Isolation of the Mouse Aldose Reductase Promoter and Identification of a Tonicity-responsive Element*

Sylviane Daoudal, Colette Tournaire, Alain Halere, Georges Veyssiére, and Claude Jean‡

From the Laboratoire de Reproduction et Développement, CNRS, URA 1940, Université Blaise Pascal-Clermont-Ferrand II, 63177 Aubière Cédex, France

Aldose reductase (AR; EC 1.1.1.21) is an oxidoreductase that catalyzes the NADPH-dependent conversion of glucose to sorbitol, the first step of the polyol pathway. AR is of great interest due to its implication in the etiology of diabetic complications. In renal medullary cells, AR also plays an osmoregulatory role by accumulating sorbitol to maintain the intracellular osmotic balance during antiuresis. We have previously cloned the AR cDNA from mouse kidney, and we report here the isolation of the mouse AR gene promoter. Transient transfection of chloramphenicol acetyltransferase reporter constructs containing various 5′-flanking regions of the mouse AR gene in CV1 cells led to the identification of a sequence spanning base pairs −1053 to −1040, required for an enhancer activity in hypertonic compared with isotonic cell culture conditions. This sequence is similar to the toxicity-responsive element first characterized in the betaine-γ-aminoxylic acid transporter promoter.

The osmotic balance between intracellular and extracellular compartments of cells is critical for the maintenance of cellular homeostasis. Exposure to anisotonic media initiates a response that counteracts volume perturbations by complex mechanisms involving changes in the intracellular concentrations of active organic solutes (osmolites) such as sorbitol, inositol, betaine, myo-inositol, and glycerolmonophosphorylcholine (1–5). Among the organic osmolites, sorbitol has received special attention since it is a beneficial factor during antidiuresis and yet appears to be detrimental in diabetes (6, 7). For example, by accumulating sorbitol, renal medullary cells maintain both their volume and their intracellular medium unperturbed under hyperosmotic stress. In target tissues of diabetes such as kidney, nerve, and eye, sorbitol accumulation exerts a hyperosmotic effect that contributes to some complications of diabetes mellitus (see Ref. 8 for review).

Sorbitol is formed by the reduction of glucose by the enzyme aldose reductase (AR; EC 1.1.1.21). AR is a ubiquitous “housekeeping” enzyme probably functional in all cells (9, 10). An osmoregulatory role of AR has been suggested by studies in cell lines derived from renal inner medulla showing that an increase in the osmolality of the medium is associated with increases in cellular sorbitol levels, AR activity, and AR gene expression (3, 4, 11). Reduction of AR by hypertonic media was demonstrated also in kidney mesangial cells (12), glomerular endothelial cells (13), Chinese hamster ovary cells (12), lens epithelial cells (14), and human embryonic epithelial cells (15). The molecular mechanism of transcriptional regulation of the AR gene in response to hypertonicity is still unknown.

Nucleotide and deduced amino acid sequences for mouse AR (mAR) have been recently reported (16, 17). We report here the isolation and sequence of the 5′-flanking region of the mAR gene and its functional characterization.

EXPERIMENTAL PROCEDURES

Genomic Cloning—Genomic DNA was isolated from mouse Balb/c liver for PCR amplification of mAR gene intron-2 (Toq polymerase, Perkin-Elmer). Exon boundaries were delimited on the AR cDNA sequence (17) by homology to the rat AR sequence (18). The upstream primer used for PCR amplification matched the mAR gene exon-2 end, with the last four nucleotides identical to the beginning of rat AR gene intron-2. The sequence of this primer was 5′-GTCTCTTCTTGAGCAAGGTTAAC-3′. The downstream primer matched exon-3 (positions 287–304 on the mAR cDNA sequence). Its sequence was 5′-TTCACCATTGCTCTTGTCA-3′. PCR was performed with 5% formamide. The 640-bp amplified DNA fragment was cloned in the pGEM-T vector (Promega) and sequenced using the T7 sequencing kit (Pharmacia Biotech Inc.) according to the manufacturer’s instructions.

A second PCR was performed from this clone to amplify intron-2 without exon sequences. For this PCR, the primers were 5′-TGTGAAGATTGCTCAGGCAAGGCAAGAAGCAG-3′. This amplified DNA fragment was used to screen a genomic library obtained by partial Sau3AI digestion of Balb/c tail DNA and insertion in BamHI sites of the λEMBLL12 vector. A single positive clone of 13.8 kb (mAR 1-2) was further characterized.

