

Expression cloning for arsenite-resistance resulted in isolation of tumor-suppressor *fau* cDNA: possible involvement of the ubiquitin system in arsenic carcinogenesis

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Arsenic is a human carcinogen whose mechanism of action is unknown. Previously, this laboratory demonstrated that arsenite acts as a comutagen by interfering with DNA repair, although a specific DNA repair enzyme sensitive to arsenite has not been identified. A number of stable arsenite-sensitive and arsenite-resistant sublines of Chinese hamster V79 cells have now been isolated. In order to gain understanding of possible targets for arsenite's action, one arsenite-resistant subline, As/R28A, was chosen as a donor for a cDNA expression library. The library from arsenite-induced As/R28A cells was transfected into arsenite-sensitive As/S5 cells, and transfectants were selected for arsenite-resistance. Two cDNAs, *asr1* and *asr2*, which confer arsenite resistance to arsenite-hypersensitive As/S5 cells as well as to wild-type cells, were isolated. *asr1* shows almost complete homology with the rat *fau* gene, a tumor suppressor gene which contains a ubiquitin-like region fused to S30 ribosomal protein. Arsenite was previously shown to inhibit ubiquitin-dependent proteolysis. These results suggest that the tumor suppressor *fau* gene product or some other aspect of the ubiquitin system may be a target for arsenic toxicity and that disruption of the ubiquitin system may contribute to the genotoxicity and carcinogenicity of arsenite.

Introduction

Arsenic, a common environmental toxicant, is a well documented human carcinogen which, paradoxically, does not produce cancers in most animal carcinogenesis bioassays (1–4). Arsenic compounds do not induce significant gene mutations (5), but arsenite (the most likely carcinogenic form) can act as a comutagen by inhibiting DNA repair (6,7) and can induce sister chromatid exchanges (8,9), gene amplification (10,11), aneuploidy (12), and chromosome aberrations (9,13). Since the mechanism of arsenic's carcinogenicity is not well understood, it was thought that identifying genes overexpressed in arsenite-resistant cells might lead to insight into the cellular targets of arsenite. Previously we reported the isolation and partial characterization of arsenite-resistant and -hypersensitive sublines of Chinese hamster V79 cells (14,15). We also reported that arsenite tolerance was inducible by low concentrations of arsenite or antimonite in V79 cells and in As/R28A cells, an arsenite-resistant subline (14), but not in arsenite-hypersensit-

Abbreviation: FBR-MuSV, Finkel-Biskis-Reilly murine sarcoma virus.

ive V79 cell variants or in any human cells (16). We have also demonstrated that the arsenite resistant phenotype is dominant to arsenite sensitivity in cell fusion experiments (14). This made it feasible to clone genes responsible for arsenite-resistance by expression cloning of cDNA from arsenite-induced As/R28A cells transfected into arsenite-sensitive As/S5 cells, selecting for increased arsenite resistance. We report here the isolation of cDNAs isolated by this method, and the identification of one of these genes as the tumor suppressor *fau*.

Materials and methods

Cell culture, treatments and cytotoxicity assay

Chinese hamster V79 cells were obtained from American Type Culture Collection (Rockville, MD). The arsenite-resistant cell line As/R28A was established in this laboratory previously (14). Both V79 cells and As/R28A cells were maintained in F12 medium containing 5% fetal calf serum, 100 µg/ml penicillin and 100 µg/ml streptomycin, without arsenite, as a monolayer culture. Cytotoxicity was determined by colony formation. Exponentially growing cells were trypsinized, counted, and replated at 500 cells/60 mm dish. Arsenite was added to the medium immediately after seeding, and remained in the medium throughout the incubation. The cells were incubated for 10 days without changing medium, fixed with methanol and stained with Giemsa. The number of colonies is scored and survival is defined as the ratio of the colony number in the treated group to that in the control group.

