GHF-1–promoter-targeted immortalization of a somatotropic progenitor cell results in dwarfism in transgenic mice

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During pituitary development, the homeo domain protein GHF-1 is required for generation of somatotropes and lactotropes and for growth hormone (*GH*) and prolactin (*PRL*) gene expression. *GHF-1* mRNA is detectable several days before the emergence of GH- or PRL-expressing cells, suggesting the existence of a somatotropic progenitor cell in which *GHF-1* transcription is first activated. We have immortalized this cell type by using the *GHF-1* regulatory region to target SV40 T-antigen (Tag) tumorigenesis in transgenic mice. The *GHF*–Tag transgene caused developmental entrapment of somatotropic progenitor cells that express *GHF-1* but not *GH* or *PRL*, resulting in dwarfism. Immortalized cell lines derived from a transgenic pituitary tumor maintain the characteristics of the somato/lactotropic progenitor in that they express *GHF-1* mRNA and protein yet fail to activate *GH* or *PRL* transcription. Using these cells, we identified an enhancer that activates *GHF-1* transcription at this early stage of development yet is inactive in cells representing later developmental stages of the somatotropic lineage or in other cell types. These experiments not only demonstrate the potential for immortalization of developmental progenitor cells using the regulatory regions from cell type-specific transcription factor genes but illustrate the power of such model systems in the study of developmental control.

[*Key Words:* Transgenic mice; transcription factor GHF-1; POU homeo domain; progenitor cell; targeted oncogenesis.]

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Understanding the regulatory hierarchies that control development of cell lineages in mammalian organogenesis has been difficult owing to a paucity of tractable experimental systems. Traditional approaches employed in invertebrate systems, such as genetic analysis or single cell ablation, are often not practical in mammals, which therefore require novel strategies.

The anterior pituitary is a useful model system for studying mammalian organogenesis (for review, see Voss and Rosenfeld 1992). During the development of this gland, five endocrine cell types arise in a specific temporal pattern (Simmons et al. 1990). On embryonic day 11 (e11) of the rat, prior to the emergence of Rathke's pouch (the anlagen for the anterior pituitary) from an out-pocketing of the oral ectoderm, transcription of the gene encoding the α-subunit of the glycoprotein hormones becomes detectable. This represents the first discernible step in commitment of the developing anterior pituitary. It is followed by expression of the pro-opiomelanocortin (POMC) gene in developing corticotropes. Cells that express the three β subunits of the glycoprotein hormones emerge in turn: thyrotropes expressing thyroid-stimulating hormone (TSH) β on e14, and gonadotropes expressing luteinizing hormone (LH) β on e15–16 and follicle-stimulating hormone (FSH) β on e17. Two additional cell types, somatotropes, which express growth hormone (GH) arising on e17 in the rat (e15 in the mouse), and lactotropes, which express prolactin (PRL) arising postnatally (Hoeffler et al. 1985; Leong et al. 1985; Nogami et al. 1989; Dollé et al. 1990; Simmons et al. 1990), are thought to be derived from a common precursor, the somatomammotrope (for review, see Karin et al. 1990). This cell, which exists during embryonic development and in small numbers in the adult, transiently produces both GH and PRL before commitment to a final differentiated phenotype (somatotrope or lactotrope).

The genes for *GH* and *PRL* evolved from a common ancestor (Cooke et al. 1981; Cooke and Baxter 1982) and share common developmental regulatory signals, in that both promoters have multiple binding sites for the pituitary-restricted POU homeo domain transcription factor,

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GHF-1 (also termed Pit-1; Bodner and Karin 1987; Lefevre et al. 1987; Bodner et al. 1988; Ingraham et al. 1988; Nelson et al. 1988). Transfection analyses and in vitro transcription (Bodner and Karin 1987; Lefevre et al. 1987; Nelson et al. 1988; Mangalam et al. 1989) have shown these sites to be important for tissue-specific expression of both genes in somatotrope/lactotrope cell lines such as GC, GH_3, or 235 cells (Bancroft 1981; Reymond et al. 1984). The POU homeo domain family of transcriptional regulatory proteins includes several cell type-specific and ubiquitous transcriptional activators, many of which are involved in cell fate determination (Herr et al. 1988; Rosenfeld 1991). Several members of this class are involved in developmental regulation of specific genes and act in concert with other factors to initiate commitment toward terminal differentiation (Ruvkun and Finney 1991).

GHF-1 function is absolutely required for development of the somatotropic lineage. Mice with naturally occurring mutations or deletions of the *GHF-1* gene fail to develop somatotropes and lactotropes and exhibit anterior pituitary hypoplasia and dwarfism (Li et al. 1990; Castrillo et al. 1991). In addition to activation of the *GH* and *PRL* genes, *GHF-1* is also required for expansion of the somatotropic lineage (Castrillo et al. 1991) and maintenance of thyrotropes (Li et al. 1990). During development of the mouse anterior pituitary, *GHF-1* transcription is detected at least 2 days before the emergence of somatomammotropic cells expressing *GH* and *PRL* transcripts, indicating the potential existence of a *GHF-1*-expressing progenitor for this lineage. This progenitor cell expresses high levels of *GHF-1* transcripts and low levels of GHF-1 protein (Dollé et al. 1990), but the mechanism responsible for inefficient translation of *GHF-1* transcripts at this stage of development is not known. Increased expression of GHF-1 protein correlates with the onset of *GH* gene expression (Dollé et al. 1990).

