Arsenic acts as a toxicant, a carcinogen, and an effective chemotherapeutic agent, but its mechanisms of action are unclear. We have previously shown that treatment of U937 cells with 5 μM sodium arsenite inhibits cell cycle progression through each cell cycle phase, including S phase. Cdc25A dual specificity phosphatase controls entry into and progression through S phase by dephosphorylating sites of inhibitory phosphorylation on cyclin E-cdk2 (Thr14 and Tyr15). Immunoblotting reveals that a 3-h treatment of U937 cells with 5 μM sodium arsenite results in a dramatic decrease in cdc25A protein levels. Immunoprecipitation experiments confirm that cyclin E-cdk2 is more phosphorylated at Thr14 and Tyr15 in the presence of arsenite, and kinase activity assays reveal a decrease in cyclin E–associated cdk2 activity. Therefore, arsenite-dependent cdc25A depletion could contribute to S phase inhibition. There exists an S phase checkpoint known to be mediated by proteasomal cdc25A degradation. However, cycloheximide half-life assay reveals that cdc25A is actually stabilized in arsenite-treated cells. Real-time RT-PCR shows that cdc25A mRNA levels are substantially decreased with arsenite treatment, and actinomycin D half-life assay reveals no change in message stability. Decreased cdc25A message translation is shown by sucrose density gradient polysomal analysis to be an unlikely cause for the profound arsenite-dependent reduction in cdc25A protein levels. Studies are ongoing to establish the mechanism by which 5 μM arsenite decreases cdc25A message abundance, but we surmise that, given the lack of effect on mRNA stability, an inhibition of gene transcription is likely involved.

Key Words: arsenite; S phase inhibition; cell cycle; cdc25A; U937.

Although arsenite is known to be very toxic, it has been an active ingredient in folk remedies for more than 2400 years (Antman, 2001). In the 1970’s, investigators in China confirmed that arsenic trioxide (a form of arsenite) had therapeutic value for the treatment of malignancies, especially acute promyelocytic leukemia (APL) (Antman, 2001; Zhang et al., 2001). It has been suggested that arsenite is effective against this particular malignancy because it is able to induce degradation of the oncogenic fusion protein responsible for leukemogenesis, promyelocytic leukemia protein-retinoic acid receptor alpha (Cai et al., 2000; Chen et al., 1996). Although some cytostatic mechanisms of arsenite may be specific to APL, it is clear that arsenite can suppress growth in other cancer cell models, including the U937 myeloid leukemia cell line (Cai et al., 2000; Chen et al., 1996; McCabe et al., 2000; McCollum et al., 2005; Wang et al., 1998). This suggests more general mechanisms of arsenite action and broadens the applicability of arsenite chemotherapy.

We have previously published data demonstrating that low concentrations of arsenite (≤ 5 μM), corresponding to blood plasma levels in chemotherapy patients, slow cell cycle progression through each cell cycle phase in U937 cells without decreasing cell viability (McCollum et al., 2005; Shen et al., 1997). We were particularly interested in the observation that arsenite not only slowed progression at the traditional cell cycle “checkpoints” at G1 and G2 phases but also throughout S phase. About 50% of U937 cells in an asynchronous culture are in S phase. Given the high proportion of S phase cells, it is likely that an effect of arsenite on S phase progression contributes significantly to overall growth inhibition. For this reason, we became especially interested in potential protein targets of arsenite that play a role in S phase delay.

A well-studied S phase checkpoint is mediated by phosphorylation and proteasomal degradation of the dual specificity phosphatase cdc25A (Busino et al., 2004; Sagata, 2002; Zhao et al., 2002). Active cdc25A removes inhibitory phosphates from cyclin E-cdk2, which allows this kinase to mediate the recruitment of the essential replication factor cdc45 to DNA replication origins (Busino et al., 2004; Donzelli and Draetta, 2003; Sagata, 2002; Zou and Stillman, 1998). When the S phase checkpoint is engaged, cdc25A is rapidly degraded, inhibitory phosphorylation of cyclin E-cdk2 prevents the recruitment of cdc45, and DNA replication is slowed or halted (Busino et al., 2004; Donzelli and Draetta, 2003; Sagata, 2002;
Zhao et al., 2002). Knowing that arsenite treatment slows progression through S phase (McCollum et al., 2005), we speculated that cdc25A may be a target of arsenite. We investigated arsenite’s effect on cdc25A protein levels and the regulatory mechanisms responsible for mediating this effect.

