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Osteoblast-Specific Expression of Growth Hormone Stimulates Bone Growth in Transgenic Mice

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Growth hormone (GH) is an important regulator of postnatal growth, acting on a wide variety of target tissues. Here, we show that local production of GH in osteoblasts is able to stimulate bone growth directly without significant systemic effects. Mice were made transgenic by microinjection of an osteocalcin-human GH (osteocalcin-hGH) gene construct in which approximately 1,800 bp of the rat osteocalcin promoter was fused to the hGH gene. Five lines of transgenic mice, each with measurable amounts of serum hGH (ranging from 1 to 1,000 ng/ml), were analyzed. Northern (RNA) blot hybridization showed that the hGH transcript was detectable only in the bone. Further characterization of hGH mRNA distribution by in situ hybridization revealed that in neonates the most intense signal was found in periosteal osteoblasts and in odontoblasts, while in adults, trabecular and endosteal osteoblasts were favored. In one transgenic line (992-1), hGH was expressed at a much lower level and had minimal systemic effects; however, the local concentrations of hGH in bone were sufficient to stimulate bone growth in these animals.

The growth and maintenance of skeletal tissues are regulated by the combined actions of systemic hormones, such as parathyroid hormone and growth hormone (GH), and local factors, including cytokines and growth factors. GH, in particular, is an important regulator of postnatal skeletal growth (for a review, see reference 14), possibly exerting its effects by stimulating insulin-like growth factor 1 (IGF-1) production in the liver and other target tissues (6, 7, 33). Although systemic IGF-1 may contribute to linear growth, it seems likely that GH control of the local generation of IGF-1 is important also (10, 31). For example, GH has been shown to stimulate osteoblast proliferation in culture (9), an effect inhibited by antisera to IGF-1. Similarly, GH potentiates the effects of IGF-1 on cultured epiphyseal chondrocytes (17). In order to assess the in vivo role of locally acting factors, such as GH, in bone formation, we have established a system for expressing exogenous genes in the bone microenvironment by using a promoter from a bone-specific gene.

Osteocalcin, or bone Gla protein, is an abundant bone matrix-associated protein, constituting approximately 1% of the total noncollagenous protein in the bone. Osteocalcin is synthesized by bone cells as a 10-kDa precursor protein which undergoes posttranslational γ-carboxylation (4, 28). The mature form of the protein has a molecular mass of ~5.7 kDa and is capable of binding to hydroxyapatite in a calcium-dependent fashion (29). Although the function of osteocalcin is unknown, osteocalcin levels in serum correlate with bone turnover and have been used as a biochemical marker of bone formation (16). Most importantly, osteocalcin appears to be a unique product of osteoblasts (2, 24). We have taken advantage of the apparent tissue specificity of the osteocalcin gene expression to produce transgenic mice which express the human GH (hGH) gene in osteoblasts. In addition to providing insights into the mechanism of action of GH in bone, this type of approach should prove generally useful in understanding normal and pathological interactions between bone cells.

MATERIALS AND METHODS

DNA constructs and transgenic mice. The gene for rat osteocalcin was obtained by screening a partial *Hae*III genomic library constructed in the λ phage Charon 4A. Two synthetic oligonucleotides complementary to the rat cDNA sequence (4) were used as probes, and three positive plaques were isolated and characterized. A 4-kb fragment of the gene was subcloned into pUC18 and sequenced. The codon corresponding to the initiating methionine was mutagenized by polymerase chain reaction, and *Xba*I and *Bsm*I restriction sites were added. The hGH gene was blunt-end ligated into the *Bsm*I site after treatment of the ends with the Klenow fragment of DNA polymerase. The hGH fragment used includes all five exons and approximately 550 bases 3′ to the poly(A) addition signal. This hGH fragment has been used previously for the generation of hGH-expressing transgenic mice (34, 35). The entire construct was excised from the vector by using *Kpn*I and *Sph*I, gel purified on a low-melting-point agarose gel, and then further purified by using sodium iodide-glass bead extraction (GeneClean; Bio 101). After spectrophotometric quantitation, the DNA fragment was diluted in 10 mM Tris–0.1 mM EDTA to a final concentration of 1 ng/ml for microinjection. Transgenic animals were produced as described elsewhere (12, 35). Positive offspring were detected by either Southern or dot blot analysis of tail DNA preparations. Lines of transgenic mice were established by backcrossing to BALB/c mice.