Expression cDNA library

A mammalian expression cDNA library from arsenite-induced (15 µM for 8 h) arsenite-resistant cell line As/R28A was established using mammalian expression vector pCEP4-VP (Figure 1). This vector can be maintained in the cells extrachromosomally after transfection so that episomal plasmids can be rescued. Mammalian expression vector pCEP4 was obtained from Invitrogen (San Diego, CA) and a *NotI* site was created at the *KpnI* site by first destroying the *KpnI* by restriction with *KpnI* followed by modification with Klenow fragment to generate a blunt end. A *NotI* linker was ligated to the blunt end and the linear plasmid was recircularized after *NotI* restriction. The 6 bp *NheI/HindIII* fragment of pCEP4 was replaced with a 200 bp *NheI/HindIII* fragment from pBR322 for future efficient restriction by *NotI* and easy agarose gel electrophoresis to confirm complete digestion during vector-primer preparation. To obtain a *KpnI* site, the 17 bp *XhoI/HindIII* fragment of pCEP4 was replaced with the 51 bp *XhoI/HindIII* fragment from pUC19 in which the *EcoRI* had been replaced with *XhoI* beforehand. After reconstruction, three essential restriction sites for vector-primer cDNA synthesis are aligned in the order of *KpnI*, *HindIII*, and *NotI* with *NotI* next to the promoter region.

Supercoiled plasmid DNA was isolated and purified by acidic phenol extraction (17). Total cellular RNA was isolated by the method of Chomczynski and Sacchi (18). The mRNA was prepared using a conventional oligo-dT cellulose column (Sigma, St Louis, MO). High efficiency cloning and full length cDNA were accomplished by a vector-primer cDNA synthesis approach. Purified pCEP4-VP was linearized with *KpnI* and subjected to dT tailing with terminal deoxynucleotidyl transferase and dTTP. The dT-tailed plasmid DNA was digested with *HindIII* and the plasmid DNA was purified by Sephacryl S-400 column to remove small DNA fragments. Now the vector-primer had oligo-dT at one end and a *HindIII* site at the other end, and was ready for cDNA synthesis. One microgram of prepared pCEP4-VP vector primer was annealed to 10 µg of mRNA from As/R28A. The first strand of cDNA is synthesized with Superscript M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD). The second strand of cDNA is synthesized by nick-translation with *Escherichia coli* polymerase I and RNase H. The nicks are sealed by *E.coli* ligase. The double stranded DNA is modified with T4 polymerase to generate blunt ends and ligated to *NotI* linker with T4 DNA ligase. The vector-cDNA was digested with *NotI* to generate sticky ends.

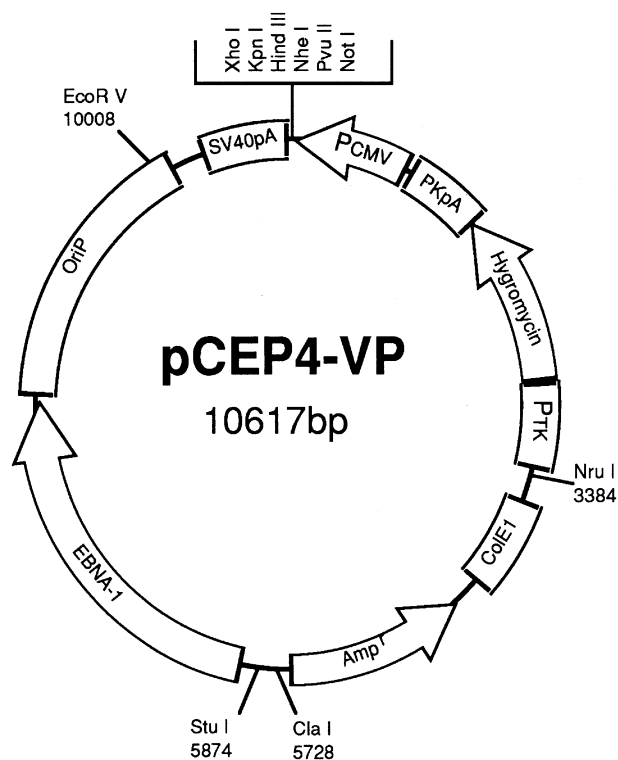


Fig. 1. pCEP4-VP, an expression vector suitable for vector-primer cDNA synthesis.

After fractionation by Sephacryl S-400 spin column, the fragments <271 bp are eliminated. The vector-cDNA was self-recircularized with T4 DNA ligase and ready for transformation.

