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Expression of the human growth hormone variant gene in cultured fibroblasts and transgenic mice

(anterior pituitary/transient expression/metallothionein)

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ABSTRACT **The nucleotide sequence of the human growth hormone variant gene, one of the five members of the growth hormone gene family, predicts that it encodes a growth hormone-like protein. As a first step in determining whether this gene is functional in humans, we have expressed a mouse metallothionein I/human growth hormone variant fusion gene in mouse L cells and in transgenic mice. The growth hormone variant protein expressed in transiently transfected L cells is distinct from growth hormone itself with respect to reactivity with anti-growth hormone monoclonal antibodies, behavior during column chromatography, and isoelectric point. Transgenic mice expressing the growth hormone variant protein are 1.4- to 1.9-fold larger than nontransgenic controls, suggesting that the protein has growth-promoting properties.**

Structure–function relationships within the human growth hormone (hGH) gene family are likely to play critical roles in growth and development. The hGH gene family consists of five highly homologous members (1–3), which are encoded within 50 kilobases (kb) on band q22-q24 of chromosome 17 (4, 5) and are categorized based on their resemblance to hGH or to chorionic somatomammotropin (CS). The *GHA* gene (now designated *GH1*) encodes pituitary hGH and is expressed in the somatotrophs of the anterior pituitary (6). The hGH variant gene (*GHB*; now designated *GH2*) also encodes a GH-like polypeptide (3, 7, 8), and recent evidence suggests that low levels of GHB mRNA are present in term placenta (9). Two of the CS-like genes (*CSA* and *CSB*; now designated *CSH1* and *CSH2*) express identical forms of CS in the placental syncitium (7), and the third (*CSHP1*) is a pseudogene (2, 3). Any two of the five genes in the family are at least 92% homologous, suggesting a relatively recent series of gene duplications (2, 3). The hGH gene family members are also related to the prolactin gene (10), with hGH and prolactin sharing ≈35% amino acid homology.

Of the five members of the hGH gene family, only the function of hGH itself (the product of the *GHA* gene) has been well characterized. The GHA protein (hGH) has both growth-promoting properties and a variety of metabolic properties (11–13), and a homozygous deletion of only the *GHA* gene has been reported to cause isolated hGH deficiency type IA (14, 15). CS may play a role in both intermediary metabolism and mammary gland development (16), but individuals with homozygous deletions of the genes for CS appear normal (17). It is possible that the structural homology of the hGH gene family members allows for some functional overlap.

An important step towards understanding the molecular biology and physiology of the hGH gene family is to determine the function(s) of the hGH variant and its morphological and developmental pattern of expression in the body. Hamer and coworkers (18, 19) have shown that the *GHB* gene can be expressed in monkey kidney cells infected by simian virus 40/*GHB* recombinants and that the protein produced resembles hGH, based in part on one-dimensional tryptic maps. We have extended these results by producing GHB in transiently transfected cells and have studied the function of GHB by generating a series of transgenic mice. In this report, we present further characterization of the protein and demonstrate that transgenic mice expressing *GHB* grow to almost twice the size of control mice.

MATERIALS AND METHODS

Plasmid Constructions. The *Mt-1/GHB* fusion gene was constructed by ligating a 1.8-kb *Eco*RI–*Bgl* II fragment containing promoter sequences from the mouse metallothionein I gene, *Mt-1*, (20) to a 2.1-kb *Bam*HI–*Eco*RI *GHB* gene fragment (prepared by using a partial *Bam*HI digestion), which contains structural sequences from the start of transcription at the *Bam*HI site to ≈500 base pairs (bp) past the poly(A) addition site (3). The ligated material was digested with *Eco*RI and inserted into the *Eco*RI site of the plasmid vector pUC12 (21). The resulting plasmid is referred to as pXGH4. The *Mt-1/GHA* fusion gene was constructed as described (22) and is referred to as pXGH5. Standard techniques of molecular cloning utilized to construct and isolate these plasmids were performed as described (23).

