Metallothionein–Human GH Fusion Genes Stimulate Growth of Mice

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Summary. The promoter or regulatory region of the mouse gene for metallothionein-I was fused to the structural gene coding for human growth hormone. These fusion genes were introduced into mice by microinjection of fertilized eggs. Twenty-three (70 percent) of the mice that stably incorporated the fusion genes showed high concentrations of human growth hormone in their serum and grew significantly larger than control mice. Synthesis of human growth hormone was induced further by cadmium or zinc, which normally induce metallothionein gene expression. Transgenic mice that expressed human growth hormone also showed increased concentrations of insulin-like growth factor I in their serum. Histology of their pituitaries suggests dysfunction of the cells that normally synthesize growth hormone. The fusion genes were expressed in all tissues examined, but the ratio of human growth hormone messenger RNA to endogenous metallothionein-I messenger RNA varied among different tissues and different animals, suggesting that expression of the foreign genes is influenced by site of integration and tissue environment.

Growth of vertebrates is mediated in part by the cascade of polypeptide hormones depicted in Fig. 1. This pathway emanates from the hypothalamus which responds to neurotransmitters by liberating either somatostatin or growth hormone–releasing factor into the portal circulation; these polypeptide hormones impinge on the pituitary to either inhibit or stimulate, respectively, the synthesis and secretion of growth hormone (GH) (*1*). Growth hormone is released periodically from the pituitary. The amplitude of the cycles of release is more striking in males than in females (*2*); the significance of this difference in secretory patterns on sexual differentiation is only beginning to be appreciated (*3*). Nevertheless, in both sexes GH is thought to stimulate the liver to produce insulin-like growth factor I (IGF-I) (*4*), a polypeptide hormone, also called somatomedin C, which shows homology to proinsulin (*5*). IGF-I is thought to mediate growth by activating receptors on peripheral tissues (*6*).

In a previous study we showed that it was possible to manipulate this pathway by introducing rat GH (rGH) genes into fertilized mouse eggs (*7*). Most of the mice that incorporated the gene into their chromosomes, called transgenic mice, grew larger than normal. The success of this approach depended on the fusion of the rGH structural gene to the mouse metallothionein-I (MT-I) gene promoter, a technique used previously to obtain expression of microinjected thymidine kinase genes (*8*). This promoter is from a "housekeeping" gene which is expressed in most cells and is regulated by a variety of environmental stimuli (*9*). One class of stimuli includes certain heavy metals, such as cadmium and zinc, which are postulated to bind to regulatory proteins that interact with promoter sequences located in the region 40 to 180 base pairs (bp) upstream of the transcription start site (*10*). The consequence of using this particular fusion gene was that rGH was produced in the same tissues as MT-I, instead of the pituitary, with the result that circulating rGH reached levels several hundred times higher than normally achieved (*7*). This extrapituitary production of GH is depicted as the GH shunt in Fig. 1. Some of the transgenic mice grew to almost twice the size of their normal littermates.

We have extended these studies, as reported here, by showing that the more distantly related human GH gene (hGH) is also capable of promoting accelerated growth of mice. This gene and its products are more easily distinguished from the endogenous mouse counterparts allowing certain technical advantages over our initial constructions with the rGH gene. The genetic engineering of mice with a hGH shunt and the regulation of this modified GH cascade are described below.

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Growth of Mice-Bearing MThGH Fusion Genes

Two different constructions were prepared in which the mouse MT-I promoter was fused to the hGH_N structural gene that was isolated from one of a pair of cosmids that include the entire GH gene family (*11*). These fusion genes are designated MThGH; our previous constructions with rGH are hereafter designated MTrGH. In both cases, the unique Bam HI site that is located just upstream of the initiation codon of hGH was utilized (*12*); in one construction (plasmid 111, Fig. 2) it was fused to a Bam HI linker sequence inserted at the +6 position of the MT-I gene (*13*), in the other (plasmid 112, Fig. 2) we utilized the existing Bgl II site located at +64 of the MT-I gene. The latter construction also includes a piece of phage λ DNA on the 3′ side of the hGH gene, a remnant from a previous construction that is presumably inconsequential. In both cases, the linear fragments indicated by the interior arrows were isolated and about a thousand copies were injected into the male pronucleus of fertilized mouse eggs. In one set of experiments the eggs and sperm were from the inbred C57 mouse line, in a second set the eggs and sperm were from C57 × SJL hybrids. A total of 101 mice developed from these eggs: 6.3 percent of the C57 eggs surviving injection developed into mice compared to 10.4 percent of the hybrid eggs. Retention of the hGH gene sequences in these mice was scored by "tail blots" (*14*) in which tail DNA was denatured, spotted onto nitrocellulose, and hybridized with a 1-kb Pvu II probe that spans most of the hGH structural gene (see Fig. 2). A total of 33 out of 101 of the animals were positive for hGH DNA. To quantitate the number of hGH sequences, we determined the DNA content of the samples, spotted samples in duplicate along with normal

