

Arsenic Mediates Cell Proliferation and Gene Expression in the Bladder Epithelium: Association with Activating Protein-1 Transactivation

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ABSTRACT

Although the mechanism of action has not yet been defined, epidemiological studies have demonstrated an association between elevated arsenic levels in drinking water and the incidence of urinary bladder transitional cell carcinomas. In the current studies, we demonstrate that mice exposed to 0.01% sodium arsenite in drinking water develop hyperplasia of the bladder urothelium within 4 weeks of exposure. This was accompanied by the accumulation of inorganic trivalent arsenic, and to a lesser extent dimethylarsinic acid, in bladder tissue, as well as a persistent increase in DNA binding of the activating protein (AP)-1 transcription factor. AP-1 transactivation by arsenic also occurred in bladders of transgenic mice containing an AP-1 luciferase reporter. Consistent with these *in vivo* observations, arsenite increased cell proliferation and AP-1 DNA binding in a human bladder epithelial cell line. Gene expression studies using RNase protection assays, reverse transcription-PCR, and cDNA microarrays indicated that arsenite alters the expression of a number of genes associated with cell growth, such as *c-fos*, *c-jun*, and *EGR-1*, as well as cell arrest, such as *GADD153* and *GADD45*. The proliferation-enhancing effect of arsenic on uroepithelial cells likely contributes to its ability to cause cancer.

INTRODUCTION

It is well established that exposure to trivalent and pentavalent forms of arsenic, which occurs worldwide primarily through occupational and environmental exposure, causes characteristic skin alterations, including hyperkeratosis and skin cancer. Recent epidemiological studies conducted in Taiwan (1, 2), Argentina (3), Chile (4), and Japan (5) also indicate a connection between arsenic exposure from contaminated drinking water and an increase in the incidence of urinary bladder cancer. In this respect, it has been estimated that at the present EPA arsenic water standard of 50 $\mu\text{g/liters}$, the internal cancer risks may be comparable to those of environmental tobacco smoke and radon in homes (6), and several large epidemiological studies in populations in the United States are being planned to investigate the association between arsenic exposure and increased incidences of bladder cancer (7, 8). It is known that about 350,000 individuals in the United States drink water with levels above the standard, and more than 2.5 million people use water containing more than 25 $\mu\text{g/liter}$ (6).

Although several hypotheses have been proposed, the mechanism responsible for arsenic carcinogenesis has not been established. Increasing evidence indicates that arsenic acts on signaling pathways that regulate cell proliferation rather than causing direct DNA damage because arsenic exhibits its mutagenic activities only at concentrations high enough to also produce cell damage (9). Recently, arsenic has been shown to modulate the mitogen-activated protein kinase cascade in several cell systems, resulting in the activation of transcription

factors, such as AP-1² (10–13). AP-1 mediates many biological effects of tumor promoters and is an important regulator of cell growth. The ability of arsenic to interact with protein thiol groups on key regulatory proteins and subsequently alters of their activities is likely to contribute to this effect (10, 11). Accordingly, it has been demonstrated that arsenic can induce a moderate increase in keratinocyte cell proliferation, as evidenced by increases in thymidine incorporation (14), cell cycling (15), labeling of the proliferating cell marker Ki-67 (15), ornithine decarboxylase activity (16), and expression of oncogenes and growth factors such as *c-fos*, *c-jun*, *c-myc*, and transforming growth factor α (14, 17). Using both *in vitro* and *in vivo* models, the present studies were conducted to determine whether arsenic produced molecular and histological changes in urinary bladder epithelium consistent with epigenetic mechanisms for carcinogenesis, in this case cell proliferation.

MATERIALS AND METHODS

Experimental Animals. C57BL/6 male mice carrying the 2 \times TRE-luciferase transgene, originally developed by Rincon and Flavell, were crossed with a DBA2 (SASCO, Omaha, NE) female mice as described previously (18). The F₁ offspring were screened by quantifying both basal and PMA-induced levels of luciferase activity as an indicator for the presence of the AP-1-luciferase reporter gene. To assay for AP-1 activity, bladders were removed, placed in lysis buffer (200 $\mu\text{l}/10$ mg of tissue) and left overnight at 4°C. The luciferase activity of the tissue supernatant obtained after lysis was measured by a luminometer with AP-1 activity expressed relative to the level of luciferase activity of control groups (18). Female C57BL/6 mice were obtained from Charles River (Portage, MI). All animals were housed at National Institute for Occupational Safety and Health facilities in compliance with American Association for Accreditation of Laboratory Animal Care approved guidelines for the humane treatment of laboratory animals. Animals were maintained on a 12-h light/dark cycle and were provided chow and water *ad libitum*. Groups of 8-week-old mice were provided 0.002 or 0.01% arsenic, as sodium arsenite (Sigma Chemical Co., St. Louis, MO) in their drinking water for up to 16 weeks and sacrificed by CO₂ asphyxia. Urine, blood, and the urinary bladder were collected under aseptic conditions. Mice receiving arsenic-treated water revealed a moderate reduction in water consumption. To account for any pathological effects related to reduced water intake, a paired water group was added. This group was provided an amount of control water equal to that consumed by animals in the experimental group. After 16 weeks of exposure, the paired water group of mice were sacrificed, the bladders were examined histologically, serum albumin levels were quantitated, and hematocrits were determined.

Histology. Bladders were removed and fixed by immersion in 10% neutral-buffered formalin and processed for paraffin embedding. Each paraffin block was step-sectioned and stained with H&E. For scanning electron microscopy, the specimens were fixed in 2.5% glutaraldehyde, washed in phosphate buffer, and postfixed in 2% osmium tetroxide. The specimens were dehydrated with graded alcohol, placed in hexamethyldisilazane, and air dried. After coating

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² The abbreviations used are: AP, activating protein; DMA, dimethylarsenic; EGF, epidermal growth factor; EGR, early growth response; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; MMA, monomethylarsenic; PCNA, proliferating cell nuclear antigen; PMA, phorbol myristate acetate; RT, reverse transcription; TRE, TPA responsive element; TPA, 12-O-tetradecanoylphorbol-13-acetate.

with gold/palladium, the specimens were examined using a JEOL 6400 (Tokyo, Japan) scanning electron microscope. For transmission electron microscopy, the samples were fixed as described for scanning microscopy and embedded in LX-112 epon (Ladd Research Industry, Burlington, VT). Ultrathin sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (JEOL 1220).