In addition to the *GH* and *PRL* genes, *GHF-1* is also involved in controlling its own transcription through positive autoregulation (Chen et al. 1990; McCormick et al. 1990). The minimal *GHF-1* promoter fragment required for maximal cell type-specific expression in committed somatotrope cell lines (GC or GH_3 cells) consists of one *GHF-1*-binding site and another pituitary-specific element centered around the TATA box (McCormick et al. 1991). Because *GHF-1* transcription is detected before the appearance of GHF-1 protein (and, therefore, *GHF-1* cannot be responsible for developmental activation of its own gene), another mechanism must be responsible for the highly specific and restricted activation of this gene in somatotropic progenitors on e13 in the mouse (Dollé et al. 1990). Identification of the mechanism responsible for the initial activation of *GHF-1* is essential for understanding the regulatory hierarchies that control the development of the anterior pituitary. Elucidation of this mechanism requires the isolation of the somatotropic progenitor.

Immortalization of cells at specific stages of differentiation can lead to an understanding of lineage relationships and has been informative in studies of hematopoietic cell lineages. Oncogenesis can be targeted to specific cell types in transgenic mice using upstream transcriptional regulatory sequences of specialized genes (Stewart et al. 1984; Hanahan 1989). Previously, we established immortal pituitary cell lines by directing expression of the SV40 large T antigen (Tag) oncoprotein to anterior pituitaries of transgenic mice using the regulatory region of the earliest pituitary marker, the glycoprotein hormone α-subunit gene (Windle et al. 1990), as well as a later marker of the gonadotrope lineage, the LH β-subunit gene (J.J. Windle, D.B. Whyte, and P.L. Mellon, in prep.). Remarkably, these cells are apparently frozen at the step in development at which the specific regulatory region is first activated and maintain the characteristics representing sequential developmental steps in the gonadotropic lineage (Sealfon et al. 1990; Windle et al. 1990; Horn et al. 1991, 1992; Mellon et al. 1991; Schoderbek et al. 1992; Tsutsumi et al. 1992). Given that immortalization targeted by the regulatory regions of genes for sequentially expressed known cellular products results in cells representing different steps in a developmental lineage, we reasoned that it might be possible to target successively earlier progenitor cells using the regulatory regions of genes encoding the transcriptional regulators that activate the expression of such cell type-specific markers.

To test this possibility, we targeted the putative somatotropic progenitor using the regulatory region of the *GHF-1* gene to express Tag. Transgenic animals expressing a *GHF*–Tag transgene are severely dwarfed and develop pituitary tumors that express *GHF-1* yet fail to express *GH* or *PRL* . Cell lines derived from such a pituitary tumor exhibited the characteristics of the somatotropic progenitor and provided a culture system that allowed the identification of a developmental stage-specific enhancer controlling the expression of *GHF-1*, which is inactive in later developmental stages.

Results

Targeting expression of Tag using the regulatory region of GHF-1

To target oncogenesis to the putative somatotropic progenitor, we utilized the 5′-flanking region of the *GHF-1* gene to direct expression of Tag in transgenic mice. Although transfections into a somatotropic cell line, GC, had indicated that as little as 200 bp of 5′-flanking region was sufficient for cell-specific expression and hormonal regulation of the *GHF-1* promoter (Chen et al. 1990; McCormick et al. 1990, 1991), we created transgenic mice using a larger fragment of the rat *GHF-1* 5′-flanking region (2.5 kb; also shown to be fully active in GC cells) to express Tag (Fig. 1). Analysis of three independent lines of −2.5*GHF*–Tag transgenic mice revealed no pituitary tumors, nor was expression of Tag RNA detectable in the pituitary (Fig. 2). Paradoxically, all three transgenic lines consistently developed Tag-expressing intestinal tumors at varying ages of onset from 7 to 14 months (Table 1; Fig. 2). Adjacent nontumorous intestinal tissue did not