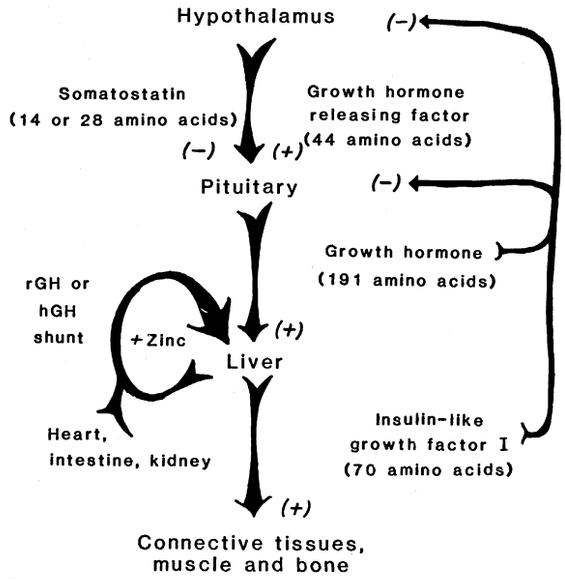

Fig. 1. Growth hormone (GH) cascade. GH participates in the regulation of growth as depicted in this diagram. The hypothalamic neuropeptides somatostatin and GH-releasing factor act on the pituitary to inhibit or stimulate, respectively, the release of GH. GH stimulates the hepatic production of insulin-like growth factor I (IGF-I) which in turn acts on peripheral tissues to stimulate their growth. This endocrine system is regulated at several different levels; possible points of feedback regulation by GH or IGF-I are indicated. The rGH or hGH shunt refers to the extrapituitary production of rGH or hGH as achieved by gene transfer. These foreign GH genes are insensitive to normal feedback mechanisms.

human DNA as standard, hybridized as above, and determined the radioactivity. In Table 1, the transgenic animals are listed according to MThGH gene copy number, which varied from 0.9 to 455 copies of the MThGH gene per cell, because we are not sure that there is a meaningful difference in the expression of the two fusion gene constructs in either inbred or hybrid mice.

Table 1 also summarizes the growth ratio of these transgenic animals compared to sex-matched littermates. A majority (23 out of 33) of the transgenic animals grew more than 18 percent larger than their littermates; several were twice as large. There is no correlation between growth rate and MThGH gene copy number as some of the largest animals had only a few copies, but most (seven out of ten) of the transgenic animals that did not grow larger had less than three copies.

To explore the basis of enhanced growth of these transgenic animals, we measured the amount of hGH in the serum by radioimmunoassay (Table 1). The circulating hGH levels ranged from undetectable to 64 μg/ml compared to normal values of 10 to 100 ng of mouse GH per milliliter (*15*). All of the transgenic mice that grew larger than controls had immunoreactive hGH in their serum, but the relations between growth rate and circulating hGH levels are crude. It appears that less than 100 ng of hGH per milliliter is sufficient to stimulate nearly maximal growth, but it is perplexing that some animals with intermediate or high levels of circulating hGH do not grow as well as others. A most interesting case is Hyb-182-4♀; this animal apparently expresses the gene, since substantial amounts of immunoreactive hGH are present in the serum, but it still fails to grow more than normal.

The growth rate of the largest transgenic mouse, which had integrated two copies of MThGH gene per cell and had a moderate level of circulating hGH (250 ng/ml), is shown in Fig. 3A together with a typical normal littermate of the same sex. These two animals are shown on the cover at 24 weeks of age. Mice expressing MThGH genes are already larger than littermates at weaning (~ 5 weeks) and they grow rapidly until 11 to 13 weeks; during this time the growth rate is typically two to three times that of normal mice of this strain. To establish more accurately when GH first begins to be effective, we bred mouse C57-173-3♂ and compared the growth rates of offspring that did and did not receive the MThGH genes from their father. Figure 3B shows that accelerated growth begins between 16 and 22 days after birth. We have not yet systematically examined MThGH gene expression during fetal development, but we do know that these genes are expressed before birth. Thus, we suspect that the mice became sensitive to GH 2 to 3 weeks after birth. This experiment also documents that the enhanced growth rate is heritable, a point that has also been established with the transgenic mice expressing MTrGH fusion genes (*16*).

