Journal of Cellular Biochemistry 43:59–66 (1990)

Developmental Changes in Levels of Growth Hormone mRNA in Zucker Rats

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Levels of pituitary growth hormone (GH) messenger RNA (mRNA) were compared in groups of genetically obese (fa/fa) and lean (Fa/-) littermate male Zucker rats at four different ages, 3, 5, 9, and 11 weeks, in order to determine the earliest age at which a difference between the two groups could be detected. No difference was seen in three-week-old animals. Five weeks of age was the earliest time at which the level of GH mRNA was significantly decreased in the obese rats; this decrease was present at all subsequent ages. Mean serum growth hormone levels were lower in obese animals at all ages, but the differences were not statistically significant because of the large individual variation associated with the pulsatile nature of GH release. The earliest occurrence of differences in GH mRNA level is later than some of the obesity associated abnormalities present in adipose tissue. The earliest time of the GH mRNA differences can be associated with the time when decreased protein deposition is initially seen in the obese rats. Because of this association, decreased GH mRNA may enhance the development of obesity.

Key words: obesity, genetically obese Zucker rat, growth hormone, messenger RNA, adipose tissue

The genetically obese Zucker rat (fa/fa) [1] manifests a variety of endocrinological abnormalities, one of which is the impaired release of growth hormone (GH). Studies of plasma samples using both single point determinations [2] and pulsatile 6 h patterns [3] have shown that the obese male rats have lower mean GH and fewer peaks of lower magnitude than age-matched lean controls. In an attempt to determine the molecular basis of this abnormality, we [4] have compared levels of GH mRNA in the pituitary glands of obese and lean Zucker rats. Adult male obese rats have significantly less pituitary GH mRNA than lean littermates, as measured in individual pituitaries by dot blot analysis and in pooled pituitary samples by slot blot and Northern hybridization analyses.

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; GHRH, growth hormone releasing hormone; mRNA, messenger RNA; PRL, prolactin; SEM, standard error of the mean.

Received July 10, 1989; accepted January 17, 1990.

The obesity in this mutant is visibly distinguishable by body shape, at about the time of weaning, but an obesity associated defect in thermogenesis and an increase in body fat content can be demonstrated as early as the first week of life [5]. The animals used in our previous study [4] were fourteen weeks of age, a time at which obesity is well-developed. Therefore, the present study was undertaken to answer the following question: What is the earliest age at which levels of GH mRNA differ between groups of lean and obese rats of the Zucker strain?

In addition to GH, two other mRNA levels were studied in the same tissue. In order to rule out a generalized pituitary defect, prolactin (PRL) mRNA was measured. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured as an example of a constitutive gene, which should not differ between lean and obese animals.

METHODS

Animals

The animals used in these studies were obtained from a breeding colony at Northeastern Ohio Universities College of Medicine. Six to eight pairs of genetically obese (fa/fa) and lean littermate (Fa/-) male Zucker rats were used at each of the following ages: 3, 5, 9, and 11 weeks. At ages greater than 3 weeks, the obese individuals could be easily distinguished. For the 3-week-old animals, retrospective determination of the phenotype [6] was done by measuring the lipid content of individual inguinal fat pads from 69 3-week-old rats. The lipid content of the animals chosen as the obese group was 7.5 times greater than those chosen as the lean group. The animals were sacrificed by decapitation. Pituitaries were rapidly removed, frozen in liquid nitrogen and stored at −70°C until assayed.

Total RNA Extraction

Total pituitary RNA was extracted as described [4]. Briefly, pituitaries were thawed and homogenized in 240 μl of cold buffer (10 mM Tris-HCl, 1 mM EDTA, and 0.05% Nonidet P-40, pH 8) [7]. The homogenates were incubated on ice for 1 min, centrifuged (12,000g, 4 min at 4°C), and 200 μl of supernatant removed. Aliquots of supernatant (20 μl), in triplicate, were transferred to separate sterile tubes for the cytoplasmic dot blot analysis; the rest (140 μl) was adjusted to 100 mM NaCl, 100 mM Tris-HCl (pH 8), and 1% sodium dodecylsulfate (SDS), extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). Each time the aqueous phase was collected by centrifugation (12,000g, 5 min at room temperature). The final aqueous phase was adjusted to 250 mM sodium acetate (pH 5.2) and the RNA precipitated by adding three volumes of ethanol and storing at −20°C overnight. The RNA was recovered by centrifugation (12,000g, 15 min at 4°C), washed once with 70% ethanol, dried in vacuum, then redissolved in 100 μl of 0.05% diethylpyrocarbonate (DEPC)-treated water. Total RNA was quantitated by the absorbance at 260 nm ($1A_{260}$ unit = 40 μg/ml of RNA) as well as by the ethidium bromide agarose plate method [8].

