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Transcription Factor Nrf2 Regulates Promoter Activity of Mouse Aldose Reductase (AKR1B3) Gene

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Abstract. Transcription factor Nrf2 regulates gene expression of drug metabolizing enzymes such as glutathione *S*-transferase via the antioxidant response element, ARE. Aldose reductase (AR), a member of the aldo-keto reductase (AKR) superfamily, metabolizes various endogenous and exogenous aldehydes. The AR gene 5'-flanking region contains a multiple stress response region (MSRR) composed of two putative AREs (ARE1 and ARE2), an AP1 site, and a tonicity response element (TonE). As this region is highly conserved among species, we examined the involvement of Nrf2 in transcriptional regulation of the AR gene. β-Naphthoflavone, an Nrf2 activator, elevated the level of AR mRNA in HepG2 cells and increased the promoter activity of the mouse AR (AKR1B3) gene. The promoter activity of the AKR1B3 gene, containing MSRR, was also augmented by overexpression of Nrf2. Deletion and mutation analyses indicated that both ARE1 and the AP1 site were essential for the responsiveness to Nrf2, while ARE2 was nonfunctional. The presence of an ARE1 binding protein complex was revealed by electrophoretic mobility shift assay. These findings indicate that Nrf2 regulates the AKR1B3 promoter activity via ARE1 and the AP1 site.

Keywords: aldose reductase, aldo-keto reductase, Nrf2, AP1, antioxidant response element

Introduction

Aldose reductase (AR; EC 1.1.1.21) is an NAD(P)H-dependent monomeric oxidoreductase and a member of the aldo-keto reductase (AKR) superfamily (1). The AKR superfamily consists of more than one hundred proteins with $(\alpha/\beta)_8$ barrel structure. A systematic nomenclature system for the AKR superfamily was adopted, and each AR has been designated as AKR1B1 (human), AKR1B3 (mouse), or AKR1B4 (rat) (1). AR has been studied as a key enzyme in the pathogenesis of various diabetic complications (2). It is the rate-limiting enzyme in the polyol pathway where glucose is converted to sorbitol by AR, followed by NAD^+-dependent conversion to fructose by sorbitol dehydrogenase. Under hyperglycemic conditions, acceleration of the polyol pathway leads to a condition similar to hypoxia, provoking early tissue damage in the target organs associated with diabetic complications (2, 3). In addition, AR also functions as a metabolizing enzyme toward various endogenous and exogenous aldehydes, quinones, and alcohols. AR detoxifies acrolein, a metabolite of cyclophosphamide, 4-hydroxynonenal produced during lipid peroxidation, and the dihydrodiol form of benz(a)pyrene metabolites (4 – 8).

It is generally known that expression of the AR gene is activated by osmotic stress (9, 10). The tonicity response element (TonE) within the 5'-flanking region of the AR gene is responsible for hyperosmotic stress (11). The TonE binding protein (TonEBP) has been identified to be a *rel*-like transcription factor and designated as a member of the nuclear factor of activated T-cell (NFAT) family, NFAT5 (12, 13). Transcription factor NF-κB also recognizes TonE and activates the AR promoter in response to TNF-α (14). An AP1site is localized adjacent to TonE, although its involvement in AR gene regulation has not been clarified (11, 15). As AR expression is enhanced by oxidative substances such as hydrogen peroxide, nitric oxide, oxidized low-density lipoprotein, and advanced glycation endproducts (AGEs) (16 – 18), AR can also be designated as an oxidative stress-inducible protein.

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Nrf2 (NF-E2-related factor 2) is a member of the Cap'n'Collar subfamily of basic region-leucine zipper (bZip) transcription factors. Nrf2 forms a heterodimer with small Maf proteins and binds to the antioxidant response element (ARE) (19, 20). The core sequence of ARE, 5'-TGACNNNGC-3', was found in the promoter regions of drug metabolizing enzymes such as glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase (QR) (21 – 23). Several groups of xenobiotics including phenolic antioxidants, flavonoids, and isothiocyanate, such as β-naphthoflavone (β-NF) and 2(3)-tert-butyl-4-hydroxyanisole (BHA), are known to activate Nrf2 and induce drug metabolizing enzymes (24). Because *nrf2*-null mice showed severe impairment in the induction of several phase II drug metabolizing enzymes, Nrf2 appears crucial for ARE-mediated gene expression (22). Nrf2 is also involved in the induction of anti-oxidative stress proteins such as γ-glutamylcysteine synthetase and heme oxygenase-1 (25, 26). In Nrf2-deficient macrophages, induction of anti-oxidative stress genes by electrophiles or reactive oxygen species was abrogated (22). These findings suggest that Nrf2 plays a key role in the anti-oxidative stress response. Recently, the expressions of some of the genes encoding anti-inflammatory proteins, growth factors, and NADPH regenerating enzymes were also identified to be Nrf2-dependent by microarray analyses of Nrf2-knockout mice (27, 28). Among these genes, an aldose reductase-related gene was shown to be regulated by Nrf2 (27).

