Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 590-595

METALLOTHIONEIN-II AND FERRITIN H mRNA LEVELS ARE INCREASED IN ARSENITE-EXPOSED HeLa CELLS

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Received October 11, 1994

Summary: Arsenite is extremely toxic and, though non-mutagenic, is a carcinogen. To determine the effects of arsenite on changes in cell physiology, we searched for genes in HeLa cells whose mRNAs are more abundant after cellular exposure to arsenite. A cDNA subtraction was performed between cDNA synthesized from HeLa cells grown in the absence and presence of 5 μM sodium arsenite. Isolation and sequencing of three clones that showed a higher hybridization signal to RNA from arsenite-exposed cells, versus unexposed cells, revealed that two of the cDNAs coded for human ferritin H chain and the other coded for metallothionein-II. These results suggest the possibility that arsenite exposure may lead to increased levels of oxygen radicals, which augmented metallothionein and ferritin can act to detoxify.

Arsenic is extremely toxic and has no known nutritional value. Epidemiological studies have linked arsenic exposure to cancer in humans (1). Although arsenite is non-mutagenic using bacterial and mammalian tester strains (2), it potentiates the mutagenicity of UV and alkylating agents (3). In addition, arsenite exposure induces sister chromatid exchanges and chromatid aberrations in human peripheral lymphocytes and fibroblasts (2,4), as well as augments X-Ray- and UV-induced chromosomal damage (4). Arsenite ion interacts with the sulfhydryl groups of amino acids, thereby inhibiting thiol-dependent enzymes (4,5). It has also been suggested that arsenite ion may inhibit DNA repair via inactivation of DNA ligase (3,4), consistent with its non-mutagenic but genotoxic properties. Arsenite ion exposure was found to lead to DNA-protein cross-links and DNA strand breaks (5), suggesting additional mechanisms of DNA damage and carcinogenicity for this metalloid.

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Abbreviations: DMEM, Dulbelcco's Modified Eagle's Medium; dNTP, dinucleotide triphosphate; IPTG; isopropyl-β-D-thiogalactoside; kb; kilobase pairs; MOPS, 3-[N-morpholino]propane-sulfonic acid; MT, metallothionein; Xgal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside.

0006-291X/94 $5.00

Eukaryotic cellular defense systems to toxic metal exposure include the modulation of gene expression. Arsenite ion exposure induces the synthesis of some heat shock proteins (6). Heme oxygenase was also shown to be induced by arsenite exposure in human cells (6,7). Heme oxygenase catalyzes the oxidative degradation of protoheme to biliverdin and other products, which are effective peroxide radical scavengers. Hence, it was suggested that heme oxygenase acts to protect against oxidative damage (7), caused directly or indirectly by arsenite. When rats were fed arsenite, hepatic MT was augmented (8). In rats and mice, DNA damage in the lung caused by dimethylarsinic acid, a major metabolite of inorganic arsenicals, was due to production of dimethylarsenic peroxyl radical and other active oxygen species (9). In addition, arsenite stimulates the synthesis of a 31-kDa nuclear membrane protein in an SV40-transformed Balb/c cell line (10).

In order to identify genes in HeLa cells whose expression is induced, or mRNA stability augmented, in the presence of sodium arsenite, a subtraction "library" was created between cDNA made from cells exposed to 5 μM sodium arsenite versus unexposed cells. Described here is the method used to subtract, clone, and screen the cDNAs, and the identification of two genes, whose mRNA is more abundant after a 24 h exposure to arsenite, as MT-II and the ferritin H chain.

MATERIALS AND METHODS

Cell culture. HeLa cells were grown on plates in DMEM (pH 7.0) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Burlington, Canada), 2.5 μg/ml amphotericin B (Fungizone, Squibb, Montreal, Canada), 10 units/ml penicillin and 100 μg/ml streptomycin (Pen-Strep, Gibco-BRL, Burlington, Canada) at 37°C with a 5% CO_2 atmosphere until a cell density of $6.7X10^5$ cells/ml was reached. The cells were then transferred to DMEM (pH adjusted to 5.5 or 7.0 with 12 N HCl) or to DMEM (pH 7.0) containing 5 μM sodium arsenite. After 24 h, the cells were trypsinized and pelleted.

DNA manipulations. Hydrolysis with *Eco*R1, and calf intestinal alkaline phosphatase treatment, was performed as previously described (11). Labelling of DNA with [α-^{32}P]dNTPs (> 3000 Ci/mmol; ICN Biomedicals, Mississauga, Canada) was accomplished with the random prime method using 100 ng of cDNA (12).

