The Human Growth Hormone Transgene: Expression in Hemizygous and Homozygous Mice (43096)

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Abstract. **Female transgenic mice carrying the mouse metallothionein-I/human growth hormone (hGH) fusion gene are sterile. Transmission of the transgene has been limited to the male germ line, resulting in the production of hemizygous (He) progeny containing only a single (paternal) copy of the gene. Using ovary transfer, we have developed procedures for producing homozygous (Ho) TG mice, viz., male TG mice were mated with control (non-TG) females carrying ovaries donated by female TG mice. In both He and Ho TG animals, serum levels of hGH were higher (1.5-fold) in males than in females, tended to decrease with age of the animal, and were increased (about 5-fold) by zinc induction. However, in comparison to He animals of the same sex, the Ho TG mice attained a greater body weight and had more than 2-fold higher levels of liver hGH-mRNA and serum hGH, both under basal conditions and in response to zinc induction. That is, the expression of the transgene was qualitatively similar in He and Ho TG mice, but the level of transgene activity was greater in the Ho animals. We interpret this to indicate that both copies (maternal and paternal) of the transgene were active and expressed additively (or cooperatively) in the Ho TG animal.** [P.S.E.B.M. 1990, Vol 194]

Transgenic mice carrying the mouse metallothionein-I/human growth hormone (*mMT-I/hGH*) fusion gene (TG mice) exhibit high levels of serum hGH (1, 2), enhanced somatic growth (1–3), and female sterility (4–6). Due to the female sterility, the *mMT-I/hGH* transgene has been transmitted exclusively via the male germ line, resulting in the production of progeny containing only a single, paternal copy of the transgene, i.e., hemizygotes (He). Transgenic animals, homozygous (Ho) with respect to a number of transgenes have been reported, e.g., β-S-globin gene (7), *PHT-1* gene (8), and myelin basic protein gene (9). In the case of human GH transgenes, mice carrying the *HuGH* transgene are fertile (both males and females) and matings between He *HuGH* progenitors have produced Ho *HuGH* offspring (10). However, the *HuGH* transgene is not expressed in either He or Ho animals (6, 10).

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In the present communication, we describe procedures for producing TG mice which contain both maternal and paternal copies of the *mMT-I/hGH* fusion gene. So far as we know, these are the first reported examples of Ho TG mice that express a human GH transgene. We also present data regarding the relative expression of the transgene in Ho and He TG mice as evidenced by somatic growth, tissue levels of hGH-mRNA, and serum hGH under various conditions. These findings may help to elucidate the regulation of endogenous GH gene expression in humans and other animals. A preliminary account of this work has appeared (11).

Materials and Methods

TG Mice. TG founder animals ($B6C3F_2$ hybrid of C57BL × C3H) were produced as described previously (12). The TG mice used in the present study were the offspring of founder animal no. 6, carrying one copy of the *mMT-I/hGH* fusion gene (3). The animals were obtained by mating TG males (10th generation) with non-TG female recipients ($B6C3F_1$) of ovaries donated by TG females (see below). Progeny displaying one of three different genotypes were obtained: Ho, He, and non-TG, containing, respectively, two, one, and zero

copies of the hGH transgene. Copy number was determined by slot blot analysis of DNA isolated from tail biopsies as described previously (3). The assignment of copy number was routinely validated by phenotypic analysis of the offspring produced by selected genotypic mating combinations.

Animals were housed at a controlled temperature (21°C) and light cycle (5 AM:7 PM, light:dark) and provided a balanced diet (Purina 5001 Rodent Chow; Purina Mills, Inc., St. Louis, MO) and acidified tap water *ad libitum*. In some cases (as indicated), animals were provided a 25 m*M* $ZnSO_4$ drinking solution in place of tap water.

