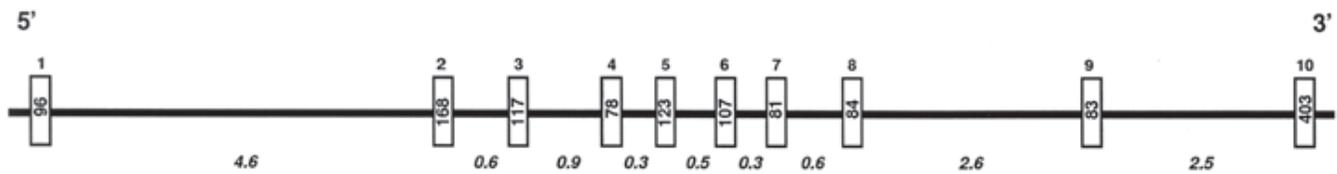


Figure 1. The mouse aldose reductase gene. Schematic diagram of AR gene depicting 10 exons (boxes) and introns 1-9 (solid line). Sizes of exons are noted within the boxes and given in bp, while intron sizes (italicized) are given in Kb. Genbank accession numbers are U89140 (5' flanking region), U89141 (exon 1), U89142 (exon 2), U89143 (exon 3), U89144 (exon 4), U89145 (exon 5), U89146 (exon 6), U89147 (exon 7), U89148 (exon 8), U89149 (exon 9), and U89150 (exon 10).

Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer (Foster City, CA). Sequences of these primers were: 5'-cggctcgagctgtaactctccag-3' and 5'-ttctggaagctctttcaccatgc-3'.

Polymerase Chain Reaction (PCR)— Mouse AR intron sizes were determined by analyzing PCR products of a positive P1 genomic clone. Primers were constructed from mouse AR exonic sequences (Table I). The PCR reaction mixture consisted of 500 ng P1 template, 10 μ M each dNTP, 0.5 μ M each primer, 1X VENT buffer, 1X BSA, and 2U Vent DNA Polymerase (New England Biolabs, Beverly, MA) in a total reaction volume of 100 μ l. For introns 2 through 9, the thermal cycling program consisted of one cycle at 94 °C for 2 min followed by 20 cycles at 94 °C for 1 min and 72 °C for 10 min, and terminating with a final extension at 72 °C for 10 min in a Perkin-Elmer 9600 thermal cyler (Foster City, CA). Intron 1 was amplified by one cycle at 95 °C for 3 min, 65 °C for 2 min, and 70 °C for 7.5 min followed by 14 cycles at 95 °C for 1 min, 65 °C for 30 s, and 70 °C for 7 min, and ending with a final extension at 70 °C for 10 min. Products were resolved and analyzed on a 1% agarose gel in 1X TBE.

Sequencing and Analysis of a P1 Genomic Clone— The mouse AR genomic sequence was determined utilizing the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) on the previously mentioned PCR products and direct fluorescent or conventional dideoxy sequencing [34] of the P1 genomic clone. The fluorescent sequencing reaction of the PCR products contained 1 μ l (500 ng-1 μ g) of template, 8 μ l of assay mixture, 5 pmol of primer, and H₂O up to 20 μ l. The thermal cycling parameters were set at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min for a total of 25 cycles. Products were purified on STE Select-D G-50 spin columns (5 Prime - > 3 Prime, Inc., Boulder, CO). Sequence samples were prepared, run on a Perkin-Elmer 310 Genetic Analyzer, and analyzed as per manufacturer's instructions (Perkin Elmer).

For direct fluorescent sequencing of the P1 genomic clone, 6 μ g of template and 20 pmol primer were utilized. The reaction conditions consisted of 30 cycles at 95 °C for 15 s, 50 °C for 15 s, and 60 °C for 4 min. The reaction volume was brought to 100 μ l by addition of H₂O, and products were purified and analyzed as previously noted above. In conventional sequencing, primers were kinase labelled, utilizing γ^{32} P-ATP (Dupont NEN, Boston, MA), and sequence reactions were run according to the manufacturer's protocol using a fmolSequencing System (Promega, Madison, WI). Products were then resolved on a 6% acrylamide sequencing gel (National Diagnostics, Atlanta, GA).

Nucleotide comparisons between mouse, rat (Genbank accession number U70876), and human (Genbank accession number U70877) AR genes were carried out using the Pustell Dot Matrix program in MacVector 3.5 program (International Biotechnologies, Inc., New Haven CT). The transcriptional trans-acting factor binding sites were analyzed with the Tfdsites. subseq. 7.0 program within MacVector 3.5 program. Percent sequence similarities of the promoters were analyzed by the Gap program within the Wisconsin Package, version 8, Genetics Computer Group (GCG) software, Madison, WI.