Tissue Culture and Transfections. Mouse L cell fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum. L cells (≈5 × 10^5) were plated on 10-cm tissue culture dishes and allowed to grow for 3 days to ≈30% confluence. DEAE-dextran-mediated transfections were performed essentially as described (22, 24). Briefly, the medium was aspirated from the dish, and the cells were washed once with phosphate-buffered saline (PBS). The desired amount of DNA was precipitated with ethanol and resuspended in 60 μl of Tris-buffered saline (0.15 M NaCl/5 mM KCl/1.5 mM Na_2HPO_4/2.5 mM Tris base/1 mM $CaCl_2$/0.5 mM $MgCl_2$, pH 7.5; the $CaCl_2$ and $MgCl_2$ were prepared together as a 100× stock solution, which was added slowly to the other components to avoid precipitation) and then added to 120 μl of warm (30–40°C) Tris-buffered saline containing DEAE-dextran at 5 mg/ml. This mixture was then combined with 3 ml of 10% Nu-Serum (Collaborative Research, Waltham, MA) and added to the plate. After incubation for 4 hr at 37°C in 5% CO_2/95% air, the DNA–DEAE-dextran–Nu-Serum was removed and replaced by 5 ml of 10% (vol/vol) dimethyl sulfoxide in PBS. This was incubated for 1 min at room

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Abbreviations: GH, growth hormone; hGH, human GH; CS, chorionic somatomammotropin; nt, nucleotides.

temperature, the dimethyl sulfoxide was aspirated, and 10 ml of the appropriate medium was added to the dish. The cells were incubated at 37°c in 5% CO_2/95% air for several days, and aliquots of the medium were collected at various times as described in the text. Total RNA was prepared from mouse L cells, and blot-hybridization analysis was carried out as described (25). The resulting nitrocellulose filter was prehybridized overnight and then hybridized to a ^{32}P-labeled *Mt-1/GHB* genomic probe (an 802-bp *Sac* I fragment from position −211 to +591 with respect to the transcriptional start site; see Fig. 1).

Protein Analyses. GH levels were measured with a two-site radioimmunoassay under the conditions recommended by the manufacturer (Hybritech, San Diego, CA). Radiolabeling of L cells transiently transfected (as above) was performed as follows: 5 days after transfection, medium was removed and replaced with 7.5 ml of methionine-free minimal essential medium containing 2% calf serum; 2 hr later, 5 ml of the identical medium supplemented with 0.2 mCi (1 Ci = 37 GBq) of [^{35}S]methionine was added to each plate, and aliquots of medium were collected 23 hr later. Fifty microliters of each sample was electrophoresed on a denaturing 12% polyacrylamide gel. Radioimmunoassays using affinity-purified iodinated GHA were performed as follows (S.B., unpublished data): 100 μl of standard or unknown was incubated with 100 μl of monoclonal antibody (mAb) 352, 033, or 665 [ref. 26; diluted 1:2 × 10^6 (mAB 352), 1:3.2 × 10^6 (mAB 033), and 1:3 × 10^6 (mAB 665) in PBS, pH 7.0/0.2% bovine serum albumin] for 2 hr at 37°C; ^{125}I-labeled GHA (≈4000 cpm) was added, the mixture was incubated overnight at 4°C, and bound radiolabel was separated by using a solid-phase goat anti-mouse IgG (Immunobeads, Bio-Rad). Interassay variability was 12%, and intraassay variability was 7%. Human, bovine, and murine GH were purified by A. F. Parlow of Harbor–University of California, Los Angeles, Medical Center and distributed by the National Hormone and Pituitary Program. Gel filtration was performed with 1.6 × 70 cm columns of Sephadex G-75 in PBS (pH 7.0). Samples of 2 ml were applied to the column, and 2.5-ml fractions were collected and assayed for GH immunoreactivity with antibody 665. Isoelectric focusing was performed as described (27).

Transgenic Mice. A 2.8-kb *Kpn* I–*Eco*RI fragment containing the *Mt-1/GHB* fusion gene was isolated from pXGH4 sequences by preparative gel electrophoresis and electroelution. Fertilized mouse eggs for microinjection were recovered *in cumulus* from the oviducts of (C57BL/6 × C3H)F_1 females that had mated with (C57BL/6 × C3H)F_1 males several hours earlier. Approximately 1000 copies of the fusion gene fragment were microinjected into the male pronucleus of each fertilized egg. Microinjected eggs were implanted into the oviducts of 1-day pseudopregnant ICR foster mothers and were carried to term (28). Total genomic DNA was prepared from mouse tails as described (29). Total cellular RNA was isolated from tissue samples by the guanidinium isothiocyanate/CsCl technique (30). For RNA blot analysis, 10 μg of total RNA from each sample was subjected to electrophoresis on a 1.2% agarose/formaldehyde denaturing gel and transferred to nitrocellulose filters. The filter was then prehybridized, hybridized to a ^{32}P-labeled 802-bp *Sac* I fragment from within the *Mt-1/GHB* fusion gene, washed, and exposed to x-ray film.

