Differential control of murine aldose reductase and fibroblast growth factor (FGF)-regulated-1 gene expression in NIH 3T3 cells by FGF-1 treatment and hyperosmotic stress

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Aldose reductase (AR) is an NADPH-dependent aldo–keto reductase implicated in cellular osmoregulation and detoxification. Two distinct murine genes have been identified that are predicted to encode proteins with significant amino acid sequence identity with mouse AR: mouse vas deferens protein and fibroblast growth factor (FGF)-regulated-1 protein (FR-1). Here we report that the AR and FR-1 genes are differentially regulated in NIH 3T3 fibroblasts. FGF-1 stimulation of quiescent cells induces both AR and FR-1 mRNA levels, but the effect on FR-1 mRNA expression is significantly greater. FGF-1 treatment also increases FR-1 protein expression, as determined by Western-blot analysis using FR-1-specific polyclonal antiserum.

Calf serum stimulation of quiescent cells increases AR mRNA expression but not FR-1 mRNA expression. Finally, when NIH 3T3 cells are grown in hypertonic medium, AR mRNA levels are significantly increased whereas FR-1 mRNA levels are only slightly up-regulated. These results indicate that the AR and FR-1 genes are differentially regulated in murine fibroblasts by two different growth-promoting agents and by hyperosmotic stress. Therefore these structurally related enzymes may have at least some distinct cellular functions; for example, although both AR and FR-1 activity may be important for the metabolic changes associated with cellular proliferation, AR may be the primary aldo–keto reductase involved in cellular osmoregulation.

INTRODUCTION

The aldo–keto oxidoreductases are a diverse family of monomeric enzymes that catalyse the NADPH-dependent reduction of various carbonyl substrates (reviewed in [1]). Aldose reductase (AR; EC 1.1.1.21), one of the most extensively characterized members of this family, can catalyse the NADPH-dependent conversion of glucose into sorbitol, the first step in the polyol pathway of carbohydrate metabolism (reviewed in [2]). In most circumstances, there is only a small amount of glucose flux through this pathway; however, when blood glucose levels are elevated (e.g. in diabetes mellitus) there is enhanced polyol pathway activity and, as a consequence, intracellular sorbitol accumulation. This produces a hyperosmotic effect which is believed to be important for the development of several diabetes-associated complications (reviewed in [2–4]). Indeed, recent experiments using transgenic mice have provided direct evidence that AR-mediated polyol accumulation can promote diabetic cataracts [5,6] and neuropathy [7]. In addition to cellular osmoregulation, AR is likely to play an important role in several other processes, including the oxidation of steroids within reproductive tissues [8] and the cellular catabolism of cytotoxic aldehydes [9,10].

Three distinct murine genes have been identified that are predicted to encode aldo–keto oxidoreductases with significant amino acid sequence identity with rat and human AR. In 1990, Pailhoux et al. [11] reported the isolation of a cDNA clone encoding a 316-amino acid polypeptide which they named mouse vas deferens protein (MVDP). This protein has approx. 70 %

amino acid sequence identity with rat and human AR. Subsequent studies have indicated that the MVDP gene is regulated by androgens [11–13] and is expressed primarily, but not exclusively, in the epithelium of the vas deferens [14,15]. It is at present not known whether MVDP is in fact a functional aldo–keto reductase. In 1994, our group reported the identification of a fibroblast growth factor (FGF)-1-inducible gene in murine NIH 3T3 cells predicted to encode a 316-amino acid aldo–keto reductase with approx. 82 % sequence identity with MVDP and approx. 69 % identity with rat and human AR [16]. This gene, named FGF-regulated (FR)-1, is expressed in a tissue-specific manner in the adult mouse, with highest mRNA levels detected in adrenal gland, heart, intestine, lung, ovary and testis [15,16]. Wilson et al. [17] have demonstrated recently that recombinant FR-1 exhibits NADPH-dependent reductase activity with D,L-glyceraldehyde as a substrate and that zopolrestat, a potent AR inhibitor, can also inhibit FR-1 activity. Furthermore these investigators were able to determine the crystal structure of the FR-1–NADPH–zopolrestat ternary complex at 1.7 Å resolution. The third and most recently identified murine aldo–keto reductase gene reported to date encodes a 316-amino acid protein with approx. 97 % sequence identity with rat AR, approx. 85 % identity with human AR and approx. 69 % identity with mouse MVDP or FR-1 [18,19]. This gene is likely to be the murine homologue of rat and human AR and is expressed primarily in the kidney, skeletal muscle and testis of adult mice. Recombinant mouse AR protein is enzymically active, demonstrating reductase activity for various aldo sugars but not for steroids [18].

