Growth Hormone (GH) Binding and Effects of GH Analogs in Transgenic Mice (43740)

ANDRZEJ BARTKE,*[,1] DANIEL TURYN,† CLAUDIO C. AGUILAR,† ANA I. SOTELO,† RICHARD W. STEGER,* XIAO Z. CHEN,‡ AND JOHN J. KOPCHICK‡

Department of Physiology, Southern Illinois University, Carbondale, Illinois 62901; UBA-CONICET,† Buenos Aires, Argentina; and Edison Animal Biotechnology Center,‡ Ohio University, Athens, Ohio 45701*

Abstract. **Overexpression of human (h) or bovine (b) growth hormone (GH) in transgenic mice is associated with marked (2- to 12-fold) and significant increase in hepatic binding of GH and prolactin (PRL). This is due to an increase in the number of GH and PRL receptors (GHR, PRLR) per mg of microsomal protein without changes in binding affinity. Comparison of results obtained in transgenic animals expressing bGH with a mouse metallothionein (MT) or a rat phosphoenolpyruvate carboxykinase (PEPCK) promoter suggests that effects of bGH on hepatic GHR and PRLR do not require GH overexpression during fetal life and, within the dose range tested, the effects on PRLR are not dose dependent. The increase in hepatic GHR was accompanied by significant increases in plasma GH-binding protein (GHBP) and in mean residence time of injected GH. Thus life-long elevation of peripheral GH levels alters the availability of both free GH and GHR. Site-directed *in vitro* mutagenesis was used to produce hGH and bGH analogs mutated within one of the sites involved in binding to GHR and PRLR. Mutating hGH to produce amino acid identity with bGH at Position 11, 18 (within Helix 1), 57, or 60 (within the loop between Helix 1 and 2) did not affect binding to GHR *in vitro*, or somatotropic activity in transgenic mice *in vivo* but reduced lactogenic activity in Nb_2 cells by 22%–45%. Mutations of bGH designed to produce amino acid identity with hGH at one to four of the corresponding positions in the bGH molecule did not interfere with binding to GHR or somatotropic activity *in vivo*, and failed to produce significant binding to PRLR but resulted in alterations in the effects on the hypothalamic and anterior pituitary function in transgenic mice. Apparently region(s) outside the domains examined are essential for lactogenic activity of hGH, and different portions of the GH molecule are responsible for its diverse actions *in vivo*.**

[P.S.E.B.M. 1994, Vol 206]

Multiple physiological actions of growth hormone (GH) include stimulation of synthesis of GH receptors (GHR) and GH-binding protein (GHBP). The relationship between these effects of GH is of particular interest because GHBP originates either from differential transcription of the GHR gene or from cleavage of extracellular domain of GHR. Biological actions of GH are closely related to the actions of another pituitary hormone, prolactin (PRL). Growth hormone and PRL, as well as GHR and PRL receptors (PRLR) exhibit significant structural homology (1, 2); GH can induce both GHR and PRLR (3, 4); and human GH (hGH) interacts with both GHR and PRLR in rodents (5). Against this background, we decided to examine the effects of bovine GH (bGH) and hGH on hepatic GHR and PRLR, circulating GHBP and pharmacokinetics of GH. For these studies, we have used transgenic mice, which provide a unique model of exposure to high levels of heterologous hormones starting during fetal or early postnatal life and continuing throughout the entire life span without complications due to immunogenicity of injected or infused heterologous GH in "conventional" animals.

We are also interested in the interaction of different domains of the GH molecule with GHR and/or PRLR. The recent evidence that GH action involves simultaneous binding of one GH molecule to two GHR (6, 7) and suggestions that a similar mechanism may apply also to the actions of PRL (8) provide new

[1] To whom requests for reprints should be addressed at Department of Physiology, Southern Illinois University, School of Medicine, Carbondale, IL 62901–6512.

0037-9727/94/2063–0190$10.50/0

framework for analyzing these interactions and for determining why hGH but not bGH can exert lactogenic effects. We are approaching this problem by examining the consequences of site directed *in vitro* mutagenesis of nonhomologous regions of hGH and bGH.

