

Zinc depletion reduced Egr-1 and HNF-3 β expression and apolipoprotein A-I promoter activity in Hep G2 cells

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Cui, LiBin, Norberta W. Schoene, Lei Zhu, Jessica C. Fanzo, Ali Alshatwi, and K. Y. Lei. Zinc depletion reduced Egr-1 and HNF-3 β expression and apolipoprotein A-I promoter activity in Hep G2 cells. *Am J Physiol Cell Physiol* 283: C623–C630, 2002. First published April 24, 2002; 10.1152/ajpcell.00308.2001.—We examined the influence of zinc status on expression of certain transcription factors involved in regulation of apolipoprotein A-I (apoAI) expression in human hepatoblastoma Hep G2 cells. A low zinc basal medium (zinc deficient, ZD) consisting of DMEM and 10% Chelex100-treated fetal bovine serum was used to deplete cellular zinc over one passage. Cells were also cultured for one passage in medium supplemented with 0.4 (ZD0.4), 4.0 (zinc normal, ZN), 16.0 (zinc adequate, ZA), or 32.0 μ M zinc (zinc supplemented, ZS). Compared with ZN cells, cellular zinc levels were 43 and 31% lower in ZD and ZD0.4 cells but 70 and 146% higher in ZA and ZS cells, respectively. Supplementa-tion of 0.4 μ M zinc significantly increased DNA contents per plate, from 65% in ZD cells to 83% in ZD0.4 cells compared with ZN cells. Addition of >4 μ M zinc in medium did not further increase DNA contents. The proportion of cells in G₁/S and S phase was about fourfold higher and threefold lower, respectively, in ZD cells compared with ZN and other groups. Nuclear Egr-1 protein was markedly decreased in ZD and ZD0.4 cells. Moreover, hepatocyte nuclear factor (HNF)-3 β was severely degraded in ZD and ZD0.4 cells. In contrast, HNF-4 α remained stable in all groups and was not significantly lower in ZD and ZD0.4 cells. Furthermore, downregulation of *trans*-acting factor Egr-1 and cleavage of HNF-3 β were associated with reduction of apoAI promoter activity in zinc-deficient Hep G2 cells. Thus zinc is critical in transcriptional regulation of apoAI gene expression in hepa-tocytes.

hepatocyte nuclear factor-4; cell cycle; zinc supplementation; G₁/S arrest

APOLIPOPROTEIN A-I (apoAI) is the major protein component of plasma high-density lipoprotein (HDL) particles and has been implicated in the protection against atherosclerosis (27, 33). ApoAI is synthesized mainly in the intestine and liver, and its expression can be modulated by hormones, growth factors, and nutrients, including the micronutrient zinc (29, 36). Zinc is an

essential trace element involved in variety of cell structural and regulatory functions. Several lines of evidence have established a strong correlation between zinc and plasma HDL level. Reduction of HDL has been documented in numerous studies in zinc-deficient animals (1, 15–17) and zinc-deficient humans (12). Moreover, previous studies in our laboratory (36, 37) have demonstrated that alterations in cellular zinc status regulate plasma apoAI protein level and hepatic apoAI expression both in vitro and in vivo. However, the molecular mechanisms responsible for the zinc deficiency-induced reduction in hepatic apoAI expression are unknown.

An increasing number of transcription factors have been found to be involved in the regulation of apoAI gene expression (29). These transcription factors include members of a steroid/thyroid nuclear receptor superfamily, such as hepatocyte nuclear factor (HNF)-4 α (11); apoAI regulatory protein (ARP)-1 (6); RXR α and RAR/RXR heterodimers (26); the HNF-3/forkhead family of transcription factors, such as HNF-3 α and HNF-3 β (10); early growth response factor (Egr-1) (14); and, more recently, transcription factor Sp1 (7, 13, 40, 41). These *trans*-acting factors bind different *cis*-acting elements. Functional analysis of apoAI gene promoter by site-directed mutagenesis and in vitro cotransfection studies identified that three *cis*-acting elements, located between nucleotides –222 and –110 upstream from the transcription start site, are essential and sufficient for liver-specific expression of apoAI gene in Hep G2 cells (34). These *cis*-acting elements, designated site A (–214 to –192), site B (–169 to –146), and site C (–134 to –119), bind different hepatocyte-enriched transcription factors (10, 11). Sites A and C bind HNF-4 α , ARP-1, RXR α , and RAR/RXR heterodimers. Site B binds HNF-3 α and HNF-3 β (10). In addition, two Egr-1-responsive elements flanking site A, denoted as E1 (–189 to –181) and E2 (–221 to –213), are also involved in the regulation of the hepatocyte-specific apoAI gene expression (14). Moreover, the activation of the hepatic expression of apoAI gene

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is controlled by synergistic interaction between these transcription factors (11, 13).