Abbreviations used: AR, aldose reductase; FGF, fibroblast growth factor; FR, FGF-1-regulated; GST, glutathione S-transferase; MAP, multiple antigen peptide; MVDP, mouse vas deferens protein.

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Here, we report that AR and FR-1 gene expression in murine NIH 3T3 fibroblasts is differentially regulated by FGF-1 treatment, calf serum treatment and hyperosmotic stress. We also describe the generation of anti-FR-1 polyclonal antiserum and show that FGF-1 stimulation of quiescent fibroblasts promotes FR-1 protein accumulation.

MATERIALS AND METHODS

Cell culture

Murine NIH 3T3 cells (American Type Culture Collection) were maintained, expanded and serum-starved as described [21]. For the growth-stimulation experiments, serum-starved cells were treated for various lengths of time with either 10 ng/ml recombinant human FGF-1 (gift from W. Burgess, Holland Laboratory) in combination with 5 units/ml heparin (Upjohn) or with 10% calf serum (Hyclone Laboratories). Cells were harvested by trypsin/EDTA treatment. For the hyperosmotic stress experiments, cells were first grown to approx. 80% confluency in normal culture medium. The medium was then replaced with either fresh isotonic medium (110 mM NaCl, 25 mM glucose) or fresh medium supplemented with 190 mM NaCl, 190 mM raffinose, 190 mM sorbitol, 275 mM glucose, 275 mM 3-*O*-methylglucose or 275 mM mannitol. The cells were then cultured for various lengths of time in either the isotonic or hypertonic medium and then harvested as described above.

In vitro transcription assays

The mouse AR plasmid (pDIRECT/ALR2; gift from J. Mark Petrash, Washington University School of Medicine) and the mouse FR-1 plasmid (pGEM3Zf+/FR-1 [16]) were each linearized by *Hind*III digestion. Sense strand transcripts were then synthesized *in vitro* using T7 RNA polymerase and the Ribomax large-scale RNA production system (Promega). RNA concentrations were determined by UV light absorbance at 260 nm.

RNA isolation and Northern-blot hybridization analysis

Total cellular RNA was isolated using RNA Stat-60 (Tel-Test Inc.) according to the manufacturer's instructions. RNA concentrations were calculated by measuring UV light absorbance at 260 nm. Each RNA sample (10 µg) was denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was electroblotted on to Zetabind nylon membranes (Cuno Inc.) and cross-linked by UV irradiation using a Stratalinker (Stratagene). Northern-blot hybridization analysis was performed as described [22] using either the 1.1 kb *Hind*III–*Nco*I fragment of pDIRECT/ALR2 or the 1.3 kb *Eco*RI fragment of pGEM3Zf+/FR-1 (see above for sources of plasmids). The probes were radiolabelled with [α -³²P]dCTP (3000 Ci/mmol; Amersham) using a random-primed DNA-labelling kit (Boehringer-Mannheim) according to the manufacturer's instructions. The blots were air-dried and autoradiography was performed using Kodak X-Omat AR film. Hybridization signal intensities were quantified by densitometry using the BioImage whole-band analyzer (Millipore).

FR-1 expression and purification

Recombinant FR-1 was obtained by expression of the cDNA in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein. The expression plasmid pGEX-KG/FR-1 was

constructed by ligation of an *Nco*I–*Sal*I restriction fragment of pGEM3Zf+/FR-1 into the bacterial expression vector pGEX-KG (gift from R. Friesel, Holland Laboratory [23]). Overnight cultures of *Escherichia coli* HB101 (Gibco/BRL) transformed with either pGEX-KG or the pGEX-KG/FR-1 plasmid were diluted 1:10 in Luria broth and grown at 37 °C for 1 h. Isopropyl β -thiogalactoside (Gibco/BRL) was then added to a final concentration of 0.2 mM, and 3–4 h later cells were pelleted and resuspended in PBS supplemented with 1 mM PMSF (Sigma) and 1 mg/ml lysozyme (Sigma). Cells were lysed by incubation at 37 °C for 5 min, Triton X-100 (Sigma) was added to a 1% (v/v) final concentration, and mild sonication was used to reduce viscosity. The lysates were then subjected to two cycles of centrifugation at 10000 *g* at 4 °C for 5 min. The supernatants were then incubated with hydrated glutathione–Sephacrose 4B (Pharmacia) at 4 °C for 30 min with end-over-end mixing. The beads were collected by brief centrifugation at 500 *g* and washed 5 times with PBS containing 1% (v/v) Triton X-100. For protease cleavage of the GST–FR-1 fusion protein, the beads were washed once with 50 mM Tris/HCl, pH 8.0/150 mM NaCl/2.5 mM CaCl₂ and then incubated in 0.5 ml of this buffer containing 10 units/ml bovine thrombin (Armour) and 30 mM 2-mercaptoethanol (Sigma) at room temperature for 30 min with end-over-end mixing. The beads were collected by brief centrifugation and the supernatant (containing FR-1) was stored at –80 °C. The FR-1 concentration was determined using the BCA protein assay reagent (Pierce Chemical Co.). All samples were analysed by SDS/PAGE followed by staining with Coomassie Blue.