Fluorescent in situ Hybridization (FISH)— Chromosomal localization of the mouse AR gene was performed by the FISH technique (Genome Systems, Inc.). DNA from the mouse clone was labelled with digoxigenin by nick translation. The probe, combined with sheared mouse genomic DNA, was hybridized to mouse metaphase chromosomes in a solution containing 50% formamide, 10% dextran sulfate, and 2X SSC. Hybridized slides were incubated with fluorescent antidigoxigenin antibodies followed by counterstaining with 4', 6-diamidino-

TABLE I. PRIMERS USED FOR PCR ANALYSIS OF MOUSE AR GENE STRUCTURE

Exon	Oligo Sequence (5' -> 3')	Orientation
1	ccagccatctggaactcaacaacggc	F
2	gcttcaccaacctgctccttgagcttc	RC
2	tctctggccagggtgactgaggccgtg	F
3	cttgaaccccggtggccagtg	RC
3	agcgacctgcagctggactacctgg	F
4	tcactaggtatcacgttccctgagg	RC
4	tcagggaacctgatcctagtgcacc	F
5	tgaagagggttgaagttggagacacc	RC
5	gctatggaacaactagtggatg	F
6	agggcctgtcaggagaaccaag	RC
6	atcgagtgccaccctacctaac	F
7	ctgggctgtagtatttattgtac	RC
7	caagcctgaagatccgtctctc	F
8	cttcaagttctcagcaatgog	RC
8	gtgctgatccggttcccattc	F
9	ctcatcaaggcgacaccctcc	RC
9	gtctttgactttgaggtgagcag	F
10	ggctactggtactgccctccag	RC

Oligonucleotide sequence is given for each exon specific primer. Orientation of primer relative to the mouse AR cDNA sequence is given as either the forward (F) or reverse complement (RC) sequence.

2-phenylindole (DAPI).

Primer Extension— The mouse AR transcription initiation start site was determined using total RNA derived from brain and testis (Clontech) and utilizing the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Gibco/BRL, Gaithersburg, MD). Ten pmol of mouse AR exon 1 specific primer was kinase labelled in 1X kinase buffer, 2.5 μ Ci/ μ l γ^{32} P-ATP, and 10 U T4 kinase (Gibco/BRL) in a total volume of 10 μ l. The reaction was incubated at 37 °C for 30 min and then terminated at 90 °C for 2 min. Labelled oligo was purified on Microspin G-50 Columns (Pharmacia Biotech, Piscataway, NJ). Labelled primer was added to 10 μ g of total RNA from brain and testis as well as to control yeast total RNA (Sigma Chemical Company, St. Louis, MO) and incubated at 70 °C for 10 min. The denatured products were added to 1X PCR buffer, 2.5 mM MgCl₂, 10 mM DTT, and 400 μ M each dNTP and incubated at 42 °C for 2 min. Two hundred units of Superscript II RT were then added followed by incubation at 42 °C for 50 min. The reaction was terminated at 70 °C for 15 min followed by addition of 10X DNA Sequencing Stop Solution (Promega). Products were run adjacent to a DNA sequencing ladder on a 6% sequencing gel. The exon 1 specific primer utilized in the primer extension and sequencing reactions was 5'-gtgggcatcttggtgccgttgtagttcc-3'. Sequencing template was derived from the subcloned 1.2 Kb mouse AR promoter/exon 1 in the pGL3 basic plasmid (Promega).

Subcloning Promoter Constructs— Promoter deletion constructs 1 through 4 were designed utilizing the mouse AR promoter and exon 1 sequences. Synthetic oligonucleotides, containing unique restriction enzyme sites, were designed from the mouse promoter or exon 1 sequence and used in a PCR amplification assay. DNA fragment products, purified from the Wizard PCR Purification System (Promega), were



Figure 2. Chromosomal localization of mouse AR gene. The gene is localized to the telomeric region of chromosome 6 as shown by fluorescent *in situ* hybridization with the mouse AR genomic DNA probe (arrows).

directionally subcloned into a phosphatase (Promega) pGL3 basic Luciferase Reporter Vector (Promega) by standard ligation (Takara, Biomedicals, Madison, WI). Ligands, including previously prepared rat and human AR deletion constructs [13], were then used to transform *Escherichia coli* cells (JM 109, Promega) according to the manufacturer's specifications.

