ELSEVIER

Molecular and Cellular Endocrinology 147 (1999) 149–159

MCE
Molecular and Cellular Endocrinology

Growth hormone and the expression of mRNAs for matrix proteins and oncogenes in bone

Mohammed A. Salih *, Paul B. Orhii, Cang Chen, Dike N. Kalu

Department of Physiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7756, USA

Received 25 June 1998; accepted 17 September 1998

Abstract

To examine the effects of growth hormone (GH) on the expression of the mRNAs of bone matrix proteins, three experiments were carried out with 3-month-old female Sprague–Dawley rats. In the first experiment rats were given a single subcutaneous injection of recombinant human GH (8 mg rhGH/kg b. wt.), sacrificed 15 min, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h later, and RNA isolated from cancellous bone from the distal femoral metaphysis. Growth hormone increased the level of type I collagen mRNA by 187, 417, and 509% over the control level at 15 min, 1 h and 2 h, respectively; the mRNA levels declined to 119 and 99% at 4 and 8 h, respectively, and then rose again to 351 and 423% over the control level at 16 and 24 h, respectively. Osteocalcin mRNA transcript increased by 89, 90, 325, 342, 361, and 407% over the control level at 15 min, 1 h, 2 h, 4 h, 8 h and 16 h, respectively, and fell to 66% at 24 h. The level of IGF-I mRNA increased by 45, 83, 120, 140, and 175% over the control level at 2, 4, 8, 16, and 24 h, respectively. In the second experiment, following the administration of rhGH (8 mg/kg b. wt.) bone osteocalcin mRNA increased by 127, 177, 361, and 413% over the control level at 30 min, 1 h, 2 h and 4 h, respectively; IGF-I mRNAs increased by 38, 33, 87, and 437 at 30 min, 1 h, 2 h and 4 h, respectively, but the levels did not become significant until 2 h; c-fos mRNA increased significantly at 30 min, and c-jun and c-myc mRNAs did not increase until 4 h. In the third experiment, animals were given a single injection of rhGH (8 mg/kg b. wt.) and the animals were bled at timed intervals and acid ethanol-extractable serum IGF-I determined. Serum IGF-I increased significantly only at 12 h following rhGH administration. Our data indicate that GH stimulates a rapid increase in the expression of mRNAs for the bone matrix proteins, type I collagen and osteocalcin, by a mechanism that appears to be independent of IGF-I, the early response oncogenes or an increase in osteoblast number. © 1999 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Type I collagen; Osteocalcin, IGF-I; c-fos; c-jun; c-myc

1. Introduction

Growth hormone (GH) has important bone anabolic properties. It stimulates both longitudinal and circumferential growth of bone (Sontag et al., 1980; Sontag, 1986; Isaksson et al., 1987; Andreassen et al., 1995). However, the mechanism of action of GH on bone is not well understood. It is generally considered that GH enhances linear bone growth by stimulating hepatic production of IGF-I and the differentiation of chondrocytes which also produce IGF-I. The latter then acts in the classical hormonal fashion and/or in a paracrine-autocrine fashion to increase chondrocyte proliferation and activity (Salmon and Daughaday, 1957; Daughday, 1981; Isaksson et al., 1982; Hochberg et al., 1989). Growth hormone continues to stimulate bone formation after linear growth has ceased. Some observations indicate that GH increases the proliferation and activity of osteoblasts which are the bone forming cells (Stracke et al., 1984; Ernst and Froesch, 1988; Slootweg et al., 1988; Martin and Waters, 1991; Nishida et al., 1992; Kassem et al., 1993). It is uncertain whether, in vivo,

* Corresponding author.

0303-7207/99/$ - see front matter © 1999 Published by Elsevier Science Ireland Ltd. All rights reserved.
PII: S0303-7207(98)00206-8

the latter actions are mediated directly or via IGF-I, and it is equally unclear whether the stimulatory effects of GH on osteoblast proliferation and activity are tightly linked or are dissociable. Although GH has been shown to modulate the expression of mRNAs of proteins that are involved in osteoblastic bone formation (Bikle et al., 1995; Monsonego et al., 1995; Turner, 1995), its mechanism of action on the gene expression of bone matrix proteins in vivo has received little attention. Recent observations that GH-receptor interaction leads to the activation of cytoplasmic signalling systems that are involved in the regulation of gene transcription (Carter-Su et al., 1996) have yet to be incorporated into the mechanism of the bone anabolic action of GH.

The exploration of the mechanism of action of GH is complicated by many factors including, the heterogeneity of the GH receptor, the presence of soluble receptor-related GH binding proteins in plasma, and the lack of suitable models for studying the actions of GH both in vitro and in vivo (Herington, 1994). In vitro actions of GH may not always faithfully reflect events occurring in vivo, and in vivo effects of GH administered long term to animals may not necessarily be mediated directly by GH. Therefore, the following studies were undertaken to examine the *acute* in vivo actions of GH on the expression of the mRNAs for the bone matrix proteins, type I collagen and osteocalcin, and to examine whether the effects of GH are mediated directly, through IGF-I, or through the expression of the early response oncogenes, c-fos, c-myc and c-jun.

2. Materials and methods

2.1. Animals:

Ninety-day-old female Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN) and used for the following studies when they were 95 days old. On arrival at our institution the rats were housed in a room maintained at 26°C on 14 h light, 10 h dark cycles. During the experimental period they were fed a Teklad diet (Madison, Wl) that contained 0.93% calcium, 0.65% phosphorus and 3.0 units of vitamin D/g and allowed free access to drinking water. rhGH (somatotropin) was kindly provided by Genetech, (San Francisco, CA) in a lyophilized powder form that had the following composition per vial: somatotropin (5.0 mg), mannitol (45.0 mg), glycine (1.7 mg), sodium phosphate (1.75 mg). The pH of the hormone solution was adjusted to 7.4 before it was freeze-dried. The hormone powder was reconstituted in sterile water just before use. To investigate the in vivo actions of GH on the gene expression of bone matrix proteins, three experiments were carried out. In experiment 1, rats were divided into eight groups of nine animals per group. Each rat received a single injection of rhGH (8 mg/kg b. wt.), subcutaneously, and the animals were sacrificed 15 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h later. In experiment 2, rats were divided into four groups of nine animals per group and the animals received similar injections of rhGH (8 mg/kg b. wt.) as in experiment 1 and were sacrificed 30 min, 1 h, 2 h and 4 h later. In experiment 3, rats were divided into nine groups of six animals per group and animals received similar injections of rhGH as in experiments 1 and 2 (8 mg/kg b. wt.) and were decapitated and blood collected 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h later. In all experiments, control animals were given solvent vehicle (with the same chemical composition as in the hormone powder) and sacrificed immediately 0 h). To avoid the complicating effects of circadian rhythms, rhGH was administered such that all animals were sacrificed at 11:00 h The short-term nature of these studies was designed to preclude the complicating effect of changes in cell numbers or composition that can result from long term GH therapy. The dose of rhGH used is based on an earlier report that doses in that range are required to elicit the skeletal effects of rhGH in rats (Jorgensen et al., 1995). All animals were treated in accordance with the guidelines of our Institutional Animal Care and Use Committee and the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Extraction of RNA from femoral cancellous bone

