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Expression of a mutated bovine growth hormone gene suppresses growth of transgenic mice

(*in vivo* antagonists/dwarf mice/amphiphilic α-helix)

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ABSTRACT **To determine the importance of the third α-helix in bovine growth hormone (bGH) relative to growth-related biological activities, the following experimental approach was used: (*i*) mutagenesis of helix III of bGH to generate an idealized amphiphilic helix; (*ii*) *in vitro* expression analyses of the mutated bGH gene in cultured mouse L cells; (*iii*) mouse liver membrane binding studies of wild-type and mutated bGH; and (*iv*) expression of the mutated gene in the transgenic mouse. An altered bGH gene (pBGH10Δ6-M8) was generated that encodes the following changes: glutamate-117 to leucine, glycine-119 to arginine, and alanine-122 to aspartate. The plasmid pBGH10Δ6-M8 was shown to be expressed in, and its protein product secreted by, mouse L cells. The altered hormone possessed the same binding affinity to mouse liver membrane preparations as wild-type bGH. Transgenic mice containing the mutated bGH gene, however, showed a significant growth-suppressed phenotype. The degree of suppression was directly related to serum levels of the altered bGH molecule.**

Bovine growth hormone (bGH) is a single-chain polypeptide composed of 191 amino acids with a molecular mass of ≈22,000 daltons (1–4). It is produced and secreted by cells of the anterior pituitary gland. A variety of growth hormone (GH) genes and cDNAs have been cloned, including those derived from human (5), rat (6), cow (7), pig (7), and chicken (8). The bGH gene consists of five exons and four introns and encodes a protein containing 217 amino acids (9). Twenty-six amino acids encoded by exon I and part of exon II direct the synthesis of the bGH signal peptide, which is not retained in the mature hormone. The NH_2 terminus of the secreted form of bGH is heterogeneous, with ≈50% of the molecules beginning with alanine and the remainder starting with phenylalanine (10). This heterogeneity is likely due to differences in utilization of the signal-peptide cleavage site. Four cysteine residues are found in mature bGH that form two disulfide bonds. One forms a large loop between amino acid residues 53 and 164. The other, found between residues 181 and 189, forms a small loop.

GHs found in various species have been reported to be involved in growth promotion (11, 12) as well as other processes including lipid, nitrogen, mineral, and carbohydrate metabolism (13–19). The mechanism by which GH exerts its multiple biological effects is not well understood. The GH molecule may possess multiple active domains (20), may contain distinct amino acid sequences within the molecule that are responsible for the various biological activities (21), or may bind to multiple receptors (22).

The three-dimensional structure of porcine GH (pGH) has been analyzed at a 2.8-Å resolution by x-ray diffraction techniques (23). According to the crystal structure, pGH contains four antiparallel α-helical regions. Since there is >90% amino acid sequence identity between bGH and pGH, it is likely that bGH has a similar three-dimensional structure. By aligning these α-helical structures into a two-dimensional Edmundson wheel projection (24), it is clear that an amphiphilic α-helical segment exists between amino acid residues 109 and 126 in the third α-helical region (ref. 25; Fig. 1). However, amino acids 117, 119, and 122 are positioned so that an idealized amphiphilic α-helix is not generated.

In this study, we have used the following approach to determine whether this amphiphilic α-helix is important in the growth-related properties of bGH: (*i*) mutagenesis of the third α-helix of bGH so as to generate an idealized amphiphilic α-helix (Fig. 1); (*ii*) expression analyses of the wild-type and mutated bGH genes in cultured mouse L cells; (*iii*) mouse liver membrane binding studies comparing wild-type and mutated bGH; and (*iv*) production of transgenic mice containing wild-type and mutated bGH genes.