Two putative ARE-like sequences, ARE1 and ARE2, are found adjacent to TonE and AP1 site in the 5'-flanking region of the AR gene (11, 15, 29, 30). The arrangement of these elements is highly conserved among animal species, suggesting that this region is important for the regulation of AR gene expression. Thus, we designated this region as the multiple stress response region, MSRR (Fig. 1). Since AR has a role in the metabolism of xenobiotics and the consensus sequences of ARE are located within its promoter region, Nrf2-mediated regulation of AR gene expression is speculated. We, thus, investigated the involvement of Nrf2 in the regulation of mouse AR (AKR1B3) promoter activity.

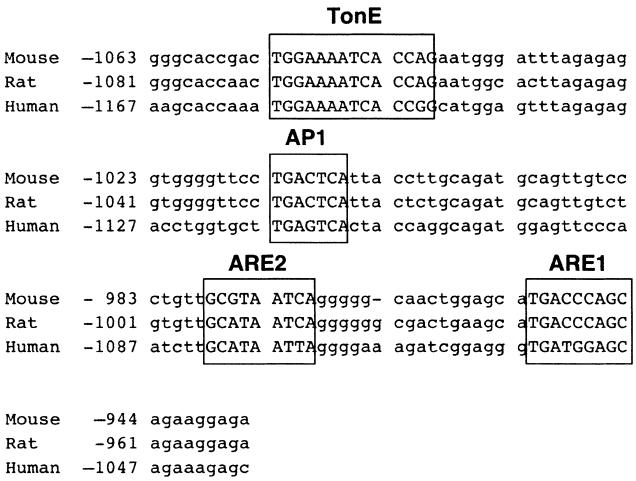

Fig. 1. Conserved elements in multiple stress response region (MSRR) of the aldose reductase (AR) gene among animal species. TonE, tonicity response element; AP1, AP1 binding site; ARE, antioxidant response element.

Materials and Methods

Reagents

β-Naphthoflavone (β-NF) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Anti-Nrf2 antibody (C-20), raised against the C-terminus of Nrf2 protein, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit IgG and bovine serum albumin were products of Sigma Chemical Company (St. Louis, MO, USA). Other reagents were of the highest grade available.

Plasmids

Luciferase reporter constructs containing the promoter region of the mouse AR gene were described previously (15). To obtain enhancer regions of the AR promoter, the polymerase chain reaction (PCR) was performed with −1.06-AR-luc as a template using the following primers: 5'-tgtatcttatggtactgtaactg-3' (GL1 primer) and 5'-gaagatctccttctgctgggtcatgct-3' (Z2 primer) for pMSRR-luc (−1063 to −936, pTonE-AP1-ARE-luc); GL1 primer and 5'-gaagatctgcaaggtaatgagt cag-3' for pTonE-AP1-luc (−1063 to −994); 5'-ggggtaac cagaatgggatttagagagg-3' and Z2 primer for p3A-luc (−1044 to −936, pAP1-ARE-luc). The mutant enhancer elements were prepared by PCR with p3A-luc as a template using GL1 primer and the following primers: 5'-gaagatctccctgattacgcaacaggg-3' for p3AΔARE1-luc (−1044 to −967), 5'-gaagatctccttctgctgggtcctgct-3' for p3AmARE1-luc, and 5'-gaagatctccttctgctgggtcatgctccag ttgcccccggattacgcaa-3' for p3AmARE2-luc. The AP1 mutants were prepared by PCR between Z2 primer and the following primers: 5'-ggggtaccagaatgggatttagagaggt ggggttccggactca-3' for p3AmAP1-1-luc and 5'-ggggtac-cagaatgggatttagagaggtggggttcctgactcctta-3' for p3AmAP1-2-luc. The amplified fragments were digested with KpnI and BglII and subcloned into the pGL2-promoter vector (Promega Corp., Madison, WI, USA). To construct 2xARE-luc, the following oligonucleotides with ARE consensus sequences and SalI flanking sequences were synthesized: 5'-tcgagactgaag