The procedure used for isolating RNA from cancellous bone is basically as we described previously (Salih et al., 1993). Briefly, each femur was removed quickly following sacrifice, cleaned of soft tissues and cut at the mid-diaphysis with a dental-drill fitted with a saw. Bone marrow was flushed out repeatedly from the distal half of the femur with ice-cold alpha minimum essential medium (a-MEM) (Sigma, St. Louis, MO) and the marrow discarded. The bone was then cut longitudinally with the saw and the interior of the bone flushed out again several times with ice-cold a-MEM to remove any remaining, removable marrow and the marrow discarded. Cancellous bone tissue which had been rid of most of its marrow was scraped from the secondary spongiosa about 1–2 mm from the growth plate and towards the mid-diaphysis. Pooled cancellous bone tissue was homogenized in a 4.0 M solution of guanidinium isothiocyanate and RNA was precipitated by ultra-centrifugation in a 5.7 M solution of cesium chloride (Chirgwin et al., 1979). The pelleted RNA was resuspended in a resuspension buffer (10 mM Tris, 10 mM EDTA and 0.5% SDS), ethanol precipitated and dissolved in DEPC-treated water and stored at −80°C

until needed for analysis. The integrity of isolated RNA was examined by spectrophotometric determination of the concentration ratios of its nucleic acid to protein ratios and by examining the intactness of the 28S and 18S rRNA bands following formaldehyde denatured agarose gel electrophoresis.

2.3. Probes:

Rat 363 bp of osteocalcin cDNA insert that contained the complete reading frame for rat osteocalcin was a gift from Dr Lydia C. Pan (Central Research Division, Pfizer, Groton, CT) (Pan and Price, 1985). We subcloned the cDNA into the expression vector, pSKII (Stratagene, La Jolla, CA). Antisense RNA was prepared from the latter and used as probe to examine cancellous bone RNA extracts for osteocalcin mRNA transcripts by Northern blot analysis. Riboprobes for type I collagen, IGF-I, c-fos, c-jun, c-myc and beta actin, were generated using the following plasmids:(a) pGEM3 containing the full-length cDNA for rat type-l alpha collagen, a gift from Dr Barbara Smith (Boston University, Boston, MA) (Fine et al., 1990); (b) pGEM 3-z containing the full length cDNA for IGF-I, a gift from Dr L. Murphy (University of Manitoba, Canada) (Murphy et al., 1987); (c) DNA templates for pTRI-c-fos/exon2-mouse, pTRI-jun A, pTRI-c-myc and pTRI-beta actin which were purchased from Ambion (Austin, TX). Plasmids containing the appropriate inserts were linearized and mRNA transcripts were generated using an in vitro transcription system from Promega (Madison, Wisconsin) according to the manufacturer's instructions.

2.4. Northern blot analysis

The mRNAs for osteocalcin, type I collagen, IGF-I and c-fos were determined by Northern blot analysis using standard procedures as we described previously (Sambrook et al., 1989; Salih et al., 1993). Briefly, 20 μg of RNA were fractionated on formaldehyde denatured agarose gel and transferred by capillary absorption to a pre-soaked nitrocellulose membrane filter as described by Thomas, (1980). An RNA ladder (BRL, Gaithersburg, MD) was prestained with ethidium bromide (1 mg/ml) and used as molecular weight markers. RNA on filter was fixed by UV irradiation for 1 min, baked at 80°C under vacuum for 2 h and stored under vacuum until required for analysis. Filters were prehybridized for 24 h at 60°C in a solution containing 50% formamide, 5 × SSP, 0.1% SDS, 2 × Denhardt's solution and 100 μg/ml yeast tRNA. Labelled riboprobes were added to the prehybridization solution and hybridization continued for 48 h. Following hybridization, the filters were washed extensively with 2 × SSPE containing 0.5% SDS at 68°C. They were then washed twice with a solution of 0.1 × SSPE containing 0.5% SDS at 68°C for 20 min per washing. The washed filters were incubated in Phospholmage cassettes (Sunnyvale, CA) in order to visualize radioactive mRNA riboprobe hybrids. mRNA hybrids were corrected for any differences in sample loading using 28S rRNA or β-actin as internal standards.

2.5. RNA protection assay

The mRNAs for c-jun, c-myc and the internal standard, β-actin were determined by RNA protection assay using standard procedures (Sambrook et al., 1989). Briefly, 20 μg of RNA were added to a premixed solution of ^{32}P-labelled antisense probes for c-jun, c-myc and β-actin (20 000 cpm/probe). For positive and negative controls, 50 μg of yeast tRNA were added. The mixture of RNAs and probes was pelleted, denatured at 95°C for 2 min and hybridized at 68°C for 10 min. Following hybridization, the mixture was treated with RNase A/T for 30 min at 37°C in order to digest unprotected single-stranded RNA fragments. Protected RNA fragments were recovered by ethanol precipitation and size fractionated by electrophoresis using a 5% urea-denatured polyacrylamide gel. ^{32}P-labelled RNA molecular size markers were prepared and run on outside lanes as described by the manufacturers (Ambion, Austin, TX). At the termination of electrophoresis, the polyacrylamide gel was dried (BioRad, Hercules' CA) and incubated in Phosphorimage cassettes in order to visualize the radioactive protected mRNA-probe fragments.

2.6. Serum IGF-I assay

Serum IGF-I was determined as in our previous studies (Kalu et al., 1994). Briefly, IGF-I was extracted from serum with the acid ethanol extraction procedure, and immunoreactive IGF-I was measured on diluted serum extracts with an RIA assay kit obtained from Nichols Institute (San Juan Capistrano, CA).

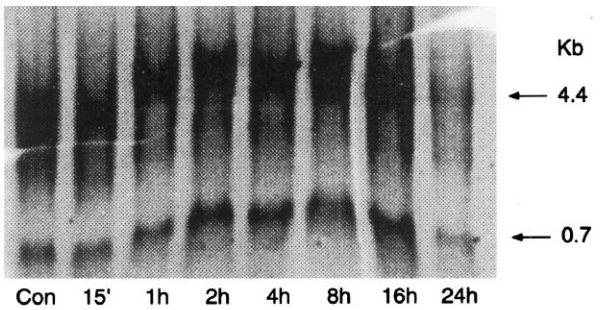
2.7. Statistics

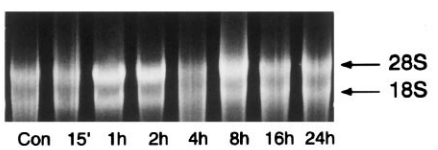
Data analysis involved estimation of means, standard errors and analysis of variance (ANOVA) (Fisher, 1935). ANOVA was performed using Statview Statistical package (Abacus Concepts, Berkeley, CA) on a Macintosh Ilsi computer. When the ANOVA indicated significant differences among means, the differences between the groups were evaluated by Fisher's protected least-significant difference multiple comparison procedure (Snedecor and Cochran, 1967). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Experiment 1.

Female Sprague–Dawley rats were given a single injection of rhGH (8 mg/kg b. wt.) and sacrificed 15 min, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h after the hormone was administered. Control animals received solvent vehicle and were killed immediately (0 h). RNA was isolated from cancellous bone from the secondary spongiosa of the femoral distal metaphysis and the mRNAs for osteocalcin, type I collagen and IGF-I determined.





