Zinc Transporters in the Rat Mammary Gland Respond to Marginal Zinc and Vitamin A Intakes during Lactation

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ABSTRACT Marginal intake of zinc and vitamin A is common during lactation and a deficiency of one micronutrient can result in a secondary deficiency of the other. However, the resistance of milk zinc (Zn) concentration to changes in dietary Zn or vitamin A indicates tight regulation of mammary gland Zn transport. Although several mammalian proteins have been identified and implicated in Zn transport, the mechanisms responsible for mammary gland Zn transport and their regulation by dietary Zn and vitamin A are unknown. In this study, we identified mammary gland Zn transporters and determined effects of marginal Zn and vitamin A intakes on their levels. Rats were fed a control (25 mg Zn/kg, 4 retinol equivalents (RE)/g), a low Zn (10 mg Zn/kg), a low vitamin A (0.4 RE/g), or a low Zn (10 mg Zn/kg) and vitamin A (0.4 RE/g) diet throughout lactation. ZnT-1, ZnT-2 and ZnT-4 were identified in the mammary gland and localized to the serosal membrane (ZnT-1) or intracellularly (ZnT-2 and ZnT-4) by immunostaining. Rats fed a low Zn or low vitamin A diet had lower ZnT-1 protein and higher ZnT-4 mRNA expression and protein levels compared with controls. There was a significant interaction between dietary Zn and vitamin A on zinc transporter mRNA expression and protein levels. Although total mammary gland Zn was not affected, mammary gland metallothionein levels were lower in rats fed low Zn and higher in rats fed low vitamin A, suggesting different mechanisms regulating zinc transporter levels. These results indicate that milk Zn level is maintained through coordinated regulation of mammary gland zinc transporters and documents an effect of vitamin A on zinc homeostasis at the molecular level during lactation. J. Nutr. 132: 3280–3285, 2002.

KEY WORDS: • zinc • vitamin A • zinc transport • mammary gland • lactation • rats

Marginal intake of zinc (Zn) is common in industrialized and developing countries. Consequently, marginal zinc deficiency is believed to be more prevalent than once thought (1); however, a lack of sensitive indicators of zinc nutriture precludes accurate assessment of zinc status. During lactation in humans and rodents, milk zinc concentration is maintained over a wide range of dietary Zn intake, and decreases only after plasma Zn concentration is reduced (2,3). Many intervention studies attempting to improve zinc nutriture have been aimed at lactating women; however, most of these studies have failed to show an effect of increased maternal zinc intake on milk zinc concentration despite improved serum zinc concentrations (4,5). The failure of maternal zinc supplementation to alter milk zinc concentration suggests that mammary gland zinc transport is tightly regulated.

Inadequate vitamin A intake is prevalent in nonindustrialized countries, and effects of vitamin A deficiency on zinc metabolism have been documented. Severe vitamin A deficiency decreased intestinal zinc absorption and altered tissue mineral concentrations, whereas vitamin A supplementation increased liver metallothionein (MT) concentration in rats (1,6–8); however, the mechanisms responsible for these effects of dietary vitamin A on zinc metabolism are unclear. Furthermore, marginal zinc and vitamin A deficiency often coexist in many populations and may result in distinct effects on zinc metabolism, thus interfering with homeostatic zinc regulation and potentially confounding intervention studies.

Recently, several proteins have been described that participate in zinc trafficking across membranes; some of these have been identified in mammals (9). Five mammalian genes involved in zinc transport have been identified and their protein products are referred to as ZnT-1, -2, -3, -4 and -5. ZnT-1–ZnT-4 are structurally similar; they have six transmembrane domains and a histidine-rich domain that is believed to play a key role in zinc binding. ZnT-1 has been proposed to export zinc from cells, is localized to the plasma membrane and is expressed ubiquitously (10). ZnT-2 facilitates the vesicular localization of zinc into endosomal vesicles in small intestine, kidney and testes (11), although its physiologic importance is unknown. ZnT-3 is abundant in the hippocampus and cortex (12), responsible for the accumulation of zinc in synaptic vesicles and has been proposed to serve a neuromodulatory role. ZnT-4 is expressed in many tissues (13), although the mechanism of ZnT-4–mediated transport has not been determined. A point mutation in ZnT-4 that results in a premature termination codon is believed to be responsible for the murine lethal milk phenotype (1m).2 Milk produced by 1ml/1m females...
contains insufficient zinc to support the needs of suckling mice (14); however, a direct relationship between this mutation and low zinc concentration in milk has yet to be confirmed through the use of an in vivo model. ZnT-5 is unique in that it has 15 transmembrane domains and is highly expressed in pancreatic β-cells associated with secretory granules (15). Additionally, other genes encoding putative zinc transporters in humans (hZIP) have been identified as a result of gene sequence homology with known zinc transporters found in plants and yeast (16). However, the role these transporters play in regulating zinc homeostasis remains to be determined. Intracellularly, zinc trafficking is controlled primarily by MT (17), a high affinity intracellular zinc binding ligand that is regulated directly by zinc, and changes in MT reduction-oxidation activity (18) or intracellular levels of MT (17) are believed to alter the concentration of "labile" zinc within the cell.