MATERIALS AND METHODS

Plasmids and *in Vitro* Mutagenesis. Plasmid pBGH10Δ6 was used as the parental plasmid in this study (Fig. 2). It encodes wild-type bGH and contains intron A (while lacking introns B, C, and D) fused to the mouse metallothionein I transcriptional regulatory sequences. pBGH10Δ6-M8 was derived from pBGH10Δ6. Mutations within the amphiphilic α-helix between amino acid residues 109 and 126 were accomplished by segment-directed mutagenesis in which two oligonucleotides containing the designed mutations were synthesized by using a DuPont Coder 300 DNA synthesizer. The two oligonucleotides encoded the following changes: Glu-117 → Leu, Gly-119 → Arg, and Ala-122 → Asp. Also, one silent mutation was designed in these oligonucleotides that generated a *Bam*HI restriction site. This unique *Bam*HI site was used in plasmid screening. The oligonucleotides were hybridized and inserted between the *Tth*III I and *Xma* I sites by standard procedures (26). A plasmid, pBGH10Δ6-M8, was selected and purified, and mutations were confirmed by Sanger dideoxy sequence analysis (27). The mutated protein encoded by this DNA was termed bGH-M8.

Cell Culture, Transient Expression, and Immunoblotting. Mouse L cells (thymidine kinase-negative and adenine phosphoribosyltransferase-negative) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% Nu-Serum (Collaborative Research). Transfection protocols using mouse L cells have been described (28).

Twenty microliters of culture fluids from day 5 posttransfected mouse L cells and 6 μl of mouse serum from Mt/bGH transgenic animals were analyzed by sodium dodecyl sulfate (SDS)/PAGE (15% or 12.5% polyacrylamide) as described (29). After electrophoresis, the proteins were transferred to

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Abbreviations: GH, growth hormone; bGH, bovine GH; hGH, human GH; pGH, porcine GH.

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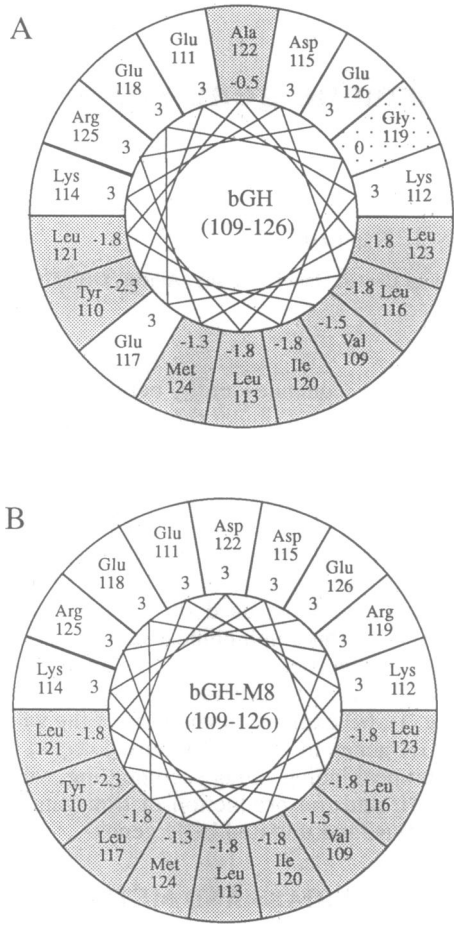


FIG. 1. Axial projection of the third α-helix (residues 109–126) of the wild-type (*A*) and mutated (*B*) bGH. Amino acid positions and corresponding hydrophilicity values are given. Amino acid residues in the open boxes are hydrophilic (top half of the wheel), and those in the shaded boxes are hydrophobic (bottom half). The glycine residue (residue 119) is depicted with dots.

nitrocellulose filters and probed with rabbit polyclonal bGH antiserum as described (28).

Binding Studies. Mouse liver membrane binding studies using C57BL/6J × SJL hybrid mice were performed as described (29). ^{125}I-labeled bGH (^{125}I-bGH) was obtained from Cambridge Medical Diagnostics (Billerica, MA).