RESULTS

Production and Characterization of the GHB Protein. We have demonstrated (22) that hGH (GHA) and several other secreted proteins can be produced in large quantities by transiently transfected mouse L cells. To express the GHA and GHB proteins, appropriate fusion genes were constructed (Fig. 1); the plasmid containing the *Mt-1/GHB* fusion gene is pXGH4, and the *Mt-1/GHA*-containing plasmid is pXGH5.

Total cellular RNA was prepared 4 days after transient transfection of L cells with 10 μg of either pXGH4 or pXGH5 by the DEAE-dextran method. The RNA was then subjected to blot-hybridization analysis (Fig. 2 *Left*). RNA prepared from cells transfected with either plasmid contained a hybridizing band of ≈900–950 nucleotides (nt), which corresponds to the expected fusion mRNAs [predicted to contain 64 nt of *Mt-1* 5′ untranslated region, 817 nt of *GHA* or *GHB*, and a poly(A) tail]. Based on this result, we conclude that similar amounts of GHA and GHB fusion mRNAs are produced in L cells transiently transfected with equal amounts of the plasmids. This similarity in mRNA levels can be attributed to the presence of the identical *Mt-1* promoter in both fusion genes and to the high homology between the GHA and GHB mRNAs. It also suggests that the transcriptional rates of the fusion genes are approximately the same and that the stabilities of the fusion mRNAs are also comparable. Both RNA samples contain an additional hybridizing band of ≈1500 nt. The origin of this band may be due to an upstream promoter or to aberrant or partial processing of a correctly initiated transcript.

To determine if the fusion mRNAs could direct synthesis and secretion of GHA and GHB, aliquots of medium were taken daily from dishes of L cells transiently transfected with 1 μg of either pXGH4 or pXGH5. The level of GHA or GHB secreted into the medium was determined with a commercially available radioimmunoassay in which the amount of hGH specifically bound by a first mAb is measured by using a second, ^{125}I-labeled mAb directed against a different hGH epitope. GHA production (solid line in Fig. 2 *Center*) was first detectable at ≈24 hr after transfection and accumulated rapidly in the medium during the next 3 days. GHB expression (dashed line in Fig. 2 *Center*) could not be detected by this assay. Since the *Mt-1/GHB* fusion gene is properly transcribed, this result suggests that, if GHB is synthesized and secreted properly, it does not bind efficiently to one or both of the anti-GHA mAbs used in this assay.

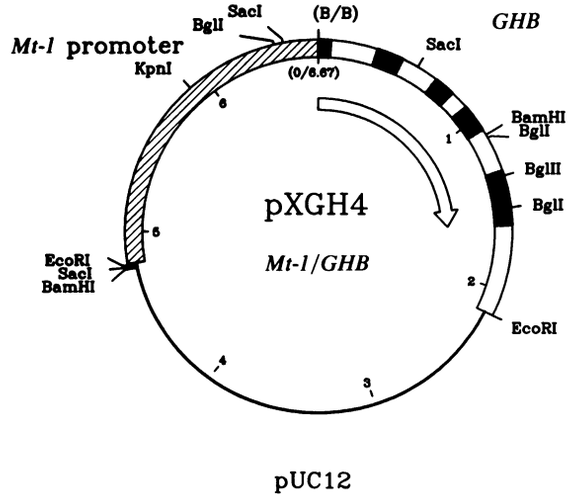

FIG. 1. Structure of plasmid containing the *Mt-1/GHB* (pXGH4) fusion gene. The hatched box in the plasmid represents the promoter and 5′ flanking sequences of the mouse *Mt-1* gene, and the black and white boxes represent the exons and introns respectively of the human *GHB* gene. B/B is the site at which the *Mt-1 Bgl* II site was ligated to the *GHB Bam* HI site (and which now cannot be cut with either enzyme). Numbers inside the plasmid represent the distance in kb from B/B, and the clockwise arrows indicate the predicted start site and direction of transcription of the fusion gene.

