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Modulation of Growth Axis Gene Expression by In Utero and Lactational Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) in the Weaning Holtzman Rat

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While the *in utero* and lactational effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on both male and female reproductive systems appear to be severe, little is known about its effects on the developing growth axis. The objective of this study was to describe changes in growth axis gene expression that accompany exposure to TCDD during *in utero* and lactational development. Pregnant Holtzman rats were administered 1 μg TCDD/kg maternal body weight or vehicle control on gestational day 15 by gavage. Using ribonuclease protection assays, we compared mRNA levels measured in 21-d-old female pups exposed to TCDD with levels measured in control animals for the following genes: somatostatin, growth hormone-releasing hormone (GHRH), hypothalamic and pituitary galanin (GAL), growth hormone (GH), and insulin-like growth factor-I (IGF-I). Serum GH concentrations measured by radioimmunoassay were significantly increased, although GH mRNA levels were unchanged from controls by TCDD exposure. Hypothalamic GAL mRNA was decreased in TCDD-treated animals, whereas pituitary GAL mRNA in TCDD-treated animals was not altered. GHRH mRNA was increased in hypothalami from TCDD-exposed animals. IGF-I mRNA in the liver was decreased to 67% of controls. These data indicate that the growth axis is sensitive to the effects of TCDD delivered during critical periods of development. The alterations observed in growth axis gene expression with exposure to TCDD add to the body of data demonstrating a potent effect of this compound on the fetal and neonatal endocrine system.

Key Words: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GH; GHRH; somatostatin; IGF-I; galanin; in utero; lactation.

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Introduction

The environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin), formed during various incineration procedures (Rappe et al., 1979), is a potent disruptor of vertebrate homeostasis (Peterson et al., 1993). TCDD is a promoter of animal neoplasias (Poland and Knutson, 1982), although the adverse effects to humans remains largely unknown (Collins et al., 1992). In addition to its carcinogenicity, TCDD induces a variety of other toxic responses, for example: severe weight loss, thymic atrophy and immune suppression (Greenlee et al., 1985, Smialowicz et al., 1994), fetotoxicity, and teratogenicity (Schewtz et al., 1973). Evidence suggests that the actions of TCDD are mediated by a soluble cytoplasmic receptor termed the aromatic hydrocarbon receptor (AhR; reviewed in Hankinson, 1995). AhR acts as a ligand-activated transcription factor to induce perturbations in target gene expression (Burbach et al., 1992).

The endocrine responses of adult mammals given acute doses of TCDD vary depending on the hormone and the species, strain, age, and sex of animals studied. In adult male Sprague-Dawley rats given acute graded doses of 0–100 μg TCDD/kg, prolactin (PRL) and testosterone were decreased, whereas growth hormone (GH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and adrenocorticotropic hormone (ACTH) were not altered (Moore et al., 1985, 1989). In adult female rats, TCDD affects the estrogen signaling system, either via induction of cytochrome P450 enzymes that metabolize estrogen (Gierthy et al., 1993), or by decreasing estrogen receptors. For example, in Long-Evans rats, uterine and heptic estrogen receptors were decreased (Romkes et al., 1987), whereas in the Sprague-Dawley strain of rat, TCDD increased hepatic estrogen receptors (Hruska and Olson, 1989). Whereas it is unknown which effects are direct and which are secondary, it is possible that small doses of TCDD result in a cascade of responses, resulting in the appearance of systemic endocrine disruption (Umbreit

and Gallo, 1988). Contrary to the data in the adult model, where certain species are relatively resistent to TCDD-induced toxicity (e.g., hamster [Henck et al., 1981]), TCDD appears to have similar potency among species when the exposure occurs *in utero* and through lactation (Peterson et al., 1993).

Exposure to TCDD during critical periods of development results in a range of endocrine dysfunction in both male and female rats. For example, male Holtzman rats born to dams given a single oral dose of 1 μg TCDD/kg maternal body weight on gestational day 15 exhibited altered sexual differentiation, feminized and demasculinized sex behavior, female-like LH patterns of secretion, decreased anogenital distance, and decreased number of tesiticular and epididymal sperm (Mably et al., 1992a, 1992b, 1992c; Bjerke et al., 1994). Gray et al., (1995) replicated these studies using Long-Evans rats, finding alterations in sex behavior and decreased sperm counts. It was also reported that male pups treated in a similar fashion had a 3.6-d delay in puberty as measured by preputial separation (Gray et al., 1995). Testosterone concentrations were normal in rats exposed to TCDD, as were in vitro testosterone synthesis and androgen receptor levels (Gray et al., 1995). Changes in the testosterone levels early in development do not seem to be a causative factor in the alterations to the male reproductive system.