In this study, we focused on several zinc transporters that we believed might participate in the regulation of mammary gland zinc homeostasis. We used a rat model to identify mammary gland zinc transporters and determine their localization by immunohistochemistry. Furthermore, the assumption that marginal intake of zinc and vitamin A deficiency and the documented interactions of zinc and vitamin A, we examined effects of marginal zinc and vitamin A intake on zinc transporter gene expression and protein levels, and intracellular zinc partitioning was assessed by changes in MT protein levels.

**MATERIALS AND METHODS**

**Animals.** This study was approved by Animal Research Services at the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Virgin Sprague-Dawley rats (n = 24; ~250 g) were obtained commercially (Simonsen, Gilroy, CA) and maintained in stainless steel hangling cages. Rats (n = 6/diet) were randomly assigned to consume 1 of 4 experimental diets ad libitum. Purified experimental diets (AIN-93G) differed only in vitamin A and zinc levels with groups receiving one of the following: 1) a diet low in zinc (ZD, 10 mg Zn/kg); 2) a diet low in vitamin A (AD, 0.4 retinol equivalents [RE]/g); 3) a diet low in zinc and vitamin A (DD, 10 mg Zn/kg, 0.4 RE/g); or 4) a control diet (C, 25 mg Zn/kg, 4 RE/g) (19). Rats were fed diets for 70 d before mating, throughout gestation and 10 d into lactation. On postnatal d 2, litters were culled to 10 pups. On postnatal d 10, dams were removed from pups for 4 h, anesthetized (intraperitoneal, 1.6 mg xylazine/kg and 33 mg ketamine/kg), and fasted 6/diet) were randomly assigned to

**Zinc and retinol analysis.** Plasma was digested at room temperature with 0.1 mol/L trace mineral–free nitric acid (Fisher Scientific). Mammary glands were minced and rinsed three times in fresh isotonic saline at room temperature for 10 min each to remove sequestered milk. Whole milk and milk-dried, minced mammary gland were digested with concentrated nitric acid and wet-ashed using a modification of Clegg et al. (20). Zinc was analyzed by flame atomic absorption spectroscopy on a Spectroflame Model 1000 (Spectro Analytical Instruments, Jarrell Ash, Franklin, MA). Plasma and mammary gland retinol was extracted and analyzed by HPLC as described previously (19).

**Identification of zinc transporter transcripts by polymerase chain reaction (PCR).** Total mammary gland RNA was extracted in TRIzol following manufacturer’s instructions. mRNA was isolated from total RNA (MicroFast Track Kit, Invitrogen, Carlsbad, CA) and digested with EcoRI (Amersham Pharmacia Biotech). Restriction digests were separated and purified as described above. Glyceraldehyde phosphatedehydrogenase (GAPDH) cDNA (a generous gift from Katti Jessen, University of California, Davis) was used as a normalization control. cDNA probes were labeled with 32P (cDNA Labeling Kit, Amersham Pharmacia Biotech) and desalted (S-200 MicroSpin Columns, Amersham Pharmacia Biotech). Equal amounts of mRNA from individual mammary glands (10 µg) were denatured in 3-morpholinopropanesulfonic acid (MOPS) sample buffer containing ethidium bromide (Sigma) and electrophoresed through a 0.8% agarose gel containing MOPS-EDTA buffer (Sigma, St. Louis, MO) for 30 min at room temperature, protected from light (Molecular Probes, Eugene, OR) and visualized using the Chemi-doc Gel QuantiFication System (Bio-Rad, Hercules, CA).

