GH gene expression in the submaxillary gland in normal and Ames dwarf mice

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Abstract

High local GH-releasing hormone (GHRH) levels are capable of inducing transdifferentiation in salivary cells to synthesize GH. However, the factors implicated in this process remain unknown. To study this subject, normal and Ames dwarf mice were implanted in the submaxillary gland with a slow release pellet releasing 21 µg GHRH (1–29)-NH₂/day for 2 months. Control animals received placebo pellets at the same site. After 60 days, heart blood was collected and submaxillary glands were removed. Circulating levels of GH and IGF-I were significantly decreased ($P < 0.05$) in dwarf mice in comparison with controls, and GHRH treatment did not modify either of these two parameters. Controls carrying GHRH pellets showed a significantly higher GH content ($P < 0.05$) in the submaxillary gland than the placebo-treated normal mice. There were no differences between the IGF-I concentrations of placebo- and GHRH-treated salivary tissue

from normal mice. Analysis of GH mRNA by RT-PCR followed by Southern blot revealed that GH transcripts were present in the salivary gland samples carrying the placebo pellets in both normal and dwarf mice. The expression of GH was significantly ($P < 0.05$) increased by the GHRH pellets in salivary tissue from normal mice, but not in submaxillary glands from dwarf mice. Pit-1 mRNA was not detected in the GHRH-treated glands of normal and dwarf mice by RT-PCR or by Southern blot. Using these highly sensitive methods, we have been able to detect the transcription of both GH and Pit-1 in pituitaries from Pit-1-deficient Ames dwarf mice. The present experiment demonstrates that salivary tissue synthesizes GH when it is exposed to the influence of GHRH. Both basal and GHRH-induced salivary GH expression appear to be independent of Pit-1.

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Introduction

In previous studies from our group, we have reported that adult rat salivary tissue is capable of synthesizing certain pituitary hormones when stimulated by hypothalamic factors both *in vivo* and *in vitro* (Tresguerres *et al.* 1999a,b). This effect seems to be due to a transdifferentiation process of salivary tissue cells.

Rat parotid gland cells in culture, submitted to hypothalamic extracts or synthetic hypothalamic hormones, were able to synthesize and secrete some pituitary hormones including luteinizing hormone (LH), prolactin, and growth hormone (GH) (Tresguerres *et al.* 1999a, Fernandez *et al.* 1994). In these experiments, the effect was less marked when synthetic factors were used, probably indicating the existence of unknown enhancers in the crude hypothalamic extract. The capability of adult rat salivary cells to produce pituitary hormones was confirmed by *in vivo* studies, in which rat submaxillary glands were also able to synthesize GH after local GH-releasing

hormone (GHRH) stimulation (Tresguerres *et al.* 1999b). However, the factors implicated in the GH-induction process and the characteristics of this salivary GH remain unknown.

GHRH-induced GH synthesis in the pituitary has been found to be mediated by the transcription factor Pit-1, through the activation of the GH gene (Karin *et al.* 1990, Ruvkun 1992). Pit-1 also activates the genes of prolactin and of the β -subunit of thyrotrophin (Fox *et al.* 1990, Steinfeld *et al.* 1991), and controls the differentiation of somatotropes, lactotropes and thyrotropes during pituitary development (Andersen & Rosenfeld 1994). Ames dwarf mice are an experimental model of pituitary dwarfism caused by an autosomal recessive mutation that produces a dramatic deficiency in Pit-1 (Sornson *et al.* 1996). As a consequence of this, these animals are GH and prolactin deficient, exhibit a hypothyroid condition and lack the pituitary cell types responsible for production of these hormones (Bartke 1964, Slabaugh *et al.* 1981).

The present study was undertaken to evaluate the implication of Pit-1 in the locally GHRH-induced transdifferentiation process in submaxillary glands of adult normal and Ames dwarf mice. In addition, salivary GH mRNA has also been analyzed by both Southern blot hybridization and restriction endonuclease digestion.