Transgenic Mouse Production. The procedure for production of transgenic mice by direct microinjection of DNA into the male pronucleus of fertilized mouse eggs has been described (30). The linear DNA fragment used for microinjection was a 3-kilobase (kb) molecule located between the *Eco*RI sites in pBGH10Δ6, which includes the mouse metallothionein I transcriptional regulatory sequences and the bGH coding region (Fig. 2). DNA extraction from mouse tails, slot blots, and serum bGH determinations were performed as described (30).

RESULTS

***In Vitro* Mutagenesis.** A mutated bGH gene (pBGH10Δ6-M8) was generated that encodes three amino acid substitutions: Glu-117 → Leu, Gly-119 → Arg, and Ala-122 → Asp as seen in Fig. 2. These changes were confirmed by Sanger dideoxy sequence analysis.

***In Vitro* Expression.** The plasmids pBGH10Δ6 and pBGH10Δ6-M8 were transiently transfected into cultured mouse L cells. The culture fluids were collected and analyzed by immunoblotting. Protein bands of ≈22 kDa were observed (Fig. 3). Wild-type bGH (Fig. 3, lane B) derived from pBGH10Δ6 and mutated bGH derived from pBGH10Δ6-M8 (Fig. 3, lane C) revealed discrete bands that possess the proper apparent molecular mass.

Binding Studies. Culture fluids lacking serum were collected from cells transfected with either pBGH10Δ6 or pBGH10Δ6-M8 DNA. After lyophilization of the culture medium, the bGH concentration was determined by spectrophotometric analyses of the immunoblots. Competitive receptor binding studies were carried out as described. A Scatchard analysis on the data from competitive binding experiments is shown in Fig. 4. Mean dissociation constants (K_d) of wild-type bGH and bGH-M8 were calculated from four separate experiments in which each experiment was carried out in triplicate. The computer program LIGAND was used for K_d determinations (31). The results indicated that there were no significant differences in K_d values between wild-type bGH (10.47 ± 1.15 nM) and bGH-M8 (9.82 ± 1.71 nM) as determined by the Student *t* test.

Transgenic Mouse Production. Transgenic mouse lines that contained wild-type and pBGH10Δ6-M8 genes were produced by standard microinjection techniques (30). Offspring generated were assayed for bGH DNA by slot-blot hybridization analysis (data not shown). Mouse lines generated contained bGH or bGH-M8 DNA ranging from 1 to ≈25