For immunohistochemistry, 6- μ m sections were prepared from paraffin blocks and placed on microscope slides. Slides were deparaffinized, and the antigen was unmasked using 10 mM sodium citrate buffer (pH 6.0) at 95°C for 5 min. The endogenous peroxidase activity was blocked by peroxidase block (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Slides were treated for 20 min with 1% normal goat serum and incubated overnight at 4°C with a 1:1000 dilution of rabbit antihuman c-jun or c-fos polyclonal antibodies (Santa Cruz Biotechnology Inc.) or a 1:100 dilution of monoclonal anti-PCNA antibody (Vector Laboratories, Inc., Burlingame, CA). After being washed, the samples were incubated for 1 h with a 1:200 dilution of biotinylated goat antirabbit or antimouse IgG antibody, respectively (Vector Laboratories; 1:200) followed by incubation for 1 h with ABC buffer (Vectastain Elite kit, Vector Laboratories, Inc.). Ten mg of diaminobenzidine were dissolved in 20 ml Tris-imidazole buffer (50 mM Trizma, 10 mM imidazole, pH 7.2) containing 3.3 μ l of 30% H₂O₂. Samples were incubated in this buffer for 6 min before rinsing with Tris-imidazole buffer and counterstained with Harris's hematoxylin (EM Diagnostic Systems, Gibbstown, NJ). Results are presented as the percentage of positively stained nuclei counted in four random areas of the slide.

Arsenic Determination in Tissues. Urinary bladders from control or arsenic-treated mice were quick-frozen in acid-free vials and stored at -70°C. The tissue samples were digested by addition of 6 N HCl at 80°C for 16 h in a specially designed reaction vessel. Analyses of arsenic tissue levels were performed by Battelle Marine Sciences Laboratory (Seqium, WA) using a complex atomic absorption method (19). Briefly, arsenic, MMA, and DMA were converted to volatile arsines by addition of 4% NaBH₄ solution. The volatiles were purged from the sample onto a cooled glass trap packed with 15% OV-3 on Chromasorb WAW-DMCSO. The trapped arsines were thermally desorbed to increase boiling points and carried as an inert gas stream into the quartz furnace of an atomic absorption spectrophotometer. The first arsine desorbed was AsH₃, which represents total inorganic arsenic in the sample, whereas MMA and DMA were desorbed and detected several minutes later. To determine the concentration of arsenite (iAs³⁺), another aliquot of sample was placed in the reaction vessel, and Tris buffer was added. This procedure was repeated to determine only the arsine produced from arsenite. Quality control was established through calibration and testing of the hydride generation, purging, and detection systems.

Cell Cultures. UROtsa, a SV40 immortalized human urothelium cell line, was obtained from Dr. G. Petzoldt, (University College, London, United Kingdom). The cell line does not acquire characteristics of transformed cells including growth in soft agar or development of tumors in nude mice (20). The cells were grown at 37°C/5% CO₂, in RPMI 1640 culture medium supplemented with 10% FBS and 2 mM L-glutamine (Life Technologies, Gaithersburg, MD), referred to as complete medium.

Cell Mitogenesis. UROtsa cells were seeded in 96-well, flat-bottomed culture plates at a concentration of 2 \times 10³ cells/well. The following day (approximately 40% confluent) the culture medium was replaced with RPMI 1640 medium supplemented with 1% FBS, and the cells were exposed to different concentrations of sodium arsenite for 48–72 h. During the last 4 h of incubation, [³H]thymidine (6.7 Ci/mmol; DuPont New England Nuclear) was added at a concentration of 0.5 μ Ci/well. The cells were detached from the plates by addition of 0.1% trypsin and collected onto glass-fiber filters using an automated cell harvester (Skatron, Sterling, VA). Cellular incorporation of [³H]thymidine was determined by liquid scintillation counting.

Cell Cycle Analysis. A flow cytometry, propidium iodide method was used to determine the cell cycle. Briefly, UROtsa cells in complete medium were seeded at a concentration of 2 \times 10⁶ cells/ml into Falcon Petri dishes (100/15 mm, Becton Dickinson Labware, Lincoln Park, NJ). The medium was replaced with RPMI 1640 medium with 1% FBS, reincubated for 24 h, and treated with sodium arsenite for an additional 24 h. The cells were removed with 0.1% trypsin, and the pelleted cells were fixed overnight in 70% ethanol at 4°C. The cells were stained with 50 mg/ml propidium iodide (Sigma) in the presence of 100 units/ml RNase A (Sigma) to degrade double-stranded RNA. Analyses were conducted by flow cytometry (Becton Dickinson Immunocytometry

Systems, FACScan with Quest 1.2 software, Sunnyvale, CA), and the data were evaluated by Modfit LT 2.0 (Verity Software House Inc.).

Nuclear Extracts and EMSA. Nuclear proteins were prepared from aliquots of 1 \times 10⁷ cells or frozen samples of bladder tissue according to the method of Schreiber *et al.* (21). DNA binding reactions and EMSAs were performed as described previously (22). Briefly, the 5' ends of the double-stranded oligonucleotides were labeled with [³²P]ATP (DuPont New England Nuclear) using 6–10 units of T4 polynucleotide kinase (United States Biochemical Corp./Amersham Pharmacia Biotech, Piscataway, NJ). Binding reactions (30 μ l) were performed on ice for 30 min in reaction mixtures containing 10 μ g of nuclear proteins, 20 mM Tris-HCl (pH 7.8), 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 5 mM DTT, 50 μ g/ml BSA, 2 μ g of poly(dI-dC)·poly(dI-dC), 10% glycerol, and approximately 0.1 ng (2 \times 10⁵ cpm) of specified probe. For detection of AP-1 DNA-binding activity, an oligonucleotide was obtained from Santa Cruz Biotechnology containing an AP-1 consensus sequence, 5'-CGC TTG ATG ACT CAG CCG GAA-3'. Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel. Gels were electrophoresed at 125 V in 50 mM Tris-50 mM boric acid/1 mM EDTA, dried, and autoradiographed overnight. The autoradiograms were scanned with a computerized laser densitometer (Eagle Eye II image analysis system, Stratagene, La Jolla, CA). These results were examined using the One Dscan gel analysis software and the NIH Image 1.54 analysis software. For characterization of DNA binding activity, nuclear protein extracts were preincubated for 1 h prior to the addition of labeled probe with either excess unlabeled oligomers for 30 min or 2 μ g/ml of antibodies (Santa Cruz Biotechnology).

