Identification of a Novel cis-Element Required for the Constitutive Activity and Osmotic Response of the Rat Aldose Reductase Promoter*

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A new and essential cis-element AEE (aldose reductase enhancer element), necessary for the constitutive activity and the osmotic stress response of rat aldose reductase transcription in a rat liver cell line, has been identified. In transient transfection assays, an increase in promoter activity, up to 3.8-fold, was observed with osmotic stress (600 mosm/kg H2O) using a luciferase reporter gene construct containing aldose reductase promoter sequence from −1,094 base pair (bp) to +23 bp. A deletion between −1,071 and −895 bp reduced the constitutive activity and abolished the osmotic response of the promoter. Exonuclease III mediated in vitro DNA footprinting and dimethyl sulfate in vivo footprinting revealed DNA protection of a 32-bp region and two guanosines (G) within this region protected from methylation, respectively. Electrophoretic gel mobility shift assays using whole liver cell extracts showed protein binding, under both normal and stressed conditions. Deletion of the sequence between the two guanosines protected by in vivo dimethyl sulfate DNA footprinting (GAAGAGTG) in a luciferase construct (−1,094 bp to +23 bp) abolished the constitutive promoter activity. One copy of AEE fused to the thymidine kinase promoter gave a maximum constitutive activity of 7.7-fold and a maximum osmotic response activity of 6.7-fold.

The elevation of renal medullary extracellular NaCl and urea during antidiuresis has been known to be adjusted intra-cellularly by accumulation of solutes such as sorbitol, myo-inositol, betaine, and glycerophosphorylcholine. Aldose reductase (AR),1 an enzyme which catalyzes the reduction of various sugars to alcohols, including glucose to sorbitol, is highly expressed in kidney inner medulla (1, 2), and is believed to play a role in normal renal osmoregulation. A similar role for AR has been reported in plants that accumulate sorbitol during seed development (3). Contrary to the role of AR in osmoregulation, AR-mediated accumulation of sorbitol from excess glucose in diabetic patients has been suggested to be one of the triggering events in diabetic complications, including cataract, retinopathy, neuropathy, and nephropathy (4–6). The inhibition of this enzyme in diabetic animal models, such as galactose-fed rats has been successful in retarding or preventing such complications (7).

Elevation of AR enzymatic activity and protein synthesis in cultured rabbit renal inner medulla cells (PAP-HT25) exposed to hypertonic medium was first reported by Moriyama et al. (2) followed by additional findings that AR induction by hypertonic stress also occurs in other cell types and species by Kaneko et al. (8). Wang et al. (9) analyzed the human AR promoter up to −609 bp from the transcriptional start site using HepG2 cells (9). DNA protection in a CGGAA(A/G) motif (−186 to −146), and a GC-rich region (−87 to −31) was observed by in vitro DNase I footprinting, but no response to osmotic stress was observed with this promoter region in transfection studies. Ferraris et al. (10) observed a 40-fold increase in promoter activity with a −3.6 kilobase to +34-bp rabbit AR construct under osmotic stress (10). Three recent reports describe the involvement of a tonE-like osmotic response element of the human AR promoter at position −1,235 bp (11), the rabbit AR promoter at −1,106 bp (12), and the mouse AR promoter at −1,053 bp (13). A signal transduction study revealed that the p38 or SAPK/JNK pathway are not necessary for the transcriptional regulation of the AR promoter through osmotic response element (14).

In this paper, we describe the identification of a novel cis-element necessary for the constitutive activity and the osmotic response activity of the rat AR promoter which is located in the proximity of the previously described tonE-like element. This novel AEE element is shown to be occupied in vivo in both transiently transfected templates and in the endogenous promoter, suggesting that a stable association with transcription factors at this site is required for effective rat AR promoter activity.