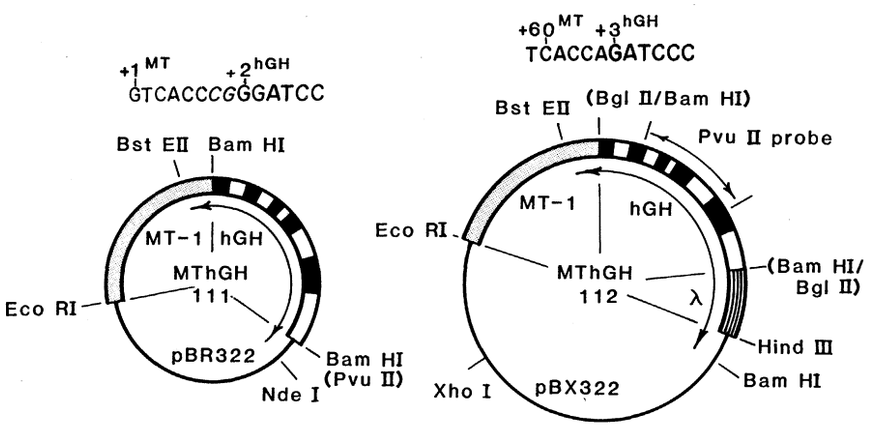

Fig. 2. Construction of metallothionein-human growth hormone (MThGH) plasmids. For MThGH 111, a Bam HI linker sequence (CGGGATCCCG) was inserted between the +6 position of mouse MT-I gene and the Pvu II site of pBR322. Then a 2.15-kb Bam HI fragment including the hGH structural gene was excised from a 2.65-kb Eco RI subclone of hGH_N in the polylinker of pUC12 and inserted into the Bam HI site of the MT-I vector to give a 6.3-kb plasmid. The sequence at the MT-I/hGH junction is shown [see (*12*) and (*13*) for more sequence information]; the seventh and eighth nucleotides are derived from the Bam HI linker. For MThGH 112, a MT-I vector was used in which the Bgl II site of MT-I (at position +64) was followed by a 0.7-kb BgI II-Hind III fragment of phage lambda in a pBR322 derivative called pBX322 because of the Xho I linker in the copy control region. The same 2.15-kb hGH Bam HI fragment was inserted into this vector to generate an 8.9-kb plasmid. The sequence at the MT-I/hGH junction is shown. The stippled region represents 5′ flanking sequences of MT-I; solid boxes represent hGH exons; open boxes represent hGH introns and 3′ flanking sequences; striped box represents phage lambda sequences; and the solid line represent pBR322. The inner arrows indicate the DNA fragments that were isolated and microinjected into mouse eggs. The location of the Pvu II probe used to detect transgenic mice is indicated by the outer arrows.

Consequences of High Levels of Circulating hGH

Because the hGH is produced in these transgenic animals from fetal stages onward, their immune system presumably recognizes hGH as self. Thus, long-term stimulation by hGH delivered in this manner may be more effective than delivery based on injection or continuous infusion into newborn or adult animals.

Since GH is thought to mediate growth indirectly via the stimulation of IGF-I (*4*), we anticipated that animals growing rapidly in response to hGH would have elevated IGF-I levels in their serum. Table 2 summarizes the results from four transgenic animals from which we obtained blood samples successively over a period of a month after supplementing their water with 25 m*M* $ZnSO_4$. The hGH concentrations of individual animals were relatively constant over this period but varied from animal to animal. IGF-I levels were elevated about two- to threefold and were inversely correlated with the high-circulating hGH concentrations. This inverse relationship might help explain the lack of correlation between hGH concentrations and growth.

Because of the long-term exposure to elevated concentrations of hGH, we were curious to know whether histological examination of the pituitaries of these transgenic animals would reveal abnormalities. Pituitaries of three transgenic mice were examined after applying Slidders' strain (*17*) which differentiates many of the pituitary cell types. Two of the mice (Hyb-197-5♀ and Hyb-184-5♂) had few (less than 1 percent) acidophilic cells representing somatotrophs and lactotrophs which synthesize GH and prolactin, respectively, while the third transgenic mouse (C57-173-2♀) had only 25 percent of the normal number of acidophilic cells. (Fig. 4, A and B). The transgenic pituitaries resembled those of *lit/lit* mice (Fig. 4C) which have a genetic deficiency in GH production (*18*).

Regulation of hGH Synthesis by Heavy Metals

Because these fusion genes carry the mouse MT-I gene promoter, we were interested to know whether MThGH messenger RNA (mRNA) would be inducible by heavy metals such as cadmium or zinc. To quantitate MThGH mRNA levels we used a solution hybridization protocol which is sensitive enough to detect less than one molecule of mRNA per cell (*19*). As a probe we used a Taq 1 fragment (272 bp corresponding to parts of exons 4 and 5) isolated from a homologous, human placental lactogen complementary DNA (cDNA) clone (*20*). This fragment was nick-translated and the cDNA strand was purified as described previously (*19*). Control experiments revealed that it hybridized equally well to human placenta and human pituitary RNA under standard hybridization conditions. To measure induction by heavy metals, we subjected the mice to a partial hepatectomy and then switched them to a water supply containing 25 m*M* $ZnSO_4$. Two weeks later we subjected the mice to another partial hepatectomy. Table 1 shows that in four transgenic mice treated in this way, Zn treatment increased the amount of MThGH mRNA between 3- and 170-fold. Thus, the foreign genes seem to be regulated in a manner similar to the endogenous MT-I genes (*21*). The induction in MThGH mRNA also resulted in an increase in circulating hGH (Table 1). Hepatectomy alone stimulates MT gene expression (*9*), but controls have shown that this stimulation subsides within 2 weeks (*14*).