Materials and Methods

Animals and accommodation

Female Ames dwarf mice (*df/df*) of 1.5–2 months of age and their normal siblings (*DF/–*) were used in this experiment. The mice were born and raised in the breeding colony at Southern Illinois University at Carbondale, Carbondale, IL, USA. The animals were housed four to five per cage in a room with controlled light (12 h light:12 h darkness cycle, lights on at 0630 h), temperature ($22 \pm 1^\circ\text{C}$) and humidity (40%). Mice were given free access to a nutritionally balanced diet (Tekland; Harlan Sprague–Dawley Inc., Madison, WI, USA) and tap water.

Experimental designs

The studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. The protocol for animal use was also approved by the committee of Southern Illinois University at Carbondale with the reference number 95–034.

Thirty-four mice were implanted with a slow release pellet (Innovative Research of America, Toledo, OH, USA) in the right submaxillary gland under light anesthesia, and divided into the following four groups. One group of controls ($n=7$) and another group of dwarf ($n=10$) mice received a pellet loaded with 1.25 mg GHRH (1–29)-NH₂ (Geref; Serono, Madrid, Spain) allowing a continuous peptide release of 21 µg/day for 60 days. Two further groups of normal mice ($n=7$) and dwarf mice ($n=10$) received a placebo pellet.

After 60 days, the submaxillary glands from normal mice bearing the pellet were dissected under ether anesthesia. Glands were weighed and halved: one half was immediately frozen in liquid nitrogen and kept frozen at -80°C until analyzed by molecular biology techniques, and the other half was kept at -20°C for hormone content measurements using radioimmunoassay (RIA). Because of the small size of the submaxillary gland in Ames dwarf mice we decided to use all the tissue treated with GHRH or placebo pellets for molecular biology studies, since the analysis of salivary GH gene expression was the main objective of the present experiment. Blood was drawn from the heart, centrifuged and the corresponding plasma stored at -20°C for GH and insulin-like growth factor-I (IGF-I) determination using specific RIA methods. The mice were killed by decapitation and their

pituitary glands carefully removed and also kept frozen at -80°C until analyzed.

Hormonal and protein determinations

GH, IGF-I, GHRH and protein contents were measured in homogenates of submaxillary glands. GH and IGF-I were also determined in plasma samples.

The homogenates were obtained by sonication (Branson Sonifier 450) in 0.01 M phosphate sodium buffer, pH 3.5. One half of this extract was centrifuged for 15 min at 1500 g at 4°C and used to measure GH, IGF-I and total protein contents, and the other half was boiled for 5 min, centrifuged and the supernatants lyophilized and stored frozen until analyzed for GHRH.

Mouse GH determinations were carried out using an RIA method adapted from that reported by Lima *et al.* (1993) for rat GH. Mouse GH-AFP 10783-B, generously provided by Dr A F Parlow (Harbor-University of California, Torrance, CA, USA), was used as a reference preparation and also for iodination using the lacto-peroxidase method (Thorell & Johansson 1971). The antibody used was anti-rGH-S5 supplied by the National Hormone and Pituitary Program (NIDDK, Baltimore, MD, USA). The sensitivity of the standard curve was 5 pg/tube. All samples were included in the same assay, and the intra-assay coefficient of variation was 4.9%.

Plasma IGF-I levels and IGF-I concentrations in the homogenates of the submaxillary glands were determined by a double-antibody RIA using a non-equilibrium technique (Daughaday *et al.* 1980). IGF-binding proteins (IGFBPs) in both types of samples were separated by acid-ethanol extraction before the assay, according to the method of Daughaday *et al.* (1980). Human biosynthetic recombinant IGF-I lot A52-EPD-182 provided by Eli Lilly Co. (Indianapolis, IN, USA) was used as the standard and also for iodination, using in this case the chloramine T method as previously described (Daughaday *et al.* 1980, Chatelain *et al.* 1983). Polyclonal rabbit anti-IGF-I antibody UB3-T89 was also supplied by NIDDK. The minimum amount of hormone detectable in the assay was 2 pg/tube and the inter- and intra-assay coefficients of variation were 12.9 and 7.8% respectively.