Generation and purification of anti-FR-1 serum

An FR-1 peptide was synthesized on an octa-branched core matrix using the multiple antigen peptide (MAP) approach described by Tam [24]. Briefly, the peptide LLPETVNMEETPYDAEY (residues 300–316 of FR-1 [16]) was synthesized on Fmoc (fluoren-9-ylmethoxycarbonyl)-MAP resin (eight-branch; Applied Biosystems), cleaved from the resin with trifluoroacetate, and then purified by reverse-phase HPLC. A New Zealand White rabbit was first injected with approx. 0.5 mg of the antigen in complete Freund's adjuvant (Calbiochem) and then boosted 4 times with approx. 0.3 mg of the antigen in incomplete adjuvant (Calbiochem). For affinity purification of the antiserum, recombinant FR-1 protein was immobilized on to a methanol-pretreated ProBlot membrane (Applied Biosystems) by immersing the membrane in 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 0.05% (v/v) Triton X-100 and 2.5 µg/ml FR-1 at room temperature for 4 h. The membrane was washed with the above solution (minus protein) for 10 min to remove unbound FR-1 and then blocked by immersion in 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 4% (w/v) BSA (Boehringer-Mannheim) at room temperature for 2 h. An aliquot of antiserum was then diluted 2-fold using 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 0.05% (v/v) Triton X-100 and incubated with the membrane at 4 °C for 18 h on a rocking platform. The membrane was then washed twice (5 min each) with the above solution, and antiserum eluted by immersion in 0.2 M glycine, pH 2.8, with vigorous agitation for 15 min. The antiserum was then neutralized using 1 M Tris/HCl, pH 8.0, and stored at –20 °C.

Western-blot analysis

Recombinant FR-1 protein was purified as described above and recombinant rat AR protein was a gift from D. Carper (National Eye Institute, Bethesda, MD, U.S.A.). Cellular lysates were

prepared as described [21] and the amount of protein in each clarified lysate was determined using the BCA protein assay reagent. Equivalent amounts of each protein sample (0.2 μ g of each recombinant protein or 50 μ g of NIH 3T3 cell protein) were mixed with 2 $\times$ sample buffer [125 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol], heated at 95 $^{\circ}$ C for 4 min, and subjected to SDS/PAGE using either a 15% (recombinant protein) or 12% (cellular lysates) polyacrylamide/SDS slab gel. Low-molecular-mass protein standards (Bio-Rad Laboratories) were also included on the gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting. The membranes were stained with Ponceau S (Sigma) to verify that equivalent amounts of protein were present in each gel lane and then incubated in TBST/milk [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% (v/v) Tween 20, 5% (w/v) non-fat dry milk] at 4 $^{\circ}$ C for 18 h. The membranes were then incubated in TBST/milk containing either a 1:1000 dilution of anti-FR-1 serum or a 1:250 dilution of anti-AR serum (also a gift from D. Carper) at room temperature for 2 h, washed twice with TBST/milk, and then incubated in TBST/milk containing a 1:20000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad Laboratories) at room temperature for 1 h. Bound secondary antibody was detected using an enhanced chemiluminescence kit (Amersham). The blots were air-dried and autoradiography was performed using Kodak X-Omat AR film.

