Transgenic Mice That Overexpress Metallothionein-I Resist Dietary Zinc Deficiency[1,2]

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ABSTRACT Transgenic mice that overexpress metallothionein-I (MT-I) accumulate more MT-I and zinc in major organs than do control mice. The effects of overexpression of MT-I on resistance to dietary zinc deficiency were examined by feeding transgenic and control mice a zinc-deficient (0.5–1.5 μg/g) or a zinc-adequate (50 μg/g) diet and by measuring effects on pregnancy. When pregnant mice were maintained under conditions of dietary zinc deficiency, the number of resorptions and teratogenic defects of fetuses was greatly reduced in transgenic compared with control mice. Differences between transgenic and controls were not apparent at d 8 of pregnancy (d 1 = vaginal plug) but were apparent by d 14. This result suggests that the larger maternal zinc pool in the transgenic females allows fetal development to progress normally for a longer period of time. However, neither transgenic nor control zinc-deficient mice could complete pregnancy. Pancreatic MT concentrations were the greatest in zinc-adequate transgenic mice. Moreover, there was >10-fold more MT per gram wet weight in the pancreas of transgenic mice than in any other organ examined. Pancreatic MT concentrations were an exceptionally sensitive indicator of zinc deficiency. Pancreatic MT declined 99.8% and zinc declined to basal levels by d 14 of pregnancy when transgenic and control mice were fed a zinc-deficient diet, whereas MT concentrations in other organs decreased only modestly. We suggest that the larger pool of zinc MT in the transgenic mice provides a biologically important labile pool of zinc during periods of zinc deficiency. J. Nutr. 126: 825–833, 1996.

INDEXING KEY WORDS:

• *metallothionein* • *transgenic mice*
• *zinc deficiency* • *pregnancy* • *pancreas*

Zinc is an essential metal in many biochemical processes (Vallee and Falchuk 1993, Walsh et al. 1994). It is required for the activity of hundreds of enzymes and DNA-binding proteins. It also modulates the activity of peptide hormones (e.g., growth hormone) and neurotransmitter receptors (Vallee and Falchuk 1993). In addition, zinc exerts antioxidant activity by protecting sulfhydryl groups and stabilizing cell membranes. Alcoholism, sickle cell disease, renal disease and penicillamine therapy and diets rich in phytate can each lead to zinc deficiency. Acrodermatitis enteropathica is a human genetic disorder of zinc absorption that results in severe zinc deficiency. As would be predicted from the diverse functions of zinc, a deficiency of this essential metal results in a wide spectrum of physiological effects, including disorders of the skin, neurological, immune and reproductive systems (Apgar 1985, Walsh et al. 1994).

Zinc deficiency exerts profound effects on reproductive processes (Vallee and Falchuk 1993, Walsh et al. 1994). In males, it leads to reduced spermatogenesis and fertilization capacity of spermatozoa. In females, it can cause reduced secretion of gonadotropins and altered ovarian development (Apgar 1985). Zinc requirements increase during the latter half of pregnancy coincident with the rapid growth of the fetus (Walsh et al. 1994), and in birds, fish and mammals, including humans, embryonic development is jeopardized by zinc deficiency (Apgar 1985). The rat is the most well-studied species with regard to effects of zinc deficiency on pregnancy (Vallee and Falchuk 1993). During the

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preimplantation period, zinc deficiency alters blastocyst morphology and reduces the implantation success rate. During the postimplantation period, it causes an increased incidence of resorption of the implantation site, and those embryos that survive show a high incidence of craniofacial and limb bud abnormalities and growth retardation (Apgar 1985).

Zinc metabolism is thought to be controlled by uptake, storage in peripheral tissues and secretion. Zinc absorption occurs via active transport in the brush boarder of the intestinal mucosa (Oestreicher and Cousins 1989). Uptake responds to dietary zinc and increases during periods of zinc deficiency and during pregnancy (Walsh et al. 1994). A zinc transporter that may serve to efflux zinc from the cell has recently been cloned (Palmiter and Findley 1995), and a candidate transmucosal zinc carrier protein has been isolated from the rat intestine (Hempe and Cousins 1991). The physiological roles of these proteins in zinc metabolism are unknown. Under normal conditions, nearly as much zinc is lost in the bile pancreatic secretions as is absorbed by the intestine (Walsh et al. 1994). Thus, it has been suggested that the pancreas plays a major role in zinc homeostasis (McClain 1990, Walsh et al. 1994). However, mechanisms regulating zinc secretion as well as those regulating use of zinc by peripheral tissues are not well understood.