Site-Directed Mutagenesis— Site-directed mutagenesis was performed on the 1.2 Kb mouse AR promoter (construct 1) utilizing the Morph Site-Specific Plasmid DNA Mutagenesis Kit (5 Prime -> 3 Prime, Inc.) as per manufacturer's instructions. The mutant clones contained either the AEE-like deletion (5'-gacgagtg-3') or the TonE-like deletion (5'-tggaaaatcaccag-3'). Mutations were verified by fluorescent dye terminator cycle sequencing.

Cell Line Transfection and Reporter Gene Assay— A mouse lens epithelial cell line, α TN4 [7], was cultured at 37 °C and 5% CO₂ in D-MEM/F-12 medium (Gibco-BRL) supplemented with 10% fetal bovine serum, 1% insulin-selenium, 2 mM L-glutamine, gentamycin (40 μ g/ml), and fungizone (0.5 μ g/ml). When cells reached 60% confluency, 9 μ g of mouse, rat, or human AR promoter construct was cotransfected with 1 μ g of pSV β -galactosidase vector (Promega) utilizing the Profection Mammalian Transfection Systems kit (Promega). Cells were transfected for 48 h at 37 °C, fresh medium or medium supplemented with 100 mM NaCl (500 mOsmol/kg H₂O) was then applied, and cells were incubated an additional 18 h at which time they were lysed, harvested, and assayed using the Dual Light kit (Tropix, Bedford, MA) according to the manufacturer's protocol.

Electrophoretic Mobility Shift Assay— Double-stranded oligonucleotides containing the mouse TonE-like element (5'-

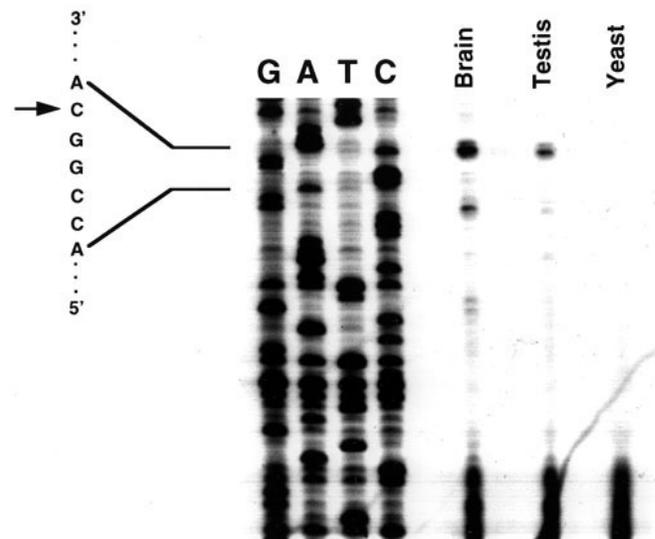


Figure 3. Primer extension of mouse AR transcription start site. Total RNA from different tissues was hybridized with a kinase-labelled mouse AR exon 1 specific primer and reverse transcribed, terminating at the cap site. The GATC sequence ladder to ascertain nucleotide length is shown along with results from brain, testis, and yeast (control). The major product, which is approximately 74 bp in size and terminates at a cytosine residue, is specified by an arrow and shown in the autoradiograph.

gcaccgactggaatcaccagaatgggattta-3') or the AEE-like element (5'-gggtgttgacagtgccaaaattgccgtc-3') as well as oligos, lacking the TonE-like element (5'-gcaccgacaatgggattta-3'), or AEE-like element (5'-gggtgttgccaaaattgccgtc-3'), were synthesized. The forward and reverse complement primers were combined in a final concentration of 10 pmol/ μ l double-stranded DNA. Ten pmol of oligonucleotide was kinase labelled and then purified with a Microspin G-25 column (Pharmacia) as per manufacturer's protocol. The α TN4 cells were grown to 100% confluency in 100 mm diameter dishes. Fresh medium was then added to control plates while the osmotically-stressed cells received 100 mM NaCl-supplemented medium. Cells were incubated for an additional 18 h. Cells

were then rinsed twice in PBS, harvested, resuspended in 2 packed cell volumes of buffer (20 mM Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 M EDTA, 0.5 mM DTT, 2 mM AEBSF-protease inhibitor, and 25% glycerol v/v), snap-frozen in dry ice/ethanol, thawed, homogenized, and centrifuged at 34,000 g for 1 h. Whole-cell extracts (2-6 μ g of protein) were combined with binding buffer (200 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM EDTA, 5 mM DTT, and 50% glycerol v/v) and 4 μ g poly dI-dC. The mixture was incubated at 4 °C for 30 min. The 0.1 pmol ³²P-labelled oligomer (10⁴ cpm) was then added and incubated at 25 °C for 30 min. Products were resolved on a 6% acrylamide gel (National Diagnostics).

