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# Dominant dwarfism in transgenic rats by targeting human growth hormone (GH) expression to hypothalamic GH-releasing factor neurons

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**Expression of human growth hormone (hGH) was targeted to growth hormone-releasing factor (GRF) neurons in the hypothalamus of transgenic rats. This induced dominant dwarfism by local feedback inhibition of GRF. One line, bearing a single copy of a GRF–hGH transgene, has been characterized in detail, and has been termed Tgr (for Transgenic growth-retarded). hGH was detected by immunocytochemistry in the brain, restricted to the median eminence of the hypothalamus. Low levels were also detected in the anterior pituitary gland by radioimmunoassay. Transgene expression in these sites was confirmed by RT–PCR. Tgr rats had reduced hypothalamic GRF mRNA, in contrast to the increased GRF expression which accompanies GH deficiency in other dwarf rats. Endogenous GH mRNA, GH content, pituitary size and somatotroph cell number were also reduced significantly in Tgr rats. Pituitary adrenocorticotrophic hormone (ACTH) and thyroid-stimulating hormone (TSH) levels were normal, but prolactin content, mRNA levels and lactotroph cell numbers were also slightly reduced, probably due to feedback inhibition of prolactin by the lactogenic properties of the hGH transgene. This is the first dominant dwarf rat strain to be reported and will provide a valuable model for evaluating the effects of transgene expression on endogenous GH secretion, as well as the use of GH secretagogues for the treatment of dwarfism.**

*Keywords*: dominant dwarfism/GRF gene/growth hormone/hypothalamus/transgenic rat

## Introduction

Growth hormone (GH) is the major endocrine regulator of post-natal growth in mammals. GH gene expression (Barinaga *et al.*, 1985a,b), synthesis (Bilezikjian and Vale, 1984; Fukata *et al.*, 1985) and secretion (Guillemin *et al.*, 1982; Rivier *et al.*, 1982; Spiess *et al.*, 1983) are stimulated by the hypothalamic peptide GH-releasing factor (GRF) which also has a powerful trophic effect on somatotrophs (Billestrup *et al.*, 1986), the pituitary cells which produce GH. This system is subject to direct feedback control by GH; hypothalamic GRF gene expression is increased in GH deficiency (Chomczynski *et al.*, 1988) and reduced by GH excess (De Gennaro *et al.*, 1988), probably mediated by GH receptors in the arcuate nucleus (Burton *et al.*, 1992; Lobie *et al.*, 1993), the major site of GRF synthesis in the brain.

Advances in our understanding of these basic physiological mechanisms have emerged from studies of rodents bearing spontaneous mutations which result in an altered pituitary GH production and growth phenotype. Since the ultimate cause of dwarfism is GH deficiency, they can invariably be stimulated to grow by direct replacement therapy, either with exogenous GH (Charlton *et al.*, 1988), or by a transgene expressing GH (Hammer *et al.*, 1984). The most obvious example is the dwarf *dr/dr* rat, which carries a mutation in the rat GH gene resulting in a truncated, inactive product (Takeuchi *et al.*, 1990). We have also characterized a spontaneous GH-deficient dwarf (*dw/dw*) rat (Charlton *et al.*, 1988), whose recessive mutation has not yet been identified. This animal shows a specific partial GH deficiency, and grows in response to GH or insulin-like growth factor (IGF)-1 treatment (Skottner *et al.*, 1989). The GRF receptor appears normal, and GRF treatment elicits small GH secretory responses (Carmignac and Robinson, 1990). However, the GH output is too small to elicit a significant increase in growth, even with prolonged administration of GRF. GH deficiencies also arise from other mutations. For example, the Snell and Jackson dwarf mice bear recessive mutations in the *Pit-1* gene (Li *et al.*, 1990), resulting in a defect in the development of somatotrophs, lactotrophs [prolactin (PRL)-producing cells] and thyrotrophs [thyroid-stimulating hormone (TSH)-producing cells], and show multiple pituitary hormone deficiencies (Roux *et al.*, 1982). In contrast, the Little (*lit/lit*) mouse (Eicher and Beamer, 1976) shows a specific GH deficiency, resulting from a recessive mutation in the GRF receptor gene (Godfrey *et al.*, 1993; Lin *et al.*, 1993).

Whilst all of these dwarf animals will grow in response to GH, all bear mutations which render them unsuitable for testing the growth-promoting capacity of GH secretagogues which may be of therapeutic value in the treatment of children with short stature (Thorner *et al.*, 1990). However, it is now possible to create new models of altered growth rate by transgenesis. Numerous lines of transgenic mice have been generated which express GH under the control of heterologous promoters, usually resulting in increased growth given a significant level of peripheral GH expression (Palmiter *et al.*, 1982; Morello *et al.*, 1986; Bchini *et al.*, 1991). There have been two interesting exceptions to this phenotype, in which dominant dwarfism results from human GH (hGH) expression in the CNS (Hollingshead *et al.*, 1989; Banerjee *et al.*, 1994). This arises from unregulated hGH transgene expression

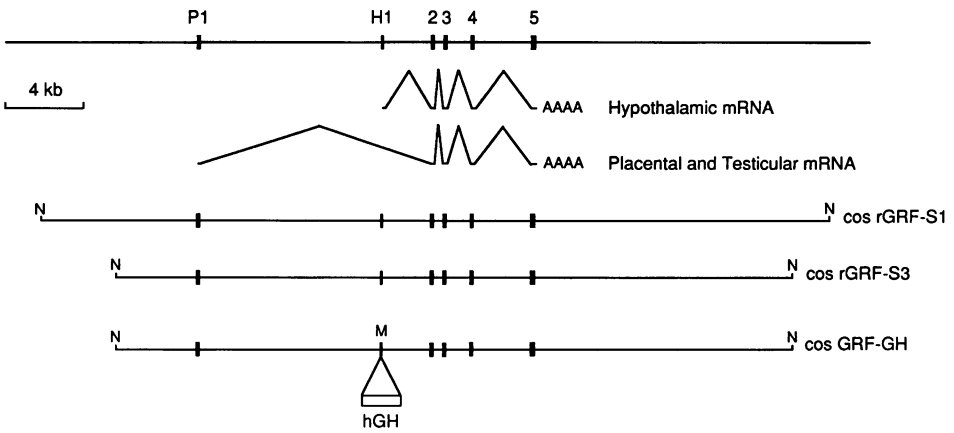


**Fig. 1.** Structure of the rat GRF gene locus. Thin lines represent introns, 5′- and 3′-flanking sequences; filled boxes represent exons. Transcripts arising from the GRF gene are represented below, as are cosmids cos rGRF-S1, -S3 and cos GRF-GH, with the hGH gene represented by the open box. The *Mlu*I site (M) used for insertion of the hGH gene and the vector *Not*I sites (N) used for excision of the insert are indicated.

exerting an inappropriate negative feedback via hypothalamic GH receptors to suppress GRF activity, which in turn reduces endogenous mouse GH production. Unfortunately, the size of these dwarf mice renders studies of physiological GH secretion and GRF responsiveness difficult to perform *in vivo*.