The metallothioneins (MT)[4] play a central role in intracellular zinc homeostasis as suggested by Bremner (1991) and Cousins (1985). The MT are cysteine-rich metal-binding proteins that are predominantly isolated as $zinc_7$-MT complexes, although copper is also detected (Andrews 1990). The MT gene transcription is rapidly induced by zinc, and MT is expressed in major organs (liver, pancreas, kidney and intestine) that play roles in nutrient homeostasis, as well as in the reproductive tract during pregnancy (Andrews 1990, Andrews et al. 1993). In the mouse, four MT genes have been cloned (MT-I to MT-IV) (Palmiter et al. 1993b), but MT-I and MT-II are the most widely distributed MT isoforms, and MT-I is about twice as abundant as MT-II (Andrews 1990). In adult rats, zinc deficiency decreases MT concentrations dramatically in the pancreas and modestly in the kidney with little effect on the liver or brain (Onosaka and Cherian 1982). Maternal zinc supply is a major determinant of MT concentrations in neonatal rats (Andrews et al. 1987a). Despite these correlations suggesting a role for MT in zinc homeostasis, establishing a direct role for MT has not been accomplished. MT can activate apoenzymes that require zinc in vitro (Bremner 1991, Kagi 1991), but MT is not essential for biosynthesis of zinc-containing proteins (Masters et al. 1994).

As an approach to this problem, we have utilized a transgenic mouse strain that overexpresses MT-I transgenes (MT-I*) (Palmiter et al. 1993a) to examine the relationship between MT concentrations and effects of zinc deficiency. These transgenic mice express elevated concentrations of MT-I mRNA and protein in most major organs (Iszard et al. 1995), including liver, kidney, intestine and pancreas. MT-I is also overexpressed in the reproductive tract of both male and female mice (Iszard et al. 1995). During early pregnancy, MT-I is expressed in the uterus, deciduum, placenta and visceral yolk sac (Andrews et al. 1993), and evidence, to date, suggests that overexpression of the MT-I* transgenes in these transgenic mice occurs only in those cell types previously shown to actively express the endogenous MT-I gene (Dalton et al. 1995). The results of our studies herein establish that, based on several criteria, overexpression of MT protects against zinc deficiency in mice by providing a larger pool of bioavailable zinc.

MATERIALS AND METHODS

Animals and diets. All experiments involving animals were conducted in accordance with NIH guidelines for the care and use of experimental animals, and all animal experiments were approved by our Institutional Animal Care and Use Committee. Transgenic mice were derived from heterozygous males that carry 56 copies of a minimally mutated MT-I* gene on a B6/SJL F1 background (Palmiter et al. 1993a). These mice were outbred to CD-1 females (48–60 d old; Charles River Breeding Laboratories, Raleigh, NC), and the heterozygous transgenic and nontransgenic control littermates were identified by slot blot hybridization. Control and transgenic littermates were compared experimentally with each other and with CD-1 mice.

Mouse diets were all purchased from Harlan Teklad (Madison, WI). Zinc concentrations in the diets were as follows: zinc-deficient (Zn-D), 0.5–1.5 μg/g; zinc-adequate (Zn-A), 50 μg/g. These diets each contained ~18 μg/g Cu, and were otherwise identical (**Table 1**).

Zinc concentration of diets, deionized-distilled water and vacuum-dried tissue samples was determined by atomic absorption spectrophotometry performed in the University of Kansas Environmental Health Sciences Support Facility.

Experimental designs

Experimental Design 1. CD-1 females, or heterozygous transgenic and control male mice were housed three per cage on cedar bedding and had free access to the Zn-A or the Zn-D feed and deionized-distilled water. Under these conditions, substantial recycling of zinc via feces and bedding is expected to occur, thus this experimental condition was defined as moderate

[4] Abbreviations used: d, day of pregnancy; HO, heme oxygenase-I; MT-I, metallothionein-I; MT, metallothionein(s); PCR, polymerase chain reaction; Zn-A, zinc-adequate diet; Zn-D, zinc-deficient diet.

TABLE 1

Composition of diets[1]

Ingredient	Concentration
	g/kg diet
Egg white solids, spray-dried	200.0
Dextrose, monohydrate	634.2
Corn oil	100.0
Cellulose	30.0
Salt mixture[2]	25.7
Vitamin mixture[3]	10.0[4]
Biotin	0.004
Ethoxyquin (antioxidant)	0.02

[1] Shown is the Zn-deficient diet. In the Zn-adequate diet, zinc carbonate ($ZnCO_3$, 0.0893 g/kg) replaced an equal mass of cellulose.

[2] Salt profile (g/kg diet): $CaHPO_4$, 19.767; $MgSO_4$, 2.4752; KCl, 2.2882; NaCl, 0.7781; $FeSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.1662; $CuSO_4$, 0.0151; KIO_3, 0.0004; $CrK(SO4)_2 \cdot 12H_2O$, 0.02.

[3] Vitamin profile (mg/kg diet): *p*-aminobenzoic acid, 110.1; ascorbic acid, coated (97.5%), 1016.6; biotin, 0.44; vitamin B-12 (0.1% trituration), 29.7; calcium pantothenate, 66.1; choline dihydrogen citrate, 3496.9; folic acid, 1.98; inositol, 110.1; menadione, 49.5; niacin, 99.1; pyridoxine HCl, 22.0; riboflavin, 22.0; thiamin HCl, 22.0; dry retinyl palmitate, 39.65; dry cholecalciferol, 4.4; dry *dl*-α-tocopheryl acetate, 242.3.