RNA isolation, cDNA synthesis and subtraction. RNA was isolated using the RNaid II Kit (Bio 101, Mississauga, Canada). The subtraction method was performed according to Schraml *et al.* (13), except for the modifications described here. The cDNA from unexposed cells was synthesized directly on oligo$(dT)_{25}$ Dynabeads (Dynal Inc., Lake Success, USA) according to Schraml *et al.* (13), except that the cDNA synthesis buffer was modified to contain 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 20 mM dithiothreitol. From 2.16 mg of total cellular RNA, it was estimated that 8.6 μg of single-stranded driver cDNA, bound to the Dynabeads, was synthesized (12). Double-stranded tracer cDNA, from RNA extracted from arsenite-exposed cells, was synthesized using a Time Saver cDNA synthesis kit with the addition of *Eco*R1/*Not*I linkers (Pharmacia Biotech, Montreal, Canada). In a 500 μl screw-cap polypropylene tube, 0.5 μg of denatured, double-stranded tracer cDNA was mixed with 8.6 μg of driver Dynabead-bound cDNA (driver:tracer ratio of 34:1) in hybridization buffer [0.75 M NaCl, 25 mM Hepes (pH to 7.5 with 2 N NaOH), 5 mM EDTA, 0.1% (w/v) sodium dodecyl sulfate] at 68°C for 72 h. The supernatant fluid, containing annealed non-subtracted cDNA (enriched in double stranded induced/augmented cDNAs from exposed cells), was magnetically separated from the Dynabead-bound cDNA and precipitated using 60 mM ammonium acetate, 20 μg glycogen and 70% (v/v) ethanol. After incubation at -20°C for 16 h, the DNA was pelleted by centrifugation at 15000 x g for 30 min at 4°C and resuspended in 10 μl of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

Cloning of subtracted cDNA. Five μl of the subtracted cDNA was ligated to 50 ng of pUC119 (14), previously hydrolyzed with *Eco*R1 and treated with calf intestinal alkaline phosphatase at 15°C for 15 h (12). An aliquot of the ligation reaction was electroporated into 40 μl of

electrocompetent *E. coli* strain DH5αF' (15) according to the manufacturer's protocol (Bio-Rad Laboratories, Mississauga, Canada) using the Bio-Rad Gene Pulser apparatus set at 25 μFarads, 2.5 kV and 200 ohms, and then plated on LB agar (12) containing 40 μg/ml ampicillin, 1 mM IPTG and 40 μg/ml Xgal.

Screening procedure. Total cellular DNA was isolated from 1-cm^2 patches of white clones using the rapid cleared lysate procedure (16) and subjected to electrophoresis through 0.75% agarose gels. The DNA in the gels was then denatured and bidirectionally transferred (17) to Hybond-N membranes (Amersham Ltd., Oakville, Canada). One filter was hybridized (18) with [α-^{32}P]-labelled cDNA synthesized from RNA isolated from unexposed cells. The other filter was hybridized (18) to [α-^{32}P]-labelled cDNA synthesized from RNA isolated from sodium arsenite exposed cells.

Northern blot and slot blot analysis. For northern blotting, 5 μg of RNA was subjected to electrophoresis through 1% denaturing agarose gels (12), except that the RNA was prestained by the addition of 100 μg/ml ethidium bromide to the loading buffer, and the running buffer contained 7.4% (v/v) formaldehyde, 40 mM MOPS-NaOH (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. After electrophoresis, the gel was soaked in 20XSSC [3 M NaCl, 0.3 M sodium citrate (pH 7.0)] for 20 min and the RNA transferred to a Hybond-N membrane for 16 h (12). RNA was treated (12) and applied through a Bio-Rad Bio-dot apparatus onto a Hybond-N membrane. The filters were hybridized (19) to [α-^{32}P]-labelled probes. The probes were isolated after hydrolysis of pUC119 plasmids containing cloned cDNAs with *Eco*R1 and gel purifying the cDNA fragment (12). The actin probe was isolated as a 1.15 kb *Pst*1 DNA fragment from plasmid 91 (20). The probes were removed from the filters by washing twice in 0.5 N NaOH at 37°C.

DNA sequencing. For DNA sequencing, single-stranded phagemid DNA was prepared from the pUC119-based plasmids (14). Dideoxy-sequencing reactions with [α-^{35}S]dATP (500 Ci/mmol; Du Pont Canada Inc., Mississauga, Canada) were performed with a Sequenase kit using the universal primer (U.S. Biochemical Corp., Cleveland, USA).