We decided to exploit this GH negative feedback mechanism more specifically, by targeting expression of hGH to the GRF neurons of the hypothalamus. Our approach differed from previous studies in two key features. The transgenic mice lines expressed hGH in relatively broad areas of the CNS (Hollingshead *et al.*, 1989) and peripheral sites (Banerjee *et al.*, 1994). Since attempts to target transgene expression to GRF neurons using short GRF gene 5′-flanking sequences have not been successful (Botteri *et al.*, 1987; Giraldi *et al.*, 1994), we used a cosmid transgene with larger pieces of DNA flanking the rat GRF gene to maximize the chances of appropriate tissue-specific expression of the hGH transgene. Secondly, we chose to use rats rather than mice, to create a model that would be amenable to detailed physiological analysis using methodology developed for the rat (Clark *et al.*, 1986). This report describes the production and initial physiological characterization of such a line of transgenic growth-retarded (Tgr) rats. Some of these results have been presented recently in abstract form (Flavell *et al.*, 1995; Wells *et al.*, 1995).

## Results

### *Isolation of genomic clones for the rGRF gene*

Screening of a rat genomic DNA cosmid library with a rat GRF (rGRF) cDNA (Mayo *et al.*, 1985) resulted in the isolation of two independent cosmid clones (cos rGRF-S1 and -S3) which hybridized strongly on Southern blots. Restriction mapping and comparison with previously cloned genomic fragments (generously provided by Dr Kelly Mayo) revealed that both cosmids contained the entire rGRF gene with cos rGRF-S1 containing 20 kb of 5′- and 12 kb of 3′-flanking sequence, and cos rGRF-S3 containing 16 kb of 5′- and 14 kb of 3′- flanking sequence (Figure 1). In total, these cosmids span 42 kb of genomic DNA of the rGRF gene.

**Table I.** Growth rates of rGRF–hGH founders

| Rat # | Sex | Tg status | Body weights (g) at week | | | | |
|---|---|---|---|---|---|---|---|
| | | | 6 | 8 | 10 | 12 | 14 |
| FP62 | F | – | 131 | 176 | 212 | 228 | 238 |
| FP64 | F | – | 120 | 176 | 214 | 226 | 240 |
| FP65 | F | – | 121 | 182 | 214 | 237 | 244 |
| FP66 | F | +6 | 95 | 141 | 165 | – | 222 |
| FP101 | M | +1 | 83 | 124 | 157 | 184 | 190 |
| FP128 | M | +16 | 43 | 76 | 102 | 128 | 158 |
| FP129 | M | +/– | 156 | 237 | 277 | 331 | – |

Individual body weights for the four rRGF–hGH founders are shown, together with their genotype and copy number. Three wild-type female littermates of FP 66 are shown for comparison. Founder #129 had a partial copy of the transgene and grew at a normal rate for male rats.

### *Generation of GRF–GH transgenic rats*

A genomic fragment containing the 5′-untranslated region (UTR), exons 1–5, introns, the 3′ UTR and polyadenylation sequence of the hGH gene (DeNoto *et al.*, 1981) was inserted into the 5′ UTR of the rGRF gene hypothalamic exon 1 in cos rGRF-S3 to make cos GRF-GH (Figure 1). The resulting 38 kb rGRF–hGH insert fragment was microinjected into fertilized AS rat eggs. Surviving eggs were transfered into the oviducts of pseudopregnant recipients, and the resulting pups were assayed for the presence of the transgene by Southern blot analysis with an hGH probe of genomic DNA prepared from tail biopsy. Four pups (66, 101, 128, 129) were found to have incorporated transgene DNA (data not shown) and were mated with wild-type AS rats. Two of the founders, 101 and 129, produced litters from which lines subsequently were established. Founder 66 (female) produced only one litter which died shortly after birth, and founder 128 (male) produced no offspring. Southern blot analysis of founder 129 indicated that this animal bore a partial copy of the transgene which lacked the entire rGRF 5′-flanking sequences and the 5′ end of the hGH gene, which would not make hGH mRNA. Founder 66 contained six copies of the transgene, 101 a single copy and 128 ~16 copies. The growth rate of all founders was monitored; the three founders with intact transgenes all exhibited dwarfism, whilst founder 129 did not (Table I).

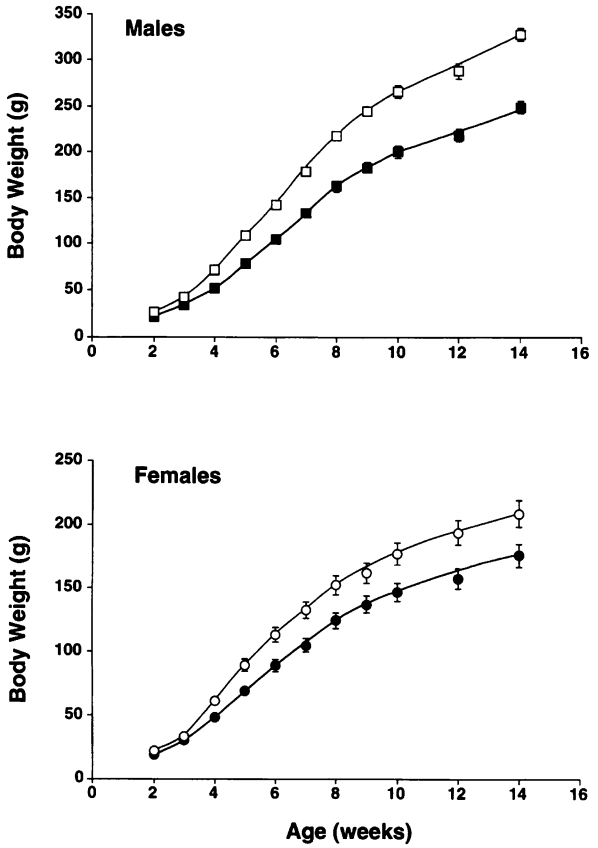


**Fig. 2.** Growth curves for wild-type (open symbols) and Tgr (closed symbols) male and female littermates. Groups of 6–8 animals were weighed weekly for up to 14 weeks. Data shown are mean ± SEM. Tgr rats were significantly smaller than their wild-type littermates by 3 weeks of age.

### *Growth rate of Tgr rats*

Tgr animals from line 101 and their wild-type littermates were weighed at weekly intervals. Adult hemizygous Tgr rats were ~60–70% of the weight of their sex-matched littermates (Figure 2). This dwarfism became significant ($P<0.001$) in both sexes by 3 weeks of age. Body length was also reduced in adult male Tgr rats (nose–tail length 329 ± 5 mm) compared with wild-type animals (362 ± 3 mm, $n$ = 7,7; $P<0.01$). In the rat, growth is sexually dimorphic. In the normal rats, this sex difference became significant ($P<0.05$) by 4 weeks of age, whereas this difference was not significant until 7 weeks in Tgr rats. The rate of weight gain continued more slowly in Tgr males than in wild-type males (Δ wt between 6 and 12 weeks was 115 ± 4 versus 150 ± 4 g, $P<0.05$), whereas the rate of weight gain in female Tgr animals was more similar to that of wild-type females (Δ wt 69 ± 3 in Tgr versus 79 ± 5 g in wild-type females, n.s.)

Homozygote animals did not show any greater growth retardation than the hemizygotes (data not shown). Subsequent studies were therefore performed on paired groups of hemizygote dwarfs and their non-transgenic littermates.