Cytoplasmic Dot Blot Analysis

The supernatants were adjusted to a final concentration of 7.5% formaldehyde and 6 × SSC (1 × SCC = 150 mM NaCl, 15 mM Na citrate) [7], then incubated at 60°C

for 15 min. A series of twofold dilutions was made with 15 × SCC and 100 μl aliquots were applied onto nylon membranes (Genescreen Plus, N.E.N., Beverly, MA, USA) using a minifold apparatus (S and S, Keene, NH, USA). Membranes were baked for 2 h at 68°C, prehybridized, then hybridized with ^{32}P-labeled GH cDNA [9] or PRL cDNA [10] overnight at 42°C [8]. The membranes were then washed at 68°C in 1 × SSC, 0.1% SDS. A ^{32}P-labeled GAPDH cDNA [11] was used to quantify GAPDH mRNA as an example of constitutive mRNA, i.e., an internal control; in this instance the wash was 2 × SSC, 0.1% SDS at 50°C for 1 h. Membranes were used to expose Kodak XAR-5 film at −70°C. The hybridization signals were quantitated by either densitometric scanning or liquid scintillation counting of the hybridized spots.

Northern Blot and Slot Blot Analysis

For Northern blot analysis, 4 μg of pooled total RNA/genotype/age was denatured [8], mixed with ethidium bromide (1 μl of 1 mg/ml), and fractionated on a 1.5% agarose gel containing 0.66 M formaldehyde in 1 × MOPS buffer (5 × MOPS = 200 mM morpholinopropanesulphonic acid, pH 7; 50 mM sodium acetate, pH 5.2; 5 mM EDTA, pH 8 in DEPC-treated H_2O). After electrophoresis, 18S and 28S rRNA were visualized by UV-induced fluorescence to check the uniformity of total RNA loading. The RNA was transferred to a nylon membrane by capillary transfer. The membrane was baked, hybridized with GH [^{32}P]cDNA, washed, then used to expose XAR-5 film as described above.

For the slot blot analysis, 3 μg of pooled total RNA/genotype/age was denatured in 4% formaldehyde, 6 × SSC at 60°C for 15 min, serially diluted twofold with 15 × SSC, then applied onto nylon membrane using a slot blot apparatus (BRL, Gaithersburg, MD, USA). The membranes were baked, prehybridized, washed, then used to expose XAR-5 film. Quantitation was done by densitometric scanning.

The cDNAs were labelled with [^{32}P]dCTP (3,000 Ci/mmol, ICN, Irvine, CA) using random hexanucleotide primers [12] to a specific activity of about 10^9 CPM/μg of DNA.

The linearity of cytoplasmic dot blot assay was determined by regression analysis. To test the interaction between dilutions and the genotype, a two-way analysis of variance was done using genotype and dilution as factor 1 and factor 2, respectively. Separate analyses were done for rats of different ages.

Serum GH Measurement

Truncal blood was collected at the time of decapitation and stored at 4°C for at least 2 h. Serum was obtained by centrifugation at 3,000g for 20 min at 4°C and assayed with materials provided by NIADDK and a murine GH antibody according to methods described by Renier et al. [13]. For each age, a t-test was used to compare the lean vs. obese group.

RESULTS

Cytoplasmic Dot Blot Analysis

Obese animals had lower GH mRNA levels ($P < 0.01$) than lean controls at 5, 9, and 11 weeks of age. There was, however, no significant difference in GH mRNA levels between lean and obese animals at 3 weeks of age (Fig. 1). Regression analysis of