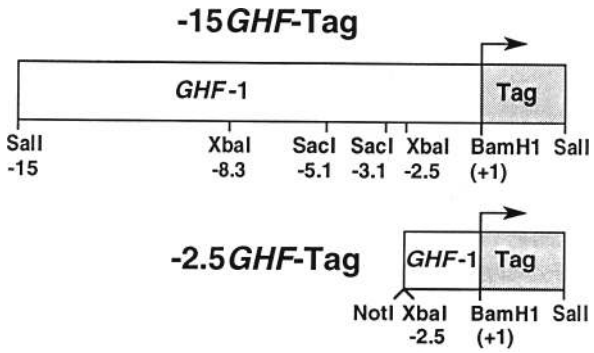

Figure 1. Structure of the hybrid transgenes −15*GHF*–Tag and −2.5*GHF*–Tag. The −15*GHF*–Tag construct contains *GHF-1* sequences from a *Sal*I site at −15 kb to an artificially generated *Bam*HI site at +1 of the rat *GHF-1* gene (McCormick et al. 1990). The −2.5*GHF*–Tag construct contains sequences from the *Xba*I site at −2.5 kb to the *Bam*HI site at +1. The regulatory regions were fused to the structural gene for SV40 Tag (Windle et al. 1990). The locations of several restriction enzyme recognition sites within the *GHF-1* promoter are indicated.

express Tag mRNA (data not shown). Furthermore, these intestinal tumors do not express the endogenous mouse *GHF-1* mRNA (Fig. 2), and histological analysis indicated that they were leiomyosarcomas of smooth muscle origin (data not shown). These tumors most likely result from ectopic expression of the transgene in cells of the gut that are unrelated in their embryonic origin to the endocrine cells of the anterior pituitary. Regardless, the lack of expression in the pituitary indicates that the proximal 2.5 kb of 5′-flanking region of the *GHF-1* gene was not sufficient to target somatotrope-specific expression in vivo.

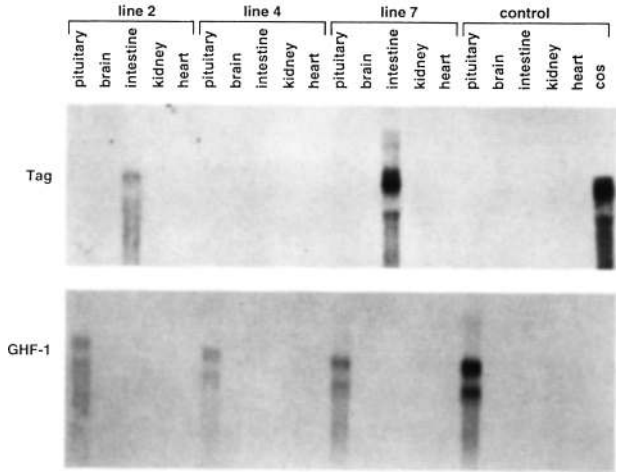

Figure 2. Expression of Tag and *GHF-1* mRNAs in −2.5*GHF*–Tag mice. Total RNAs from the indicated tissues of −2.5*GHF*–Tag transgenic mice from lines 2, 4, and 7 were analyzed by Northern blot hybridization with a radiolabeled probe for Tag (*top*) or *GHF-1* (*bottom*). The low levels of Tag expression in the intestinal tumors of line 4 were not detectable by Northern analysis but were evident in polymerase chain reaction (PCR) analysis (data not shown).

Table 1. *Incidence of intestinal tumors in −2.5GHF–Tag mice*

Transgenic mouse line	Average age at tumor development (months)	Tumor incidence (%)
−2.5*GHF*–Tag-2	9.5	100
−2.5*GHF*–Tag-4	14.7	50
−2.5*GHF*–Tag-7	7.0	100

In contrast, a larger fragment of the rat *GHF-1* 5′-flanking DNA (15 kb) specifically targeted Tag expression to the anterior pituitary (−15*GHF*–Tag transgene; Fig. 1). Three of the four −15*GHF*–Tag transgenic mice obtained were phenotypically dwarf compared to their nontransgenic, sex-matched littermates (Fig. 3A). The growth rate was reduced, and the weight was ~40% of nontransgenic littermates at 6 weeks of age (Fig. 3B). Two of the transgenic mice survived to ages at which normal mice are reproductive (15 and 18 weeks of age). However, neither was fertile, preventing derivation of pedigrees. The testes in both of these male animals ap-

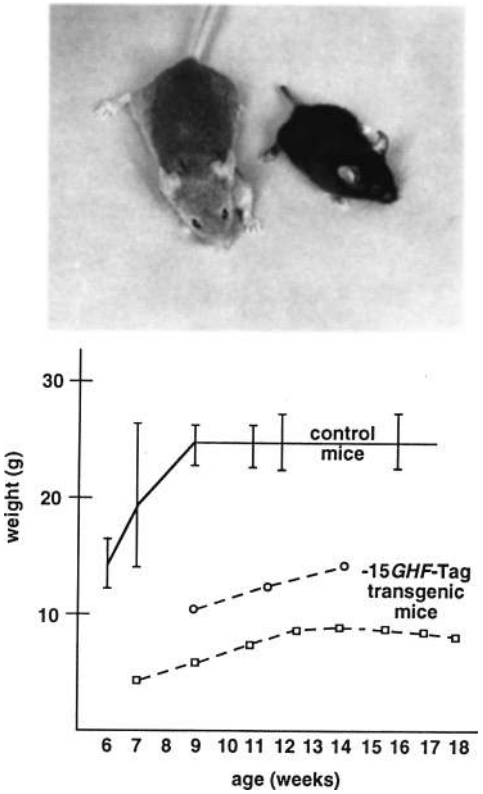

Figure 3. Transgenic −15*GHF*–Tag mice exhibit a dwarf phenotype. (*Top*) The black transgenic mouse on the *right* is shown with its nontransgenic littermate at 14 weeks of age. (*Bottom*) −15*GHF*–Tag transgenic mice exhibit retarded growth. Two transgenic animals were ~40% of the weight of their sex-matched nontransgenic littermates at 9 weeks of age and were, at most, 52% of normal weight at sacrifice.

