Effects of an S84E Mutation of Bovine Growth Hormone in Transgenic Mice

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The ability of mutant bovine growth hormones (bGH) to serve as either agonist or antagonist has been demonstrated in transgenic mice. We have prepared two transgenic strains of FVB/N mice, one expressing wild-type bGH and a second with a glutamic acid mutation at serine 84 in helix 2. Comparison of their phenotypes to those of nontransgenic littermates indicates that wild-type bGH induces a previously described phenotype for hyper-somatotrophic mice. In contrast, the replacement of the side chain hydroxyl at serine 84 with acetic acid produced a phenotype that expressed bGH at appreciable concentrations, but failed to elicit the phenotype observed with either an agonist or an antagonist of bGH. These results indicate that serine 84 is crucial for the activity of bGH despite this site being distal to the receptor binding surfaces. Exp Biol Med 231:296–302, 2006

Key words: somatotrophin; bovine; transgenic mouse; IGF-1

Introduction

Growth hormone and prolactin are members of a cytokine/growth factor protein family that share structural features and similar receptor mechanisms for initiation of a wide variety of biological activities. The genes for these proteins are derived from a common ancestral gene by successive tandem duplications (1). Despite their common origins, these two proteins have diverged significantly. For example, bovine growth hormone (bGH) and prolactin share only a 23% amino acid identity in sequence homology (Genbank accession nos. P01239, P01246). Both proteins are class I cytokines that stimulate distinct responses in target cells by the dimerization of either somatotrophic or lactogenic receptors (2, 3). Both proteins function as classic hormones when secreted from acidophilic cells in the anterior pituitary (4, 5). Secretion from other tissues in which the proteins serve autocrine and/or paracrine functions has also been documented (6). Pituitary cells that secret either growth hormone or prolactin are derived from a pool of precursor cells that may share common processes of storage and secretion of the respective hormones (7).

Pituitary forms of both growth hormone and prolactin are posttranslationally modified by phosphorylation (8, 9). Phosphorylated forms of prolactin are found in rat (10), chicken (11), and bovine pituitaries (12). In the cow, this can represent the majority of pituitary prolactin.[2] We have isolated phosphorylated bovine prolactin (13, 14); it has a sharply reduced biological activity both *in vitro* (15) and *in vivo* (16). In the cow the major site of phosphorylation is serine 90 (13), a residue whose homologue is conserved in all other prolactins and growth hormones. The phosphorylation site in bovine prolactin resides in the middle of a salt bridge that appears to be critical for the functional activity of bovine prolactin (17). Mimicry of phosphorylation by mutation of serine 90 to glutamic acid produces a recombinant protein with a similar reduction in biological activity when compared to the phosphorylated protein isolated from the pituitary (18). Prolactin phosphorylation is mediated by a zinc-dependent protein kinase that is associated with the endoplasmic reticulum and Golgi apparatus in the bovine anterior pituitary (19).

Similarly, growth hormone is also found in a phosphorylated form in the anterior pituitaries of the chicken, turkey (20), sheep (21), rat (22), and cow (12). The functional significance of growth hormone phosphorylation and the site of phosphorylation remain to be established. A zinc-dependent kinase located in the bovine pituitary phosphorylates bGH (23). This kinase may be the same as the prolactin kinase, because the two activities have similar K_ms and both require zinc for full activity. Serine 84 in bGH is the conserved homologue of the serine 90 phosphorylation site of bovine prolactin. Serine 84 is within a region of modest sequence conservation, where the

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immediate sequence (QSWLGP) does not retain the functionally important salt bridge (17) present in bovine prolactin (RSWNDP).

Growth hormone was one of the early genes placed into the context of the mouse genome (24). The transgenic mouse has served as a remarkably durable system to examine the biology of growth hormone excess in living mammals. Early studies demonstrated that excess growth hormone produced gigantism and associated pathologies, including a failure to reproduce (25, 26) and renal lesions (27). The model has also been successfully used to determine the consequences of structural changes on biological functions. The replacement of glycine 119 in bGH by arginine antagonized the endogenous mouse growth hormone (28); the offspring of these mice displayed an impaired stature (29). Similarly, mutations that reduce biological activity without displaying either agonist or antagonist activities should not perturb the normal biology of the mouse.