DNA and RNA hybridization analysis. Mouse genomic DNA was prepared as previously described (35) and either dot blotted onto nitrocellulose (Schleicher & Schuell) or digested with restriction enzymes, electrophoresed through agarose gels, and then transferred to nylon membranes (Hybond N+; Amersham), all as recommended by the manufacturers. RNA from mouse tissues was prepared by the method of Chomczynski and Sacchi (5), electrophoresed

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through formaldehyde agarose gels, and transferred to nylon membranes as described above. For both the Northern (RNA) hybridization analysis and the in situ hybridization analysis, an 850-bp hGH cDNA that covers the entire protein-encoding region was used. For the Northern analysis of mouse GH mRNA, a 50-base oligonucleotide complementary to the mouse GH cDNA was used. cDNA probes were labeled by random primed extension, and oligonucleotide probes were end labeled with T4 polynucleotide kinase (19). [α-^{35}S]CTP-labeled RNA probes for in situ hybridization were generated by in vitro transcription reactions using SP6 and T7 RNA polymerases (Promega). Hybridizations were carried out overnight under standard conditions (19).

GH and IGF-1 determinations. Blood was obtained from mice by puncture of the orbital sinus, and the serum fraction was serially diluted for assay. Serum hGH was assayed by enzyme-linked immunosorbent assay (ELISA) using polyclonal antiserum (Hybritech). For IGF-1 determinations, sera were acid extracted and then subjected to radioimmunoassay (Genentech assay services).

Histology. Mice were sacrificed by cervical dislocation, and tissues were removed and fixed in 10% neutral buffered formalin 24 h prior to demineralization. Bones were demineralized by using either an ion-exchange resin (American Histology Reagent Co.) or buffered EDTA (0.1 M Tris [pH 7.5], 10% EDTA, 7.5% polyvinylpyrrolidone) and embedded in paraffin. For in situ hybridization, 2-μm sections were mounted onto poly-L-lysine-coated slides. The paraffin was removed by using xylene; then sections were rehydrated through graded alcohols and incubated in prehybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris [pH 8], 5 mM EDTA, 10% dextran sulfate, 10 mM dithiothreitol, 0.02% Ficoll 400-polyvinylpyrrolidone-bovine serum albumin) prior to the addition of SP6- or T7-generated sense and antisense RNA probes. After overnight hybridization at 50°C, the slides were washed in multiple changes of 0.5× SSC (1× SSC is 0.15 M sodium chloride plus 15 mM sodium citrate) at room temperature, treated with 1.0 μg of RNase A per ml, and washed under high-stringency conditions (0.1× SSC; 60°C). Slides were then dehydrated in xylene, dipped in photographic emulsion (Kodak NTB2), and left in lightproof containers at 4°C for appropriate time intervals. After development of the emulsion, slides were examined by bright- and dark-field microscopy.

Statistics. All values are given as means ± 1 standard deviation. Probabilities of differences between groups were assessed by using a two-tailed *t* test.

RESULTS

Transgene expression. Seven lines of transgenic mice harboring the construct shown in Fig. 1 were produced as described above. Of these founder animals, three lines (992-1, 993-2, and 994-8) were selected for further analysis. Serum hGH levels for these founder animals, shown in Table 1, ranged from ~1 ng/ml in the 992-1 line to ~1 μg/ml in the 994-8 line. This variation in expression levels has been reported by other investigators (26, 27, 34, 35). As there is no obvious correlation with gene copy number, these differences may be due to permissive effects exerted by the chromosomal locus into which the gene is integrated. Serum hGH levels in transgenic progeny from each of these founders were consistent with that in the founder animal, although in the 992-1 line, the serum hGH levels for the F_1 animals were somewhat higher than that for the founder, ranging between 5 and 10 ng/ml.

