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Retinol binding protein expression is induced in HepG2 cells by zinc deficiency

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Abstract Zinc (Zn) deficiency is often associated with low plasma vitamin A (retinol) concentrations. It has been suggested that the reduction in plasma retinol is secondary to reduced liver retinol binding protein (RBP) synthesis. In the present study, RBP expression was determined in HepG2 cells cultured in either Zn adequate media or chelated media containing varying concentrations of Zn. Levels of RBP mRNA increased in a time- and Zn concentration-dependent manner such that 0.5 μM Zn-treated cells exhibited a >7.5-fold increase while cells treated with 15 μM Zn were increased 2.9-fold at 72 h compared to controls. RBP protein also progressively increased by 72 h to levels >8-fold and 3-fold higher than controls, in 0.5 μM and 15 μM Zn-treated cells, respectively. The increase in RBP occurred without any change in DNA concentration between groups through 72 h. The Zn deficiency-induced elevations in RBP transcript levels could be reversed within 24–48 h of repletion in Zn adequate media. Thus, the reductions in plasma retinol observed in Zn deficiency are in part a direct consequence of the deficiency.

Key words: Retinol binding protein; Zinc deficiency; Mouse; Hepa 1-6 cell

1. Introduction

Zinc (Zn) is essential to the function of numerous proteins including its role as a component of the catalytic site(s) of hundreds of enzymes [1]. Zn is also an essential component of transcription factors wherein it serves an integral structural role in DNA-contacting Zn finger motifs [2]. This later property suggests a possible role of Zn as a regulatory ion in gene expression [3]. Indeed, recent studies have observed changes in transcript levels of several genes in response to changes in Zn status [4–8].

The effects of Zn deficiency in both humans and experimental animals have been clearly established and are known to result in abnormal development and growth failure [9]. Another common finding in Zn deficiency is low plasma vitamin A (retinol) levels which often occur despite normal to elevated liver vitamin A (reviewed in [10]), suggesting that Zn deficiency impairs hepatic vitamin A mobilization. Since Zn supplementation in deficiency has been shown to improve indicators of vitamin A status including restoration of plasma retinol to within the normal range [11], these Zn deficiency-induced effects on vitamin A may in some cases be reversible.

In humans, retinol is the major form of vitamin A in the circulation where it is bound to the carrier molecule retinol binding protein (RBP) [12]. Synthesized primarily in the liver, RBP is integral to systemic vitamin A homeostasis, binding a single retinol molecule and facilitating its delivery to, and subsequent uptake by, vitamin A-dependent tissues [13,14]. Reductions in plasma RBP levels have been observed in Zn deficiency [10] and there is a positive correlation between plasma concentrations of retinol and/or RBP with plasma Zn [11]. In aggregate, studies suggest that the reduction in the circulating retinol concentration in Zn deficiency is a consequence of reduced hepatic RBP synthesis and/or a reduction in the release of retinol-RBP (holoRBP) from the liver. The mechanism by which this occurs is, however, unknown.

Although Zn deficiency has been shown to result in an overall depression of hepatic protein synthesis [15], mRNA levels of a number of genes have been shown to be either increased [4,6,8] or decreased [5,7,16,17] as a consequence of Zn deficiency. Although Zn deficiency is associated with reductions in plasma retinol and RBP, a study in rats observed an increase in the relative abundance of liver RBP transcripts as a consequence of Zn deficiency [15]. Whether this increase in RBP mRNA manifested as an increase in RBP protein, or is reversible with Zn supplementation, was not assessed. Pair-fed animals, however, also exhibited an increase in hepatic RBP mRNA, although to a lesser extent than in the Zn-deficient group [15]. This observation, together with those of other studies in which pair-fed animals also exhibited reductions in plasma retinol (reviewed in [10]), suggests that the effects of Zn deficiency on vitamin A mobilization may not be a specific, direct response to Zn depletion, but rather may reflect indirect effects secondary to Zn deficiency-induced anorexia. It should be noted, however, that pair-fed animals also have reduced Zn status as evidenced by reductions in plasma and bone Zn concentrations compared to ad libitum, Zn adequate-fed controls [15]. This highlights a limitation to interpretation of results from whole animal studies wherein there is an inability to definitively distinguish the direct effects of Zn deficiency from the indirect effects resulting from decreased food intake and associated hormonal alterations. Cell culture models allow for the study of Zn deficiency directly, in the absence of these confounding factors.