**Preparation of cDNA probes and determination of mRNA relative abundance by Northern Blotting.** PCR transcripts were separated by gel electrophoresis through a 2% low melt agarose gel, excised, isolated, purified (GeneClean Kit, BIO101, Vista, CA) and sequenced to confirm identity. Northern blots were hybridized at 68°C in the absence of stringent conditions for each filter with 0.1% SDS at 68°C for 1 h and a final extension at 68°C for 3 min; 20T-2: 5'-CATGCCCA-GAATTTGCATG, 3'-GTCCCAATGGTGTAATGGC; 94°C for 3 min, and a final extension at 68°C for 3 min; 20T-4: 5'-TCTGTAAGAGT-GTACCCAGA, 3'-CACAGCTGTCAAGGACTCCA; 30 cycles at 94°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 3 min. PCR transcripts were separated on a 2% agarose gel and identified by staining in SYPR Gold (1:10,000 dilution in Tris-Acetate-EDTA buffer, Sigma, St. Louis, MO) for 30 min at room temperature, protected from light (Molecular Probes, Eugene, OR) and visualized using the Chemi-doc Gel QuantiFication System (Bio-Rad, Hercules, CA).

**Preparation of mammary gland and milk proteins.** Mammary gland protein was isolated following a modification of McMahon and Cousins (9). Mammary gland (500 mg) was homogenized for 20 s in 10 mL Hepes-EDTA buffer (10 mm Hepes, pH 7.4/1 mmol/L EDTA/250 mmol/L sucrose/protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonyl fluoride, trans-eptoxysuccinyl-l-leucyl-amido(4-guanidino)butane, bestatin,
leupeptin, aprotinin, and sodium EDTA (Sigma) with an ice-cold polytron homogenizer. The homogenate was centrifuged for 5 min at 300 × g followed by 30 min at 21,000 × g at 4°C. The supernatant (soluble protein) was removed and the crude membrane fraction (pellet) was resuspended in 0.5 mL homogenization buffer and stored at −80°C. Protein concentration was determined by the Lowry assay (21).

Production of antibodies to ZnT-1, ZnT-2 and ZnT-4. Peptides, predicted from the published mRNA sequences of ZnT-1 (GTRPOVHSKGE, LifeTechnologies) ZnT-2 (GKFNFIHTMTIQEGYSEDMKSCQECQGPSE, Genemed Synthesis, South San Francisco, CA) and ZnT-4 (QLIPGSSKWEEVQSKA, Genemed Synthesis,) were synthesized with an additional cysteine residue for conjugation to keyhole limpet hemocyanin (KLH) at the C-terminal end. Sequences were verified by amino acid analysis and mass spectrometry. KLH-conjugated peptides were injected into New Zealand White rabbits (1 mg peptide/rabbit) for polyclonal antibody production. Antibody specificity was verified by peptide competition analysis. Briefly, membrane protein (20 μg) from mammary gland at d 10 lactation was resolved and transferred as described below. After blocking, blots were incubated with primary antibody (1:10,000) ≥ 1 g/L peptide for 1 h. After incubation with secondary antibody, blots were visualized as described below.

Western blotting/Slot blotting. Equal amounts of mammary gland protein (20 μg) were resolved through 10% polyacrylamide SDS-PAGE gels under reducing conditions (ZnT-1, ZnT-2, ZnT-4) and transferred to nitrocellulose for 1 h at 300 mA or vacuum-applied to nitrocellulose (MT). Blots were blocked overnight at 4°C with 50 g/L nonfat milk in PBS-T. Blots were incubated with zinc transporter antibody (1:10,000), or a mouse monoclonal antibody to MT (1:2,000, Dako, Carpinteria, CA) for 1 h and washed 3 times in PBS-T. Blots were incubated with donkey-anti-rabbit immunoglobulin (IgG) conjugated to horseradish peroxidase (ZnT-1, ZnT-2, ZnT-4, Amersham Pharmacia Biotech) in 50 g/L nonfat milk or sheep-anti-mouse IgG conjugated to horseradish peroxidase (MT, Dako). Blots were visualized with chemiluminescence (Super Signal Femto, Pierce) and quantified using the Chemi-doc Gel Quantification System (Bio-Rad).