The validation of both the IGF-I assay and the IGFBP extraction procedure in plasma samples has already been reported by our group (Rol de Lama *et al.* 1998). Similarly, a further validation procedure was performed with the homogenates of the submaxillary glands in order to confirm that the remaining IGFBPs did not interfere with the assay, according to the 'Recommendations from the 3rd International Symposium on Insulin-like Growth Factors' (Bang *et al.* 1995). The correlation between the IGF-I reference curve and the dilution series of extracted homogenates was assessed, observing a high parallelism between them. Secondly, high recovery of labelled IGF-I was also demonstrated (85%) when the radioactive

preparation was preincubated with several samples before acid-ethanol extraction. Separation of extracted homogenates with dextran-coated charcoal showed that they were 99.3% free of IGFs compared with 90% in the unextracted homogenates.

GHRH content in submaxillary homogenates was assayed by a specific RIA, using GHRH (1–29)-NH₂ as the standard and iodination by the lactoperoxidase method. Antibody anti-GHRH was prepared in our laboratory by immunizing rabbits with GHRH (1–29)-NH₂ coupled with hemocyanin with carbodiimide and mixed with Freund's complete adjuvant (Difco Labs, Detroit, MI, USA) as previously described for other peptides (Goodfriend *et al.* 1964). GHRH antibody did not show a cross-reaction with glucagon, parathyroid hormone, corticotrophin-releasing hormone, somatostatin, LH-releasing hormone or gastrin, and was used at a final dilution of 1:42 000. The assay was performed following the protocol used by our group for measurement of the same peptide in the hypothalamus (Cardinali *et al.* 1994). The level of detection of the GHRH assay was 5 pg/tube with inter- and intra-assay coefficients of variation of 14.4 and 12.1%.

Total protein content in submaxillary homogenates was determined by the brilliant blue method (Bradford 1976). Coomassie brilliant blue G-250 (Sigma, St Louis, MO, USA) dissolved in ethanol and phosphoric acid was used as a colouring reagent and the reference curve was prepared with bovine serum albumin (BSA) (Sigma). The sensitivity of this assay was 2 µg/tube and the inter- and intra-assay coefficients of variation were 5.05 and 1.84%.

RNA isolation and cDNA synthesis by reverse transcription

Total RNA was extracted from a portion of a mouse submaxillary gland kept at –80 °C by a single step acid guanidium thiocyanate-phenol-chloroform method as described elsewhere (Chomczynski & Sacchi 1987). The integrity of each RNA sample was examined by gel electrophoresis, using a 2% agarose gel with ethidium-bromide staining. The quantity of total RNA was assessed by spectrophotometric absorbance at 260 nm (Shimadzu UV-1201).

In addition, RNA was isolated from pituitary glands of normal and Ames dwarf mice. Pituitaries from normal mice were used as a positive control for the amplified product.

Three micrograms of total RNA were utilized for the synthesis of cDNA by reverse transcription (RT), using 18 IU avian myeloblastosis virus reverse transcriptase (Promega Corporation, Madison, WI, USA), 1.5 µM oligo(deoxythymidine)_{1–15} primer (Life Technologies Ltd, Paisley, Strathclyde, UK), 50 mM KCl, 10 mM MgCl₂, 50 mM Tris-HCl pH 8.3, 10 mM dithiothreitol, 0.2 mM of each deoxy-NTP, and 50 IU ribonuclease inhibitor (Recombinant RNasin; Promega). The reaction mixture was incubated at 42 °C for 1 h and at 52 °C for 30 min.

PCR and Southern blot hybridization

The cDNA was later amplified by polymerase chain reaction (PCR) to study mouse GH, Pit-1 and γ -actin genes, using specific primers synthesized by Life Technologies Ltd. γ -Actin gene was used as an example of ubiquitous gene expression to normalize GH signal (Matsubara *et al.* 1995).

The mouse GH primers were: sense 5' ACTGCTTG GCAATGGCTAC 3' (51–69 nucleotides (nt)) and anti-sense 5' ATCTTCCAGCTCCTGCATC 3' (505–523 nt), which amplify a 473 base pair (bp) fragment (Linzer & Talamantes 1985). The Pit-1 primers were: sense 5' TGG AGTGATGGCAGGCAGTTTAA 3' (201–223 nt) and antisense 5' TTTCACCCGTTTTTCTCTCTGCCT 3' (796–819 nt), which amplify a 618 bp fragment (Li *et al.* 1990). The γ -actin primers were: sense 5' ATCTGGC ACCACACCTTCTACAATGAGCTGCG 3' (234–265 nt) and antisense 5' TCAGGGGGAGCAATGATCTT AATCTTCATCGTG 3' (950–982 nt), which amplify a 748 bp fragment (Peter *et al.* 1988). All these primers were tested in a BLASTN 1.4.11 computer program to verify the complete match with the corresponding gene sequence (Altschul *et al.* 1990).