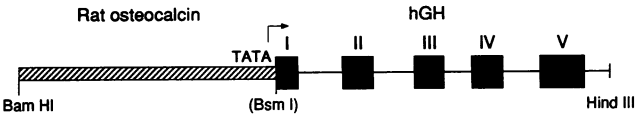

FIG. 1. Osteocalcin-hGH gene construction. The osteocalcin-hGH gene was constructed as described in Materials and Methods. The 5′ region of the rat osteocalcin gene, including 1,710 bp 5′ of the TATA box and 35 bp of the first exon, is crosshatched. These osteocalcin regulatory regions were linked to the hGH gene so as to include all five hGH exons (black boxes) with 60 bases 5′ of the translation start codon and 550 bases 3′ of the poly(A) addition signal. The start site for the transcription (arrow) and the *Bsm*I site destroyed by the introduction of the hGH gene are indicated.

Initially, Northern hybridization was used to demonstrate tissue-specific expression of the transgene. Total RNA isolated from tissues of control and transgenic animals was analyzed for the presence of the hGH message. Figure 2 shows that only the skeletal tissues express the hGH transcript in the 994-8 animals. Similar results were obtained for all other lines examined, and the relative abundance of the hGH mRNA correlated well with the serum hGH levels. Using a sensitive RNase protection assay, we have occasionally seen an hGH transcript in the brain in one line of mice (data not shown). However, there is no evidence that this low level of expression affects the transgenic phenotype. To further localize the hGH expression, in situ hybridization experiments with 994-8 and 992-1 adults and neonates were performed (Fig. 3). A strong hybridization signal was detected in the 2-day-old 994-8 pups (Fig. 3B), with intense labeling of periosteal and endosteal osteoblasts in sections of long bones. Trabecular osteoblasts were also labeled, although the signal was reduced. Intense hybridization in developing tooth germs was also noted (data not shown), presumably originating from odontoblasts, which are known to express osteocalcin (3).

When the adult 994-8 sections were examined, a different pattern of expression emerged. In the femur and the tibia, weak hybridization of the hGH probe was noted for periosteal osteoblasts, while most of the hybridization signal was seen in endosteal (Fig. 3D) and trabecular (not shown) osteoblasts. In contrast, sections of the skull (Fig. 3E) showed that both periosteal and endosteal osteoblasts express high levels of the transgene mRNA. The results obtained from RNA in situ hybridization experiments performed on the 992-1 adult animals also demonstrated that expression was detectable in endosteal and trabecular osteoblasts (Fig. 3H) but at a much reduced level, consistent with the Northern hybridization results (data not shown). No hybridization was detected in bone from nontransgenic animals (Fig. 3F). When mouse osteocalcin cRNA probes

TABLE 1. Serum hGH levels in rat osteocalcin-hGH-expressing transgenic founder animals

Founder	Serum hGH (ng/ml)[a]
992-1	1.4 ± 0.67
991-3	295 ± 55
993-2	362 ± 36
994-8	1,023 ± 56
995-4	124 ± 7

[a] Level of hGH in the circulation of adult founder mice as measured by ELISA. The data from four separate bleeds of each animal are expressed as means ± standard deviations.

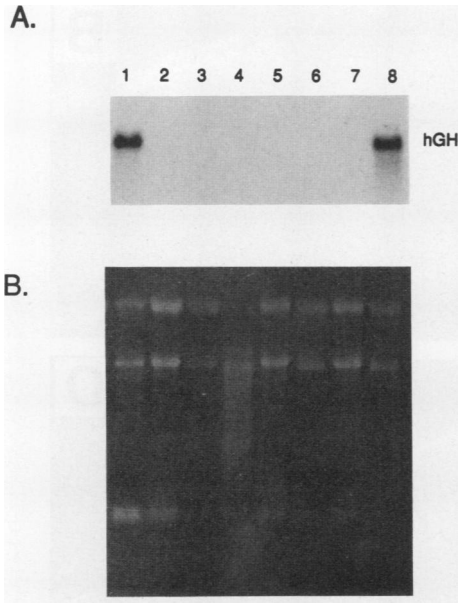

FIG. 2. Northern hybridization analysis of transgene expression in the 994-8 transgenic line. Total RNAs from selected tissues were electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled hGH probe. (A) Autoradiograph (24-h exposure); (B) ethidium bromide-stained gel. Lanes contain RNAs from mouse tissues as follows: 1 and 8, pooled long bone; 2, brain; 3, heart; 4, lung; 5, liver; 6, kidney; 7, spleen.