EXPERIMENTAL PROCEDURES

Reporter Gene Construction—The rat AR promoter was isolated from a phage rat genomic library (CLONTECH) (15). The 5′-flanking sequence of the gene was sequenced up to −3.6 kb (LARK Technologies, Houston, TX). The promoter fragments were amplified by PCR and subcloned into the pGL3 luciferase reporter vector (Promega, Madison, WI) and sequenced. Five additional reporter constructs were made by ligation of 5′-KpnI-GAGGGGTGTTGGAAGAGTGCCAAATTTCCGC-CATT-KpnI-3′ sequence in both sense and antisense orientation, 5′-KpnI-AGGGGTGTTGGAAGAGTGCCAAATTTCC-KpnI-3′, 5′-KpnI-GGTTTGAGAAGTGCCAAATTTCC-KpnI-3′, 5′-KpnI-GGTTTGAGAAGTGCCAAATTTCC-KpnI-3′ to the 5′ end of the thymidine kinase (TK) promoter (−105 to −52 bp) in the pGL3basic luciferase reporter vector (16).

Cell Culture, Transfection, and Osmotic Shock—A normal rat liver cell line (Clone 9, ATCC, Rockville, MD) which expresses AR and responds to osmotic stress (data not shown) was cultured in 35-mm
diameter culture wells (Falcon 3046) in Ham's F-12K medium (Life Technologies, Inc.), 10% fetal bovine serum (Life Technologies, Inc.), and 50 μg/ml gentamicin (Life Technologies, Inc.). When cells were 60% confluent, 1.7 μg of luciferase construct plasmid and 0.7 μg of pSV-β-galactosidase vector were transfected by the CaCl2 precipitation method (Profection mammalian transfection system, Promega) without osmotic shock. After 48 h, six of the wells received normal medium and the other six received hypertonic medium (medium supplemented with 150 mM NaCl; 600 mosm/kg H2O). Cells were harvested after 18 h, followed by luciferase assay and β-galactosidase assay according to the manufacturer's instructions (Dual-Light, Tropix, Bedford, MA).

In Vivo Exonuclease III-mediated Footprinting—Exonuclease III (ExoIII)-mediated footprinting was carried out as described by Archer et al. with slight modification (17–19). Cells were cultured in 10-cm diameter dishes up to 60% confluency and were then transfected with 10 μg of the luciferase construct 4, which contains 1,094 bp of upstream AR promoter sequence. After 6 h, the cells were washed with medium and grown an additional 48 h. Cells were scraped and washed with phosphate-buffered saline followed by suspension in 5 ml of homogenization buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine, 5% sucrose). The suspension was homogenized in a Dounce homogenizer and 1 ml of 1 M sucrose solution (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 10% sucrose) was added. After centrifugation at 1,400 × g for 20 min the pelleted nuclei were washed once with wash buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine). Isolated nuclei (4.5 × 107) were resuspended in 200 μl of enzyme digestion buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, 5% glycerol, 1 mM dithiothreitol) and treated at 30 °C for 15 min with 200 units of Asp718 or HindIII restriction enzyme (Boehringer Mannheim) and 650 units of ExoIII (Boehringer Mannheim). One ml of proteinase K buffer (10 mM Tris-HCl, pH 7.6, 10 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K) was added and incubated at 37 °C for 2 h to digest the nuclei. DNA was purified by phenol/chloroform extraction followed by ethanol precipitation. The DNA was resuspended and treated at 30 °C for 30 min with 50 units of mung bean nuclease (Boehringer Mannheim) in mung bean buffer (50 mM NaOAc, pH 5, 30 mM NaCl, 1 mM ZnSO4). Twenty micrograms of isolated DNA was used for linear PCR. Primers used for linear PCR were 5'-AGTTCCTCTCAAGAACAATTGGCGGAA-3' (−877 bp to −952 bp, primer 1) for the coding strand and 5'-AGTTGCCCTCAAGAACAATTGGCGGAA-3' (−3250 bp to −3250 bp, primer 2) for the luciferase construct 9 (−877 bp to −952 bp).
−901 bp, primer 2) for the noncoding strand. For control experiments construct 4 was digested with HindIII and linear PCR was performed with primer 1 (Fig. 2, lane 1) or the construct 4 was digested with Asp718 and linear PCR was performed with primer 2 (Fig. 2, lane 3).