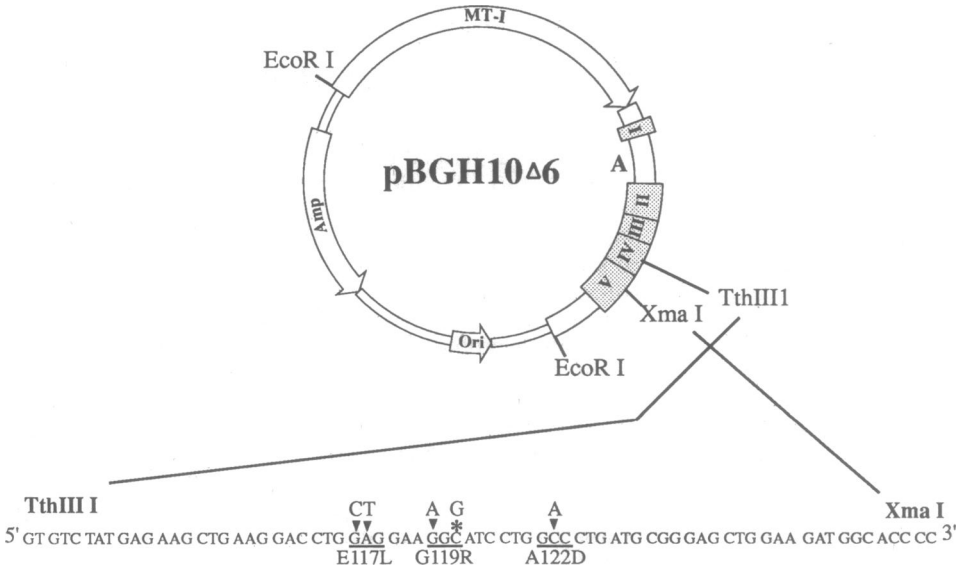


FIG. 2. General strategy of oligonucleotide-directed site-specific mutagenesis. pBGH10Δ6, the parental vector, contains the mouse metallothionein I transcriptional regulatory sequences (MT-1) fused to the bGH gene, which contains five exons (shaded boxes I–V) and intron A. This fusion gene was incorporated into pBR322 at the *Eco*RI site. The pBR322 origin of replication is indicated (Ori). The oligonucleotide sequence between the *Tth*III I and *Xma* I restriction sites is shown. Substitution mutations are indicated by arrows and encode the following changes: Glu-117 → Leu (E117L), Gly-119 → Arg (G119R), and Ala-122 → Asp (A122D). A silent substitution is also indicated (*), which generated a unique *Bam*HI site (GGATCC).

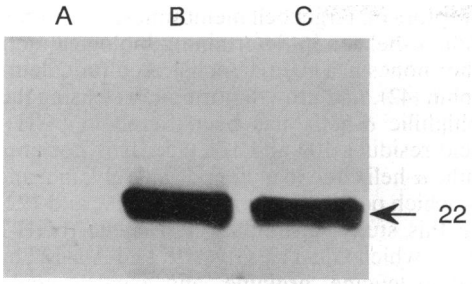


FIG. 3. Immunoblot analysis of transiently transfected mouse L cells. Culture fluids were analyzed by SDS/15% PAGE followed by immunoblotting with anti-bGH antiserum. Lanes: A, culture fluid from mock-transfected cells; B, culture fluid from cells transfected with wild-type bGH gene; C, culture fluid from cells transfected with pBGH10Δ6-M8. The arrow indicates migration of trypsin inhibitor (not shown), which possesses a molecular mass of 22 kDa.

copies. Sera from transgenic animals were collected and analyzed for bGH expression by immunoblotting (Fig. 5). Serum bGH concentrations ranged from 0.5 to 6 μg/ml for wild-type bGH transgenic mice and from 0.6 to 5 μg/ml for bGH-M8. These values are consistent with previous reports (32). All mice that expressed either wild-type or bGH-M8 in their serum were monitored for growth rate. The mean growth ratio (mean body weight of transgenic mice per mean body weight of corresponding nontransgenic littermates) for wild-type bGH transgenic mice was ≈1.2–1.6 for females and 1.4–1.6 for males (Fig. 6). The mean growth ratio for bGH-M8 transgenic mice was ≈0.8 (Fig. 6). There were significant differences ($P < 0.01$) between the body weights of pBGH10Δ6-M8 transgenic mice and their nontransgenic littermates as determined by the Student *t* test at all time points as well as a strong correlation between the serum levels of bGH-M8 and body weights (Fig. 7).

DISCUSSION

The majority of information defining functional domains in GH has been obtained through protease digestion experiments in which GH peptide cleavage products were purified and analyzed for a particular biological activity. It was found that enzymatic cleavage of bGH in the large loop between amino acid residues 134 and 150 did not affect its bioactivity (33). A fragment of bGH from amino acid residue 96 to residue 133 retained significant tibia growth-promoting activity, whereas the fragments 1–95 and 134–191 had much less activity (34). By using synthetic peptides, it was found that

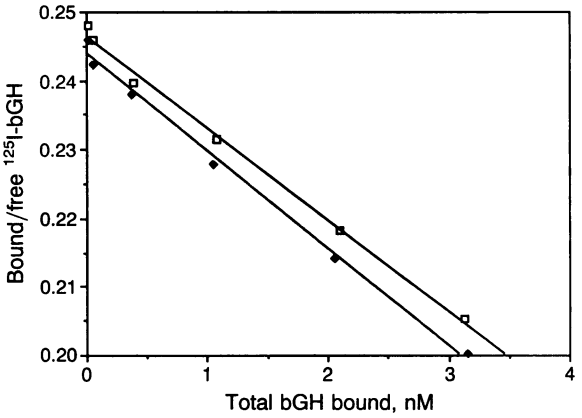


FIG. 4. Scatchard plots of data from competitive binding experiments for wild-type bGH (□) and bGH-M8 (◆) using mouse liver membrane preparations. The ordinate represents the ratio of bound to free bGH, and the abscissa represents the concentration of total bGH bound. Each point represents the mean of four experiments, each of which was carried out in triplicate.