were used, similar patterns of expression were seen (Fig. 3J). Figure 4 confirms that the cells expressing the hGH transcript are osteoblasts; this is a short (24-h) exposure of an in situ hybridization experiment, shown at a higher magnification and in bright field. The silver grains representing the hGH mRNA (appearing as black spots) are seen only over the osteoblasts lining the bone spicule and not over either the bone marrow or the mesenchymal tissue. On the basis of Northern and in situ hybridization data, we conclude that the osteocalcin promoter directs expression of the hGH mRNA specifically to osteoblasts and that this pattern of expression parallels that of the endogenous osteocalcin message.

Animal growth. The transgenic lines expressing high levels of hGH (994-8 and 993-2) showed accelerated growth rates (in both body weight and bone length) comparable to those previously reported for hGH-expressing transgenic mice (data not shown) (26, 27). The 992-1 line, however, showed an unusual pattern of growth (Table 2). Differences in growth became obvious at a gross level by 6 weeks of age. Measurements of body length in young (2-month-old) 992-1 adults showed that they were significantly longer than nontransgenic littermates (data not shown). X-ray measurements showed increased tibial and femoral lengths for the 992-1 animals (Table 2). These differences were more marked in 4-month-old animals (data not shown). Organ weights for the 992-1 animals generally showed no significant differences from those of control animals at 2 months, although some increases were noted as the animals aged. It is not clear why the brains of the transgenic females appear to be larger than those of their littermates. It should be noted, however, that this apparent difference disappears when the values are expressed as a proportion of body weight. These unusual growth patterns suggest that the local production of hGH in osteocalcin-hGH-expressing transgenic mice stimulates bone growth and that, in the 992-1 animals, this is due primarily to the local effects of hGH on bone tissue.

Systemic markers of GH action. To determine whether the osteoblast-derived hGH was affecting parameters other than bone growth, we measured several systemic markers of hGH action. An important target organ for GH is the liver, where it has a number of effects, including regulation of the synthesis and secretion of IGF-1, cholesterol, and albumin (20, 27, 34). None of the hGH-expressing transgenic lines showed elevated IGF-1 levels in the face of high levels of circulating hGH, consistent with measurements from other hGH-expressing transgenic mice characterized in our laboratory (34). The levels seen in the 992-1 animals (259.6 ± 48.4 ng/ml; n = 10) were not significantly different from control values (225.6 ± 58.4 ng/ml; n = 10). GH affects the splicing of IGF-1 mRNA (25, 30), changing the relative abundance of alternatively spliced transcripts in the liver. We do not see substantial changes in absolute levels of IGF-1 mRNA in the liver, although transgenic lines 993-2 and 994-8 (but not 992-1) do show differences in the relative proportion of IGF-1 transcripts (Fig. 5). Figure 5 also shows that the levels of IGF-1 mRNA are increased in the bones of the 993-2 and the 994-8 transgenic mice. However, we have been unable to reproducibly demonstrate increased levels of IGF-1 mRNA in the bones of the 992-1 transgenic mice by

TABLE 2. Bone lengths, organ and body weights, and serum cholesterol and albumin concentrations in 8-week-old 992-1 transgenic and littermate control mice[a]

Mice (n)[b]	Length (mm)		Wt (g)			Concn in serum	
	Femur	Tibia	Kidney	Brain	Body	Cholesterol (mg/dl)	Albumin (g/dl)
Male							
Transgenic (6)	14.25 ± 0.62[c]	16.94 ± 0.7[d]	0.39 ± 0.03[e]	0.42 ± 0.01[e]	25.4 ± 1.3[e]	143 ± 4.8[c]	3.25 ± 0.6[e]
Nontransgenic (4)	13.25 ± 0.42	15.63 ± 0.34	0.36 ± 0.03	0.41 ± 0.01	23.3 ± 1.6	113 ± 13.8	2.65 ± 0.51
Female							
Transgenic (4)	14.75 ± 0.3[c]	15.9 ± 0.18[d]	0.26 ± 0.01[e]	0.43 ± 0.01[c]	19.9 ± 1.5[e]	106 ± 4.36[e]	3.68 ± 0.04[e]
Nontransgenic (4)	12.92 ± 0.7	14.71 ± 0.4	0.22 ± 0.02	0.40 ± 0.01	17.7 ± 1.3	105 ± 7.25	3.43 ± 0.25