Characterization of the Genomic Clone mAR 1-2—A restriction map of the mAR 1-2 clone was obtained by digestion with 16 different enzymes from Boehringer Mannheim. Digestion products were electrophoresed on a 0.8% agarose gel and transferred to nylon filters (Hybond-N, Amersham Corp.). Hybridization of filters with the cDNA probe led to the orientation of the clone and to the localization of the 5′-end of the gene. Digestion of the mAR 1-2 clone with SphI/NotI released a 9-kb and a 4.8-kb fragment, which were subcloned separately in the pGEM7Zf(−) vector from Promega (see Fig. 2). An EcoR1 fragment of 1.9 kb was selected from the 9-kb clone, subcloned into the pGEM7Zf(−) vector, and fully sequenced (see Fig. 2). Sequence data were analyzed using BISANCE programs at CITI 2 (Paris) (19). The transcription start site was positioned by homology to the rat AR gene (18).

Genomic Southern Analysis—Mouse Balb/c liver genomic DNA was digested with 13 different enzymes (listed in the Fig. 1 legend) and subjected to Southern blot analysis. The filter was first hybridized with the 32P-labeled mAR cDNA (17) under the same conditions as described for the intron-2 probe.

Construction of CAT Fusion Plasmids—All constructs were obtained by cloning PCR-amplified fragments from the 9-kb clone in the promoterless basic plasmid pBLCAT3 (20). PCR products were isolated from...
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RESULTS

Isolation and Characterization of the mAR Promoter—A first screening of a mouse genomic DNA library with the mAR cDNA (17) revealed the presence of many AR pseudogenes. This is why an intron-specific probe was required to isolate the functional mAR gene. By comparison with the rat AR gene (18), intron-2 was observed to be the 5′-shortest intron of the gene (600 bp in the rat sequence), so it was selected to target the 5′-end of the gene with its promoter and upstream regulatory sequences. The mAR gene intron-2 was amplified by PCR (see “Experimental Procedures”). The probe derived from this intron-2 sequence was used to hybridize a Southern blot of mouse genomic DNA digested with enzymes that cut infrequently in the genome. Fig. 1A shows a single hybridizing band in each digest in contrast to the complex pattern observed when the same filter was hybridized with the mAR cDNA probe (Fig. 1B). The intron-2-specific probe was further used to screen a λEMBL12 genomic DNA library. A single positive clone (AAR 1-2; insert size of 13.8 kb) was isolated. According to the restriction map and to the cDNA hybridization pattern (data not shown), AAR 1-2 was supposed to span the 5′-end of the gene (up to intron-2) and 9 kb of 5′-flanking region. Digestion of AAR 1-2 with StuII/Xhol released a 9-kb and a 4.8-kb fragment, which were subcloned in the pGEM7Zf(-) vector (Fig. 2). The 1.9-kb EcoRI fragment released from the 9-kb clone was fully sequenced. It contains 141 bp of intron-1, exon-1 (104 bp), and 1.7 kb of 5′-flanking sequences including the promoter.

This fragment shows 85 and 49% sequence identity to the rat (18) and rabbit (23) AR gene promoters, respectively. When multialigmentation was carried out in the 650-bp equivalent sequenced region of the human AR promoter (24), the sequence identity of the mAR promoter to rat, rabbit, and human sequences was 74, 54, and 52%, respectively. The putative transcription start site was defined by homology to the rat sequence. The A of the initiation codon corresponds to nucleotide +39. Exon-1 is identical to that published for the mAR cDNA (17). Only the first three nucleotides of the 5′-untranslated region were missing in the cDNA sequence. One untranslated region were missing in the cDNA sequence. One