### *Expression of the GRF–GH transgene*

Initial attempts to detect hGH mRNA in Tgr hypothalamus by RNase protection, or hGH protein by radioimmunoassay (RIA) of hypothalamic extracts were unsuccessful. We therefore examined expression of the hGH transgene

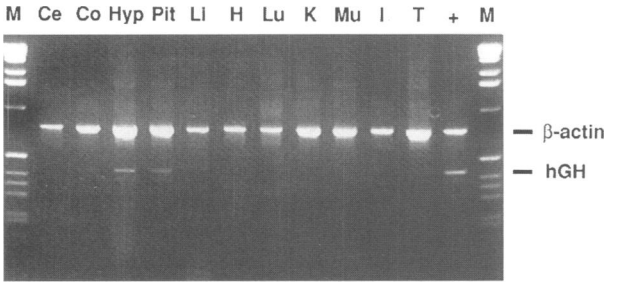


**Fig. 3.** RT–PCR analysis of hGH transgene expression in brain and peripheral tissues of Tgr rats. cDNA was prepared from 1 μg of total RNA from Tgr rat tissues and subjected to RT–PCR with hGH- and rat β-actin specific primers. Products were analysed on a 2% agarose gel. Markers (M) were 1 kb DNA ladder (Gibco-BRL), tissues are: Ce, cerebellum; Co, cortex; Hyp, hypothalamus; Pit, pituitary; Li, liver; H, heart; Lu, lung; K, kidney; Mu, muscle; I, intestine, T, testis; +, positive control (GH3 rat pituitary cells transiently transfected with an hGH gene driven by the cytomegalovirus promoter).

by reverse transcription–polymerase chain reaction (RT–PCR) using hGH gene-specific primers. Rat β-actin was also amplified as a control. The expected 428 bp PCR product was observed in the hypothalamus and pituitary of *Tgr* animals (Figure 3). With extended RT–PCR analysis, small amounts of hGH product were also detectable in extracts of cortex, cerebellum, lung and testis, but no product was visible in liver, heart, kidney, muscle or intestine (not shown). Immunocytochemistry was used to examine the site of hGH expression in Tgr brain sections. Immunoreactive hGH was only detectable in the median eminence of the hypothalamus (Figure 4A and B). A similar distribution of hGH immunoreactivity was also found in the median eminence of founder 66. Serial sections examined for hGH and rat GRF precursor (Monts *et al.*, 1996) by immunocytochemistry showed that both peptides exhibited an identical distribution in the median eminence of Tgr animals (not shown). hGH immunoreactivity was not detected in other parts of the brain, nor in brains from wild-type animals. A few hGH-positive cells were also detected in the anterior pituitary of Tgr animals and of founder 66.

### *Expression of the endogenous rGRF, rGH and rPRL genes*

Hypothalamic GRF mRNA levels were examined in wild-type and Tgr littermates at post-natal day 42 (P42) by RNase protection analysis (Figure 5A). The level of GRF mRNA was decreased in Tgr hypothalamus compared with wild-type littermates. This contrasts with an up-regulation of GRF mRNA levels in the *dw* dwarf rat compared in the same assay. Measurements of hypothalamic GRF peptide content by RIA also showed a slight reduction in Tgr rats (1.15 ± 0.09 versus 1.37 ± 0.09 ng), but this did not reach statistical significance ($P$ = 0.08).

Expression of the GH and PRL genes in the anterior pituitary of wild-type and Tgr littermates was examined through post-natal development by S1 nuclease protection analysis, with rat β-actin mRNA levels determined as a control (Figure 5B). GH gene transcription rises gradually from birth in wild-type and Tgr animals.When normalized to β-actin levels, rGH mRNA levels were reduced in Tgr pituitaries, from post-natal day 2 (P2) and at subsequent

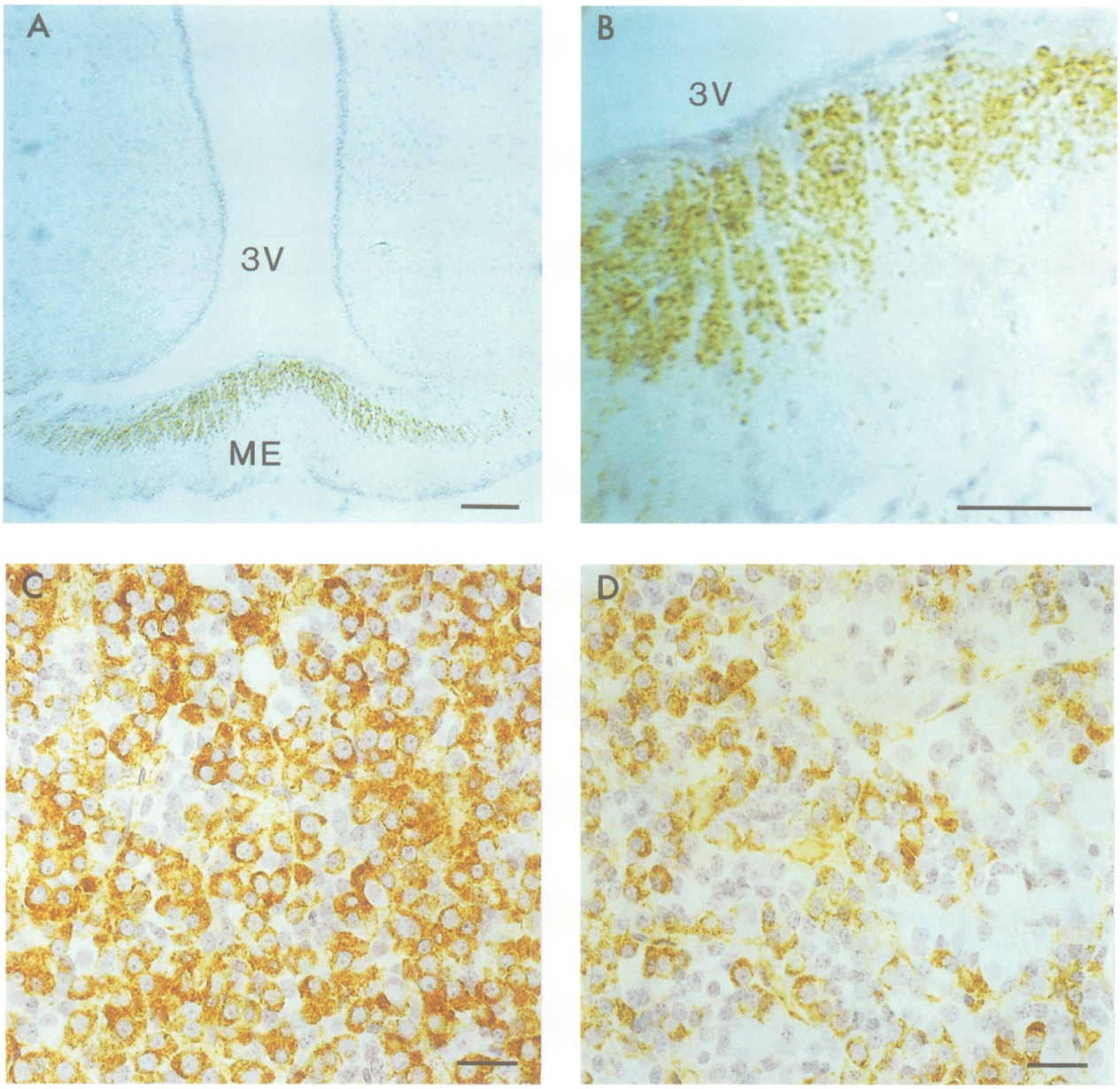


**Fig. 4.** Immunocytochemistry of hypothalamic human GH and pituitary rat GH in the Tgr rat. Human GH immunoreactivity is restricted to the hypothalamic median eminence (**A** and **B**) of a Tgr rat. Immunopositive GH cells in wild-type rat anterior pituitary (**C**) are much more abundant than in the Tgr pituitary gland (**D**). 3V = third ventricle, ME = median eminence, scale bar = 100 μm.

time points, compared with non-transgenic littermates, except for P42 female pituitary. The reduction in mRNA levels between normal and Tgr animals became less pronounced with age, such that GH mRNA levels are comparable at P42 when measured per μg of pituitary RNA. rPRL mRNA levels were also reduced in Tgr pituitary compared with wild-type pituitaries.