RESULTS

cDNA subtraction, cloning and screening. In an effort to find genes whose expression, or mRNA stability, is augmented by cellular exposure to sodium arsenite, a cDNA subtraction was performed between cDNAs synthesized from 5 μM arsenite exposed versus unexposed cells. The cDNA from the unexposed cells was synthesized directly on ferric Dynabeads (Fig. 1A) with the subsequent separation of non-subtracted, double-stranded cDNA using a magnet, thus allowing the direct cloning of the remaining non-subtracted, double-stranded cDNAs (Fig. 1B) (13). By comparing the transformation efficiency of cloned cDNAs ligated before subtraction to that after subtraction, it was estimated that an approximate 21-fold enrichment was achieved (not shown). To screen for cloned cDNAs that encoded augmented mRNAs after a 24 h exposure to 5 μM arsenite, 234 non-subtracted cDNAs, cloned into pUC119 (14), were hybridized to total radiolabelled cDNA synthesized from unexposed versus exposed cells (Fig. 1C). Three plasmids that showed a difference in hybridization between the two probes, designated pAG60, pAG61, and pAG62, were further analyzed.

RNA analysis. To quantify the increase in mRNA abundance after arsenite exposure, the individual cDNAs were isolated, purified, radiolabelled, and used to probe northern and dot blots of total RNA from exposed and unexposed cells (Fig. 2). In addition, the blots were stripped of radioactivity and hybridized to an actin probe, used as an internal control. RNA was extracted from unexposed cells grown at pH 5.5 and pH 7.0 to rule out possible pH effects. Densitometric scanning of the blots (11) revealed that plasmids pAG60 and pAG61 contained a cDNA that hybridized to an approximately 0.8-0.9 kb mRNA whose abundance is augmented at least 2-fold

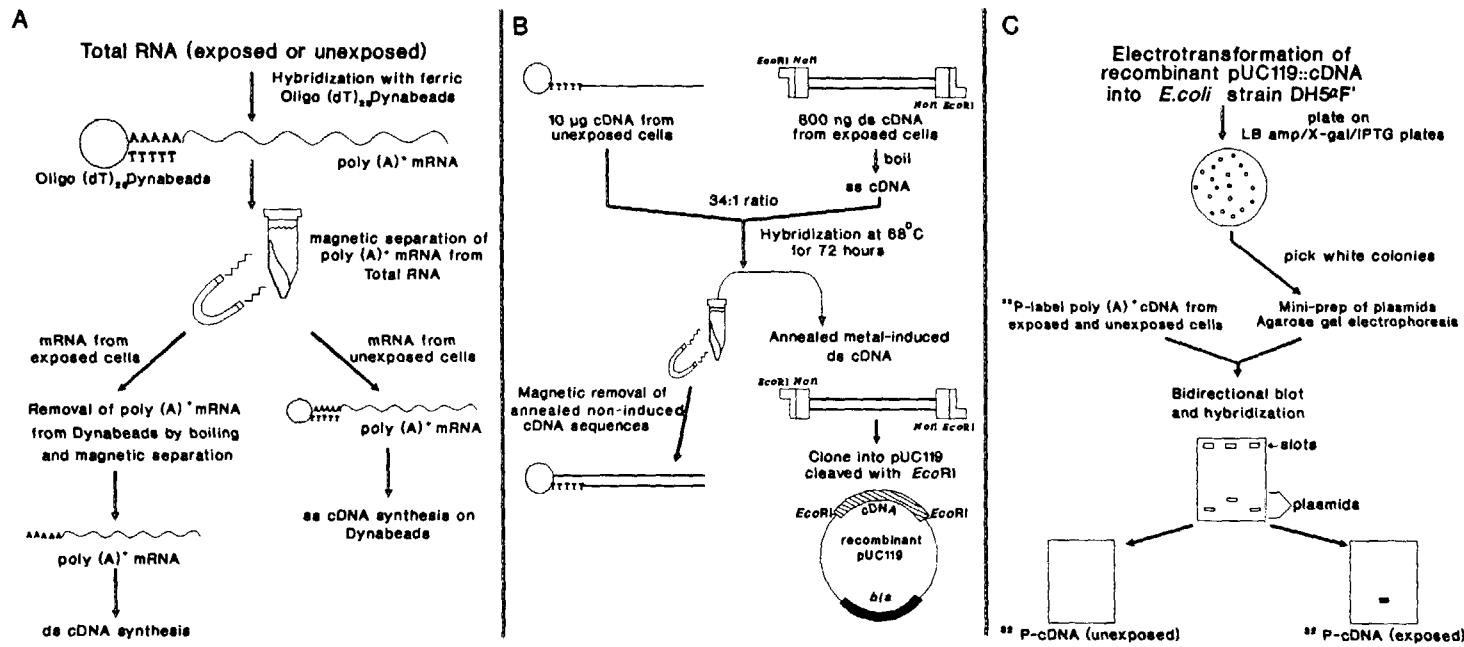

Figure 1. Outline of the method for (**A**) poly(A)$^+$ mRNA isolation, (**B**) subtraction and cloning and (**C**) screening of transformants. For details, see Materials and Methods.