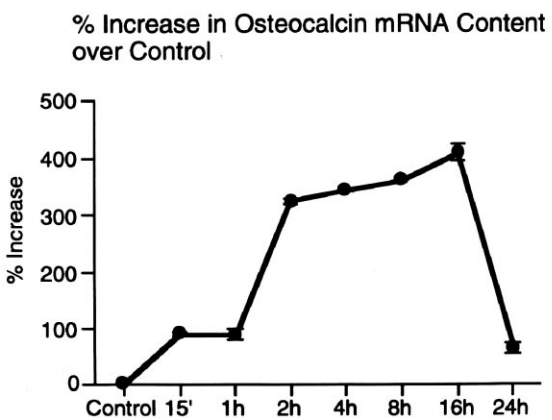
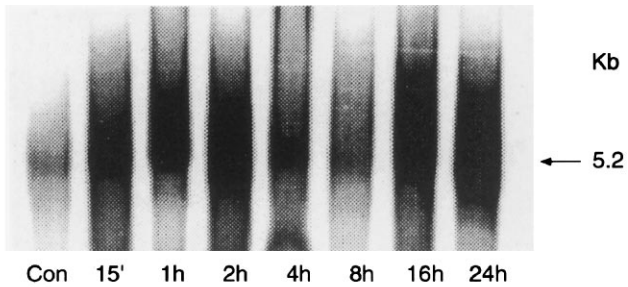
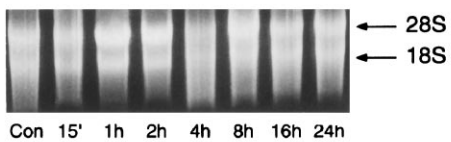


Fig. 1. Northern blot analysis of the effects of rhGH on the expression of osteocalcin mRNA in cancellous bone cells in the distal metaphysis of the femur of 3-month-old female Sprague–Dawley rats. Twenty micrograms of femoral RNA (experiment 1) were fractionated by denatured gel electrophoresis, transferred to a membrane filter and hybridized with a ^{32}P-labeled antisense riboprobe for osteocalcin. The top panel shows two bands with molecular sizes of 4.4 and 0.7 kb. The 0.7 kb osteocalcin mRNA transcript was quantified with the aid of a phospholmager using an Image Quant program (Molecular Dynamics, Sunnyvale, CA). Each time point (bottom panel) is a mean of data from three pools of samples and each pool consisted of femoral cancellous RNA isolated from three animals. Vertical bars are standard errors. The agarose gel (middle panel) was stained with ethidium bromide and the intensity of 28S rRNA was used to normalize for equal loading.





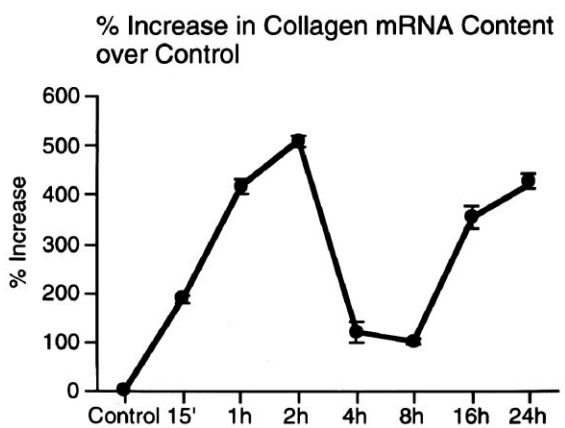


Fig. 2. Northern blot analysis of the effects of rhGH on the expression of type I collagen mRNA in cancellous bone cell in the distal metaphysis of the femur of 3-month-old female Sprague–Dawley rats. Twenty micrograms of femoral RNA (experiment 1) were fractionated by denatured gel electrophoresis, transferred to a membrane filter and hybridized with a ^{32}P-labeled antisense riboprobe for type I collagen. The probe recognized the expected 5.2 kb collagen mRNA transcripts (top panel) The intensity of the 5.2 kb band was quantified with the aid of a Phospholmager using an Image Quant program (Molecular Dynamics, Sunnyvale, CA). Each point (bottom panel) is a mean of data from three pools of samples and each pool consisted of RNA isolated from the right and left femurs of three animals. Vertical bars are SEs. The agarose gel (middle panel) was stained with ethidium bromide and the 28S rRNA was used to normalize for equal loading.

3.1.1. Osteocalcin mRNA

Osteocalcin antisense riboprobe recognized osteocalcin mRNA transcripts 4.4 and 0.7 kb in size (Fig. 1). rhGH caused a progressive and significant increase in the level of the 0.7 kb mRNA transcript by 89, 90, 325,

343, 361, 407% over the control level 15 min, 1 h, 2 h, 4 h, 8 h and 16 h, respectively, following the administration of the hormone, and by 24 h the level of the 0.7 kb mRNA transcript had declined to 66% (Fig. 1).

3.1.2. Type I collagen mRNA

Type I collagen antisense riboprobe recognized the expected 5.2 kb mRNA transcript for type I alpha collagen (Fig. 2). Growth hormone administration had a biphasic effect on the expression of type I collagen mRNA. The level of the mRNA increased by 187, 417, and 509% at 15 min, 1 h and 2 h, respectively above the control level, and then declined to 119 and 99% at 4.0 and 8 h, respectively. The mRNA level then rose again to 351 and 423% at 16 and 24 h, respectively, following the injection of rhGH.

3.1.3. IGF-I mRNA

^{32}P-labelled antisense riboprobe for IGF-I recognized both the large (7.5 and 4.7 kb) and the small (1.8 and 1.0 kb) isoforms of IGF-I mRNA transcripts (Thissen and Underwood, 1992) (Fig. 3). GH increased the levels of the IGF-I mRNA transcripts by 45, 83, 120, 140, and 175% above the control level 2, 4, 8, 16, and 24 h, respectively, following the administration of the hormone. Note that 2 h was the earliest time point at which IGF-I mRNA was analyzed in this study. Earlier samples were inadvertently missed. It was in part for this reason that the next experiment was performed to obtain additional information on the time course of the effects of rhGH on the expression of IGF-I mRNAs at earlier time periods.

3.2. Experiment 2.

Female Sprague–Dawley rats were given a single injection of rhGH (8mg/kg b. wt.) and sacrificed 30 min, 1 h, 2 h and 4 h after the hormone was administered. Control animals received solvent vehicle and were killed immediately (0 h). RNA was isolated from cancellous bone from the secondary spongiosa of the femoral distal metaphysis as in experiment 1, and the mRNAs for IGF-I, osteocalcin and the early response oncogenes, c-fos, c-myc and c-jun, determined.

3.2.1. Osteocalcin mRNA

^{32}P-labeled antisense riboprobe for osteocalcin hybridized intensely with the 0.7 kb osteocalcin transcript and faintly with the 2.7 and 4.4 kb transcripts (Fig. 4). Similar to the findings in experiment 1, rhGH caused a progressive increase in the level of the 0.7 kb osteocalcin mRNA transcript from 127% at 30 min to 177, 361, and 413% at 1, 2 and 4 h, respectively following the administration of the hormone. The non specific mRNA transcript in the 4.4 kb band was only faintly expressed. The reason is unclear but may relate to differences in the lots of animals used for the different experiments since variations in the number of bands hybridizable to osteocalcin RNA probes have been linked to variations in the lots of animals used in the studies (Rahman et al., 1993).