### *Pituitary hormone content*

Pituitary rGH protein levels were examined by RIA through post-natal development in wild-type animals and their Tgr littermates (Figure 6). When compared with normal animals, Tgr pituitary rGH levels were significantly reduced from 9 days of age and all subsequent time points in both males (Figure 6) and females (not shown). Pituitary weights were also markedly reduced in Tgr animals (150 days, 6.0 ± 0.5 versus 10.1 ± 0.6 mg in wild-type littermates, $P<0.001$). Levels of other pituitary hormones were examined by RIAs of the same pituitary homogenates taken from 23 day wild-type and Tgr littermates (Table II). GH showed the most dramatic reduction, to 20% of wild-type levels. PRL and luteinizing hormone (LH) levels were also reduced, though LH levels were not reduced when pituitaries from older animals were examined (data not shown). Levels of adrenocorticotrophic hormone (ACTH) and TSH were unaffected. Pituitary hGH levels were also determined by an assay specific for hGH and found to be low, but consistently detectable in extracts of Tgr pituitary but not from their wild-type littermates (Table II).

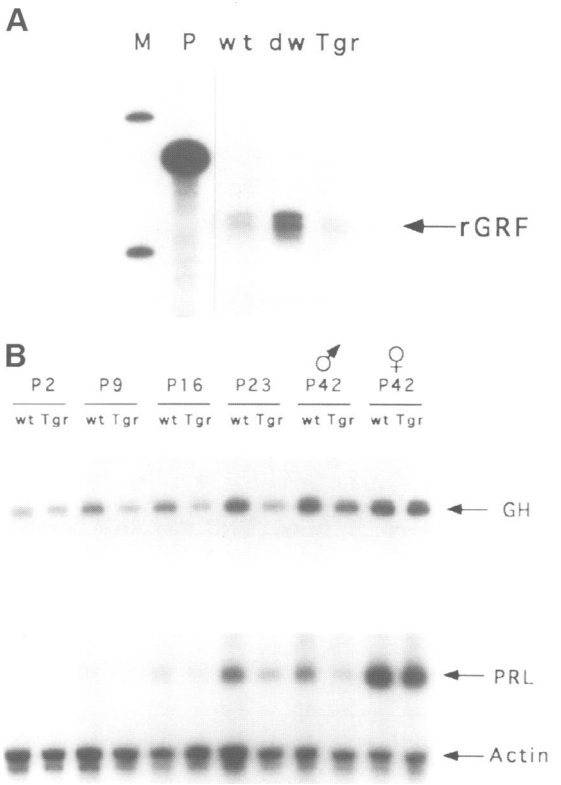


**Fig. 5.** (**A**) Expression of GRF in hypothalami of AS dwarf, wild-type and transgenic rats. RNase protection analysis of 20 μg of total cellular RNA from hypothalami of AS dwarf (dw), wild-type (wt) and transgenic (Tgr) rats at post-natal day 42 (P42). M is $^{32}$P end-labelled *Msp*I-digested pUC19. P is undigested probe. (**B**) Expression of GH, PRL and β-actin in the anterior pituitary of wild-type and Tgr rats through post-natal development. S1 nuclease protection analysis of 3 μg of total cellular RNA from pooled ($n$ = 5–13) normal (wt) and transgenic (Tgr) pituitaries at post-natal day 2 (P2), day 9 (P9), day 16 (P16), day 23 (P23) and day 42 (P42) males and females, with probes specific for rGH, rPRL and β-actin. M is $^{32}$P end-labelled *Msp*I-digested pUC19.

### *Immunocytochemistry for rGH and rPRL*

Immunocytochemistry using rGH-specific antibodies was performed on sections from 150 day Tgr and wild-type littermate pituitaries (Figure 4) and the percentage of GH-immunoreactive cells was determined. The proportion of pituitary cells identified as somatotrophs was reduced markedly in Tgr rats (31 ± 1% versus 45 ± 1% in wild-type rats, $P<0.001$), whereas the lactotroph numbers identified by immunocytochemistry in the same sections were unaffected (26 ± 1 versus 28 ± 2%, $n$ = 6 per group).

### *Release of endogenous GH in Tgr dwarf rats*

The secretory pattern of rat GH was measured by automated serial blood microsampling in conscious, chronically catheterized adult wild-type and Tgr male rats. Two examples are shown in Figure 7, and analysis of secretory profiles from a larger group of animals is shown in Table III. Whilst GH pulse frequency was not significantly altered, GH peak amplitude, peak area and total GH secretion were all markedly reduced in the Tgr animals, compared with their non-transgenic littermates.

## Discussion

Our primary aim was to generate a novel dwarf rat with pituitary GH deficiency but still responsive to GH

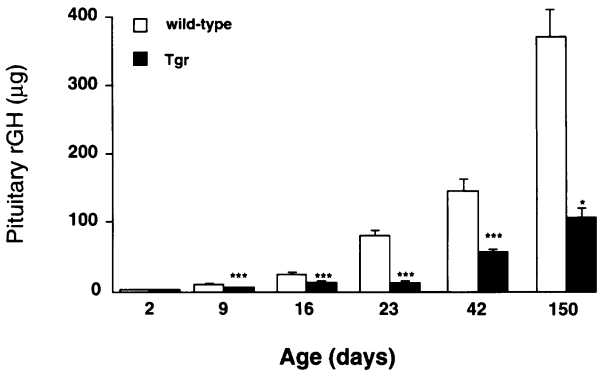


**Fig. 6.** Pituitary rGH content through post-natal development in wt and Tgr rats. Rat GH levels were measured by RIA in wt and Tgr pituitary extracts at post-natal days 2, 9, 16, 23, 42 and 150. The first three groups are from both sexes, whereas the last three groups are from males only. Results are expressed as mean ± SEM, $n$ = 6–22; *, $P<0.05$; ***, $P<0.001$.