catgacccagcagaaggagag-3' and 5'-tcgactctccttctgctgggt catgcttcagtc-3'. The oligonucleotides were annealed, phosphorylated using T4 polynucleotide kinase, and cloned at the SalI site of pGL2-promoter vector. A luciferase construct with a tandem repeat of ARE sequence was selected among the obtained constructs by nucleotide sequencing. The Nrf2 expression plasmid (pNrf2) was a gift from Dr. Cecil B. Pickett (Schering-Plough Research Institute, NJ, USA) (23). To generate a dominant-negative Nrf2, a DNA fragment containing a Cap'n'Collar homology region and basic leucine-zipper domain (amino acids 386 to 580) was amplified by PCR using the primers 5'-gccgccgccatggctgctgtgcacgaatcc cag-3' (sense) and 5'-cctcccgaacctagtttttctttgtatctggc-3' (antisense). The amplified fragment was first ligated into pGEM-T Easy vector and next subcloned into pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA).

Northern blotting

Northern blotting was performed as described previously (16). Briefly, total RNA was size-fractionated in 1% agarose gels containing 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, and 2.2 M formamide (pH 7.0). RNA was transferred onto a Hybond-N^+ nylon membrane (Amersham-Pharmacia Biotech., Tokyo) and immobilized by UV irradiation. The human AR cDNA probe (31) was labeled with [α-^{32}P]-dCTP by random priming and purified with a G-25 spin column. The blotted membrane was hybridized with a 1×10^6 cpm /ml radiolabeled probe at 42°C in a buffer containing 5 × SSPE, 1% SDS, 5 × Denhardt's solution, and 50% formamide. The blot was first rinsed with 2 × SSC containing 0.5% SDS and then washed twice with 0.1 × SSC containing 0.5% SDS for 30 min at 55°C. The radioactive signals were analyzed using a BAS 2000 Bioimaging Analyzer (Fuji Film, Tokyo).

Cell culture and transfection

Human hepatoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under an atmosphere of 95% air and 5% CO_2. The cells were seeded in 6-well-plates one day before the transfection. For transfection using Lipofectin (Invitrogen Corp.), the cells were incubated with a DNA-Lipofectin mixture for 4 h and then grown in DMEM containing 10% FBS. To normalize the transfection efficiency, the β-galactosidase expression plasmid pSV-β-GAL was co-introduced to the cells.

Reporter assay

The cells were harvested 48 h after the transfection and lysed in reporter lysis buffer (Promega Corp.). Luciferase activity was measured with a luciferase assay system (Promega Corp.) with a Micro Lumat LB96P Luminometer (Berthold Japan, Co. Ltd., Tokyo). β-Galactosidase activity was spectrophotometrically measured with a β-galactosidase enzyme assay system (Promega Corp.). Luciferase activity was normalized by the β-galactosidase activity for each sample.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as described previously (32). Oligonucleotide probes containing AREs and the AP1 site (109 bp) were prepared by digesting the corresponding pGL3-luciferase constructs, p3A-luc and p3AmARE1-luc, with BglII and KpnI. The oligonucleotide probes were dephosphorylated and then labeled at their 5'-ends using [γ-^{32}P]ATP (ICN Biomedicals) and T4 nucleotide kinase. The HepG2 nuclear extracts were prepared as described previously (32). The nuclear extracts and the labeled probe were incubated at 4°C for 30 min, resolved in a polyacrylamide gel, and analyzed by a BAS2000 Bioimaging Analyzer.

Results

Induction of AR by an Nrf2 activator β-NF

As HepG2 has been used for the analyses of AREs in the promoters of rodent GST and QR (23, 33), the effect of β-NF, an Nrf2 activator, was examined in this cell line to elucidate whether the transcription factor Nrf2 participates in the AR gene expression. In HepG2 cells, β-NF is known to induce catalytic and regulatory subunits of γ-glutamylcysteine synthetase in an Nrf2-dependent manner (34). As shown in Fig. 2A, the level of AR mRNA was increased in the cells treated with β-NF. The effect of β-NF on the regulation of mouse AR (AKR1B3) promoter activity was next analyzed using a reporter plasmid containing up to −1063 bp upstream of the 5'-flaking region of AKR1B3 gene (−1.06 AR-luc) (15). This region included two putative AREs, TonE, and AP1 (Fig. 2B). As shown in Fig. 2C, β-NF treatment significantly augmented the promoter activity. The 2xARE-luc construct was used as a positive control. These results suggested the involvement of transcription factor Nrf2 in the AR gene regulation.