Given the effects of Zn deficiency on vitamin A, and the recent evidence that Zn status can affect the expression of specific genes, the present study was undertaken to determine whether Zn status directly affects the expression of RBP. Us-

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ing human RBP-expressing HepG2 cells as a model, in the present study we characterized the time-dependent effects of different concentrations of Zn in the culture media on RBP mRNA and protein levels as well as the response to Zn repletion. We observed that culture of HepG2 cells in low Zn media results in increases in RBP mRNA and protein, with the magnitude and timing of these increases being correlated to the extent of media Zn depletion. In addition, the Zn deficiency-induced elevations in RBP transcript levels were reversed by switching cells from Zn-deficient to Zn-supplemented media. These results support the concept that the alterations in vitamin A levels observed in Zn deficiency are in part a direct consequence of the deficiency.

2. Materials and methods

2.1. Materials

Human hepatoma (HepG2) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco (Grand Island, NY, USA). Phenylmethylsulfonyl fluoride and diethylenetriaminepentaacidic acid (DTPA) were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

HepG2 cells were maintained in DMEM supplemented with 7.5% FBS and antibiotics (50 U/ml penicillin/streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 in air. For experiments, cells were seeded in 100 mm^2 tissue culture dishes at 1×10^6 cells/dish in DMEM/7.5% FBS (designated as standard medium). Following a 12 h initial attachment period, the standard media were removed by aspiration and discarded, and the cells were carefully washed with phosphate-buffered saline (PBS). The media were then replaced with either chelated media containing 0.5, 15, 50 μM Zn or unchelated standard media (control) as indicated, and the cultures continued for the indicated time periods. Duplicate or triplicate dishes were used per media Zn concentration/time point and each experiment was repeated at least twice.

Since FBS is the primary source of Zn in culture media, Zn-deficient FBS was prepared by chelation with DTPA as described previously [18]. Using this method, the concentration of Zn in the chelated serum was reduced to 2.5 μM as determined by inductively coupled plasma atomic absorption/emission spectroscopy analysis (Trace Scan, Thermo Jarrell Ash Corp., Franklin, MA, USA). Copper and iron concentrations were fixed at 1 μM and 5 μM, respectively, to remove these metals as variables in these studies. The chelated FBS was diluted with DMEM (DMEM has <0.1 μM Zn) to a final concentration of 3 mg of protein/ml; a protein concentration matched to that of non-chelated, non-dialyzed standard media (7.5% FBS). The concentration range of the individual Zn-deficient media used was 0.4–0.65 μM Zn. Portions of this media were supplemented with $ZnSO_4$ to concentrations of 15 and 50 μM Zn. Analysis of the retinol concentration of the different Zn media by high performance liquid chromatography indicated a uniform concentration of 0.0113 ± 0.0017 μM, thereby precluding differences in media retinol as a variable.

2.3. RNA analysis

Total RNA was isolated using the guanidine isothiocyanate method [19] at the indicated intervals. The total RNA was then quantitated, denatured and electrophoresed in a 1% agarose-formaldehyde gel, with subsequent transfer to a Hybond N nylon membrane (Amersham, Arlington Heights, IL, USA) according to standard procedures [20]. Procedures for prehybridization, hybridization and membrane washing were performed as we have described previously [21]. Membranes were hybridized with randomly ^{32}P-labeled cDNA probes [20] for RBP and β-actin (as a normalization control). Following exposure against film, the relative amounts of specific mRNAs were determined by densitometric analysis and normalized to the β-actin signal.

2.4. Protein analysis

To assess RBP protein levels, HepG2 cells were plated and treated as above. At designated times, cells were rinsed in ice-cold PBS and then placed in a hypotonic solution (10 mM Tris) and homogenized using a Dounce homogenizer. The cell lysate was then centrifuged ($15\,000\times g$ for 5 min) and the resultant supernatant fraction was collected. The protein concentration of the supernatant was determined using the BCA assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Protein preparations were then subjected to size separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gels [20], transferred onto polyvinyl diethyl fluoride membranes and incubated in a blocking solution (5% w/v Blotto) followed by an overnight incubation with goat anti-human RBP antibody (Dako, Carpenteria, CA, USA). After washing, membranes were incubated with horseradish peroxidase-labeled rabbit anti-goat IgG for 1 h [20]. The enzyme bound to RBP was visualized using enhanced chemiluminescent detection using the ECL kit (Amersham) according to the manufacturer's instructions. Relative quantities of RBP and albumin (as a control) were determined by densitometric analysis.