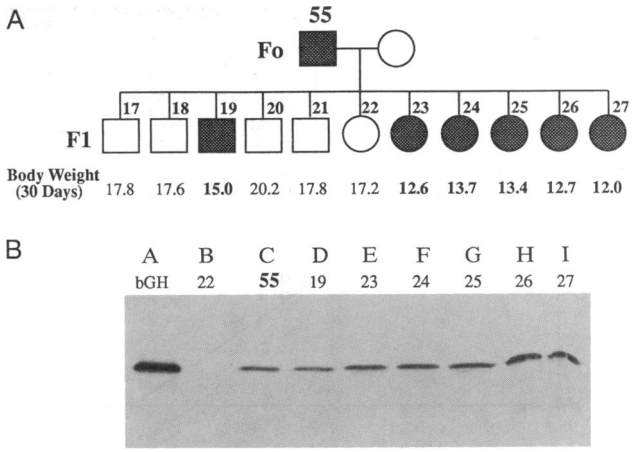


FIG. 5. Immunoblot analysis of sera from a transgenic mouse line, TG-M8. (*A*) Transgenic mouse pedigree from founder (Fo) mouse 55 (Fo shaded box). Mice positive for pBGH10Δ6-M8 DNA are shown by shading. Also, body masses are indicated at 30 days after birth. (*B*) Immunoblot analysis of sera from transgenic mice. Lanes: A, 10 ng of purified bGH, which was used as a positive control as well as for bGH serum concentration determinations; B, serum from a pBGH10Δ6-M8-negative mouse (F1-22); C, serum from founder mouse 55; D–I, sera from siblings F1-19, F1-23, F1-24, F1-25, F1-26, and F1-27, respectively.

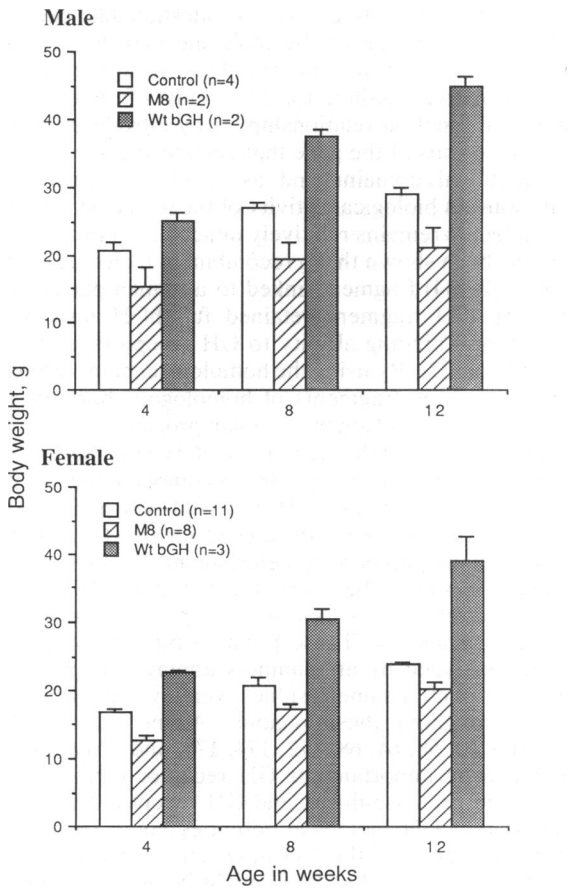
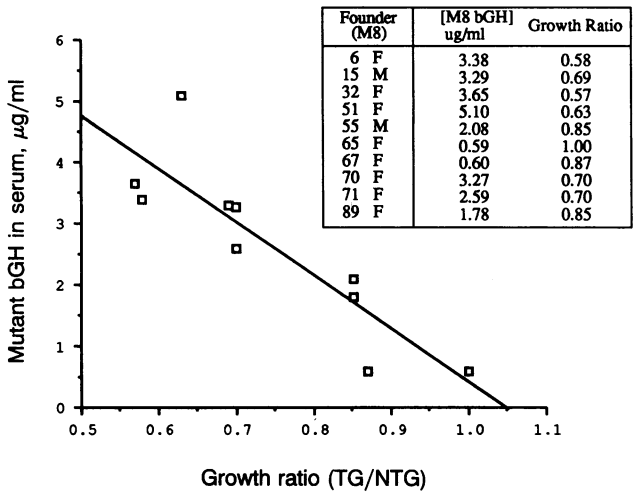