In this study, we examined the influence of cellular zinc status on the expression of several of these transcription factors known as important modulators of the apoAI gene expression in hepatocytes. We found that the expression of certain members of these transcription factors were readily downregulated by zinc depletion in the human hepatoblastoma Hep G2 cells and that the downregulation of Egr-1 and degradation of HNF-3 β may be the underlying mechanism leading to the depressed apoAI gene expression in zinc-deficient liver cells.

MATERIALS AND METHODS

Cell culture and treatment. The human hepatoblastoma cell line Hep G2 was purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from Life Technologies (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 U/ml penicillin, and 20 μ g/ml streptomycin sulfate. Medium was replaced every 2 days, and 6.5 days of culture constitute one passage. Nearly confluent cells at the end of *passage 80* were subcultured at the ratio of 1:8 for the initiation of experimental treatment.

Chelex100 resin (Bio-Rad, Hercules, CA), a divalent ion-chelating resin, was used to remove zinc from FBS before FBS was added to DMEM (25). The resin was first neutralized to physiological pH with 0.25 M HEPES, pH 7.4, and then mixed with FBS at a 1:4 ratio (wt/vol) at 4°C for 2 h. The Chelex100 resin was separated from FBS by centrifugation, followed by filtration through a 0.2- μ m filter for sterilization and removal of residual Chelex100 resin. The zinc concentration in this chelexed FBS was 1.6 μ M, as determined by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA). The DMEM with 10% chelexed FBS, containing <0.2 μ M zinc, was termed the zinc-depleted (ZD) medium. For the other treatments groups, zinc was added to the media in the form of ZnSO₄ so that the only difference between these media was the zinc concentration. The ZD0.4 medium was prepared by addition of 0.4 μ M ZnSO₄ to ZD medium. Previous study has shown that addition of at least 0.4 μ M ZnSO₄ to ZD medium is required to prevent depression of Hep G2 cell growth observed in severe zinc deficiency (37). The zinc-normal (ZN) and the zinc-adequate (ZA) media were prepared by adding 4.0 and 16.0 μ M ZnSO₄ to ZD medium to mimic the zinc level observed in normal culture medium or in human plasma, respectively. The zinc-supplemented (ZS) medium that contained the ZD medium and 32 μ M ZnSO₄ was used to represent plasma zinc levels attainable by oral zinc supplementation in humans. The Hep G2 cells were cultured overnight in ZN medium before being changed to their respective media. Cells were then grown in ZD, ZD0.4, ZN, ZA, or ZS media for 6.5 days (one passage). The cells were harvested for cellular zinc and DNA content, nuclear protein extraction, and cell cycle analysis.

Cellular zinc and DNA content determination. Cells were collected with medium by scraping from tissue culture plates. Cell suspensions were then centrifuged at 500 *g* for 5 min at 4°C, and cell pellets were washed twice with phosphate-buffered saline (PBS). Cells were resuspended into PBS and sonicated on ice. An aliquot of the sonicated cell suspension was used to measure cellular zinc content by flame atomic absorption spectrophotometry (Hitachi) against the standard

curve of 0.05–1.0 ppm. The certified zinc solutions (Fisher Scientific, Springfield, NJ) were compared with Bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. From the same sample, a small aliquot of the cell lysate was used to measure cellular DNA content by using diphenylamine (35). Cellular zinc level was expressed as cellular zinc per microgram of DNA because a linear relationship between cellular DNA and cell number was previously established (35).

Cell cycle analysis. DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS) by using a FACScalibur cytometer (Becton Dickinson, San Jose, CA) as previously described (8). Hep G2 Cells were grown in ZD, ZD0.4, ZN, ZA, and ZS media for one passage, trypsinized, washed in PBS (Ca²⁺, Mg²⁺ free), and fixed in 70% ethanol. Cells were then stained with propidium iodide. Flow cytometry and FACS analysis (FACScanner; Becton Dickinson) were used to quantify the distribution of DNA fluorescence and intensity. Flow cytometric data files were collected and analyzed by using the CELLQuest program (Becton Dickinson). A total of 10,000 cell events were collected for DNA analyses. Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). The calibration standard LinearFlow green and the DNA QC Particle kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

Nuclear extract preparation. Hep G2 cell nuclear extract preparations were prepared based on the method described by Schreiber (28). Cells were washed with cold Tris-buffered saline (TBS), scraped off the dish, and collected by centrifugation at 1,500 *g* for 5 min at 4°C. The cell pellets were suspended in five times the original packed cell volume of *buffer A* containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 mM benzamidinium-HCl. Cells were allowed to swell on ice for 15 min. After the addition of 10% Nonidet P-40 to attain a final concentration of 0.5%, the cell mixture was vigorously mixed for 20 s. The nuclei were collected by centrifugation immediately at 13,000 *g* for 1 min at 4°C. The supernatant was removed, and the nuclear pellets were resuspended in cold *buffer C* containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 1 mM benzamidinium-HCl, and 1 mM PMSF. The nuclear suspension was vigorously mixed at 4°C for 15 min, followed by centrifugation at 13,000 *g* at 4°C for 5 min to collect the nuclear extracts. The nuclear extracts were frozen and stored at -80°C. Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad) with BSA as standard.