Ovary Transplant. Ovary transplants were conducted using procedures described by Tanioka *et al.* (13), modified as follows. TG ovary donors (2–5 months old) were anesthetized with Avertin (14) and both ovaries were excised with dissecting scissors and watchmaker forceps[1]. The excised ovaries were placed in a drop of phosphate-buffered saline solution in a culture dish and kept on ice. Non-TG recipients of the TG ovaries (either littermates or females of the same age and strain, $B6C3F_1$; The Jackson Laboratory, Bar Harbor, ME) were anesthetized (Avertin) and a 2-cm incision was made on the lower lumbar region of each animal exposing the ovarian complex. Recipient animals were placed under a dissecting microscope (×10) and the bursa (ovarian capsule) was broken (midline) using watchmaker forceps to expose the ovary. The ovaries were removed with sharp scissors. TG ovaries were then implanted into the vacated ovary site (replacing the excised ovaries) and covered with the bursa which was then sutured together (one stitch) using 6-0 silk with a c-3 needle (Ethicon Inc., Somerville, NJ). The ovarian complex was replaced in the body cavity and the incision was closed with sterile wound clips (Clay Adams, Parsippany, NJ). Approximately 2 weeks after surgery, the TG ovary recipients were mated with non-TG males and the offspring were analyzed for *hGH* gene integration and expression (hGH, hGH-mRNA); ovary recipients producing TG offspring were subsequently mated to TG males (as described above).

DNA and RNA Analysis. Total genomic DNA was isolated from the tails of offspring produced by TG ovary recipients and the number of copies of integrated transgene was determined by quantitative slot blot (15) and Southern hybridization (16). Liver biopsies (approximately 200 mg) were performed on Ho and He TG animals, and on control (non-TG) animals, before and after treatment with zinc (25 m*M*, 8–14 days). Total liver RNA was isolated using a guanidinium one-step procedure (17). The RNA (20 μg/lane) was electrophoresed through a 1% agarose-formaldehyde gel and transferred onto a nylon membrane (18). The membrane was hybridized with a randomly primed (^{32}P-labeled) DNA probe prepared from hGH genomic sequences (1.4 Kb *Bam*HI *Bgl*/II fragment). After hybridization, washing, and drying, the membrane was exposed to a Kodak x-ray film. The relative intensity of each sample was determined by densitometry (Gilford Response, Ciba-Corning Diagnostic Corp., Oberlin, OH) of the developed autoradiograph.

hGH Assay. Blood samples were collected from a tail vein and hGH levels were measured in 100-μl aliquots of serum using a solid phase double monoclonal antibody radioimmunoassay under conditions recommended by the manufacturer (Hybridtech, Inc., San Diego, CA).

Statistical Analysis. Group data are expressed as means ± SE. Differences between paired means were analyzed using Student's *t* test. Multiple interactions were analyzed by a two-factor analysis of variance, followed by Cicchetti's multiple range test (19).

Results

Production of Ho TG Mice. TG ovaries were bilaterally implanted into 12 non-TG recipient mice. Of this total, seven of the implants were successful, i.e., matings between TG ovary recipient females and non-TG males produced offspring containing the *hGH* gene.

As indicated in Table I, matings between He TG males and female TG ovary recipients ($n = 7$) gave rise to progeny ($n = 52$) of the following genotypes: Ho ($n = 13$), He ($n = 19$), and non-TG ($n = 20$). In matings between TG males and He TG ovary recipients, 24 of 39 (62%) of the offspring were found to carry the *hGH* transgene. Similarly, 8 of 13 (62%) of the offspring derived from matings between TG males and Ho TG ovary recipient females were TG. Transmission of the transgene via the male germ line gave rise to 37 of 82 (45%) TG offspring (Table I, F10 and F11).

Copy number of the TG mice was determined by DNA slot blot analysis as illustrated in Figure 1. The intensity of the hybridization signal corresponds to copy number, i.e., darker slot blots (a, c, h, i, and j) represent Ho TG animals, lighter slot blots (b, d, e, and f) represent He TG animals, and negative slot blots (g) represent non-TG animals, containing, respectively, two, one, and zero copies of the hGH transgene. In validation of the slot blot analysis, when Ho males were subsequently mated with non-TG females, 100% of the progeny (8 of 8) were determined to be He TG. Significantly, Southern blot analysis of this line of animals indicates that the transgene is very stable, with no indication of rearrangement or duplication of the gene throughout the generations.

Body Weight. Growth curves (Fig. 2) demonstrate that over the period from 30 to 150 days of age, the

[1] In most cases, He females served as ovary donors; on two occasions, first generation Ho females (bred by mating TG males with TG ovary recipient females) served as ovary donors.