The GH PCR reactions were carried out using an aliquot of 5 µl of the resulting RT mixture from submaxillary gland samples and 1.5 µl from pituitary, 1 IU Taq polymerase (EcoTaq; Ecogen SRL, Madrid, Spain), 16.6 mM (NH₄)₂ SO₄, 2.5 mM MgCl₂, 67 mM Tris-HCl pH 8.8, 0.2 mM of each deoxy-NTP and 0.5 µM of each specific primer, in a total volume of 50 µl. Thirty-five cycles of PCR were run using a thermal cycler (Programmable Thermal Controller PTC-100, MJ Research Inc.) according to the following step program: 1 min of denaturation at 94 °C, 1 min of annealing at 53 °C, and 1.5 min of extension at 72 °C. Amplification was completed with an additional extension step at 72 °C for 15 min. Pit-1 and γ -actin PCR were performed following the same protocol as that described for GH but the number of cycles and the temperature of the annealing process were changed. For Pit-1, 10 µl cDNA from salivary glands and 5 µl from pituitaries were submitted to 40 cycles using 63 °C as the annealing temperature. α -Actin PCR was performed with 5 µl cDNA for every sample, running 35 cycles at 70 °C.

Two kinds of negative control were run in each RT-PCR reaction to confirm that no contamination had occurred during the course of both procedures. In the first control assay, total RNA was omitted in the RT and, in the second one, cDNA was omitted in the PCR method. The experiment was considered useful only when no band was observed in the negative control lane on the agarose gel. In addition, to avoid false identification of PCR products derived from co-amplified genomic DNA contaminants, PCR primers were designed from two adjacent exons for each of the genes analyzed.

The PCR products were visualized in 2% agarose gel electrophoresis with ethidium-bromide staining under UV light (Fisher FB-TIV-88).

The agarose gel was blotted onto a Hybond-N+ nylon membrane (Amersham Life Science, Amersham, Bucks., UK) under 0.5 M NaOH overnight. The prehybridization was performed for 3 h at 57 °C for GH, Pit-1 and γ -actin in 50 $\times$ Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA), 20 $\times$ SSPE, 10% SDS and 10 mg/ml denatured salmon sperm DNA according to the procedure of Sambrook *et al.* (1989). Hybridization was carried out in the same buffer as that in the prehybridization step using a ³²P-labelled specific probe for 20 h at 57 °C for GH (probe sequence 5' TTGCTTCGCTTC TCGCTGCT 3', 359–378 nt from Linzer & Talamantes 1985), Pit-1 (5' AGCAGGTCGGAGCTTTGTAC 3', 593–612 nt from Li *et al.* 1990) and γ -actin (5' TCTATG AGGGCTACGCCCTT 3', 475–494 nt from Peter *et al.* 1988). After the hybridization, the membrane was washed five times in 6 $\times$ SSC for 20 min at the corresponding temperature. The blots were exposed overnight to X-ray film at –80 °C with two intensifying screens and developed by standard procedures.

The resultant autoradiography was analyzed and quantitated using Hewlett Packard Descan II and PC Image computer programs. The intensity of the GH signal was normalized to the γ -actin band and plotted as arbitrary units.

Restriction endonuclease digestion

Ten microlitres of PCR products from normal pituitaries and from submaxillary glands were digested by Aval (Promega) using 2 IU of the restriction enzyme. The reaction mix was incubated for 5 h at 37 °C. Restriction products, together with undigested samples, were electrophoresed in a 2% agarose gel stained with ethidium bromide and examined as described above.

Statistical analysis

Results are presented as means $\pm$ s.e.m. Statistical analysis was performed by two-factor analysis of variance. Student's *t*-test was used when values of only two groups were compared. The significance was determined using *P*<0.05.

Results

Plasma GH and IGF-I levels

As shown in Table 1, plasma concentrations of GH and IGF-I were significantly decreased (*P*<0.05 in both cases) in Ames dwarf mice with respect to those seen in controls. GHRH treatment did not modify either of these two parameters in normal or dwarf mice (Table 1).