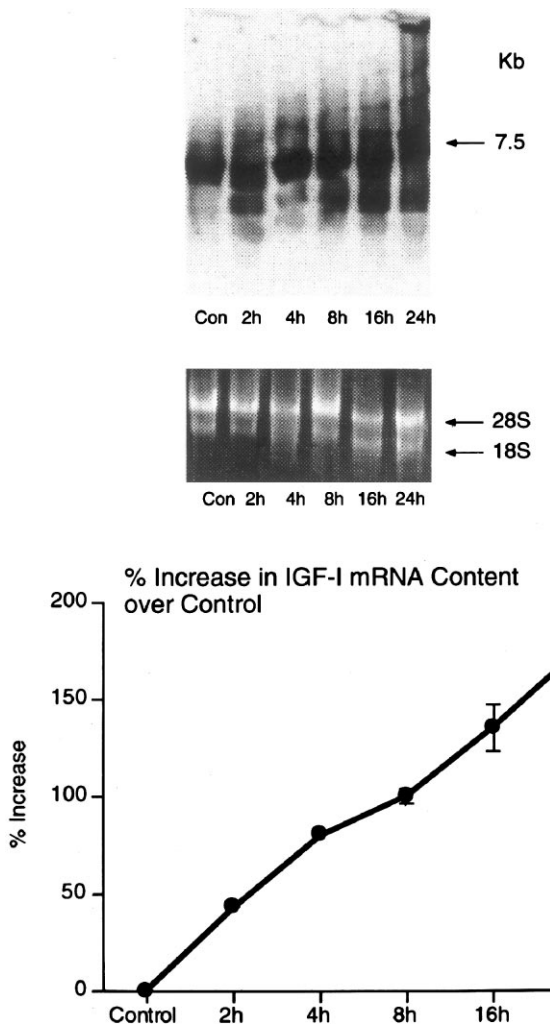


Fig. 3. Northern blot analysis of the effects of rhGH on the expression of IGF-I mRNA in cancellous bone cells in the distal metaphysis of the femur of 3-month-old female Sprague–Dawley rats. Twenty micrograms of femoral RNA (experiment 1) were fractionated by denatured gel electrophoresis, transferred to a membrane filter and hybridized with a ^{32}P-labeled antisense riboprobe for IGF-I. The probe recognized the low (1.8 kb) and high molecular weight (7.5 kb) mRNAs transcripts for IGF-I (top panel). The intensities of all bands were quantified with the aid of a Phospholmager using an Image Quant program (Molecular Dynamics, Sunnyvale, CA). Each point (bottom panel) is a mean of data from three pools and each pool consisted of RNA isolated from the right and left femurs of three animals. Vertical bars are SEs. The agarose gel (middle panel) was stained with ethidium bromide and 28S rRNA was used to normalize for equal loading.

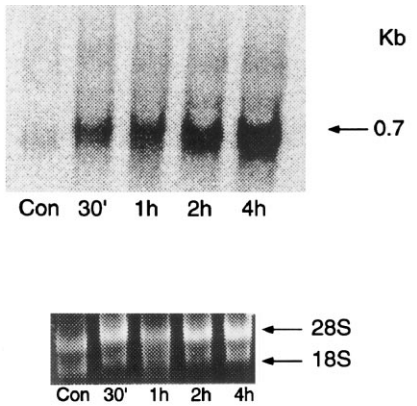


Fig. 4. Northern blot analysis of the effects of rhGH on the expression of osteocalcin MRNA in cancellous bone cells in the distal metaphysis of the femur of 3-month-old female Sprague–Dawley rats. Twenty micrograms of femoral RNA (experiment 2) were fractionated by denatured gel electrophoresis, transferred to a membrane filter and hybridized with a ^{32}P-labeled antisense riboprobe for osteocalcin. The top panel shows a band of hybridized mRNA transcript (0.7 kb) which was quantified with the aid of a phospholmager using an Image Quant program (Molecular Dynamics, Sunnyvale, CA). Each time point (bottom panel) is a mean of data from three pools of samples and each pool consisted of femoral cancellous RNA isolated from three animals. Vertical bars are standard errors. The agarose gel (middle panel) was stained with ethidium bromide and 28S rRNA was used to normalize for equal loading.

3.2.2. IGF-I mRNA

Similar to the findings in experiment 1, rhGH administration increased the expression of cancellous bone IGF-I mRNA. The increase was 38, 33, 87, and 437% at 30 min, 1 h, 2 h and 4 h, respectively above the control level, but did not reach statistical significance until 2 h following the injection of the hormone (Fig. 5).

3.2.3. mRNAs of the early response oncogenes

^{32}P-labeled antisense riboprobe for c-fos recognized the expected 2.2 kb mRNA transcript for c-fos in Northern analysis (Fig. 6). rhGH administration increased c-fos mRNA over 5-fold 30 min following the injection of the hormone, and the level of the mRNA declined towards the control level with time. c-jun and c-myc mRNAs were investigated with RNA protection assay. RNase digestion of preformed hybrids between bone RNA and ^{32}P-labeled antisense riboprobes for c-jun and c-myc gave protected fragments with the expected molecular sizes of 205 and 326 nucleotides, respectively (Fig. 7). The levels of these protected fragments for c-jun and c-myc did not increase over baseline levels until 4 h following rhGH administration.

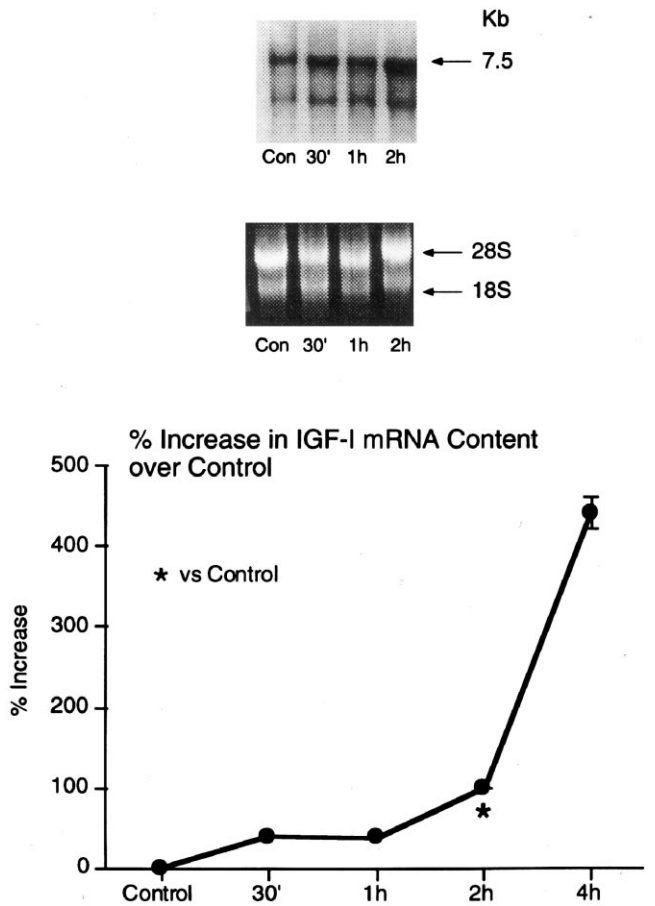


Fig. 5. Northern blot analysis of the effects of rhGH on the expression of IGF-I mRNA in cancellous bone cells from the distal metaphysis of the femur of 3-month-old female Sprague–Dawley rats. Twenty micrograms of femoral RNA (experiment 2) were fractionated by denatured gel electrophoresis, transferred to a membrane filter and hybridized with a ^{32}P-labeled antisense riboprobe for IGF-I. The probe recognized two transcripts (top panel), the predominant 7.5 kb transcript and a second 4.7 kb transcript. The intensities of the predominant 7.5 kb bands were quantified with the aid of a Phospholmager using an Image Quant program (Molecular Dynamics, Sunnyvale, CA). Each point (bottom panel) is a mean of data from three pools of samples and each pool consisted of RNA isolated from the right and left femurs of three animals. Vertical bars are SEs. The agarose gel (middle panel) was stained with ethidium bromide and 28S rRNA was used to normalize for equal loading.