**Table II.** Pituitary hormone content in wild-type and Tgr rats at post-natal day 23

| Hormone content (μg/pituitary) | Wild-type (wt) | Transgenic (Tgr) |
|---|---|---|
| Rat GH | 36.3 ± 3.3 | 6.9 ± 0.4*** |
| Rat PRL | 0.85 ± 0.08 | 0.49 ± 0.04*** |
| Rat LH | 3.3 ± 0.5 | 2.1 ± 0.3* |
| Rat TSH | 1.7 ± 0.1 | 1.6 ± 0.1 |
| Rat ACTH | 13.4 ± 1.0 | 13.4 ± 0.9 |
| Human GH | n.d. | 0.0003 ± 0.00003 |

The rat pituitary hormones GH, PRL, LH, TSH, ACTH and the human GH transgene product were measured by specific RIAs in the same homogenates obtained from wild-type (wt) and transgenic (Tgr) 23-day old littermates. Results are expressed as mean ± SEM, $n$ = 11 (wt), $n$ = 22 (Tgr). n.d. not detectable * $P<0.05$; *** $P<0.001$.

secretagogues. Although existing dwarf strains are useful in studies of GH or IGF-1 action (Skottner *et al.*, 1989; Gargosky *et al.*, 1995), neither show significant growth responses to GH secretagogues. The primary stimulus for GH release is hypothalamic GRF, but no GRF-deficient mice made by homologous recombination or transgenic ablation have been reported. Destruction of GRF neurons by neonatal monosodium glutamate (MSG) treatment produces dwarf rats which grow in response to GRF (Clark and Robinson, 1985), but this treatment is non-specific and toxic. Two transgenic mouse experiments suggested a different strategy to us. A dominant dwarf phenotype was reported in mice expressing hGH in the cerebral cortex driven by the mouse mammary tumour virus long terminal repeat (Hollingshead *et al.*, 1989). A similar phenotype was also reported in a mouse line expressing hGH in the CNS under the control of the tyrosine hydroxylase promoter (Banerjee *et al.*, 1994). Both these lines showed reduced GRF and pituitary GH (Hollingshead *et al.*, 1989; Szabo *et al.*, 1994), suggesting that the dwarfism resulted from central hGH activating the feedback pathway by which pituitary GH inhibits its own secretion (Katakami *et al.*, 1987).

GH controls its own production via feedback on GH receptors located in the arcuate and periventricular nuclei, repressing GRF and increasing somatostatin expression, respectively (Burton *et al.*, 1992; Lobie *et al.*, 1993).

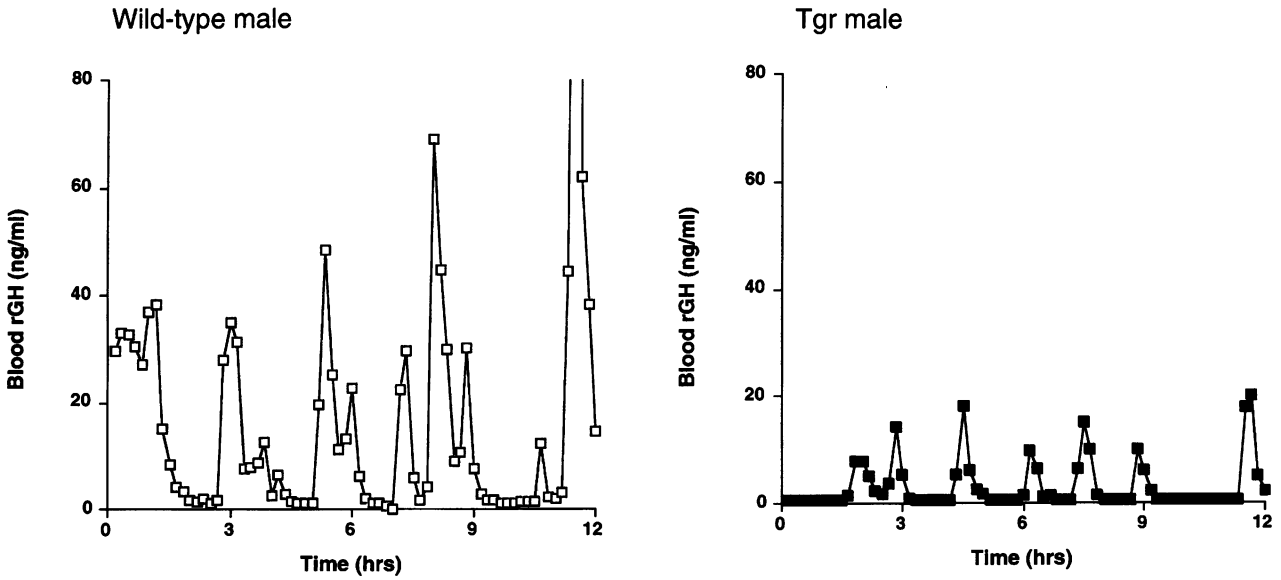


**Fig. 7.** Blood rGH secretory profiles in individual wild-type (□) and Tgr (■) freely-moving conscious male rats.

However, GH receptors are expressed in several other sites in the CNS, where their function is poorly understood. To maximize physiological control of transgene expression, we chose to express hGH under the control of a specific hypothalamic promoter. The GRF gene was chosen since its expression is largely restricted to neurons in the arcuate nucleus of the hypothalamus (Bloch *et al.*, 1983) and these represent a major target for GH feedback in the rat. We chose to make rats rather than mice since a dwarf phenotype in rats is still compatible with extensive cannulation, sampling and infusion procedures which are not feasible in dwarf mice. Although most knowledge of the physiological control of GH release by the hypothalamus derives largely from studies in the rat, few transgenic rats with modifications in the GH axis have been reported (Ikeda *et al.*, 1995).

Previous attempts to target GRF neurons in transgenic mice used relatively short flanking sequences of the human (Botteri *et al.*, 1987) or mouse (Giraldi *et al.*, 1994) GRF genes expressing SV40 large T antigen. No hypothalamic expression was obtained, though ectopic expression produced thymic hyperplasia or adrenal medullary tumours. Appropriate transgene expression is achieved more reliably with larger constructs that contain the regulatory elements necessary for position-independent, copy number-dependent expression (Grosveld *et al.*, 1987; Jones *et al.*, 1995). Accordingly, we isolated cosmids spanning 42 kb of the rGRF gene locus. In the rat, GRF is also expressed in extrahypothalamic tissues such as placenta (Gonzalez-Crespo and Boronat, 1990; Margioris *et al.*, 1990) and testis (Berry and Pescovitz, 1988). These transcripts arise from promoters 10 kb upstream of the hypothalamic promoter and splice into the second exon of the hypothalamic GRF mRNA (Gonzalez-Crespo and Boronat, 1991). To avoid extrahypothalamic expression, the hGH gene was inserted into the 5′ UTR in the hypothalamic first exon of the GRF gene. Any transcripts arising from upstream GRF promoters, which could theoretically splice into the second exon of the hGH gene, would not produce bioactive hGH.

**Table III.** PULSAR analysis of GH secretion in wild-type and Tgr male rats

| GH secretory parameters | Wild-type male | Tgr male |
|---|---|---|
| Total secretion (ng/ml×12 h) | 168.6 ± 24.2 | 69.2 ± 10.6** |
| Peak frequency (peaks/12 h) | 10.2 ± 0.9 | 7.8 ± 0.8 |
| Peak interval (h) | 1.24 ± 0.11 | 1.51 ± 0.17 |
| Peak amplitude (ng/ml) | 39.1 ± 5.8 | 20.4 ± 3.6* |
| Peak area (ng/ml×h) | 12.8 ± 1.5 | 6.7 ± 1.1** |

GH secretory profiles were obtained from conscious, chronically catheterized male wild-type ($n$ = 5) and Tgr rats ($n$ = 6) and subjected to PULSAR analysis as described in Materials and methods. Data shown are mean ± SEM *$P$<0.05; **$P$<0.01 versus wild-type.