We have chosen to create two transgenic mouse lines to determine the effects of glutamic acid at serine 84 in bGH. The first line of transgenic mice expressed wild-type bGH and displayed the classic large-mouse phenotype. The second transgenic line expressed S84E bGH and displayed a normal phenotype by all measures. We conclude from these studies that serine 84 in bGH is critical for biological activity. Its replacement by glutamic acid has a severely reduced agonist activity and displays no detectable antagonist activities in this model system.

Materials and Methods

A pT7–7 phagemid was created (30) by insertion of an *f1* origin into the pT7–7 plasmid that was kindly provided by S. Tabor (Harvard Medical School, Boston, MA). This phagemid (pT7–7 f(+)) was used for cloning and production of single-stranded DNA in RZ1032 *E. coli* for mutagenesis by the method of Kunkel *et al.* (31). The pbGH10D16 plasmid was a gift from John Kopchick (Edison Animal Biotechnology Center, Ohio University, Athens, OH). This plasmid contained the mouse metallothionein-I promoter (MT) ligated to the bGH gene without introns b and c (Fig. 1). Intron A was included to increase expression of bGH mRNA (32). pbGH10D16 was cut with *Eco R1* and cloned into this site in the pT7–7 phagemid. Positive clones were selected by ampicillin resistance in the DH5α strain of *E. coli*, characterized by restriction digests, and completely sequenced (33) to confirm colonies with the desired sequence and orientation. The selected phagemid was called pT7–7-MTbGH. This phagemid was prepared and transfected into the RZ1032 strain of *E. coli* for preparation of