Overexpression of Nrf2 enhanced AKR1B3 promoter activity

To verify whether Nrf2 regulates AKR1B3 gene expression, an Nrf2 expression plasmid pNrf2 and the reporter plasmid −1.06 AR-luc were co-transfected into HepG2 cells. Overexpression of Nrf2 significantly increased the AKR1B3 promoter activity in a dose-dependent manner (Fig. 3A). This augmentation was

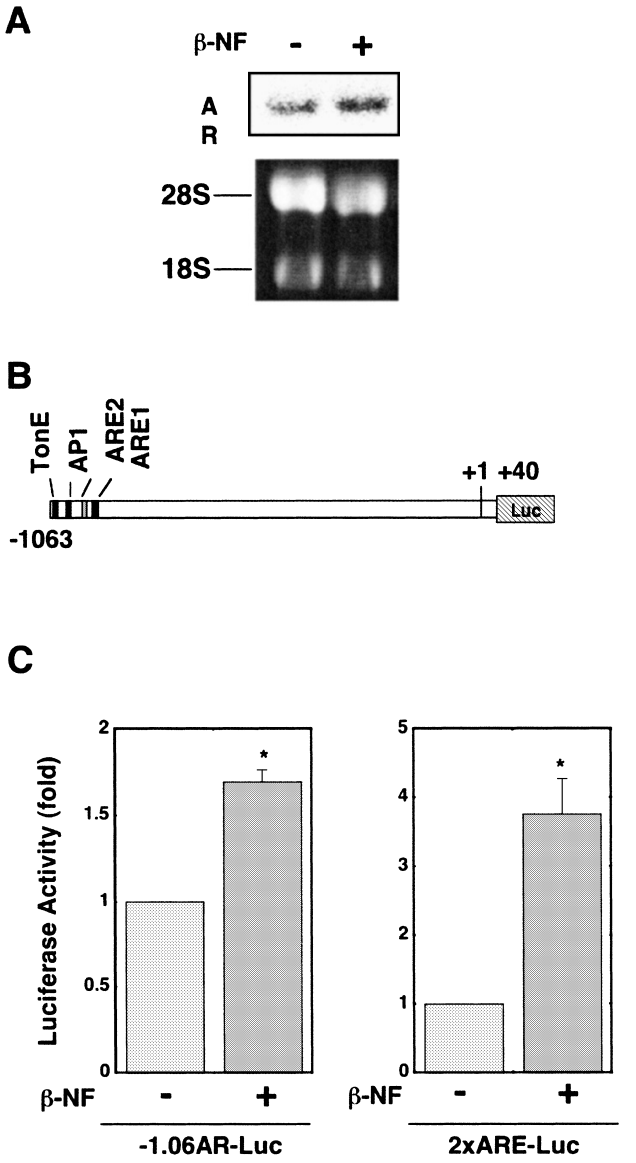

Fig. 2. β-NF induced AR expression in HepG2 cells. A, HepG2 cells were treated with 5 μM β-NF for 8 h. Total RNA was prepared and subjected to northern blotting (upper panel). Lower panel shows the ethidium bromide-stained ribosomal RNAs. B, a schematic structure of the −1.06-AR promoter-luc construct. C, β-NF augmented the mouse AR (AKR1B3) promoter activity. HepG2 cells were transfected with −1.06 AR-luc. After 24 h, the cells were treated with 10 μM β-NF for 24 h. As a positive control, the 2xARE-luciferase construct was examined. Bars represent the mean ± S.E.M. obtained from three experiments. *P<0.05 (analyzed by Student's t test).