[4] Corn starch (46.66%) serves as a diluent for the vitamin mixture.

dietary zinc deficiency. To determine effects on MT mRNA levels, tissues from three mice were collected on the indicated days of the study (up to d 27), pooled and processed immediately as described below. To determine effects on MT protein concentrations, tissue samples were collected and frozen in nitrogen and later assayed individually using samples from at least three mice. To assess effects on pregnancy, after 3 wk under these experimental conditions, CD-1 females (68–90 d old) were mated with CD-1 males or with heterozygous transgenic or control males, as indicated, and the morning of a vaginal plug was designated as d 1 of pregnancy. All mice used were plugged within the first 4 d of wk 4 of the diet. Mice were maintained under these experimental conditions until d 14 at which time the pregnancy rate, the total number of implantation sites and the number of resorbed and nonresorbed implantation sites per uterus were determined visually, and the morphology of nonresorbed fetuses was determined by examination under a dissecting microscope.

Experimental Design 2. Female heterozygous transgenic and control littermates (68–90 dys old) were housed in pairs in cages with stainless steel false bottoms to reduce recycling of zinc (Cook Mills and Fraker 1993). Mice had free access to the Zn-A or Zn-D feed and deionized-distilled water. Water bottles were washed in 4 mol/L HCl and rinsed in deionized water to remove zinc (Cook Mills and Fraker 1993). This experimental condition was defined as severe dietary zinc deficiency. After 3 wk under these experimental conditions, some mice were killed (n = 6), and the thymus weight (pooled weight), body weight gain of each mouse and pancreatic Zn and MT concentrations in two groups of three mice each were determined. To determine effects on pregnancy, after 3 wk under these experimental conditions, some mice (n as indicated) were mated with CD-1 males. All mice used were plugged within the first 4 d of wk 4 of the diet. Mice were maintained under these experimental conditions until d 14 at which time the pregnancy rate, the total number of implantation sites and the number of resorbed and nonresorbed implantation sites per uterus were determined visually, and the morphology of nonresorbed fetuses was determined by examination under a dissecting microscope.

Experimental Design 3. CD-1, heterozygous transgenic and control females (68–90 d old) were mated with CD-1 males or heterozygous transgenic males. On d 1 (vaginal plug) mice were placed under conditions of severe dietary zinc deficiency as previously described in Experimental Design 2. Daily intake of food was monitored in most experiments, and restricted groups that received the Zn-A diet in amounts equivalent to the mean of that consumed by the Zn-D transgenic or control mice were included as pair-fed controls. Effects of dietary zinc on pregnancy were examined on d 8 and d 14. On d 8 the percentage of animals with visible uterine implantation sites (pregnancy rate), the number of implantation sites per uterus and the gross morphology of these sites were recorded. On d 14 the pregnancy rate, the total number of implantation sites and the number of resorbed and nonresorbed implantation sites per uterus were determined visually, and the morphology of nonresorbed fetuses was determined by examination under a dissecting microscope. The genotype of the fetuses was determined by using the polymerase chain reaction (PCR) as described below. Effects of these experimental conditions on pancreatic MT mRNA and protein concentrations were determined on d 5, d 9 and d 14 in transgenic, control and CD-1 females that were pregnant or were found to be nonpregnant when killed. The MT mRNA concentrations were determined in two samples consisting of tissue pooled from three mice each that was processed immediately, as described below. The MT protein concentrations were determined in tissue samples collected and frozen in nitrogen and later assayed individually using six mice per point.

Cd-heme assay for MT

Pancreas, liver, kidney and testes were collected from individual mice or pooled from three mice and frozen in liquid nitrogen. Steady-state concentrations of total MT were quantitated using the Cd-Heme exchange assay (Eaton and Cherian 1991).

RNA isolation and Northern blot hybridization

Pancreas samples from three mice (0.7 g) were collected and immediately homogenized in 5 mol/L guanidine isothiocyanate (Han et al. 1987), and RNA was prepared as described (McMaster et al. 1992). Liver RNA was isolated by SDS/phenol/chloroform extractions as described (Andrews et al. 1987b). Total RNA (2 μg in 5 μL) was size-separated by electrophoresis in a 1.5% agarose-formaldehyde gel, transferred and cross-linked to nylon membranes (Dalton et al. 1994). Northern blots were prehybridized, hybridized and washed as described (Dalton et al. 1994). Hybrids were detected by autoradiography at −70°C with intensifying screens and quantitated by radioimage analysis using the radioanalytic image system (Ambis Systems, San Diego, CA). In all experiments, duplicate gels were stained with acridine orange to verify integrity and equal loading of RNA.