Table I. Transmission of MT-I/hGH Fusion Gene through TG Ovary Transplant Females and TG Males

	No. of recipients	No. of litters	Offspring		
			Ho TG	He TG	Non-TG
TG ovary transplant females mated with TG males					
Type of TG ovary transplanted					
Hemizygous	5	6[a]	9	15	15
Homozygous[b]	2	3[a]	4	4	5
Total	7	9	13 (25%)	19 (37%)	20 (38%)
Non-TG females mated with TG males (male germ line)					
F10 and F11 generations	—	13	—	37 (45%)	45 (55%)

[a] One ovary recipient had two litters.
[b] Progeny of hemizygous ovary recipient mated with TG male.

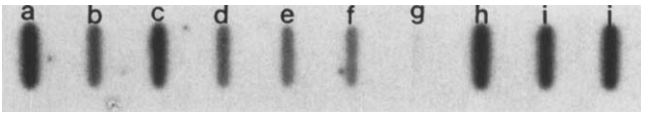

Figure 1. Representative slot blot analyses of DNA isolated from tail biopsies of Ho, He, and non-TG mice. Darker blots (a, c, h, i, and j) represent Ho TG; lighter blots (b, d, e, and f) represent He TG; and blot g represents non-TG. DNA was probed using a 2.1-kb *Bam*HI/*Eco*RI hGH genomic DNA fragment.

somatic growth of Ho TG mice exceeds that of He TG mice which exceeds that of non-TG mice. Furthermore, for each of the genotypes, the body weight of males exceeds that of females. Analysis of the data at 90 days, pooled with respect to genotype (Ho + He) and gender (male + female), indicates that: (i) the body weight of Ho TG animals (males + females, $n = 12$) is significantly greater than that of He animals (51.3 ± 1.2 vs 43.6 ± 1.1 g, $P < 0.001$) and (ii) the body weight of male TG mice (He + Ho, $n = 12$) is greater than that of female TG mice (49.7 + 1.6 vs 45.2 ± 1.4 g, $P < 0.05$). Although female Ho TG mice attained greater

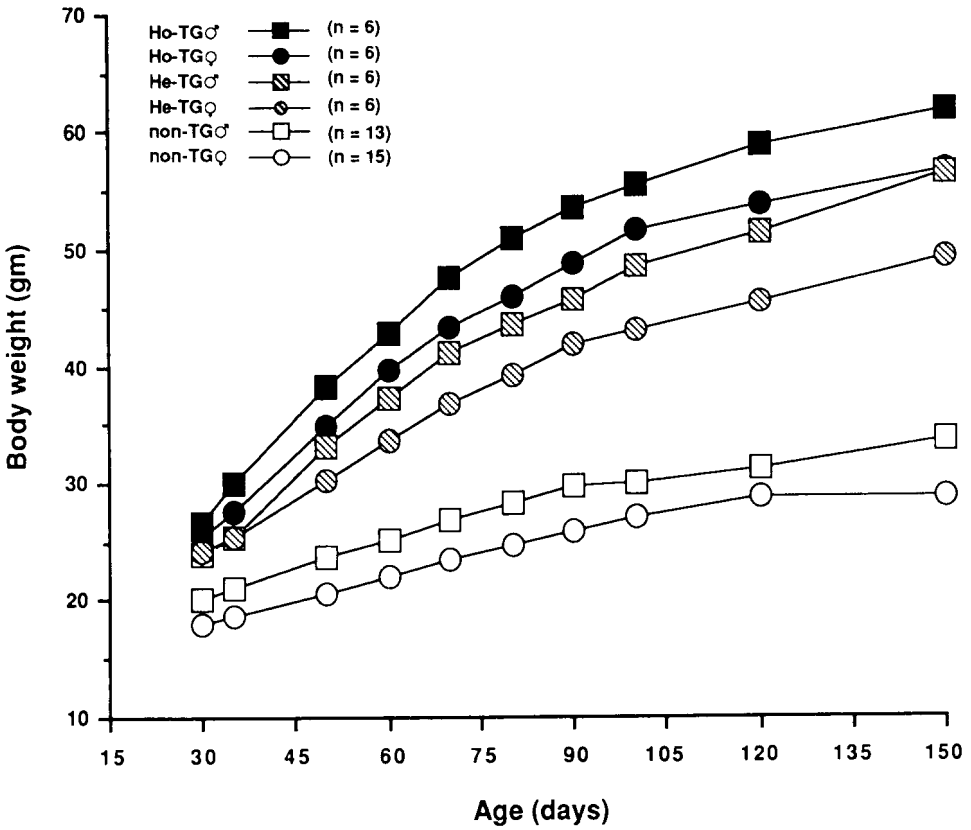

Figure 2. Growth curves of Ho, He, and non-TG mice from 30 to 150 days of age. Note that for each of the genotypes (Ho, He, and non-TG) body weight of males is greater than females and that body weight of Ho TG, regardless of sex, is greater than that of He TG.