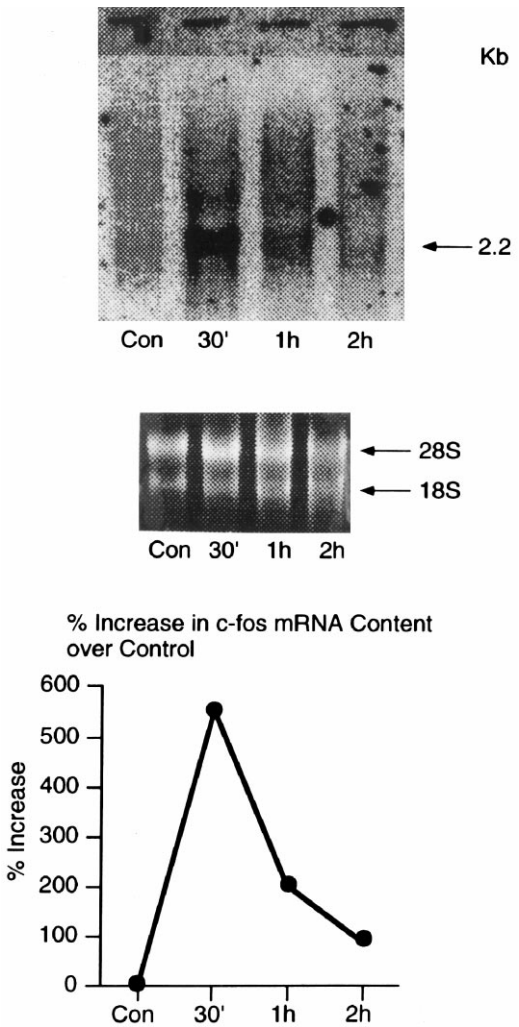


Fig. 6. Northern blot analysis of the effects of rhGH on the expression of c-fos mRNA in cancellous bone cells from the distal metaphysis of the femur of 3-month-old female Sprague–Dawley rats. Twenty micrograms of femoral RNA (experiment 2) were fractionated by denatured gel electrophoresis, transferred to a membrane filter and hybridized with a ^{32}P-labelled antisense riboprobe for c-fos. The probe recognized the expected 2.2 kb mRNA transcript for c-fos (top panel). The intensities of the 2.2 kb bands were quantified with the aid of a Phospholmager using an Image Quant program (Molecular Dynamics, Sunnyvale, CA). Each point (bottom panel) is a mean of data from three pools of samples and each pool consisted of RNA isolated from three animals. Vertical bars are SEs. The agarose gel (middle panel) was stained with ethidium bromide and 28S rRNA was used to normalize for equal loading.

3.2.4. Summary of the induction kinetics of bone mRNAs:

In Fig. 8 we compared the induction times of the various bone mRNAs. Growth hormone administration caused a rapid increase (15–30 min) in the induction of the mRNAs for osteocalcin and type I collagen. The mRNA for c-fos also increased significantly within 30 min, while the induction of the mRNAs for IGF-I and the rest of the early response oncogenes lagged behind those of the bone matrix proteins.

3.3. Experiment 3.

Female Sprague–Dawley rats were given a single injection of rhGH (8 mg/kg b. wt.), decapitated and bled 15 min. 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h after the hormone was administered. Control animals received solvent vehicle and were sacrificed immediately (0 h).

3.3.1. Serum IGF-I

Two to 4 h following rhGH administration serum IGF-I levels were decreased by about 10% (Fig. 9). Subsequently, serum IGF-I increased but the levels were significantly higher than that of controls only at 12 h following hormone administration ($P < 0.002$). Although the levels of serum IGF-I appear high for humans, these levels are normal for the rat as we and others have previously reported (Sontag et al., 1980; Kalu et al., 1994).

4. Discussion

Studies designed to examine the mechanism of action of GH have employed both short term in vitro and long term in vivo approaches (D'Ercode et al., 1984; Schlechter et al., 1986; Maor et al., 1989; Bichell et al., 1992; Izumi et al., 1995). Both approaches have shortcomings. In vitro effects of GH on transformed cell lines may not always faithfully reflect its in vivo actions on normal cells, and in vivo effects of long term administration of GH to animals may not necessarily be

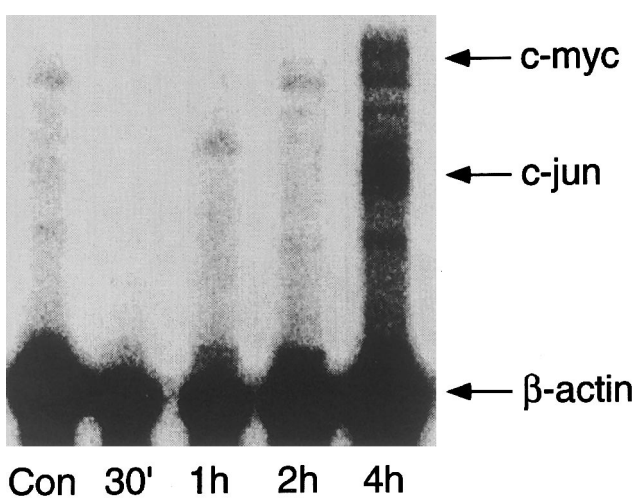


Fig. 7. Analysis of total RNA from cancellous bone of the distal metaphysis of the femur for mRNAs of c-myc and c-jun using RNA protection assays. Twenty micrograms of femoral RNA (experiment 2) were hybridized with ^{32}P-labelled probes for c-myc, c-jun, and β-actin, and fractionated by urea denatured polyacrylamide gel electrophoresis. The protected fragments for c-myc, c-jun and β-actin had the expected molecular sizes of 205, 316 and 125 nucleotides, respectively.