Western blot analysis. Nuclear proteins (40 μ g) were separated on a 10% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBS, pH 7.6, and 0.1% Tween 20 and then incubated overnight at 4°C with anti-Egr-1 (sc-189), anti-HNF-3 β (sc-6554), or anti-HNF-4 α (6556) (all from Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed with TBS and 0.1% Tween 20 and then incubated with horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology). Antibody was detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) by following the manufacturer's instructions. Prestained TriChromRanger Marker from Pierce was used as SDS-PAGE standard. Equal loading was monitored by incu-

bating the membrane with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris·HCl, pH 6.7) at 52°C for 60 min and then detected with anti-histone H1-specific antibody (sc-8030; Santa Cruz Biotechnology). Protein bands were quantified by using laser densitometry (Bio-Rad).

Preparation of luciferase construct. The human apoAI genomic DNA used for the preparation of apoAI promoter constructs was kindly provided by Dr. Lawrence Chan (Baylor College of Medicine) (38). A 3-kilobase *HindIII-HindIII* fragment of apoAI promoter from nucleotide -2500 to +397 was isolated and inserted into the *HindIII* site of plasmid pGL3-basic (Promega, Madison, WI) to generate construct -2500AI-LucB. Subsequently, the construct -2500AI-LucB was digested with restriction enzyme *NcoI*, filled in with Klenow DNA polymerase, and then digested with restriction enzyme *HindIII*. The resulting 696-base pair DNA fragment of human apoAI promoter (-298 to +397) was isolated from agarose gel and cloned into the *SmaI-HindIII* sites of plasmid pGL3-Basic, resulting in the construct -298AI-LucB. The construct -256AI-LucB, containing *SmaI-HindIII* fragment (-256 to +397) of the apoAI promoter was created by deleting a *SmaI* fragment from -2500AI-LucB. The -41AI-LucB construct containing apoAI promoter segment -41 to +397 was generated by deleting the *SmaI-PstI* fragment from -256AI-LucB. Plasmid DNA used for transient transfections was prepared by using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA).

Transient transfection and luciferase assay. Hep G2 cells were transfected by using Lipofectamine 2000 reagent according to the protocol provided by the manufacturer (Life Technologies). Hep G2 cells in DMEM with 10% FBS without antibiotics were seeded at a density of 2×10^5 cells/well in 24-well plates the day before transfection. Transfections were performed in triplicate with 800 ng of the luciferase reporter constructs and 25 ng of an internal control plasmid, pRL-SV40 (Promega). Five hours after transfection, the cells were washed with DMEM and the medium was changed to DMEM containing 10% chelexed FBS plus 0, 4.0, 16.0, or 32.0 μ M zinc. The cells were cultured for another 48 h. Luciferase activity was measured in the Luminometer TD-20/20 (Promega) by using the Dual-Luciferase reporter assay system (Promega) according to instructions provided by the manufacturer.

Statistical analysis. Statistical analyses were performed by using SAS 8.1 Windows software release (SAS Institute, Cary, NC). The data were analyzed with one-way ANOVA, and the means were further analyzed by least significant differences. Values were expressed as means \pm SE, with a statistical probability of $P < 0.05$ being considered significant.

RESULTS

Zinc depletion reduced cellular zinc level and DNA content. Hep G2 cells were cultured for one passage (6.5 days) in zinc treatment media, and cellular zinc levels as well as the DNA contents were measured as described in MATERIALS AND METHODS. A significant reduction in cellular zinc level was observed in cells from the zinc depletion treatments (Fig. 1). Compared with ZN cells, cellular zinc levels were significantly lower in ZD and ZD0.4 cells (57.2 ± 5.3 and 68.7 ± 1.8 vs. $100.0 \pm 8.7\%$) but significantly higher in ZA cells ($170.3 \pm 6.9\%$) and ZS cells ($246.4 \pm 12.9\%$). Cellular zinc concentrations were expressed per cellular DNA to