peared underdeveloped, suggesting that expression of the transgene inhibited normal sexual development or maturation. In contrast to the −2.5*GHF*–Tag transgenic mice, two dwarf founder mice with the −15*GHF*–Tag transgene developed pituitary tumors. These animals were sacrificed at ~15–18 weeks of age following the appearance of neurological signs consistent with pituitary tumors (Windle et al. 1990). The other two animals died at 5 and 8 weeks of age without manifestation of an external neurological phenotype.

RNA analysis revealed expression of both Tag and *GHF-1* in pituitary tumors but not in other transgenic tissues (Fig. 4). However, neither *GH* or *PRL* mRNA was detectable in the pituitary tissue of the transgenic animals. Furthermore, immunohistochemistry of the pituitary tumors revealed staining with GHF-1 and Tag antisera but not with GH antiserum (data not shown; background staining was observed with PRL antiserum but was easily distinguished from the bright punctate staining seen with the positive control pituitaries). Although it is not likely that every cell destined for the somatotropic lineage in the developing transgenic pituitary became transformed, the remaining cells apparently were overwhelmed by tumor growth. Thus, expression of the

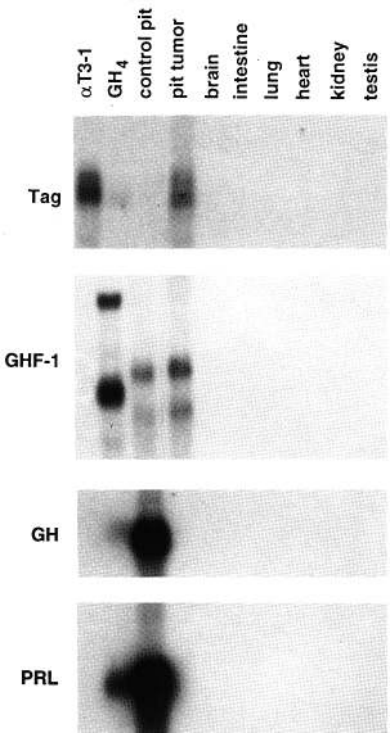

Figure 4. Gene expression in −15*GHF*–Tag-induced pituitary tumors. Five micrograms of total RNA from the indicated tissues from a −15*GHF*–Tag mouse was analyzed by Northern blot hybridization using radiolabeled cDNA probes for Tag, *GHF-1*, *GH*, and *PRL* . The αT3-1 cells are derived from Tag-induced transgenic mouse tumors and are pituitary gonadotrope progenitors (Windle et al. 1990). The positive control for GHF-1 RNA is from rat GH_4 cells that express transcripts of different lengths from mouse cells or mouse pituitary.

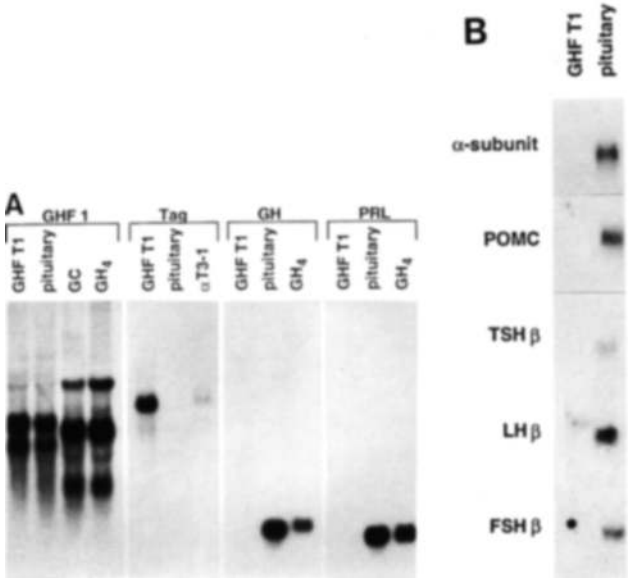

Figure 5. Gene expression in GHFT1 cells. (*A*) GHFT1 cells exhibit a somatotropic progenitor phenotype. Five micrograms of total RNA from the indicated cell lines was analyzed by Northern blot and hybridization to radiolabeled rat *GHF-1*, Tag, *GH*, and *PRL* cDNA probes. Longer exposures of Northern blots (up to 2 weeks) hybridized with either *GH* or *PRL* probes confirmed these results (data not shown). Size differences between *GHF-1* RNAs in the mouse-derived GHFT1 and rat-derived GC and GH_4 cells are the result of species differences, probably in the use of polyadenylation sites. (*B*) GHFT1 cells do not express RNAs characteristic of nonsomatotropic pituitary cells. Five micrograms of total RNA from the GHFT1 cell line or from control pituitaries was analyzed by Northern blot hybridization to radiolabeled cDNA probes for the α-subunit gene of the glycoprotein hormones, POMC, the TSHβ subunit, the LHβ subunit, and the FSHβ subunit.