**MATERIALS AND METHODS**

**Reagents.** Fresh stock solutions of sodium m-arsenite (i.e., NaAsO₂) (Sigma Chemical Co., St Louis, MO) (2mM in Hanks’ balanced salt solution) were prepared before every experiment and filter sterilized. The following reagents were also purchased from Sigma Chemical Co: 5-Bromo-2’-deoxyuridine (BrdU), pepsin, bovine serum albumin (BSA), polyoxyethylene-sorbitan monolauroate (Tween 20), propidium iodide (PI), epoxomicin, cycloheximide, actinomycin D, and RNase A. Concentrated HCl was purchased from Fisher Scientific (Fair Lawn, NJ). Purified mouse α-bromodeoxyuridine, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG-specific mAb was purchased from BD Biosciences PharMingen (San Diego, CA). 1X PBS was purchased from Invitrogen Life Technologies (Carlsbad, CA). Glycerol and dithiothreitol (DTT) were purchased from J.T. Baker (Phillipsburg, NJ).

**Cell culture.** U937 cells were obtained from the American Type Culture Collection (Manassas, VA), and maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 1% L-glutamine, and 0.1% gentamycin. RPMI 1640, FBS, glutamine, and gentamicin were obtained from Invitrogen Life Technologies. Cells were maintained in logarithmic growth at a density of 10⁶ cells/ml at 37°C with 5% CO₂. For all experiments, the seeding cell density was 2 x 10⁵ cells/ml.

**Flow cytometric analysis of cell cycle kinetics.** U937 cells (2.0 x 10⁶ cells/10 ml) were pulse labeled by incubation with 10μg BrdU for 15 min at 37°C in a humidified atmosphere with 5% CO₂. They were washed once with 1X PBS and cultured for 0–12 h with or without arsenite. After labeling, they were washed once with 1X PBS and cells were collected with 70% ethanol overnight at 4°C. Fixed cells were washed once with 1X PBS and incubated with 0.2 mg/ml pepsin/2N HCl/1X PBS for 20 min at 37°C. Following two washes with 1X PBS/0.5% BSA/0.5% Tween 20, cells were treated with 1X PBS/2% FBS for 20 min at room temperature. Cells were incubated with 0.5 μg purified mouse α-BrdU/10 μl in the dark overnight at 4°C and washed in 1X PBS/0.5% BSA/0.5% Tween 20. Cells were incubated with 0.5 μg FITC-conjugated rat anti-mouse IgG1 mAb/10 μl in the dark at 4°C and washed in 1X PBS/0.5% BSA/0.5% Tween 20. They were then incubated in 500 μl PBS containing 10 μg/ml PI in the presence of RNase A (100 U/ml final) for at least 30 min at room temperature. PI fluorescence (i.e., DNA content) and FITC fluorescence (BrdU incorporation) were determined by flow cytometry on a FACScalibur (Becton Dickinson). A minimum of 20,000 cells per sample were analyzed. Data were collected and analyzed using CellQuest software.

**Immunoblotting.** U937 cells were cultured for 2-6 h with or without 5μM arsenite and/or 1μM epoxomicin. Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (50 μg protein per lane on a 10% polyacrylamide gel) followed by transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were probed with mouse anti-cdc25A antibody (Santa Cruz #sc-7369) at a 1:1000 dilution in 1X TBS/0.1% Tween 20 (1 h at room temperature). Secondary antibody was HRP-conjugated goat anti-mouse IgG as before. Blots were developed, imaged, and analyzed as described above.