Tissue Specific Expression of MThGH Genes

Given that foreign genes can be expressed in transgenic animals, one of the salient questions is whether these genes are regulated properly. The mouse MT-I gene can be considered a "housekeeping" gene in that it is expressed in nearly all tissues (with the exception of thymus) and it is inducible by heavy metals in most of these tissues, although the extent of induction varies considerably from tissue to tissue (*21*). A reasonable model is that the MT-I gene is regulated by a metal-binding protein that interacts with the promoter. Thus, in the simplest case, we expected that proper regulation

Table 1. Expression of MThGH genes in transgenic mice. All mice that were positive for hGH gene sequences are listed. Animals were either inbred (C57-) or hybrids (Hyb-) developing from C57 × SJL eggs fertilized by C57 × SJL sperm.

Animal	Plasmid*	Gene copy number/cell†	Liver hGH mRNA (molecules/cell)‡			Serum hGH (ng/ml)§			Relative growth (ratio)¶
			Control	+Zn	+Cd	Control	+Zn	+Cd	
C57-173-2♀	111	455	902	2,730		9,600	130,000		1.82
C57-173-3♂	111	405				120			1.43
C57-168-5♂	112	91	15	210		90	4,000		1.26
Hyb-194-2♀	112	47.3				< l.d.			0.96
Hyb-182-3♂	111	45				3,700	14,600		1.67
Hyb-182-2♀	111	44				64,000			1.30
C57-168-6♀	112	38.5			818	3,500		27,800	[1.74]
Hyb-185-2♂	111	34				80			1.24
C57-167-2♀	112	18.5			1,242	4,600		18,900	(1.55)
Hyb-186-4♀	111	18				8,200	143,000		2.14
Hyb-197-3♀	112	12.2				25			0.96
C57-168-2♀	112	11.7				100			1.30
Hyb-184-5♂	111	10.4				520	18,000		1.70
Hyb-186-3♀	111	10.2	2	345		80	6,400		1.34
C57-167-5♂	112	6.8				10			1.18
Hyb-180-1♂	111	6.3				3,000			1.55
C57-168-4♀	112	6.1				45			1.20
Hyb-198-3♀	112	6.1				190			1.02
Hyb-186-5♂	111	4.1			990	6,500		19,800	(1.95)
Hyb-186-1♂	111	3.5			657	2,900		4,500	(1.84)
Hyb-198-2♀	112	2.6				100			(2.16)
Hyb-182-4♀	111	2.3				1,200			0.97
Hyb-184-1♀	111	2.0				250	11,900		2.37
Hyb-184-7♀	111	2.0				30			0.96
C57-161-1♀	112	1.6				40			1.29
Hyb-194-3♀	112	1.4				< l.d.			0.93
Hyb-194-6♂	112	1.4				< l.d.			0.99
Hyb-194-8♀	112	1.3				80			1.03
C57-168-3♀	112	1.2				75			1.52
Hyb-184-2♂	111	1.1				275			2.03
C57-170-1♂	111	0.9				< l.d.			0.87
Hyb-194-4♂	112	0.9				60			1.01
Hyb-197-5♀	112	0.9	1	12		20	35		1.77

*About 1000 copies of linear DNA fragments isolated from MThGH plasmids 111 or 112 (see Fig. 2) were microinjected into the male pronucleus (*8*). †Tail DNA (3 μg) was denatured in base, spotted onto nitrocellulose, baked, hybridized with the nick-translated Pvu II probe (see Fig. 2) as described (*14*), and washed. The radioactivity was then measured by scintillation counting. For quantitation we assumed that human DNA has five homologous sequences (*11*) per haploid genome of 3.2 pg. ‡MThGH mRNA was measured by solution hybridization (*19*) with a ^{32}P-labeled cDNA derived from a 272-bp Taq I fragment that covers parts of exons 4 and 5 of human placental lactogen (*20*). A partial hepatectomy was performed on four of the mice (control values) before we supplemented the water with 25 m*M* $ZnSO_4$ for 2 weeks, at which time a second partial hepatectomy was performed (+Zn). Four other animals were injected twice with $CdSO_4$ (1 mg/kg) 18 and 4 hours before they were killed (+Cd); see Table 3. Experiments were performed when the animals were 7 to 21 weeks old. §Human GH was measured in triplicate by radioimmunoassay on serum samples (up to 10 μl) drawn before (control) or after treatment with $ZnSO_4$ or $CdSO_4$. < l.d., less than lower than limit of detection (~ 10 ng/ml) with standard assay. ¶The relative weights of transgenic mice compared with sex-matched littermates at 16 weeks of age are shown. Weights in parentheses were taken at 10 weeks, weights in brackets were taken at 6 weeks.