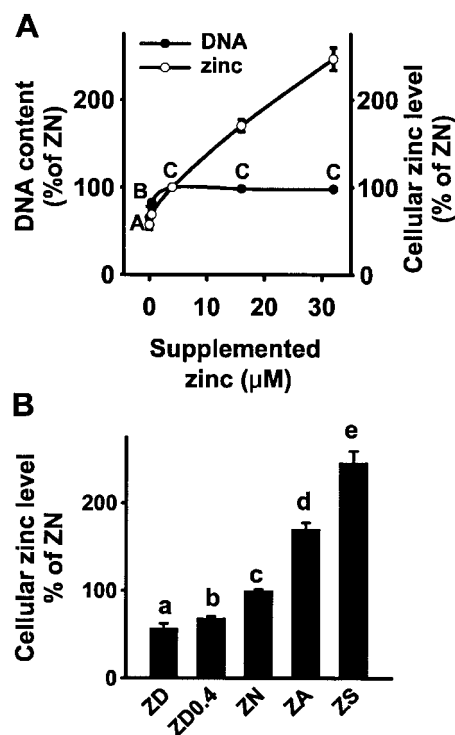


Fig. 1. Cellular zinc levels and DNA content in Hep G2 cells. Cells were cultured in zinc-deficient (ZD; DMEM plus 10% chelexed FBS), ZD0.4 (ZD plus 0.4 μ M zinc), zinc-normal (ZN; ZD plus 4.0 μ M zinc), zinc-adequate (ZA; ZD plus 16.0 μ M zinc), and zinc-supplemented (ZS; ZD plus 32.0 μ M zinc) media for one passage (6.5 days). Cellular zinc was measured by atomic absorption spectrophotometry and DNA content by diphenylamine. Cellular DNA contents per plate as well as zinc concentration per microgram of DNA were expressed as a percentage of ZN controls. Values represent means \pm SE from 4 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); treatments with the same letters indicate no significant difference.

correct for any differences in cell numbers between plates.

In addition to causing changes in the cellular zinc status, the zinc depletion treatments also significantly decreased the DNA value per plate. DNA value per plate for ZD and ZD0.4 cells was 65 and 83% of ZN cells, respectively (Fig. 1). No difference in DNA content was detected among ZN, ZA, and ZS cells (Fig. 1). Because a linear relationship exists between cellular DNA and cell number (35), the reductions in DNA value mean lower cell counts, which suggests that DNA synthesis and cell proliferation may have been affected by zinc status in ZD and ZD0.4 cells.

Zinc depletion blocked the progression of Hep G2 cells from G_1 to S phase. Previous studies have clearly demonstrated that zinc plays important roles in cell proliferation (18). However, little is known about how zinc deficiency affects the cell cycle of the liver cells. Therefore, we studied the cell cycle distribution of Hep G2 cells after one passage of zinc treatment. The cell cycle analysis on data derived from three separate experiments indicated that zinc depletion significantly blocked the progression of ZD cells from G_1 to S phase (Fig. 2). Of the ZD cells, only 2.7% were in S phase.

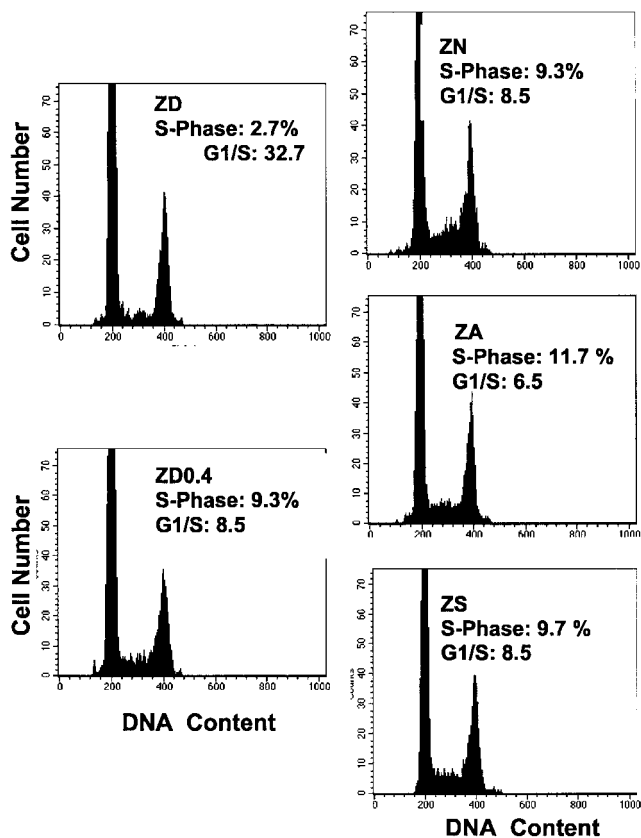


Fig. 2. Cell cycle analyses. DNA content of Hep G2 cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in ZD, ZD0.4, ZN, ZA, and ZS, ZD media for one passage. Washed cells were fixed in ethanol and stained for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. A total of 10,000 cell events were collected for DNA analyses. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. The calibration standard LinearFlow green and the DNA QC particle kit were used for verification of instrument performance. Histograms are representative of 3 independent experiments. The proportions of cells in S phase and the G₁-to-S ratios (G₁/S) are indicated for each medium.