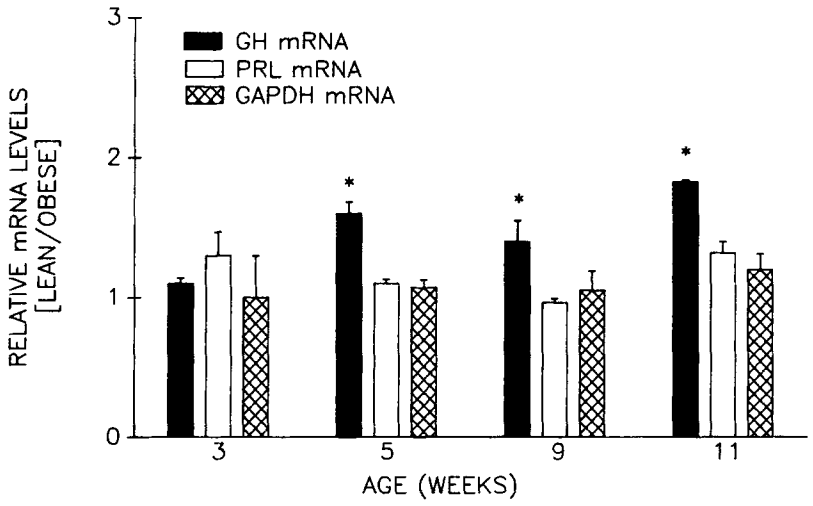

Fig. 1. Levels of pituitary GH, PRL, and GAPDH mRNA expressed as a ratio of lean to obese within each age group (3, 5, 9, and 11 weeks). Data are taken from dot blot analysis performed on cytoplasmic samples from individual animals; quantitation was done by densitometric scanning or liquid scintillation counting. Each group consisted of 6–8 animals. Bar indicates standard error of the mean. *, $P < 0.01$.

cytoplasmic dot blot data confirmed the linearity of the assay. The difference in GH mRNA levels between lean and obese rats was given by the divergence of the angle of slopes (Fig. 2A,B). No significant difference was found in PRL mRNA and GAPDH mRNA levels between lean and obese animals at any ages studied (Fig. 1).

Northern Blot Analysis

Northern blot analysis (Fig. 3) was done on pooled total RNA from each genotype at each age in order to corroborate the results of GH mRNA levels determined by the cytoplasmic dot blot assay. A 1 kb GH mRNA band was detected in each lane. The amount of GH mRNA was greater in the "lean" lanes as compared to that in the corresponding "obese" lanes for all ages, except 3 weeks, which showed no difference.

Slot Blot Analysis

Slot blot analysis (Fig. 4) was carried out to quantitate the relative levels of GH mRNA in each genotype across the different age groups for an intergroup comparison. No difference in GH mRNA levels was observed between lean and obese animals at 3 weeks of age. At 5 weeks of age and older, although GH mRNA levels increased with age in both genotype, the lean showed relatively greater increase than the obese animals.

Serum GH Levels

While serum GH levels were lower in obese animals at all ages studied (Table I), the differences were not statistically significant.

DISCUSSION

The decrease in pituitary GH mRNA level, previously reported to be present in 14-week-old male obese Zucker rats in comparison to their lean littermates [4], is under