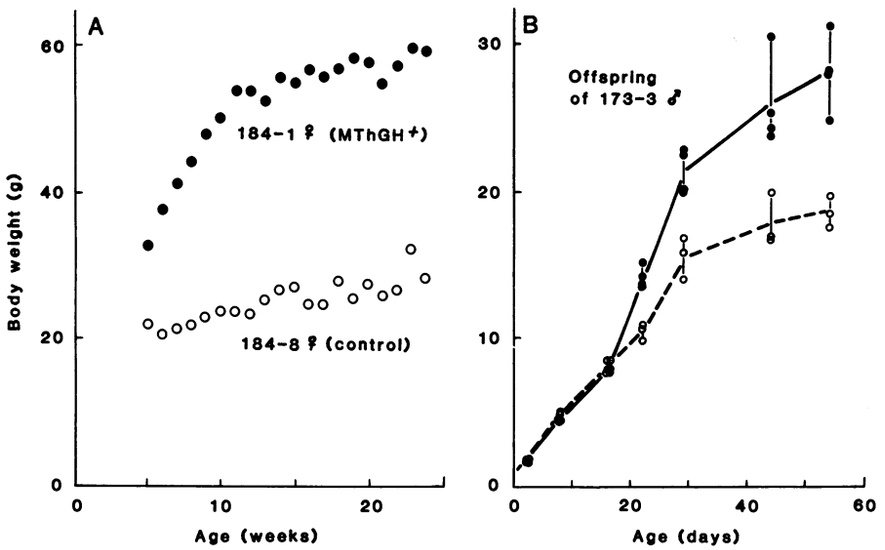

Fig. 3. Comparative growth of transgenic mice expressing MThGH genes and controls. (A) Body weights of a transgenic female (Hyb-184-1, solid circles) and a control female littermate (Hyb-184-8, open circles) were recorded weekly after weaning. The water was supplemented with 25 m*M* $ZnSO_4$ at 17 weeks. (B) A transgenic male (C57-173-3) was outbred producing seven pups in the first litter. The mice were marked and their weights were recorded periodically; subsequently hybridization of tail DNA with the Pvu II probe revealed that four offspring inherited the MThGH genes (solid circles) and three did not (open circles). Individual body weights are indicated; significant ($P < 0.05$) differences in weights of mice with and without MThGH genes were evident at 22 days and older (Student's *t*-test). A second litter was similarly analyzed and showed the same growth relationships.

would be reflected in a constant ratio of MThGH mRNA to the endogenous mouse MT-I mRNA when comparing one tissue to another.

Table 3 shows the results of MThGH and MT-I mRNA determinations from eight tissues of four transgenic animals that were stimulated with $CdSO_4$. When one compares the MThGH to MT-I mRNA ratios in different tissues of the same transgenic animal, it is apparent that they are far from constant, varying from 32- to 187-fold. The extreme example is animal C57-167-2♀, which expresses the MThGH gene well in the liver but hardly at all in the kidney. Since both mRNA's are measured in triplicate from the same total nucleic acid preparation and the endogenous MT-I mRNA levels are relatively constant from animal to animal, these different ratios are unlikely to be due to mRNA quantitation errors. In these comparisons, the differences in mRNA ratios could be due either to tissue specific differences in transcriptional response of the endogenous MT-I genes and the foreign MThGH genes or to differences in the relative stability of the two mRNA's from tissue to tissue. If the latter were true, then one might expect the ratios in the same tissue of different animals to be constant. When these comparisons are made (Table 3) we observe that the ratios are similar within a given tissue for most, but not all, of the animals.

Our interpretation of these results is that there is a systematic hierarchy of MThGH mRNA production in different tissues relative to the endogenous MT-I mRNA: liver > testis > heart > (lung, spleen, intestine) > (kidney, brain). This hierarchy is the product of differential transcription and stability of MThGH mRNA relative to MT-I mRNA. Superimposed on this hierarchy are some strong position effects that are tissue specific. These position effects show up as extraordinarily high or low MThGH to MT-I mRNA ratios; for example, the kidney, brain, and heart of animal Hyb-186-1♂ express MThGH genes extraordinarily well, and the intestine expresses them poorly. Likewise, the heart, testis, and spleen of animal Hyb-186-5♂ express MThGH genes well. These results might be the consequence of chromosomal integration near a gene that is expressed in a comparable tissue specific manner.