Mouse MT-I and rat heme oxygenase-I (HO) (A. Smith, University of Missouri-Kansas City, Kansas City, MO) cDNAs were inserted into pSP6 or pGEM vectors (Promega Biotec, Madison, WI) and used as templates for the synthesis of ^{32}P-labeled cRNA probes as described (Melton et al. 1984). Probes had specific radioactivities of ~30 mBq/μg.

Genotyping of fetuses by PCR

The PCR was used to determine the genotype of individual fetuses based on the MT-I transgene using primers and reaction conditions as described (Lee et al. 1996).

Statistical analysis

Statistical significance was determined using analysis of variance and Student's t-test, χ^2 analysis and 2-way ANOVA, as indicated. Differences were considered significant when the P-value was < 0.05. Values are given as means ± SD and P-values are presented in the text and tables.

RESULTS

Effects of zinc deficiency on pregnancy in control and transgenic mice. Mice were analyzed according to Experimental Design 1 in which substantial recycling of zinc is expected to occur. Prolonged maintenance of mice under these experimental conditions of moderate zinc deficiency resulted in some parakeratosis and hair loss after 60 d, particularly in the control animals, but it had no other apparent adverse effects. Reproductive functions under these experimental conditions were examined in CD-1 mice. At d 14 of pregnancy no effects on pregnancy rate or on the number of implantation sites per uterus were noted, and there was no difference in postimplantation embryo lethality (resorbed embryos) (**Table 2**). However, an effect on morphogenesis was noted. Zinc deficiency increased the number of embryos with craniofacial, limb bud and neural tube defects from 1% to 13% ($P < 0.01$). Nonetheless, under these experimental conditions mice could give birth to live young. These results showed that morphogenesis of the postimplantation embryo is a sensitive indicator of moderate zinc deficiency.

TABLE 2

Effects of 3 wk of moderate dietary zinc deficiency on subsequent pregnancy in CD-1 mice

Diet[1]	n	Pregnancy rate[2]	Implantation sites per uterus[3]	Resorption rate[4]	Abnormal morphology[5]
		%		%	
Zn-A	12	92	14 ± 3	2 (3/159)	1 (1/88)
Zn-D	12	92	12 ± 3	3 (5/159)	13 (13/100)*

[1] This experiment was performed according to Experimental Design 1. Zinc concentrations in the diets were as follows: Zn-A (zinc-adequate diet), 50 μg/g; Zn-D (zinc-deficient diet), 0.5–1.5 μg/g.

[2] Mice were killed on d 14 of pregnancy, and the number of mice actually pregnant divided by the number of mice plugged equals the pregnancy rate.

[3] The total number of implantation sites per uterus (mean ± SD).

[4] The number of implantation sites that had obviously resorbed divided by the total number of implantation sites.

[5] Craniofacial, neurotube and limb bud development were examined under a dissecting microscope, and the number of obviously abnormal embryos was recorded and is divided by the total number of embryos examined. * $P < 0.01$; χ^2 analysis.

Female transgenic and control mice were studied by using Experimental Design 2 in which recycling of zinc is reduced. At midpregnancy after 3 wk of severe zinc deficiency, the number of implantation sites per uterus was not affected (not shown). However, an effect on fetal viability (resorption rate) by d 14 was noted (**Table 3**). In zinc-deficient control mice, 57% of the implanted embryos had died by d 14, and the majority of the remaining embryos were malformed. In contrast, a significantly lower ($P < 0.001$) percentage (21%) of the fetuses in the zinc-deficient transgenic females died by d 14 (Table 3), and the majority of the viable embryos appeared morphologically normal (not shown). Despite these differences between control and transgenic mice at d 14, none of these zinc-deficient mice successfully carried pregnancy through parturition.

Effects of severe dietary zinc deficiency beginning on d 1 of pregnancy were examined in transgenic and control mice according to Experimental Design 3. Reproductive functions were examined on d 14 (**Table 4**). In parallel experiments, transgenic and control mice were pair-fed [4 g food/(mouse × day)]. The pregnancy rates in the pair-fed mice were generally low in these experiments. However, zinc deficiency beginning on d

TABLE 3

Effects of 3 wk of severe dietary zinc-deficiency on subsequent pregnancy in transgenic and control mice

Strain	Diet[1]	n	Pregnancy rate[2]	Resorption rate[3]
			%	
Transgenic	Zn-A	6	100	1 (1/86)
	Zn-D	9	100	21 (12/56)
Control	Zn-A	6	100	2 (1/54)
	Zn-D	12	75	57* (65/114)

[1] This experiment was carried out according to Experimental Design 2. Dietary zinc levels were as follows: Zn-A (zinc-adequate diet), 50 μg/g; Zn-D (zinc-deficient diet), 0.5–1.5 μg/g.

[2] The number of mice actually pregnant at d 14 divided by the number of mice plugged equals the pregnancy rate.

[3] The number of implantation sites that had obviously resorbed divided by the total number of implantation sites. * $P < 0.001$ (*t*-test) relative to Zn-D transgenic.