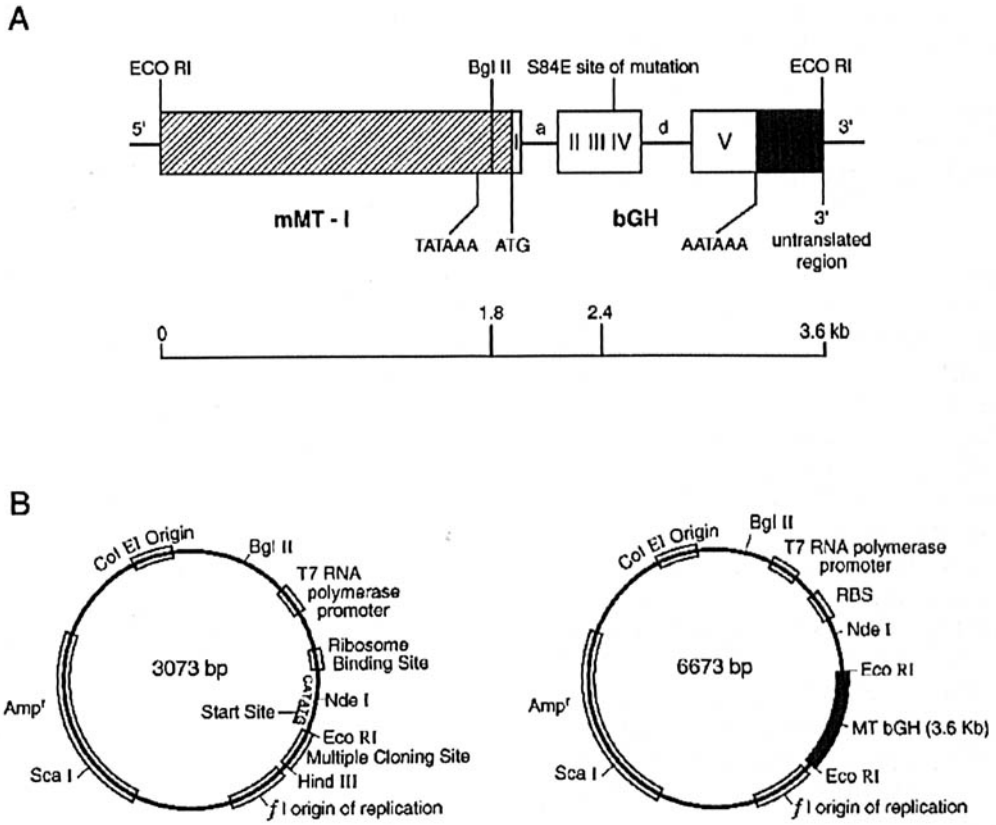

Figure 1. Maps of the metallothionein promoter-bGH gene, pT7–7, and PT7–7-MTbGH phagemids. The metallothionein promoter was fused to the coding sequence for mature bGH that contains intron A (upper panel). *Eco R1* sites were used to ligate this construct into the pT7–7 phagemid (lower left panel) to produce pT7–7-MTbGH phagemid (lower right). Mutagenesis was performed in this vector to produce pT7–7-S84E-MTbGH. The wild-type and mutant inserts were removed with *Eco R1* and prepared for injection.

single-stranded DNA that subsequently was used to prepare a S84E mutation by the method of Kunkel *et al.* (31). Selection of mutant colonies was aided by the inclusion of a translationally silent *AvaI* site in the primer. This mutagenic primer had the following sequence: 5′ CTG CAG GGG ***CCC GAG*** CCA **CTC** CTG GAT GAG GAG CAG 3′. The ***CCC GAG*** is the *AvaI* restriction site, and the **CTC** codes for the serine to glutamic acid substitution at position 84 of bGH. Phagemid DNA from clones containing the *AvaI* site was sequenced (33) to confirm the entire insert sequence containing the desired changes. The S84E mutant bGH phagemid was called pT7–7-S84E-MTbGH.

The 3.6 kb DNA fragment contained in either pT7–7-MTbGH or pT7–7-S84E-MTbGH was cut from the phagemid with *Eco RI*, isolated by electrophoresis on low-melting agarose, and purified by a Gene Clean Spin Kit (Bio101, La Jolla, CA). The DNA was precipitated and washed with ethanol, and a solution at 3 μg/ml was prepared in 5 m*M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA.

Two strains of transgenic mice were produced in collaboration with the Transgenic Animal Facility of the Neurobiotechnology Center at The Ohio State University. All mice were treated according to our Institutional Laboratory Animal Care and Use Committee-approved protocol. Donor mice of the FVB/N strain were superovulated by an intraperitoneal injection of 10 IU of pregnant mare's serum gonadotropin (PMSG, Vernie; Fisher Scientific, Hampton, NH) followed 48 hrs later by an intraperitoneal injection of 10 IU of human chorionic gonadotropin (Vernie; Fisher Scientific). Mice were killed by cervical dislocation and oocytes were collected and rinsed with M2 media (HEPES-buffered modified Krebs-Ringer solution) and transferred to M16 media (similar to Witten medium) at 37°C and 5% CO_2 until they were used for microinjection.

Viable oocytes were selected for microinjection of the DNA encoding either bGH or the S84E mutant into the pronuclei of zygotes. Injection of 1 to 2 pL of DNA was performed at a magnification of 400× using phase-contrast Nomarski optics. Fifteen to 20 injected zygotes were transferred to the infundibula of synchronized recipient C3B6F1 female mice. All animals were kept under barrier conditions and were provided food *ad libitum* and water containing 25 m*M* zinc sulfate.

Three-week-old transgenic mice were genotyped by Southern blot analysis using DNA collected from tail clips. DNA was extracted as described by Blin and Stafford (34). Twenty to 30 μg of sample DNA was digested with *Pst*1, separated by electrophoresis on 1% agarose gel in 89 m*M* Tris borate and 1 m*M* EDTA, transferred under alkaline conditions onto Nitran Plus membranes (Schleicher and Schuell, Keene, NH) and prehybridized for 1 hr at 42°C in 1.08 *M* NaCl, 60 m*M* $NaPO_4$, 6 m*M* EDTA, pH 7.7, 2.5× Denhardt's solution, 50% formamide, 1% sodium dodecyl sulfate, and 0.2 mg/ml Herring sperm DNA. A 585-base pair probe was prepared from *Pst*1 digests of pT7–7-MTbGH separated by agarose gel electrophoresis and purified with a Gene Clean Spin Kit. This probe contained contiguous sequences for exons II, III, and IV of the transgene and contained no intron sequence. The probe was labeled with ^{32}P using the High Prime DNA Labeling Kit (Roche, Indianapolis, IN). Hybridization was performed overnight in the same buffer containing the probe at an activity of greater than 10^9 cpm/μg DNA. The matrix was washed twice for 15 min at room temperature in 7× SSC (0.15 *M* NaCl, 15 m*M* Na citrate, pH 7.0) and twice for 20 min at 55°C in 0.1× SSC containing 1% sodium dodecyl sulfate. The matrix was exposed to an imaging plate of a Phosphoimager (Molecular Dynamics, Sunnyvale, CA) and the image was digitally recorded. Positive mice were identified by the presence of a strong band at approximately 585 bases. Founder animals were bred to nontransgenic FVB/N mice to initiate transgenic lines.