Tag transgene was specifically directed and confined to the pituitary when under the control of the 15-kb regulatory region of the *GHF-1* gene, indicating the presence of tissue-specific regulatory sequences in the region from −15 to −2.5 kb upstream of the start of *GHF-1* transcription.

Establishment of an immortal somatotrope progenitor cell line

The pituitary tumors were cultured immediately upon removal from the sacrificed transgenic animals, as described previously (Windle et al. 1990). After 2 months of repeated passage on plastic culture dishes to remove fibroblasts, an immortalized cell population emerged (termed GHFT1). Northern blots of RNA from GHFT1 cells demonstrate that the characteristics of the original pituitary tumor have been maintained, that is, these cells express both Tag and *GHF-1* mRNAs but fail to express *GH* or *PRL* mRNAs (Fig. 5A). In contrast, the established somatotropic/lactotropic cell lines GC and GH_4 express *GHF-1*, *GH*, and *PRL* mRNAs, as these cells represent more differentiated somatotropes and/or so-

matomammotropes (Tashjian et al. 1968, 1970; Bancroft 1981). The level of *GHF-1* mRNA in GHFT1 cells is comparable to that in GC cells. Furthermore, the GHFT1 cells do not express other mRNAs characteristic of differentiated anterior pituitary phenotypes (Fig. 5B) such as the common α-subunit gene of the glycoprotein hormones (Chin et al. 1981), the corticotrope-specific POMC gene (Eberwine and Roberts 1984) or the β-subunit genes of TSH (Gurr et al. 1983), LH (Tepper and Roberts 1984), and FSH (Maurer 1987).

Morphologically, GHFT1 cells do not resemble other established cell lines of the somatotropic lineage such as GC, GH_3, and GH_4 cells. Different GHFT1 subclones vary in morphology from rounded nonadherent cells to relatively flat, adherent cells. Immunocytochemistry demonstrates the presence of nuclear staining for GHF-1 and Tag and the absence of staining for GH and PRL in the GHFT1 cells (Fig. 6). However, the staining for GHF-1 is less intense than the staining in GC cells. The GHFT1 cells are therefore unique in possessing characteristics of an early developmental phenotype. *GHF-1* mRNA is expressed, but activation of the *GH* or *PRL* genes [or potentially the TSHβ gene (Li et al. 1990)] have yet to occur.

There is a demonstrable lag period between the onset of *GHF-1* transcription in the mouse pituitary at e13.5 and the detection of the GHF-1 protein at e15.5 (Dollé et al. 1990). Although GHFT1 cells expressed as much *GHF-1* mRNA as GC cells did (Fig. 5A), indirect immunofluorescence of GHFT1 cells suggested that they express lower amounts of GHF-1 protein (Fig. 6). To compare the levels of GHF-1 more precisely, a Western blot of whole-cell extracts from GHFT1 and GC cells was probed with a GHF-1-specific antibody. The characteristic 33- and 31-kD GHF-1 doublet (Castrillo et al. 1989) was apparent in whole pituitary extracts (not shown), and in extracts of both GHFT1 cells and GC cells (Fig. 7A). As expected, it was absent from extracts of Rat6 fibroblasts. Expression of GHF-1 protein appears to be at least 10-fold lower in GHFT1 cells than in GC cells. The decreased GHF-1 signal in GHFT1 cells is probably not the result of reduced immunoreactivity of the anti-rat GHF-1 antiserum with the mouse GHF-1 protein because these antibodies were raised against a peptide that is 100% conserved between the rat and mouse proteins (Bodner et al. 1988; Li et al. 1990). DNase I protection assays indicate that the GHF-1 protein in GHFT1 nuclear extracts is fully capable of specific binding to the well-characterized *GHF-1*-binding sites in the rat *GH* gene and produces protection patterns identical to those produced by 235 and GC cell nuclear extracts (Fig. 7B). Because these assays are performed in protein excess, they do not reflect the relative abundance of the GHF-1 protein in the various cell lines.