Production and Characteristics of Transgenic Mice

Transgenic mice utilized for the studies of GHR, GHBP, and pharmacokinetics of injected GH were derived from lines expressing hGH or bGH with mouse metallothionein-1 (MT) promoter or bGH with rat phosphoenolpyruvate carboxykinase (PEPCK) promoter. Each line was derived from a single founder produced by microinjection of the corresponding hybrid gene (MT-hGH, MT-bGH, PEPCK-bGH) into male pronuclei of recently fertilized mouse ova (9–11).

Transgenic male descendants of these founders were kindly made available to us by Drs. T. E. Wagner and Y. S. Yun and used to develop and maintain lines by crossing transgenic males, in every generation, with normal C57 BL/6 × C3H F_1 females. This breeding system rapidly reduces differences in genetic background between the lines and provides a virtually unlimited supply of approximately equal numbers of hemizygous transgenic mice and their genetically normal litter-mates. In transgenic animals, expression of heterologous GH starts during fetal life or postnatally, corresponding to the regulation of the MT and the PEPCK promoter, respectively (9, 12). Production of heterologous GH in these transgenic animals is ectopic, with most of the hormone present in circulation being presumably derived from the liver and the kidney (PEPCK) or the liver, kidney, and intestine (MT). In adult transgenic mice plasma hGH levels are approximately 20 ng/ml in MT-hGH animals, while plasma bGH levels are approximately 30 ng/ml in MT-bGH, and approximately 300 ng/ml in PEPCK-bGH mice. Thus data obtained from these lines allow comparisons between the actions of lactogenic plus somatotropic and purely somatotropic GH (MT-hGH versus MT-bGH), and provide some information on the effects of the dose and the developmental onset of exposure to GH excess (MT-bGH vs PEPCK-bGH). Phenotypic consequences of GH overexpression in transgenic mice from the various lines include: major enhancement of postweaning growth and adult body weight (9–12), increase in plasma levels of IGF-I (11, 13), suppression of endogenous (mouse) GH production in the pituitary (14-16), increase in plasma corticosterone levels (17) which apparently reflects enhanced ACTH release (18), alterations in norepinephrine and dopamine turnover in different regions of the hypothalamus and in the release of gonadotropins and PRL from the anterior pituitary (19–22), female sterility (in all MT-hGH and most PEPCK-bGH mice; 11, 19, 23), early development of mammary tumors in MT-hGH females, and drastically reduced life span in PEPCK-bGH and MT-hGH animals (24). In spite of elevated GH and corticosterone levels, plasma glucose concentration is normal or near normal (25, 26) while insulin levels may be substantially elevated (26, Balbis and Turyn, unpublished).

Effects of GH Overexpression on Hepatic GHR and PRLR

As expected from the well-documented ability of GH to stimulate synthesis of GHR (3, 4, 27), overexpression of either hGH or bGH in transgenic mice was associated with a significant increase in the binding of [^{125}I]bGH to liver microsomes. These changes were due to an increase in GHR number without changes in affinity (28). Percent increase in GH binding in transgenic versus control animals was similar in males and in females (28). Comparison of bGH binding to microsomal preparations from the livers of MT-hGH and MT-bGH mice indicated that hGH is more effective in the induction of GHR (28).

In each of the lines examined, overexpression of heterologous GH was associated also with an increase in the binding of oPRL by liver microsomes (28). These actions of hGH and bGH in transgenic animals were much more pronounced in males (approximately 1000%–1200% increase) than in females (approximately 200%–300% increase), leading to complete disappearance of the usual sexual dimorphism in hepatic PRL binding (28). These effects are fully consistent with our previous studies of hepatic hGH binding in a different line of MT-bGH transgenic mice (29) and with the report of increased PRLR in the liver of transgenic mice overexpressing ovine GH (4). However, our evidence that bGH is equally or perhaps slightly more effective than hGH in inducing hepatic PRLR is new and unexpected.