Addition of only 0.4 μ M zinc to ZD medium (ZD0.4) normalized the proportion of cells in G₁/S₁ and S phase to those of ZN, ZA, and ZS cells.

Egr-1 nuclear protein levels are regulated by cellular zinc status. Nuclear Egr-1 protein was detected by Western blot by using specific Egr-1 antibodies sc-110 and sc-189 (Santa Cruz, CA). In our test system, sc-189 gave much less nonspecific bands than antibody sc-110, and therefore sc-189 was used in this study. Nuclear Egr-1 protein in ZD and ZD0.4 cells were decreased to a level of only 2.3 ± 6.2 and $7.1 \pm 7.7\%$ of ZN cells, respectively (Fig. 3). Egr-1 level in ZA cells is comparable to that in ZN cells (Fig. 3). ZS cells had a higher level of Egr-1 compared with ZN cells (190.3 ± 19.12 vs. $100 \pm 3.0\%$; Fig. 3).

Cleavage of HNF-3 β but not HNF-4 in zinc-deficient Hep G2 cells. Transcription factors HNF-4 and HNF-3 β are critical for expression of the apoA1 gene in Hep G2 cells (11). To determine whether cellular zinc status affects the expression of HNF-3 β and HNF-4, we

performed Western blots using anti-HNF-3 β (sc-6554) and anti-HNF-4 α (sc-6556) specific antibodies (Santa Cruz, CA). We found that the HNF-3 β protein was severely degraded in ZD and ZD0.4 cells (Fig. 4). This degradation led to a marked reduction in the intact HNF-3 β protein level in the nuclei of ZD and ZD0.4 cells (8.0 ± 5.6 and $28.2 \pm 18.6\%$, respectively) compared with ZN cells ($100 \pm 7.0\%$). Three degradation fragments of HNF-3 β protein with molecular masses of 39, 34, and 16 kDa were recognized by the anti-HNF-3 β antibody sc-6554, which was raised against peptide located in the COOH-terminal end of the HNF-3 β molecule. No degradation of HNF-3 β protein was observed in ZN, ZA, and ZS cells, and nuclear HNF-3 β protein levels in ZN, ZA, and ZS cells remained unchanged.

In contrast to the cleavage of HNF-3 β , we did not observe any degradation of HNF-4 α protein in ZD or ZD0.4 cells, as well as in ZN, ZA, and ZS cells. Levels of nuclear HNF-4 α protein in ZD and ZD0.4 cell were comparable to that in ZN cells (Fig. 5).

Zinc depletion reduced apoA1 promoter activity in Hep G2 cells. Because Egr-1 and HNF-3 β nuclear protein levels were significantly reduced in zinc-depleted Hep G2 cells, apoA1 promoter activity was next evaluated by using the luciferase reporter construct -298AI-LucB, which contains the apoA1 promoter segments -298 to +397 including both the HNF-3 β -responsive

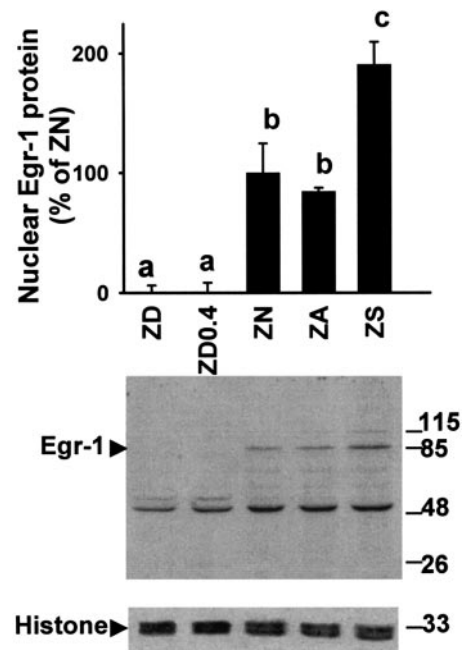


Fig. 3. Relative early growth factor Egr-1 nuclear protein levels in Hep G2 cells. Cells were cultured in ZD, ZD0.4, ZN, ZA, and ZS media for one passage. The nuclear Egr-1 protein was detected with anti-Egr-1 antibody (sc-189) and SuperSignal West Pico chemiluminescent substrate by following the manufacturer's instructions. Equal loading was monitored with anti-histone H1 antibody (sc-8030) by using the same membrane, as described in MATERIALS AND METHODS. Quantification was by densitometry. Values are means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); treatments with the same letters indicate no significant difference.