body weight than male He TG animals, the difference was not significant (90 days, 48.9 ± 1.5 vs 45.7 ± 1.8 g, $P > 0.05$). In comparison to non-TG animals of the same sex, both Ho TG mice and He TG mice showed increased body weight (approximately, 1.8- and 1.6-fold, respectively).

Expression of the Transgene: Serum Levels of hGH. Serum levels of hGH were determined in He TG mice ($n = 6$; 3 males, 3 females) at 1, 2, 3, 5, and 7 months of age. As illustrated in Figure 3, serum concentrations of the peptide were highest at 1 month of age (the youngest animals tested) and then gradually declined with increasing age. We have not fully characterized age-related changes in serum hGH in Ho TG mice; however, levels of the hormone were higher in 1.5-month old vs 3-month-old Ho TG (33.5 ± 2.2 vs 24.1 ± 1.6 ng/ml, $n = 5$/group, $P < 0.01$).

Figure 4 compares hGH levels in 3-month-old (male and female) Ho and He TG mice. Serum hGH levels were highest in Ho TG males (27.8 ± 1.0 ng/ml) and progressively declined in Ho females (18.4 ± 1.7 ng/ml), He males (11.7 ± 0.6 ng/ml), and He females (7.9 ± 1.2 ng/ml). Analysis of variance (two factor) indicates that the main effects (gender, copy number) were significant ($P < 0.0001$) and that there was also a significant group interaction ($P < 0.05$). Cicchetti's post-hoc multiple range test (19) indicates that all comparisons between paired means were significant ($P < 0.05$) except for He males versus He females ($P > 0.05$). In both He and Ho TG animals (Fig. 5), zinc administration (25 m*M*, 8–14 days) resulted in an approximately 5-fold increase in serum levels of hGH ($P < 0.001$).

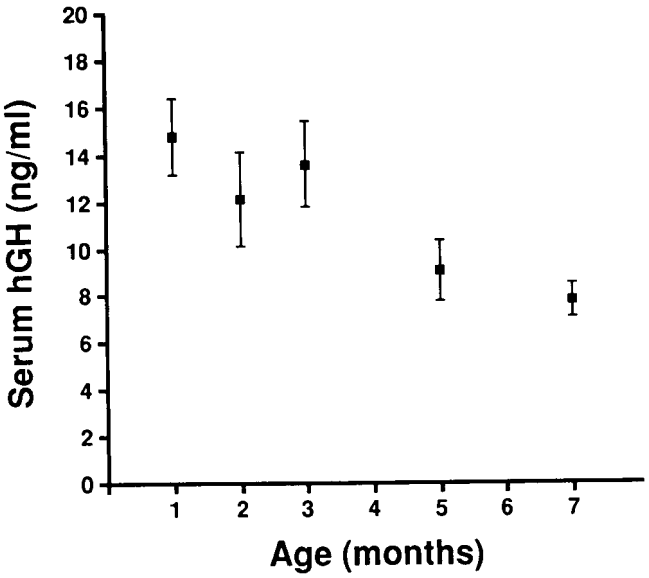

Figure 3. Effect of age on serum hGH levels in He TG mice ($n = 6$; 3 males, 3 females); data are mean ± SE. Note gradual decline in hGH levels during period from early to late adulthood.