RESULTS AND DISCUSSION

Differential regulation of AR and FR-1 mRNA expression by cell-growth promoters

In a previous report, we demonstrated by Northern-blot hybridization analysis that FGF-1 stimulation of quiescent NIH 3T3 fibroblasts increased FR-1 mRNA levels and that this

Figure 1 Hybridization specificity of the murine AR and FR-1 cDNA probes

Equivalent amounts of *in vitro* transcribed mouse AR (MAR) RNA and mouse FR-1 RNA were loaded in duplicate on an RNA gel and then analysed by Northern-blot hybridization using the 32 P-labelled cDNA probes indicated on the left. In this and the subsequent Northern-blot Figures, only the region of the autoradiogram that contained a hybridization signal is shown. The bottom panel is a photograph of the ethidium bromide (EtBr)-stained RNA gel demonstrating that equivalent amounts of sense RNA were present in each gel lane.

Figure 2 Effect of treatment with FGF-1 or calf serum on AR and FR-1 mRNA levels in NIH 3T3 fibroblasts

Serum-starved cells were either left untreated (0') or treated with FGF-1 (A) or calf serum (CS; B) for the indicated time periods. RNA was isolated and equivalent amounts of each sample analysed by Northern-blot hybridization using the 32 P-labelled cDNA probes indicated on the left. The bottom part of each panel is a photograph of the ethidium bromide-stained RNA gel demonstrating that equivalent amounts of rRNA were present in each gel lane.

cellular response required *de novo* RNA and protein synthesis [16]. FGF-2 treatment also induced FR-1 mRNA expression but treatment with other polypeptide mitogens (e.g. platelet-derived growth factor) or with calf serum (which contains numerous growth-promoting factors) had no significant effect. In order to determine whether FGF-1 stimulation of NIH 3T3 cell growth also induced AR mRNA expression, we obtained a murine AR cDNA clone to use as a Northern-blot hybridization probe. The murine AR and FR-1 cDNAs have approx. 65% nucleotide sequence identity; thus one would predict that they should only recognize their corresponding mRNAs when used as probes in high-stringency hybridization experiments. In our initial experiments, we confirmed that this was indeed the case by Northern-blot analysis using *in vitro* transcribed AR and FR-1 mRNA (Figure 1).

We then examined AR and FR-1 mRNA levels in FGF-1- or calf serum-treated NIH 3T3 cells by Northern-blot hybridization analysis. Serum-starved cells were either left untreated or treated with FGF-1 or calf serum for different lengths of time and then harvested. RNA was isolated and equivalent amounts analysed by Northern-blot hybridization. The AR and FR-1 transcripts were each approx. 1.4 kb in size (consistent with previous reports [16,18]) and were detected in both quiescent and growth-promoter-stimulated cells. FGF-1 treatment increased both AR and FR-1 mRNA expression levels, although FR-1 mRNA induction was more pronounced (Figure 2A). Increased AR and FR-1 mRNA levels were first apparent at 4 h, with maximal AR mRNA levels (approx. 5.8-fold induction) and FR-1 mRNA levels (approx. 13.8-fold induction) detected at 24 h after FGF-1 addition. Calf serum treatment also increased AR mRNA expression, with an approx. 2.9-fold level of induction detected at 8 h after serum addition; however, FR-1 mRNA levels were only slightly increased (Figure 2B). The observed changes in AR and FR-1 mRNA levels after FGF-1 or calf serum addition do not appear to reflect unequal loading of the RNA samples since similar amounts of 28 S and 18 S rRNA were present in each gel lane. We attempted to confirm that equivalent amounts of RNA were loaded in each gel lane by reprobing the Northern blots

Figure 3 Expression and purification of recombinant murine FR-1

Bacterial cells transformed with either pGEX-KG (encoding GST alone) or pGEX-KG/FR-1 (encoding the GST–FR-1 fusion protein) were grown and recombinant proteins purified by affinity chromatography on immobilized glutathione. Thrombin cleavage was also performed on an aliquot of the GST–FR-1 fusion protein. Samples were analysed by SDS/PAGE followed by staining with Coomassie Blue. Molecular masses of protein size standards (in kDa) are shown on the left and the positions of the recombinant proteins are noted on the right.

with a β -actin cDNA clone, but we found that β -actin mRNA expression was up-regulated after mitogenic stimulation of quiescent cells (results not shown). This result is consistent with previous reports demonstrating that β -actin is a growth-factor-regulated immediate-early response gene (reviewed in [25]).