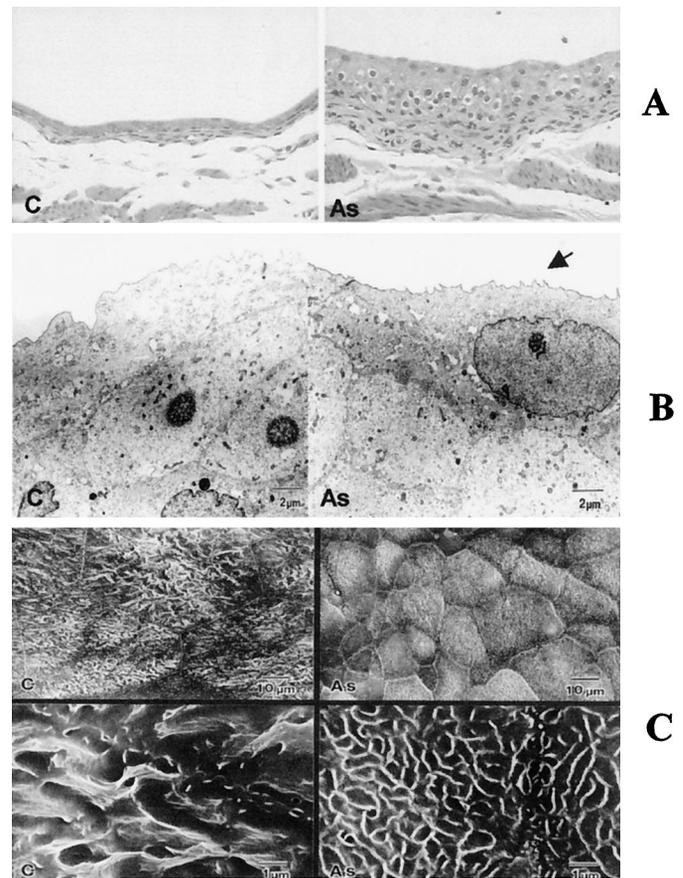


Fig. 1. Microscopic analysis of urinary bladders. A, histopathology of urinary bladder from mice given either vehicle (C) or 0.01% sodium arsenite (As) in their drinking water for 4 weeks. Full thickness bladder sections were cut, and 6- μ m sections were prepared and stained with H&E. Mice treated with arsenic revealed mild transitional cell hyperplasia. The magnification is \times 20. B, transmission electron microscopy of a bladder of a mouse administered either vehicle (C) or 0.01% sodium arsenite (As) in its drinking water for 4 weeks. Bladders from treated animals showed pleomorphic microvilli consistent with mild simple hyperplasia. C, scanning electron microscopy of a bladder from a mouse given either vehicle (C) or 0.01% sodium arsenite (As) in its drinking water for 4 weeks. Bladders from controls revealed flat polygonal cells of uniform size, whereas cells from treated animals had cells that were slightly raised and of variable sizes, with distinct cell borders, giving a cobblestone appearance.

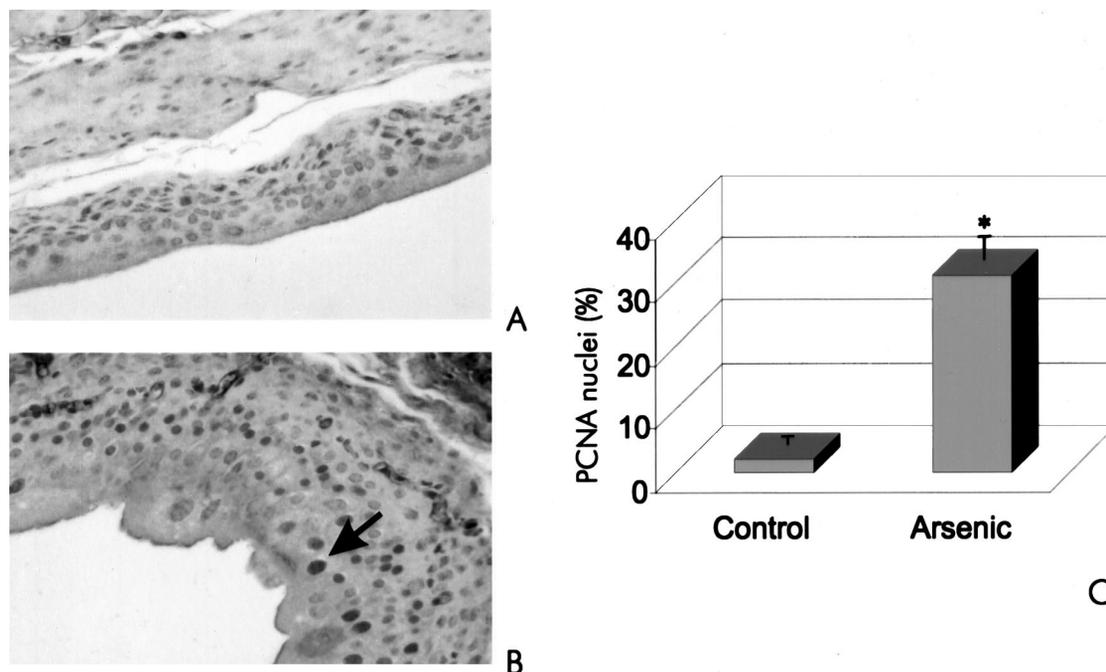


Fig. 2. PCNA immunostaining of the bladder from a control and arsenic-treated mouse. Mice were administered either control drinking water (A) or 0.01% sodium arsenite drinking water (B) for 16 weeks. Darker areas, PCNA-stained nuclei. C, data are presented as percentage of positively stained nuclei per total nuclei counted. *, significantly different from control at $P < 0.05$.

Gene Expression Studies. RNA from cell cultures was extracted using the Rneasy total RNA kit (Qiagen, Santa Clarita, CA). RNA purity and concentration were assessed by determining A_{260}/A_{280} absorption. cDNA was synthesized from 1 μg of RNA. PCR amplification was performed as described previously using commercially available PCR primers for *c-jun*, *c-fos*, and G3PDH from Clontech Laboratories Inc. (Palo Alto, CA) and 5- μl aliquots of cDNA, corresponding to 100 ng of RNA (22). RNA concentrations and PCR cycles were titrated to establish standard curves, to document linearity, and to permit semiquantitative analysis of signal strength as described previously (14). When appropriate, the specificity of the PCR bands was confirmed by restriction enzyme analysis of the amplified cDNA, which generated restriction fragments of the expected size (data not shown). The PCR products were visualized by UV illumination after electrophoresis through 2.0% agarose (Ultra-Pure, Sigma) at 60 V for 80 min and staining in Tris borate/EDTA buffer (89 mmol/liter Tris, 89 mmol/liter boric acid, 2.5 mmol/liter EDTA, pH 8.2) containing 0.5 mg/ml ethidium bromide. The gels were analyzed using the Eagle Eye II image analysis system (Stratagene) and NIH Image 1.54 software. The area under the curve was normalized for G3PDH content.