Similar effects occur in female rats. Serum estrogen concentrations were significantly reduced and ovarian, uterine, pituitary, and hypothalamic estrogen receptor expression was altered on postnatal day 21 in female offspring of dams treated with TCDD on day 15 of gestation (Chaffin et al., 1996). The onset of puberty in females exposed *in utero* and via lactation was delayed as measured by day of vaginal opening (Gray and Ostby, 1995). This exposure paradigm resulted in pups of both sexes that exhibited reduced body weight vs their control counterparts on postnatal day 21–22 (Mably et al., 1992a, Gray and Ostby, 1995).

Whereas adult male rats did not exhibit alterations in a single-time point measurement of serum GH following TCDD exposure (Moore et al., 1989), the mechanisms of decreased neonatal body weight may involve alterations in the growth axis. In the rat, GH synthesis and secretion is regulated by two hypothalamic factors, GHRH, which is stimulatory and somatostatin, which is inhibitory (Brazeau et al., 1972; Wehrenberg et al., 1982). Control of GH secretion from the anterior pituitary is also at least partly regulated by the peptide GAL, which acts as a GH secreteagoge (Bauer et al., 1986). In turn, circulating GH acts to stimulate IGF-I production at the liver, which is a regulator of linear growth, especially during puberty, and metabolic function (Sara and Hall, 1990).

The effects of *in utero* and lactational exposure to TCDD on the growth axis remain uncharacterized. As part of our efforts to survey the endocrine changes induced by this exposure paradigm to TCDD, we describe alterations in the growth axis genes for GH, somatostatin, GHRH, GAL, and IGF-I in the 21-d-old female rat.

Results

Pregnant female dams given 1 μg TCDD/kg body weight on gestational day 15 did not exhibit signs of overt toxicity, and pups were born at the expected time. Offspring mortality, as assessed by visual inspection, was 0 and 10% in control- and TCDD-exposed pups, respectively. *In utero* and lactational exposure to TCDD had varied effects on hypothalamic mRNA levels in female offspring sacrificed on day 21 of age. GHRH mRNA from TCDD-exposed animals was increased compared to vehicle-exposed rats (Fig. 1) whereas somatostatin mRNA was decreased by TCDD exposure (Fig. 2). Hypothalamic GAL mRNA was also decreased by TCDD exposure (Fig. 3).

TCDD exposure did not alter pituitary mRNA levels for GH or GAL in TCDDexposed female pups (Figs. 4 and 5). Serum GH concentrations were 3.4 ng/mL ± 0.7 ($n = 7$) in nonexposed females and 6.5 ng/mL ± 1.4 ($n = 7$; $p < 0.05$, t-test) in TCDD-exposed animals.

In order to assess the potential for TCDD to alter the physiologic actions of GH, we examined the regulation of IGF-I mRNA in the liver. Treatment of the dams with TCDD resulted in a 33% decrease of hepatic IGF-I mRNA in exposed vs nonexposed 21-d-old female pups (Fig. 6).

Discussion

Previous studies have shown that *in utero* and lactational exposure to TCDD on gestational day 15 results in male and female pups with lower body weight and delayed puberty (Mably et al., 1992a; Gray et al., 1995; Gray and Ostby, 1995). Control of linear growth and metabolism is influenced by multiple endocrine factors, most important are somatostatin, growth hormone-releasing hormone, and galanin at the hypothalamic level, and growth hormone from the anterior pituitary, and IGF-I from the liver. Disruption at any point in this pathway may lead to altered cellular metabolism or growth abnormalities that may result in phenotypic changes such as growth retardation. We have shown here that exposure of female rats to TCDD during *in utero* and lactational development results in changes to mRNA levels of GHRH, hypothalamic GAL, somatostatin, and IGF-I, and no change in GH mRNA or pituitary GAL mRNA.