2.5. DNA analysis

DNA concentrations were ascertained fluorometrically using a commercially available kit (CyQuant, Molecular Probes, Eugene, OR, USA). DNA was quantitated subsequent to DNase-free RNase A/T1 pretreatment of cell extracts as described by the manufacturer. A concentrated DNA standard consisting of sheared calf thymus DNA at 3 mg/ml was diluted to 1 μg/ml in a proprietary cell lysis buffer and was used immediately to generate a standard curve. Fluorescence measurements were made using a LS 50B spectrofluorimeter equipped with a plate reader (Perkin Elmer Corp., Norwalk, CT, USA) subsequent to the addition of the CyQuant GR intercalator and a 30 min incubation period. An excitation wavelength of 485 nm and an emission wavelength of 530 nm were chosen and both slits were set at 2.5 nm width. A 515 nm emission filter was selected to reduce spectral noise.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with subsequent post-hoc comparisons by Scheffe were performed using Statview (Brainpower Inc., Calabazas CA, USA). A P value of ≤0.05 was considered statistically significant. Values are presented as the mean ± S.E.M. unless otherwise indicated.

3. Results

3.1. Effect of Zn-deficient media on relative RBP transcript levels in HepG2 cells

A comparison of HepG2 RBP mRNA normalized to β-actin levels following culture in standard, Zn adequate media versus Zn-deficient (0.5 μM Zn) media is shown in Fig. 1. Compared to controls in Zn adequate standard media, cells maintained in the 0.5 μM Zn media exhibited a gradual increase in RBP mRNA levels which was apparent by 48 h, and amplified by 72 h at which time RBP mRNA levels were $>320\%$ higher than controls. The levels of β-actin normalized RBP mRNA in cells grown in standard (control) media did not change over the duration of the experiment.

3.2. Effect of Zn-deficient media on DNA concentration and RBP transcript levels in HepG2 cells

Zn deficiency can be associated with compromised cell viability in vitro [22,23] and in vivo [24]. Thus, time-course experiments were repeated and the DNA content in the dishes was quantified along with RBP and β-actin mRNA levels. A second group treated with 50 μM Zn media was added for comparison. As depicted in Fig. 2A, treatment with either 0.5 μM or 50 μM Zn media resulted in no change in DNA concentration through 72 h compared to cells in Zn adequate, standard media. When β-actin-normalized RBP mRNA values were further normalized to DNA (Fig. 2B), the pattern with respect to RBP transcript levels between the control and

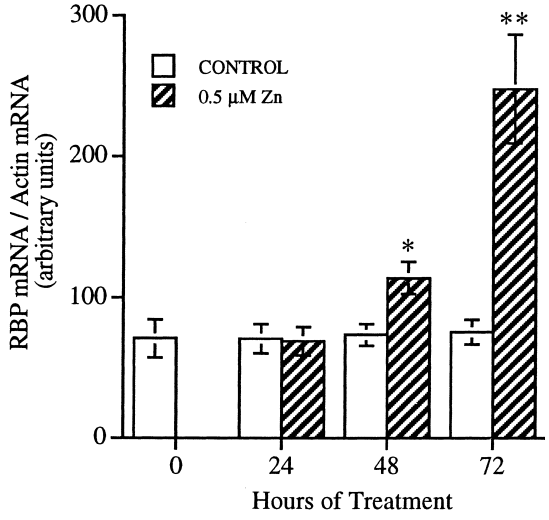

Fig. 1. Effect of Zn-deficient media on RBP mRNA levels in HepG2 cells. Cells were grown in either control, Zn adequate (standard) media or 0.5 μM Zn media for the indicated time periods. Each bar represents the mean ± S.D. from two independent experiments. Asterisks indicate significant differences from control cells (*$P < 0.05$; **$P < 0.01$; one-way ANOVA test).

low Zn group was very similar to that seen in our initial experiments. By 72 h, cells in 0.5 μM Zn media had RBP transcript levels that were ≥330% of controls. RBP mRNA levels in the 50 μM Zn media group were equivalent to controls at all time points.