Analysis of differential gene expression was performed on UROtsa cells using the Atlas human cDNA expression array (Clontech, Palo Alto, CA). Briefly, UROtsa cells were allowed to grow to 70% confluency and treated with 10 or 50 μM sodium arsenite or 20 ng/ml PMA for 2 h. Poly(A) RNA was prepared using Oligotex resin (Qiagen), and 1 μg of mRNA was reverse transcribed from each test sample with superscript (Perkin-Elmer) in the presence of [α - ^{32}P]dATP (NEN Life Science Products, Boston, MA). The unbound ^{32}P was removed by gel filtration in Chroma Spin-200 columns (Clontech). The blots were incubated with 2×10^6 cpm/ml of buffer and hybridized overnight at 68°C to a human cDNA microarray consisting of 588 human genes under highly regulated transcriptional control, as indicated in the manufacturer's instructions (Clontech). After high stringency washes in $2 \times$ standard saline citrate-SDS, the membranes were exposed and recorded using a phosphorimaging system and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Gene expression images were normalized to housekeeping genes provided in the array. Changes in expression between control and treatments were analyzed using ArrayVision program (Imaging Research Inc., Catherines, Ontario, Canada). Replicate experiments were performed. Only genes that demonstrated significant and consistent changes in expression between control and treatment were reported.

Arsenic-induced changes in gene expression were verified by either RT-

PCR or RNase protection assay for randomly selected genes. Analysis of RNA samples by RNase protection assay was performed using a commercial kit (RiboQuant kit, PharMingen, San Diego, CA) according to the manufacturer's instructions. ^{32}P -Labeled antisense RNA probes were produced from commercial DNA templates (*in vitro* transcription kit, PharMingen). The samples were electrophoresed on a sequencing gel, which was quantitated using a phosphorimaging system and ImageQuant software (Storm, Molecular Dynamics).

Statistical Analysis. All experiments were replicated and representative findings are shown. Statistical significance was determined by one-way ANOVA. When the F value was significant, the means were compared using Fisher's post hoc analysis. In all statistical comparisons, $P < 0.05$ was used to indicate a significant difference.

RESULTS

Within 4 weeks of exposure to 0.01% sodium arsenite in their drinking water, all experimental mice developed mild hyperplasia of the urinary bladder epithelium (Fig. 1A). The urinary bladder epithe-

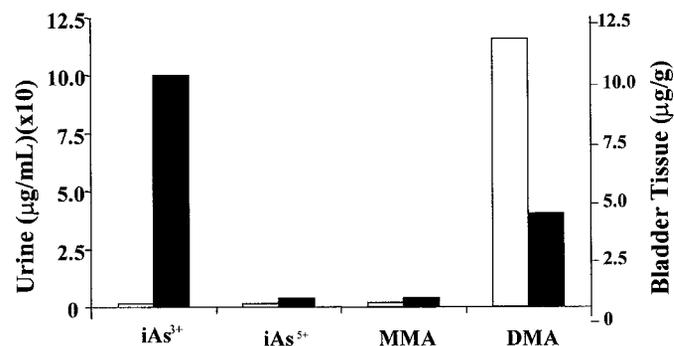


Fig. 3. Urinary and bladder tissue metabolites of arsenic. Mice were treated with 0.01% sodium arsenite (iAs³⁺) in their drinking water for 16 weeks. Urine (\square) and tissue (\blacksquare) were collected, and metabolites were determined as described in "Materials and Methods." Each value represents mean a of 2-4 animals. The control levels of arsenic in the bladder tissue and urine were similar to the assay detection limits (3 ng/g or ng/ml), except that urinary concentrations of DMA were 88 ± 15 ng/ml.

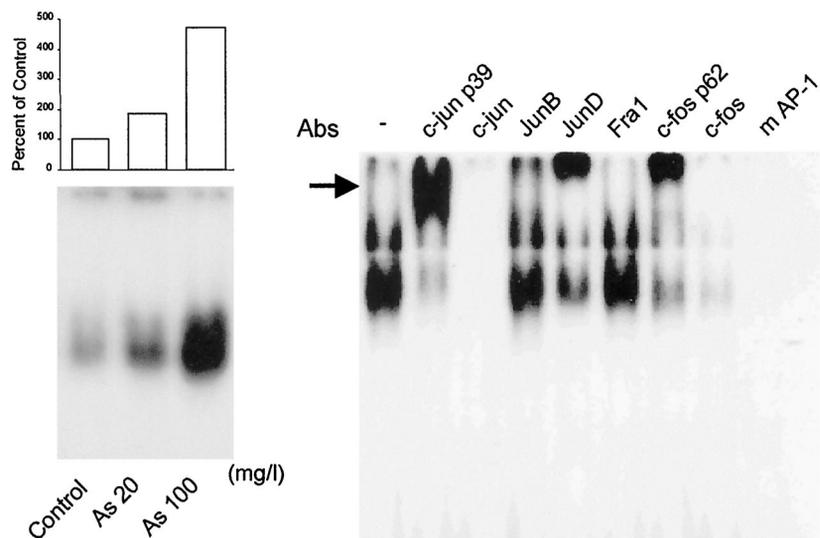


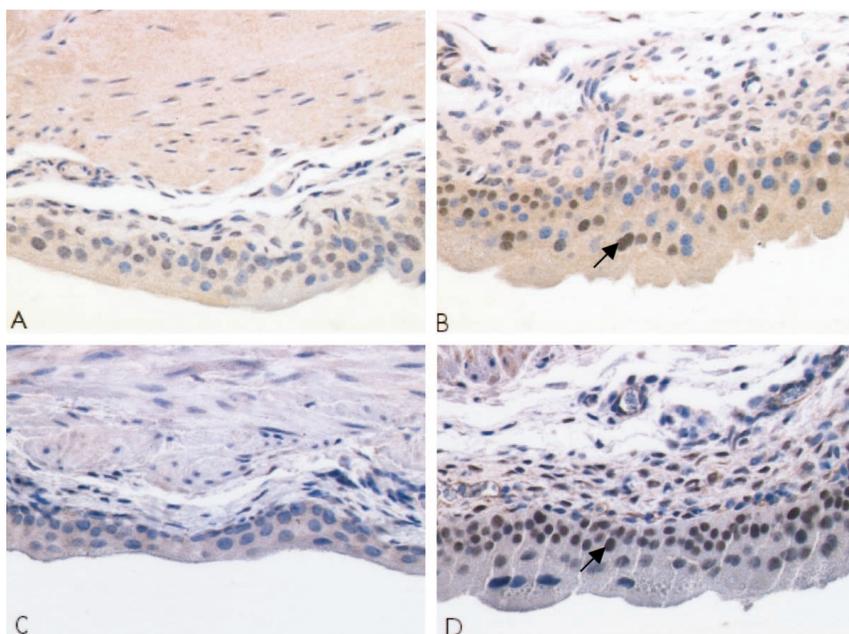
Fig. 4. AP-1 DNA binding activity in the mouse urinary bladders measured by EMSA. Mice were given drinking water or water containing 0.002% or 0.01% sodium arsenite for 16 weeks. Nuclear protein extracts were isolated from the urinary bladder and probed using a ^{32}P -labeled double-stranded oligonucleotide containing the AP-1 consensus sequence. The gels were autoradiographed and scanned using a computerized laser densitometer. Data are presented as the percent of the response in control animals. For immunochemical characterization, nuclear extracts were preincubated (1 h at 4°C) with $2\ \mu\text{g}$ of antibodies specific to c-fos or c-jun subunits. Arrow, supershifted band. The nuclear extract from the identical treatment was also probed with a mutated AP-1 probe (*mAP-1*) to help establish specificity.