Table 1 Circulating GH and IGF-I plasma levels at the end of the experiment in Ames normal and dwarf mice receiving a placebo or GHRH pellet in the submaxillary gland. Values represent the means $\pm$ s.e.m. The number of animals in each group is shown in parentheses

	GH (ng/ml)	IGF-I (ng/ml)
Groups		
Normal+placebo (7)	2.9 $\pm$ 1.2	264.6 $\pm$ 16
Normal+GHRH (7)	2.3 $\pm$ 1.3	250.8 $\pm$ 11
Dwarf+placebo (10)	0.67 $\pm$ 0.1*	56 $\pm$ 4.3*
Dwarf+GHRH (10)	0.7 $\pm$ 0.11*	47 $\pm$ 7.7*

**P*<0.05 vs normal mice.

Hormonal contents and weight of submaxillary glands

GH content in submaxillary glands (ng/mg protein) of normal mice implanted with GHRH pellets was significantly higher (*P*<0.05) than in glands treated with placebo pellets (Fig. 1A). However, there were no significant differences in IGF-I contents (ng/mg protein) between placebo- and GHRH-treated salivary glands (Fig. 1B).

GHRH levels in submaxillary glands treated with this peptide were measured in order to prove that GHRH had been released from the pellets and was present in the tissue. High amounts of radioimmunoassayable GHRH in

Figure 1 Concentrations of (A) GH and (B) IGF-I (ng/mg protein) in submaxillary glands of normal mice implanted with a GHRH or placebo pellet. Values represent the mean $\pm$ s.e.m. and the number of animals per group was seven. **P*<0.05 vs placebo-treated mice.

Figure 2 Expression analysis of (A) GH and (B) γ -actin mRNA by RT-PCR in pituitary and submaxillary glands of normal and Ames dwarf mice. The molecular size of the PCR products was 473 bp for GH mRNA and 749 bp for γ -actin. Lane 1: pituitary from normal mice. Lanes 2 and 3: GHRH-treated submaxillary glands from normal mice. Lane 4: placebo-treated submaxillary glands from normal mice. Lane 5: GHRH-treated submaxillary glands from dwarf mice. Lane 6: placebo-treated submaxillary glands from dwarf mice. Lane 7: 100 bp ladder.

the GHRH-treated glands were found with an average of 24 ± 15 ng/mg protein (ranging from 2.6 to 69 ng/mg protein). The levels of GHRH in glands bearing a placebo pellet were undetectable.

The weight of submaxillary glands was similar in normal mice treated with GHRH or placebo pellets (GHRH-treated: 70 ± 6.9 mg; placebo-treated: 70 ± 4.3 mg). A similar weight in GHRH- and placebo-treated submaxillary glands could also be observed in Ames dwarf mice (GHRH-treated: 23 ± 3 mg; placebo-treated: 19 ± 1.5 mg).

Analysis of GH expression

GH and γ -actin mRNA levels in the pituitaries and submaxillary glands of Ames mice were analyzed by RT-PCR followed by Southern blot. The expected 473 bp GH band was obvious after amplification of cDNA from normal pituitaries. In addition, a band of the same size, although with less intensity than in the pituitary, was observed in the samples obtained from submaxillary glands of GHRH-treated normal mice (Fig. 2A). Surprisingly, a faint but detectable GH signal could also be seen in the submaxillary glands from placebo-treated normal mice and from placebo- or GHRH-treated dwarf mice (Fig. 2A). The expression levels of the γ -actin gene were constant in all the tested tissues (Fig. 2B).

Figure 3 (A) GH and (B) γ -actin expression in pituitary and submaxillary glands of normal and Ames dwarf mice after Southern blot hybridization. Lane 1: pituitary gland from normal mice. Lane 2: placebo-treated submaxillary gland from normal mice. Lanes 3–4: GHRH-treated submaxillary gland from normal mice. Lane 5: placebo-treated submaxillary gland from dwarf mice. Lane 6: GHRH-treated submaxillary gland from dwarf mice. (C) GH mRNA levels normalized to γ -actin (arbitrary units) in salivary tissues presented as means $\pm$ S.E.M. GH mRNA level relative to γ -actin in pituitary from normal mice was 5.16 arbitrary units. The number of samples was seven in both groups of normal mice and ten in those of Ames dwarf mice. * $P < 0.05$ vs the rest of the groups.