Body weights of a subset of transgenic mice, including those containing wild-type and S84E bGH, as well as nontransgenic littermates, were measured to the nearest 0.1 g and recorded every 5 days between 35 and 120 days of age. At the time of sacrifice, blood was collected from the orbit of mice receiving ether anesthesia. The samples were clotted for 30 min at 4°C and centrifuged and the serum collected and stored frozen. A double antibody procedure for bGH was used with reagents kindly provided by the National Hormone and Pituitary Program. Highly purified bGH was iodinated by Iodogen (Pierce Chemical Co., Rockford, IL) using carrier-free ^{125}I-iodine (ICN, Costa Mesa, CA) to a specific activity of 32 μCi/μg. Duplicate 20-μl samples were assayed. The sensitivity of this assay was 5 ng/ml. In assays of nontransgenic mouse serum, no bGH was detected by this assay method. An IGF-1 immunoassay kit was purchased from Mediagnost (catalog no. IGF-R20; Tuebingen, Germany, http://www.mediagnost.de/content/e/pdf/IGFR20_e.pdf). Serum was diluted to dissociate IGF-1 from IGF-binding proteins followed by addition of an excess of IGF-2 to occupy the sites on the binding proteins as described by Blum and Breier (35). Duplicate samples were assayed for IGF-1 activities.

Sections of liver, kidney, spleen, adrenals, pituitary, lungs, heart, pancreas, testes, and ovary were taken from four S84E-MTbGH transgenic founders (one male and three females, age 390 days) and seven animals (six males and one female, age 300 days) of the F1 generation. Tissues were also taken from two male and one female founder MTbGH mice (age 300 days). Tissues were fixed in formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and evaluated by light microscopy.

Morphometry of renal glomeruli was performed in four S84E-MTbGH transgenic animals of the F1 generation of line #47 (age 300 days) and compared to five gender-matched nontransgenic littermates. Three longitudinal sections of the left kidney were used in each animal to measure the Area Fractions (A_A) of glomerular tuft/renal cortex. A_A values were derived from point counting using

the Stereologer system (Systems Planning and Analysis, Alexandria, VA) coupled to a Laborlux S microscope (Leitz, Wetzlar, Germany). One-way ANOVA was used to compare area fractions between experimental groups.

Results

PT7–7-MTbGH and PT7–7-S84E-MTbGH were shown by restriction digestion and sequencing to contain the appropriate coding sequence. Three founder animals (one male and two females) were identified by Southern blotting to be among 30 animals produced by pronuclear injection of MTbGH DNA. Mating for a period of 6 months did not produce offspring from any of the MTbGH founder mice. The male was capable of mating, as evidenced by vaginal plugs, but no females became pregnant. Because of markedly reduced fertility, progeny were not obtained from these animals.