GHRH mRNA was increased in TCDD-exposed animals compared with nonexposed animals. There is one consensus dioxin response element at –92 in the first 450 base pairs of the upstream region relative to the transcriptional start site of GHRH (Mayo et al., 1985). This leads us to hypothesize that a direct effect of TCDD on GHRH is possible (Montiminy et al., 1984; McLane and Whitlock, 1994).

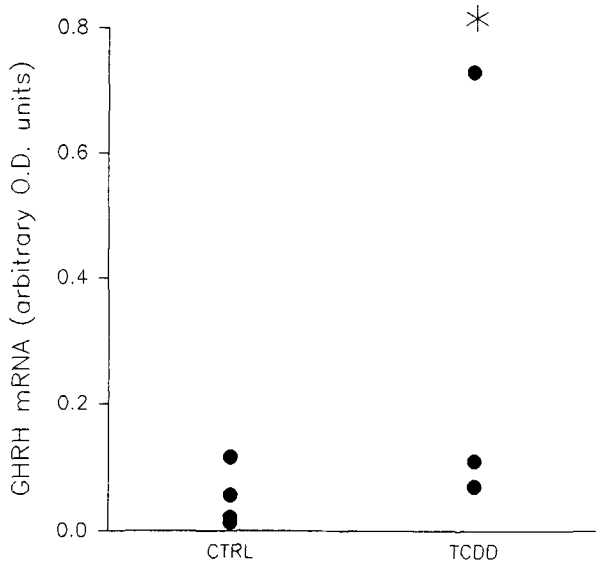

Fig. 1. *In utero* and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increases GHRH mRNA. Levels of growth hormone-releasing hormone (GHRH) messenger RNA from hypothalami of 21-d-old females exposed perinatally to vehicle (CTRL, $n = 4$) or TCDD ($n = 3$) were measured by RNase protection assay (*see* Methods). Values represent the distribution of data (normalized to 18s rRNA) points that was tested by Mann-Whitney U test.

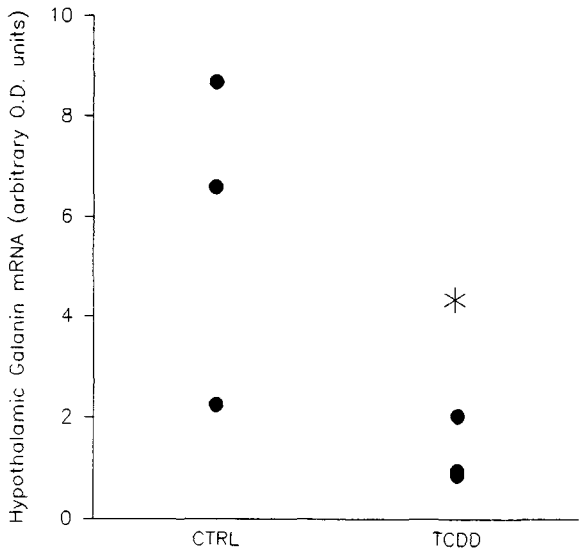

Fig. 3. *In-utero* and lacational exposure to TCDD reduces hypothalamic GAL mRNA levels in 21-d-old female rats. Levels of GAL messenger RNA from hypothalami of 21-d-old females exposed perinatally to vehicle (CTRL, $n = 4$) or TCDD ($n = 3$) were measured by RNase protection assay (*see* Methods). Values represent the distribution of data (normalized to 18s rRNA) points that was tested by Mann-Whitney U test.

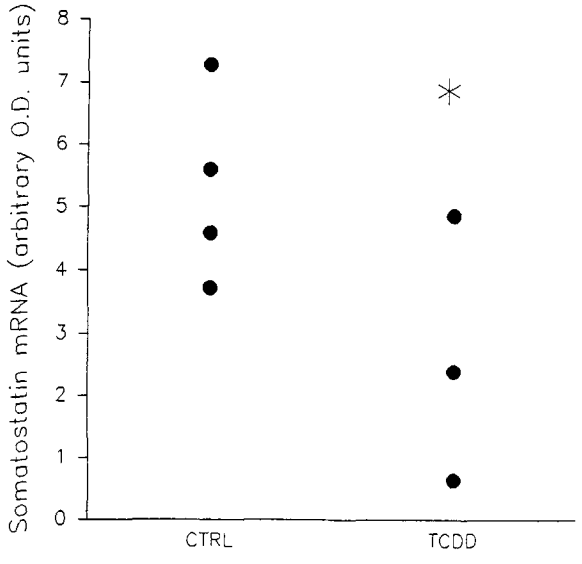

Fig. 2. *In-utero* and lacational exposure to TCDD reduces somatostatin mRNA levels in 21-d-old female rats. Levels of somatostatin messenger RNA from hypothalami of 21-d-old females exposed perinatally to vehicle (CTRL, $n = 4$) or TCDD ($n = 3$) were measured by RNase protection assay (see Methods). Values represent the distribution of data (normalized to 18s rRNA) points that was tested by Mann-Whitney U test.