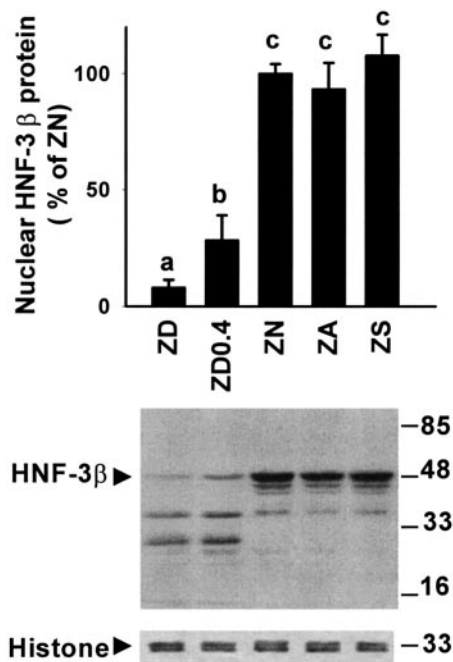


Fig. 4. Relative hepatocyte nuclear factor (HNF)-3 β nuclear protein levels in Hep G2 cells. Cells were cultured in ZD, ZD0.4, ZN, ZA, and ZS media for one passage. Nuclear proteins (40 μ g) were loaded on each lane, and HNF-3 β was detected with HNF-3 β antibody (sc-6554) and SuperSignal West Pico chemiluminescent substrate by following the manufacturer's instructions. Equal loading was monitored with anti-histone H1 antibody (sc-8030) by using the same membrane, as described in MATERIALS AND METHODS. Quantification was by densitometry. Values are means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); treatments with the same letters indicate no significant difference.

element site B (-169 to -146) (10) and the Egr-1-responsive elements E1 (-189 to -181) and E2 (-221 to -213) (14). Compared with that in ZN cells, the apoAI promoter activity in ZD cells was reduced by 40% (Fig. 6). Furthermore, the apoAI promoter activities were elevated to 132 and 146% in ZA and ZS cells, respectively, compared with ZN cells (Fig. 6). These results are consistent with previous studies showing that increasing the cellular zinc levels elevated the abundance of apoAI mRNA in Hep G2 cells (37).

DISCUSSION

Recent evidence shows that micronutrient zinc has profound influence on the liver-specific expression of apoAI gene (36, 37). However, little is known about the mechanism by which apoAI expression in hepatocytes are regulated in response to intracellular zinc status. In this study, we have examined the influence of the intracellular zinc status on the expression of several nuclear transcription factors known to be important *trans*-acting factors of liver-specific promoter of apoAI gene. We report that downregulation of early growth response factor Egr-1 and hepatocyte nuclear factor HNF-3 β contributes in part to the depressed apoAI promoter activity in zinc-depleted Hep G2 cells.

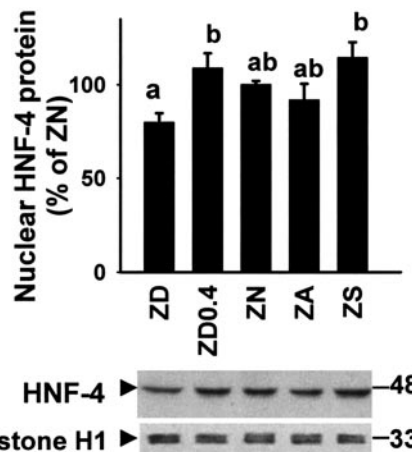


Fig. 5. Relative HNF-4 nuclear protein levels in Hep G2 cells. Cells were cultured in ZD, ZD0.4, ZN, ZA, and ZS media for one passage. The nuclear HNF-4 α protein was detected by using 40 μ g of nuclear protein with anti-HNF-4 α antibody (sc-6556) and SuperSignal West Pico chemiluminescent substrate by following the manufacturer's instructions. Equal loading was monitored with anti-histone H1 antibody (sc-8030) by using the same membrane, as described in MATERIALS AND METHODS. Quantification was by densitometry. Values are means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); treatments with the same letters indicate no significant difference.

Low zinc media consisting of DMEM and 10% chelated FBS have been used successfully to deplete intracellular zinc in several experimental systems (24, 25, 37, 38). Besides zinc ion, Chelex100 resin is capable of sequestering other divalent metals. However, because chelated FBS was used in both ZD and the other zinc-supplemented groups, the only difference among all treatment media was the level of zinc.