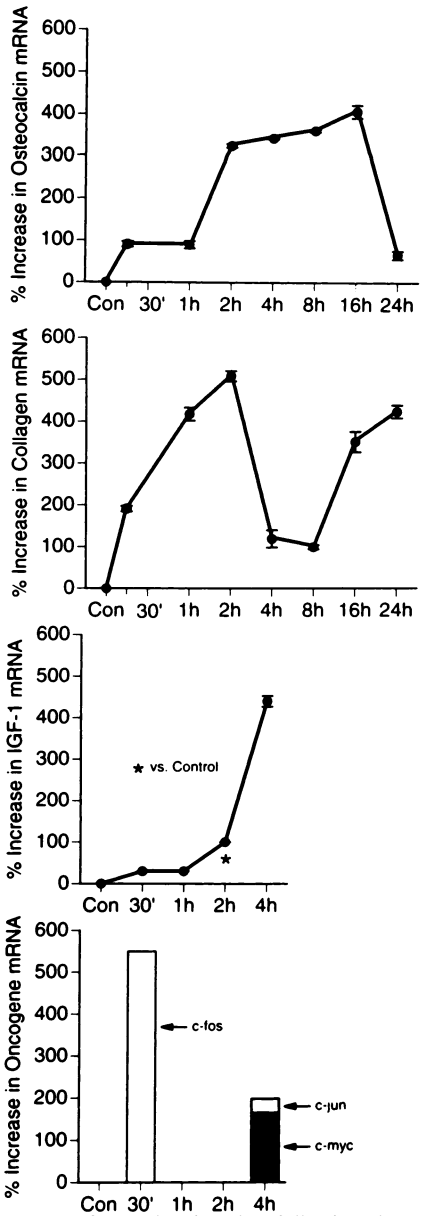


Fig. 8. A summary figure showing that following rhGH administration the increase in bone osteocalcin mRNA and bone type I collagen mRNA preceded the increase in the mRNAs for bone IGF-I and the early response oncogenes, c-fos, c-jun and c-myc. This is a composite of data from Figs. 2, 3 and 6–8.

mediated directly by the hormone. Furthermore, long term GH therapy may lead to changes in cell number and/or composition and complicate the interpretation of findings. In this study, we used an in vivo model that allowed us to examine in intact normal rats the kinetics of acute effects of rhGH on the expression of the mRNAs of bone matrix proteins and other proteins that have been implicated in the mediation of GH action. We specifically studied cancellous bone rather than whole bone because bone is a heterogeneous tissue and different bone envelopes may respond differently to various challenges (Kalu, 1991; Turner et al., 1992). The short term nature of our study precludes complications due to alterations in cell number or composition. However, although we rigorously washed out marrow from cancellous bone, we cannot completely rule out the possibility that our cancellous bone preparations contained small amounts of trapped marrow or stromal cells.

We previously reported that RNA extracted from femoral cancellous bone contains osteocalcin mRNA transcripts with molecular sizes of 4.4, 2.7 and 0.7 kb (Salih et al., 1993). Regardless of the type of osteocalcin cDNA probe we used for mRNA hybridization, the condition of hybridization, or the stringency of washing of filters employed, the three transcripts were always detected in RNA we isolated from cancellous bone (Salih et al., 1993). As in our previous report with osteocalcin cDNA probe (Salih et al., 1993), osteocalcin antisense riboprobe used in the current study recognized osteocalcin mRNA transcripts other than the 0.7 kb transcript. Since the latter transcript is reported to be the authentic osteocalcin transcript (Pan and Price, 1985), our current investigation of the mechanism of the effect of rhGH on bone osteocalcin was restricted to the evaluation of the 0.7 kb mRNA transcript using antisense osteocalcin riboprobe.

The molecular mechanism by which GH mediates its bone anabolic actions is uncertain. At least three modes of action have been proposed. First, GH stimulates the production of IGF-I which in turn mediates the action of GH on bone either by acting as a classical hormone through the circulation or by acting in a paracrine/autocrine fashion (Daughday, 1981; Izumi et al., 1995). In this study rhGH caused a significant increase in cancellous bone IGF-I mRNA 2 h following the administration of the hormone. In contrast, the induction of the mRNAs for the bone matrix proteins, osteocalcin and type I collagen, occurred within 15–30 min and preceded the induction of bone IGF-I mRNA. Therefore, it is unlikely that an increase in bone IGF-I is responsi-

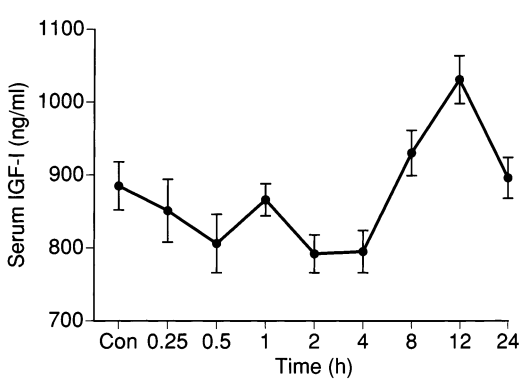


Fig. 9. Effects of rhGH administration on serum IGF-I levels in intact female Sprague–Dawley rats. Each point is a mean of data from six animals. Vertical bars are SEs. $P < 0.05$, con versus 2 h, $P < 0.01$, con versus 12 h.

ble for GH induced increase in the mRNAs for bone matrix proteins which occurred earlier. Plasma IGF-I is not likely to be the mediator of the early (15–30 min) action of rhGH on the expression of the mRNAs for bone matrix proteins. Our view is supported by two previous studies in which the level of serum IGF-I did not increase significantly until 3–12 h following GH administration to rats (Schlechter et al., 1986; Murphy et al., 1987). Similarly, in our current study the level of serum IGF-I did not increase significantly until 12 h following GH administration to rats. Furthermore, in rats IGF-I mRNA in liver, which is the main source of circulating IGF-I, did not increase significantly until 2 h following rhGH administration (D'Ercode et al., 1984). Therefore, it appears that rhGH stimulates, at least, the early expression of mRNAs for bone matrix proteins by a mechanism that appears to be independent of its action on the expression of IGF-I. In situ hybridization studies are required to confirm our conclusions.

An alternative mechanism by which GH may mediate its actions is through the expression of the early response oncogenes, c-fos, c-jun and c-myc (Muller, 1986; Murphy et al., 1987; Gurland et al., 1990; Rotwein et al., 1994). The products of these early response oncogenes were shown to regulate the transcription of a variety of genes by acting either as individual proteins or complexed with each other (Spiegelman et al., 1988; Slootweg et al., 1991; Sumantran et al., 1992). It has been proposed that the induction of these early response oncogenes is a component of the mechanism by which GH mediates some of its actions (Berczi et al., 1987; Murphy et al., 1987). However, in our study, it is unlikely that the early (15–30 min) stimulatory effect of rhGH on the expression of the bone matrix protein mRNAs studied is mediated through oncoproteins for at least two reasons. First, rhGH administration caused a rapid increase (15–30 min) in the induction of the mRNAs for osteocalcin and type I collagen. Second, although c-fos mRNA increased significantly within 30 min, the induction of the mRNAs for the rest of the early response oncogenes studied lagged well behind those of the bone matrix proteins.

Having excluded the possibility that the early increase in bone matrix mRNAs for osteocalcin and type I collagen following rhGH administration is secondary to increased expression of IGF-I and the early response oncogenes, c-fos, c-jun and c-myc, a third possibility is that the effect of rhGH is mediated directly. Support for the latter includes the rapid induction of the mRNAs for the bone matrix proteins, and several previous reports that GH acts directly to regulate the transcription of genes such as those for c-fos, c-jun (Muller, 1986; Gurland et al., 1990; Slootweg et al., 1991), the serine protease inhibitor, Spi 2.1 (Yoon et al., 1987), the cytochrome P450 enzyme, 2CI2 (Tollet et al., 1990) and IGF-I (Roberts et al., 1986; Xu et al., 1995). Although the mechanism through which GH may mediate its direct actions is still debatable, there is sufficient evidence to suggest three possible mechanisms. The first is that GH may mediate its effects directly by binding to a putative GH response element (Billestrup et al., 1992). Second, GH may act directly by activating nuclear transcription factors such as STAT 1, 3 and 5 (Gaisgaard et al., 1996; Silva et al., 1996; Smit et al., 1996). Third, GH may mediate its direct actions by binding to some nuclear GH binding proteins to form a complex that can activate gene transcription directly (Lobie et al., 1991, 1992). It is clear that additional studies are required to validate our conclusion that GH may act directly to stimulate the expression of the mRNAs for the bone matrix proteins, osteocalcin and type I collagen.