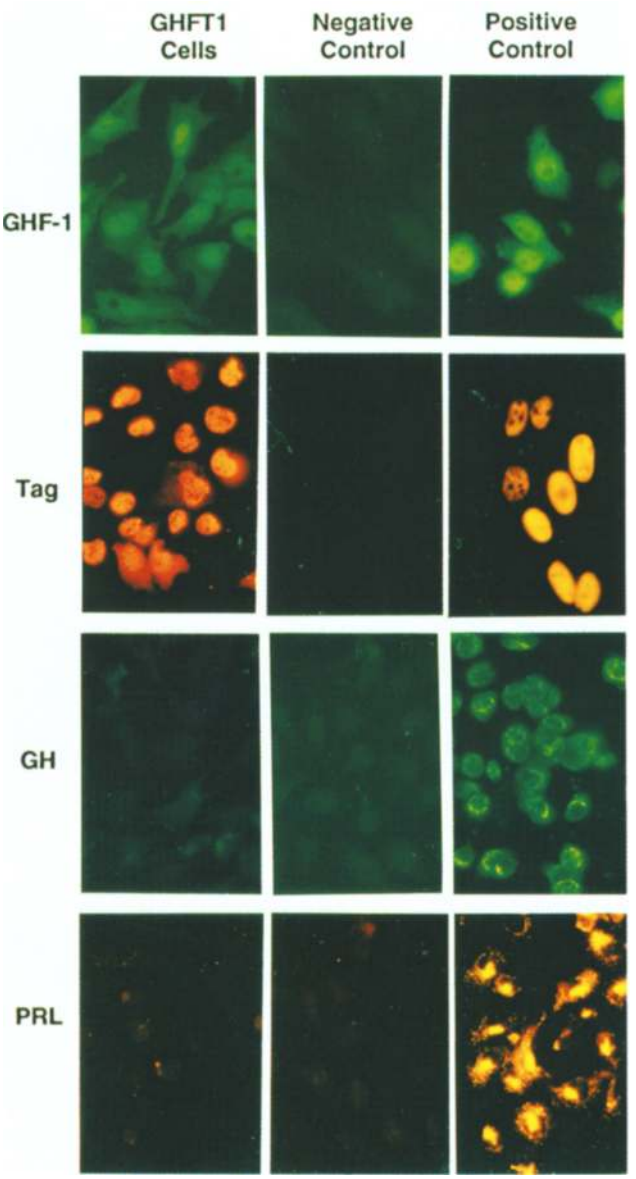

Figure 6. Immunohistochemical analysis of GHFT1 cells. Cells were fixed and immunostained with fluoroscein-labeled antibodies to GHF-1 or GH , or rhodamine-labeled antibodies to Tag or PRL . (From left to right) Cells stained with anti-GHF-1: GHFT1, Rat2 fibroblasts, GH_3; cells stained with anti-Tag—GHFT1, Rat2, HF (SV40-transformed human fibroblasts); cells stained with anti-GH: GHFT1, HeLa, GC; cells stained with anti-PRL: GHFT1, HeLa, 235 prolactinoma.

GH *and* PRL *promoters are inactive in GHFT1 cells*

To investigate whether the lack of *GH* and *PRL* expression in GHFT1 cells is determined at the level of transcription, transfections were performed with luciferase (Luc) reporter genes containing the rat *GH* and *PRL* promoters into somatotrope (GC) and lactotrope (235) cell lines (Fig. 8A). While the GH–Luc reporter gene was efficiently expressed in GC cells and the PRL–Luc reporter gene was efficiently expressed in 235 cells, neither reporter gene was active in GHFT1 cells. Similar results were obtained with a GH–chloramphenicol acetyltransferase (CAT) reporter gene (data not shown). The lack of *GH* promoter activity in GHFT1 cells is unlikely to be

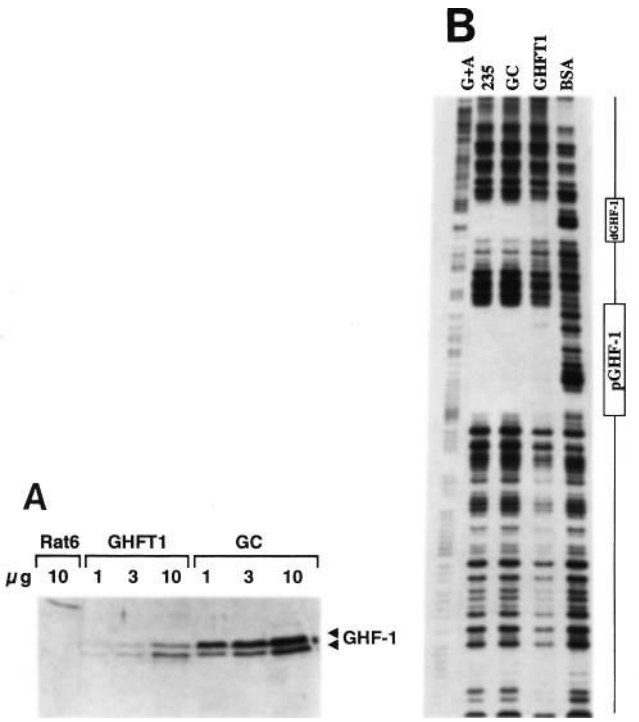

Figure 7. GHF-1 protein is expressed in GHFT1 cells. (*A*) Whole-cell extracts (1, 3, or 10 μg) were separated by SDS-PAGE and analyzed by Western blotting using anti-GHF-1 antibodies (Bodner et al. 1988). A Rat6 fibroblast whole-cell extract (10 μg) was included as a negative control. (*B*) DNase I footprinting demonstrates that GHF-1 from GHFT1 cells binds to the *GH* promoter region. A 312-bp fragment from the rGH promoter was incubated with 2.5 μg of nuclear protein extract from GHFT1, 235, or GC cells and digested with DNase I. The position of the proximal (p) and distal (d) *GHF-1*-binding sites in the rGH promoter (Lefevre et al. 1987) are indicated. The Maxam–Gilbert G+A and the BSA control digestion ladder are shown for reference. Note that these assays were performed under conditions of nuclear extract excess and thus are not reflective of the relative levels of GHF-1-binding activity.