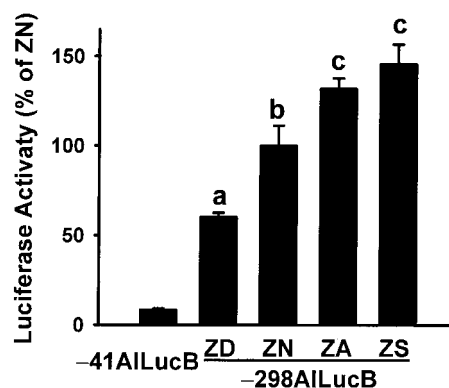


Fig. 6. Effects of zinc on apolipoprotein A-I promoter activity. Hep G2 cells were transfected in DMEM with 10% FBS by using Lipofectamine2000 with 800 ng of luciferase reporter construct -298AI-LucB, together with 25 ng of pRL-SV40, to calibrate transfection efficiency. -41AI-LucB was used as basal promoter control. Sixteen hours after transfection, cells were cultured in ZD, ZN, ZA, or ZS media for another 48 h. Luciferase activity was measured by using the Dual-Luciferase reporter assay system. Values represent means \pm SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); treatments with the same letters indicate no significant difference.

Consistent with previous studies in Hep G2 cells, zinc depletion led to a reduction in DNA content per plate (37). Addition of only 0.4 μM zinc significantly restored the DNA content per plate (Fig. 1), indicating that minimal changes of cellular zinc status have profound influence on cell proliferation and DNA synthesis. On the other hand, the zinc depletion-reduced DNA content in zinc-depleted Hep G2 cells may also reflect the possibility that some of these cells were undergoing apoptosis (4, 20). However, we were unable to observe any significant increase in caspase3/7 activities in ZD or ZD0.4 cells compared with ZN cells, although addition of 16 and 32 μM zinc did result in reduction of caspase3/7 activities in a dose-dependant manner (unpublished data). Furthermore, poly(ADP-ribose) polymerase (PARP), cleaved by caspase3 in HeLa cells depleted of cellular zinc by the membrane-permeable chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (3), was found not to be degraded in either ZD or ZD0.4 cells in our test system (unpublished data). Recently, Nakatani et al. (20) reported that depletion of intracellular zinc by TPEN induced apoptosis in hepatocytes, whereas depletion of cellular zinc by another membrane-impermeable chelator, diethylenetriamine pentaacetic acid (DTPA), did not induce apoptosis. Moreover, a recent report by Fanzo et al. (5) demonstrated that in normal human bronchial epithelial cells, zinc depletion with 0.4 μM zinc culture medium did not alter cell growth. Thus the reduction in DNA content per plate of zinc-deficient Hep G2 cells may not be caused by apoptosis.

We found that the cell cycle progression of Hep G2 cells was readily altered by depressed intracellular zinc status. An elevated percentage of the ZD cells was found to be in G_1 phase, and the proportion of S phase cells was markedly reduced by zinc depletion. This result suggests that zinc is critical for the progression of Hep G2 cells from G_1 to S phase. Previously, a blockage of G_1/S phase transition was observed in zinc-deficient rodent fibroblasts (2). However, the mechanism of how zinc depletion impairs the G_1 -to-S phase transition remains unclear. In mammalian cells, cell cycle progression is coordinated by tightly regulated expression and activation of cell cycle-associated proteins, namely, cyclins and cyclin-dependant kinases (cdks) and their complexes. In hepatocytes, upregulated expression of cyclin D1 and Cdc2 has been associated with S phase progression (19, 32). Moreover, the accumulation of cyclin D1 and transition of G_1 to S phase in hepatocytes are correlated with growth factor-stimulated activation of mitogen-activated protein kinase (MAPK) kinase/extracellular signal-regulated kinase (ERK) signaling cascade (19). In that respect, studies by Lefebvre et al. (18) have shown that in rodent fibroblasts the reduction in cell proliferation by zinc chelator DTPA is associated with decreased activation of ERK1/2 by insulin-like growth factor-1 (IGF-1). Further studies designed to investigate how cellular zinc regulates the activation of MAPK signaling pathways are being performed in our laboratory to fully understand the role of zinc in cell cycle progression.

In the present study, zinc-depleted ZD and ZD0.4 cells exhibited significantly lower levels of Egr-1 protein than did ZN, ZA, and ZS cells. The observed downregulation of Egr-1 protein may affect the cell cycle progression of the ZD cells. Egr-1 is a zinc-finger, DNA-binding transcription factor that is rapidly and transiently induced in response to a variety of extracellular stimuli, such as growth factors, cytokines, injury, hypoxic stress, and extracellular zinc (22). Egr-1 has been proposed to play important roles in modulating cell proliferation (13, 27, 30). In the early phase of liver regeneration after partial hepatectomy, Egr-1 is dramatically induced during the transition of cells from the G_0 to G_1 phase, resulting in upregulation of the phosphatase of regenerating liver-1 (PRL-1) gene expression, which has been implicated in cell growth regulation (23). Moreover, a dominant-negative form of Egr-1 blocks the entry of cells from G_1 to S phase and reduces the survival of cells exposed to ionizing radiation (9). Thus the impaired progression of zinc-deficient ZD Hep G2 cells from G_1 to S phase may have resulted from the depressed level of Egr-1.