3.3. Changes in RBP mRNA and protein levels in response to varying concentrations of media Zn

Given the effect of Zn deficiency on RBP mRNA levels, further experiments were conducted to examine the dose-dependent relationship between media Zn concentration and RBP transcript levels. As depicted in Fig. 3A, levels of β-actin normalized RBP transcripts exhibited both time and Zn concentration dependency. RBP transcripts in the 0.5 μM Zn group progressively increased to 375% and 760% of control levels at 48 and 72 h, respectively. Levels of RBP mRNA in the intermediate (15 μM Zn) group increased in a pattern similar to that of the 0.5 μM group but were only 290% higher than controls at 72 h. Cells treated with 50 μM Zn had RBP mRNA values which were similar to untreated controls at all time points.

Because of the effect of varying media Zn concentrations on RBP transcript levels, additional experiments were conducted to determine whether this was manifested by changes in cellular RBP protein levels. As shown in Fig. 3B, cells treated with 0.5 μM Zn media had significantly elevated RBP protein levels by 24 h (6.2-fold relative to untreated controls) which increased through 72 h to >8-fold higher than controls. Cells treated with 15 μM Zn media exhibited a similar progressive increase in RBP protein levels that was evident by 48 h and which peaked at 72 h (3-fold higher than controls). Cellular RBP protein concentrations were similar in the controls (maintained in unchelated standard growth media) and cells treated with media containing 50 μM Zn. For comparison, albumin concentrations were also determined. In contrast to RBP, no discernible time- or media Zn-dependent pattern was observed for albumin (Fig. 3C).

3.4. Zn-deficient media-induced increases in RBP mRNA can be abrogated by switching to Zn replete media

Following an initial 72 h culture period in either standard media or 0.5 μM Zn media, cells were switched to Zn replete media (chelated media supplemented to 50 μM Zn) for an additional 24 or 48 h period after which time RBP mRNA levels were determined. As shown in Fig. 4, cells maintained continuously in the 0.5 μM Zn media exhibited the predicted pattern of increase in RBP mRNA following the initial 72 h exposure period. However, cells switched from the 0.5 μM Zn to the 50 μM Zn (replete) media and grown for 24 or 48 h had RBP mRNA levels equivalent to (after 24 h), or even slightly lower (after 48 h) than cells maintained continuously in standard growth media.