The identity of GH amplification products as well as the differences in the intensity among samples were confirmed by Southern blot hybridization with an internal radio-labelled oligonucleotide. This highly sensitive technique indicates that PCR products of GH from submaxillary glands were identical to the corresponding pituitary GH cDNAs. Representative Southern blot data comparing GH mRNA from normal pituitaries and from submaxillary glands treated with placebo or GHRH pellets are shown in Fig. 3A. The γ -actin band corresponding to these samples is presented in Fig. 3B.

GH and γ -actin signals obtained by Southern blot analysis were used to quantitate GH expression. The mean value of GH mRNA normalized to γ -actin in salivary tissues from normal mice was significantly ($P < 0.05$) increased in the GHRH-treated group with respect to that found in placebo-treated glands. However, there were no

Figure 4 RT-PCR analysis of GH and Pit-1 expression in pituitary and submaxillary gland. GH mRNA levels (473 bp) are shown in lanes 1 and 2, whereas lanes 4, 5, 6 and 7 represent Pit-1 expression (618 bp). Lanes 1 and 4: pituitary from normal mice. Lanes 2 and 5: pituitary from dwarf mice. Lane 6: submaxillary gland from placebo-treated normal mice. Lane 7: submaxillary gland from GHRH-treated normal mice. Lane 3: molecular size markers.

differences between the values observed in glands bearing GHRH or placebo pellets in dwarf mice, which were similar to the levels found in placebo-treated glands from normal mice (Fig. 3C). The GH mRNA level relative to γ -actin in pituitary from normal mice was 5.16 arbitrary units.

In addition to these studies, analysis of the presence of GH mRNA in pituitaries from normal and Ames dwarf mice was performed. As can be seen in Fig. 4, the 473 bp band was detectable after RT-PCR in the normal pituitary (lane 1) and also in a sample from pooled dwarf pituitary glands (lane 2), although in this case the signal was less intense. GH mRNA was confirmed by Southern blot hybridization in both tissues.

Study of Pit-1 gene expression

Pit-1 mRNA levels were also monitored by RT-PCR followed by Southern blot in pituitary and submaxillary gland samples from normal and dwarf mice. The amplification of cDNA from normal pituitaries showed a marked Pit-1 band of the predicted size (618 bp) (Fig. 4, lane 4). A less intense but still clear Pit-1 signal was present in pooled pituitaries of dwarf mice (Fig. 4, lane 5). Pit-1 mRNA was absent from the submaxillary gland samples from placebo- (Fig. 4, lane 6) or GHRH-treated (Fig. 4, lane 7) normal mice, and from salivary tissue samples from Ames dwarf mice. The same results appeared after the corresponding Southern blot of the PCR products.

Restriction endonuclease analysis of GH gene expression

GH PCR products were submitted to enzymatic digestion with *Ava*I. As can be observed in Fig. 5, PCR samples

Figure 5 Agarose gel analysis of GH PCR products digested with *Ava*I yielding two bands of 203 and 270 bp. Lane 1 and 8: 100 bp ladder. Lane 2: undigested pituitary sample. Lane 3: digested pituitary sample. Lanes 4 and 6 represent undigested salivary samples from two GHRH-treated mice, and the corresponding digested products are shown in lanes 5 and 7.

from normal pituitaries (lane 2) were completely digested into two fragments of 203 bp and 270 bp respectively (lane 3). The same pattern of bands appeared when samples from submaxillary glands were tested (undigested samples: lanes 4 and 6; digested samples: lanes 5 and 7), confirming that submaxillary GH transcript is similar to that found in the normal pituitary gland.

Discussion

Adult rat salivary tissue submitted to the influence of high local GHRH concentrations was capable of synthesizing GH both *in vivo* and *in vitro* (Tresguerres *et al.* 1999a,b). The findings presented here confirm that such a GH-induction process also takes place in submaxillary cells from mice under the same experimental conditions. Moreover, using highly sensitive techniques such as RT-PCR followed by Southern blot, we have been able to demonstrate that salivary tissue also basally transcribes GH mRNA.