[a] Data are means ± standard deviations.
[b] For cholesterol and albumin concentration data, n = 10.
[c] Significantly different from corresponding value for nontransgenic mice ($P < 0.05$).
[d] Significantly different from corresponding value for nontransgenic mice ($P < 0.01$).
[e] No significant difference from corresponding value for nontransgenic mice.

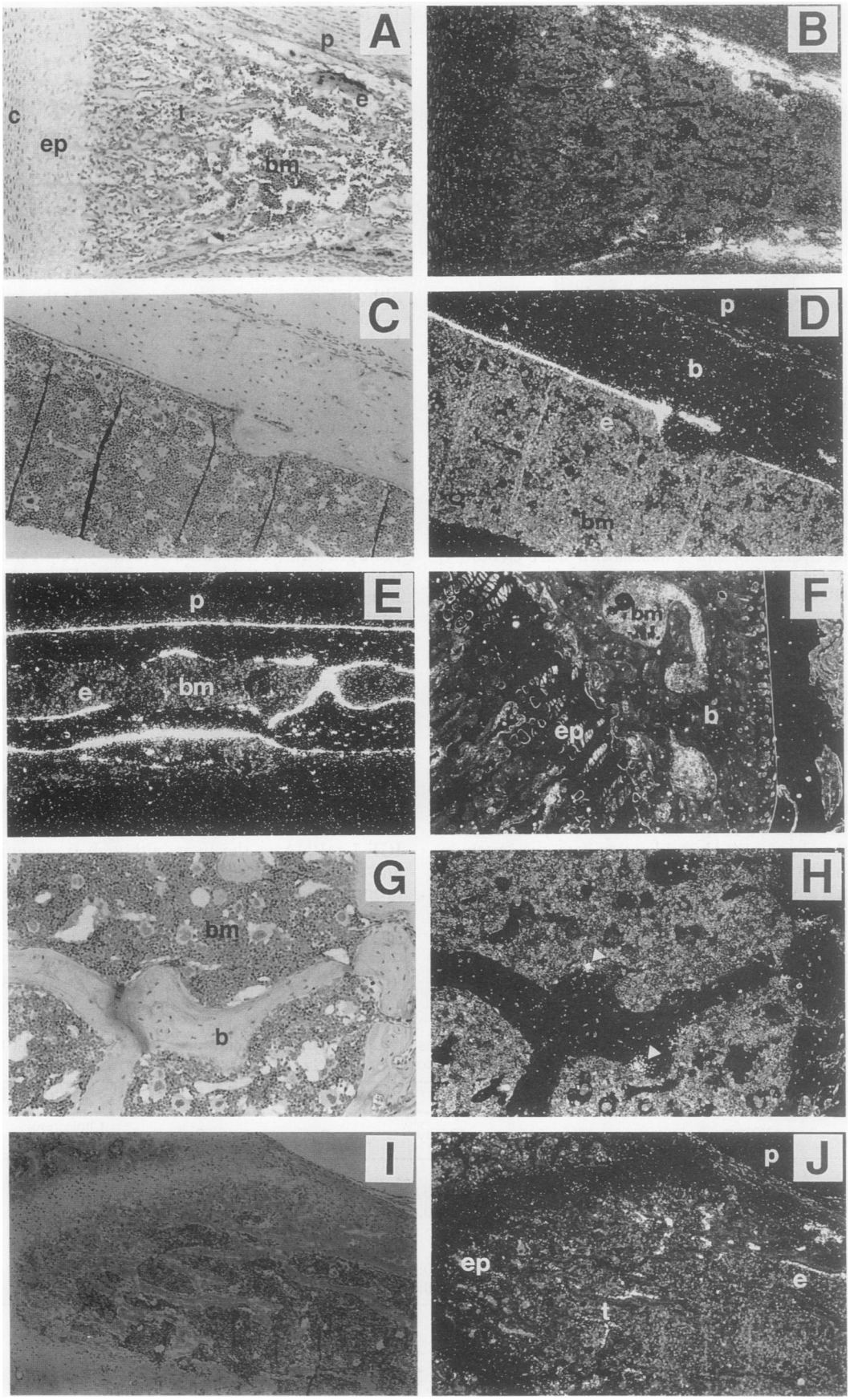
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FIG. 3. In situ localization of hGH and osteocalcin mRNA. Sections were photographed by using bright-field microscopy (A, C, G, and I) to demonstrate morphology and dark-field microscopy (B, D, E, F, H, and J) to emphasize areas of hybridization. (A through H) Hybridization to hGH cRNA probes. (A and B) Long-bone tissue of a two-day-old 994-8 transgenic mouse; (C and D) femur tissue of an adult 994-8 transgenic mouse; (E) skull tissue of an adult 994-8 transgenic mouse; (F) femur tissue of an adult nontransgenic mouse; (G and H) femur tissue of an adult 992-1 transgenic mouse; (I and J) femur tissue of an adult nontransgenic mouse, probed with osteocalcin cRNA. Magnifications, ×250 (A through E, I, and J) and ×625 (F through H). Abbreviations: b, bone; p, periosteum; e, endosteum; t, trabecular osteoblasts; bm, bone marrow; c, cartilage; ep, epiphyseal growth plate. Osteoblasts actively transcribing the transgene in the 992-1 transgenic mice (H) are indicated (arrowheads).