**Coimmunoprecipitation.** U937 cells were cultured for 2-6 h with or without 5μM arsenite. After washing twice with 1X PBS, cells were incubated in lysis buffer (150mM NaCl, 10mM sodium phosphate, pH 7.2, 10% glycerol, 5mM EDTA, 0.2mM Na₃VO₄, 50mM NaF, 1% Halt Protease Inhibitor Cocktail [Pierce #78410] and 1% Nonidet P-40 [NP-40]) on ice for 30 min. Lysates were cleared twice by centrifugation. Five hundred microgram protein was incubated with 20 μl mouse anti-cyclin E (HE111) agarose conjugate (Santa Cruz #sc-248 AC) antibody overnight at 4°C with end-over-end agitation. Beads were washed 4X with wash buffer (same as lysis buffer without the NP-40). Beads were incubated with 50 μl elution buffer (50mM Tris, pH 6.8, 0.005% bromophenol blue, 12.5% glycerol, 1% SDS) at room temperature for 5 min. Supernatants were removed from beads, supplemented with 3 μl 2-mercaptoethanol, boiled for 5 min, and subjected to SDS-PAGE (20 μl per lane on a 10% polyacrylamide gel) followed by transfer to PVDF membranes. Membranes were probed with rabbit anti-phospho-cdk2 thr14/tyr15 (Sigma #C0231) at a 1:800 dilution or with mouse anti-cdk2 (Stressgen #KAM-CC107) at a 1:5000 dilution or HRP-conjugated goat anti-mouse IgG (Santa Cruz #sc-2055) at a 1:2000 dilution in 5% nonfat dry milk in 1X TBS/0.1% Tween 20 (overnight at 4°C). The secondary antibody used was either HRP-conjugated goat anti-rabbit IgG (Santa Cruz #sc-2054) at a 1:5000 dilution or HRP-conjugated goat anti-mouse IgG (Santa Cruz #sc-2055) at a 1:2000 dilution in 5% nonfat dry milk in 1X TBS/0.1% Tween 20 (1 h at room temperature). Blots were developed, imaged, and analyzed as described above.

**Kinase assay.** U937 cells were cultured for 2-6 h with or without 5μM arsenite. Cyclin E-cdk2 complexes were coimmunoprecipitated as outlined above. After beads were washed 4X with wash buffer, they were equilibrated 3X with ice-cold kinase buffer (150mM NaCl, 7.5mM MgCl₂, 0.5mM DTT). Beads were then incubated in 40 μl reaction buffer (25μM ATP, 0.0625 μM/cell [γ-32P]-ATP, and 1 μg/ml histone H1 in kinase buffer) for 15 min at 37°C. Supernatant was separated from the beads, which were incubated with 50 μl elution buffer (50mM Tris, pH 6.8, 0.005% bromophenol blue, 12.5% glycerol, 1% SDS) at room temperature for 5 min. Ten microliter boiling 5X sample buffer (312.5mM Tris, pH 6.8, 0.015% bromophenol blue, 25% glycerol, 10% SDS) was added to the supernatants. Elution buffer was removed from beads, combined with the supernatants, and supplemented with 3 μl 2-mercaptoethanol before boiling for 5 min. Supernatants were then subjected to SDS-PAGE (20 μl per lane on a 10% polyacrylamide gel) followed by transfer to PVDF membranes. Membranes were air dried after transfer and exposed to a Molecular Dynamics Storage Phosphor Screen overnight. Images were collected using a Molecular Dynamics PhosphorImager SI-475 and analyzed with Image Quant 5.2 software. Membranes were then rewetted with 100% methanol, blocked in 5% nonfat dry milk in 1X TBS/0.1% Tween 20 (1 h at room temperature), and probed with mouse anti-cyclin E as described above.

**Cdc25A half-life assay.** U937 cells were cultured for 2 h with or without 5μM arsenite. Cycloheximide (25 μg/ml) was added to the cultures for 0–60 min. After washing twice with 1X PBS, cells were incubated in lysis buffer (150mM NaCl, 10mM sodium phosphate, pH 7.2, 10% glycerol, 5mM EDTA, 0.2mM Na₃VO₄, 50mM NaF, 1% Halt Protease Inhibitor Cocktail [Pierce #78410] and 1% NP-40) on ice for 30 min. Lysates were cleared twice by centrifugation. Five hundred microgram protein was incubated with 5 μg rabbit anti-cdc25A (Bethyl Laboratories, Montgomery, TX #A300-076a) antibody 2 h at 4°C with end-over-end agitation. Protein-antibody complexes were added to 50 μl packed protein-A agarose beads (Calbiochem, San Diego, CA #IP06) and...
incubated for 2 h at 4°C with end-over-end agitation. Beads were washed 4× with wash buffer (same as lysis buffer without the NP-40). Beads were boiled in 50 μl elution buffer (50mM Tris, pH 6.8, 0.005% bromphenol blue, 12.5% glycerol, 1% SDS) for 5 min and subjected to immunoblotting as described above.