The above results demonstrate that (i) FGF-1-stimulated cells express elevated levels of both the AR and FR-1 mRNAs, but these two transcripts have different maximal induction levels, (ii) calf serum-stimulated cells express significantly elevated levels of AR mRNA but not FR-1 mRNA, and (iii) although both FGF-1 and calf serum treatment of quiescent cells can induce AR mRNA levels, the effect of each growth-promoting agent is distinct. The results reported here on the effect of FGF-1 or calf serum on FR-1 mRNA expression in NIH 3T3 fibroblasts are consistent with our previous study [16]. FGF-1 and the related mitogen FGF-2, which can also induce FR-1 [16] and AR (results not shown) mRNA levels in these cells, have been shown to regulate AR gene expression in two other experimental systems. Peek et al. [26] reported that increased AR mRNA expression was associated with FGF-2-mediated rat lens epithelial-cell terminal differentiation, and Jacquin-Becker and Labourdette [27] reported that AR gene expression was up-regulated after FGF-1 or FGF-2 treatment of rat astrocytes. Taken together with our results, it appears that FGF-1- or FGF-2-stimulated cellular proliferation as well as differentiation can be accompanied by elevated AR and/or FR-1 gene expression. The physiological role of increased AR or FR-1 expression in these contexts is at present not clear, but these aldo-keto reductases may be important for the profound changes in cellular metabolism that are associated with these complex processes.

Regulation of FR-1 protein expression by FGF-1 treatment

We next investigated whether the increased expression of FR-1 mRNA in FGF-1-stimulated NIH 3T3 cells resulted in a detectable increase in FR-1 protein levels. To address this issue, we had to generate an immunological reagent that would recognize FR-1 but not AR. This was accomplished using the following approach. First, we expressed FR-1 as a GST fusion protein in

Figure 4 Reactivity and specificity of the anti-FR-1 polyclonal antiserum

(A) The amino acid sequence identity between the FR-1 peptide antigen and the corresponding C-terminal regions of MVDP, mouse AR (MAR), rat AR (RAR) and human AR (HAR) is shown. The numbers at the top refer to the amino acid positions and residues that are conserved between FR-1 and each of the other proteins are boxed. (B) Equivalent amounts of recombinant FR-1 and recombinant rat AR (RAR) were loaded in duplicate on a protein gel and then subjected to SDS/PAGE and Western-blot analysis using either anti-FR-1 (top panel) or anti-RAR (bottom panel) polyclonal antiserum and the enhanced chemiluminescence detection system. Molecular masses of protein size standards (in kDa) are shown on the left of each panel.

bacteria and then used recombinant protein for both affinity purification of crude antiserum and for testing antiserum specificity. The full-length FR-1 cDNA was subcloned into the pGEX-KG expression vector and the parental and recombinant plasmids individually transformed into bacterial cells. Cultures were treated with isopropyl β -thiogalactoside, and GST itself as well as the GST–FR-1 fusion protein were purified from bacterial lysates by affinity chromatography on glutathione–Sephadex. An aliquot of the immobilized GST–FR-1 fusion protein was then cleaved with thrombin to obtain recombinant FR-1. The purified GST, GST–FR-1 and FR-1 proteins were the appropriate sizes (approx. 26, 62 and 36 kDa respectively) as determined by SDS/PAGE and Coomassie Blue staining (Figure 3). Second, we immunized rabbits with an octa-branched synthetic MAP corresponding to the C-terminal 17 amino acid residues of the predicted FR-1 protein. This peptide was chosen because murine AR, MVDP and FR-1 have a relatively low degree of sequence identity in their C-terminal regions (Figure 4A). Western-blot analysis using affinity-purified anti-FR-1 serum indicated that it recognized FR-1 but not rat AR, which has the same C-terminal sequence as mouse AR (Figure 4B). In contrast, polyclonal antiserum generated against recombinant rat AR protein recognized both FR-1 and rat AR. This latter finding indicates that previous Western blot, ELISA or immunohistochemical studies that utilized antiserum generated using purified AR protein as the antigen could be detecting AR as well as AR-related proteins that may be expressed in the cells or tissues under investigation.

Western-blot analysis was then performed to investigate whether FGF-1 treatment of NIH 3T3 cells resulted in elevated

Figure 5 Effect of FGF-1 treatment on FR-1 protein levels in NIH 3T3 fibroblasts

Serum-starved cells were either left untreated (0') or treated with FGF-1 for the indicated time periods. Cell lysates were prepared and equivalent amounts of protein were subjected to SDS/PAGE and Western-blot analysis using anti-FR-1 polyclonal antiserum and the ECL detection system. Molecular masses of protein size standards (in kDa) are shown on the left.