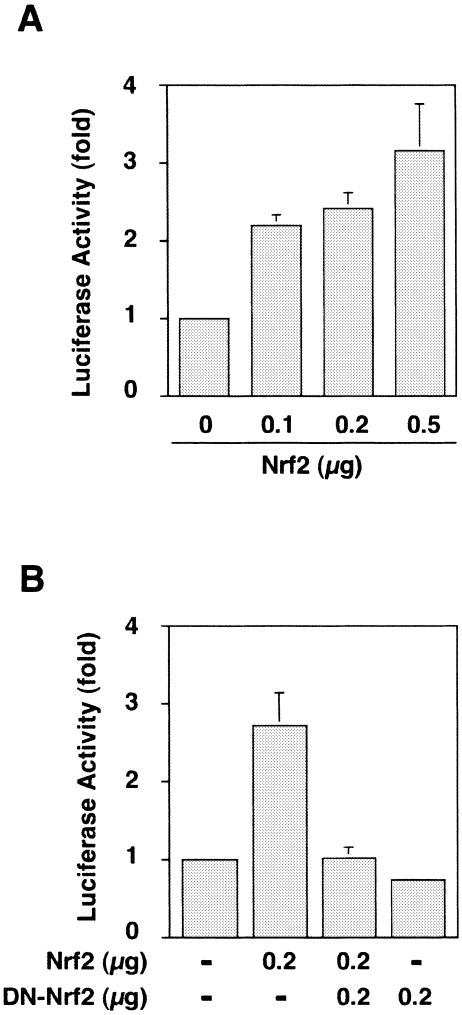

Fig. 3. Overexpression of Nrf2 enhanced AR promoter activity. A, the −1.06-AR promoter-luc construct (0.5 μg) was co-transfected with increasing amounts of an Nrf2 expression plasmid, pNrf2. B, dominant-negative Nrf2 suppressed Nrf2-induced increase in the AR promoter activity. HepG2 cells were co-transfected with 0.5 μg of the −1.06-AR promoter-luc, a dominant-negative Nrf2 expression plasmid (DN-Nrf2) and pNrf2 (Nrf2). The total amount of DNA was adjusted by addition of an appropriate amount of the empty plasmid vector pcDNA3. The values indicate the fold induction relative to the activity in the absence of Nrf2 and DN-Nrf2. Bars represent the mean ± S.E.M. obtained from three experiments.

suppressed by the co-expression of dominant negative Nrf2 (Fig. 3B). Similar results were obtained when a mouse fibroblast cell line, NIH/3T3, was used (data not shown). These results suggested that Nrf2 was involved in the regulation of AKR1B3 gene expression.

ARE1 and AP1 sites were essential for the Nrf2-induced increase in AKR1B3 promoter activity

To identify the region responsible for the Nrf2-mediated activation, reporter constructs containing various lengths of the AKR1B3 gene 5'-flanking region were generated (Fig. 4). In the presence of two putative AREs, Nrf2-mediated activation was markedly diminished when TonE and the AP1 site were deleted. Thus, the region including TonE and the AP1 appeared to be responsible for Nrf2-mediated activation. However,

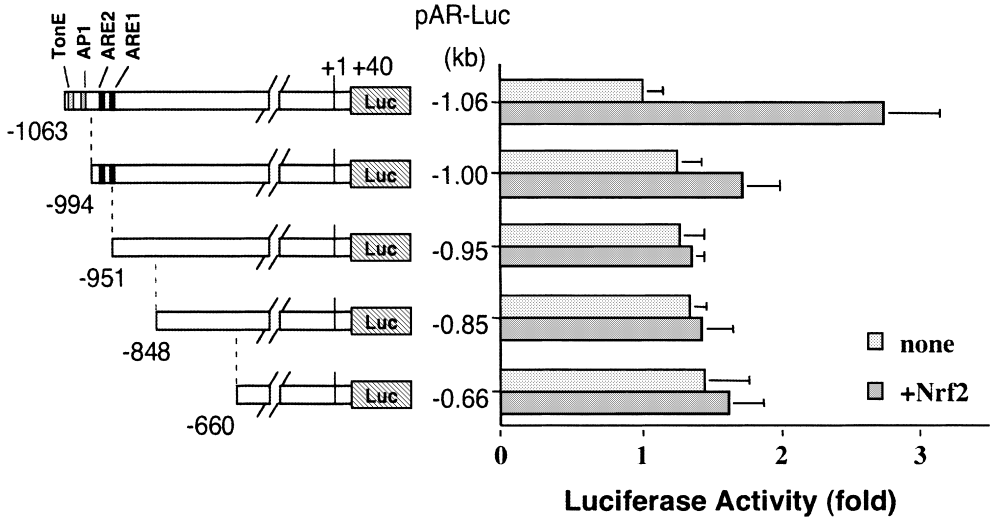

Fig. 4. Deletion analysis of the 5'-flanking region of AR gene. HepG2 cells were co-transfected with 0.2 μg of pNrf2 and 0.5 μg of various AR-promoter-luc constructs. The values indicate the fold induction relative to the activity with −1.06-AR-luc in the absence of Nrf2. Bars represent the mean ± S.E.M. obtained from three experiments.

this region alone (pTonE-AP1-luc: −1063 to −994) was insufficient for the Nrf2-induced activation (Fig. 5A). It is therefore speculated that AREs may be necessary in addition to TonE and the AP1 site. As shown in Fig. 5A, the responsiveness to Nrf2 was observed when the reporter construct containing TonE, AP1 site, and two AREs (pMSRR-luc: −1063 to −936) was examined. Enhanced promoter activity by Nrf2 was still observed with the construct p3A-luc lacking TonE. These findings indicated that the region encompassing the AP1 site and AREs (−1034 to −936) were important for the responsiveness to Nrf2.