Linear PCR product were resolved in a 6% sequencing gel (Sequagel-6, National Diagnostics, Atlanta, GA).

Dimethyl Sulfate in Vivo Footprinting—Cells were treated with 0.1% dimethyl sulfate for 90 s in both normal and hypertonic medium. DNA was extracted and cleaved by piperidine. Ligation-mediated (LM)-PCR was performed as described previously (20, 21). Primers used for analysis were primer 2 (shown above), 5′-TGAACAGGCAGAATCCCATA-3′ (2747 to 2766 bp, primer 3), and 5′-CTGAAATAATCGGAGTTGC-CCCA-3′ (2864 to 2886 bp, primer 4). After LM-PCR, labeled products were resolved in a 6% sequencing gel (Sequagel-6, National Diagnostics, Atlanta, GA). At least two independent DNA preparations were used.

Whole Cell Extract Preparation for Electrophoretic Mobility Shift Assay (EMSA)—Whole cell extracts were prepared as described previously (22). Rat liver cells were cultured in three 10-cm diameter dishes to 90% confluency. The cells were then grown in normal medium or hypertonic medium for 30 min or 3 h. After stress, cells were pelleted, washed with phosphate-buffered saline, and resuspended in 2 packed cell volumes of Buffer C (20 mM Hepes, pH 7.9, containing 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% (v/v) glycerol, 2 mM proteinase inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride). The cells were snap frozen in ethanol/dry ice. Frozen cells were quickly thawed for homogenization and centrifuged for 1 h at 34,000 × g. The supernatant was used for EMSA without dialysis.

EMSA—Whole cell extracts (2–6 μg of protein) were incubated with 0.1 pmol of 32P-labeled oligonucleotide (105 cpm/reaction mixture) in EMSA binding buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 5% (v/v) glycerol, and 2 μg of poly(dI-dC) for 30 min at 4 °C. The oligomers used were: probe A, 5′-GAGGGGTGTTGGAAGAGTGCCAAATTTCCGCCATT-3′; probe A8, 5′-GAGGGGTGTTGAGGAGACACCAAATTTCCGCCATT-3′. For the competition experiments, extracts were preincubated at 4 °C for 30 min with a × 100 excess of unlabeled oligomers prior to the addition of labeled probe.

RESULTS

Identification of a Region Controlling the Constitutive Transcription and Osmotic Response of the Rat AR Promoter—Various luciferase reporter constructs of the rat AR promoter were made and transfected into rat liver cells to measure the promoter activity under normal and hypertonic conditions (Fig. 1). Rat AR constitutive promoter activity was constant for constructs 1 (23,104 to 2923 bp) and 2 (23,371 to 2923 bp), while further deletions increased the activity up to construct 5 (21,052 to +23 bp) which gave the highest constitutive activity. Deletion to construct 6 (2895 to +23 bp) had a significant effect on the reduction of constitutive promoter activity.

Induction of promoter activity by osmotic response of construct 1 was 4.4-fold while deletion to construct 2 resulted in an increase of 11.8-fold (Fig. 1). Further deletion to construct 4 (1,094 to +23 bp) gave the highest absolute promoter activity by osmotic response (100.00 under hypertonic conditions). Construct 5 (−1,052 to +23 bp) which lacks the tonE element still...
maintained an osmotic response of 2.0-fold.