The basal GH expression by the submaxillary gland observed in our experiment is in agreement with other studies which describe extrapituitary GH synthesis in normal rat and human tissues as, for example, in the liver, kidney, muscle, gastrointestinal tract, lung and lymphocytes (Kyle *et al.* 1981, Weigent *et al.* 1991). The existence of an almost ubiquitous transcription of the GH gene has been suggested in a recent review by Harvey & Hull (1997), and other authors have also commented that basal or low-level expression of GH could be seen in non-pituitary cells (Tuggle & Trenkle 1996). The binding of ubiquitous transcription factors to the GH promoter or the absence of inhibitory elements which normally repress GH expression in non-pituitary cells could explain this

extrapituitary GH transcription (Chelly *et al.* 1989, Tuggle & Trenkle 1996). In addition to all these data supporting our findings, the submaxillary gland has been found to produce several hormones (renin, angiotensin II, glucagon, somatostatin, gastrin), and growth factors (epidermal growth factor, nerve growth factor and IGF-I) (Barka 1980, Ryan *et al.* 1992).

The basal transcription of the GH gene in mouse salivary tissue appears to be Pit-1 independent according to our results, since it was also found in submaxillary gland samples from Pit-1-deficient Ames dwarf mice. GH gene expression independent of Pit-1 has already been described in murine immune system cells (Weigent & Blalock 1994, Kooijman *et al.* 1997), and pituitary-type prolactin gene transcription in human uterine cells has also been shown to be Pit-1 independent (Gellersen *et al.* 1995). Nevertheless, the subject cannot yet be fully explained.

When GHRH was locally administered at very high doses in normal mice, GH expression in the submaxillary gland was markedly increased, thus confirming the GHRH-dependent GH induction process previously described in the rat by our group (Tresguerres *et al.* 1999b). Several studies have previously suggested that GHRH could induce GH synthesis in tissues other than the pituitary (Leveston *et al.* 1981). This effect of GHRH on GH transcription was not observed in salivary tissue from Pit-1-deficient dwarf mice, which suggests that its influence on GH was at least partially mediated by Pit-1. However, we were also unable to detect Pit-1 expression by RT-PCR followed by Southern blot in salivary glands from normal mice treated with GHRH which had shown elevated GH contents. Therefore, other factors, such as those previously mentioned in relation to the control of the basal extrapituitary GH expression, could be implicated. Nevertheless, the absence of GHRH-induced GH expression in Ames mice remains to be clarified until new information becomes available.

While previous studies have failed to detect GH and Pit-1 mRNA in the pituitary glands of Ames dwarf mice (Slabaugh *et al.* 1981, Cheng *et al.* 1983), several recent reports have described the existence of GH- and Pit-1-positive cells (Andersen *et al.* 1995, Gage *et al.* 1995, 1996) and Pit-1 mRNA (Camper *et al.* 1990) in pituitary glands from these animals. In agreement with the latter findings we have confirmed the expression of GH and Pit-1 in pooled pituitaries from Ames dwarf mice using RT-PCR followed by Southern blot. The differences in detecting GH and Pit-1 could be attributable to the different sensitivity of the methods employed by the researchers or the variations in different colonies of the dwarf mice.

In the present study, we have reported low but detectable levels of circulating GH and IGF-I. This observation contrasts with the results in the literature (Cheng *et al.* 1983, Chandrashekar & Bartke 1993) in which these two hormones were not detectable by RIA in the circulation of the same kind of animals. Different methods used to

measure these hormones could explain this discrepancy in the results. In this regard, Chandrashekar & Bartke (1993) used cryoprecipitation to eliminate IGF-I binding proteins present in the plasma, whereas we used acid-ethanol extraction. Regarding plasma GH levels, a higher sensitivity in our mouse GH could explain the differences. Thus, Cheng *et al.* (1983) reported a sensitivity of 100 pg/tube against 5 pg/tube registered by us.

In conclusion, the present experiment confirms that adult submaxillary glands synthesize GH when they are exposed to the influence of high local GHRH concentrations. In addition, we have demonstrated that salivary tissue basally transcribes GH. Both basal and GHRH-induced salivary GH productions appear to be independent of Pit-1, although the reason why GHRH does not induce GH gene expression in Ames mice remains to be determined.

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