RESULTS

Genomic Organization and Structure of the Mouse Aldose Reductase Gene—A P1 mouse genomic library was screened by PCR amplification with AR exon and intron specific primers. A putative AR clone from this genomic library was then PCR amplified and sequenced, and the gene structure was analyzed. The mouse AR gene is approximately 14.2 kb and contains 10 exons and 9 introns (Figure 1). The genomic organization is conserved upon comparison with the rat, human, and rabbit AR genes [8,35,36]. The exons range in size from the smallest, exon 4 (78 bp), to the largest, exon 10 (403 bp). Introns range in size from the smallest, introns 4 and 6 (0.3 kb), to the largest, intron 1 (4.6 kb). The acceptor and donor splice junctions conform to consensus sequences noted for eukaryotic genes [37] with the exception of exon 7 which has an extra G (CAGG/G). Intervening sequences 6 and 9 interrupt codons coding for Trp and Ser, respectively, whereas introns 1, 2, 3, 4, 5, 7, and 8 are flanked by intact codons Lys/Ser, Lys/Leu, Lys/Pro, Thr/Ala, Gln/Ile, Gln/Val, and Lys/Val, respectively.

The P1 genomic AR DNA was utilized for chromosomal localization of the mouse AR gene. Fluorescent in situ hybridization results of mouse metaphase chromosomes indi-

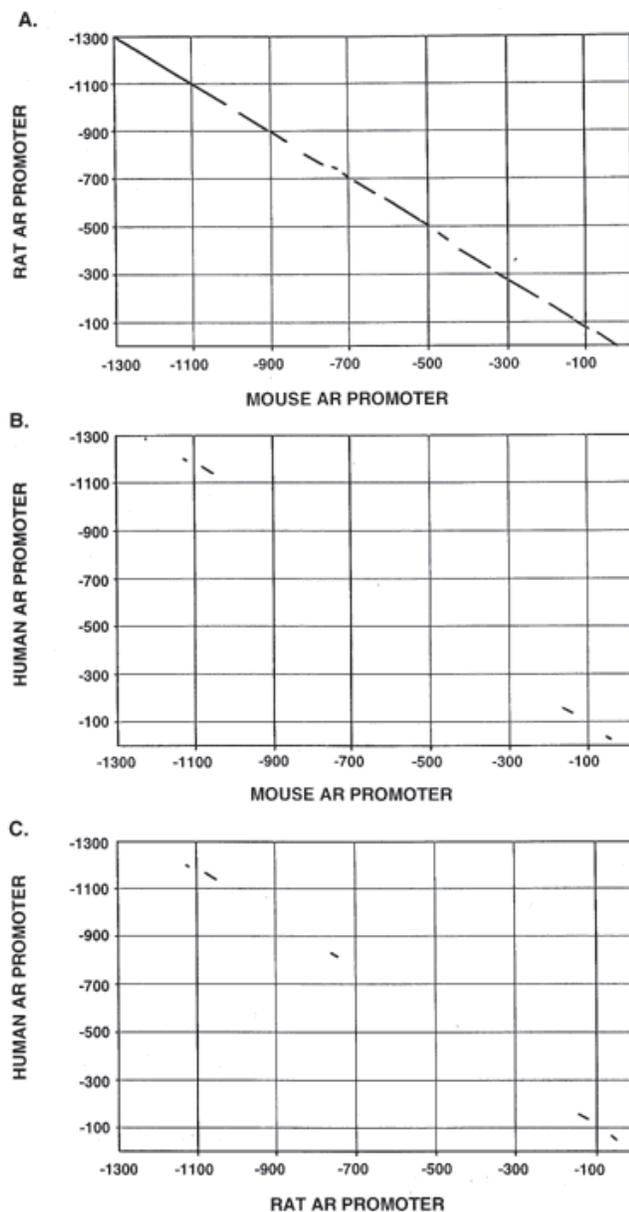


Figure 4. Dot matrix comparisons of 1300 bp of AR promoter sequences. Parameters for comparison are 30 bp with 65% similarity. (A) Mouse versus rat sequence. (B) Mouse versus human sequence. (C) Rat versus human sequence.