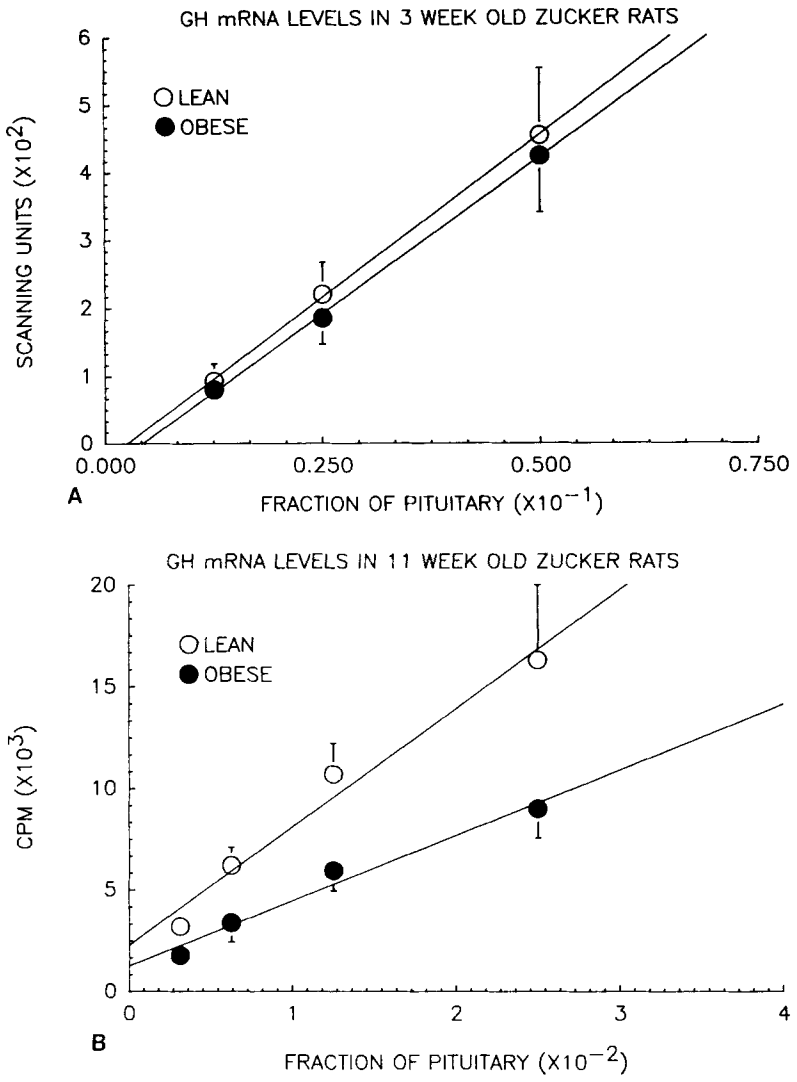

Fig. 2. **A:** Relationship between GH mRNA levels, as quantitated by densitometric scanning, and cytoplasmic fraction of individual pituitaries (n = 6); data are taken from 3-week-old animals. **B:** Relationship between GH mRNA levels, as quantitated by scintillation counting, and cytoplasmic fraction of individual pituitaries (n = 8); data are taken from 11-week-old animals. By regression analysis, the difference between the groups is statistically significant at eleven weeks but not at three weeks.

developmental control. At 3 weeks of age the levels of GH mRNA do not differ between groups of lean and obese animals. The earliest age at which the difference can be measured is 5 weeks; this difference is then present at all later ages studied. Because depressed GH mRNA appears later than other obesity associated abnormalities [5], the GH gene can be ruled out as the site of primary defect in the fatty rat. The fact that GH plays a role in protein deposition, however, suggests that reduced GH mRNA may enhance the development of obesity. The timing of the change in relative level of GH

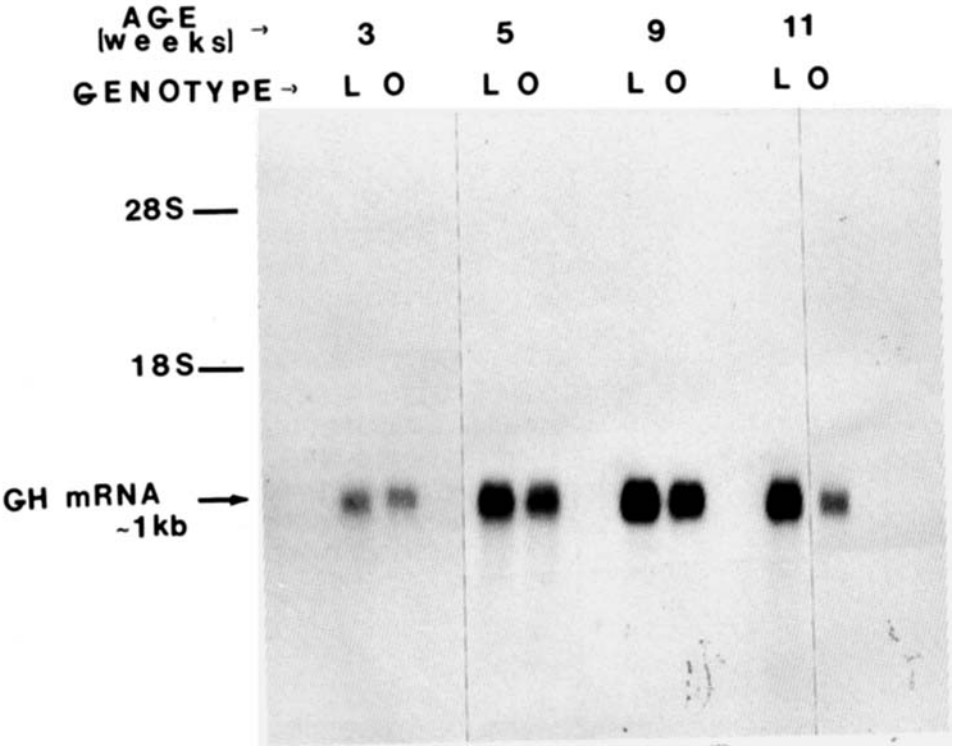

Fig. 3. Northern blot analysis of pooled samples of pituitary RNA hybridized to [^{32}P]GH cDNA. Comparisons are made between groups of lean (L) and obese (O) rats at 3, 5, 9, and 11 weeks of age. Each lane was loaded with 4 μg of total RNA/genotype/age.

mRNA (between 21 and 35 days) is compatible with the time at which the obese animals show an absolute decrease in body protein deposition (between 31 and 42 days) [14,15].

The functional relevance of decreased pituitary GH mRNA can be assessed by measuring circulating GH levels. While there were no statistically significant differences in serum GH between lean and obese rats, the means, and the medians (data not shown) were always lower for the obese animals. Lack of statistical significance can be attributed to the large variation within each group (note large SEM), which is a result of the pulsatile nature of GH release [16].