FIG. 6. Growth rate comparison between male and female founder transgenic mice containing wild-type bGH (pBGH10Δ6) or pBGH10Δ6-M8 genes and their littermates at ages 4, 8, and 12 weeks after birth. Numbers of the experimental animals for each group are indicated (*n*). The body weights of the experimental animals are expressed as the mean ± SD.



Founder (M8)	[M8 bGH] ug/ml	Growth Ratio
6 F	3.38	0.58
15 M	3.29	0.69
32 F	3.65	0.57
51 F	5.10	0.63
55 M	2.08	0.85
65 F	0.59	1.00
67 F	0.60	0.87
70 F	3.27	0.70
71 F	2.59	0.70
89 F	1.78	0.85

FIG. 7. Relationship between serum bGH-M8 concentrations and the growth ratio of 10 founder transgenic mice (TG)/nontransgenic (NTG). The ordinate represents bGH-M8 concentrations in serum. The abscissa represents the growth ratio of TG/NTG at 30 days of age. The correlation coefficient ($r = -0.87$) is highly significant ($P < 0.01$). (*Inset*) Raw data used to plot and calculate the correlation coefficient.

the NH_2 terminus of hGH (1–15) possessed insulin-like activities (35), whereas the COOH terminus (177–191) had antiinsulin activity (36).

The information obtained through analyses of protease-generated peptide fragments is questionable because the native conformation of the molecule may be dramatically disrupted in addition to the problems with fragment purity. An alternative method for the study of bGH structure–biological function relationships is by introduction of mutations in regions of the gene that encode specific amino acids or functional domains and assay of the effect of these mutations on biological activity of the molecule. In this way, the molecule remains relatively intact with minimal changes.

It has been shown that a recombinant molecule containing a hGH-(1–134) fragment linked to a human placental lactogen-(141–191) fragment retained full hGH immunological activity and binding affinity to GH receptors isolated from rabbit liver (37). By using the homolog-scanning mutagenesis technique, gene fragments of homologous hormones—i.e., human placental lactogen or human prolactin—were systematically substituted throughout the hGH gene, thus producing various chimeric hormones (38). A comparison of the binding affinities of these mutant GHs and wild-type hGH to a cloned liver hGH receptor led to the conclusion that there were three discontinuous polypeptide determinants in hGH involved in receptor binding. They were located at the NH_2 terminus, COOH terminus, and within a loop between amino acid residues 54 and 74. These putative binding domains were further analyzed by an alanine-scanning mutagenesis technique in which alanine residues were systematically substituted throughout these regions. Amino acid residues at positions 10, 58, 64, 68, 172, 174, 175, and 176 of hGH were shown to be important for GH receptor binding (39). Together the protease-digest and GH receptor-binding results suggest that GH biological activities could be ascribed to different regions of the GH molecule. In particular, amino acid residues 95–133 of bGH might be an active part of the molecule responsible for growth, which is distinct from receptor binding domains of the peptide.