using either Northern hybridization or RNase protection analysis. Serum albumin and cholesterol levels are markedly elevated in both the 994-8 and the 993-2 lines (data not shown), as previously described for other hGH-expressing transgenic animals (18, 20, 30, 34), but not in the 992-1 line.

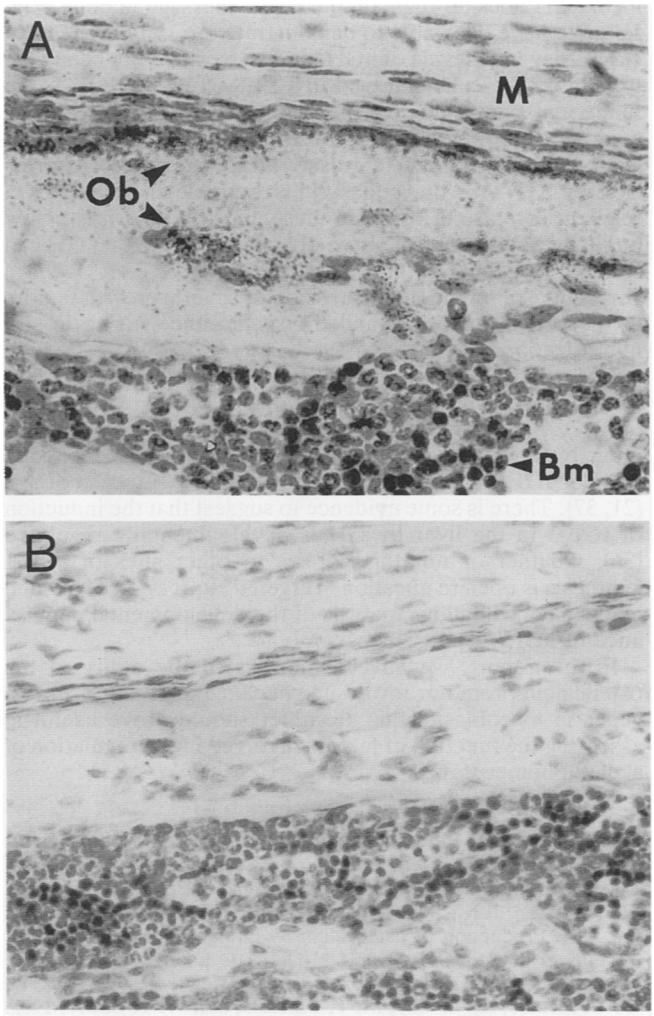

FIG. 4. In situ localization of hGH mRNA. Sections were photographed at a higher magnification (×435) and by using bright-field microscopy in order to demonstrate that the cells containing the hGH transcript are osteoblasts. In bright-field micrographs, the silver grains appear as dark spots. (A) Section through a long bone of a 2-day-old transgenic pup hybridized with an hGH antisense probe. Ob, osteoblasts; Bm, bone marrow; M, mesenchymal tissue. (B) Section through a long bone of a 2-day-old pup hybridized with a sense strand hGH probe.