Statistical analysis. Results are presented as mean ± SD for the number of samples reported. Statistical analysis was performed using Prism Graphpad version 3.02 (San Diego, CA). Tests for interaction were made using two-way ANOVA and post-tested using the Bonferroni test. Significant effect of diet was determined by one-way ANOVA and post-tested using the Tukey test. Significance of difference was demonstrated at P < 0.05.

RESULTS

Antibody specificity. Specificity of antibodies generated against ZnT-1, ZnT-2 and ZnT-4 peptides was confirmed by the disappearance of specific immunoreactive bands after peptide coincubation (Fig. 1). Two immunoreactive bands were identified for ZnT-1 at approximately 46 kDa which responded similarly to peptide coincubation. One immunoreactive band for ZnT-2 (~46 kDa) and ZnT-4 (~42 kDa) was identified.

Identification and localization of zinc transporters in the mammary gland. Expression of ZnT-1, ZnT-2 and ZnT-4 was identified by RT-PCR performed on mRNA isolated from the mammary gland of control rats at d 10 of lactation. Immunostaining of mammary gland from control rats at d 10 of lactation was used to determine the general location of these zinc transporters. ZnT-1 was localized to the serosal membrane in multiple cell types of the mammary gland. ZnT-2 and ZnT-4 were localized intracellularly and primarily in the mammary epithelial cells lining the alveoli lumen (Fig. 2).

Effect of diet on plasma, milk and mammary gland zinc and retinol. Although there were no effects of diet on plasma, mammary gland or milk Zn concentration, there was a significant interaction between zinc and vitamin A on plasma Zn concentration such that in rats fed adequate dietary Zn, plasma Zn concentration was dependent upon dietary vitamin A (Table 1). There was a significant effect of zinc intake on plasma retinol (ROH) and a significant effect of vitamin A intake on mammary gland ROH. Additionally, mammary gland ROH concentration was dependent upon vitamin A intake in rats fed marginal Zn.

Effect of diet on zinc transporter mRNA expression and protein levels and MT protein levels. There were effects of diet on zinc transporter mRNA expression (Figs. 3A and B) and protein level (Figs. 4A and B) in the mammary gland. One ZnT-1 transcript (~2.6 kb) was identified in the mammary gland of lactating rats and there was no effect of diet on mRNA expression. Two immunoreactive bands were identified as ZnT-1 protein. Both bands responded similarly to dietary treatment. The effect of diet was more pronounced for the larger 80 kDa band; therefore, it was used for subsequent analysis. ZnT-1 was lower in rats fed the marginal Zn or vitamin A diets (P < 0.0001) compared with control rats and there was an interaction between zinc and vitamin A intake on ZnT-1 protein level (P = 0.04).

Two ZnT-2 transcripts (~4.5 and 2.3 kb) were identified in the mammary gland. Both bands responded similarly to dietary treatment and we chose the larger 4.5 kb for subsequent analysis. Rats fed the marginal Zn and vitamin A diet had higher ZnT-2 mRNA expression than rats fed other diets, and there was an interaction between Zn and vitamin A intake on ZnT-2 mRNA levels. There was no effect on ZnT-2 protein level.

Two ZnT-4 transcripts (~5.4 and 1.9 kb) were identified in the mammary gland. Both bands responded similarly to dietary treatment and we chose the larger 5.4 kb for subsequent analysis. Rats fed the marginal Zn or vitamin A diet had higher ZnT-4 mRNA expression and protein levels than controls and rats fed the diet marginal in zinc and vitamin A.

FIGURE 1 Representative Western blot of rat mammary gland membrane protein (20 μg) incubated with peptide-derived antibody with and without coincubation with ZnT-1, ZnT-2 or ZnT-4 peptide.
There was an interaction between zinc and vitamin A intake on ZnT-4 mRNA expression \((P < 0.0001)\).

There were also effects of zinc and vitamin A intakes on the amount of MT protein such that rats fed marginal Zn diets had lower \((P = 0.0002)\) and rats fed marginal vitamin A diets had higher \((P < 0.0001)\) MT protein levels in the mammary gland (immunoblot not shown). Additionally, there was an interaction between zinc and vitamin A intake on MT protein levels \((P = 0.02)\) in the mammary gland.