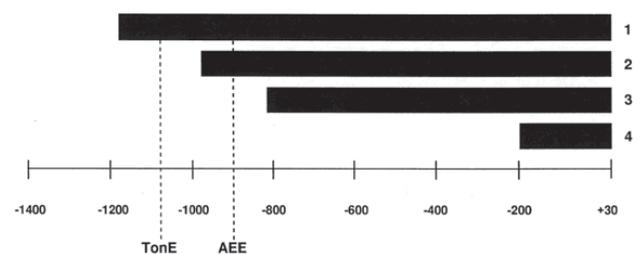


Figure 5. Design of aldose reductase promoter-deletion constructs. Constructs range in size from 1.2 to 0.22 Kb and include the promoter region and 30 bp of exon 1. The 1.2 Kb construct (1) contains the AEE-like and TonE-like elements. The 1 Kb construct (2) lacks the TonE-like *cis*-acting element while the 0.84 Kb (3) and 0.22 Kb (4) constructs also lack the AEE-like element. Location of TonE-like and AEE-like elements are indicated by dashed lines.

cate that this gene is located on chromosome 6 (Figure 2). The AR gene is located at a position which is 17% of the distance

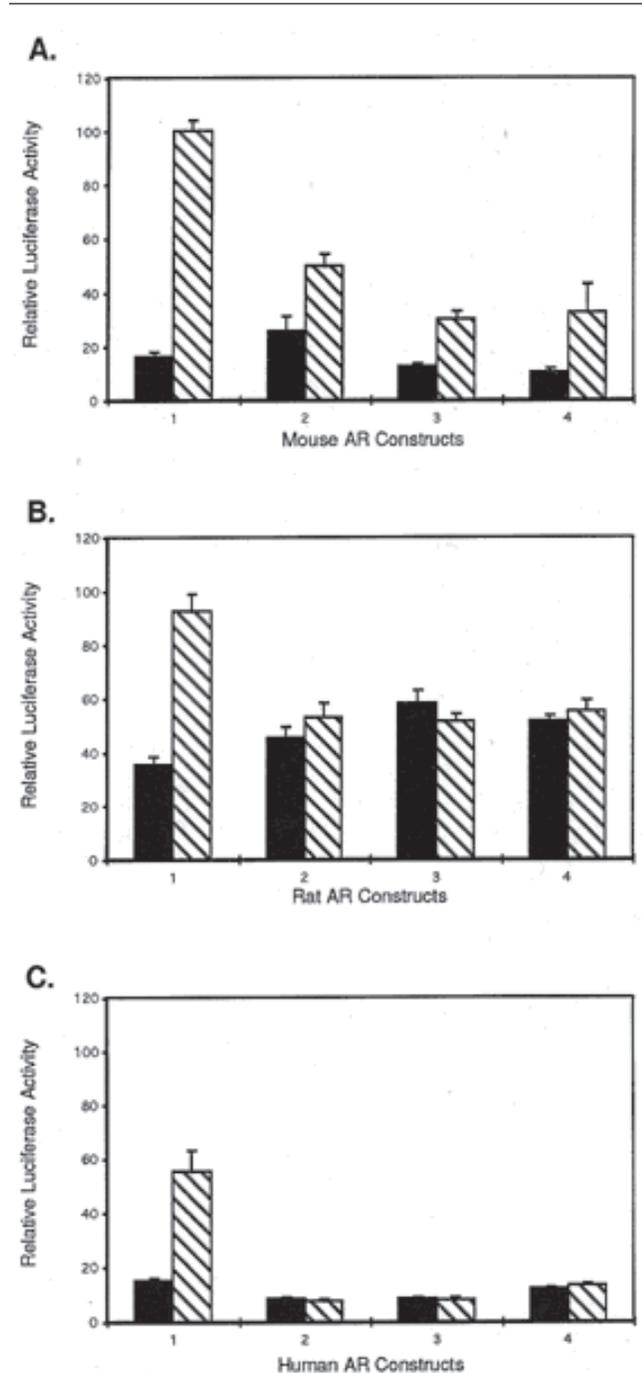


Figure 6. Aldose reductase promoter constructs and luciferase reporter gene activities in a mouse lens epithelial cell line. Relative luciferase activities for mouse (A), rat (B), and human (C) AR promoter constructs are depicted where basal activity is represented by black boxes while osmotic-stress values are denoted by striped boxes. Values represent the mean of six independent transfections (unless otherwise noted in Table II), \pm standard deviation indicated by error bars. Relative luciferase activity indicates absolute values from fluorescence emission normalized relative to control pSV β -galactosidase transfection activity and represented as a percentage upon comparison of all promoter activities. Background luciferase activity (not shown) from the promoter-less pGL3 basic vector was negligible.

from the heterochromatic-euchromatic telomere boundary of chromosome 6. This position corresponds to band 6B1. A total of 80 metaphase cells were screened with 74 showing label specificity.