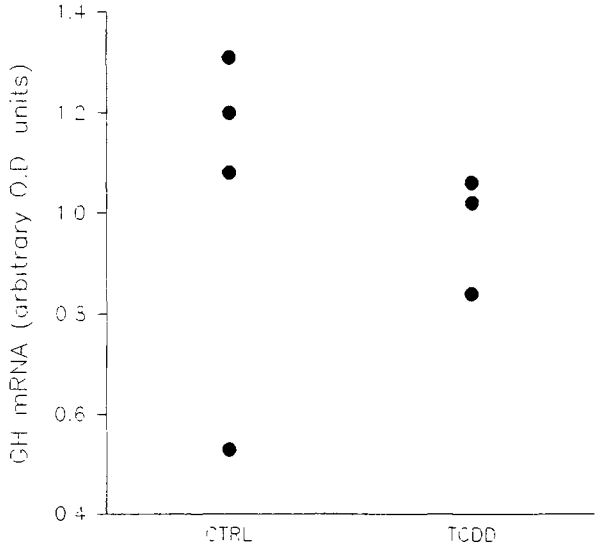

Fig. 4. *In-utero* and lacational exposure to TCDD does not alter pituitary GH mRNA levels in 21-d-old female rats. Levels of GH messenger RNA from pituitaries of 21-d-old females exposed perinatally to vehicle (CTRL, $n = 3$) or TCDD ($n = 3$) were measured by RNase protection assay (*see* Methods). Values represent the distribution of data (normalized to 18s rRNA) points that was tested by Mann-Whitney U test.

Both somatostatin and GHRH may be regulated by other factors, such as GH, and perhaps GAL. The increase in GHRH mRNA and decrease in hypothalamic GAL mRNA may be related by a feedback mechanism although the relationship of hypothalamic GAL to either somatostatin or GHRH remains obscure (Gabriel et al., 1988; Ottiecz et al., 1988; Sato et al 1991). It is possible that a decrease in hypothalamic GAL mRNA may have caused an increase

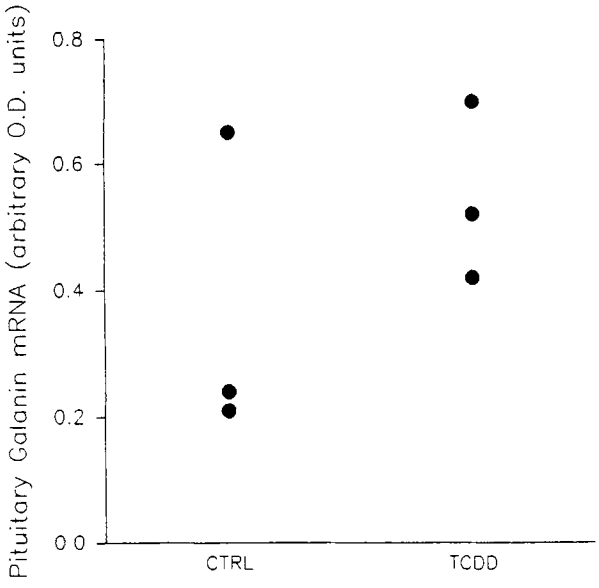

Fig. 5. *In-utero* and lacational exposure to TCDD does not alter pituitary GAL mRNA levels in 21-d-old female rats. Levels of GAL messenger RNA from hypothalami of 21-d-old females exposed perinatally to vehicle (CTRL, $n = 3$) or TCDD ($n = 3$) were measured by RNase protection assay (*see* Methods). Values represent the distribution of data (normalized to 18s rRNA) points that was tested by Mann-Whitney U test.