lium from mice receiving control water was composed of differentiated, 1–2-cell-thick transitional cells, whereas the bladders from animals on arsenic drinking water had 6–8-cell-thick transitional cells consistent with mild hyperplasia. There was no microscopic evidence of inflammation or necrosis. Hyperplastic urothelial cells did not form papillary structures but progressed toward the lumen of the bladder. Occasional squamous metaplasia without keratinization was observed in some hyperplastic areas. There was no evidence of microcrystalluria, calculi, or amorphous precipitates in any of the tissues examined. Amorphous urinary precipitates that contain calcium phosphate can result from administration of high doses of some organic sodium salts and lead to bladder tumors in some animal species (23). Ultrastructural changes caused by arsenic in the uroepithelium, analyzed by transmission electron microscopy, revealed the presence of pleomorphic projections thought to be microvilli formation on the luminal

surface (Fig. 1B). Although not definitive, previous morphological studies have suggested that the occurrence of microvilli reflects a higher metabolic activity associated with epithelial cell proliferation (24). Scanning electron microscopic examination of bladders from control mice revealed flat, polygonal cells with protruding microvilli on the luminal surfaces (Fig. 1C). In contrast, bladders from arsenic treated animals had slightly raised, variable size cells with distinct cell borders, lending a cobblestone appearance. Although there was a slight (approximately 20–25%) decrease in water intake in arsenic-treated mice, the paired water group showed no effects on bladder histology, serum albumin levels, or hematocrits, indicating that pathological changes were caused by arsenic.

The ability of arsenic to induce hyperplasia of the urinary bladder epithelium was confirmed by immunostaining for PCNA. After 16 weeks of treatment with sodium arsenite, the percentage of positive

Fig. 5. Jun or Fos immunoreactivity in the urinary bladder of mice exposed to arsenic in their drinking water for 16 weeks. Bladder from control (A) or arsenic-treated (B) mice immunostained with a 1:1000 dilution of polyclonal antibodies to c-jun. Bladder from control (C) or arsenite-treated (D) mice immunostained with a 1:1000 dilution of polyclonal antibodies to c-fos. Arrows, representative positively stained cells.



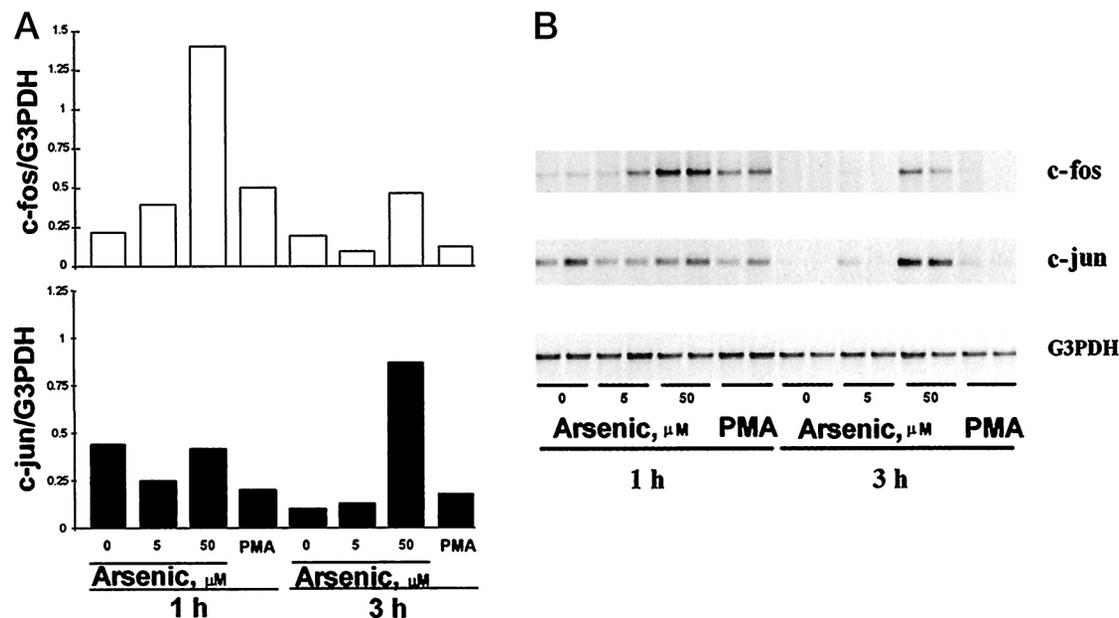


Fig. 6. *c-jun* and *c-fos* expression in UROtsa cells treated for 1 or 3 h with sodium arsenite. A, image analysis of ethidium bromide-stained RT-PCR products after normalization relative to the density of each corresponding band for G3PDH. B, RT-PCR was determined as described in "Materials and Methods." Cultures were performed in quadruplicate, and representative samples are shown.

stained nuclei in the urinary bladder epithelium was significantly increased from 2% in controls to 31% in treated mice (Fig. 2). The increased percentage of PCNA-positive cells after 4 weeks of exposure to arsenic exposure was similar to that observed at 16 weeks (data not shown).

Studies in humans and mice have demonstrated that urinary excretion is the major pathway for elimination of inorganic arsenite and that

Table 1 Effects of iAs^{3+} on UROtsa cell growth

Treatment	$[^3H]TdR$ incorporated (cpm $\times 10^3$)	Percentage of cells in cycle		
		G ₀ -G ₁	S	G ₂ -M
None	2.3	72	13	15
iAs^{3+} (2 μM)	2.9 ^a	68 ^a	17 ^a	15
iAs^{3+} (4 μM)	3.0 ^a	66 ^a	18 ^a	16

^a Significantly different from controls at $P < 0.05$.