Primer extension was performed to map the transcriptional start site of the mouse AR gene (Figure 3). The cap site was located 30 bp upstream from the ATG translation initiation site in mouse brain and testis. Total RNA from yeast served as a control. By comparison, in human liver, the major transcription start site was mapped to 31 bp upstream of the translation initiation codon [36] while in rat lens, the cap site was found 35 bp upstream of the ATG site [38]. The 3' UTR is 363 bp long with a poly A signal, AATAAA, located at 345-350 bp. This element is probably functional in AR mRNA processing since the mouse AR cDNA contains a poly A tail 14 bp downstream from this signal (Genbank accession number L39795) [39].

Analysis of 1300 bp of the mouse AR 5' flanking region revealed 85% and 60% sequence identity with rat and human promoters, respectively. Segments of the mouse promoter share high degrees of sequence similarity with the rat promoter (Figure 4a) [13]. In contrast, much less sequence similarity is observed upon comparison of mouse and human promoters (Figure 4b) or rat and human promoters (Figure 4c). The mouse AR promoter contains a TATA box (TATTTAAA) at -38 to -31 (relative to the cap site) and CCAAT boxes at -74 to -70 and -107 to -103. These *cis*-acting elements also are found in the rat and human AR promoters in addition to other putative trans-acting factor binding sites such as Sp1, AP1, AP2, CRE, and Hsp's. These 5' flanking regions also possess a TonE-like element (5'-TGGAAAATCACCAG-3' in mouse at -1060 to -1047), which is a hypertonicity responsive element found and studied in the canine betaine transporter promoter [40]. A second trans-acting factor binding site, which was observed and

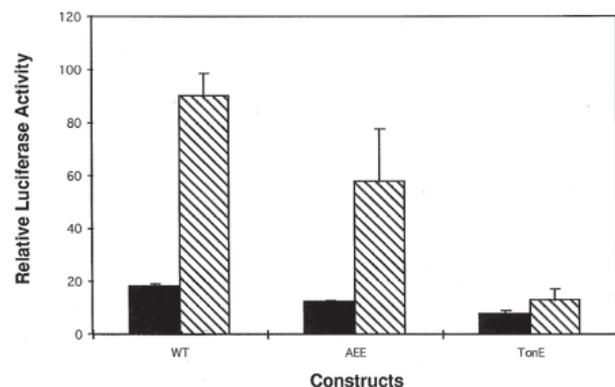


Figure 7. Mouse AR promoter basal and osmotic-stress activities of site-directed mutants. Relative luciferase activity is shown with basal promoter activities depicted as black boxes and osmotic-stress activities are represented by striped boxes. Constructs include the 1.2 Kb mouse AR promoter-plasmid construct 1 (WT) and the mutant-plasmids either lacking the AEE-like element (AEE) or the TonE-like element (TonE). Values represent the mean of three independent transfections, \pm standard deviation indicated by error bars.

characterized, is a rat aldose reductase enhancer (AEE) element [13]. This *cis*-acting element has been shown to bind *trans*-acting factors derived from C9 rat liver cells when grown under isotonic or hypertonic conditions. In the mouse AR promoter, the AEE-like element (5'-GACGAGTG-3') is located at -907 to -900.

Mouse, Rat, and Human AR Promoter Constructs and Reporter Gene Activity Assays— Mouse α TN4 cells were cotransfected with promoter deletion (Figure 5) or site-directed mutant-luciferase constructs and a pSV β -galactosidase construct. Cells were lysed, harvested, and assayed for reporter gene activity. The mouse, rat, and human AR basal promoter activities in α TN4 cells were compared (Figure 6a-c, Table II, top panel). The overall basal activity was greatest for the rat AR promoter compared to mouse and human with relative luciferase values ranging from 35.2 to 58.0, 10.1 to 25.7, and 8.4 to 14.6, respectively. Basal activity profiles showed up to a 2-fold change with sequential deletions. The osmotic-stress induction patterns for the three species were also compared. Promoter activities were greatest for construct 1. Relative luciferase activities for the osmotic-stress induction ranged from 6.1, 2.6, and 3.8-fold for mouse, rat, and human AR promoters, respectively. Deletion of the AR promoter to 1.0 Kb (construct 2) eliminated the osmotic response in the rat and human, while the mouse osmotic induction decreased from 6.1-fold to about 2.0-fold. In the mouse AR promoter, constructs 3 and 4 still elicited an osmotic induction of 2.4 and 3.2-fold, respectively. Further analysis of the effects of the AEE-like and TonE-like elements on the mouse AR promoter activity were analyzed with site-directed mutants (Figure 7, Table II, bottom panel). Deletion of the AEE-like or TonE-like elements

reduced the basal AR promoter activity by 1.5 and 2.5-fold, respectively. Promoter activities for the AEE-like and TonE-like mutants decreased from 90 (WT) to 57.7 and 12.9, respectively, under hypertonicity. Compared to the WT mouse AR promoter activity which showed an osmotic induction of 4.9-fold, the AEE-like and TonE-like mutants demonstrated inductions of 4.7 and 1.7-fold, respectively.