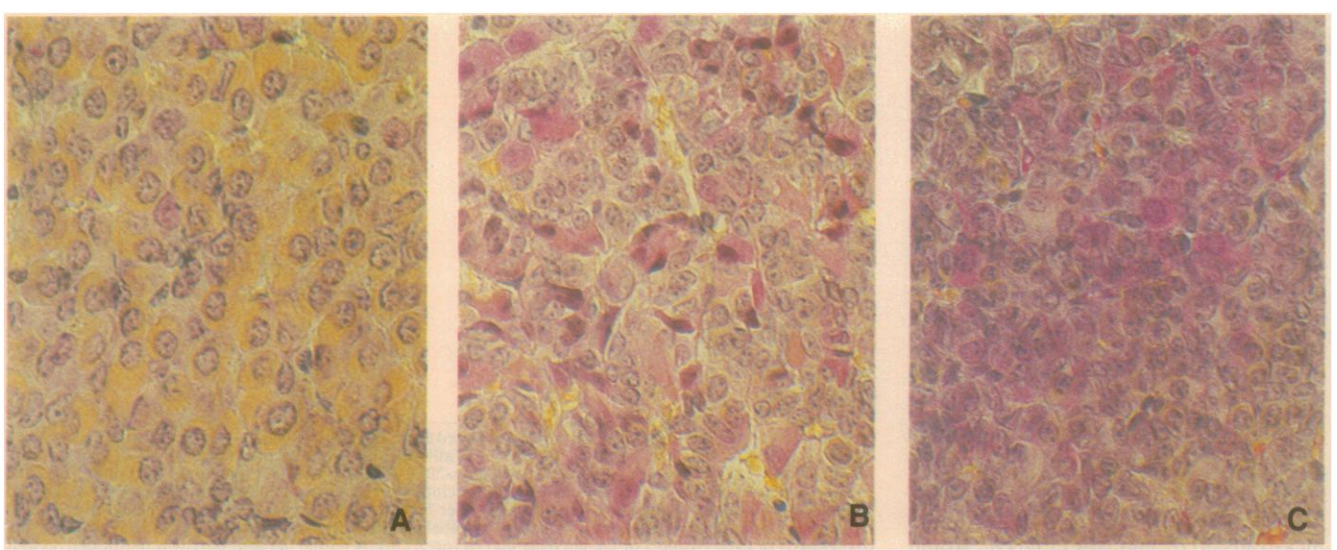

Fig. 4. Histology of pituitaries from normal transgenic and *lit/lit* (little) mice. Pituitaries were put in Bouin-Hollande fixative (*30*). They were then embedded in paraffin and serial sections (5 μm) were prepared and stained according to Slidders' method (*17*), where acidophilic cells stain yellow. (A) Control male mouse. (B) Transgenic mouse Hyb-184-5♂. (C) *Lit/lit* female mouse (×600). The number of acidophilic cells was counted in four random sections of each pituitary. The results, expressed as the number of acidophilic cells per 33 μm^2 ± standard deviation were control, 171 ± 16; control female, 132 ± 16; *lit/lit* male, 13 ± 8; *lit/lit* female, 10 ± 6; transgenic mouse Hyb-197-5♀, 1 ± 1; C57-173-2♀, 33 ± 5; Hyb-184-5♂, 1 ± 1. In sections from *lit/lit* mice the amount of acidophilic cytoplasm was also greatly reduced compared to controls.

Implications and Conclusions

This report complements and extends our previous work on the expression of MTrGH fusion genes in mice. It shows that enhanced growth does not depend on a highly homologous peptide because rat and human GH differ in 67 out of 191 amino acids (*22*). The maximum size (about twice normal) achieved with expression of MThGH genes is similar to that achieved with MTrGH genes. This growth is obtained in some transgenic mice with less than 100 ng of hGH per milliliter (Table 1) which is somewhat higher than the average circulating level of mice GH (mGH) (*15*). Thus, it appears that both mGH and hGH are of similar potency when binding to mGH receptors.

Growth is thought to result from activation of hepatic GH receptors which in turn stimulate the synthesis and secretion of IGF-I, a 70–amino acid peptide hormone that circulates to peripheral tissues to stimulate their growth (*4*). We have shown that IGF-I concentrations are elevated about two- to threefold, consistent with the hypothesis that IGF-I plays an important role in the GH cascade. The mice appear to be normally proportioned, rather than acromegalic, presumably because of the continuous exposure to increased hGH; but how this apparent allometric growth is coordinated is not clear. We do not know, for example, whether all tissues contain receptors for IGF-I or whether some of the tissues respond to yet other growth factors released in response to elevated IGF-I.