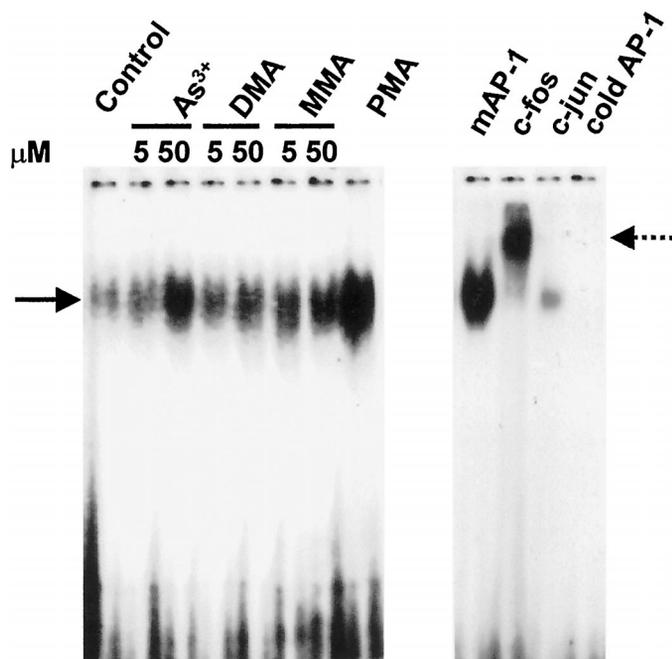


Fig. 7. AP-1 DNA binding activity in UROtsa cells treated with arsenic. Nuclear extracts from UROtsa cells were treated for 2 h with vehicle, sodium arsenite, DMA, or MMA and evaluated by EMSA using a ^{32}P -labeled double-stranded oligonucleotide probe containing the AP-1 consensus sequence. A mutated AP-1 probe (*mAP-1*) and a 50-fold excess of the unlabeled consensus probe were used to establish specificity. For immunohistochemical characterization, nuclear extracts were preincubated (1 h at 4°C) with 2 μg of antibodies to *c-fos* or *c-jun* subunits. As a positive control, cells were incubated for 2 h with 50 ng/ml PMA, and AP-1 activity was determined on isolated nuclear proteins.

DMA is the major urinary metabolite (25). Consistent with these observations, urinary metabolites in rodents exposed to sodium arsenite for 16 weeks in their drinking water consisted almost exclusively of DMA with a minimal contribution by MMA, iAs^{3+} , or iAs^{5+} (Fig. 3). In contrast to urine, quantitative chemical analyses demonstrated that trivalent arsenite was the predominant form of arsenic contained within bladder tissue, with over 10 $\mu g/g$ of the parent compound present compared to 4.5 $\mu g/g$ of DMA and only trace amounts of the other metabolites.

Increased DNA binding of the AP-1 transcription factor is often associated with the regulation of genes involved in cell proliferation (26). Furthermore, the expression of *c-jun*, a member of the AP-1 complex, correlates with rapid cell turnover in urinary bladder transitional cell carcinomas (27, 28). To determine whether arsenic modulates AP-1 activities in the urinary bladder, nuclear proteins were isolated from the bladders of mice exposed to 0.002 or 0.01% sodium arsenite in their drinking water for 16 weeks, and EMSAs were conducted. Bladder tissue from mice exposed to arsenic demonstrated a dose-dependent increase in nuclear AP-1 DNA binding activity (Fig. 4). Increased DNA binding activity was not obtained when a mutated AP-1 probe was substituted for the consensus AP-1 probe, establishing the specificity of the arsenic-induced AP-1 complex. The subunits constituting the AP-1 complex activated by arsenic was determined immunohistochemically using antibodies against known members of the family. The complex was abrogated using antibodies broadly reactive with members of *c-fos* or *c-jun* family and was supershifted by antibodies specific for the *c-jun* p39 and *c-fos* p62 subunit. The AP-1 complex was only slightly supershifted by antibody against JunD and unaltered by antibodies specific for JunB and Fra1, indicating that the

Table 2 Effect of arsenic on gene expression by cDNA array

Genes	10 μM As	50 μM As	20 ng/ml PMA	Function
<i>Waf-1</i>		+++ ^a	+++	An inhibitor of cyclin-dependent kinases
<i>BCL-2 binding component 6</i>		+++		Antiapoptotic
<i>BCL-2 binding athanogene 1 (BAG-1)</i>		+++		Antiapoptotic
<i>Serine/threonine protein kinase NIK</i>		+++	+	Nuclear factor κB -inducing kinase
<i>Death-associated protein kinase 1</i>		+++	+	Apoptosis
<i>Pig 7</i>	++	+		Redox-related gene, unclear
<i>c-fos</i>	+++	+++	+++	Transcription factor; proliferation/apoptosis
<i>GADD153</i>	+++	+++		Growth arrest
<i>GADD45</i>	+	+++	+	Growth arrest
<i>RAD</i>	+	+++		Repair-associated proteins
<i>Rho8 (RhoE) protein</i>	+	+++		Actin cytoskeleton reorganization
<i>Bone morphogenetic protein 2α</i>		+++	+++	Bone morphogenic, unclear other
<i>Heparin-binding EGF-like growth factor</i>		+++	+++	Similar to EGF; mitogenic; transformation
<i>Early growth response protein 1</i>	+	+++	+++	Transcription factor; role in proliferation
<i>Interleukin 11 or 12</i>		+++	+++	Immunomodulatory
<i>Intergrin-linked kinase</i>	---	---		Involved in mediating signal transduction
<i>Laminin B1</i>	--	--		Involved in cell attachment and chemotaxis
<i>Semaphorin</i>	--	--		Lymphocyte aggregation
<i>Homeobox HOX 4A homeodomain protein</i>	--	---		Regulation of cell adhesion processes
<i>Tyrosine-protein kinase receptor HTK</i>	---	---		Involved in mammary carcinogenesis
<i>Leukocyte interferon-inducible peptide</i>	--	---		Mediates interferon effects

^a + and -, 2-fold difference; ++ and -- 3-fold difference; +++ and ---, greater than 4-fold difference.

complex is composed primarily of c-jun p39/c-fos p62 heterodimers. Consistent with these observations, immunohistochemical examination using polyclonal antibodies revealed an increase in c-jun and c-fos immunostaining in the nuclei of bladder epithelium from exposed mice indicating that the increase in AP-1 activities was associated with an increase in immunoreactive proteins (Fig. 5). The percentage of cells that stained positive for c-jun and c-fos proteins from bladders of arsenic treated mice was 38 ± 8 and $76 \pm 12\%$, respectively, compared to approximately $2 \pm 1\%$ in control mice. The ability of arsenic to induce AP-1 activation *in vivo* was confirmed using TRE-luciferase reporter transgenic mice, in which sodium arsenite exposure resulted in a greater than 2-fold increase in AP-1 activity in bladder tissue, as measured by luciferase activity. PMA, a strong activator of AP-1 when applied to the skin, induced a 3-fold increase in bladder AP-1 activity after intrabladder installation (20 mg/ml; 2 h).