Comparison of results obtained in MT-bGH and PEPCK-bGH mice suggested that the effects of bGH on hepatic GHR and PRLR are not influenced by prenatal bGH gene expression and, within the range of doses tested, the effects on PRLR are not related to the dose (i.e., peripheral levels) of bGH. Similarity of results obtained in two lines derived from different founders and expressing a completely different gene construct (MT-bGH versus PEPCK-bGH) argues against involvement of insertional mutagenesis in the induction of the observed effects. However, the role of systemic versus locally produced (i.e., hepatic) GH, and the possible involvement of IGF-I in the induction of GHR and PRLR by heterologous GH cannot be evaluated on the basis of our data.

Pharmacokinetics of GH and Plasma GHBP Levels in Transgenic Mice

In transgenic MT-bGH and PEPCK-bGH mice, similar to their normal siblings, injected [^{125}I]hGH was rapidly taken up by the liver, kidney, and spleen. Results of simultaneous administration of cold hGH or bGH provided evidence that the kidney and spleen uptake was nonspecific, while the liver uptake was specific and consistent with binding to both GHR and PRLR (30, 31). Complexes of labeled hGH with both GHR and PRLR were detected in the liver of MT-bGH transgenic mice injected with [^{125}I]hGH (30, 31). Disappearance of labeled hGH from plasma followed a multiexponential curve with little change in overall $t_{1/2}$ in MT-bGH mice and a significant prolongation of $t_{1/2}$ in PEPCK-bGH animals, as compared with their normal siblings (30). Similarly, the mean residence time was not altered in MT-bGH, and significantly increased in PEPCK-bGH transgenic animals (167 ± 24 vs 55 ± 5 min; $P < 0.001$; [30]). We feel that differences between results obtained in transgenic mice from these two lines reflect differences in plasma GHBP levels, and more specifically, in the ratios between the concentrations of GHBP-GH complexes and total GH.

Chromatographic analysis of serum incubated with iodinated hGH or bGH provided evidence for a 2- to 3-fold increase in the levels of GHBP in transgenic MT-bGH and PEPCK-bGH animals as compared with their normal siblings (Turyn, unpublished). These findings were confirmed by measurement of GHBP levels performed in samples from the same animals in another laboratory using a specific GHBP radioimmunoassay (Engbers, Talamantes, and Bartke, unpublished observations). In normal mice, approximately one-third of GHBP was complexed with GH, while in transgenic animals, most of GHBP was bound. Concomitant increases in the amount of total GHBP and in the proportion of GHBP that was bound lead to a massive increase in the amount of GHBP-GH complexes in the serum of transgenic versus normal mice. This increase was approximately 4-fold in MT-bGH and approximately 10-fold in PEPCK-bGH mice. The percentage of GH bound in circulation was significantly reduced in PEPCK-bGH transgenics compared with normal mice but not altered in MT-bGH animals. Thus, effects of GH on GHBP levels were, in general, parallel to the effects of GH on hepatic GHR. As was the case with the changes in GHR, our data do not allow us to distinguish between the effects of hepatic versus systemic GH or between direct and IGF-I-mediated effects of GH. Results obtained in different lines of transgenic mice indicate that life-long exposure to either modest (MT-bGH) or massive (PEPCK-bGH) elevation of circulating GH levels is associated with a significant increase in serum GHBP concentration. However, this increase is proportionally much smaller than the increase in peripheral GH levels; thus, the amount of free GH in circulation increases much more than the amount of total GH. Further research is needed to determine to what extent these changes in GH binding may contribute to phenotypic and physiological consequences of life-long GH excess in transgenic animals.