1 had no effect on the number of implantation sites per uterus at d 14 in any of these experimental groups (Table 4). In contrast, a dramatic difference between control and transgenic mice was noted in the resorption rate of postimplantation embryos ($P < 0.001$). Transgenic mice were unaffected by zinc deficiency and only 8% of the implanted embryos in the Zn-D pair-fed; and 4% in the freely fed Zn-D group had died by d 14, whereas in the Zn-D control mice, ~60% of the implanted embryos had died by d 14 in both the freely fed and pair-fed groups. Furthermore, none of the fetuses from Zn-D transgenic females were morphologically abnormal, whereas 55% of the surviving fetuses in Zn-D control females were morphologically abnormal and exhibited craniofacial, limb bud and neural tube defects (Table 4). Interestingly, gross examination of implantation sites at d 8 revealed no differences between Zn-D control and transgenic mice. In Zn-D control females, there were 15 ± 3 ($n = 3$) implantation sites per uterus, and none (0/46) of the d 8 implantation sites appeared to be grossly abnormal. As in the previous experiment, neither control nor transgenic females successfully carried pregnancy through parturition under these severe zinc deficiency conditions.

The low rate of fetal resorption and malformation in the transgenic mice indicates that transgenic embryos are not preferentially resistant to zinc deficiency, because only 50% of them are transgenic. However, to further test that possibility, CD-1 females were mated with heterozygous transgenic males and analyzed according to Experimental Design 3. Embryos (d 14) that had not been resorbed were genotyped by DNA analysis. In the Zn-A group 55% (22/40) of the embryos were transgenic and in the Zn-D group (44%) (22/50) of the surviving d 14 embryos were transgenic ($P > 0.05$).

Effects of zinc deficiency on MT and zinc concentrations in control and transgenic mice

The effects of moderate dietary zinc deficiency (Experimental Design 1) on concentrations of MT in major organs in heterozygous male transgenic and control littermates were examined. The MT concentrations were significantly greater ($P < 0.05$) in each of the major organs examined in transgenic males compared with controls (**Table 5**). During the course of zinc deficiency,

TABLE 4

Effects of severe dietary zinc deficiency beginning on d 1 of pregnancy in transgenic and control mice

	Diet[1]	n	Pregnancy rate[2]	Implantation sites per uterus[3]	Resorption rate[4]	Abnormal morphology[5]
			%		%	*
Strain						
Transgenic	Zn-D	13	77	12 ± 3	4 (5/112)	
Control	Zn-D	9	100	12 ± 5	60 (65/109)*	
Pair-fed						
Transgenic	Zn-A	6	33	13	8 (2/26)	0 (0/24)
	Zn-D	6	50	12 ± 0	8 (3/36)	0 (0/33)
Control	Zn-A	5	60	12 ± 2	3 (1/36)	0 (0/35)
	Zn-D	6	83	13 ± 2	57 (36/63)*	55 (15/27)

[1] Mice were analyzed according to Experimental Design 3. On d 1 mice were fed the Zn-A (zinc-adequate; 50 μg/g) or Zn-D (zinc-deficient; 0.5–1.5 μg/g) diet. In parallel experiments, transgenic and control mice received the zinc-adequate diet in amounts equivalent to average of that consumed by the zinc-deficient mice (pair-fed).

[2] The number of mice actually pregnant on d 14 divided by the number of mice plugged equals the pregnancy rate.

[3] The total number of implantation sites per uterus for each mouse (mean ± SD).

[4] The number of implantation sites that had obviously resorbed divided by the total number of implantation sites.

[5] Craniofacial, neurotube and limb bud development were examined under a dissecting microscope, and the number of obviously abnormal embryos was recorded and is divided by the total number of embryos examined. * $P < 0.001$ (χ^2) relative to Zn-D transgenic.

TABLE 5

Effects of moderate dietary zinc deficiency on steady-state levels of metallothionein in transgenic and control male mice

Strain	Organ[3]	Metallothionein[1] Day of diet[2] 0	9	18	27
		nmol/g tissue			
Transgenic	Pancreas	215.0 ± 16*	196.0	185.0	103.0
	Liver	3.0 ± 0.8*	5.8	10.0	5.2
	Kidney	2.7 ± 1.0*	1.8	2.0	2.4
	Testes	17.8 ± 3.3*	22.0	19.0	16.0
Control	Pancreas	8.8 ± 2.1	5.5	0.8	1.2
	Liver	1.0 ± 0.7	2.5	2.2	2.1
	Kidney	0.6 ± 0.7	nd	1.2	nd
	Testes	3.4 ± 0.4	nd	5.5	nd

[1] Transgenic and control male littermates were analyzed according to Experimental Design 1. Samples were analyzed in triplicate for MT concentrations using the Cd-heme exchange assay (Eaton and Cherian 1991). Values are expressed as nmol MT per g organ wet weight ± SD for the d 0 point. Other values are the means of a triplicate assay. nd, not determined. * $P < 0.05$ (*t*-test) relative to control mice.