RNA isolation. U937 cells were cultured for 2–6 h with or without 5μM arsenite. For mRNA half-life analysis, cells were cultured in the presence of actinomycin D (5 μg/ml) for 0–3 h with or without 5μM arsenite. After washing with 1× PBS, cells were lysed in a solution of 4M guanidine isothiocyanate, 25mM sodium citrate, pH 7.0, and freshly added 0.72% 2-mercaptoethanol. Lysates were extracted with one volume acid phenol-chloroform-isomyl alcohol (25:24:1, pH 6.6). Lysates were incubated with 1/8 volume 10× reaction buffer (20mM MgCl₂, 200mM Tris-HCl, pH 8.3) and 1/8 volume amplification grade DNaseI (Ambion #2224) for 15 min at room temperature. During this incubation, 1/8 volume 2M sodium acetate, pH 4.0, was added. Lysates were extracted with 1.25 volumes acid phenol-chloroform and precipitated with 1.25 volumes 100% isopropanol for 30 min at –20°C. RNA pellets were washed with 70% ethanol and stored in nuclease-free water. RNA integrity was verified by electrophoresis through a 1.2% agarose gel in the presence of 6.7% formaldehyde.

Real-time RT-PCR. The iScript One-Step RT-PCR Kit with SYBR Green (BioRad) was used for real-time RT-PCR analysis. For each 20 μl reaction, 10 ng total RNA (isolated as described above) was mixed with 2× SYBR Green RT-PCR Reaction Mix, 750μM specific primers, and iScript Reverse Transcriptase for One-Step RT-PCR. The reactions were carried out in triplicate, and the samples were analyzed on a Roto-Gene 2000 real-time light cycler from Corbett Research (Phenix Corporation, Hayward, CA). The oligonucleotide primer sequences used in these experiments were human Cdc25A, 5'-GCACCTGGGCTAGTTGAAG-3' and 5'-CATGGGCTTCTCTGGA-TTA-3'; human β-actin, 5'-CTCTTCAGGCTCTTCTCCT-3', and 5'-AGC-ACGTGTTGGGCTACAG-3'.

Polysomal analysis. U937 cells were cultured for 4 h with or without 5μM arsenite. Cycloheximide (0.01 volume 9 mg/ml) in polysomal PBS (125mM NaCl, 10mM Na₂HPO₄, 30mM K₂HPO₄) was added to the cell culture and incubated at 37°C for 10 min. After washing twice with ice-cold polysomal PBS containing 90 μg/ml cycloheximide, supernatants were removed and cell pellets were snap-frozen in dry ice bath and stored at –80°C. Linear sucrose (15–45%) gradients were prepared in 25mM Tris-HCl, pH 7.4/25mM NaCl/5mM MgCl₂ supplemented with 1mM DTT and 300 U/ml RNasin. Cell pellets were thawed in the presence of 1mM DTT and 300 U/ml RNasin in 10mM NaCl/10mM Tris-HCl, pH 7.4/15mM MgCl₂ and lysed by the addition of 1.2% each sodium deoxycholate and Triton X-100. Postnuclear supernatants were mixed 1:1 with polysomal buffer (25mM Tris-HCl, pH 7.4/10mM MgCl₂/25mM NaCl/0.05% Triton X-100/0.14M sucrose) supplemented with 1mM DTT and 300 U/ml RNasin and layered onto sucrose gradients. Gradients were centrifuged in a Beckman SW27 rotor at 27,000 rpm for 225 min at 4°C. Fractions were collected from the bottom down in 3 ml volumes. Ass measurements were recorded to track the distribution of rRNA. Total RNA was isolated from each fraction and analyzed by real-time RT-PCR as described above except that DNase treatment was unnecessary.