Fig. 1. Southern blot analysis of mouse genomic DNA using the mAR gene intron-2-specific probe (A) or the mAR cDNA probe (B). In lanes 1–13, 15 μg of mouse Balb/c liver genomic DNA were digested with XhoI, EcoRI, PstI, Ncol, XmaI, ClaI, HindIII, KpnI, SacI, SpeI, NotI, BamHI, and Spel, respectively. Hybridization was carried out as described under “Experimental Procedures.” The single band observed for any digestion with the intron-2-specific probe (A) contrasts with the complex pattern given by the cDNA probe hybridization that results from the existence of many aldose reductase pseudogenes in the mouse genome.
c-Myc, and the cAMP response element (25)) are present in the mAR promoter. Several potential cis-acting steroid response sequences corresponding just to the right half-site consensus sequence of the estrogen response element and the androgen/glucocorticoid/progesterone response element were identified in the 5'-flanking region of the mAR gene. The most important feature is the presence, 1053 bp upstream of the putative transcription start site, of a sequence similar to the toxicity-responsive element (TonE), described by Takenaka et al. (26), in the promoter of the canine betaine transporter (BGT1) gene. This sequence, located near an AP1 site, was shown to be essential for the osmotic regulation of the BGT1 gene. A likely sequence arrangement was observed in the 5'-region of the mAR gene (TonE-like at position -1053 and an AP1 site at position -1014; Fig. 2). This sequence organization is well conserved among the different AR genes (Fig. 2B). This region

**Fig. 2.** A, schematic diagram of the genomic clone AAR 1-2 and the nucleotide sequence of the 1.9-kb EcoRI fragment. Endonuclease restriction sites used to subclone the 9-, 4.8-, and 1.9-kb fragments are as follows: E, EcoRI; S, SalI; X, XhoI. The organization of the promoter elements is shown in the 1.9-kb clone. The putative transcription start site is indicated by an asterisk in the nucleotide sequence. The TonE-like sequence, AP1 site, CCAAT and TATA motifs, and translation start site are underlined. The nucleotide sequence of the 1.9-kb clone has been entered in GenBank™ under accession number U36489. B, comparison of the TonE-like sequences and AP1 sites present in the 5'-flanking region of the rat (18), mouse, and rabbit (23) AR genes with the TonE described in the canine BGT1 promoter (26). Positions are given above each sequence according to the transcription start site.
was further investigated to study the response of the mAR gene to hypertonicity.

Response of the mAR Gene to Hypertonicity—The response of the mAR gene to hypertonicity was studied by transient transfections of reporter gene constructs in CV1 cells. As previously shown (27, 28), NaCl added to make the medium hypertonic is a potent inhibitor of cell proliferation. After 24 h of exposure to hypertonic medium (H; 500 mosm/kg of H2O), CV1 cells appeared to be less confluent than in isotonic medium (I; 330 mosm/kg of H2O). CV1 cells cultured in either isotonic medium (I; 330 mosm/kg of H2O) or hypertonic medium (H; 500 mosm/kg of H2O) for 24 h (C). Twenty micrograms of total RNAs were loaded per well and hybridized with the mAR cDNA and the 18 S rRNA cDNA. Accumulation of AR mRNAs was markedly increased under hypertonic culture conditions (2.6-fold).

To test the ability of mAR sequences to direct hypertonicity-induced stimulation, constructs containing 5'-flanking sequences from the mAR gene linked to the indicator CAT gene were transfected in the CV1 cells. All the constructs transfected in CV1 cells exposed to isotonic medium supported detectable levels of expression of the CAT gene in the range of 9.98–43.89%, indicating that this is indeed a functional promoter (Fig. 4). Deletion of the −1586/−998 region results in a reduction of the basal promoter activity of p998CAT and p142CAT. It is interesting to note that the API site is absent from these two constructs. The p1586CAT and p1067CAT constructs showed a hypertonicity-dependent enhancement of transcriptional activity. In cells exposed to hypertonic medium, CAT activity increased 3-fold over the level of basal activity measured under isotonic conditions (Fig. 4). This effect seems to be promoter-specific since, under the same conditions, the activity of the pSV2CAT plasmid control is not stimulated. To identify the sequences involved in the response to hypertonicity, subfragments were analyzed. As shown in Fig. 4, deletions up to nucleotides −998 and −142 resulted in a loss of the transcriptional activation by hypertonic stress. The hypertonic/isotonic CAT activity ratios of p998CAT and p142CAT are significantly different from those of p1586CAT and p1067CAT (p < 0.05), whereas they are not significantly different from that of pSV2CAT. The TonE-like sequence at position −1053 is the most likely candidate to be involved in the response to hypertonicity. To determine the role of this 5'-TGGAATTCCACCGAG-3' sequence present in the p1067CAT and p1586CAT constructs, it was mutated to 5'-TGTCCGCTTACCCAG-3' in p1067mCAT. No enhancer activity was observed with this construct. The hypertonic/isotonic ratio calculated for p1067mCAT was significantly different from those of p1586CAT and p1067CAT (p < 0.05), but was similar to that reported for pSV2CAT. These results, showing that if the TonE-like sequence is mutated or deleted, the response to hypertonicity is lost, strongly suggest that this motif might function as a hypertonicity-responsive element.