The effects of arsenic on AP-1 activity were examined *in vitro* using UROtsa cells, a human immortalized, nontransformed urothelial cell line. Initially, RNA was prepared from arsenic-treated UROtsa cells and examined by RT-PCR for *c-fos* and *c-jun* gene expression (Fig. 6). A significant increase in *c-fos* expression occurred within 1 h of arsenite treatment, followed by a rapid decline, whereas *c-jun* expression was also up-regulated, with a peak response at 3 h. These time points and slight differences in maximum expression are consistent with published data (11, 12). EMSAs were conducted to determine whether the increase in expression of these genes was associated with AP-1 binding activity. UROtsa cells exposed to a 5 or 50 μM concentration of sodium arsenite showed a dose-response increase in AP-1 DNA binding activity (Fig. 7). PMA was used as a positive control. Both MMA and DMA induced AP-1 DNA binding activity in UROtsa cells, but they were less potent than the parent form. DNA binding was specific for AP-1, because preincubation of the nuclear extracts with excess cold AP-1 inhibited the response, whereas preincubation with mutated AP-1 had no effect on binding activity. Consistent with findings in urinary bladders from mice exposed to arsenic, the DNA binding complex was composed primarily of c-jun and c-fos subunits, because the complex was supershifted by antibodies to c-fos (Fig. 7, dotted arrow) and abrogated by antibodies to c-jun. Arsenic-induced AP-1 DNA binding was neither inhibited nor supershifted by antibodies against fos B, fra 1, fra 2, jun B, or jun D (data not shown).

In accordance with the hyperplasia in mouse urinary bladders

caused by arsenic exposure, it was demonstrated that arsenic can stimulate the growth of UROtsa cells *in vitro*. As shown in Table 1, exposure to $i\text{As}^{3+}$ for 72 h induced a significant, albeit modest, increase in thymidine uptake in UROtsa cells. Additionally, cell cycle analysis of UROtsa cells with propidium iodide-stained nuclei revealed that arsenic induced an increase in the number of cells in S-phase and a concomitant decrease in G_0/G_1 .

To obtain a general profile of the genes regulated by arsenic in uroepithelial cells, mRNA obtained from control and arsenic-treated UROtsa cells were analyzed using a cDNA microarray. Of the 588 human genes examined, 13 of 22 (2.2%) were differentially expressed after 2 h of exposure to 10 μM sodium arsenite, whereas an additional 9 (3.7%) were increased after exposure to 50 μM sodium arsenite (Table 2; Fig. 8). The microarray analysis was conducted in triplicate, and only genes modulated by arsenic more than 2-fold in all three replicates are described. This percentage of genes expressed is similar to that altered in EVV304 cells treated with homocysteine (29) and HepG2 hepatoma cells treated with β -mercaptoethanol (30), both of which are thiol-containing agents that affect cell growth. Among the genes that are associated with cell growth that were consistently increased by greater than 2-fold at 10 μM sodium arsenite were *c-fos*, *Pig 7*, *Rho 8* and *EGR-1*. A number of genes associated with stress responses were also increased, including *GADD45* and *GADD153*. Several genes were up-regulated only at the high arsenic concentration. Most of these were also associated with mitogenic activity and cell cycle regulation, such as heparin-binding EGF-like growth factor, bone morphogenic protein 2 α , and *Waf-1*. Arsenic also increased the expression of *BCL-2 binding component* and *BCL-2 binding athanogene 1* genes, the expression of which was involved in inhibition of apoptosis. The expression of several genes from this panel was down-regulated by arsenic (Table 2). PMA was included in the array studies as a positive control for evaluation of gene expression (not all of the genes induced by PMA are not listed in Table 2). To help establish the reliability of the cDNA microarray system in identification of differentially expressed genes, randomly selected genes modulated by arsenic in UROtsa cells were analyzed by an RNase protection assay (Fig. 8D). Consistent with the results from cDNA microarray, arsenic induced an increase in *GADD45* (4-fold), *c-fos* (9-fold), and *WAF-1* (2-fold) but did not affect *p53* expression. The arsenic-induced up-regulation of other genes, such as *EGR-1*, *GADD153*, and *Pig7* was confirmed by RT-PCR (data not shown).

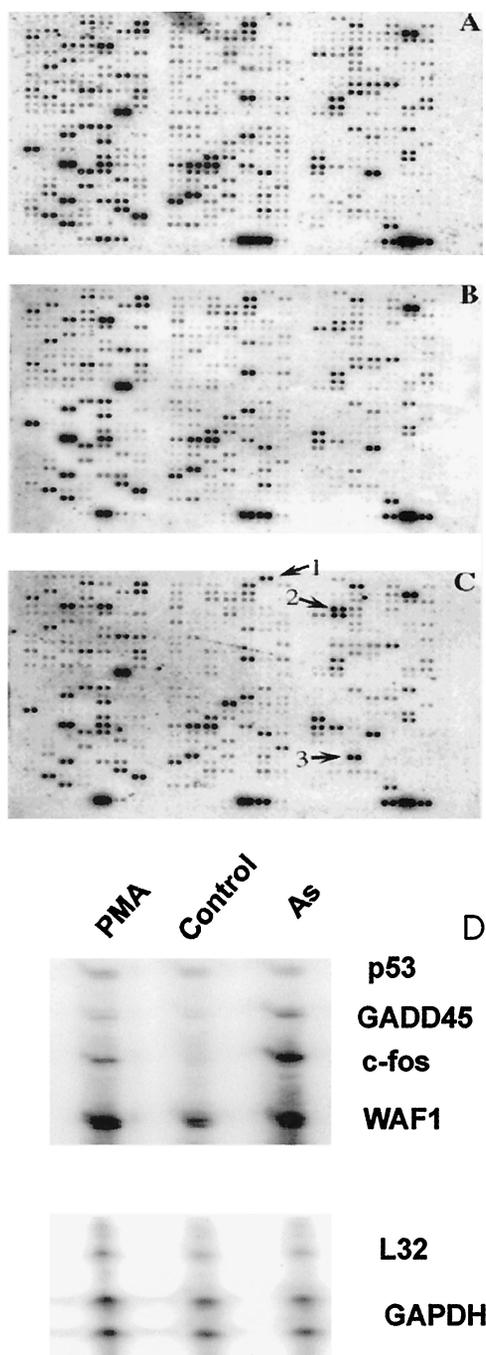


Fig. 8. Gene expression in arsenite-treated UROtsa cells as analyzed by cDNA microarray. Cells were cultured in the presence of vehicle (A), 10 μM sodium arsenite (B), or 50 μM sodium arsenite (C) for 2 h. ^{32}P -Labeled cDNA probes generated from poly(A)⁺ RNA were hybridized to cDNA microarrays as described in "Materials and Methods." Arrows in C represent *c-fos* (arrow 1), *GADD153* (arrow 2), and *EGR-1* (arrow 3). Relative changes in gene expression were determined by normalizing the hybridization signals to the signals obtained from the housekeeping genes included with the cDNA array. RNase protection assay was conducted on RNA, isolated from UROtsa cells treated with sodium arsenite (50 μM) or PMA (20 ng/ml) for 2 h (D).