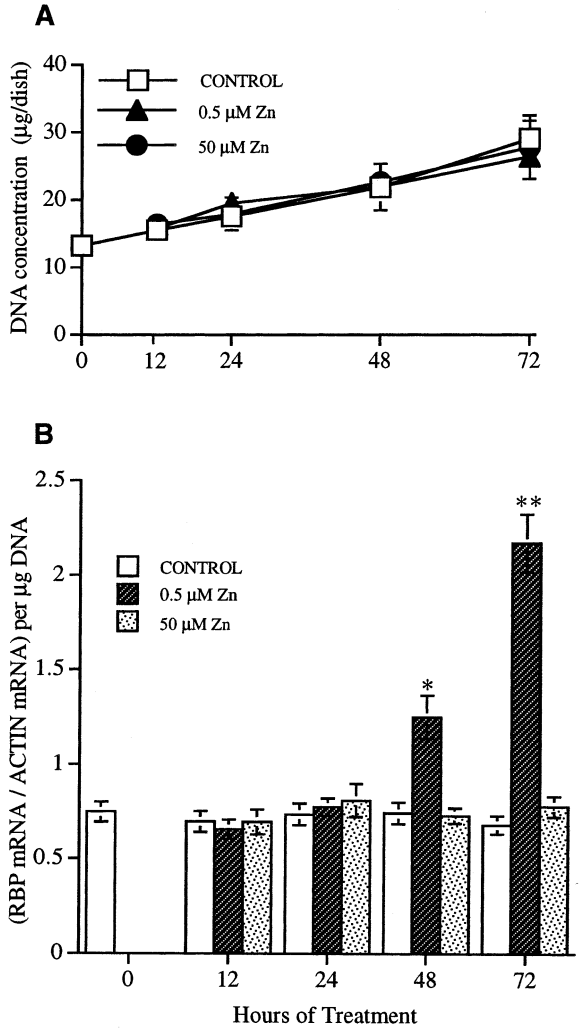

Fig. 2. Effect of Zn-deficient media on the DNA concentration and relative RBP mRNA levels in HepG2 cells. (A) Assessment of DNA concentration in cells grown in either control Zn adequate standard media, 50 μM Zn, or 0.5 μM Zn media for the indicated time periods. (B) Effect of Zn-deficient media on HepG2 cell RBP mRNA levels with normalization to β-actin and DNA. Cells were grown in either standard Zn adequate media, 50 μM Zn, or 0.5 μM Zn media for the indicated time periods. Each symbol/bar represents the mean ± S.E.M. from two independent experiments. Asterisks indicate significant differences from control cells (*$P < 0.05$; **$P < 0.01$; one-way ANOVA test).

4. Discussion

While it is known that Zn deficiency can be associated with low plasma retinol concentrations and an elevation in hepatic RBP, the mechanism(s) underlying the effect of Zn deficiency on vitamin A metabolism are unknown. In the present study, RBP-expressing HepG2 cells cultured in low Zn media (0.5 μM Zn) had progressively elevated levels of RBP mRNA beginning at 48 h and continuing to 72 h, relative to cells grown in standard (control) media. Elevations in transcript levels of other genes have been observed in response to Zn deficiency in animals [8] and in cell cultures depleted of Zn [6]. Our finding of elevated RBP transcripts in Zn deficiency is in agreement with that of Kimball et al. [15] who reported that rats fed a Zn-deficient diet had increased liver RBP mRNA levels compared to ad libitum, Zn sufficient-fed animals. However, since hepatic RBP mRNA levels were also increased in pair-fed animals (albeit to a smaller extent), Kimball and coworkers concluded that the effect of Zn deficiency on RBP was indirect, resulting from the inanition that accompanied the Zn deficiency [15]. Thus, an objective of the present study was to determine if the effect of Zn deficiency on RBP was direct.