Receptor Binding and Biological Activity of hGH and bGH Analogs

It is well documented that hGH exhibits lactogenic activity in rodents, while bGH does not. In transgenic mice, the effects of bGH and hGH expression on various neuroendocrine and reproductive functions are often different and, in some cases, opposite (15, 19–22, 32–34). In an attempt to identify structural differences between the bGH and hGH molecules that are responsible for differences in their receptor binding and biological activity, we are examining a series of bGH and hGH analogs generated by site directed *in vitro* mutagenesis. For these studies we have selected two sites within Helix 1 (11 and 18 in hGH, corresponding to 12 and 19 in bGH) and two sites within the loop region between Helix 1 and 2 (57 and 60). Importance of amino acid residues at these particular sites, in the ability of hGH to bind to PRLR and exert lactogenic effects, can be suspected from comparisons of the structure of bGH, hGH, and hPRL (1, 3), and from previous studies (3). The analogs generated were expressed and studied in mouse L cells using transient transfection and Western blotting analysis. Media conditioned by transfected cells were tested for binding and biological activities using mouse liver microsomal preparations, cells expressing porcine GHR, and Nb_2 rat lymphoma cell line.

Examination of a series of bGH analogs with single mutations (A12D, Q19H), double mutations (T57S:A70T, A12D:Q19H), triple mutations (A12D:T57S:A60T, Q19H:T57S:A60T), and quadruple mutations (A12D:Q19H:T57S:A60T) revealed that all of them bound to GHR with the same binding constant (K_d) as wild type bGH. Two of these analogs, A12D:T57S:A60T and A12D:Q19H:T57S:A60T exhibited very low binding to PRLR but none was significantly mitogenic in Nb_2 cells. These results suggest that additional modifications of the bGH molecule are necessary to endow it with lactogenic activity.

These bGH analogs were fused with the MT promoter-regulator region and used to produce transgenic mice. All of the resulting lines exhibited enhanced postweaning growth, increased adult body weight and increased weights of the liver, the spleen, and the kidneys, as expected from the *in vitro* studies of binding to GHR. However, measurements of plasma LH and

PRL levels, and hypothalamic turnover of norepinephrine and dopamine in these animals revealed some alterations resembling the consequences of hGH rather than bGH expression as well as changes which differed from the effects of both wild type bGH and wild type hGH. On the basis of results obtained recently in a different series of bGH mutants, we have suggested that different regions of the GH molecule may be involved in producing its various effects (35). Results of the preliminary studies described above are fully consistent with this conclusion and may extend it to biological effects normally associated with lactogenic, rather than somatotropic activity. It is interesting to speculate that different profiles of biological activity of the various GH analogs may be related to differential effects of the introduced mutations on their ability to bind to different forms of GHR and/or PRLR. It also appears to be possible that the hGH-like effects of some of the examined bGH mutants could be related to their ability to bind to PRLR in some but not other target organs and/or to bind simultaneously to one GH and one PRL receptor.

Four single (D11A, H18Q, S57T, T60A), three double (D11A:H18Q, H18Q:S57T, D11A:T60A) and one triple (D11A:H18Q:Q22H) mutations of hGH were also generated, expressed and tested *in vitro* for receptor and biological activity. All mutants bound to GHR and exhibited somatotropic activity in cells expressing pGHR. However, lactogenic activity, measured in Nb_2 cells was reduced by 22% to 45%. The greatest reduction of lactogenic activity was detected in analogs mutated at position 18 (H18Q) either singly, or in conjunction with one or two additional substitutions. These observations are consistent with the previously reported evidence for the importance of histidine at Position 18 for binding of the hGH molecule to the PRLR (8). Persistence of partial lactogenic activity in all hGH analogs listed above suggests that positions other than the four examined (11, 18, 57, and 60) must be importantly involved in the interaction of hGH with PRLR. Assuming that lactogenic actions of hGH (and presumably also PRL) are mediated by interaction of each ligand molecule with two receptors, we can suspect that the failure of mutations in one of the putative sites of receptor binding to abolish lactogenic activity of hGH was due to continued ability of the second site to interact with PRLR.

Findings discussed in this chapter, including recent unpublished data from our laboratories were obtained from studies supported by the NIH (DK-42137 and HD20001), the Cooperative State Research Service, U.S. Department of Agriculture, under agreement No. 89-37240-4584, Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina (CONICET) and Fundacion Antorchas, Buenos Aires, Argentina. The help and encouragement of Dr. S. T. E. Wagner and J. S. Yun are gratefully acknowledged.