Five (Nos. 2, 17, 31, 43, and 47) of the 50 FVB/N pups derived from S84E-MTbGH DNA injected into pronuclei were identified as transgenic by Southern blot (Fig. 2). Mating of S84E-MTbGH transgenic founder mice to nontransgenic partners produced offspring within the first month. Evaluation of serum concentrations for bGH by radioimmunoassays in F1 mice identified three founder mice that were able to transmit S84E bGH to their offspring (Nos. 17, 31, and 47). In the F1 generation, three of eight mice of line 17 and 5 of 15 mice of line 47 were transgenic for S84E-MTbGH. These percentages indicated that these founders were mosaics. From the 14 line 31 progeny,

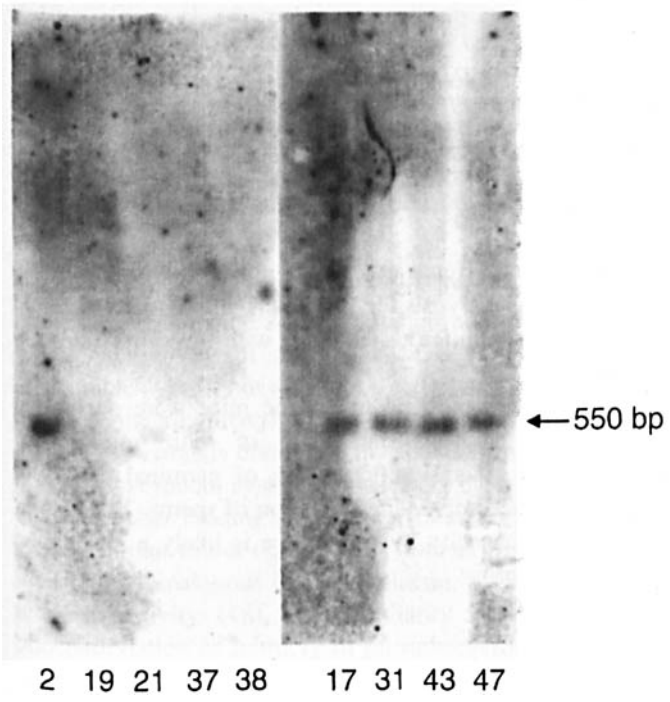

Figure 2. Southern blot of DNA from nontransgenic and transgenic mice tails. Thirty micrograms of DNA from nontransgenic or transgenic mice was digested with *PstI* and run on a 1% agarose gel, transferred to a Nitran Plus matrix, and probed with a ^{32}P-labeled probe. Images were captured with a Phosphorimager.

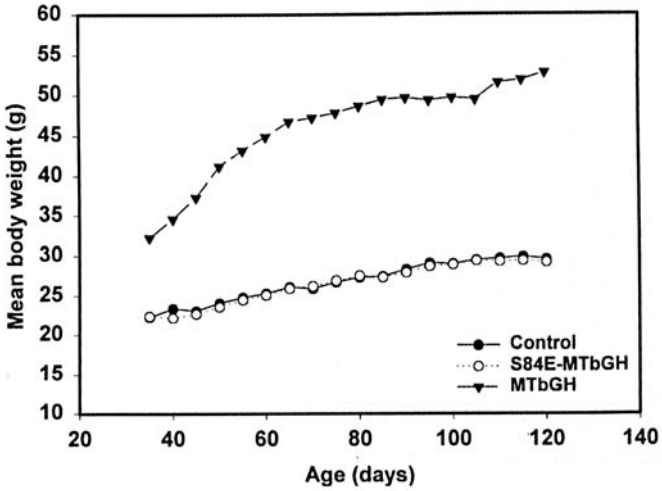

Figure 3. Body weights of wild-type bGH transgenic mice, S84E bGH transgenic mice, and nontransgenic littermates. Note that the standard deviation for the weight of the pups at each time point was approximately the size of the symbol representing the mean value.

serum was available for only five mice, two of these mice were transgenic.

The body weights of MTbGH, S84E-MTbGH, and nontransgenic littermates were recorded to the nearest 0.1 g every 5 days between 35 and 120 days of age (Fig. 3). At 35 days of age, the MTbGH transgenic mice were already heavier than either the S84E-MTbGH transgenic mice or their control littermates. By 120 days of age, the weight of the MTbGH mice was approximately twice that of either the S84E-MTbGH transgenic mice or their littermate controls ($P < 0.001$). Comparison of the S84E-MTbGH transgenic mice with their control littermates showed no difference in weights or rates of growth, indicating that S84E-bGH has a markedly reduced somatotrophic activity and does not function as a potent antagonist.