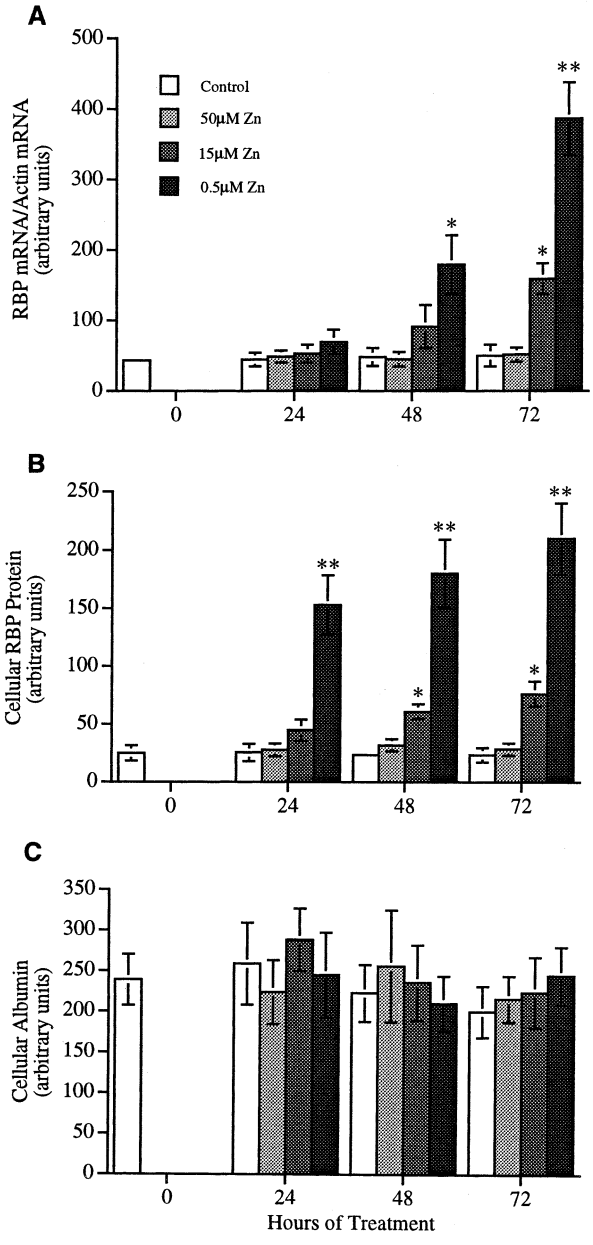

Fig. 3. Effect of varying media Zn concentrations on RBP mRNA, RBP and albumin protein levels in HepG2 cells. (A) Effect of decreasing media Zn concentrations on RBP mRNA. (B) Effect of decreasing media Zn concentrations on cellular RBP protein. (C) Effect of decreasing media Zn concentrations on cellular albumin levels. Cells were grown in either standard (control) media or 50 μM, 15 μM or 0.5 μM Zn containing media for the indicated times. Actin-normalized RBP mRNA levels were determined by Northern blot, intracellular RBP and albumin protein concentrations were determined by Western blot analysis as described in Section 2. Each bar represents the mean ± S.E.M. from two independent experiments. Asterisks indicate significant differences from control cells (*$P < 0.05$; **$P < 0.01$; one-way ANOVA test).

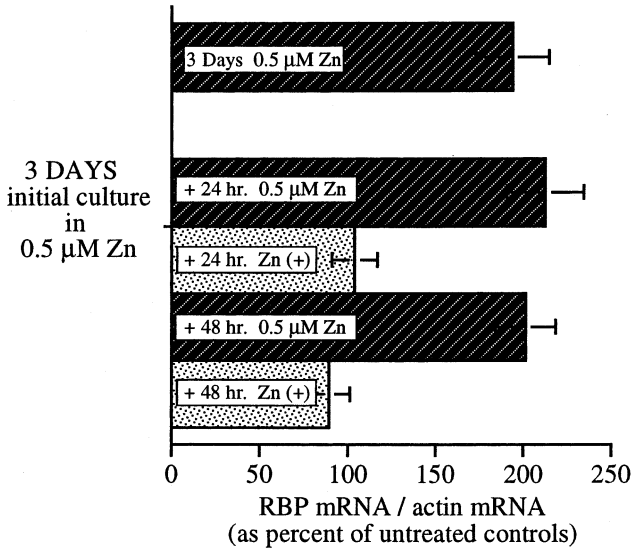

Fig. 4. Effect of Zn repletion on the Zn-deficient media-induced increase in RBP mRNA levels in HepG2 cells. Cells were initially grown in either standard Zn adequate media or 0.5 μM Zn media for 72 h (3 days). Following this initial treatment period, the media were removed, the cells rinsed and the media replaced with either chelated media supplemented to 50 μM Zn [Zn(+)] or 0.5 μM Zn media, and the cells allowed to grow for an additional 24 or 48 h as indicated. RBP mRNA levels were then determined relative to β-actin as described in Section 2. Each bar represents the mean ± S.E.M. from two experiments and is shown as a percent of RBP mRNA levels from cells grown continuously in standard growth media.

Although Zn deficiency has been reported to promote cell proliferation [25,26], we [27] and others (see [9] for review) have shown that Zn deficiency is associated with decreased growth in animals as well as decreased cell viability in vitro [22,23] and in vivo [24]. Zn deficiency has also been shown to induce apoptosis [18,22,28,29]. Although Zn deficiency-induced apoptosis has been observed by 24 h in 3T3 [18] and rat osteoblast cells [29] in vitro, we observed no differences in DNA concentration during the 72 h culture period between the 0.5 μM Zn-, 50 μM Zn- and standard media-treated cells. Normalization of RBP mRNA levels to DNA demonstrated a significant elevation in the abundance of RBP transcripts in the 0.5 μM Zn cells beginning at 48 h and persisting through 72 h. Cells cultured in 50 μM Zn media had RBP transcripts per μg DNA equivalent to controls. Whether the increase in abundance of RBP mRNA in Zn-deficient cultures is a result

of an increase in the rate of RBP gene transcription, an increase in RBP mRNA half-life, or both remains to be ascertained.