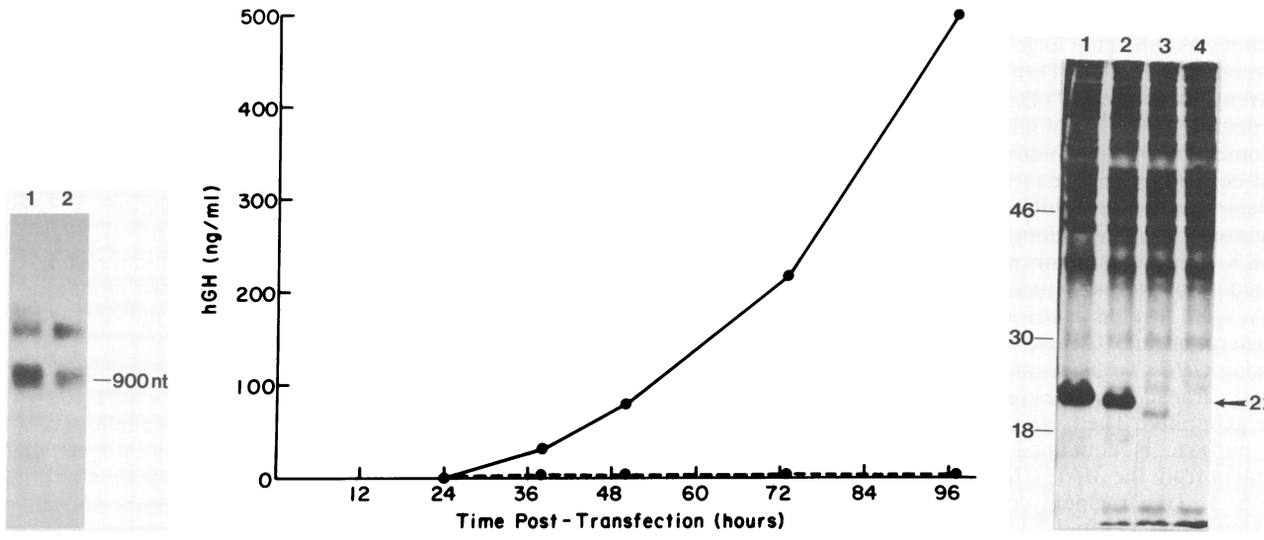

FIG. 2. (*Left*) Blot-hybridization analysis of total cellular RNA prepared from transiently transfected cells. Mouse L cells were transfected with 10 μg of pXGH4 or pXGH5 using the DEAE-dextran protocol, and RNA was prepared 4 days later, separated on a 1.2% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with an 802-bp *Sac* I fragment of the *Mt-1/GHB* gene as described. Lanes contained 10 μg of total RNA prepared from cells transfected with pXGH4 (lane 1) or pXGH5 (lane 2). In this experiment the hybridizing bands are ≈900 nt, although in other experiments (not shown), the bands are closer to 950 nt; this minor discrepancy may be due to small differences in the quality of RNA preparations but most likely reflects typical experiment-to-experiment variability in electrophoresis. (*Center*) Detection of GHA (—) and GHB (- - -) in the medium of transiently transfected cells. Mouse L cells were transfected with 1 μg of pXGH4 or pXGH5 as in *Left*, and aliquots of the medium were taken at the indicated times after transfection and assayed by using a commercially available radioimmunoassay. (*Right*) Radiolabeling of transiently expressed GHA and GHB. Mouse L cells were mock-transfected (lane 4) or were transfected with 10 μg of pXGH4 (lane 3) or pXGH5 (lane 2) and were radiolabeled as described. Rat GH4 cells, a well-characterized pituitary tumor cell line known to secrete a 22-kDa rat GH (arrow), were radiolabeled as above but were not transfected (lane 1). Aliquots of medium were electrophoresed on a denaturing 12% polyacrylamide gel, and size markers (in kDa) are indicated.

The ability of the GHB fusion mRNA to direct translation of a secreted product was investigated by performing a radiolabeling experiment. L cells transiently transfected with 10 μg of either pXGH4 or pXGH5 were cultured in the presence of [^{35}S]methionine for ≈1 day. Aliquots of medium from both transfections were collected and analyzed by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis followed by autoradiography (Fig. 2 *Right*). Medium from cells transfected with either pXGH4 or pXGH5 contained a radiolabeled protein that was not present in the medium of mock-transfected L cells treated as above.