attributable to an influence of the Tag oncoprotein because cotransfection of a GH–CAT reporter into GC cells together with a Tag expression vector resulted in modest enhancement of expression, rather than repression (Fig. 8B).

The GHF-1 *gene contains a tissue- and stage-specific enhancer*

The inability of the −2.5*GHF*–Tag transgene to be expressed in the anterior pituitary was surprising in light of previous studies indicating that this fragment, or as little as the first 200 bp of the *GHF-1* 5′-flanking region, was equally sufficient for maximal cell type-specific expression and hormonal regulation in determined somatotrope/lactotrope cell lines such as GC, GH_3, and 235 (Chen et al. 1990; McCormick et al. 1990, 1991; K. Klausing, unpubl.). To further investigate the regulation of *GHF-1* transcription in GHFT1 cells, we compared the activity of the minimal −200 *GHF-1* promoter region in GHFT1 cells with that in GC cells. The minimal *GHF-1* promoter was at least fivefold less active in GHFT1 cells than in GC cells (Fig. 9A). As was the case with the *GH* promoter, no effect was observed in *GHF-1* promoter activity owingto cotransfection with a Tag expression vector in GC cells (Fig. 8B).

The pituitary-specific expression of the −15*GHF*–Tag transgene, on the other hand, suggested the presence of cell type-specific regulatory elements in the region between −2.5 and −15 kb upstream of the *GHF-1* start site. To identify potential enhancer elements in this region, restriction fragments encompassing the entire region were subcloned upstream to the minimal *GHF-1* promoter and activity was assessed by transfections into GHFT1 and GC cells. Fragments spanning positions −2.5 to −8.3 kb and −3.1 to −5.3 kb markedly increased the activity of the minimal *GHF-1* promoter (Fig. 9A), suggesting the presence of an enhancer. Remarkably, this enhancer was inactive in GC cells. Other fragments of the 5′-flanking region between −2.5 and −15 kb but outside of the −3.1- to −5.3-kb region failed to enhance expression (data not shown).

To determine whether the −3.1- to −5.3-kb region contains a cell type-specific enhancer element that can activate a heterologous promoter, this fragment was subcloned upstream of position −63 bp of a truncated human collagenase promoter fused to the CAT reporter

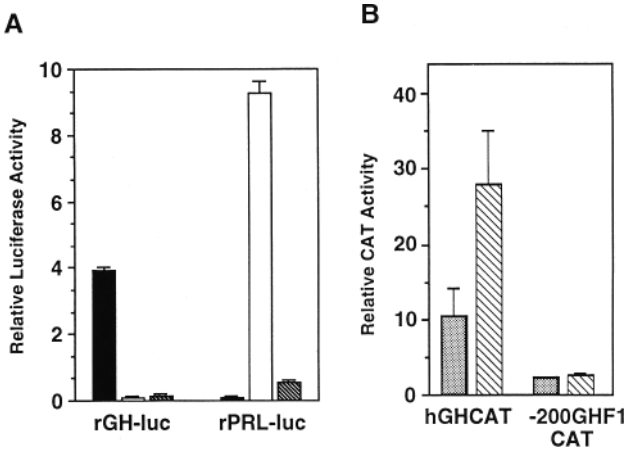

Figure 8. The *PRL* and *GH* promoters are silent in GHFT1 cells. (*A*) Luc expression vectors that contain the rat *GH* (−312rGH) and *PRL* (−422rPRL) promoters were transfected into GHFT1 (shaded bar), 235 (open bar), and GC (solid bar) cells together with an RSV–β-gal internal control. Levels of expression were determined 48 hr after transfection and were normalized relative to the level of RSV–β-gal expression. Data represent the average of four experiments ±S.E.M. (*B*) A Tag expression vector (RSV–Tag, T. Deng, pers. com.) was cotransfected with human growth factor (hGH)–CAT or −200GHF1–CAT into GC cells, and expression levels were determined 48 hr later. Expression levels were normalized relative to those of a cotransfected RSV–β-gal internal control reporter gene. Data represent the average of four experiments ±S.E.M. (Shaded bar) Control; (hatched bar) RSV–Tag.