We have noted that transgenic mice do not commence greater than normal growth until about 3 weeks of age despite the presence of excess hGH prior to that time. This observation suggests that some later step in the GH cascade is rate-limiting in newborn mice; for example, production or responsiveness to IGF-I. These results are consistent with the notion that fetal and newborn growth is controlled by other hormones (*23*). One intriguing hypothesis (*24*) is that another member of the GH gene family, namely placental lactogen (also known as chorionic somatomammotropin), stimulates fetal growth acting via IGF-II. Thus, in this view two homologous gene families have diverged so that one member of each controls fetal growth and another member of each controls adult growth. The data also show that mice become insensitive to the growth stimulating properties of hGH when they are about 3 months old.

Histological examination revealed that the number of acidophilic pituitary cells was severely reduced, presumably due to feedback inhibition by the increased concentrations of hGH or IGF-I as depicted in Fig. 1. It is not yet clear whether the somatotrophs are present but do not appear acidophilic or whether they fail to develop. The histology of the pituitary, coupled with the high levels of hGH lead us to conclude that expression of the foreign MThGH genes is responsible for the accelerated growth of the transgenic mice.

The overall efficiency of achieving transgenic animals that grow significantly larger than their littermates averaged 70 percent in these experiments, a value lower than that reported for MTrGH (*7*) but more meaningful since it is based on 33 rather than 7 animals. This level of expression is similar to that achieved with MT-thymidine kinase fusion genes (*14*). But this high frequency of expression is not universally true of MT fusion genes because MT-human α-globin and MT-β-galactosidase constructs have not worked well (*25*). Despite this high ratio of expressors, we have little control on the level of expression of the foreign genes. In the 23 animals described here, the circulating level of hGH in serum ranged from 10 to 64,000 ng/ml in the absence of exogenous heavy metals (Table 1). Some of this variability might result from the number of integrated genes, but the site of integration probably has the most profound effect. These results are similar to those observed with integrated viral genomes (*26*), but they contrast with the relative uniformity of expression of genes introduced into *Drosophila* by P element vectors (*27*). These differences might relate to either the different mechanisms of gene commitment in flies and mice (for example, DNA methylation) or to an important role of the P element itself in promoting gene expression in a uniform manner (perhaps by preventing encroachment of neighboring chromatin influences). Development of a comparable vector for mammalian gene transfer would clearly be advantageous. Nevertheless, the current methods allow some insight into the role of chromatin position on gene expression because in each animal the insertion site is different.

Table 2. Effects of high hGH concentrations on mouse IGF-I levels. Serial blood samples were collected four times between 17 and 21 weeks of growth on a diet supplemented with 25 m*M* $ZnSO_4$ in the drinking water. Serum was prepared and hGH was measured by radioimmunoassay (RIA) with a kit obtained from the NIAMDD. The RIA procedures were adapted for use with small-volume samples but otherwise were performed essentially as recommended by the NIAMDD. [^{125}I]-labeled tracers were prepared by means of Iodogen (Pierce Chemical Company). No measurable immunoreactivity of hGH could be found in the serum of control mice. IGF-I was determined by human placental membrane radioreceptor assay with pure human IGF-I being used as standard (*28*). Serum samples were extracted with acid-ethanol to enable us to assay the total IGF-I. Four serum samples were individually assayed for total IGF-I; transgenic mice had significantly ($P < 0.05$) higher levels of IGF-I than controls.

Animal	hGH (μg/ml)	IGF-I (μg/ml)
Hyb-182-3♂	14.6 ± 1.4*	1.48 ± 0.13
Hyb-184-1♀	11.9 ± 0.7	1.73 ± 0.06
Hyb-184-5♂	18.0 ± 1.5	1.21 ± 0.08
Hyb-186-4♀	143.0 ± 6.5	1.04 ± 0.12
Controls	0	0.55 ± 0.04

*Standard error of the mean, $N = 4$ (samples per animal collected about 1 week apart).

The high level of expression of MThGH genes in some animals allows quantitation of mRNA concentrations in various tissues of transgenic animals. Transgenic mice expressing herpesvirus thymidine kinase have been unsatisfactory for these studies because the concentration of thymidine kinase mRNA is too low to quantitate in most tissues (*8*). For this study, we deliberately chose four animals with high levels of circulating hGH. These four animals showed similar high levels of MThGH mRNA in the liver, but lower, variable levels of MThGH mRNA in other tissues. Considering the mass of these tissues relative to the pituitary, it is not surprising that the circulating hGH was high, averaging 4400 ng/ml prior to treatment with cadmium. The data in Table 3 show that expression of the MThGH genes is particularly favored in the liver, testis, and heart, since the ratios of MThGH mRNA to the endogenous MT-I reference mRNA are highest in these tissues. This might be due to a higher percentage of responsive cells, establishment of more efficient chromatin structure, higher levels of proteins that enhance transcription, or greater stability of the fusion mRNA in these tissues. Superimposed on a hierarchy of expression in different tissues, there are some striking departures which are attributed to tissue specific position effects. For example, if the foreign genes were integrated near a locus that was normally activated only in the kidney, then we might expect an unusually high level of MThGH expression in the kidney.