The radiolabeled pXGH4- and pXGH5-derived bands differed with respect to apparent molecular mass and intensity. The pXGH5-derived band had an apparent molecular mass of 22 kDa and was ≈10-fold more intense than the pXGH4-derived band, which had an apparent molecular mass of 21 kDa. Mature GHA and GHB were both predicted to have molecular masses of 22 kDa, and the difference in their observed mobility most likely reflected differences in their isoelectric points (see below) and SDS-binding capacities. The 10-fold difference in accumulation between GHA and GHB (as determined by densitometry) might reflect less efficient synthesis or secretion of GHB or a greater instability of GHB in the medium as compared with GHA. In any case, the *GHB* fusion gene does direct synthesis and secretion of a protein product consistent with its DNA sequence.

Medium from cells transfected with the *GHB* fusion gene was tested with a battery of three anti-GHA mAbs (26). Relative GHB cross-reactivity with each mAb was estimated by dividing the amount of culture medium containing GHA that gave 50% displacement of ^{125}I-labeled hGH by the amount of GHB-containing medium required for 50% displacement and normalizing to the absolute amount of GH protein sequences present in each medium (as determined by radiolabeling above). The estimated cross-reactivity of GHB (Table 1) ranges from 1.7×10^{-2} with antibody 033 to $2.4\ 10^{-1}$ with antibody 665.

The cross-reactivities of murine and bovine GHs with the three anti-GHA mAbs were calculated by assaying a sample containing a known amount of one of these GHs with each mAb and then dividing the measured amounts by the known amount of GH present in the sample (Table 1). Although GHB has less cross-reactivity than GHA with respect to all three antibodies, the cross-reactivity of GHB is substantially higher than both murine and bovine GH. The relative specificity of the mAbs for the GHB protein as compared to mouse GH is important because it suggests that GHB expressed in the serum of a transgenic mouse at even a moderate level can be distinguished from endogenous mouse GH by using any of the mAbs.

The molecular mass of the anti-GH cross-reacting material produced by L cells transfected with either the *GHA* or *GHB* fusion gene was determined by gel filtration on Sephadex G-75 columns (data not shown). Fractions collected from the

Table 1. Relative cross-reactivities of GHB, mouse GH, rat GH, and bovine GH with anti-GHA mAbs

GH	Relative cross-reactivity with mAb		
	mAb 352	mAb 033	mAb 665
GHB vs. GHA	7.7×10^{-2}	1.7×10^{-2}	2.4×10^{-1}
Murine GH vs. GHA	5.0×10^{-5}	5.0×10^{-5}	1.5×10^{-4}
Bovine GH vs. GHA	$<5.0 \times 10^{-5}$	$<5.0 \times 10^{-5}$	6.0×10^{-5}
GHB vs. murine GH	1540	340	1600
GHB vs. bovine GH	>1540	>340	4000

GH levels were measured with a nonequilibrium radioimmunoassay using affinity-purified iodinated GHA. Relative cross-reactivities of GHB and murine and bovine BHs with the anti-GHA mAbs were determined by assaying a known amount of these GHs and then dividing the measured amounts by the known amounts (the actual amount of GHB assayed was determined by performing a radiolabeling experiment as described in the text). Relative cross-reactivities of GHB compared with murine and bovine GHs with the GHA mAbs were calculated by dividing GHB cross-reactivity with each mAb by murine and bovine cross-reactivity with each mAb.

columns were assayed with mAb 665. When GHA-containing medium was subjected to gel filtration, ≈70–75% of the GHA was recovered as a 22-kDa monomer and the remainder as an apparent dimer of ≈44 kDa. The GHB gel filtration profile was distinct from that of GHA, with only 25% recovered as monomer and the remainder as an apparent dimer. When the GHB-containing culture medium was subjected to immunoisoelectric focusing (with mAb 665), three peaks of GH immunoreactivity were found. The major peak was located at pH 8.8, and two minor peaks, most likely representing deamidation products analogous to those observed with GHA, were located at pH 8.35 and pH 8.5.