DISCUSSION

Limited evidence exists that arsenic is a complete carcinogen, because it fails to initiate neoplastic disease in classical one- or two-stage models (31) and is only mutagenic at doses that are highly cytotoxic (9). This is in contrast to the carcinogenicity of other metals, such as cadmium and chromium, which appear to act as classic genotoxic and mutagenic agents (32). Increasing evidence indicates

that arsenic acts at the level of tumor promotion by modulating cell growth. In this respect, in keratinocytes, arsenic enhances mitogenesis, the expression of nuclear and cell membrane proliferation markers, and the expression of early immediate response genes the products of which are involved in cell growth (11, 14, 16, 17). Urinary bladder, like the skin, represents a major target for arsenic-induced cancers in humans. As evidenced in the present studies, human uroepithelial cells exposed *in vitro* to sodium arsenite respond by enhanced cell growth demonstrated by increased percentage of cells in S phase and DNA synthesis. Consistent with these observations, hyperproliferation is evident within 4 weeks of treatment in the uroepithelium after *in vivo* exposure.

The metabolism of arsenic follows similar pathways in both humans and laboratory animals; in these pathways, pentavalent arsenic is first reduced to the trivalent form, which is subsequently methylated to MMA acid and then to DMA acid (25). The majority of evidence supports that the inorganic forms, particularly trivalent arsenite, are the primary toxic metabolites associated with arsenic (33), although several reports have suggested a role for methylated metabolites. For example, exposure of rats to high doses of DMA for 32 weeks enhanced diethylnitrosamine-induced urinary bladder tumors (34), whereas exposure for 97–104 weeks produced bladder tumors without diethylnitrosamine initiation (35). In this respect, Arnold *et al.* (36) observed urothelial toxicity and hyperplasia in rats administered DMA in the feed at 100 ppm. The present studies demonstrate that, despite the predominance of DMA in the urine, sodium arsenite accumulated in bladder tissue. This would suggest that tissues that have high affinity for sodium arsenite or fail to metabolize it represent the most susceptible targets. Furthermore, in studies with UROtsa cells, sodium arsenite was a more effective inducer of cell growth and AP-1 activation than the methylated forms, although the methylated forms still possess significant activity (Fig. 7). Consistent with these observations, in collaboration with M. Styblo *et al.* of the University of North Carolina,³ it was revealed that both keratinocytes and UROtsa cells are extremely poor methylators of arsenic when compared to other cells types, such as hepatocytes or HeLa cells.

Arsenite has a strong affinity for thiol groups in macromolecules, and it has been proposed that arsenic modulates cellular phosphorylation events either through binding to c-Jun-N-terminal kinase phosphatases, resulting in prolonged c-Jun-N-terminal kinase and p38 activation (11), or through binding to cysteine residues in the EGF receptor, resulting in activation of extracellular signal-related protein kinase (10, 12). Alternatively, arsenic can inhibit glutathione reductase and diminish cellular levels of reduced glutathione (37), resulting in an altered cellular redox state. Oxidative stress has been associated with activation of genes involved in stress and proliferative responses, such as *GADD153* and *c-myc*, respectively (38, 39, 40), probably through activation of oxidant-sensitive transcription factors (41). These events have in common the ability to activate AP-1. Studies involving a variety of cell culture and animal model systems have established the importance of AP-1 activation in preneoplastic-to-neoplastic transformation. In this report, AP-1 is a critical mediator of tumor promotion because it alters gene expression in response to classical tumor promoters such as UV radiation and TPA. Similar to these properties of well-characterized tumor promoters, iAs^{3+} -induced mitogen-activated protein kinase signaling pathways have been related to increased AP-1-DNA binding in several cell systems, including HeLa cells, PC-12 cells, and keratinocytes (10, 11, 17).

The present studies provide evidence that arsenite transactivates AP-1, and this is accompanied by uroepithelial proliferation. This was

³ M. Styblo *et al.*, unpublished data.

demonstrated *in vitro* using a human bladder epithelial cell line, as well as *in vivo* after exposure of normal and TRE-luciferase reporter transgenic mice. Characterization of the arsenic-induced AP-1 DNA binding complex demonstrated that the complex consisted of c-Jun/c-Fos heterodimers; this is a common heterodimer responsible for regulating cell growth (26). Arsenic induction of AP-1 DNA binding activity is accompanied by up-regulation of *c-fos* and *c-jun* nuclear proteins in bladder epithelial cells. Of particular relevance to the present studies is a report that *c-jun* expression is as a concomitant factor associated with urinary bladder transitional carcinomas (27, 28). Because arsenic has been linked mainly to development of typical transitional carcinomas, without any unique clinical or histopathological features (42), it can be expected that genes induced by arsenic are typical of molecules involved in bladder carcinogenesis.

The results obtained from the present studies prompted us to examine for changes in specific gene expression using cDNA microarrays, on the assumption that arsenic would alter the expression of genes that regulate cell growth and arrest. We elected to examine UROtsa cells treated for 2 h with 10 or 50 μM iAs^{3+} . Gene expression is a dynamic process, and the profile presented would be influenced by the specific culture conditions, time points examined, and concentration of the test chemical. Nonetheless, it can be assumed a general profile of gene expression might emerge that would provide some insights into the mechanism(s) by which arsenic exerts its effects. The cDNA microarrays revealed consistent activation of 16 genes by the higher concentration of arsenic, 7 of which were also induced by the lower concentration tested. Furthermore, decreased expression was observed in six genes at both the high and low concentrations. In addition to previously reported early immediate genes modulated by arsenic, including *c-fos* and *c-myc* (14, 17, 38), the cDNA microarray demonstrated a strong induction of the *EGR-1* gene. *EGR-1*, which encodes zinc finger DNA binding transcription factors, has been related to cell proliferation induced by mitogens such as EGF, nerve growth factor, or serum (43). Recently, overexpression of *EGR-1* has been associated with human prostate cancer and correlated with the pathomorphological stage of malignancy (44). Functional *EGR-1* binding sites are found in the promoter domains of a large number of genes involved in cell growth, including transforming growth factor α , insulin growth factor II, *c-myc*, thymidine kinase, and cyclin D (see Ref. 44 and references therein). We also observed that arsenic, in contrast to PMA, induced genes implicated in response to cellular stress and growth arrest, such as *GADD153* and *GADD45*. Activation of these genes is an integral part of endoplasmic reticulum stress and is associated with the activation of CAAT/enhancer binding protein and the modulation of pathways leading to cell death or cell regeneration (39). Arsenic also altered genes that encode antiapoptotic proteins, such as BCL-2 binding protein and BAG-1, repair associated protein, and proteins involved in cytoskeleton reorganization. Although the precise role of these genes in arsenic-induced malignancies, if any, needs to be defined, the present studies suggest that arsenic, through AP-1, activates genes of which the products are involved in neoplastic cell proliferation and arrest. It is hypothesized that these events serve as a prerequisite for arsenic-induced carcinogenesis by increasing the growth rate of initiated or mutated cells.

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Arsenic Mediates Cell Proliferation and Gene Expression in the Bladder Epithelium: Association with Activating Protein-1 Transactivation

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