Measurement of serum concentrations of bGH by double antibody radioimmunoassay showed that no bGH could be detected in control littermates, indicating a high specificity for the bGH assay and that mouse GH would not interfere with the assay. In contrast, S84E-MTbGH transgenic mice had concentrations of the mutant bGH between 20 and 475 ng/ml, indicating a significant expression of the transgene and release of the translated bGH from transgenic cells but not at concentrations that might antagonize receptor dimerization. Interestingly, the average serum bGH concentration of founder mice was 22 ± 4 ng/ml, whereas the F1 mice averaged 134 ± 152 ng/ml ($P < 0.001$ by a double-tailed Mann-Whitney U test).

Serum concentrations of IGF-1 were measured and compared between nine F1 S84E-MTbGH transgenic mice (307 ± 41 ng/ml) and 57 control littermates (262 ± 73 ng/ml) (Fig. 4). Comparison by a two-way ANOVA showed no significant increase in IGF-1 brought about by the expression of bGH ($P < 0.078$) or by gender ($P < 0.567$). These data show that despite a measurable increase in S84E bGH, it lacks sufficient somatotrophic agonist

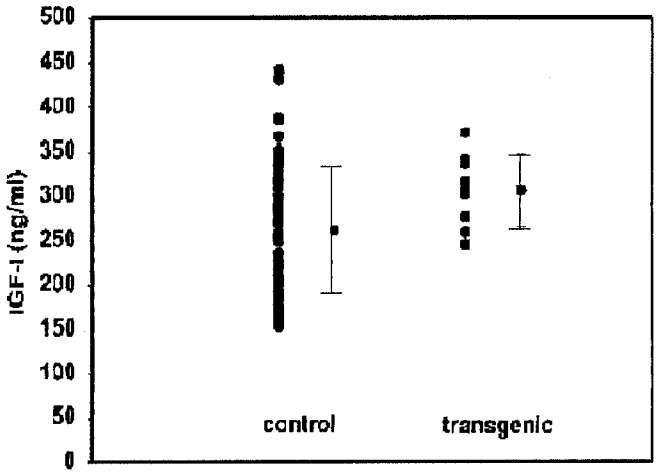

Figure 4. Serum concentrations of IGF-1 in S84E bGH transgenic mice and nontransgenic littermates.

activity to significantly increase serum IGF-1 concentrations.

The weights of organs that are enlarged in transgenic mice expressing wild-type bGH were measured in S84E-bGH transgenic mice and compared with those of control littermates. Neither hearts (S84E-MTbGH ± SD: controls ± SD, 0.20 g ± 0.04: 0.21 g ± 0.03), lungs (0.27 g ± 0.05: 0.29 g ± 0.03), spleens (0.12 g ± 0.02: 0.11 g ± 0.01), livers (1.75 g ± 0.03: 1.65 g ± 0.04), nor kidneys (0.34 g ± 0.07: 0.31 g ± 0.03) showed changes in weight as determined by a Wilcoxon's rank sum test. These data again suggest that S84E-MTbGH had dramatically reduced somatotrophic agonist activity.

The histology of organs in hGH transgenic mice is well described (36) and is largely in contrast to our observations of organs from S84E bGH transgenic mice. Histological comparisons of the 10 organs from S84E-bGH transgenic mice and their nontransgenic littermates showed no effects from the expression of S84E bGH. Histological changes were limited to the kidney and were present in two aging male transgenic founders (390 days of age). These lesions were consistent with mild chronic progressive nephropathy and affected less than 10% of the renal cortex (Fig. 5). Similar observations were made in an aging nontransgenic littermate. Morphometric analysis of area fractions of glomerular tuft/renal cortex showed no statistically significant difference ($P = 0.072$ by ANOVA) between S84E-bGH transgenic mice (mean = 0.0143, SD = 0.0019) and control nontransgenic littermates (mean = 0.0123, SD = 0.0007). These observations are in contrast to those in wild-type-bGH transgenic founder animals, in which marked renal lesions characterized by loss of tubules and glomeruli and replacement by fibrous connective tissue were observed. All glomeruli were markedly enlarged and sclerotic (Fig. 6). The average glomerular tuft featured sclerosis affecting more than 50% of the cross-sectional area. Additional lesions were noted in liver and testis of wild-type-bGH