Next, we examined whether both AREs are necessary or either of them is sufficient for the Nrf2-induced activation. As shown in Fig. 5B, either one point mutation (p3A-mARE1-luc) or deletion of ARE1 (p3A-ΔARE1-luc) resulted in a total loss of the responsiveness, suggesting that ARE1 was indispensable in the Nrf2-mediated AR promoter activation. In contrast, the mutation of the same corresponding nucleotide of the consensus sequence within ARE2 (p3A-mARE2-luc) revealed no effect on the Nrf2-mediated transactivation, suggesting that ARE2 is not essential (Fig. 5C). The substitution of the proximal A to C of the AP1 site (p3A-mAP1-2-luc) significantly attenuated the responsiveness to Nrf2 while the substitution of the distal T to G (p3A-mAP1-1-luc) was less effective. These findings verified the importance of the AP1 site and also suggested that the orientation of the AP1 site toward ARE1 was important despite the palindromic sequence of AP1. Together, both ARE1 and the AP1 site appeared to be essential for the AKR1B3 gene activation by Nrf2.

Binding of Nrf2 to ARE1 of AKR1B3 promoter

To investigate the direct interaction of Nrf2 and ARE1 of the AKR1B3 promoter, electrophoretic mobility shift assays were performed (Fig. 6A). When the probe isolated from the AKR1B3 promoter containing ARE1, ARE2, and the AP1 site (AP1-ARE) was used, several mobility-shifted bands were observed (lane 1). Among them, the band indicated by an arrow was diminished when ARE1-mutated oligonucleotide (AP1-mARE1) was used as a probe (lane 2). While the band disappeared in the presence of an excess amount of unlabeled wild-type probe (lane 3), it remained in the presence of an excess amount of unlabeled mutant probe used as a competitor (lane 4). When an excess amount of unlabeled ARE consensus sequence oligonucleotide was used as a competitor, the intensity of the band was diminished (lane 5). These data suggested that HepG2 cells contained nuclear factors that bind to ARE1 of the AKR1B3 promoter. To examine whether the ARE1-binding complex includes Nrf2 protein, effects of anti-Nrf2 antibody were examined. As shown in Fig. 6B, the anti-Nrf2 antibody markedly attenuated the binding of the nuclear factors to ARE1 (lane 2). It appeared that the antibody, recognizing the C-terminus of Nrf2 protein, prevented Nrf2 from binding to the probe since the DNA binding domain and the leucine zipper domain, necessary for dimerization, are located in the C-terminal region of Nrf2 (35). Accordingly, these results suggested the interaction of Nrf2 with ARE1 of the AKR1B3 gene.