Construct 8, which lacks 8 bp of sequence between the two guanosines protected in vivo (2915 to 2908 bp) abolished the constitutive promoter activity. Construct 9, which contains all of the sequence of construct 1 except for a 177-bp deletion between 21071 and 2895 bp, abolished the constitutive activ-

**FIG. 3.** Dimethyl sulfate-mediated in vivo DNA footprinting of the promoter region involved in increased constitutive activity and osmotic response. Dimethyl sulfate-mediated in vivo DNA footprinting was performed on rat liver cells cultured under normal conditions or under osmotic stress as described under "Experimental Procedures." Lane 1, LM-PCR of DNA from cells in hyperosmotic medium for 3 h (stress 3 h in vivo); 2, LM-PCR of DNA from cells in hyperosmotic medium for 30 min (stress 30 min in vivo); 3, LM-PCR of DNA from cells in normal osmotic medium (nonstress in vivo); 4, LM-PCR of in vitro methylated DNA (G). Horizontal nucleotide sequence shows the primer 2 location; dotted line indicates the ExoIII stop location and short solid lines indicate guanosine band pattern. Arrows A and B indicate the positions of methylation-protected guanosines bands. Bold underline G indicates the same protected guanosines in the promoter sequence.

**FIG. 4.** EMSA of the 32-bp exonuclease III-protected region with whole cell extract from rat liver cells. The ExoIII-protected 32-bp region was used as a probe to confirm the factor binding and to determine the effect of protein binding by mutation. Lane 1, probe A (no extract added); lane 2, probe A/normal osmolarity (A/n); lane 3, probe A/hyper-osmolarity for 30 min (A/s(30 h)); lane 4, probe A/hyper-osmolarity for 3 h (A/s(3 h)); lane 5, probe A/normal osmolarity/100× cold probe A (A/n comp:A); lane 6, probe A/hyper-osmolarity for 30 min/100× cold probe A (A/s(30 h) comp:A); lane 7, probe A/hyper-osmolarity for 3 h/100× cold probe A (A/s(3 h) comp:A); lane 8, probe A/normal osmolarity (A/n); lane 9, probe A/hyper-osmolarity for 30 min/100× cold probe A (A/s(30 h) comp:A); lane 10, probe A/hyper-osmolarity for 3 h/100× cold probe A (A/s(3 h) comp:A); lane 11, probe A/normal osmolarity/100× cold probe A8 (A/n comp:A8); lane 12, probe A/hyper-osmolarity for 30 min/100× cold probe A8 (A/s(30 h) Comp:A8); lane 13, probe A/hyper-osmolarity for 3 h/100× cold probe A8 (A/s(3 h) Comp:A8). Arrows A-F indicate the complexes. Probe A contains the ExoIII-protected 32-bp region, Probe B is the same as Probe A except for 8 nucleotide replacements. Protected guanosines by dimethyl sulfate methylation (Fig. 3) in Probe A sequence are indicated by an asterisk and the mutations made for probe A8 are indicated by underline. Samples in lanes 1–7 and 11–13 were incubated with probe A.
Fig. 5. The effect of thymidine kinase basal promoter activity and osmotic response when fused with the AEE element. To observe the effect of isolated AEE on a heterologous promoter, the AEE element was fused to the 5'-end of the thymidine kinase promoter and promoter activity was measured under isotonic and hypertonic conditions. A, scheme and sequence of luciferase reporter constructs of AEE (35, 28, 24, and 20 bp) fused to the thymidine kinase promoter (-105 to +52 bp). Filled arrow indicates the direction of double strand DNA flanked by KpnI restriction enzyme sites. Construct 1, pAEE35-TK (35 bp of AEE); 2, pAEE28-TK (28 bp); 3, pAEE24-TK (24 bp); 4, pAEE20-TK (20 bp); 5, pTK (0), all in sense direction. B, relative luciferase activity of the four AEE-thymidine kinase promoter constructs under normal and hyperosmotic conditions. Open bar indicates isotonic (nonstress) and filled bar indicates hyperosmotic (stress) conditions. Error bars indicate S.D. as recorded in C. C, luciferase activity ± S.D. under isotonic or hypertonic conditions. PX/pTK indicates the increase over pTK luciferase activity. H/I indicates the ratio between hypertonic and isotonic activity. n indicates the number of replicates.