Four rGRF–hGH founders were generated, three of which grew more slowly than their non-transgenic littermates. The fourth founder, which had the GRF 5′-flanking sequences and part of the hGH gene deleted, showed a normal growth rate. The two higher copy number animals did not breed successfully, suggesting that there may be a gene dosage effect of hGH on fertility (Hammer *et al.*, 1984). However, a line was established from the single copy number founder #101, which we have termed Tgr (for Transgenic growth-retarded). In this line, hGH transgene expression was low, and highly restricted within the CNS. It was detectable by RT–PCR in the hypothalamus and anterior pituitary gland of Tgr rats, whilst extremely low level expression was also observed in cerebellum, cortex, lung and testis, matching endogenous GRF expression (Matsubara *et al.*, 1995). hGH was detectable by immunocytochemistry in the median eminence of founder 66 and in Tgr rats from line 101; no other CNS site showed hGH immunoreactivity. The low level of hypothalamic hGH expression was not unexpected, since hypothalamic GRF levels are low in normal animals (Katakami *et al.*, 1987). Furthermore, since the hGH transgene is driven from the rGRF promoter, transgene hGH production from GRF cells would inhibit its own expression.

The slight amounts of hGH mRNA and protein in the pituitary of Tgr rats could have reflected inappropriate

expression of the transgene, caused by regulatory sequences within the hGH gene (Slater *et al.*, 1985) directing expression to the anterior pituitary gland. This is unlikely, as relatively few anterior pituitary cells stained for hGH whereas somatotrophs make up ~40% of the cells in the anterior pituitary. Pituitary expression of the transgene is more likely to parallel the low level expression of the endogenous GRF gene in the anterior pituitary (Matsubara *et al.*, 1995). Hypothalamic GRF content was slightly but not significantly reduced in the Tgr hypothalamus. However, GRF content measurements reflect a balance between synthesis and release. For example, hypophysectomy (Katakami *et al.*, 1987; Chomczynski *et al.*, 1988) increases GRF mRNA but reduces hypothalamic GRF content. In contrast to the increased GRF mRNA levels normally observed in GH-deficient rats (Chomczynski *et al.*, 1988; De Gennaro Colonna *et al.*, 1988; Downs *et al.*, 1990; Mizobuchi *et al.*, 1991), Tgr rats showed a significant reduction in GRF mRNA levels despite their marked pituitary GH deficiency.

GH mRNA levels and GH content and cell numbers were reduced thoughout post-natal development of Tgr rats. ACTH and TSH levels were normal, suggesting that the corticotroph and thyrotroph populations were unaffected by transgene expression. However, Tgr rats also showed a reduced PRL mRNA level and PRL content. Reduction in PRL is not simply a consequence of somatotroph hypoplasia, as PRL levels are normal in the spontaneous dwarf rat (Kineman *et al.*, 1989). Since hGH also binds to PRL receptors in the rat (Roupas and Herington, 1989), a decrease in PRL is consistent with a hypothalamic feedback inhibition of the PRL system by hGH (Steger *et al.*, 1991; Phelps and Bartke, 1995).

GH release is highly episodic in the rat (Tannenbaum and Martin, 1976), and effective analysis requires multiple serial sampling. We showed that, in chronically catheterized conscious male Tgr rats, the normal episodic pattern of GH secretion was maintained, but with a markedly reduced pulse amplitude. Other physiological studies in these animals have shown that they release GH in response to GRF, and that their dwarfism can be corrected by chronic treatment with GH secretagogues (Wells *et al.* 1995). This first specific model of GRF-responsive GH-deficient dwarfism in the rat will therefore prove invaluable in studies of the physiological control of the somatotroph by GRF and of the effectiveness of GH secretagogues in the treatment of GH deficiency.

## Materials and methods

### Screening of cosmid libraries

A rGRF cDNA clone (Mayo *et al.*, 1985) was used to screen a rat genomic DNA library (kindly provided by Dr Noel Buckley) in the cosmid vector pWE15. Positive clones were Southern blotted, and strongly hybridizing clones were restriction mapped and compared with previously isolated clones (Mayo *et al.*, 1985).

### Construction of the rGRF–hGH transgene

An 11 kb *Kpn*I fragment containing 4 kb of 5′-flanking sequence, exons 1–4 of the rGRF gene and 2 kb of intron 4 was subcloned, and an *Mlu*I site was introduced at the *Pvu*I site in the 5′ UTR of the rGRF gene. A 1.5 kb (*Bam*HI–*Ssp*I) *Mlu*I-linkered fragment containing the entire hGH gene (DeNoto *et al.*, 1981) with its own ATG initiator codon, introns, stop codon and polyadenylation signal [*Bam*HI–*Ssp*I (DeNoto *et al.*, 1981)] was inserted into the *Mlu*I site in the appropriate orientation. The 11 kb *Kpn*I fragment of cos rGRF-S3 was replaced by the 12.5 kb rGRF–GH *Kpn*I fragment to generate cos GRF-GH, and packaged using Gigapack II Plus packaging extract (Stratagene).

### Generation of transgenic rats

All experiments in the generation and analysis of transgenic rats were carried out in accordance with local and National ethical guidelines. Cos GRF-GH was digested with *Not*I and the 38 kb insert fragment was separated from the vector by centrifugation on a 5–25% salt gradient (Dillon and Grosveld, 1993). DNA (2 ng/μl) was injected into the male pronucleus of fertilized one-cell rat oocytes using standard techniques (Hogan *et al.*, 1986). Viable eggs were transferred into the oviducts of pseudopregnant surrogates under halothane anaesthesia, and tail biopsies were taken from the resulting pups using local anaesthesia. Transgenic pups were identified by Southern blot analysis using a 1 kb *Pvu*II hGH 3′ probe (DeNoto *et al.*, 1981) or a 700 bp *Bgl*II–*Hin*dIII rGRF intron 1 probe (Mayo *et al.*, 1985) for copy number determination.

### Reverse transcriptase–polymerase chain reaction

Reverse transcription on 1 μg of total cellular RNA was carried out using a cDNA Cycle Kit (Invitrogen) according to the manufacturer's instructions. Primers used were: 5′ hGH, TAGCTGCAATGGCTACAGGCT; 3′ hGH, TTAGGAGGTCATAGACGTTGCTGTC; 5′ rat β-actin, TTGTAACCAACTGGGACGATATGG; 3′ rat β-actin, GATCTTGATCTTCATGGTGCTAGG. PCR conditions used were as follows. Reactions were incubated at 94°C for 5 min then 1 unit of *Taq* DNA polymerase (Cetus) was added. PCR amplification was performed for 35 cycles of: denaturation at 94°C for 1 min; annealing at 59°C for 1 min; extension at 72°C for 50 s, followed by a final extension at 72°C for 5 min. Products were analysed by electrophoresis on a 2% agarose gel.