To prepare competent cells, *E.coli* strain Top10 (Invitrogen) were grown in 1 l LB broth to an OD₅₅₀ of 0.6, chilled on ice, washed twice with water, and twice with 10% glycerol at 0°C, and then resuspended in a total volume of 2 ml 10% glycerol and frozen in small aliquots. Competent cells are thawed on ice and mixed with DNA just prior to use. An electroporator (Invitrogen) was used with the setting: 50 µF for capacitance, 150 W for loading resistance, 1500 V for voltage. Immediately following the pulse, 1 ml of SOC medium (19) at room temperature is added to the cuvette and mixed well. The cells were transferred to a sterile tube and shaken at 37°C for 1 h before spreading. The total independent transformants for the cDNA library was about 1.5 million.

Transfection and selection of arsenite-resistant clones

Plasmid DNA, isolated by acidic phenol extraction (17), was used to transfect As/S5 cells by the calcium phosphate method (19). The medium is changed and the culture is incubated for 24 h, trypsinized and replated at 0.5×10^6 cells/100 mm dish in medium containing 200 U/ml hygromycin B. The selective medium is changed twice during 10 days' incubation. The total number of independent transformants was about 1 million. The hygromycin B-resistant transformants were trypsinized and replated at 5×10^5 cells/100 mm dish and then challenged with various concentrations of sodium arsenite for 7 days. After 7 days, the colonies growing in 10 µM (~30 colonies), 9 µM (~200 colonies) and 8 µM (~1000 colonies) arsenite were pooled and expanded, after which the episomal plasmids were rescued as follows: 10^7 cells were trypsinized, washed twice with PBS, and centrifuged. The cell pellets were resuspended in 100 µl of 50 mM Tris-HCl (pH 8.0) and 10 mM of EDTA. Two hundred microliters of alkaline lysis buffer (0.2 N NaOH, 1% SDS) was added and mixed. After incubation on ice for 10 min, 120 µl of saturated ammonium acetate was added and immediately mixed by vortex. After 15 min centrifugation at 12 000 g, the DNA in the supernatant was precipitated with ethanol, and redissolved in TE.

The episomal plasmids were introduced by electroporation into *E.coli* to form a secondary cDNA library. This library was then reintroduced into the arsenite-sensitive cells using the calcium phosphate method (19). Arsenite-resistant colonies were selected in 10 mM arsenite. Eight individual colonies were expanded to mass culture and episomal plasmid DNA was rescued as described above. These plasmids were introduced into the arsenite-sensitive and wild-type V79 cells to verify that they confer an arsenite resistant phenotype.

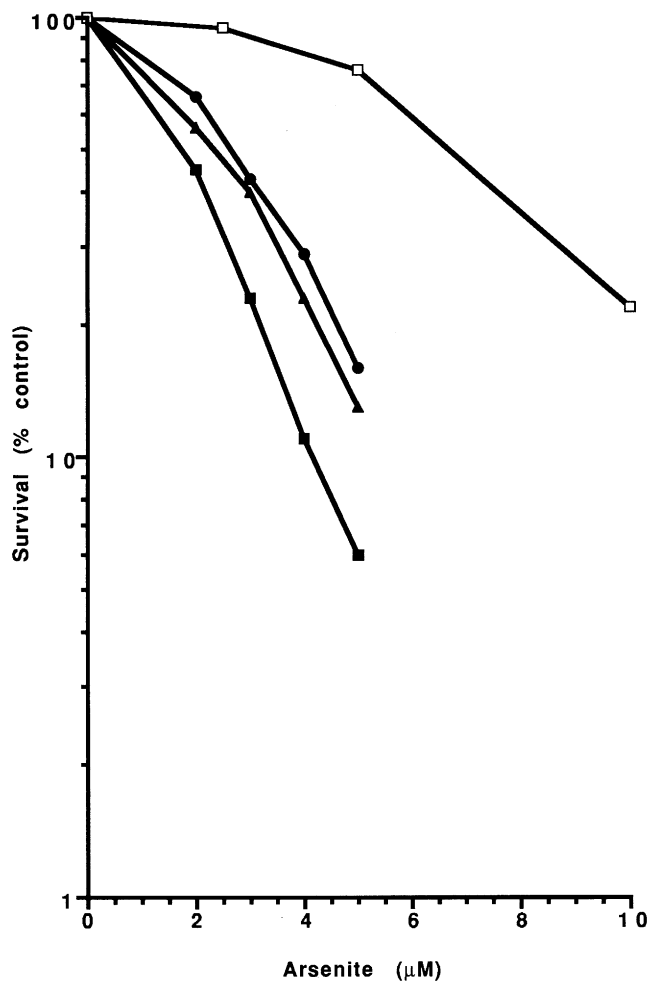


Fig. 2. Sensitivity of As/S5 transfectants and V79 cells to arsenite. ■, As/S5 transfected with vector alone; ▲, As/S5 transfected with vector containing *asr1* cDNA; ●, As/S5 transfected with vector containing *asr2* cDNA; ◻, wild-type V79 cells.