The downregulation of Egr-1 may influence the expression of the apoAI gene in ZD cells. Previous studies have shown that hepatic apoAI is expressed in a regulated fashion during development and several pathophysiological states (21, 30). The fetal liver or rapidly growing hepatocytes in the regenerating liver have been shown to have much higher levels of apoAI mRNA than the adult liver or nonproliferating hepatocytes (21). In the regenerating liver, an elevated level of Egr-1 protein has been observed (23). This enhanced Egr-1 expression may contribute, at least in part, to the observed upregulation of apoAI expression in the regenerating liver. In support of this possibility, two Egr-1 binding sites, E1 (-189 to -181) and E2 (-221 to -213), have been identified in the proximal promoter of apoAI gene, and Egr-1 was found to be required to fully overcome the repression of ARP-1 on apoAI promoter activity (14). These two Egr-1 binding sites, E1 and E2, are also found in the apoAI luciferase construct in our study. Furthermore, Zaiou et al. (39) have shown that Egr-1 is involved in the basal level as well as nephrotic syndrome-induced human apoAI gene expression in transgenic mouse models. In that study, Egr-1 knockout mice exhibited 50% reductions in plasma apoAI and hepatic apoAI mRNA, compared with those of wild-type mice.

Besides Egr-1, other transcription factors, especially HNF-3 β and HNF-4 α , have been shown to play important role as positive *trans*-acting factors in the regulation of the expression of the apoAI gene (10, 11). The binding of HNF-3 β and HNF-4 α to their corresponding *cis*-acting elements and synergistic interaction of these transcription factors are essential for the full expression of the apoAI gene in Hep G2 cells (11). In this study, we observed a severe degradation of nuclear HNF-3 β protein in zinc-depleted Hep G2 cells. In contrast, nuclear HNF-4 α protein remained intact in all the treatment groups, suggesting that the proteolysis of HNF-3 β is protein specific. Future studies must be

performed to define the specific cleavage sites in the HNF-3 β molecule and to characterize the proteases responsible for the degradation of HNF-3 β in zinc-deficient cells. Recently, Chimienti et al. (3) reported that in HeLa cells, TPEN-induced zinc depletion resulted in proteolysis of zinc-finger transcription factor Sp1 and other members of the Sp family. They also observed that the proteolysis of PARP was faster compared with the kinetics of Sp protein degradation. In contrast, we did not observe any cleavage of PARP in our zinc-depleted ZD or ZD0.4 Hep G2 cells, even though we did observe the degradation of Sp1 and Sp3 protein in ZD and ZD0.4 cells (unpublished data). This suggests that the influence of zinc depletion on the proteolysis of these nuclear proteins depends on the context of cell type and the depletion methods used.

Recently, Zheng et al. (40, 41) have shown that Sp1 is an important transcription factor in the regulation of apoAI gene expression in Hep G2 cells induced by protein kinase A, protein kinase C, and Ras-MAPK signaling cascades. Therefore, we examined nuclear Sp1 status and found that Sp1 was cleaved in ZD and ZD0.4 Hep G2 cells (unpublished data). Thus reduction in Sp1 protein level may contribute to the depressed transcription of apoAI gene in zinc-deficient Hep G2 cells. However, the Sp1-responsive element located between nucleotides -425 and -376 of the apoAI gene (40, 41) is not found in the luciferase reporter construct -298AI-LucB, which was shown to be responsive to cellular zinc status in our test system. Thus the depressed luciferase activity of the apoAI gene reporter construct -298AI-LucB observed in our ZD cells is most likely not due to the decrease in Sp1 nuclear protein. We are currently designing experiments to examine the influence of zinc status on Sp1 expression in the modulation of apoAI promoter activity in Hep G2 cells by using reporter construct containing the Sp1 binding site located between nucleotides -425 and -376.

In summary, our studies show that zinc depletion in Hep G2 cells decreases apoAI promoter activity, which is associated with a markedly depressed level of transcription factor Egr-1 and the enhanced cleavage of nuclear HNF-3 β proteins. Interestingly, the liver-specific knockout of HNF-3 β gene in a transgenic mice model, using Cre-LoxP system, did not decrease apoAI expression (31). Nevertheless, the reduction of apoAI promoter activity in our zinc-depletion system is associated with depressed levels of multiple members of transcription factor families known to be essential *trans*-acting factors of apoAI promoter activity. Most importantly, our findings suggest that multiple transcription factors, impaired by zinc deficiency, may be required to act together in modulating apoAI promoter activity.

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