DISCUSSION

The isolation and sequence of the 5'-flanking region of the mAR gene led us to underline its transcriptional regulation by hypertonicity and to identify a toxicity-responsive element in a small region spanning base pairs −1053 to −1040. The complex pattern obtained when a mouse genomic DNA Southern blot was hybridized with the mAR cDNA compared with the single band observed with the intron-2-specific probe confirms previous results obtained for human (29) and rat (18) genomes concerning the existence of AR pseudogenes.

Two half-palindromic sites for binding of the androgen/glucoorticoid/progesterone receptor (5'-TGTTGC-3') are found in the mAR sequence, but such half-sites are not known to be functional for other genes in the literature. Until now, we had no evidence concerning hormonal regulation of the mAR gene. AR mRNA levels in seminal vesicle, vas deferens, epididymis, and kidney are not altered in adult castrated mice (17). Moreover, there is no sequence similarity between the mAR and mouse vas deferens protein promoters (30). Mouse vas deferens protein, a member of the aldoketoreductase superfamily sharing 69% identity with mAR (17), is highly expressed in the vas deferens under androgenic control (31–33).

In contrast, regulation by hypertonicity is now well established for the rabbit AR gene (3, 23, 34). We report here the same regulation for the mAR gene in transfected CV1 cells and precisely localize one of the osmotic response elements proposed by Ferraris et al. (23). We demonstrated that the region spanning nucleotides −1067 to −998 upstream of the transcription start site and containing a TonE sequence similar to that described in the BGT1 promoter (26) is necessary for the response to hypertonicity. When the TonE-like sequence is mutated (p1067mCAT) or when the −1067/−998 region is deleted (p998CAT), the response to hypertonicity is lost. In the BGT1 TonE, if the middle sequence GAAA or the 3'-end sequence GTCCA is mutated, there is no longer enhancer activity under hypertonic conditions, whereas mutation in the 5'-end of the TonE does not alter its enhancer activity. Moreover, complex θ involved in the response is not competed by oligonucleotides mutated in the middle or in the 3'-end sequence of the TonE in contrast to oligonucleotides mutated in the 5'-end, suggesting that this complex results from the binding of transcription factors on the middle and the 3'-end of the TonE. Mutation in the middle of the mouse TonE-like sequence (p1067mCAT) leads to the loss of response to hypertonicity. The 3'-end of the mouse TonE-like sequence, which was originally an imperfect palindrome compared with that of the BGT1 gene, with insertion of an additional nucleotide after the four adenines seems nevertheless to be able to confer response to hypertonicity. The response obtained with the p1586CAT construct is quite modest (−3-fold), but is in good agreement with the increase in endogenous AR mRNA (2.6-fold). However, it cannot be excluded that other sequences could be involved in the response to hypertonicity.
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Deletion of the TonE-like sequence and the downstream AP1 site (p998CAT and p142CAT) resulted also in a reduction of the basal promoter activity. This last observation suggests that the AP1 site, which is absent from these two constructs, is necessary for the basal promoter activity. Moreover, in the BGT1 gene, a complex was formed when the DNA fragment containing the TonE and the AP1 site was incubated with both isotonic and hypertonic cell extracts. This complex was competed by an excess of AP1 sequence. So, in the BGT1 gene, the AP1 site is occupied even under isotonic conditions and probably participates in the basal promoter activity.

Stimulation of the mitogen-activated protein kinase cascade is required in Madin-Darby canine kidney epithelial cells to adapt to hyperosmolality (35). Although several transcription factors such as ATF-2 (which binds to the cAMP response element), c-Myc, p62TCF/Elk-1 (which belongs to the cts gene family), and AP1 (which corresponds to homodimer or heterodimer of c-Jun) have been identified as substrates for mitogen-activated protein kinase (36), none of them has been clearly implicated in the mechanism of regulation by hyperosmolality. Nevertheless, it would be of great interest to test if such cis-acting sequences present in the mAR promoter are functionally active.

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REFERENCES


![Figure 4. Transient transfection analysis of the mAR promoter activity and response to hypertonicity.](image_url)
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