Serum cholesterol levels, however, are increased in 992-1 transgenic male (but not female) mice (Table 2).

As GH down-regulates its own production in the anterior pituitary (22), transgenic mice with significant amounts of circulating hGH have reduced amounts of the endogenous mouse GH message. Figure 6 shows the results of Northern hybridization analysis of mouse pituitary RNA in which the endogenous mouse GH mRNA is down-regulated in the 994-8 animal (lane 2) but not in the 992-1 animal (lane 4).

DISCUSSION

Local and systemic factors play important roles in bone growth and development. The complexity of bone, however, has rendered in vivo manipulation of this system problematic. We have described a means of expressing heterologous genes in the bone microenvironment by using the promoter for the bone matrix protein osteocalcin. Several lines of osteocalcin-hGH-expressing transgenic mice have been produced, demonstrating that approximately 1,800 bp of the rat osteocalcin promoter is sufficient to direct expression of the transgene to osteoblasts. Using Northern hybridization analysis and in situ hybridization techniques, we were unable to reproducibly detect transgene expression in nonskeletal tissues, although we cannot formally exclude the possibility of expression below the levels of detection of the methods used.

The in situ RNA hybridization results indicate that the transgene expression is restricted to osteoblasts. In neonatal

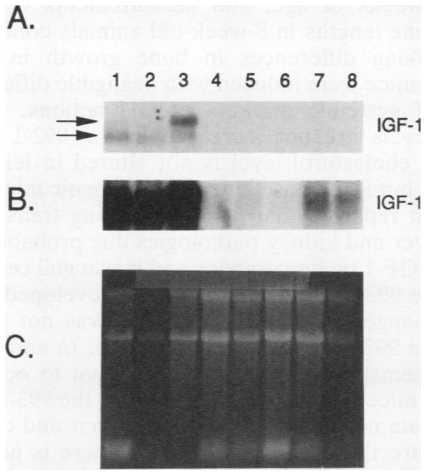

FIG. 5. IGF-1 mRNA levels in transgenic and control mice. (A and B) Autoradiograph (24-h [A] and 7-day [B] exposures); (C) ethidium bromide-stained gel. Lanes contain mRNAs from mouse tissues as follows: 1, 993-2 liver; 2, 994-8 liver; 3, control liver; 4 to 6, control bone; 7, 993-2 bone; 8, 994-8 bone. Arrows indicate different IGF-1 transcripts.

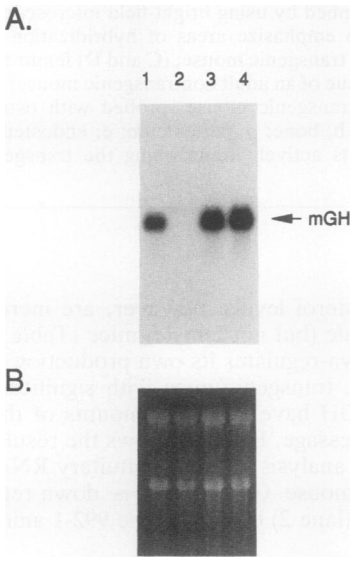

FIG. 6. Northern analysis of endogenous murine GH expression in pituitary RNAs from transgenic 994-8 (lane 2) and 992-1 (lane 4) and nontransgenic control (lanes 1 and 3) mice. (A) Autoradiograph; (B) ethidium bromide-stained gel.