Figure 6 Effect of hyperosmotic stress on AR and FR-1 mRNA levels in NIH 3T3 fibroblasts

(A) Subconfluent cells were either left untreated (0') or treated with isotonic medium (No addition) or hypertonic NaCl-supplemented medium (NaCl) for the indicated time periods. RNA was isolated and equivalent amounts of each sample analysed by Northern-blot hybridization using the 32 P-labelled cDNA probes indicated on the left. (B) Subconfluent cells were either left untreated in isotonic medium (No addition) or treated with hypertonic medium containing the indicated osmolyte for 24 h. RNA was isolated and equivalent amounts were analysed as described above. The bottom part of each panel is a photograph of the ethidium bromide-stained RNA gel demonstrating that equivalent amounts of rRNA were present in each gel lane.

FR-1 expression. Serum-starved cells were either left untreated or treated with FGF-1 for different lengths of time. Cells were harvested and equivalent amounts of protein were analysed by SDS/PAGE and Western blotting using the anti-FR-1 serum. A major immunoreactive protein of approx. 36 kDa, a size consistent with the predicted molecular mass of FR-1 [16], was detected in FGF-1-treated cells (Figure 5). FGF-1 stimulation increased FR-1 expression, and the temporal expression kinetics of FR-1 accumulation were somewhat delayed compared with that of FR-1 mRNA accumulation. These results demonstrate that FR-1 protein induction is associated with FGF-1-mediated cell cycle progression.

Differential regulation of AR and FR-1 mRNA expression by hyperosmotic stress

Previous reports have demonstrated that AR gene expression is induced when various cell types (e.g. rat astrocytes [27], human renal proximal tubule cells [28], human retinal pigment epithelial cells [29], dog lens epithelial cells [30], rabbit renal medullary cells [31], Chinese hamster ovary cells [32]) are cultured under conditions of hypertonicity. Indeed, an osmotic response element (also referred to as a tonicity-responsive element) has been identified in the promoter regions of the human [33], rabbit [34] and mouse [35] AR genes. Therefore we determined whether AR and/or FR-1 gene expression in NIH 3T3 fibroblasts was regulated by hyperosmotic stress. Cells were grown to subconfluent density in normal, isotonic culture medium and then the medium was replaced with either fresh isotonic medium or hypertonic NaCl-supplemented medium. Cells were cultured under these conditions for different lengths of time and then harvested. RNA was isolated and equivalent amounts analysed by Northern-blot hybridization. Hyperosmotic stress significantly increased AR mRNA levels but had only a small stimulatory effect on FR-1 mRNA expression (Figure 6A). AR mRNA levels gradually increased after exposure to hypertonic medium, with approx. 3.0-, 7.9- and 14.8-fold levels of induction apparent at 12, 24 and 36 h respectively.

We next investigated whether AR or FR-1 gene induction occurred in response to hyperosmolarity produced by other compounds, specifically raffinose, sorbitol, glucose, 3-*O*-methylglucose (a non-metabolizable glucose analogue) or mannitol. In this case, cells were harvested after growth in hypertonic medium for 24 h, RNA was isolated and Northern-blot hybridization analysis performed. AR mRNA levels, but not FR-1 mRNA levels, were significantly elevated by hypertonic stress induced by all of these compounds, with levels of induction similar to that noted in NaCl-supplemented medium (Figure 6B).

The changes in AR mRNA levels observed in the above experiments do not appear to reflect unequal loading of the RNA samples since similar amounts of 28 S and 18 S rRNA were present in each gel lane. We attempted to confirm that equivalent amounts of RNA were present in each gel lane by reprobing the Northern blots with a β -actin cDNA clone, but we found that β -actin mRNA expression was down-regulated in hypertonic medium (results not shown). This finding indicates that decreased expression of cytoskeletal proteins such as β -actin may be required for the changes in cell shape associated with osmotic stress. Taken together, the above results demonstrate that the mouse AR and FR-1 genes are differentially regulated by hyperosmolarity and that AR, but not FR-1, is likely to be involved in cellular osmoregulation.

In summary, the results described above indicate that, although the murine AR and FR-1 genes are co-expressed in a fibroblast cell line, they are differentially regulated by growth-promoting agents as well as by hypertonic stress. Specifically, FGF-1 treatment of quiescent cells induces both AR and FR-1 mRNA levels (albeit to different degrees), whereas serum treatment of quiescent cells or cell growth in hypertonic medium primarily results in up-regulation of AR mRNA expression. It is therefore likely that these two structurally related NADPH-dependent aldo-keto reductases have unique enzymic functions in mammalian cells.

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