One particularly bothersome aspect of our results is that there is a poor relation between gene dosage and the level of expression. A simple explanation (that is unfortunately hard to prove) is that only

one or a few genes in the tandem arrays (*8, 14*) are actually expressed. If these favored genes were at the ends of the arrays then they would be subject to neighboring chromatin influences. If, on the other hand, all genes within the array were expressed equally, it would be harder to explain the position effects because some of these arrays are hundreds of kilobases long and the genes in the middle of these arrays would be expected to have an identical environment. These considerations suggest that the use of longer DNA fragments, rather than the minimal fragments employed here which have only 400 bp of MT-I gene sequence, might effectively isolate the individual genes in the tandem arrays and thereby result in better gene dosage relationships and less influence of neighboring chromatin.

Another feature of MThGH fusion genes that is documented here is that in all animals tested the level of hepatic MThGH mRNA or serum hGH increased in response to $CdSO_4$ or $ZnSO_4$ administration (Table 1). In similar studies we have shown that $CdSO_4$ increases the rate of transcription from MTrGH genes (*16*). Thus, it appears that these MT fusion genes respond to heavy metals like their endogenous MT-I gene counterparts and proves that this transcriptional response does not depend on MT structural gene sequences or chromosomal location.

Figure 3 shows that rapid growth of transgenic mice is heritable. This is not surprising in view of our parallel studies in which we have shown that MTrGH fusion genes are transmitted in a Mendelian manner to half of the offspring during two generations and that all of the mice that inherit the MTrGH fusion gene grow two to three times faster than their normal littermates (*16*).

It is clear that transgenic animals generated by microinjection of foreign genes into fertilized eggs provide access to a number of important developmental, genetic, and endocrine questions. We are in the process of generating a homozygous line of giant mice on the C57 background. These mice should provide a valuable resource for analysis of gene inheritance and expression as well as the consequences of excess GH production on various physiological processes.

Table 3. Tissue specific expression of MThGH fusion genes. Total nucleic acids (TNA) were prepared from the indicated tissues derived from transgenic mice (7 to 13 weeks old) stimulated with $CdSO_4$ (1 mg/kg; 18 and 4 hours before they were killed). Samples were digested with proteinase K in sodium dodecyl sulfate and then extracted with phenol and chloroform (*14*). The TNA samples were used to determine mRNA levels (MT-I and MThGH) by solution hybridization to ^{32}P-labeled cDNA probes as described (*19*). The number of molecules per cell were calculated by assuming that there are 6.4 pg of DNA per cell and that MT-I mRNA and hGH mRNA are 391 and 1000 bases long, respectively. The DNA concentration was determined by a fluorescence method (*29*).

Tissue	Animal	Molecules of MT-I mRNA per cell	Molecules of MThGH mRNA per cell	Ratio of MThGH to MT-I
Liver	C57-168-6♀	2560	818	.32
	C57-167-2♀	2230	1240	.56
	Hyb-186-1♂	2490	657	.26
	Hyb-186-5♂	2310	990	.43
Kidney	C57-168-6♀	680	4.6	.007
	C57-167-2♀	283	0.9	.003
	Hyb-186-1♂	139	93	.67*
	Hyb-186-5♂	203	5	.024
Intestine	C57-168-6♀	681	33	.05
	C57-167-2♀	381	28	.07
	Hyb-186-1♂	378	5	.01
	Hyb-186-5♂	377	22	.06
Heart	C57-168-6♀	211	22	.10
	C57-167-2♀	210	33	.16
	Hyb-186-1♂	191	110	.58*
	Hyb-186-5♂	200	166	.83*
Brain	C57-168-6♀	162	2.2	.01
	C57-167-2♀	135	2.6	.02
	Hyb-186-1♂	126	37	.29*
	Hyb-186-5♂	91	1	.01
Spleen	C57-168-6♀	33	1.6	.05
	C57-167-2♀	24	1.4	.06
	Hyb-186-1♂	5	0.2	.04
	Hyb-186-5♂	10	6	.60*
Lung	C57-168-6♀	31	1.3	.04
	C57-167-2♀	40	1	.03
	Hyb-186-1♂	12	1	.08
	Hyb-186-5♂	13	1	.08
Testis	Hyb-186-1♂	166	56	.34
	Hyb-186-5♂	169	113	.67

*Marked deviation of ratio from average.

References and Notes

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