RESULTS

S Phase Transit Time Is Markedly Increased by Arsenite

Analysis of U937 cell cycle kinetics using the method described by Begg et al. (1985) and illustrated in Figure 1 reveals that DNA synthesis time is increased in arsenite-treated cultures. Relative movement is a measure of the relative DNA content of cells pulsed with BrdU and harvested at various timepoints for analysis with anti-BrdU and PI. Cells with G1 DNA content are assigned a relative movement of 0, while cells with G2/M DNA content are assigned a relative movement of 1. At the time of the BrdU pulse, the average relative movement of the BrdU-positive (S phase) population is around 0.5 (Fig. 1A, upper panel). As the length of time after the pulse increases, S phase cells move toward G2 phase, increasing their DNA content and their average relative movement approaches 1 (Fig. 1A, lower panel). S phase duration is the amount of time it takes for the average relative movement of the BrdU-positive cells to reach 1. As shown in Figure 1B, the average relative movement of arsenite-treated cultures is significantly slower to reach 1 than that of control cultures, indicating an increase in S phase duration with arsenite treatment. In fact, the average S phase duration of cells in a control culture is 10 h (± 0.7 h), compared to 13 h (± 0.2 h) in an arsenite-treated culture (Fig. 1C).

Arsenate Treatment Decreases the Abundance of cdc25A Protein

Immunoblotting revealed that cdc25A levels sharply decrease in U937 cells after 2 h of arsenite treatment (Fig. 2A). Cdc25A protein has a relatively short half-life, and its abundance is regulated by periodic synthesis and degradation via the ubiquitin-proteasome pathway (Busino et al., 2004; Donzelli et al., 2002). The protein can be stabilized by treatment with epoxomicin, a proteasome inhibitor (Meng et al., 1999). However, epoxomicin is unable to rescue cdc25A from arsenite-induced depletion at 3–6 h. (Fig. 2B).

Arsenate Treatment Increases Inhibitory Phosphorylation of Cyclin E-ecd2k and Decreases its Kinase Activity

Active cdc25A removes inhibitory phosphates from cyclin E-ecd2k (Busino et al., 2004). Arsenite-induced reduction in cdc25A protein levels should correlate with an increase in cyclin E-ecd2k inhibitory phosphorylation. Coimmunoprecipitation of cyclin E-ecd2k followed by immunoblotting with an antibody specific for cdk2 phosphorylated at Thr14 and Tyr15 revealed an increase in inhibitory phosphorylation of the complex upon arsenite treatment (Fig. 3A). As expected, kinase activity of cyclin E-bound cdk2 was decreased in arsenite-treated samples (Fig. 3B).

Arsenate Treatment Does not Induce cdc25A Protein Degradation

Cdc25A levels are rapidly reduced in response to phosphorylation of the protein, which acts as a signal to induce ubiquitination and proteasomal degradation (Zhao et al., 2002). If arsenite induces cdc25A degradation, it should reduce the half-life of the protein. To our surprise, when we measured cdc25A half-life with or without arsenite treatment, we found that it actually increases in an arsenite-dependent manner (Fig. 4).
Arsenite Treatment Decreases cdc25A mRNA Levels

We used real-time RT-PCR to evaluate the effect of arsenite on cdc25A message abundance. Cdc25A mRNA levels were rapidly diminished in response to arsenite treatment, becoming significantly lower than control levels by 3 h (Fig. 5A). This decrease corresponds with the observed decrease in protein levels shown in Figure 2. Arsenite may deplete cdc25A mRNA by decreasing either its transcription or its stability. Stability of the cdc25A mRNA in response to arsenite treatment was measured by calculation of its half-life in the presence of actinomycin D (Figs. 5B and 5C). The results clearly show that arsenite treatment does not significantly destabilize the message.

Arsenite Treatment only Slightly Decreases the Association of cdc25A mRNA with Polysomes

Decreased cdc25A protein levels may also result from decreased translation of the cdc25A message. We used sucrose density gradient centrifugation to separate populations of highly translated mRNAs incorporated into polysomes from populations of under-translated mRNAs (Meyuhas et al., 1996). We found that there was no appreciable difference in polysomal association of cdc25A mRNA between cells treated with 5μM sodium arsenite for 4 h and those that remained untreated (Fig. 6). There appears to be two distinct populations of cdc25A mRNA present in U937 cells. Both are associated with polysomes, so are presumed to be translated, but one population migrates further into a sucrose density gradient than the other. Cdc25A mRNA from arsenite-treated U937 cells displays this same bimodal distribution, but there are more mRNA molecules present in the less-translated population than in the more-translated population, which is not true for mRNA from untreated cells.