1. Nicoll CS, Mayer GL, Russell SM. Structural features of prolactins and growth hormones that can be related to their biological properties. Endocr Rev **7**:169–203, 1986.
2. Kelly P, Djiane J, Postel-Vinay M-C, Edery M. The prolactin/growth hormone receptor family. Endocr Rev **12**:235–251, 1991.
3. Knazek RA, Lin SC, Graeter RL, Wright PC, Mayer JR, Lewis RH, Gould EB, Keller JA. Growth hormone causes rapid induction of lactogenic receptor activity in the Snell dwarf mouse liver. Endocrinology **103**:1590–1596, 1978.
4. Orian JM, Snibson K, Stevenson JL, Brandon MR, Herrington AC. Elevation of growth hormone (GH) and prolactin receptors in transgenic mice expressing ovine GH. Endocrinology **128**:1238–1246, 1991.
5. Posner BI. Characterization and modulation of growth hormone and prolactin binding in mouse liver. Endocrinology **98**:645–654, 1976.
6. Cunningham BC, Ultsch M, de Vos AM, Mulkerrin MG, Clauser KR, Wells JA. Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science **254**:821–825, 1991.
7. de Vos AM, Ultsch M, Kossiakoff AA. Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. Science **255**:306–312, 1992.
8. Fuh G, Colosi P, Wood WI, Wells JA. Mechanism-based design of prolactin receptor antagonists. J Biol Chem **268**:5376–5381, 1993.
9. McGrane MM, Yun JS, Moorman AFM, Lamers WH, Hendrick GK, Arafah BM, Park EA, Wagner TE, Hanson RW. Metabolic effects of developmental, tissue and cell specific expression of a chimeric phosphoenolpyruvate carboxykinase (GTP)/bovine growth hormone gene in transgenic mice. J Biol Chem **265**:22371–22379, 1990.
10. Selden RF, Yun JS, Moore DD, Rowe ME, Malia MA, Wagner TE, Goodman HM. Glucocorticoid regulation of human growth hormone expression in transgenic mice and transiently transfected cells. J Endocrinol **122**:49–60, 1989.
11. Naar EM, Bartke A, Majumdar SS, Buonomo FC, Yun JS, Wagner TE. Fertility of transgenic female mice expressing bovine growth hormone or human growth hormone variant genes. Biol Reprod **45**:178–187, 1991.
12. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature **300**:611–615, 1982.
13. Matthews LS, Hammer RE, Brinster RL, Palmiter RD. Expression of insulin-like growth factor I in transgenic mice with elevated levels of growth hormone is correlated with growth. Endocrinology **123**:433–437, 1988.
14. Low MJ, Lechan AM, Hammer RE, Brinster RL, Habener JF, Mandel G, Goodman RH. Gonadotroph-specific expression of metallothionein fusion genes in pituitaries of transgenic mice. Science **231**:1002–1004, 1986.
15. Stefaneanu L, Kovacs K, Horvath NE, Losinski N, Mayerhofer A, Wagner TE, Bartke A. An immunocytochemical and ultrastructural study of adenohypophyses of mice transgenic for human growth hormone. Endocrinology **126**:608–615, 1990.
16. Sotelo AI, Bartke A, Turyn D. Effects of bovine growth hormone (GH) expression in transgenic mice on serum and pituitary immunoreactive mouse GH levels and pituitary GH-releasing factor binding sites. Acta Endocrinol (in press).
17. Cecim M, Ghosh PK, Esquifino AI, Began T, Wagner TE, Yun JS, Bartke A. Elevated corticosterone levels in transgenic mice expressing human or bovine growth hormone genes. Neuroendocrinology **53**:313–316, 1991.
18. Cecim M, Alvarez-Sanz MC, Milton S, Van de Kar LD. Increased plasma corticosterone level in GH transgenic mice is