In contrast to our results, in the only other report of serum GH levels as a function of age in Zucker rats [17], statistically significant differences between lean and obese groups were seen at 7, 9, and 11 weeks but not at 5 weeks; these authors also used a single-point determination, as we did. Further developmental experiments utilizing time sampling (e.g., 25 consecutive samples over a 6 h period) would clarify the discrepancies between the present study and the earlier one [17].

In adult Zucker rats, pituitary GH content is significantly lower in the obese vs. the lean rats [18], but the development sequence of this difference is not currently known. As large amounts of GH can be stored in the pituitary without being released, circulating

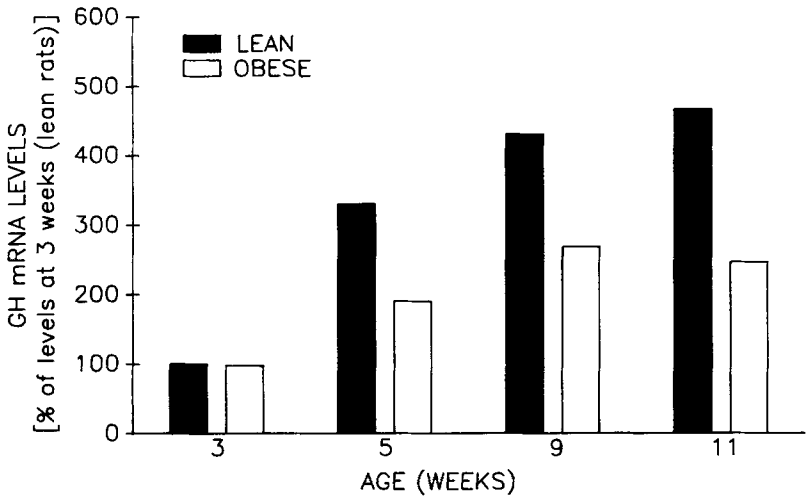

Fig. 4. Levels of pituitary GH mRNA in groups of lean and obese rats at 3, 5, 9, and 11 weeks of age, expressed as a percentage of the level measured in 3-week-old lean animals. Data are taken from slot blot analyses performed on extracted, pooled total RNA; quantitation was done by densitometric scanning.

TABLE I. Mean Level (±SEM) of Serum Growth Hormone (ng/ml) in Rats of Different Ages

	3 Weeks	5 Weeks	9 Weeks	11 Weeks
Lean	11.7 (±5)	96.5 (±71)	123.0 (±71)	234.8 (±71)
Obese	1.6 (±0.4)	81.2 (±56)	48.9 (±20)	80.0 (±73)

levels of this hormone, rather than pituitary content, would play a more important role in the effectiveness of GH at its target sites.

In normal male Sprague-Dawley rats, plasma GH levels increase between 22 and 30 days of age [19]; similarly, our data show the greatest increase in serum GH to be between 3 and 5 weeks of age. The difference in GH mRNA between lean and obese rats becomes apparent at the same time.

At the cellular level the decrease in GH mRNA could be due to either a decrease in the number of somatotrophs or a decrease in the transcriptional rate of the gene or a decrease in stability of the mRNA. Our in situ hybridization studies (in preparation) rule out the first possibility since there is no difference between the lean and obese rats in the number of somatotrophs expressing GH transcripts.

One possible explanation for the timing of age-related differences in GH mRNA could be developmental change(s) in a circulating factor (or factors) that has a direct effect on GH gene expression. Insulin is a good candidate since genetically obese Zucker rats develop hyperinsulinemia at about 3 weeks of age [20], and in vitro studies on pituitary cells have shown that insulin suppresses GH mRNA levels [21]. Alternatively, hypothalamic growth hormone releasing hormone (GHRH), a positive regulator of GH gene transcription [22,23], may play a role. Adult male obese Zucker rat have lower levels of hypothalamic GHRH than their lean littermates [18], but the developmental sequence of this difference has not yet been explored.

ACKNOWLEDGMENTS

This work was supported by a grant from the Office of Gerontology and Geriatric Medicine, NEOUCOM, and Research Challenge Funds from the Ohio Board of Regents. We wish to thank Dr. J.D. Baxter for the GH probe, Dr. R.A. Maurer for the PRL probe, and Dr. R.J. Schwartz for the GAPDH probe. We also wish to thank Drs. Pierrette Gaudreau and Paul Brazeau for performing the GH assay on the serum samples.

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