DISCUSSION

Arsenite may ultimately be useful in the treatment of a variety of cancers (Waxman and Anderson, 2001), but logical
arsenite treated compared to control according to Student’s paired t-test with a two-tailed distribution.

The cyclin E gene is one of the many genes activated during the G1 to S phase transition (Sherr, 2000). Cyclin E binding to cdk2 is necessary for phosphorylation of substrates important in DNA synthesis and S phase progression. However, cdk2-cyclin E remains inactive until inhibitory phosphates on Thr14 and Tyr15 have been removed by the cdc25A dual specificity phosphatase (Busino et al., 2004). Cdk2-cyclin E activity is associated with recruitment of the replication factor cdc45 and DNA polymerase on replication origins, without which there can be no S phase (Zou and Stillman, 1998). Cdc25A activity is required for G1 to S phase progression, so a G1 phase cell, when exposed to DNA damaging stimuli, may avoid entry into S phase by eliminating cdc25A activity, making this an important G1 checkpoint mechanism (Sherr, 2000). But also, because different replication origins fire at different times throughout S phase, continuous cdc25A activity is critical to the complete replication of the genome, and cells can slow DNA synthesis in the middle of S phase via cdc25A depletion (Busino et al., 2004; Donzelli and Draetta, 2003; Molinari et al., 2000). In this way, DNA repair has time to occur in parallel with DNA replication (Abraham, 2001). Based on the known biology of cdc25A, the observation of decreased cdc25A abundance and activity (Figs. 2 and 3) in the presence of arsenite would allow us to predict an arsenite-induced S phase delay, which is what we have shown in Figure 1 (Busino et al., 2004).

Cdc25A belongs to a family of dual specificity phosphatases (Donzelli and Draetta, 2003). The remaining members of this family, cdc25B and cdc25C, although most important in regulating the G2/M phase transition, have recently been shown to play some role in S phase progression (Boutros et al., 2006). Arsenite reduces the levels of these proteins in several biological contexts: cdc25B by an undefined mechanism (Park et al., 2000) and cdc25C via increased degradation (Chen et al., 2004). In this way, DNA repair has time to occur in parallel with DNA replication (Abraham, 2001). Based on the known biology of cdc25A, the observation of decreased cdc25A abundance and activity (Figs. 2 and 3) in the presence of arsenite would allow us to predict an arsenite-induced S phase delay, which is what we have shown in Figure 1 (Busino et al., 2004).

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However, despite this stabilization, overall cdc25A protein levels decrease in response to arsenite treatment (Fig. 2). The finding that protein half-life is not decreased is intriguing because regulation of cdc25A has, until now, largely been explained by changes in protein stability (Busino et al., 2004; Donzelli and Draetta, 2003). These results indicate that cdc25A levels may also be modulated by changes in message translation and/or abundance.