Finally, our observation that rhGH increased the expression of type I collagen mRNA in a biphasic fashion is noteworthy. Growth hormone increased skeletal type I collagen mRNA within 15 min of its administration; the increase peaked at 2 h and fell to a nadir at 4–8 h; a second increase was observed at 16 h and the increase persisted at 24 h. Although the reason for the biphasic effect is presently unclear, a plausible explanation is that rhGH acted directly to stimulate the initial increase in type I collagen mRNA, while the second increase was due to the paracrine/autocrine action of IGF-I whose bone mRNA increased progressively over 24 h following rhGH administration. Support for this interpretation is that the second peak of type I collagen mRNA was preceded, for many hours, by an increase in bone mRNA for IGF-I which has been reported to increase the expression of type I collagen mRNA in bone (S57). Whether rhGH-mediated increase in circulating IGF-I or in skeletal oncoproteins contributed to the second peak of type I collagen mRNA is unknown. We recognize that the steady state levels of mRNAs were measured in this study and that caution should be exercised in extrapolating from steady state mRNA levels to effects on gene transcription. Furthermore, GH may also act to stimulate the transition of precursor cells to mature osteoblasts. These uncertainties underline the need for additional studies on the in vivo mode of action of GH on bone.

In conclusion, we have demonstrated that a single administration of rhGH to rats increased the level of the mRNAs for the bone matrix proteins, osteocalcin and type I collagen within minutes. This early stimulatory effect of rhGH on bone matrix protein mRNAs appears to be mediated directly rather than through IGF-I or the early response oncogenes, c-fos, c-jun and c-myc. Since rhGH also caused a later increase in bone IGF-I mRNA and augments plasma IGF-I concentration, rhGH administration may, in addition, have a

later bone anabolic action that is secondary to its stimulation of IGF-I synthesis. Because of this presumed dual action, the use of GH therapy to stimulate bone formation in osteopenic states is likely to be superior to the use of IGF-I as a surrogate for growth hormone.

Acknowledgements

This study was supported in part by a University Grant Program for Osteoporosis from Procter and Gamble Pharmaceuticals and by grants from the NIH, AG13309 and K07AGO046906. Recombinant human growth hormone was generously supplied by Genetech, South San Francisco, CA.

References

Andreassen, T.T., Jorgensen, P.H., Flyvbjerg, O.H., Oxiund, H., 1995. Growth hormone stimulates bone formation and strength of cortical bone in aged rats. J. Bone Miner. Res. 10, 1057–1067.

Berczi, I., Nagy, E., Toledo, E., Muatusik, S.M., Friesen, H.G., 1987. Pituitary hormones regulate c-myc and DNA synthesis in Iymphoid tissue. J. Immun. 146, 2201–2206.

Bichell, D.P., Kikuchi, K., Rotwein, P., 1992. Growth hormone rapidly activates insulin-like growth factor I gene transcription in vivo. Mol. Endocrinol. 6, 1899–1908.

Bikle, D.D., Harris, J., Halloran, B.P., Currier, P.A., Tanner, S., MoreyHolton, E., 1995. The molecular response of bone to growth hormone during skeletal uploading: regional differences. Endocrinology 136, 2099–2109.

Billestrup, N., Montimony, M., Serup, P., Nielson, J.H., 1992. Stimulation of somatostatin and insulin gene transcription by GH, Proc. Ninth Int. Congr. Endocrinol. (Nice), Abstract P0801005, p. 280.

Carter-Su, C., Schwartz, J., Smit, L.S., 1996. Molecular mechanism of growth hormone action. Annul Rev. Physiol. 58, 187–207.

Chirgwin, J., Pryzbyla, A., MacDonald, R., Rutter, W., 1979. Isolation of biologically active ribonucleic acid from sources enriched for ribonuclease. Biochemistry 18, 5294.

Daughday, W.H., 1981. Growth hormone and the somatomedins. In: Daughday, W.H. (Ed.), Elsevier, New York, pp. 1–38.

D'Ercode, A.J., Stiles, A.D., Underwood, L.E., 1984. Tissue concentrations of somatomedin C: Further evidence of multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proc. Nat. Acad. Sci. USA 81, 935–939.

Ernst, E., Froesch, E.R., 1988. Growth hormone-dependent stimulation of osteoblast-like cells in serum-free cultures via local synthesis of insulin-like growth factor 1. Bioch. Biophys. Res. Commun. 151, 142–147.

Fine, A., Poliks, C.F., Smith, B.D., Goldstein, R.H., 1990. The accumulation of type I collagen mRNAs in human embryonic lung fibroblasts stimulated by transforming growth factor beta. Connect. Tissue Res. 24, 237–247.

Fisher, F.A., 1935. The design of experiments. Oliver and Boyd, Edinburgh.

Gaisgaard, E.D., Gouilleux, F., Groner, B., Serup, P., Nielson, J.H., Billestrup, N., 1996. Identification of a growth hormone-responsive STAT5 binding element in the rat insulin 1 gene. Mol. Endocrinol. 10, 652–659.

Gurland, G., Ashcom, G., Cochran, B.H., Schwartz, J., 1990. Rapid events in growth hormone, induction of c-fos and cjun transcription in 3T3-F442 preadipocytes. Endocrinology 127, 3187–3195.

Herington, A.C., 1994. New frontiers in the molecular mechanisms of growth hormone action. Mol. Cell. Endocrinol. 100, 39–44.

Hochberg, Z., Maor, G., Lewinson, D., Silbermann, M., 1989. The direct effects of growth hormone on chondrogenesis and osteogenesis. In: Heap, R.B., Prosser, C.G., Lemming, G.E. (Eds.), Biotechnology in growth regulation. Butterworth, London, Boston, Singapore, Sydney, Toronto, Wellington, pp. 123–187.

Isaksson, O.G.P., Jasson, J.0., Guase, J.A.M., 1982. Growth hormone stimulates longitudinal bone growth directly. Science 216, 1237–1239.

Isaksson, O.G.P., Lindahl, A., Nilsson, A., Isgaard, J. 1987. Cellular mechanisms for the stimulatory effects of growth hormone on longitudinal bone growth. In: Isaksson, O., Binder, C., Hall, K., Hokfelt, B. (Eds.), Excerpta Medica, Amesterdam, pp. 307–319.

Izumi, T., Shida, J., Jingushi, S., Hotokebuchi, T., Sugioka, Y., 1995. Administration of growth hormone modulate the gene expression of basic fibroblast growth factor in rat coastal cartilage, both in vivo and in vitro. Mol. Cell. Endocrinol. 112, 95–99.

Jorgensen, P.H., Bang, C., Andreassen, T.T., Flyvbjerg, A., Orskov, H., 1995. Dose response study of the effects of growth hormone and mechanical propertiesofskingraftwounds. J. Surg. Res. 58, 295–301.

Kalu, D.N., Arjmandi, B.H., Birnbaum, R.S., Liu, C.C., Salih, M.A., 1994. Effects of ovariectomy and estrogen on serum levels of insulin-like growth factorI and insulin-like growth factor binding protein 3. Bone Miner. 25, 135–148.

Kalu, D.N., 1991. The ovariectomized rat model of postmenopausal bone loss. Bone Miner. 15, 175–192.