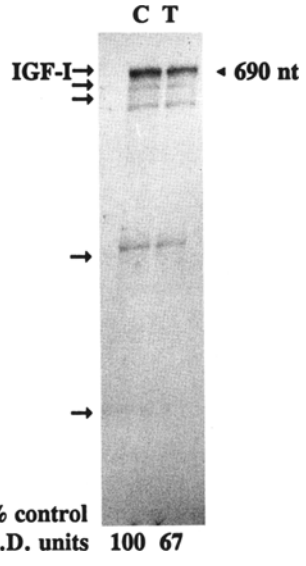

Fig. 6. *In-utero* and lacational exposure to TCDD reduces IGF-I mRNA levels in 21-d-old female rats. Autoradiogram of an RNase protection assay in which an IGF-I antisense riboprobe was hybridized to 5 μg of total RNA isolated from pooled liver tissue. Optical density (OD) units are given as a percentage of control below each lane. C, control; T, TCDD-exposed.

in GHRH mRNA and, hence, an increase in serum GH concentrations. The decrease in somatostatin mRNA by dioxin exposure may also be related to changes in hypothalamic GAL mRNA, and may in part influence the secretion of growth hormone from the pituitary. A direct effect of TCDD on somatostatin is unlikely as the somatostatin 5'-promoter and flanking regions do not contain a consensus dioxin-response element (Moniminy et al., 1984; McLane and Whitlock, 1994) between +1 and –805 base pairs.

We have demonstrated the presence of both AhR message and protein in the 21-d-old female rat hypothalamus and pituitary, so that a direct effect of TCDD on these glands is possible (Chaffin et al., 1995). Previous work in our laboratory has shown that ovarian and uterine GAL mRNA is increased (unpublished observations), so that a target of TCDD may be the GAL gene itself. The 5'-flanking region of the GAL gene does not contain a dioxin-response element, although only about 280 bases of this region in the bovine GAL gene have been sequenced (Anouar et al., 1994). A model gene for studying the interaction of dioxin response element and TCDD/AhR has been cytochrome P4501A1, which has several dioxin-response elements in the –495 to –1212 enhancer region (Lusska et al., 1993). The structure of this enhancer region demonstrates that 280 bases of the GAL promoter identified to date would not necessarily be expected to contain a dioxin-response element.

At the level of the liver, animals exposed to TCDD had 33% less IGF-I mRNA than did corresponding controls. The small increase in circulating GH would have been expected to result in an increase in hepatic IGF-I mRNA. Messenger RNA for IGF-I was, however, decreased by 33% in the liver, which may in part explain the decrease in body weight gain observed in exposed animals (Gray et al., 1995; Gray and Ostby, 1995). We hypothesize that either TCDD acted to downregulate hepatic GH receptor (either directly or via a cascade of events), uncouple the receptor and signal transduction pathway, or to directly decrease the amount of IGF-I mRNA. The GH receptor promoter/enhancer region does not contain a dioxin-response element between +95 and –385 bp (Menon et al., 1995). There are no published accounts of TCDD inducing an uncoupling of a plasma membrane receptor from its transduction pathway so this hypothesis does not seem likely. In addition, 1600 bases of the human IGF-I 5'-flanking region (Kim et al., 1991) do not contain a consensus dioxin response element, so the third alternative also does not appear likely. The possibility remains that alterations in genes not containing a dioxin response element occur as a result of indirect pathways.

The rat IGF-I gene appears to have multiple transcriptional start sites, resulting in similar mature peptides but encoding distinct pre-IGF-I molecules (Adamo et al., 1991). Variations in 5'-untranslated regions may dramatically influence the ability of the transcript to be translated. In both exposed and nonexposed animals, multiple protected fragments were observed, although there is no evidence to suggest that TCDD altered start-site usage (Fig. 6). Therefore, TCDD does not appear to regulate IGF-I expression at the level of translation efficiency in terms of message structure; this implies that levels of IGF-I mRNA

are reflected by translated concentrations of IGF-I. The lack of start-site specific regulation of the IGF-I gene by TCDD suggests that the decrease in IGF-I mRNA was a result of mechanisms not directly involving the promoter region of this gene, or perhaps through alterations in message stability.