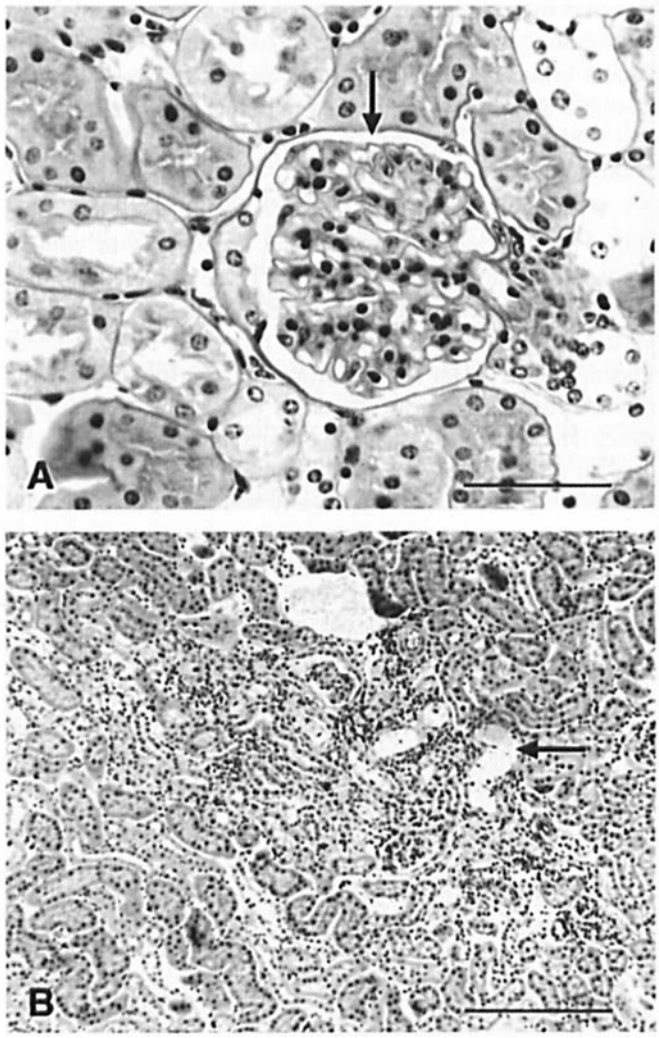

Figure 5. Histology of the kidney of a male S84E-MTbGH founder mouse (390 days old). (A) PAS stain of normal glomerulum (arrow); note minimal accumulation of PAS-positive material within the mesangium; bar, 50 μm. (B) Hematoxylin-eosin stain of focal nephropathy, loss of tubules, tubular dilation with proteinaceous casts (arrow), and collapsed glomeruli; bar, 200 μm.

transgenic mice. Hepatocytes were enlarged with karyomegaly and anisokaryosis. In the testis there was moderate atrophy of seminiferous tubules with loss of germinal epithelial cells.

The ovaries of bGH transgenic mice were normal in appearance, whereas the testes of male mice were characterized by a significant loss of germinal epithelial cells and an accompanying reduction of sperm. The loss of reproductive capacity by these mice is likely a result of a defective male.

Discussion

The transgenic mouse is an advantageous model to test the biological effects of engineered hormone structures. The phenotypic effects of specific structural changes can be produced by changes in either agonist or antagonist actions (29). In the current study, the well-characterized bGH