In Vivo Factor Binding to the Region -926 to -895 bp—Based on the luciferase gene expression experiments with construct 4, we tested transcription factor binding between -1,094 and -649 bp by using the ExoIII-mediated in vivo DNA footprinting method. This technique has been used to detect constitutive and hormone-induced factor binding to the mouse mammary tumor virus long terminal repeat (19), and is based on the fact that transcription factors upon binding, protect DNA from digestion with ExoIII nuclease. Under normal osmotic conditions, transient templates (construct 4) in isolated nuclei were digested with ExoIII and Asp718 (to supply an entry point for ExoIII) in the sense-directed digestion or with ExoIII and HindIII (to supply an entry point for ExoIII) in the antisense-directed digestion. A strong block to ExoIII digestion was detected at -926 bp for the sense strand-directed digestion and at -895 bp for the antisense strand-directed digestion (Fig. 2).

Constitutive Occupancy of the Endogeneous AR Promoter in the Region Protected by ExoIII in Vivo DNA Footprinting—To observe whether the ExoIII protected region (-926 to -895 bp) was also protected in the endogeneous gene, dimethyl sulfate in vivo footprinting was performed (20). Liver cells were grown in normal or hypertonic medium for 30 min or 3 h. The cells were then treated for 90 s with 0.1% dimethyl sulfate. As a control, DNA extracted from cells and treated with 0.1% dimethyl sulfate in vitro for 90 s was also analyzed by ligation-mediated PCR (LM-PCR) (Fig. 3). When the intensity of the bands be-
Novel cis-Element Necessary for Rat AR Promoter Activity

between the in vitro lane and the in vivo lanes were compared, two guanosines, at positions −915 and −908 bp, were strongly and reproducibly protected from methylation in cells grown in both normal or hypertonic medium as shown in comparison between lanes 4 and lanes 1–3. The relative intensity of the two guanosine bands was similar for normal and osmotic stress. This result confirms that a factor(s) binding to the region −926 to −895 bp occurs in vivo, and shows that occupancy of this site is constitutive in the endogenous rat AR promoter.

Mutation of Nucleotides between the Two Guanosines Protected by in Vivo Footprinting Inhibits Binding of Transcription Factor—DNA probes were used to further evaluate putative transcription factor binding at −926 and −895 bp in the rat AR promoter. Probe A, which encompasses the 32-bp of sequence protected by ExoIII-mediated footprinting (Fig. 2), gave several complexes in EMSA experiments (Fig. 4, lanes 2–4). These complexes were similar in normal and hyperosmotically-treated cells. In competition experiments, cold probe A caused inhibition of band complex formation (Fig. 4, lanes 5–7). Probe A8, which contains eight mutations, including the two guanosine nucleotides protected from methylation by dimethyl sulfate in vivo footprinting, did not show significant binding of the same factors (Fig. 4, lanes 8–10). This result was confirmed by a competition assay with cold mutated probe A8 which did not compete with wild type probe A (Fig. 4, lanes 11–13).

The AEE Element Confers Osmotic Inducibility and Enhances the Constitutive Activity of Thymidine Kinase Promoter—The effect on constitutive activity and osmotic response of the thymidine kinase (TK) promoter (−105 to +52 bp) was studied by fusing a portion of 35-bp ExoIII-protected region to the 5′-end of the TK promoter (Fig. 5). Construct pAEE35-TK, which contains the full 35-bp sequence of AEE, gave a 4-fold increase in basal activity as well as a 5.3-fold increase with osmotic stress. Construct pAEE28-TK (28 bp) which lacks 7 nucleotides of AEE gave the highest basal activity (7.7-fold) and the highest absolute activity under osmotic stress (100.00). Construct pAEE24-TK, which lacks 11 bp, was found to be the minimum required sequence for the functional induction of basal promoter activity and for the osmotic response. The constructs, pAEE20-TK and pTK, gave similar basal activity and no osmotic response.