mice, this expression was detected at high levels in periosteal osteoblasts, as has been reported for the endogenous osteocalcin message (11). In contrast, in adult animals, the level of transgene expression was highest in endosteal and trabecular osteoblasts. These observations suggest that the osteocalcin gene is expressed by mature osteoblasts in contact with remodeling surfaces. In the lines of mice that expressed high levels of GH (994-8 and 993-2), there was an increased rate of bone growth accompanied by a variety of systemic changes. These results are consistent with previously reported observations (20, 22, 26, 27, 34, 35). In contrast, the principal effect of locally expressed hGH in the 992-1 line was stimulation of bone growth in the absence of systemic effects. Qualitative differences in skeletal size between transgenic and control animals were readily apparent at 6 weeks of age, and measurements of tibial and femoral bone lengths in 8-week-old animals confirmed this. These striking differences in bone growth in the 992-1 transgenic mice were induced with negligible differences in a number of systemic markers of GH actions. IGF-1 and albumin levels are not increased in the 992-1 transgenic mice. The cholesterol level is not altered in female transgenic mice but is increased in male transgenic mice. Further, it has been reported that hGH-expressing transgenic mice develop liver and kidney pathologies due probably to direct effects of IGF-1 on hepatocytes and mesangial cells (18, 30). Indeed, the 993-2 and 994-8 lines both developed histopathological changes in these tissues; this was not seen, however, in the 992-1 or nontransgenic mice. In addition, alterations in hematopoiesis which are known to occur in GH transgenic mice (34) were evident only in the 993-2 and 994-8 animals (data not shown). Most significant and compelling, however, are the data indicating that there is no feedback inhibition of mouse GH mRNA levels in the pituitary glands of the 992-1 transgenic mice. Taken together, these observations strongly argue that there are minimal systemic consequences of hGH in the 992-1 mice and that the observed increase in bone length is due to local effects of hGH on bone and/or cartilage.

Isaksson and coworkers (13) have demonstrated that unilateral administration of GH into the tibial epiphyseal growth plate of hypophysectomized rats stimulates bone growth on the injected side only. Similar experiments have shown that IGF-1 is also able to stimulate local bone growth (15, 32). Finally, in situ hybridization of the rat epiphyseal growth plate with IGF-1 antisense probes showed a positive correlation between GH administration and the IGF-1 signal in hypophysectomized animals (23). The effects of hypophysectomy on GH and IGF-1 receptors and binding proteins, however, might dramatically alter the responsiveness of the growth plate to hormones. Nevertheless, results such as these argue that, at least in GH-deficient animals, GH is able to stimulate long-bone growth directly and that this is mediated in part by IGF-1. Our transgenic experiments extend these findings by demonstrating that local hGH can stimulate bone growth in animals with an intact, functioning pituitary. Initial experiments examining IGF-1 mRNA levels in osteocalcin-hGH-expressing transgenic animals show that local hGH does up-regulate the IGF-1 message in bone (Fig. 5B). We have been able to demonstrate this only in the mice expressing high levels of hGH, and as we have not been able to identify the cells involved in synthesizing the IGF-1, the functional significance of this is unclear. Unlike the transgenic mice expressing high levels of hGH, the 992-1 transgenic mice do not have a grossly increased level of expression of IGF-1 in the bones. It is possible that there is a relevant increase in IGF-1 in these mice but that it is below our detection limits. An alternative possibility is that GH can stimulate bone growth in the absence of IGF-1. We are currently examining the expression of several growth factors in the bones of normal and transgenic mice, using in situ hybridization.

The role of GH in the immediate postnatal period is less clear. Genetically GH-deficient animals (8) and hypophysectomized neonates (1) grow normally for the first 2 weeks after birth. Similarly, transgenic animals overexpressing hGH or IGF-1 also grow normally until 2 to 4 weeks of age (21, 37). There is some evidence to suggest that the induction of IGF-1 in the liver by GH is developmentally regulated (36); whether or not this applies to other sites of IGF-1 synthesis is open to question. Targeted expression of hGH in osteoblasts will allow analysis of the developmental appearance of hGH responsiveness in bone.

In this report, we have demonstrated the utility of the osteocalcin promoter for tissue-specific expression of transgenes in osteoblasts; this promoter should prove useful in assessing the function of factors involved in the regulation of skeletal homeostasis.

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