[2] Zinc concentrations in the zinc-deficient diet were 0.5–1.5 μg/g.

[3] These organs were pooled from three animals per group on the indicated day of the experiment. On d 0, three separate groups of three mice each were examined.

MT concentrations in kidney and testes were unaffected, whereas liver MT appeared to increase in both the transgenic and the control mice. The most dramatic effect of the Zn-D diet was on pancreatic MT in control mice, where concentrations were reduced 90% by d 18 compared with d 0. In contrast, pancreatic MT was only reduced 50% by d 27 of the diet in the transgenic mice. However, this represents a 13.7-fold greater loss of MT per gram pancreas in the transgenic mice (112 nmol MT/g pancreas) compared with the controls (7.6 nmol MT/g pancreas).

Effects of 3 wk of severe zinc deficiency (Experimental Design 2) on thymus weight and body weight gain, classical indicators of zinc deficiency (Cook Mills and Fraker 1993), and changes in pancreatic MT were determined (**Table 6**). Comparisons of thymic weights and body weight gain during the experiment demonstrated effects of zinc deficiency but revealed no significant differences between transgenic and control mice. Pancreatic MT protein (and MT mRNA levels; not shown) was reduced 90% in transgenic and 98% in control female mice after 3 wk of zinc deficiency ($P < 0.003$) (Table 6). Hepatic MT protein and mRNA concentrations were reduced only 75% in transgenic and control mice after 3 wk of severe zinc deficiency (not shown).

Effects of zinc deficiency on pancreatic MT and zinc concentrations during pregnancy

When pregnancy was superimposed on severe dietary zinc deficiency (Experimental Design 3), the depletion of pancreatic MT mRNA and protein were even more pronounced at d 14 ($P < 0.0001$) (**Fig. 1**). In nonpregnant transgenic mice, pancreatic MT protein concentrations had decreased 64% by d 14 of the Zn-D diet ($P < 0.0001$), whereas in pregnant Zn-D transgenic mice, pancreatic MT protein concentrations fell precipitously between d 9 and 14, to concentrations 99.8% lower than those found at the initiation of the experi-

TABLE 6

Effects of 3 wk of severe dietary zinc deficiency on thymus weight, body weight gain and pancreatic metallothionein concentrations in transgenic and control female mice

Strain	Diet[1]	Thymus weight[2]	Body weight gain[3]	Pancreas metallothionein[4]
		mg	*g/3 wk*	*nmol/g tissue*
Transgenic	Zn-A	61	2.1 ± 1.4	320 ± 99
	Zn-D	47	1.2 ± 1.1*	28 ± 48†
Control	Zn-A	55	2.5 ± 1.9	18.0 ± 7.8
	Zn-D	34	0.7 ± 1.5*	0.7 ± 0.63†

[1] Transgenic and control female littermates were analyzed according to Experimental Design 2. Zinc levels in the diets were as follows: Zn-A (Zn-adequate diet), 50 μg/g; Zn-D (Zn-deficient diet), 0.5–1.5 μg/g.

[2] The thymus weight was determined on the pooled samples ($n = 6$).

[3] Body weight gain was recorded for each mouse (mean ± SD).

[4] Pancreatic metallothionein was determined using the Cd-heme exchange assay (Eaton and Cherian 1991). Values shown are the mean nmol metallothionein per g pancreas wet weight ± SD. * $P < 0.05$ (2-way ANOVA) relative to Zn-A, but no significant difference between transgenic and control. † $P < 0.0003$ (2-way ANOVA) relative to Zn-A and between transgenic and control.