The results from the current study indicate that exposure to TCDD during *in-utero* and lactational development results in alterations in growth axis gene expression. As only message data were collected, the physiologic consequences of these perturbations are unclear; it is possible, however, that the decrease in hepatic IGF-I is a causative factor of the slower growth rate experienced by the exposed rats. Further studies are needed to elucidate the mechanism of TCDD action on the growth axis, and to determine the locus or loci of effect.

Materials and Methods

Animals and Treatments

All animals were treated according to protocols approved by our Institutional Animal Care and Use Committee. Pregnant female Holtzman rats were obtained from Harlan-Sprague Dawley Inc. (Madison, WI) on gestational day 12. Upon arrival, animals were housed individually in a constant temperature (22 ± 1°C), and humidity (55 ± 5%), and exposed to a 12 h L:12 h D lighting schedule. Animals were fed commercial food (Rat Chow 5012, Purina Mills, St. Louis, MO) and water *ad libitum*. On the morning of gestational day 15, nine pregnant rats were administered a single oral dose of TCDD (1.0 μg/kg) and nine pregnant rats received an equivalent volume of vehicle control (corn oil/acetone, 19/1, v/v, 2 mL/kg) by gavage. TCDD (98% purity) was purchased from Cambridge Isotope Laboratories (Woburn, MA).

One day after birth, litters were adjusted to five males and five females per dam to allow for similar lactational exposure to TCDD. On postnatal day 21, female pups were sacrificed by decapitation under CO_2 anesthesia. Postnatal day 21 was chosen in order to follow exposure through lactation. The hypothalami and pituitaries were removed and pooled by litter, and livers were removed, randomly pooled into groups of six without regard for litter, and frozen on dry ice. Serum samples were prepared from trunk blood collected into serum separator tubes. As this study was part of a series of experiments, only a fraction of the total sample was allocated to this experiment.

RNA Isolation

Total RNA was isolated using the acid-guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Quantity of RNA was determined using absorption at 260 nm on a spectrophotometer and quality was evaluated by electrophoresis of 1 μg of RNA through a 1% agarose gel stained with ethidium bromide and visualized using a UV-light source.

Ribonuclease Protection Assay

Complementary DNA (cDNA) probes were linearized and transcribed in vitro using SP6 or T7 RNA polymerase and labeled by the incorporation of [^{32}P]UTP. Somatostatin, GHRH, and GH cDNAs were kindly provided by Dr. Kelly Mayo, Northwestern University; GAL cDNA courtesy of Dr. Maria Vrontakis, University of Manitoba, Manitoba, Canada, and IGF-I cDNA was a gift of Dr. Derek LeRoith, NIDDK, Bethesda, MD. Loading equivalency was verified by cohybridizing 2.5 × 10^5 cpm of pT7 RNA 18s (Ambion, Austin, TX). Following transcription, we removed template DNA by using RQ1 RNase-free DNase (Promega Biotech, Madison, WI), and the transcripts were phenol:chloroform extracted. Five μg of total RNA was hybridized overnight at 44°C with 5 × 10^5 cpm of the appropriate cRNA in 80% formamide/0.4*M* NaCl/40 m*M* piperazine-N,N'-bis-[2-ethanesulfonic acid] (PIPES). Radiolabeled transcripts that remained unhybridized were removed by digestion with 10 U RNase One (Promega Biotech) in 10 m*M* Tris-Cl/300 m*M* NaCl/5 m*M* EDTA (pH 8.0) and the resulting fragments resolved on an 8% denaturing acrylamide gel. To verify that hybridization was to endogenous mRNA rather than to contaminating template DNA and to ensure the efficacy of the RNase digestion, cRNA probes were treated as above with the omission of total RNA, and with or without the addition of RNase (data not presented). All bands generated by autoradiography were quantified using a densitometer (Optimas Bioscan, Madison, WI), and were normalized to the 18s band. Data were analyzed by Mann-Whitney U-test with significance set at $p < 0.05$.

Growth Hormone Radioimmunoassay (RIA)

Serum GH concentrations were determined using a double-antibody method and reagents provided by the NIH. Within-assay coefficient of variation was less than 10%. Serum GH concentrations are expressed in terms of reference preparation NIH RP-2 in ng/mL. Assay sensitivity was 0.04 ng/tube. Ten-microliter aliquots of serum were assayed. Data were analyzed using a *t*-test and expressed as mean ± SEM. Significance was set at $p < 0.05$.

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