Electrophoretic Mobility Shift Assay— Whole-cell extracts from α TN4 cells grown under isotonic or hypertonic conditions were examined to determine the presence of *trans*-acting factors bound to mouse AEE-like or TonE-like *cis*-acting elements (Figure 8). Strong protein binding to the oligo containing the AEE-like element was observed for non-stressed and osmotically-stressed (18 h) conditions (lanes 2 and 3). In addition, under non-stressed and stressed conditions, deletion of the 8 bp AEE-like *cis*-acting core element (5'-gacgagt-3') from the radiolabelled oligonucleotide substantially decreased protein binding (lanes 4 and 5) with only a slight band (arrow B) being observed. To a lesser extent, faint protein-binding was also observed for the oligo containing the TonE-like element (lanes 6 and 7). Finally, an oligo containing the 14 bp TonE-like deletion (5'-tggaaaatcaccag-3') revealed negligible protein binding under nonstressed and osmotically-stressed conditions (lanes 8 and 9).

DISCUSSION

We have cloned and characterized the mouse AR gene. This gene, located on chromosome 6, is approximately 14.2 kb in

TABLE II. AR PROMOTER ACTIVITY

SPECIES	CONSTRUCT	ISOTONIC	HYPERTONIC	H/I
MOUSE	1	16.34 \pm 1.99	100.00 \pm 4.29	6.12
	2	25.66 \pm 5.87	49.71 \pm 4.43	1.94
	3	12.42 \pm 0.64	30.00 \pm 3.54	2.42
	4	10.11 \pm 1.59	32.46 \pm 10.78*	3.21
RAT	1	35.17 \pm 3.59	92.45 \pm 6.38	2.63
	2	45.24 \pm 4.22	52.87 \pm 5.60	1.17
	3	57.99 \pm 5.44	51.55 \pm 2.68	0.89
	4	51.23 \pm 2.53	55.36 \pm 3.93	1.08
HUMAN	1	14.64 \pm 0.83	55.19 \pm 8.35	3.77
	2	8.35 \pm 0.64	7.61 \pm 0.42	0.91
	3	8.43 \pm 0.57	8.17 \pm 0.60	0.97
	4	11.95 \pm 0.94	13.40 \pm 0.70	1.12
MOUSE (WT)		18.31 \pm 0.86	90.00 \pm 8.35	4.92
MOUSE (AEE)		12.18 \pm 0.51	57.74 \pm 19.84	4.74
MOUSE (TonE)		7.46 \pm 1.49	12.87 \pm 4.50	1.73

Relative luciferase activities of AR promoter constructs in mouse α TN4 cells grown under isotonic and hypertonic conditions. Top panel; In a representative experiment, Mouse, Rat, and Human AR promoter results indicate mean of six replicates (where *, n = 4) \pm standard deviation. Bottom panel; In a separate experiment, mouse WT, AEE, and TonE results indicate mean of three replicates \pm standard deviation. The osmotic induction is represented by the ratio of hypertonic to isotonic promoter activities (H/I).