Amphiphilic secondary structures have been proposed to be important functional domains for many peptide hormones (40). This hypothesis was based on the observation that most peptide hormones exert their biological effects through binding to receptors on target cell membranes. The importance of amphiphilic α-helices in determining biological activities of peptide hormones has been demonstrated for calcitonin (41), β-endorphin (42), and growth hormone-releasing factor (43). An amphiphilic α-helix has been found in bGH between amino acid residues 109 and 126 (25). It is not an idealized amphiphilic α-helix due to glutamic acid, glycine, and alanine residues, which occur at positions 117, 119, and 122, respectively. In this study, a mutated bGH gene (bGH-M8) was generated in which Glu-117, Gly-119, and Ala-122 have been converted to leucine, arginine, and aspartic acid, respectively. These amino acids were chosen because they have proper hydrophilic (arginine and aspartic acid) or hydrophobic (leucine) character (44), positively (arginine) or negatively (aspartic acid) charged side chains (40), and high α-helical-forming potential (45). These substitution mutations generated an idealized amphiphilic α-helix (Fig. 1). The plasmid containing the mutated gene was expressed, and the protein product was secreted by cultured mouse L cells (Fig. 3). In addition, the hormone (bGH-M8) bound to a mouse liver membrane preparation with the same affinity as wild-type bGH. Surprisingly, transgenic mice that expressed this mutated hormone were significantly smaller relative to corresponding nontransgenic littermates. We have generated 10 founder mice that express the mutated bGH gene. The growth ratio between the transgenic and nontransgenic littermates ranged from 0.58 to 1.00 (Fig. 6). The degree of suppression of growth was directly related to the serum levels of the mutated bGH (Fig. 7). Three founders have been bred that pass the trait to offspring; ≈50% of these offspring are positive for the gene and possess the corresponding small phenotype. Fig. 5 shows the F_1 generation derived from one transgenic male. We are currently rearing F_3 progeny from this founder.

Since there was no change in binding affinity of bGH-M8 to mouse liver membranes when compared with wild-type bGH, it is possible that bGH contains distinct growth-promoting and receptor-binding domain(s). The three substitution mutations in bGH (bGH-M8) located with the third α-helix may have altered either one or more critical amino acids in or the local conformation of this potential growth-promoting domain, subsequently resulting in a reduction in bGH growth–promoting activity. Amino acid sequence comparison among GHs and products of other members of the gene family revealed that Glu-117, Gly-119, and Ala-122 are relatively conserved in products of the GH gene family (46). Ala-122 is conserved only in GHs from nonprimate mammals as well as chickens, whereas Glu-117 is conserved in GHs from mammals. Gly-119 is conserved among products of all members of the GH gene family, which includes prolactins and placental lactogens. Therefore, these amino acids may be important for the biological activities of GHs.

It has been demonstrated that many activities of GH are mediated through a family of peptides known as insulin-like growth factors (IGF), in particular IGF-1, which is believed to be produced primarily in the liver following GH binding to its receptor(s) (47, 48). IGF-1 has been shown to decrease GH production in the pituitary by a classical negative feedback mechanism (49). One hypothesis to explain the growth suppression in pBGH10Δ6-M8 transgenic mice is that bGH-M8 is active as an *in vivo* antagonist to mouse GH (mGH), thereby suppressing mouse IGF-1 production. If this is true, then one would expect not only a reduction in serum mouse IGF-1 levels in bGH-M8 transgenic mice but also an increase in mGH production in the pituitary. Preliminary results from immunoblot analysis of whole pituitary glands taken from bGH-M8 transgenic mice, bGH transgenic mice, and their nontransgenic littermates suggest that the pituitary glands in those growth-suppressed mice contain higher levels of mGH relative to their nontransgenic littermates (data not shown).

In contrast, mGH levels in bGH transgenic mice were largely depressed because mouse IGF-1 levels in serum of bGH transgenic mice increased up to twice as much as levels in serum of their nontransgenic littermates (32). If our hypothesis were true, it would be the first example to our knowledge of an *in vivo* growth hormone antagonist and the first example of uncoupling of growth-promoting and receptor-binding activities of GHs.

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