DNA sequencing and data base search

A conventional enzymatic dideoxy DNA sequencing approach was used (20). Double stranded plasmid was used for DNA sequencing. The entire process was carried out according to the protocols for Sequenase 2.0 provided by USB (Cleveland, OH). Open reading frames were used for computer GenBank/EMBL and Swiss Prot. data base search using FASTA command in GCG software package (Genetic Computer Group Sequence Analysis Software Package, Version 7.0).

Northern blot analysis

Total RNA (15 mg) was subjected to electrophoresis in a 1.5% agarose gel containing 3.5% formaldehyde. RNA was transferred onto a nylon membrane and the probe (insert from recombinant plasmid) was labeled with biotin by nick translation. The mRNA was detected using the PhotoGene Nucleic Acid Detection System (BRL).

Results

Selection of cDNAs conferring arsenite resistance

A cDNA library was created from arsenite-induced arsenite-resistant cell line As/R28A, using vector-primed cDNA synthesis on plasmid pCEP4-VP (Figure 1). Five cDNA-containing plasmids rescued from the arsenite-resistant transfectants were capable of increasing arsenite resistance of wild-type and hypersensitive As/S5 cells consistently. Restriction analysis showed that two of these, named *asr1* and *asr2*, contained single inserts of ~0.5 kb and 1.11 kb, respectively.

Figure 2 shows the increased arsenite-resistance when

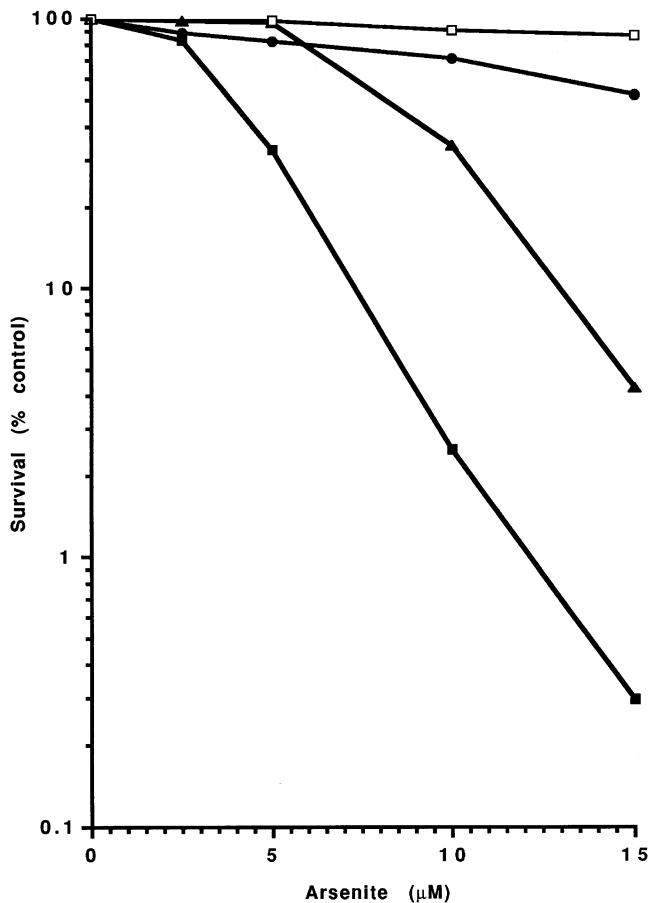


Fig. 3. Sensitivity of G12 transfectants and As/R28A cells to arsenite. ■, G12 transfectant with vector alone; ▲, G12 transfectant with vector containing *asr1* cDNA; ●, G12 transfectant with vector containing *asr2* cDNA; □, As/R28A cells.

plasmids containing these cDNAs are reintroduced into the arsenite-sensitive line As/S5A. Some protection is seen, but the cells are still more sensitive than wild-type V79, the parental cell line. An even higher level of protection from arsenite is seen if the recombinant plasmids are introduced into G12 cells, a derivative of V79 (21) (Figure 3), suggesting that the cloned sequences are not merely correcting a defect in the hypersensitive line. Here too, the transfectants are not as resistant to arsenite as the arsenite-resistant As/R28A cells from which the two cDNAs were isolated. Vector alone did not show any increase in resistance over control (untransfected) cells in either case (data not shown).