not due to a change in adrenal responsiveness to ACTH. Program 74th Annual Meeting of the Endocr Soc, p448, Abstract 1588, 1992.
19. Bartke A, Steger RW, Hodges SL, Parkening TA, Collins TJ, Yun JS, Wagner TE. Infertility in transgenic female mice with human growth hormone expression: Evidence for luteal failure. J Exp Zool **248**:121–124, 1988.
20. Steger RW, Bartke A, Parkening TA, Collins T, Buonomo F, Tang K, Wagner TE, Yun JS. Effects of heterologous growth hormones on hypothalamic and pituitary function in transgenic mice. Neuroendocrinology **53**:365–372, 1991.
21. Chandrashekar V, Bartke A, Wagner TE. Endogenous human growth hormone (GH) modulates the effect of gonadotropin-releasing hormone on pituitary function and the gonadotropin response to the negative feedback effect of testosterone in adult male transgenic mice bearing human GH gene. Endocrinology **123**:2717–2722, 1988.
22. Tang K, Bartke A, Gardiner CS, Wagner TE, Yun JS. Gonadotropin secretion, synthesis and gene expression in human-growth hormone transgenic mice and in Ames dwarf mice. Endocrinology **132**:2518–2524, 1993.
23. Yun JS, Wagner TE. Study of human growth hormone transgenic mice: Female reproductive system. 10th Korea Symposium on Science and Technology **3**(1):279–282, 1987.
24. Steger RW, Bartke A, Cecim M. Premature ageing in transgenic mice expressing different growth hormone genes. J Reprod Fertil **46**(Suppl):61–75, 1993.
25. Bartke A, Naar EM, Johnson L, May MR, Cecim M, Yun JS, Wagner TE. Effects of expression of human or bovine growth hormone genes on sperm production and male reproductive performance in four lines of transgenic mice. J Reprod Fertil **95**:109–118, 1992.
26. Balbis A, Dellacha JM, Calandra RS, Bartke A, Turyn D. Down regulation of masked and unmasked insulin receptors in the liver of transgenic mice expressing bovine growth hormone gene. Life Sci **51**:771–778, 1992.
27. Baxter RC, Zaltsman Z, Turtle JR. Induction of somatogenic receptors in livers of hypersomatropic rats. Endocrinology **111**:1020–1022, 1982.
28. Aguilar RC, Fernandez HN, Dellacha JM, Calandra RS, Bartke A, Ghosh PK, Turyn D. Somatotropic and lactotropic receptors in transgenic mice expressing human or bovine growth hormone genes. Transgenic Res **1**:221–227, 1992.
29. Chen WY, White ME, Wagner TE, Kopchick JJ. Functional antagonism between endogenous mouse growth hormone (GH) and a GH analog results in dwarf transgenic mice. Endocrinology **129**:1402–1408, 1991.
30. Turyn D, Bartke A. Pharmacokinetics of radioiodinated human and ovine growth hormones in transgenic mice expressing bovine growth hormone. Transgenic Res **2**:219–226, 1993.
31. Turyn D, Yun JS, Wagner TE, Bartke A. Specific somatotropic and lactogenic uptake *in vivo* in the livers of transgenic mice expressing bovine growth hormone gene. Growth Reg **3**:190–197, 1993.
32. Bartke A, Steger RW, Parkening TA, Collins TJ, Yun JS, Wagner TE. Influence of human and bovine growth hormones on the regulation of prolactin release in transgenic mice. In: Gupta D, Wollmann HA, Ranke MB, Eds. Neuroendocrinology: New Frontiers. Tübingen, Germany: Brain Research Promotion, pp39–48, 1990.
33. Tang K, Bartke A, Gardiner CS, Wagner TE, Yun JS. Gonadotropin secretion, synthesis, and gene expression in two types of bovine growth hormone transgenic mice. Biol Reprod **49**:346–353, 1993.
34. Stefaneanu L, Kovacs K, Bartke A, Mayerhofer A, Wagner TE. Pituitary morphology of transgenic mice expressing bovine growth hormone. Lab Invest **68**:584–591, 1993.
35. Yang C-W, Striker LJ, Pesce C, Chen WY, Peten EP, Elliot S, Doi T, Kopchick JJ, Striker GE. Glomerulosclerosis and body growth are mediated by different portions of bovine growth hormone. Lab Invest **68**:62–70, 1993.