**DISCUSSION**

The resistance of mammary gland and milk zinc concentrations to alterations in dietary zinc indicates tight regulation of zinc homeostasis in the mammary gland \((22)\), or perhaps the presence of confounding inadequacies in micronutrients such as vitamin A. In this study, our model of chronic marginal zinc

| TABLE 1 | The concentration of plasma retinol (pROH) and zinc (pZn), mammary gland retinol (gROH) and zinc (gZn) and milk zinc (mZn) in lactating rats fed control (C), low zinc (ZD), low vitamin A (AD) and low zinc and vitamin A (DD) diets

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<th>Diet</th>
<th>pROH</th>
<th>pZn</th>
<th>gROH</th>
<th>gZn</th>
<th>mZn</th>
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<tr>
<td>C</td>
<td>1.64±0.28b</td>
<td>13.8±1.5a</td>
<td>0.44±0.11a</td>
<td>0.16±0.01a</td>
<td>0.23±0.04a</td>
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<td>ZD</td>
<td>2.36±0.34a</td>
<td>16.9±3.1a</td>
<td>0.61±0.23a</td>
<td>0.17±0.01a</td>
<td>0.25±0.04a</td>
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<tr>
<td>AD</td>
<td>2.13±0.46b</td>
<td>16.9±1.5a</td>
<td>0.42±0.04ab</td>
<td>0.17±0.01a</td>
<td>0.24±0.04a</td>
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<tr>
<td>DD</td>
<td>2.43±0.32a</td>
<td>15.0±1.5a</td>
<td>0.37±0.07b</td>
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Two-way ANOVA: Zinc × Vitamin A

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<tr>
<td>Vitamin A</td>
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\(1\) Values are means ± sd, \(n = 6\). Means in a column without a common letter differ, \(P < 0.05\).
and vitamin A intake during lactation did not result in zinc or vitamin A deficiency as assessed by conventional indicators. Therefore, we believe our results may reflect zinc nutriture in many human populations and document sensitive physiologic alterations that maintain zinc homeostasis in the mammary gland as well as an interaction between zinc and vitamin A during lactation before overt zinc or vitamin A deficiency.

Previously, we documented the uptake of zinc into human mammary epithelial cells in culture to be time and concentration dependent, illustrating a saturable (i.e., transporter-regulated) and a nonsaturable component to mammary gland zinc uptake (23). The mechanisms responsible for zinc transport in the mammary gland have not previously been determined; however, we propose that ZnT-1, ZnT-2 and ZnT-4 work in coordination to maintain milk zinc concentration (Fig. 5). In this study, we have shown that regulation of milk zinc concentration is transcriptionally and post-transcriptionally controlled through alterations in ZnT-1, ZnT-2 and ZnT-4 levels in the mammary gland, which may reflect subtle changes in cellular MT levels but not total zinc or retinol concentrations. During marginally low zinc intake, ZnT-1 mRNA expression remained constant. These results are in accordance with observations made by McMahon and Cousins (9) who found that even during severe zinc deficiency, with reduced plasma zinc concentration, ZnT-1 mRNA expression in tissues was not affected. However, a diet marginally low in zinc reduced ZnT-1 protein level in the mammary gland, thus allowing the mammary gland to reduce serosal zinc export through this ubiquitous transporter during low zinc intake.

Although the mechanism behind this decrease is unknown, Gitan et al. (24) showed that increased cellular zinc in yeast increases the ubiquitination of ZRT1, a yeast zinc import protein, and results in its removal from the plasma membrane and subsequent degradation to protect the yeast from overaccumulation of zinc. Although it remains to be determined whether ZnT-1 is regulated post-translationally in a similar manner, rats fed a diet marginally low in zinc also had a lower level of mammary gland MT without alterations in total mammary gland zinc, potentially increasing the ratio of zinc to MT and increasing the concentration of "labile" zinc affecting sensitive signaling pathways.