Characteristics of *asr1* and *asr2*

After sequencing, searches of the EMBL/GenBank data base revealed that *asr1* (accession no. U41499) has a 91.1% identity in a 459 base overlap with the rat *fau* gene (Figure 4). This gene is the cellular homologue of the *fox* sequence in Finkel-Biskis-Reilly murine sarcoma virus genome (FBR-MuSV) (22). Figure 5 shows the alignment of the protein sequences of Asr1 and the rat Fau protein. These proteins differ in only two out of 133 amino acids. At position 9, rat Fau has a glutamic acid whereas Asr1 contains glycine. At position 35, rat Fau has an alanine and Asr1 substitutes serine. No homologous DNA or protein sequences were found in the data base for *asr2* (accession no. U41500).

RT-PCR amplification of the *fau* mRNA in wild-type V79 cells was carried out. Sequencing of this cDNA revealed

a sequence identical to that cloned from As/R28A cells (Figure 4).

Expression of *asr1* in Chinese hamster cells

Northern analysis of *asr1* mRNA was carried out in uninduced and arsenite-induced wild-type and As/R28A cells. Uninduced As/R28A cells appear to contain slightly less message compared with wild-type cells (data not shown). When cells were induced for 16 h with a subtoxic concentration of arsenite, the level of *fau* message did not change. This treatment with arsenite had previously been found to induce tolerance to higher concentrations of arsenite in these cells (14).

Discussion

asr1 (*fau*) and *asr2*, isolated as a result of expression cloning of arsenite-resistance genes, are able to confer arsenite resistance in both their original host (As/S5) as well as in wild-type cells. Although a consistent increase in arsenite-resistance is always observed after reintroduction of these two cDNAs into cells, the increased arsenite resistance does not reach the level of resistance seen in the donor line As/R28A (14). As/R28A was selected for arsenite-resistance in multiple steps; thus, more than one genetic change might have taken place to confer high level arsenite resistance to this cell line. The *asr1* sequence was almost a perfect match to the rat *fau* gene (Figure 4). Analysis of the promoter region of the human *fau* gene revealed that it is likely to be a housekeeping gene (23). The *fau* gene is constitutively expressed in Chinese hamster V79 cells, as would be expected of a housekeeping gene, and is not up-regulated in the arsenite-resistant As/R28A cells. We were also unable to detect any mutations in the *fau* sequence from As/R28A cells compared with that from the wild-type cells. Thus, altered expression or activity of *fau* cannot account for the arsenite resistance of As/R28A cells.

The *fau* gene encodes a 133 amino acid protein consisting of a ubiquitin-like protein fused to ribosomal protein S30. Cleavage of the *fau* gene product in the cell releases the S30 protein. The *fau* gene is the cellular homologue of the *fox* sequence in the FBR-MuSV, originally isolated from a radiation-induced mouse osteosarcoma (24). The complete mouse *fau* cDNA sequence is inversely inserted as the *fox* sequence in FBR-MuSV. The expression of the *fox* sequence as antisense of the *fau* gene increases the transforming capability of FBR-MuSV, presumably by inactivating *fau* expression (22). The *fau* gene thus functions as a tumor suppressor gene. Fau protein has also been shown to act as an immune suppressor (25), but it is not clear if this activity is related to its tumor suppressor activity.