### S1 nuclease and RNase protection analysis of tissue RNA

Tissue was taken from Southern blot-positive line 101 hemizygotes and their non-transgenic littermates and RNA was prepared by the LiCl–urea method (Auffray and Rougeon, 1980). S1 nuclease protection analysis (Berk and Sharp, 1977) was performed on 3 μg of total pituitary RNA using $^{32}$P end-labelled probes. For rGH mRNA measurement, a 292 bp *Sac*I–*Bsp*HI probe from intron 4 and exon 5 of the rGH gene (Page *et al.*, 1981) was used, giving a 161 nt protected fragment. For rPRL, a 339 bp *Acc*I–*Bgl*II rPRL cDNA fragment (Maurer *et al.*, 1981) was cloned into the *Hin*dIII site of pUC19 and a 317 bp *Pvu*II–*Bsp*EI fragment was used, giving a 133 nt protected fragment. A 320 bp *Bam*HI–*Ava*I human β-actin cDNA fragment from pHFβA-1 (Gunning *et al.*, 1983) was used for β-actin mRNA measurement, giving a 112 nt protected fragment.

RNase protection analysis (Melton *et al.*, 1984) was performed as previously described (Gabriellson *et al.*, 1995) using 20 μg of total hypothalamic RNA. Probes used were a 212 nt RNA probe complementary to the rGRF cDNA (*Eco*RI–*Hin*dIII) (Mayo *et al.*, 1985), giving a protected fragment of 205 nt. Following electrophoresis, signals were detected by autoradiography.

### Radioimmunoassay

Anterior pituitary glands were homogenized in phosphate-buffered saline (PBS) and assayed for GH, PRL, ACTH, TSH and LH by specific RIAs using reagents donated by the NIDDK reagents program. Human GH was assayed by a specific hGH RIA that did not cross-react with rat GH, as previously described (Fairhall *et al.*, 1992). Rat GRF was assayed as described by Tsagarakis *et al.* (1989).

### Immunocytochemistry

Pituitary glands and hypothalami from Tgr and wild-type rats were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 24 h prior to embedding in paraffin wax. hGH was localized in 4 μm sections of hypothalamic and pituitary tissue using an avidin–biotin (AB)–immunoperoxidase method as previously described (Brown *et al.*, 1993) but with a primary sheep anti-hGH serum (incubated at 1:30 000 overnight; Scottish Antibody Production Unit, Lanarkshire, UK). Double sequential immunostaining for rGH and rPRL in anterior pituitary sections was performed with a combined AB–immunoperoxidase–alkaline phosphatase procedure (Brown *et al.*, 1993) following overnight incubation with a monkey anti-rGH serum at 1:2000 and a rabbit anti-rPRL serum: 1:8000; NHPP, Rockville, MD, USA. AB–horseradish peroxidase complexes were visualized using a solution of 3,3-diaminobenzidine tetrahydrochloride (4 mg/10 ml) containing 3% $H_2O_2$. AB–alkaline phosphatase complexes were visualized with a 5-bromo-4-

chloro-3-indoxyl phosphate/nitro blue tetrazolium chloride mixture containing 1 mM levimisole to block endogenous phosphatase activity. Sections were counterstained with haematoxylin or neutral red. Control sections were incubated either with normal control serum in place of primary antibody, or in primary antibody pre-absorbed overnight with excess hormone (10 μg/ml). The proportion of rGH- and rPRL-immunopositive cells was determined by cell counting using an eyepiece graticule and expressed as a percentage of the total cell number of each field.

### *Blood sampling*

Serial blood samples (20 μl) were obtained from chronically catheterized conscious Tgr and wild-type adult male rats using an automated microsampling system and assayed for rat GH by RIA as previously described (Clark *et al.*, 1986). Blood GH profiles were analysed using the PULSAR algorithm (Merriam and Wachter, 1982) with the following parameters G(1) 3.98; G(2) 2.40; G(3) 1.68; G(4) 1.24; G(5) 0.93.

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## References

Auffray,C. and Rougeon,F. (1980) *Eur. J. Biochem.*, **107**, 303–314.

Banerjee,S.A., Roffler-Tarlov,S., Szabo,M., Frohman,L. and Chikaraishi,D. (1994) *Mol. Brain Res.*, **24**, 89–106.

Barinaga,M., Yamamoto,G., Rivier,C., Vale,W., Evans,R. and Rosenfeld, M.G. (1985a) *Nature*, **306**, 84–85.

Barinaga,M., Bilezikjian,L.M., Vale,W., Rosenfeld,M.G. and Evans,R. (1985b) *Nature*, **314**, 279–281.

Bchini,O., Andres,A.C., Schubaur,B., Mehtali,M., LeMeur,M., Lathe,R. and Gerlinger,P. (1991) *Endocrinology*, **128**, 539–546.

Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721–732.

Berry,S.A. and Pescovitz,O.H. (1988) *Endocrinology*, **123**, 661–663.

Bilezikjian,L.M. and Vale,W. (1984) *Endocrinology*, **115**, 2032–2034.

Billestrup,N., Swanson,L.W. and Vale,W. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 6854–6857.

Bloch,B., Brazeau,P., Ling,N., Bohlen,P., Esch,F., Wehrenburg,W.B., Benoit,R., Bloom,F. and Guillemin,R. (1983) *Nature*, **301**, 607–608.

Botteri,F.M., van der Putten,H., Wong,D.F., Sauvage,C.A. and Evans,R.M. (1987) *Mol. Cell. Biol.*, **7**, 3178–3184.

Brown,P., McNeilly,J.R., Wallace,R.M., McNeilly,A.S. and Clark,A.J. (1993) *Mol Cell Endocrinol.*, **93**, 157–165.

Burton,K.A., Kabigting,E.B., Clifton,D.K. and Steiner,R.A. (1992) *Endocrinology*, **130**, 958–963.

Carmignac,D.F. and Robinson,I.C.A.F. (1990) *J. Endocrinol.*, **127**, 69–75.

Charlton,H.M., Clark,R.G., Robinson,I.C.A.F., Porter-Goff,A.E., Cox,B.S., Bugnon,C. and Bloch,B.S. (1988) *J. Endocrinol.*, **119**, 51–58.

Chomczynski,P., Downs,T.R. and Frohman,L.A. (1988) *Mol. Endocrinol.*, **2**, 236–241.

Clark,R.G. and Robinson,I.C.A.F. (1985) *Nature*, **314**, 281–283.

Clark,R.G., Chambers,G., Lewin,J. and Robinson,I.C.A.F. (1986) *J. Endocrinol.*, **111**, 27–35.

De Gennaro Colonna,V., Cattaneo,E., Cocchi,D., Muller,E.E. and Maggi,A. (1988) *Peptides*, **9**, 985–988.

DeNoto,F.M., Moore,D.D. and Goodman,H.M. (1981) *Nucleic Acids Res.*, **9**, 3719–3730.

Dillon,N. and Grosveld,F. (1993) In Hames,B.D. and Higgins,S.J. (eds), *Gene Transcription—A Practical Approach*. IRL Press at Oxford University Press, pp. 153–187.

Downs,T.R., Chomczynski,P. and Frohman,L.A. (1990) *Mol. Endocrinol.*, **4**, 402–408.

Eicher,E.M. and Beamer,W.G. (1976) *J. Hered.*, **67**, 87–91.

Fairhall,K.M., Carmignac,D.F. and Robinson,I.C.A.F. (1992) *Endocrinology*, **131**, 1963–1969.