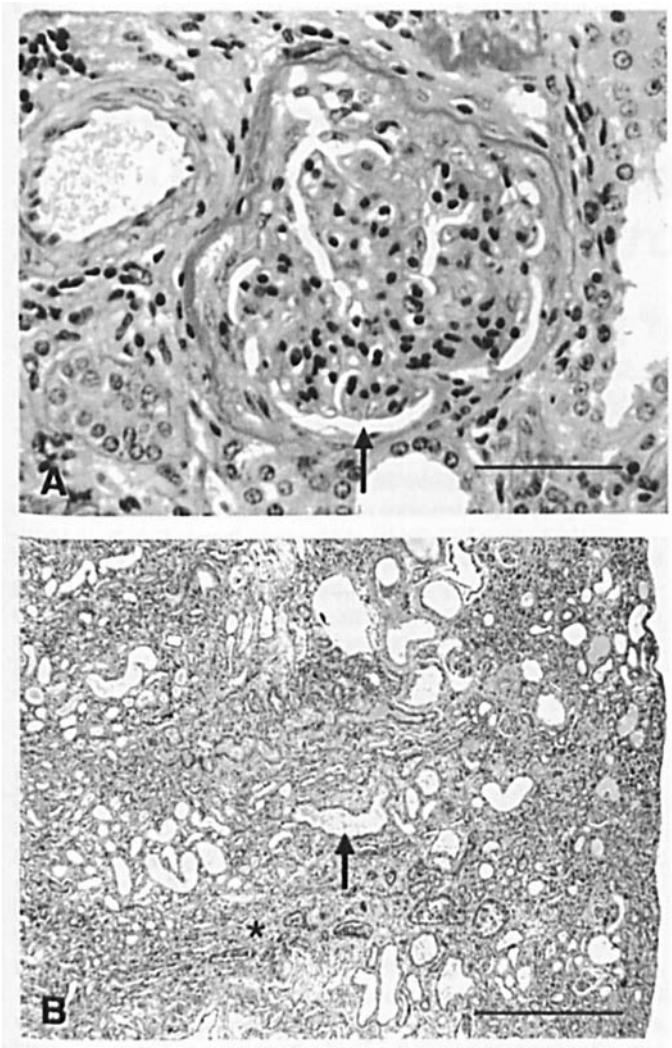

Figure 6. Histology of the kidney of a male MTbGH founder mouse (300 days old). (A) PAS stain of glomerulum (arrow); marked glomerular hypertropy, mesangial sclerosis and thickening of pericapillary and periglomerular basement membranes; bar, 50 μm. (B) Hematoxylin-eosin stain of marked interstitial fibrosis (asterisk), loss of tubules and cystic tubular dilatation (arrow); bar, 1000 μm.

transgenic mouse model was used to demonstrate that replacement of serine 84 by glutamic acid eliminates the biological activity of transgenic bGH, affecting neither measurable agonist nor antagonist activity. The replacement of the serine's hydroxyl with a two-carbon acidic group at position 84 virtually eliminates the somatotrophic activity of bGH in this model system, despite this modification being distal to either binding site for the somatotrophic receptor. This is similar to our observations both *in vitro* and *in vivo* in which an analogous bovine prolactin, S90E, had greatly reduced activity (18). The similarity of the result of phosphorylation or mimicry of phosphorylation at this site in both bGH and prolactin, the ability of a pituitary zinc-dependent protein kinase to phosphorylate both hormones, and the conservation of serine at this position suggest that this may be a common mechanism for the regulation of hormone action in this protein family.

The mechanism by which the replacement of serine 84 with glutamic acid reduced the activity of bGH is unclear. In bovine prolactin the sequence surrounding the corresponding residue S90 is within a salt bridge (RSWNDP) (Genbank no. P01239), whereas in bGH the sequence is QSWLGP (Genbank no. P01246). In bovine prolactin, a salt bridge surrounds the homologous serine 90 in a presumed helix (deduced from the structure of human prolactin PDB# 1N9D). We have shown that replacement of either member of the salt bridge reduces the activity of bovine prolactin (17). Based on this observation, we have predicted that phosphorylation of serine 90 in bovine prolactin will disrupt this section of helix 2. This does not appear to be a plausible explanation of how replacement of serine 84 with glutamic acid in bGH will alter the protein's structure with an accompanying reduction in biological activity. Residues required for salt bridge formation are not present in this sequence of bGH: the conserved residues are serine 84, tryptophan 85 and proline 88; neither member of the salt bridge is present. One possible mechanism is that replacement of serine 84 with a negatively charged residue disrupts the hydrophobic packing of tryptophan 85 with adjacent hydrophobic residues in helix 4.

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