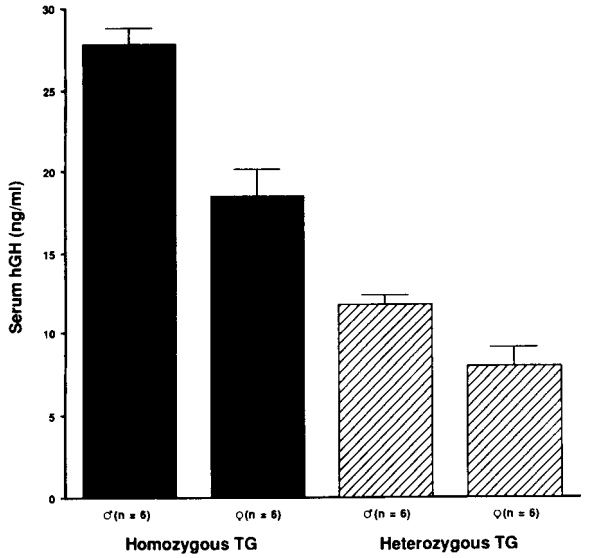

Figure 4. Serum hGH levels as a function of genotype and gender. Data (mean ± SE) were obtained on animals at 3 months of age. Analysis of variance indicates that main effects (sex, copy number) are significant ($P < 0.0001$) and that there is also a significant group interaction ($P < 0.05$). Note that for both Ho and He animals, hGH levels are higher in males than females and that for animals of the same sex, hGH levels are higher in Ho TG than in He TG animals.

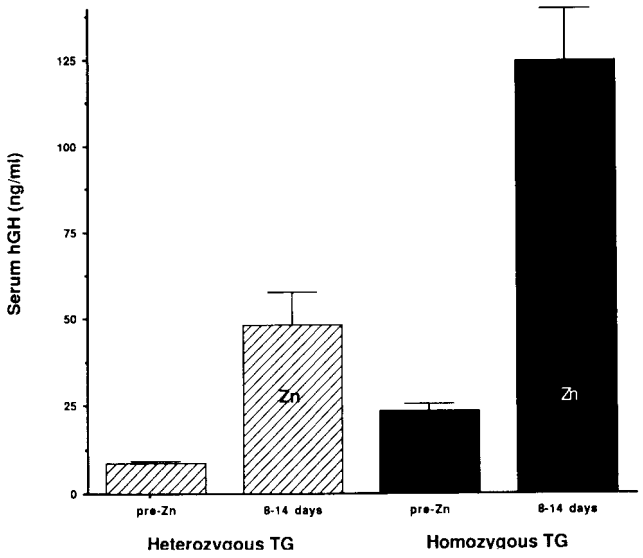

Figure 5. Effect of zinc on serum hGH levels. Data are means ± SE for Ho TG ($n = 9$) and He TG ($n = 12$), pre- and posttreatment with zinc (25 m*M* $ZnSO_4$ dissolved in drinking water, 8–14 days *ad libitum*). Differences between paired means are significant ($P < 0.001$).

Expression of the Transgene: hGH-mRNA. Tissue (liver) levels of hGH mRNA were elevated in Ho versus He TG mice (Fig. 6, Lanes 4–6 vs Lanes 7–9). Zinc administration (25 m*M*, 8–14 days) markedly increased hGH-mRNA levels in He TG mice (Fig. 6, Lanes 1–3 vs Lanes 7–9).

Discussion

Transgenic animals carrying the hGH gene are an advantageous system for studying the regulation of the

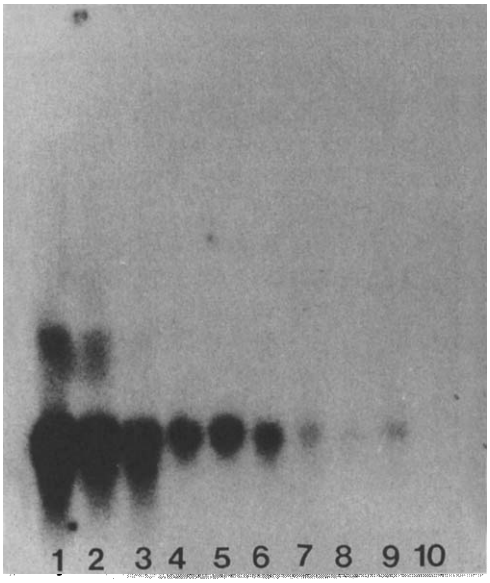

Figure 6. Northern blot analyses of total RNA from livers of TG mice. RNA was probed with a 1.4-kb *Bam*HI *Bgl* II genomic fragment. Lanes 1–3, zinc-induced He TG mice; Lanes 4–6, uninduced Ho TG mice; Lanes 7–9, Uninduced He TG mice; Lane 10, non-TG mice. Note that mRNA levels in uninduced Ho TG mice are higher than in uninduced He TG mice and that zinc treatment increases mRNA levels in the He TG animals.