Generation and Characterization of Transgenic Mice Expressing *GHB*. To determine whether GHB can function in animals, transgenic mice containing a *Mt-1/GHB* fusion gene were generated. The *GHB* fusion gene that we have used for this purpose is contained within a 2.8-kb *Kpn* I–*Eco*RI fragment from the *Mt-1/GHB* fusion gene plasmid described in Fig. 1. The fragment contains about 700 bp of *Mt-1* 5′ flanking sequences and 2.1 kb of *GHB* structural sequences. After one set of injections and implantations, a total of 45 mice were born and subjected to further analyses.

At 3 weeks of age, a small piece of tail was removed from each mouse and used for the preparation of total genomic DNA. Hybridization to this DNA probed with an 802-bp *Sac* I fragment of the fusion gene revealed that 3 of the 45 mice were transgenic. Several blot-hybridization experiments (not shown) were performed to determine the structure of the fusion genes in these 3 mice. Total genomic digests with *Bgl* I yielded an ≈1.4-kb band that hybridized to the *Sac* I probe; the size of this band corresponds to the predicted size of the fragment expected from *Bgl* I digestion of the fusion gene itself (see Fig. 1). Based on these experiments, we conclude that the genome of each of the 3 transgenic mice contains at least one integrated and structurally intact fusion gene.

The three transgenic mice, all males, were weaned 30 days after birth and their weights were monitored for the next several weeks (Fig. 3). At the time of weaning, the three mice were significantly larger than nontransgenic (C57BL/6 × C3H)F_1 males; by two months of age, they were from 40% to 90% larger than controls. This increase in size was proportional, and in all other respects the transgenic mice appeared normal. One of the three transgenic mice was extensively bred; the *Mt-1/GHA* gene was inherited in Mendelian fashion in the resulting line, the transgenic offspring displaying the transgenic phenotype. Sera from the transgenic mice were assayed by using mAb 665 (based on a standard curve generated from the pXGH4 and pXGH5 radiolabeling experiment described above) and estimated to contain GHB at 19.1 μg/ml for mouse 5, 30.9 μg/ml for mouse 16, and 17.4 μg/ml for mouse 41. Serum from control mice contained no detectable GH, confirming the low cross-reactivity of mouse GH with mAb 665 (see Table 1). These observations are similar to those for transgenic mice expressing the *Mt-1/GHA* fusion gene (31), which have been reported to contain serum levels of GHA up to 64 μg/ml. When serum from mouse 16 was subjected to gel filtration, only the GHB dimer was recovered.

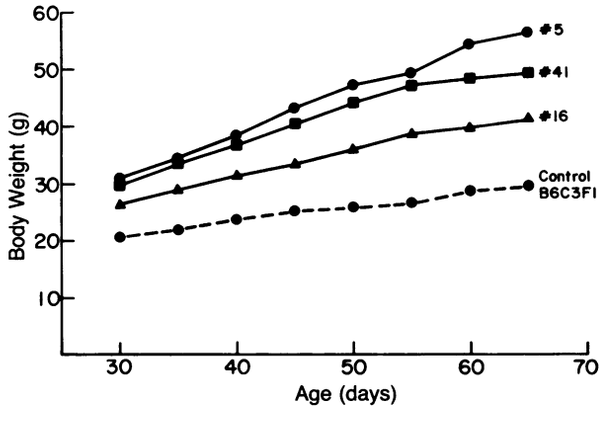

FIG. 3. Growth of transgenic mice containing the *Mt-1/GHB* fusion gene. Three of 45 mice born after one series of injections with a 2.8-kb *Kpn* I–*Eco*RI fragment containing the *Mt-1/GHB* fusion gene were found to be transgenic by Southern hybridization analysis. These 3 mice were weaned 30 days after birth and were weighed every 5th day thereafter. The dashed line represents the averaged weights of 10 (C57BL6 × C3H)F_1 males (standard errors range between 0.3 and 0.4 g for each point).

To determine the tissue specificity of fusion gene expression in these mice, RNA analyses were performed. Transgenic mouse 16 was sacrificed, and total cellular RNA was isolated from samples of several tissues by the guanidinium isothiocyanate/CsCl technique (30). RNA blot hybridizations using the 802-bp *Sac* I *Mt-1/GHB* probe showed that RNA prepared from liver (Fig. 4, lane 4) contained the most intensely hybridizing band, from brain (lane 5) and heart (lane 2) contained moderately hybridizing bands, and from kidney (lane 1) and small intestine (lane 6) contained weakly hybridizing bands. In all of these tissues, the hybridizing band was ≈900–950 nt as was expected (see above). RNA prepared from lung did not hybridize to the *Mt-1/GHB* probe. This pattern of expression is consistent with that previously reported for *Mt-1* fusion genes in transgenic mice (31–33).