Proteins are ubiquitinated prior to degradation by an ATP-dependent 26S proteasome complex, the major non-lysosomal proteolytic pathway in eukaryotes (25). Isopeptide bonds are formed between the C-terminal glycine of ubiquitin and ε-amino groups of a lysine residue on the protein to be degraded or on ubiquitins already conjugated to that protein (reviewed in refs 26,27). Like ubiquitin, the ubiquitin-like part of the Fau protein contains a C-terminal glycine and thus might also take part in isopeptide bond formation and proteolysis. Also, like ubiquitin, Fau is conserved in higher eukaryotes and is expressed in all mammalian tissues (22,23). Ubiquitin is usually recycled after targeting of the ubiquitinated protein to the proteasome, an action accomplished by deubiquitinating enzymes. Arsenite has been shown to inhibit this reaction (28). Inhibition of deubiquitination by arsenite may contribute to

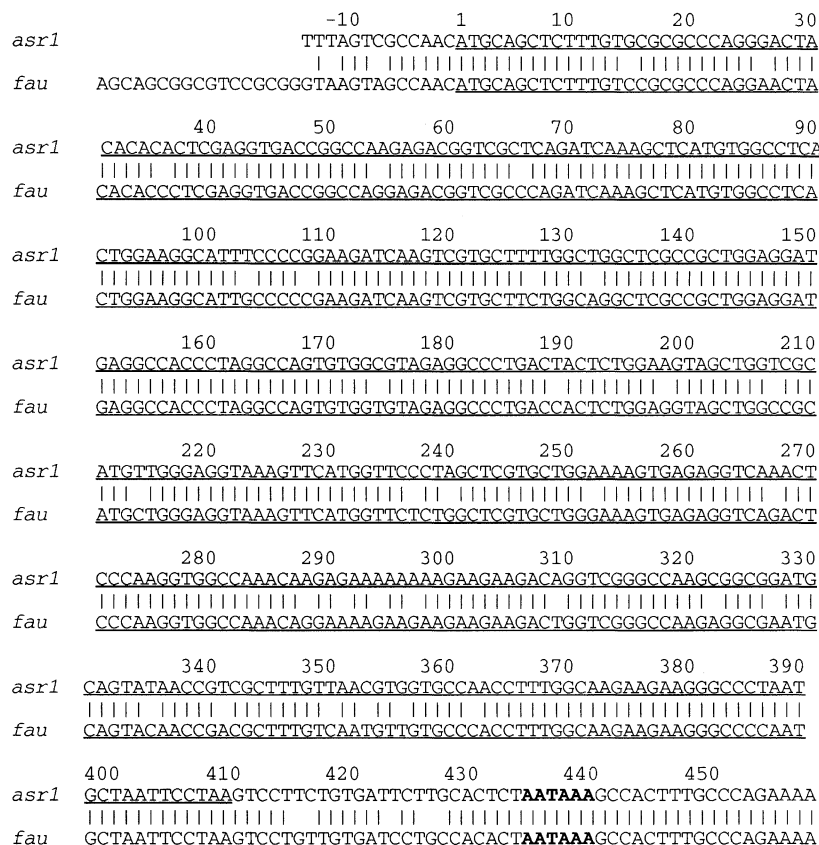


Fig. 4. Alignment of cDNA sequences of *asr1* and rat *fau*. Nucleotide sequences of *asr1* and *R.ratus fau* are compared. Open reading frame is underlined, and numbering starts from the start codon. Identical nucleotides are presented by |. Polyadenylation signal is in bold.

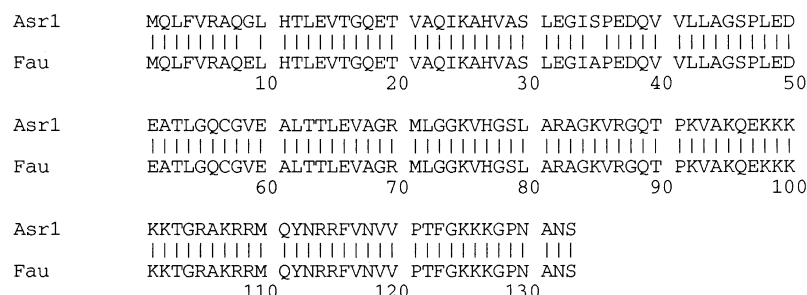


Fig. 5. Alignment of protein sequences of ASR1 protein and rat Fau. The amino acid sequences of rat Fau protein is compared with the translated sequence of ASR1. Identical amino acids are presented by |. The sequences are identical except for amino acids 9 and 35.

its toxicity and carcinogenicity by preventing proteolysis of important proteins and/or by depletion of ubiquitin pools. Overexpression of *fau* may abrogate this inhibition if cleavage of the Fau protein is less susceptible to arsenite inhibition compared with deubiquitination of other proteins.