Kassem, M., Blum, W., Ristelli, J., Mosekilde, L., Eriksen, E.F., 1993. Growth hormone stimulates proliferation and differentiation of normal human osteoblast-like cells in vitro. Calcif. Tissue Int. 52, 222–226.

Lobie, P.R., Barnard, R., Walters, M.J., 1991. The nuclear growth hormone receptor birding protein. J. Biol. Chem. 266, 226–245.

Lobie, P.R., Garcia-Aragon, J., Wang, B.S., Baumbach, W.R., Walters, M.J., 1992. Cellular localization of the growth hormone binding protein in the rat. Endocrinology 139, 3057–3065.

Maor, G., Hochberg, Z., Von der Mark, Heibegard, D., Silbermann, M., 1989. Human growth hormone enhances chodrogenesis and osteogenesis in a tissue culture system of chondroprogenitor cells. Endocrinology 125, 1239–1245.

Bernard, R., NG, K.W., Martin, T.J., Waters, M.J. 1991. Growth hormone receptors in clonal osteoblast-like cells mediate mitogenic response to GH. Endocrinology 128, 1459–1464.

Monsonego, E., Halevy, O., Gertler, A., Hurwitz, S., Pines, M., 1995. Growth hormone inhibits differentiation of avian epiphyseal-plate chondrocytes. Mol. Cell. Endocrinol 114, 35–42.

Muller, R., 1986. Cellular and viral fos gens: structure, regulation of expression and biological properties of their encoded products. Biochem. Biophys. Acta 823, 207–225.

Murphy, L.J., Greame, B.l., Friesen, H.G., 1987. Growth hormone stimulates sequential induction of c-myc and insulin-like growth factor I expression in vivo. Endocrinology 120, 1806–1812.

Nishida, S., Yamaguchi, A., Takahashi, H.E., Yoshiki, S., Suda, T., 1992. Properties of osteoprogenitor cells in bone marrow of aged and ovariectomized rats. J. Bone Miner. Res. 17, 13.

Pan, L.C., Price, P.A., 1985. The propeptide of rat bone gammacarboxyglutamic acid protein shares homology with other vitamin kdependent protein precursors. Proc. Nat. Acad. Sci. 82, 6109–6133.

Rahman, S., Oberdorf, A., Montecino, M., Tanhauser, S.M., Lian, J.B., Stein, G.S., Stein, J.L., 1993. Multiple copies of the bone-specific osteocalcin gene in mouse and rat. Endocrinology 133, 3050–3053.

Roberts, Jr, C.T., Brown, A.L., Graham, D.E., Seelig, S., Berry, S., Gabbay, K.H., Rechier, M.M., 1986. Growth hormone regulates the abundance of insulinlike growth factor I RNA in adult liver. J. Biol. Chem. 261, 10025–10028.

Rotwein, P., Gronowski, A.M., Thomas, M.J., 1994. Rapidnuclearactionsof growth hormone. Horm. Res. 42, 170–175.

Salih, M.A., Liu, C.C., Arjmandi, B.H., Kalu, D.N., 1993. Estrogenmodulates the mRNA levels for cancellous bone proteins of ovariectomized rats. Bone Miner. 23, 285–299.

Salmon, W.D., Daughaday, W.H., 1957. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J. Lab. Clin. Med. 49, 825–836.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 9.16–9.23.

Schlechter, N.I., Russel, S..M, Spencer, E.M., Nicoll, C.S., 1986. Evidence suggesting that the direct growth-promoting effect of growth hormone on cartilage in vivo is mediated by local production of somatomedin. Proc. Nat. Acad. Sci. USA 83, 7932–7934.

Silva, C.M., Lu, H., Day, R.N., 1996. Characterization and cloning of STAT 5 from IM-9 cells and its activation by growth hormone. Mol. Endocrinol. 10, 508–518.

Slootweg, M.C., Van Buul-Offers, S.C., Herman-Erlee, M.P.N., Van der Meer, J.M., Duursma, S.A., 1988. Growth hormone is mitogenic for fetal mouse osteoblasts but not for undifferentiated bone cells. J. Endocrinol. 116, RI1-RI3.

Slootweg, M.C., DeGroot, R.P., Hermann-Erlee, M.P.M., Koornneef, I., Kruijer, W., Kramer, Y.M., 1991. Growth hormone induces expression of cjun and jun B oncogens and employs a protein kinase C Signal transduction pathway for the induction of c-fos oncogene expression. Mol. Endocrinol. 6, 179–188.

Smit, L.S., Meyer, D.J., Norstedt, G., Schwartz, J., Carter-Su, C., 1996. The role of the growth hormone (GH) receptor and JAK1 and JAK2 kinases in the activation of STATS 1, 3 and 5 by GH. Mol. Endocrinol. 10, 519–533.

Snedecor, G.W., Cochran, W.G., 1967. Statistical Methods. Iowa State University Press, Ames, Iowa.

Sontag, W.E., Stegger, R.W., Forman, L.J., Meites, J., 1980. Decreased pulsatile release of growth hormone in old male rats. Endocrinology 107, 1875–1879.

Sontag, W.E., 1986. Quantitative measurements of periosteal and corticalendosteal bone formation and resorption in the mid-shaft of male rat femur. Bone 7, 63–70.

Spiegelman, B., Distel, R.J., Ro, H.S., Rosen, B.S., Satterberg, B., 1988. cfos protooncogene and the regulation of gene expression in adipocyte differentiation. J. Cell. Biol. 107, 829–832.

Stracke, H., Schultz, A., Maciler, D., Rossol, S., Schatz, H., 1984. Effect of growth hormone on osteoblasts and demonstration of somatomedin-C/IGF-I in bone organ culture. Act. Endocrinol. 107, 16–24.

Sumantran, V.N., Tsai, M.L., Schwartz, J., 1992. Growth hormone induces c-fos and cjun expression in cells with varying requirements for differentiation. Endocrinology 130, 2016–2024.

Thissen, J.P., Underwood, L.E., 1992. Translational status of the insulinlike growth factor-l mRNAs in liver of protein-restricted rats. J. Endocrinol. 132, 141–147.

Thomas, P., 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Nat. Acad. Sci. USA 77, 5201–5203.

Tollet, P., Enberg, B., Mode, A., 1990. Growth hormone (GH) regulation of cytochrome P-450 IIC12, insulin-like growth factor-1 and GH receptor messenger RNA expression in primary rat hepatocytes: A hormonal interplay with insulin, IGF-I and thyroid hormone. Mol. Endocrinol. 4, 1934–1942.

Turner, R.T., Kapeiner, S.N., Spelsperg, T.C., 1992. Tissue-specific expression of bone proteins in femora of growing rats. Am. J. Physiol. 263, E724–E729.

Turner, R.T., 1995. Effects of short-term spaceflight and recombinant growth hormone on bone growth in young rats. Aviation Space Environ. Med. 66, 763–769.

Xu, Y.P., Chedrese, J.P., Thacker, A.P., 1995. Growth hormone amplifies insulin-like growth factor I induced progesterone accumulation and P450scc mRNA expression. Mol. Cell. Endocrinol. 111, 199–206.

Yoon, J.B., Towle, H.C., Seelig, S., 1987. Growth hormone induces two MRNA species of the serine protease inhibitor gene family in rat liver. J. Biol. Chem. 262, 4284–4289.