DISCUSSION

We have studied the structure of GHB produced by transiently transfected cells and the function of GHB in transgenic mice. GHB in the medium of transfected cells shows moderate levels of cross-reactivity with anti-GHA mAbs—levels that are substantially higher than the cross-reactivities of murine and bovine GH with the same mAbs (Table 1). A greater fraction of GHB produced by transfection of mammalian cells with appropriate fusion genes is present as aggregates than similarly prepared GHA, and the isoelectric point of recombinant GHB is 8.8 compared with 4.9 for GHA. When expressed in the liver and other locations in transgenic mice, GHB causes a 1.4- to 1.9-fold increase in the size of the animals (Fig. 3).

The primary structure of GHB differs from that of GHA by 15 amino acid substitutions (out of a total of 191 amino acids in the mature proteins and 26 amino acids in the prese-

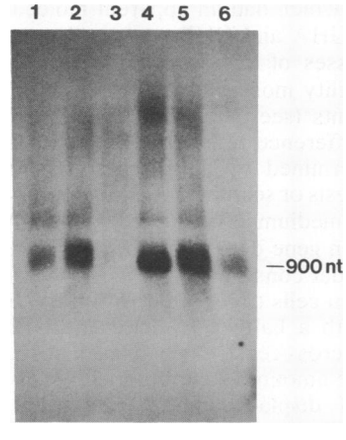

FIG. 4. Detection of Mt-1/GHB mRNA in tissues of transgenic mouse. Blot-hybridization analysis of 10 μg of total cellular RNA prepared from several tissues with the 802-bp *Sac* I *Mt-1/GHB* genomic probe was performed as described (see the Fig. 2 legend). Lanes: 1, kidney; 2, heart; 3, lung; 4, liver; 5, brain; 6, small intestine.

quence). GHB contains two fewer acidic and three more basic amino acids than GHA, and the measured isoelectric point of 8.8 for the transiently expressed protein is fully consistent with the predicted isoelectric point of 8.9 (2) based on sequence data. Although GHA and GHB have highly homologous primary structures, the mAb, isoelectric-focusing, and gel-filtration results all suggest that the two proteins have significantly different tertiary and quarternary structures.

The protein characterization we have presented and the ability to produce large quantities of GHB in the medium should facilitate the study of *GHB* expression in humans. Partially purified GHB can be used to generate mAbs, and, based on structural differences between GHA and GHB, it is reasonable to expect that some of these mAbs will bind 1–2 orders of magnitude less efficiently with GHA. The anti-GHB mAbs could then be used to assay for GHB in the serum, pituitary, placenta, and other locations in the body. If it appears that the *GHB* gene is expressed in humans, a determination of the isoelectric point of the protein should help confirm its identity.

GHB clearly functions to promote growth and development in mice. The increase in size of transgenic mice containing the *Mt-1/GHB* fusion gene is similar to that for transgenic mice containing the *Mt-1/GHA* fusion gene (31). Although GHB is growth-promoting in mice, it may not perform the same function when it is expressed in humans, or it may be expressed in a temporal and tissue-specific manner distinct from that of GHA. Patients with a homozygous deletion of only the *GHA* gene grew significantly less than average (14, 15), suggesting that *GHB* cannot by itself correct for the deficiency of *GHA* (the status of the *GHB* alleles of this patient was not studied, and it is formally possible that they were also mutant). We agree with the suggestion of Hizuka and coworkers (19) that it is possible that GHB works as a partial agonist. The possibility that a large proportion of GH oligomers found in normal human serum may be related to GHB production should also be considered.

Both GHA and GHB (19) are able to bind efficiently to GH receptors, and both promote growth in transgenic mice. A 20-kDa form of GHA, derived from alternative splicing of the GHA mRNA, has been described (34, 35), and sequence analysis shows that the GHB mRNA is also a candidate for this alternative splicing event. If the presence of GHA momomers, GHA oligomers, and the 20-kDa GHA-derived protein is in fact supplemented in humans with GHB monomers, GHB oligomers, and a 20-kDa GHB-derived protein, the study of the control of human growth and development may become even more complex than was anticipated when the existence of the five-member hGH gene family was first discovered.

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