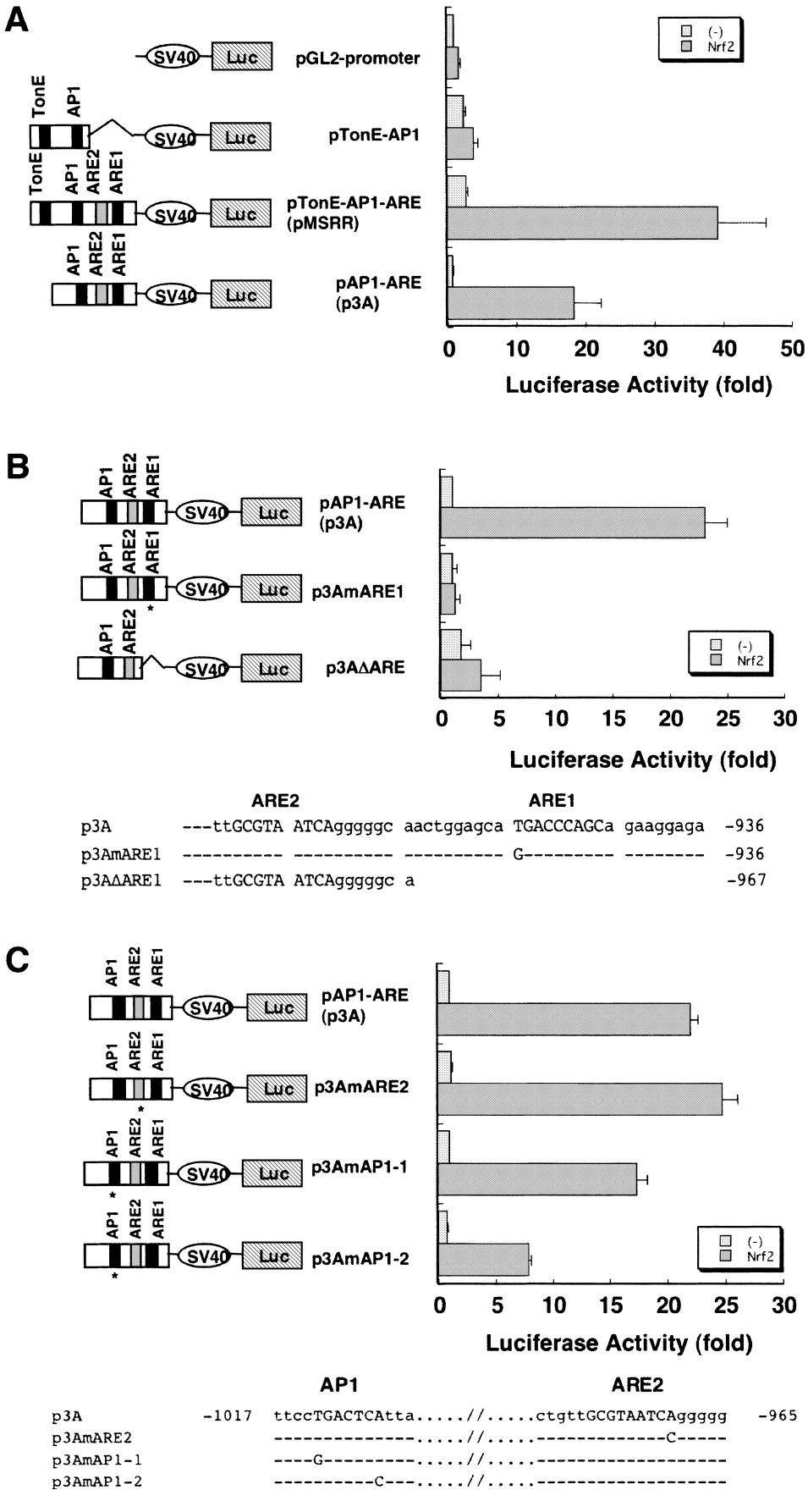

Fig. 5. ARE1 and the AP1 site were essential for the responsiveness to Nrf2. A, B, C, HepG2 cells were co-transfected with 0.2 μg of pNrf2 and 0.5 μg of mutant luciferase reporter constructs derived from multiple stress response region (MSRR) of the AR promoter. The luciferase activity was plotted after normalization to the activity of the pGL3-basic (A) or p3A-luc (B, C) in the absence of Nrf2 expression plasmid. Bars represent the mean ± S.E.M. obtained from three experiments. Asterisks indicate one point mutations in the enhancer elements.

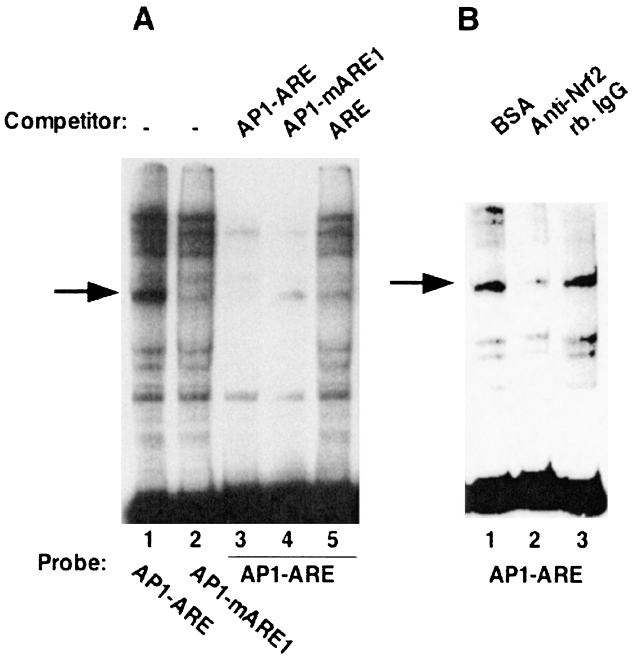

Fig. 6. Electrophoretic mobility shift assays. A, HepG2 nuclear extracts (5 μg), incubated with the radio-labeled probe containing AREs and the AP1 site of the AR promoter, were resolved in a 6% polyacrylamide gel. The arrow indicates the specific band observed with the wild type probe. B, anti-Nrf2 antibody (6 μg) was added to the reaction mixture and subjected to the assay (anti-Nrf2). The same amount of bovine serum albumin (BSA) or rabbit IgG (rb. IgG) was used for controls. A representative autoradiograph of two experiments is shown.