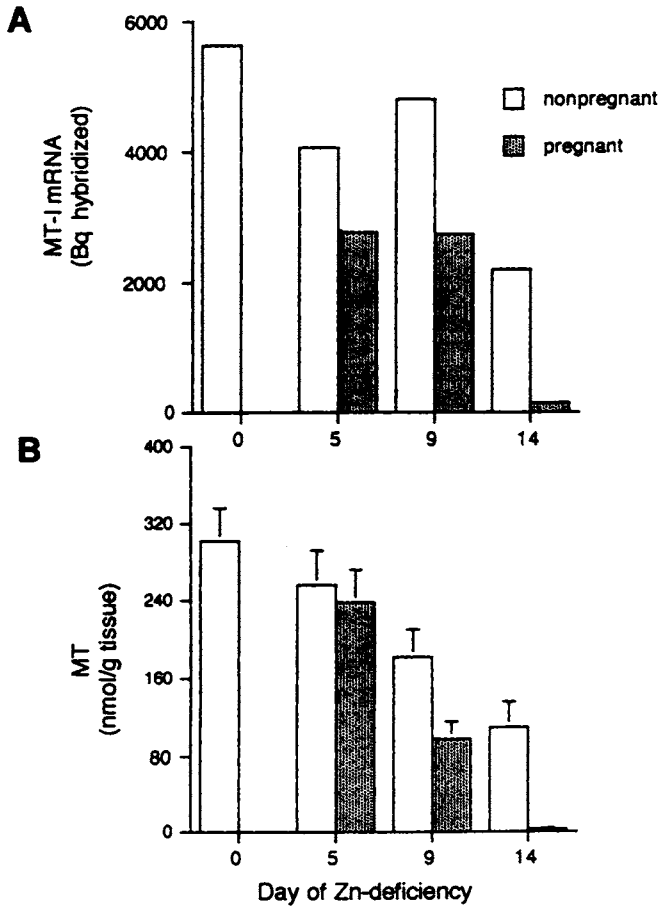

FIGURE 1 Effects of pregnancy on pancreatic metallothionein mRNA and protein concentrations in transgenic mice fed a severe zinc-deficient (Zn-D) diet. Transgenic mice were bred with CD-1 males and analyzed according to Experimental Design 3. (*A*) mice were killed and pregnancy was assessed by visual examination of the uterus. Pancreatic RNA samples from pregnant or nonpregnant mice (3 per group) were analyzed by Northern blotting as described in Materials and Methods. Hybrids were quantitated by radioanalytic analysis of membranes and values shown represent the mean of two groups of three mice each. Blots were stripped and rehybridized with an HO probe that showed a constant signal in all lanes. (*B*) pancreata were collected from individual pregnant or nonpregnant mice and assayed for MT as described in Materials and Methods. Values for MT are expressed as means ± SD (n = 6). Relative to d 0 control, pancreatic MT levels were significantly reduced in nonpregnant mice at d 14 ($P < 0.0001$, t-test). Relative to d 14 pancreatic MT in nonpregnant mice, that in pregnant mice on d 14 was significantly reduced ($P < 0.0001$, t-test).

ment (Fig. 1B). Changes in pancreatic MT mRNA concentrations mirrored those of MT protein (Fig. 1A). In these mice, concentrations of hepatic MT were not affected by pregnancy or dietary zinc deficiency (data not shown). In control mice, 14 d of zinc deficiency caused a 98% decrease in pancreatic MT ($P < 0.0002$) and a 99.2% decrease in MT mRNA in pregnant (**Fig. 2**), as well as in nonpregnant females (data not shown).

The relationship between pancreatic MT and zinc concentrations was examined in control and transgenic mice using Experimental Design 2 (**Table 7**). In transgenic mice fed the Zn-A diet, pancreatic zinc concentrations were 2.4-fold higher than those in similarly fed control mice, whereas after 3 wk of severe zinc deficiency, zinc concentrations were 30% higher in transgenic compared with control mice. Thus, transgenic mice lost more zinc from the pancreas during zinc deficiency than did control mice. In transgenic mice, MT accounted for binding of > 90% of the zinc in the pancreas under Zn-A conditions, whereas in control mice MT-bound zinc represented only 20% of the total cellular zinc pool. In transgenic mice, the loss of pancreatic MT during 3 wk of zinc deficiency accounted for all of the zinc lost from this tissue. In control mice the loss of MT accounted for 41% of the lost zinc.

DISCUSSION

Profound effects of zinc deficiency on pregnancy and embryogenesis in the mouse were noted as was predicted from studies of other species (Apgar 1985, Bedwal and Bahuguna 1994, Vallee and Falchuk 1993). Although development of preimplantation embryos in vitro is influenced by zinc (Apgar 1985, Bedwal and Bahuguna 1994), severe zinc deficiency during preg-

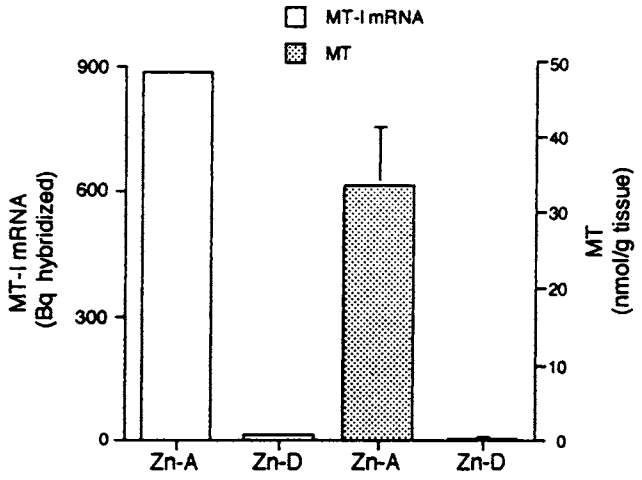

FIGURE 2 Effects of severe dietary zinc deficiency beginning on d 1 of pregnancy on pancreatic metallothionein mRNA and protein concentrations in control mice at midpregnancy. CD-1 female mice were bred with males of the same strain and then analyzed according to Experimental Design 3. Mice were fed a zinc-adequate (Zn-A) or a zinc-deficient (Zn-D) diet. On d 14, pancreatic tissue was assayed for MT as described in Materials and Methods. Values for MT are expressed as means ± SD (n = 6). The MT was significantly lower in the Zn-D mice relative to the Zn-A mice ($P < 0.0002$, t-test). The MT mRNA was detected in pancreatic RNA by Northern blotting, and hybrids were quantitated by radioanalytic analysis of membranes. Values shown represent the mean of the two samples. Duplicate gels were stained with acridine orange to examine RNA integrity and loading. Blots were stripped and rehybridized with the HO probe, which showed a constant signal in all lanes (not shown).