Dose–response studies demonstrated that the increase in RBP mRNA was dependent on the Zn concentration in the media. In cells treated with 0.5 μM Zn, the increase in RBP mRNA by 72 h was greater than in cells treated with an intermediate (15 μM) Zn concentration. That the increase in RBP transcripts was specifically due to the low Zn treatment is demonstrated by the observation that cells grown in the 50 μM Zn media had levels of RBP mRNA equivalent to that of cells grown in standard control media. The time delay before the increase in RBP mRNA and the lower extent of RBP transcript elevation in the 15 μM Zn cells may be a consequence of slower depletion and/or redistribution of Zn within intracellular pools. This dose–response relationship supports the results of previous studies that observed that even marginal Zn deficiency can affect plasma vitamin A concentrations [30].

Protein analysis by Western blot showed that cellular levels of RBP protein increased as the media Zn concentration decreased, and that this response was specific to the low Zn content of the media since no increase was observed in the cells grown in 50 μM Zn media. That the effect of Zn status is relatively specific for RBP is suggested by the observation that albumin levels were not altered by the content of Zn in the media. Our findings that the extent of increase in RBP mRNA and protein in HepG2 cells is negatively correlated with the concentration of Zn in the media supports a hypothesis that vitamin A transport is only affected below a certain threshold level of Zn.

The effect of culture in 0.5 μM Zn media on RBP transcript levels was observed to be reversible, dependent on the timing and duration of Zn repletion. Following an initial culture period in 0.5 μM Zn media, switching the cells to 50 μM Zn-supplemented media for either 24 or 48 h completely abrogated the increase in RBP mRNA observed in the cells maintained continuously in the 0.5 μM Zn. Thus, both the 24 and 48 h 'replete' cell groups had actin-normalized RBP mRNA values equivalent to that of cells that were not exposed to the low Zn media. Zn repletion of deficient cell cultures has been observed to restore Zn deficiency-induced elevations in p53 mRNA to normal levels [6] and to inhibit the apoptotic effects of Zn depletion [29,31]. In animal studies, restoration of liver and plasma vitamin A levels resulting from Zn deficiency occurred after a time-lag of several days following either dietary supplementation or a single, bolus injection of $ZnSO_4$ [32,33]. In humans, plasma retinol levels were observed to be increased at 6 months following Zn supplementation [11]. Our observation that the effects of culture in low Zn media are reversed within 24–48 h by switching to Zn-supplemented media supports the hypothesis that low Zn status directly affects processes involved in RBP synthesis. The rapidity of the response observed here, in vitro, is likely a consequence of faster delivery, cellular uptake and redistribution of the Zn in the absence of physiological factors which would likely slow these processes in vivo.

We have reported that Zn deficiency in vitro results in markedly elevated production of cellular oxidants and the subsequent induction of nuclear AP-1 complex activity [18]. Of interest here is our recent observation that the RBP gene promoter contains an AP-1 site (Jessen and Satre, submitted for publication) that may become activated under conditions of oxidant stress. The upregulation of RBP mRNA and protein levels under these oxidative conditions supports one asserted role of vitamin A as a cellular antioxidant [34], although the antioxidant role of vitamin A in vivo remains debatable [35]. Nevertheless, our findings suggest a possible early sequence of events in which low Zn status-induced increases in AP-1 activity could result in increased RBP gene transcription and elevated RBP protein.

In summary, culture of HepG2 cells in low Zn media results in increases in RBP mRNA and protein, with the magnitude and timing of these increases being governed by the severity of the reductions in media Zn. These effects on RBP mRNA and protein occurred without measurable changes in DNA concentration. In addition, the Zn deficiency-induced elevations in RBP transcript levels can be rapidly reversed by switching cells to Zn-supplemented media. These findings are consistent with the hypothesis that the reductions in plasma retinol and simultaneous elevations in hepatic vitamin A observed in Zn deficiency are a direct consequence of reduced Zn status.

Our findings of increased RBP mRNA and protein under Zn-deficient conditions may reflect an early and possibly transitory, physiological response to Zn depletion. Zn deficiency rapidly results in oxidative stress [18] while retinol displays free radical scavenging activity [34]. Thus, an increase in RBP could serve to facilitate secretion of retinol into the circulation as part of an early compensatory response to protect against oxidative damage. With continued Zn deprivation, this effect on RBP may no longer be manifest or may become masked. In whole animal studies, prolonged Zn deprivation results in hormonal alterations and reduced food intake which may affect vitamin A homeostasis and decrease plasma retinol.

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