Discussion

In the present study, we demonstrated for the first time that the promoter activity of the mouse AR (AKR1B3) gene is regulated by the transcription factor Nrf2 via ARE1 and the AP1 site located in the MSRR of the 5'-flaking region. The major line of evidence provided in this study are as follows: 1) The mouse AR promoter activity was augmented either by Nrf2 activator β-NF or by overexpression of Nrf2 protein. 2) The introduction of the dominant-negative Nrf2 plasmid to the cells completely abolished the promoter activation by Nrf2. 3) The mutational analysis revealed that ARE1 and the AP1 site are necessary for the responsiveness to Nrf2. 4) The region including ARE1 and the AP1 site was shown to interact with Nrf2 in the electrophoretic mobility shift assays, while the probe with mutated ARE1 lost its binding activity to Nrf2. These results suggested that the transcription factor Nrf2 directly interacts with the promoter region and activates the gene expression of AR. Since MSRR is highly conserved among animal species, the transcriptional regulation of AR via MSRR seems to be universal. Although two putative ARE sequences, ARE1 and ARE2, are present as an inverted repeat in MSRR, only one of these AREs, ARE1, was indispensable in the Nrf2-mediated augmentation of the promoter activity. On the other hand, the AP1 site in MSRR was shown to be necessary for the Nrf2-mediated transactivation. Interestingly, the substitution of proximal A to C in the AP1 site led to a greater decrease in the responsiveness to Nrf2, while the AP1 sequence is palindromic. Because the consensus sequences of ARE and AP1 resemble each other, an AP1 site may act as an ARE. The inverted repeat of these two ARE-like sequences with a certain distance may be important for the transcriptional activation by Nrf2. It is reported that the presence of two copies of ARE motif is necessary for maximum induction by β-NF in murine GST-A1 (36) and rat QR (37). Since the mutation in the AP1 site did not completely abolish the responsiveness to Nrf2, the AP1 site may play a supportive role as a stabilizer of the Nrf2-small Maf complex while ARE1 plays a primary role in the Nrf2-mediated transactivation.

Nrf2 is known to be involved in the transcriptional regulation of detoxification enzymes such as glutathione *S*-transferase. In addition, microarray analyses with Nrf2-knock out mice revealed that Nrf2 is also involved in the regulation of glutathione-related proteins, antioxidant proteins, NADPH-producing enzymes, and anti-inflammatory proteins (27, 28). Proteins within these categories are vital to the maintenace of a cell defense system. Nrf2 may thus play a pivotal role in cell protection against carcinogens and various toxins. The fact that Nrf2 enhanced the promoter activity of the AR gene implies that detoxification is one of the important physiological roles of AR. AR has the ability to detoxify toxic carbonyl compounds such as acrolein, a metabolite of cyclophosphamide, 4-hydroxynonenal produced during lipid peroxidation, and the dihydrodiol form of benz(a)pyrene metabolites (4 – 8). Recently, Keightley et al. demonstrated the increase of AR expression in a Chinese hamster fibroblast cell line (HA1) treated with ethoxyquin. The AR-induced HA1 cells were resistant to glycolaldehyde- or acrolein-induced cytotoxicity, whereas the inhibition of AR activity with alrestatin abolished the resistance (38). These observations strongly suggest that elevated AR expression gives a protective effect against oxidative stress. On the other hand, the resistance to anticancer drugs in tumor cells or the tolerance to ordinary medications is partly due to the elevated expression of detoxification enzymes (39). Lee et al. demonstrated that HepG2 cells overexpressing AR became more resistant to the anticancer drug daunorubicin (40). It is well-known that the Nrf2-dependent detoxification pathway is activated by various

antioxdative agents (41). Accordingly, Nrf2-dependent up-regulation of AR expression may be involved in the protection against toxic xenobiotics as well as in the resistance to such therapeutic agents as anticancer drugs in the same manner as other Nrf2-dependent detoxification enzymes. Since AR is the rate-limiting enzyme of the polyol pathway (2), the regulation of AR expression by Nrf2 may also affect the development of diabetic complications.

Further investigation on the regulatory mechanisms of AR gene expression may reveal the role of AR in the metabolism of xenobiotics as well as in the pathogenesis of diabetic complications and extend our knowledge on the pleiotropic roles of the AKR family.

Acknowledgments

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