Regulation of translation initiation occurs via several different mechanisms (Gray and Wickens, 1998). There are cis-acting elements present in the 5′ and/or 3′ untranslated regions (5′-UTR and 3-UTR) of some mRNAs that allow for modulation of translational rate. There also exist RNA-binding proteins which act to suppress translation of certain messages. A recent paper was published documenting an effect of arsenite on cytochrome P450 CYP3A23 message translation (Noreault et al., 2005). The conclusion of these authors was that arsenite inhibits the translation of this protein by altering regulatory protein binding to the 3′-UTR or 5′-UTR of its mRNA. There are also more general, less message-specific means of modulating translation initiation that directly affect the translational machinery (Gray and Wickens, 1998). Arsenite at high concentrations (20–100 μM) is known to reduce 5′ cap–mediated translation as part of the cellular stress response (Brostrom and Brostrom, 1998). However, we found no appreciable change in the overall association of cdc25A mRNA with polysomes in response to 5 μM arsenite treatment (Fig. 6). Interestingly, we did observe two populations of cdc25A mRNA with polysomes in response to 5 μM arsenite treatment (Fig. 6). The biological significance of this splicing event is unclear. The primers used in the experiments presented here were designed to detect both cdc25A mRNA
isoforms, so it is possible that the distinct populations found by polysomal analysis represent alternatively spliced messages with different translational efficiencies. Arsenite treatment of U937 cells does not significantly change the polysomal association of either population compared to control, but it does increase the magnitude of the less-translated population compared to the more-translated population within the arsenite-treated sample. If the two populations represent different cdc25A isoforms, then it is possible that arsenite decreases the abundance of the more-translated variant relative to the less translated. Future studies will seek to understand the significance of the two differentially translated populations of cdc25A mRNA, both to cell cycle regulation in general and to arsenite-induced cell cycle effects. Whatever the reason for the bimodal polysomal distribution of cdc25A mRNA, it is clear that arsenite’s effects on overall message levels are more pronounced (Fig. 5A) than its effects on translation efficiency and are thus more likely to be responsible for reducing cdc25A protein levels (Fig. 2).

The abundance of cdc25A mRNA is determined by its stability and by its transcriptional rate. The possibility that arsenite reduces cdc25A message stability has been ruled out by mRNA half-life measurement in the presence and absence of arsenite (Figs. 5B and 5C). This elimination leaves cdc25A gene transcription as the only remaining possible target of arsenite that could result in the reduction of cdc25A protein levels. Transcriptional rate depends on the ability of the transcription machinery to bind to a gene’s promoter (White, 2001). The accessibility of the promoter varies according to the chromatin state in the vicinity of the gene and the binding of transcription factors to the DNA in and around the gene. The cis and trans regulatory elements governing cdc25A gene transcription have not been fully elucidated. However, it is known that the oncogenic transcription factor c-Myc promotes cdc25A transcription (Galaktionov et al., 1996). We have observed arsenite-induced decreases in c-Myc protein levels during the same time frame as that seen with cdc25A (data not shown). We are currently investigating the possibility that arsenite-induced changes in c-Myc levels cause a decrease in cdc25A gene transcription, protein production and S phase transit.

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REFERENCES


FIG. 5. Arsenite treatment decreases cdc25A message abundance but not its stability. U937 cells were treated with 0–5 μM sodium arsenite for 2 h. (A) Ten nanograms RNA from each sample was subjected to real-time RT-PCR using the primers listed in the “Materials and Methods” section. Fold change in cdc25A mRNA levels is shown over 3 separate experiments. Fold change in actin mRNA levels was used to normalize. Error bars represent SEM. *p < 0.05, arsenite treated compared to control according to Student’s paired t-test with a two-tailed distribution. (B) Actinomycin D (5 μg/ml) was added to the medium to inhibit new RNA synthesis. Cells were harvested at 0–3 h after addition of actinomycin D to monitor cdc25A mRNA degradation by real-time RT-PCR using 10 ng total RNA per sample. The quantity of cdc25A mRNA at each timepoint is shown relative to that at the time of actinomycin D addition. The graph represents a compilation of data from three separate experiments. Error bars represent SEM. R-squared values for control and arsenite regression lines are both > 0.97. (C) Average cdc25A mRNA half-life in control and arsenite-treated cells was calculated from regression lines drawn for each individual experiment.

FIG. 6. Arsenite treatment does not appreciably change translational initiation of cdc25A message. U937 cells were treated with 0–5 μM sodium arsenite for 4 h. Postnuclear supernatants were subjected to sucrose density gradient centrifugation to distinguish populations of variably translated mRNA. Gradient fractions were analyzed by absorbance at 260 nm to determine relative RNA abundance (dotted line). The peak at fraction 4 represents monosomal rRNA, so the solid black vertical line is drawn to divide untranslated RNA (on the left) from translated RNA (on the right). Ten nanograms RNA from each fraction was subjected to real-time RT-PCR using the primers listed in the “Materials and Methods” section. Percent cdc25A mRNA in each fraction is shown in triplicate (solid line is control, dashed line is arsenite treated). Error bars represent SD. Graph is representative of three separate experiments.