**DISCUSSION**

The transfection studies of the AR promoter region between −1,071 and −895 indicate important elements necessary for the increase of AR constitutive promoter activity and osmotic response (Fig. 6). Within this region a tonE-like element (−1,071 to −1,059 bp), originally found as an osmotic response enhancer of the dog betaine transporter gene (Table 1), was found (23). The sequence and the location of the element matches other tonE-like elements recently reported in the rabbit AR promoter between −1,108 and −1,092 bp (12), and in the mouse AR promoter between −1,053 and −1,040 bp (13). The human tonE-like element has been reported at two positions, between −1,230 to −1,220 bp and −1,157 to −1,148 bp (11).

Surprisingly in the rat promoter, the deletion of the tonE-like element decreased, but did not abolish the osmotic response. Also, the lack of the tonE-like element had no effect on the high constitutive promoter activity. As the 5′-end of the promoter was deleted from construct 1 to construct 5, constitutive activity increased 7.0-fold suggesting that several negative regulatory elements may lie within this region. These results indicate that the most proximal cis-element required for the osmotic response and the constitutive promoter activity lie downstream of the tonE-like element.

The ExoIII-mediated DNA footprinting method was used to scan the region between −1,094 and −649 bp for any DNA

![Figure 6](attachment:image.png)
protection under normal osmolarity. ExoIII digestion was halted at positions −926 bp in the coding direction and at −895 bp in the noncoding direction, indicating that a 32-bp region was protected under normal osmotic conditions. This putative transcription factor binding sequence, indicated by the ExoIII method, was further studied by dimethyl sulfate in vivo DNA footprinting to determine whether the factor was bound to the endogeneous AR promoter and how it would be affected by osmotic stress. Two guanosines located at −915 and −908 bp within the 32-bp protected region were found to be protected from dimethyl sulfate methylation under both normal and hypertonic conditions. No change in the intensity of the DNA protection was observed under normal culture conditions, 30 min osmotic stress, or 3 h osmotic stress. No other sequence surrounding this ExoIII-protected region was observed protected from methylation by these experiments.

Further confirmation of transcription factor binding and delineation of the critical nucleotides involved in binding were determined by EMSA. A probe containing the 35-bp protected sequence gave several intense complexes under normal and hypotonic conditions. No match was found to this sequence in the transcription factor data base.

This novel cis-element was named aldose reductase enhancer element (AEE).

The regulatory properties of this novel cis-element were further demonstrated when various lengths of AEE were fused to the 5′-end of the thymidine kinase promoter (−105 to +52 bp). Surprisingly, construct pAEE28-TK gave a significant basal activity increase of 7.7-fold and an osmotic response of 5.7-fold, which is a higher increase than construct pAEE35-TK (Fig. 5). The minimum functional sequence for AEE was determined as 5′-GGGTGTGGGAAGTAGTGCCAAAATT-3′ by construct pAEE24-TK and pAEE20-TK.

Finally, luciferase assays were performed with constructs 8 and 9 to determine the effect of inhibition of AEE binding or inhibition of AEE plus tonE binding on promoter activity. Construct 8 was designed to only inhibit AEE binding by deleting 8 bp (5′-GAAGAGTG-G-3′) of the critical sequence determined by the EMSA experiment (Fig. 4). Both constructs 8 and 9 completely abolished the constitutive activity of the rat AR promoter, while construct 8 which includes tonE retained an osmotic response (2.5-fold). This unexpected finding suggests to us that the AEE element is critical for maintenance of constitutive activity. Deletion of AEE, which resulted in continued osmotic response, indicates the presence of other positive or negative elements.

In conclusion, we have identified a new cis-element AEE, located downstream of the tonE-like element, which has a strong effect on the constitutive activity and osmotic response of the rat aldose reductase promoter. This enhancer is capable of inducing the basal activity of a heterologous promoter by 7.7-fold and osmotic response up to 6.7-fold. From our data on AEE and considering the fact that tonE-like elements have been reported in different locations in the AR promoter (11), it is likely that several elements might be involved in the control of the osmotic stress response. Identification of the relative role of these respective elements and their connection to the signal transduction pathway will be important in understanding the process of cellular osmoregulation. The in vivo occupancy of the AEE in the endogeneous AR promoter strongly suggests that this element plays a physiological role in the regulation of AR expression.

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