TABLE 7

Pancreatic zinc and metallothionein concentrations after 3 wk of severe dietary zinc deficiency in transgenic and control mice

Strain	Diet[1]	Total zinc[2]	Zinc bound to metallothionein[3]	ΔZinc	ΔZn bound to metallothionein
		μmol/g			
Transgenic	Zn-A	2.23	2.05		
	Zn-D	0.45	0.21	1.75	1.8
Control	Zn-A	0.66	0.126		
	Zn-D	0.35	0.003	0.31	0.127

[1] Transgenic and control female littermates were analyzed according to Experimental Design 2. Zinc levels in the diets were as follows: Zn-A (zinc-adequate), 50 μg/g; Zn-D (zinc-deficient), 0.5–1.5 μg/g.

[2] Zinc levels were determined in two groups of three pancreata each by atomic absorption spectrophotometry and are expressed per wet weight of the tissue.

[3] Metallothionein levels were determined by the Cd-heme exchange assay (n = 6) (Eaton and Cherian 1991) and are expressed as μmol metallothionein per gram pancreas wet weight (see Table 6). The amount of zinc bound to metallothionein was calculated assuming seven atoms of zinc per molecule of metallothionein (Andrews 1990).

nancy had little effect on pregnancy rate or the number of postimplantation embryos per female. Major detrimental effects of zinc deficiency in vivo occur during the period of organogenesis and rapid growth (d 8–12), as shown previously in rats (Hurley et al. 1971). The majority of embryos die and are reabsorbed by midgestation, and most of the surviving embryos are malformed under conditions of severe zinc deficiency.

Mammalian MT has been hypothesized to function in zinc and copper homeostasis (Bremner 1991). Zinc is essential for numerous biological processes, but a null mutation of both the MT-I and MT-II genes is not lethal, nor do these mice exhibit symptoms of zinc deficiency when reared on a normal diet (Masters et al. 1994). We studied transgenic mice that overexpress MT-I (Palmiter et al. 1993a) to test the function of MT in zinc homeostasis and found that fetuses of these transgenic mice are markedly protected from the embryotoxic and teratogenic effects of zinc deficiency. The results suggest that MT serves as a storage depot for a pool of zinc that can be used to supply the embryo or fetus during pregnancy.

The transgenic mice employed in this study have 56 copies of the MT-I gene (Palmiter et al. 1993a). Increased transcription of these genes is assumed to lead to increased apo-MT and, therefore, the accumulation of zinc-MT in the tissues of these transgenic mice. That increased apo-MT is essential for increased hepatic uptake of zinc after LPS injection was recently demonstrated in mice with disruptions of the MT-I and MT-II genes (Philcox et al. 1995). Our findings indicate that during zinc deficiency, zinc bound to MT represents a bioavailable pool of zinc and that concentrations of MT decline as the need for zinc increases. This idea is supported by previous studies demonstrating that MT concentrations are reduced in many organs during zinc deficiency in adults and newborns (Andrews et al. 1987a, Bremner 1991). This was most dramatically demonstrated in the pancreas of transgenic mice that accumulates high concentrations of zinc as zinc-MT (Table 7). Furthermore, the loss of pancreatic zinc during periods of zinc deficiency is accounted for by the loss of MT. This is particularly striking during the last half of pregnancy when increased zinc uptake by the conceptus (Bedwal and Bahuguna 1994) is coincident with a dramatic (99.5%) decline in maternal pancreatic MT in transgenic mice. This decrease did not occur in transgenic mice that were not pregnant. Thus, pancreatic MT concentrations were remarkably sensitive to dietary zinc and zinc demands during pregnancy.

The dynamic regulation of MT in the pancreas may reflect its hypothesized role in zinc homeostasis (McClain 1990). Concentrations of pancreatic MT are a sensitive indicator of zinc status. Thus, the pancreatic zinc-MT pool is labile and may contribute to zinc efflux pathways. Under conditions of zinc deficiency, preexisting MT may provide zinc for biological processes, resulting in the gradual depletion of the zinc-MT pool. Zinc lost from the pancreas during zinc deficiency may be released directly into the bloodstream via zinc efflux (Palmiter and Findley 1995) from the basal surface of acinar cells or more likely, it may be lost via pancreatic secretion (McClain 1990). We recently detected MT in pancreatic secretions (De Lisle and Andrews et al. unpublished data). Zinc in pancreatic secretions may then be reabsorbed by the intestines (Oestreicher and Cousins 1989).

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