Ubiquitin is one of the stress proteins (29,30). The signal for induction of stress genes is thought to be abnormal protein structures. Agents which induce abnormal protein structures or which inhibit the degradation of abnormal proteins act as inducers of the stress response (31,32). Arsenite is a classic inducer of the stress response. Besides inhibiting deubiquitination, arsenite is expected to produce abnormal proteins via binding to vicinal thiols (33).

The mammalian ubiquitin system is very complex, entailing >20 E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligating) enzymes as well as numerous de-ubiquitinating enzymes (34). It is believed that different combinations of these proteins

define a high substrate specificity and play an important role in cellular regulation. Ubiquitination can also function independently of proteasome action by acting as a post-translational modifier of proteins (35). Ubiquitination can alter subcellular localization, enzymatic activity, protein-protein interactions, and organelle biogenesis (36-39). For example, the activation of NF κ B1 involves the ubiquitin system at three different steps (40). Other transcription factors are also controlled by ubiquitination. UV-induced stabilization of p53 protein is mediated by loss of ubiquitin (41), as is activation of *c-jun* after phosphorylation by mitogen-activated protein kinase (42).

There are a number of ways that the ubiquitin system has been implicated in growth control and carcinogenesis. The cyclin-dependent kinase inhibitor p27, which serves as a brake to cell division, is often deficient in tumor cells, where it is ubiquitinated and degraded, resulting in uncontrolled cell

division (43). The proto-oncogene *c-jun*, which encodes a transcription factor of the AP-1 family, has a half life of only 60–90 min, mediated by ubiquitin-dependent proteolysis. One highly transforming mutant form cannot be ubiquitinated, resulting in a longer half life. A similar story exists for the proto-oncogene *v-mos* (see ref. 27 and references therein). Cdc34, which is required for G₁ to S progression, has been identified as ubc3, an E2 enzyme (44). Lack of a deubiquitinating enzyme in a *doa4* null mutant of yeast causes defects in growth and DNA repair (45). The human oncogene *tre-2*, a homologue of *doa-4*, is an inactive form of a deubiquitinating enzyme. Wild-type Tre-2 protein normally limits the degradation of negative regulators of growth, such as p53, whereas the inactive (oncogenic) form is thought to interfere in a dominant negative fashion (45). Thus, blockage of degradation (i.e. stabilization) of p53, as well as its increased degradation, for example by human papillomavirus encoded E6 protein (46), may have oncogenic consequences. Arsenite causes increased abundance of p53 protein (47), a finding consistent with its inhibition of proteasome-dependent proteolysis. Abnormalities in p53 function will cause abnormal control of cell cycle progression, apoptosis and DNA repair (48–50). Many of the genotoxic effects of arsenite (reviewed in ref. 51) resemble those caused by abnormal p53 function.

Evidence has been accumulating that the ubiquitin system plays an important role in DNA repair. The *Saccharomyces cerevisiae rad6* mutant, which is defective in an E2 enzyme (52,53), is deficient in DNA repair. Rad6 protein acts in concert with Rad18 to bind to damaged DNA (54). It is speculated that Rad6 is needed to activate (via ubiquitination) repair enzymes or alternatively to catalyze the degradation of chromosomal proteins to make DNA lesions more accessible to repair enzymes. The human homolog to *rad6* has been isolated (55). Mouse cells expressing a temperature-sensitive E1, incubated at the non-permissive temperature, become UV-sensitive (56), suggesting interference with nucleotide excision repair. Ubiquitination of the large subunit of RNA polymerase II plays a role in transcription-coupled excision repair (57). The *S.cerevisiae* Rad23, a ubiquitin-like fusion protein, is involved in UV excision repair as well as spindle body duplication (35). The product of a human DNA repair gene involved in xeroderma pigmentosum group C is homologous to Rad23, and has ubiquitin-like domains at the N-terminus which is essential for DNA repair (58).

Arsenite treatment results in inhibition of DNA excision repair (6,59–62). So far, however, it has not been possible to identify any DNA repair enzyme which is sensitive to arsenite at doses comparable to those which inhibit DNA repair in cells (7,62,63). Thus, the effects of arsenite on DNA excision repair appear to be indirect and may be mediated by effects on cellular control of DNA repair processes, such as the ubiquitin system.

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