Marginal zinc intake dramatically increased both ZnT-4 mRNA expression and protein levels. Although the mechanisms responsible for ZnT-4 regulation remain to be determined, increased "labile" zinc, as a consequence of alterations in MT synthesis or degradation, or other physiologic adaptations affected by reduced dietary zinc, may play a role in ZnT-4 mRNA expression. ZnT-4 is most closely related to ZnT-2, (25) and a similar role for ZnT-4 in endosomal zinc transport was predicted (26). The tendency for reductions in ZnT-2 mRNA expression and protein levels (P = 0.08), concomitant with increased ZnT-4 mRNA expression and protein levels, suggests coordinated regulation to maintain milk zinc concentration. We propose that contrary to ubiquitous ZnT-1–mediated serosal zinc export and in addition to compartmentalized ZnT-2–mediated zinc transport, vesicular ZnT-4 participates in the import of zinc into endocytotic or secretory vesicles, which ultimately release zinc into milk. This integrated control of zinc transport by ZnT-1, ZnT-2 and ZnT-4 in the mammary gland would explain why a mutation in ZnT-4, which is presumed to be responsible for the lethal milk mutation in mice, reduces milk zinc by ~50% but does not eliminate milk...
zinc altogether. Our results in the mammary gland are in contrast to observations of others that reduced dietary zinc does not affect the expression of ZnT-4 mRNA in tissues such as small intestine, testes, liver, kidney or brain (27), indicating a specialized role for ZnT-4 in the mammary gland.

A number of studies have described negative effects of zinc deficiency on vitamin A status (1). In this study, we have for the first time documented specific effects of marginal vitamin A intake, in the absence of vitamin A deficiency, on zinc transport mechanisms in the mammary gland of lactating rats. During lactation, marginal vitamin A intake increased ZnT-4 mRNA expression and ZnT-4 protein levels. Although the mechanisms responsible for these effects are not understood, marginal vitamin A intake decreased total mammary gland retinol and increased MT levels without affecting mammary gland zinc concentration, suggesting that the effects on zinc transporters may be related to alterations in cellular retinol or zinc partitioning. There is evidence that vitamin A plays a role in mediating zinc transport, potentially through zinc transporter ZnT-1 by dietary zinc. Proc. Natl. Acad. Sci. U.S.A. 95: 4841–4846.

In this study, we have also for the first time documented an interaction between marginal zinc and vitamin A intake during lactation on zinc transporter mRNA expression in the mammary gland at the molecular level. However, the interaction between Zn and vitamin A intake on ZnT-2 and ZnT-4 mRNA levels was not translated into similar changes in protein levels. Interestingly, although zinc and vitamin A intake interacted to increase ZnT-2 mRNA expression, ZnT-2 protein levels had a tendency to be reduced (P = 0.06). Thus, a diet low in vitamin A and zinc resulted in reduced ZnT-1 but had no effect on ZnT-2 or ZnT-4 protein levels in the mammary gland, a different response from those in rats fed diets marginally low in Zn or vitamin A independently. The combination of lower ZnT-1 and ZnT-2-mediated zinc export in conjunction with higher ZnT-4-mediated zinc transport, potentially into intracellular secretory vesicles, appears to adequately sustain both cellular and milk zinc levels.

The results of this study indicate that diets marginal in zinc and vitamin A during lactation affect zinc transporter levels, and these changes occur in coordination to maintain adequate cellular and milk zinc concentrations. Additionally, although marginal zinc and vitamin A intakes independently appear to evoke similar homeostatic responses by the mammary gland to maintain milk zinc, these events may be regulated by different cellular mechanisms because MT was reduced by marginal Zn intake and increased by marginal vitamin A intake. Furthermore, a diet marginal in both zinc and vitamin A affected mammary gland zinc transporters differentially by decreasing ZnT-1 and not affecting ZnT-4 protein levels. These alterations are not mediated directly by reductions in total plasma zinc and retinol but most likely by subtle alterations in zinc- and retinol-responsive transcriptional, and post-transcriptional control mechanisms within the mammary gland itself. Together, these results suggest that marginal intake of vitamin A may alter intracellular retinol metabolism and cellular MT in the mammary gland, thus altering the regulation of zinc transport in the mammary epithelial cell, and may therefore play a role in mediating zinc partitioning in the mammary gland and subsequent zinc transport into milk. However, the mechanisms responsible for the uptake of zinc into the mammary gland and the effects of other nutritional inadequacies on their regulation remain to be determined.

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LITERATURE CITED