GH gene and the physiologic effects of the gene products on the host animal. Previously reported hGH transgenic models (1–6) were limited to He animals (one or more copies and insertion sites, on only one of two homologous chromosomes). In this communication, we describe a line of *mMT-I/hGH* mice consisting of both He and Ho animals; the relative expression of the hGH transgene alleles provides useful insights regarding potential interactions affecting the induction and expression of the gene.

Matings between TG males and He TG ovary recipients might be expected to produce 75% TG offspring and matings between TG males and Ho TG ovary recipients might be expected to produce 100% TG offspring. However, in both cases only 62% (24 of 39 and 8 of 13, respectively) of the progeny were found to carry the hGH transgene. This is likely due to the regeneration of functional non-TG ovarian tissue from fragments left behind during removal of the endogenous ovaries from the host animals (4, 13), i.e., TG ovary recipients may produce ova from regenerated endogenous tissue (non-TG) as well as from the implanted TG ovaries.

Our findings indicate that in Ho TG mice, both copies of the transgene are active and expressed. In Ho versus He TG animals of the same sex, serum hGH levels were elevated more than 2-fold (Fig. 4) and liver hGH-mRNA was elevated 3- to 5-fold (Fig. 6); the elevated levels of serum hGH in male versus female TG (both Ho and He, Fig. 4, and also Bartke *et al.* (20)) are most likely due to testosterone, an intriguing observation since testosterone, unlike glucocorticoids and heavy metals (21, 22), does not appear to interact with the *mMT* promoter (23). In both He and Ho TG mice, the *mMT-I/hGH* transgene is induced by heavy metals. In He TG, zinc administration led to increased (about 10-fold) liver hGH-mRNA (Fig. 6) and increased (about 5-fold) serum hGH (Fig. 5). In Ho TG mice, zinc produced a similar (5-fold) increase in serum hGH (Fig. 5). Both under basal (unstimulated) conditions and in response to zinc administration, serum levels of hGH were more than 2-fold greater in Ho TG than in He TG animals. The finding that the expression of the transgene in Ho TG animals exceeds the response expected if each of the (two) individual copies of the gene were expressed additively suggests that the two copies of the transgene may interact cooperatively and deserves further investigation.

The data presented here establish a good correlation between copy number (DNA slot blot) and expression of the transgene (hGH-mRNA, serum hGH, and body weight). On the other hand, previous studies on the *hGH* transgene (1, 3), and also other transgenes (24–27), noted little correlation between gene copy number and gene expression. Palmiter (28) suggested that only a few copies of the gene are expressed and/or that the entire gene array is very sensitive to chromosomal position. Studies on transgenic mice carrying the rat elastase (*El*) gene showed a decrease in transcription rate as the number of tandemly repeated *El* transgenes increased (29). Interestingly, this study also showed that mice transgenic for the rat *El* gene mated with mice carrying an *El* enhancer directed *hGH* gene produced progeny with two unlinked arrays of *El* enhancer driven transgenes, each of which was transcribed at the same rate as in mice bearing each gene array separately (29). All of these studies utilized He founder mice exhibiting a wide range of copy numbers and insertion sites, both of which might influence expression of the gene. In the present study the single (He TG) or double (Ho TG) copies of the gene were present in identical chromosomal locations, negating any of these extraneous effects.

Regarding the effect of copy number on the expression of endogenous *GH* genes, studies on inherited growth disorders in humans are of interest. Familial isolated GH deficiency Type 1A (30, 31) is characterized by a deletion of the DNA sequence encompassing the GH structural gene (*GH1*) and the complete absence of the hormone; only individuals with zero copies of the intact hGH gene are phenotypically affected. Although the somatic growth of individuals with one copy of the *hGH* gene (He) is within the normal range, data comparing expression of the gene in these individuals and normal individuals (Ho, two copies) are unavailable.

By using ovary transfer we have succeeded in producing Ho TG mice, i.e., animals containing two allelic copies of the *mMT-I/hGH* transgene. Both copies of the transgene are active and induced by known regulators of the gene. Significantly, expression (hGH-mRNA and hGH) of the transgene in the Ho animals exceeds that expected if the individuals copies of the gene were expressed additively. Studies designed to investigate potential interactions between multiple copies of the transgene are in progress.

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