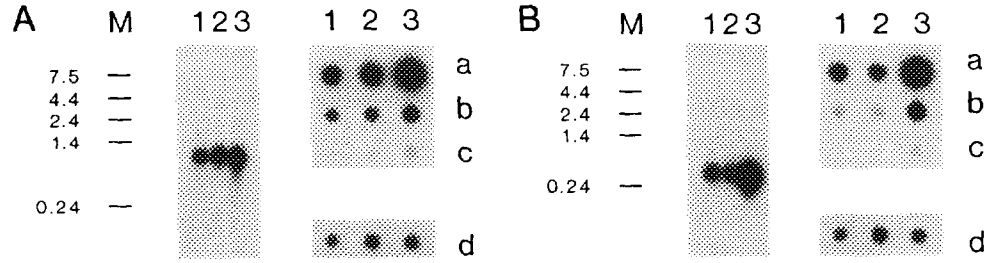

Figure 2. Northern and mRNA dot blots of cloned cDNAs. The cDNAs from plasmids pAG60 (A) or pAG62 (B) were isolated, [α-^{32}P]-labelled and hybridized to 5 µg of total cellular RNA that was subjected to electrophoresis through formaldehyde agarose denaturing gels (left panel), or hybridized to 2.5 (a), 0.25 (b) or 0.025 µg (c) of total cellular RNA on a nylon membrane dot blot (right panel). Also shown is the hybridization of [α-^{32}P]-labelled actin to 2.5 µg (d) of total cellular RNA on a nylon membrane dot blot. The RNA was isolated from HeLa cells grown at pH 5.5 (lane/slot 1), pH 7.0 (lane/slot 2) or at pH 7.0 plus 5 µM arsenite (lane/slot 3). Sizes in kb of an RNA ladder (Gibco-BRL, Burlington, Canada) marker are indicated in lanes M.

in arsenite-exposed cells (Fig. 2A), while plasmid pAG62 hybridized to a 0.3-0.4 kb mRNA whose abundance is augmented approximately 6-fold upon arsenite exposure (Fig. 2B).

Identification of the cDNAs. The three cDNAs were sequenced, and it was found that plasmids pAG60 and pAG61 contained an identical DNA sequence. When the two different cDNA sequences were compared against the DNA sequences present in the GenBank database, plasmids pAG60 and pAG61 were shown to contain the cDNA encoding the human ferritin H chain (21), while plasmid pAG62 contained a cDNA coding for human MT-II (22).

DISCUSSION

In order to find genes induced by the toxic metalloid arsenite, a cDNA subtraction was performed between cDNA from unexposed HeLa cells versus those prepared after a 24 h exposure to 5 µM sodium arsenite. After cloning and analysis of three arsenite-induced cDNAs, we found that human MT-II and ferritin H mRNA levels are elevated approximately 6- and 2-fold, respectively, in HeLa cells exposed for 24 h to 5 µM arsenite *in vitro* (Fig. 2). We do not, as yet, know if this increase is due to an increase in transcription or mRNA stability. MT exists in two distinct isoforms, designated MT-I and MT-II, that bind and sequester heavy metals, thus lowering their availability and toxicity (23). Ferritin is an iron storage protein made up of 24 subunits of H and L chains in varying ratios (21). Arsenite-ingestion was found to induce rat hepatic MT *in vivo* (8). Hepatic MT-I mRNA was increased 1.6-fold, while MT-II mRNA was induced 35-fold at 24 h post-arsenite treatment *in vivo* (8). However, the high level of apparent MT-II mRNA induction was found to be mainly due to its low initial hepatic levels.

MT is also believed to play a role in protection against oxygen radicals (24). Increased production of ferritin in cultured endothelial cells was found to increase the cells' resistance to oxidant-mediated damage (25). Hence, it is possible that MT-II and ferritin H mRNA levels were increased by a common signal, such as oxidative-mediated damage. Arsenite exposure may augment oxidative damage by directly participating in the formation of oxygen radicals (9).

Alternatively, it has been suggested that an arsenite-mediated reduction or modification in the intracellular antioxidant glutathione may be the signal, a result consistent with the observed augmentation of heme oxygenase levels in arsenite-exposed cells (7). Moreover, increased levels of glutathione correlated with arsenite resistance in a chinese hamster ovary cell line (26). Although the mechanism(s) and signal(s) for arsenite-induced increases in MT and ferritin remain to be elucidated, these results add credence to the hypothesis that arsenite toxicity may also be manifested through oxygen radical generation (or stability).

ACKNOWLEDGMENTS

We would like to thank Josée Brisebois and Djenann Saint-Dic for growing HeLa cells. A.G. was supported by a studentship from the Fonds de la Recherche en Santé du Québec (FRSQ) and a Max Binz McGill Graduate Faculty Award. C.K. was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Undergraduate Research Bursary. M.S.D. is a Research Scholar (Exceptional Merit) of the FRSQ. This research was supported by a grant (3406) from the Council for Tobacco Research USA,Inc.

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