Flavell,D.M., Wells,T., Wells,S.E., Carmignac,D.F., Thomas,G.B. and Robinson,I.C.A.F. (1995) *77th Annual Meeting*. The US Endocrine Society, Washington, Abstract P2-239.

Fukata,J., Diamond,D.S. and Martin,J.B. (1985) *Endocrinology*, **117**, 457–467.

Gabrielsson,B.G., Carmignac,D.F., Flavell,D.M. and Robinson,I.C.A.F. (1995) *Endocrinology*, **136**, 209–217.

Gargosky,S.E., Brier,B.H., Borski,R.J., Mulcahy,J. and Barnard,R. (1995) *Endocrine*, **3**, 813–817.

Giraldi,F.P., Mizobuchi,M., Horowitz,Z.D., Downs,T.R., Aleppo,G., Kier,A., Wagner,T., Yun,J.S., Kopchick,J.J. and Frohman,L.A. (1994) *Endocrinology*, **134**, 1219–1224.

Godfrey,P., Rahal,J.O., Beamer,W.G., Copeland,N.G., Jenkins,N.A. and Mayo,K.E. (1993) *Nature Genet.*, **4**, 227–232.

Gonzalez-Crespo,S. and Boronat,A. (1990) *Gene*, **93**, 321–322.

Gonzalez-Crespo,S. and Boronat,A. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 8749–8753.

Grosveld,F.G., Blom von Assendelft,M., Greaves,D. and Kollias,G. (1987) *Cell*, **51**, 975–985.

Guillemin,R., Brazeau,P., Bohlen,P., Esch,F., Ling,N. and Wehrenburg, W.B. (1982) *Science*, **218**, 585–587.

Gunning,P., Ponte,P., Okayama,H., Engel,J., Blau,H. and Kedes,L. (1983) *Mol. Cell. Biol.*, **3**, 787–795.

Hammer,R.E., Palmiter,R.D. and Brinster,R.L. (1984) *Nature*, **311**, 65–67.

Hogan,B.L.H., Constantini,F. and Lacy,E. (1986) *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hollingshead,P.G., Martin,L., Pitts,S.L. and Stewart,T.A. (1989) *Endocrinology*, **125**, 1556–1564.

Ikeda,A., Chang,K., Matsuyama,S., Nishihara,M. and Takahashi,M. (1995) *77th Annual Meeting*. The US Endocrine Society, Washington, Abstract P2-241.

Jones,B.K., Monks,R.R., Liebhaber,S.A. and Cooke,N.E. (1995) *Mol. Cell. Biol.*, **15**, 7010–7021.

Katakami,H., Downs,T.R. and Frohman,L.A. (1987) *Endocrinology*, **120**, 1079–1082.

Kineman,R.D., Chen,T.T. and Frawley,L.S. (1989) *Endocrinology*, **125**, 2035–2040.

Li,S., Crenshaw,E.B., Rawson,E.J., Simmons,D.M., Swanson,L.W. and Rosenfeld,M.G. (1990) *Nature*, **347**, 528–533.

Lin,S.-C., Lin,C.R., Gukovsky,I., Lusis,A.J., Sawchenko,P.E. and Rosenfeld,M.G. (1993) *Nature*, **364**, 208–213.

Lobie,P.E., Garcia-Aragon,J., Lincoln,D.T., Barnard,R., Wilcox,J.N. and Waters,M.J. (1993) *Dev. Brain Res.*, **74**, 225–233.

Margioris,A.N., Brockmann,G., Bohler,H.C.L., Grino,M., Vamvakopoulos,N. and Chrousos,G.P. (1990) *Endocrinology*, **126**, 151–158.

Matsubara,S., Sato,M., Mizobuchi,M., Niimi,M. and Takahara,J. (1995) *Endocrinology*, **136**, 4147–4150.

Maurer,R.A., Erwin,C.R. and Donelson,J.E. (1981) *J. Biol. Chem.*, **256**, 10524–10528.

Mayo,K.E., Cerelli,G.M., Rosenfeld,M.G. and Evans,R.M. (1985) *Nature*, **314**, 464–467.

Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.

Merriam,G.R. and Wachter,K.W. (1982) *Am. J. Physiol.*, **243**, E310–E318.

Mizobuchi,M., Frohman,M.A., Downs,T.R. and Frohman,L.A. (1991) *Mol. Endocrinol.*, **5**, 476–484.

Monts,B.S., Breyer,P.R., Rothrock,J.K. and Pescovitz,O.H. (1996) *Endocrine*, **4**, 73–78.

Morello,D., Moore,D.D., Salmon,A.M., Yaniv,M. and Babinet,C. (1986) *EMBO J.*, **5**, 1877–1883.

Page,G.S., Smith,S. and Goodman,H.M. (1981) *Nucleic Acids Res.*, **9**, 2087–2104.

Palmiter,R.D., Brinster,R.L., Hammer,R.E., Trumbauer,M.E., Rosenfeld, M.G., Birnberg,N.C. and Evans,R.M. (1982) *Nature*, **300**, 611–615.

Phelps,C.J. and Bartke,A. (1995) *77th Annual Meeting*. The US Endocrine Society, Washington, Abstract P1-18.

Rivier,J., Spiess,J., Thorner,M. and Vale,W. (1982) *Nature*, **300**, 276–278.

Roupas,P. and Herington,A.C. (1989) *Mol. Cell. Endocrinol.*, **61**, 1–12.

Roux,M., Bartke,A., Dumont,F. and Dubois,M.P. (1982) *Cell Tissue Res.*, **223**, 415–420.

Skottner,A., Clark,R.G., Fryklund,L. and Robinson,I.C.A.F. (1989) *Endocrinology*, **124**, 2519–2526.

Slater,E.P., Rabenau,O., Karin,M., Baxter,J.D. and Beato,M. (1985) *Mol. Cell. Biol.*, **5**, 2984–2992.

Spiess,J., Rivier,J. and Vale,W. (1983) *Nature*, **303**, 532–535.

Steger,W.R.W., Bartke,A., Parkening,T.A., Collins,T., Buonomo,F.C., Tang,K., Wagner,T.E. and Yun,J.S. (1991) *Neuroendocrinology*, **53**, 365–372.

Szabo,M., Butz,M.R., Banerjee,S.A., Chikaraishi,D.M. and Frohman, L.A. (1994) *Endocrinology*, **136**, 4044–4048.
Takeuchi,T., Suzuki,H., Sakurai,S., Nogami,H., Okuma,S. and Ishikawa,H. (1990) *Endocrinology*, **126**, 31–38.
Tannenbaum,G.S. and Martin,J.B. (1976) *Endocrinology*, **98**, 562–570.
Thorner,M.O., Vance,M.L., Rogol,A.D., Blizzard,R.M., Veldhuis,J.D., Cauter,E.V., Copinschi,G. and Bowers,C.Y. (1990) *Acta Paediatr. Scand. Suppl.*, **367**, 29–32.
Tsagarakis,S., Ge,F., Rees,L.H., Besser,G.M. and Grossman,A. (1989) *J. Neuroendocrinol.*, **1**, 129–133.
Wells,T., Flavell,D.M., Wells,S.E., Carmignac,D.F. and Robinson, I.C.A.F. (1995) *77th Annual Meeting*. The US Endocrine Society, Washington, Abstract P2-240.

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