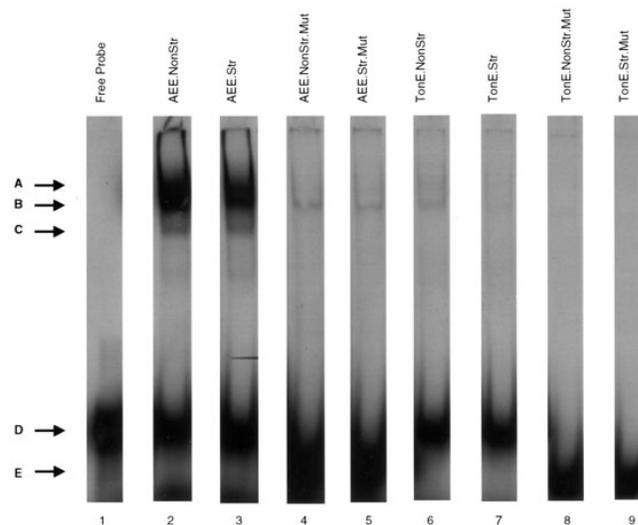


Figure 8. Electrophoretic Mobility Shift Assay (EMSA) of AEE-like or TonE-like elements with whole cell extracts from α TN4 cells. Free AEE-like probe is shown in lane 1. Complexes, indicated by arrows, are seen for AEE-like (A, B, C) and to a lesser extent for TonE-like (A, B) radiolabelled oligomers under non-stressed (NonStr) and osmotically-stressed (Str) conditions (lanes 2, 3, 6, and 7). Radiolabelled oligomers lacking only the AEE-like or TonE-like sites failed to substantially bind *trans*-acting factors under nonstressed and osmotically-stressed conditions (lanes 4, 5, 8, and 9). Free unbound probe is shown by arrows (D,E).

size, and the genomic organization is conserved among the rat, human, and rabbit AR genes. Its structure consists of 10 exons and 9 introns, and the splice junctions are similar to consensus sequences. A 1.3 Kb sequence analysis of the promoter region reveals 85%, 60%, and 62% sequence similarities between mouse and rat, mouse and human, and rat and human AR promoters, respectively. The mouse and rat AR promoters share a high degree of sequence similarity, and yet the overall basal activity is much higher for rat than for mouse in α TN4 cells. This reflects the pattern found in vivo whereby in the lens, the rat AR expression is much greater compared to the mouse. Deleting the AR promoter construct from 1.2 Kb to 0.22 Kb showed some pattern changes (up to 2-fold) in basal promoter activities for the three species. It is interesting to note that even with the smallest AR construct 4, the rat basal promoter activity was 5-fold greater than that of the mouse. The *cis*-acting elements within this DNA region that govern AR enhancement for the rat or AR repression for the mouse remain to be elucidated.

In C9 rat liver cells, it has been shown that a rat AR enhancer element (AEE) is essential for basal and osmotic response activities [13]. Deletion of 8 bp of this element completely abolished constitutive rat AR promoter activity in the C9 cells. In the mouse α TN4 cells, the AR promoter deletion construct 3, lacking the AEE-like element, revealed a 2.1-fold drop in basal activity compared to construct 2 only for the mouse. Upon closer inspection with the mouse AEE-like mutant, only a very slight decrease in basal activity and osmotic induction was observed compared to WT. However, the 8 bp core AEE-like element sufficed to strongly bind trans-acting factors under nonstressed and osmotic-stress conditions. The sole binding of AEE protein to its *cis*-acting element appears insufficient for the up-regulation of AR basal or osmotic-stress promoter activity.

A major transcriptional control element involved in the up-regulation of AR promoter activity under hypertonic conditions is the TonE element, first discovered and characterized in the canine betaine transporter gene [40]. To date, this

cis-acting element has been evaluated in rabbit [9], human [10], rat [13], and mouse [11] AR promoters in transfected PAP-HT25 rabbit renal medulla, HepG2 human liver, C9 rat liver, and CV1 monkey kidney cell lines, respectively. Our results confirm the importance of TonE in the osmotic response previously observed with the mouse AR promoter in monkey kidney cells [11]. In addition, in our system, the TonE-like element also seems to participate in basal activity enhancement. However, deletion of the TonE-like element did not entirely eliminate the osmotic response or basal promoter activity, indicating the involvement of additional factors in AR transcriptional regulation in the mouse.

In summary, transcriptional regulatory elements in the mouse, rat, and human AR promoters which account for the major differences in AR expression, have yet to be clearly defined. However, in mouse lens epithelial cells, while the AEE-like element (Figure 9) alone does not affect AR promoter activity, the TonE-like element contributes to the up-regulation of mouse AR under both basal and osmotic stress conditions. In addition, trans-acting factors were shown to interact with both of these *cis*-acting elements in α TN4 cells. Thus, the transcriptional regulation of AR gene expression appears to consist of complex mechanisms involving multiple *cis*-acting elements and trans-acting factors, perhaps specific to a given species.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Paul Russell for kindly providing the α TN4 cell line and Ms. Sharon Kiang for excellent technical assistance.

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Figure 9. Sequence alignment of mouse, rat, and human AR promoter regions containing AEE and TonE *cis*-acting elements (boxed consensus sequences). The mouse and human AEE elements differ by one base pair compared to the rat AEE sequence. The mouse and rat TonE element sequences are identical. They differ from the human TonE element by one base pair.

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