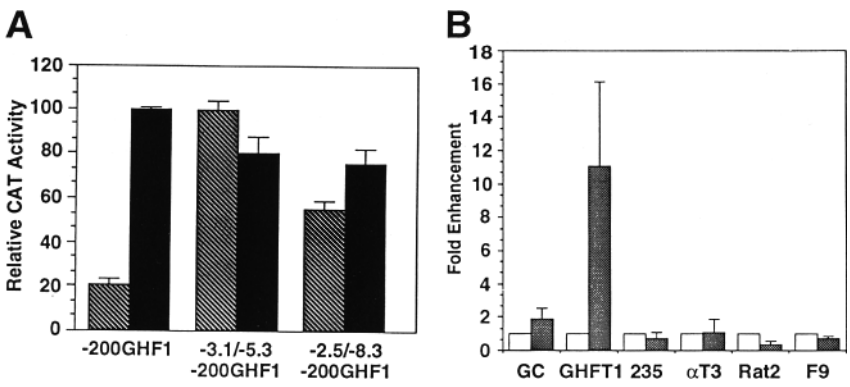

Figure 9. Identification of a tissue- and stage-specific enhancer in the *GHF-1* gene active early in the somatotrope lineage. (*A*) Fragments of the 5′-flanking region of the *GHF-1* gene were cloned 5′ to the minimal 200-bp GHF1 promoter (−200GHF1–CAT), and resulting reporter plasmids were transfected into GHFT1 (shaded bar) or GC (solid bar) cells as indicated. Transfection efficiency was monitored by parallel transfection of an RSV–CAT reporter plasmid. The maximal level of −200GHF–CAT expression in GC cells was arbitrarily set as 100%. The results shown are from four experiments ±S.E.M. (*B*) Transfection analysis with the −3.1 to −5.1-kb fragment (*Sac*I fragment) of the *GHF-1* gene cloned 5′ to the heterologous −60 collagenase promoter (Col–CAT). The enhancer-containing reporter as well as the original reporter plasmid were transiently transfected into GHFT1, GC, 235, αT3-1, Rat2, and F9 cells. The results shown are the average of four experiments ±S.E.M. (Open bar) Col–CAT; (stippled bar) −3.1/−5.3 Col–CAT.

gene (Col–CAT; Angel et al. 1987). As shown in Figure 9B, the *GHF-1*-derived fragment enhanced CAT expression at least 10-fold in transfected GHFT1 cells but not in a variety of other cell lines of pituitary and nonpituitary origin. Most important, the *GHF-1* enhancer was inactive in committed somatotropes (GC; data not shown for GH_3), lactotropes (235), or a gonadotropic progenitor (Windle et al. 1990). The lack of activity of the enhancer in the gonadotrope progenitor cell line αT3-1 is noteworthy, because this cell line also expresses Tag, indicating that the activity of this enhancer is not attributable to *trans*-activation by Tag. These results indicate that the *GHF-1* enhancer is stage specific and functions only in somatotropic progenitor cells.

Discussion

Transcriptional regulation occupies a central role in the control of cell type-specification and organogenesis. A key regulatory step in anterior pituitary development is the activation of the POU homeo domain gene *GHF-1* (for review, see Karin et al. 1990; Voss and Rosenfeld 1992). During mouse development, the *GHF-1* gene is activated exclusively in the ventral part of Rathke's pouch, the anterior pituitary anlagen, on e13 (Dollé et al. 1990), and is required for subsequent expression of the *GH* and *PRL* genes and expansion of the somatotropic lineage. By targeting oncogenesis using 15 kb of the *GHF-1* 5′-flanking region fused to SV40 Tag, we produced transgenic mice that develop pituitary tumors. Although one might expect that transformation of the somatotropic lineage would result in gigantism by analogy to the effects of *GH* overproduction in pituitary adenomas (Felig et al. 1987) or in metallothionein–GH (Palmiter et al. 1982) or metallothionein–GRF (Mayo et al. 1988) transgenic mice, the *GHF*–Tag transgenic mice were dramatically dwarfed.

Naturally occurring mutations of the *GHF-1* gene result in dwarfism by preventing formation of somatotropes (Li et al. 1990; Castrillo et al. 1991). Likewise, ablation of somatotropes by *GH*-promoter-targeted expression of toxic genes results in dwarf mice (Behringer et al. 1988; Borrelli et al. 1989). In the case of *GHF*–Tag transgenic mice, targeted Tag expression resulted in immortalization of a somatotropic progenitor that expressed *GHF-1* but not *GH* or *PRL* . Thus, immortalization of this cell type prevented further differentiation into determined somatotropes or lactotropes. The deficiency in *GH* production occurred well before a massive tumor was produced, as indicated by the early dwarf phenotype of the −15*GHF*–Tag transgenic mice. The severe growth retardation of these mice suggests that they have never produced normal amounts of *GH* . Therefore, the dwarfism is unlikely to be a direct consequence of tumor formation (additionally, transgenic mice bearing tumors of gonadotrope origin do not exhibit dwarfism; Windle et al. 1990). Rather, it is more likely to be caused by the failure of the immortalized progenitor to undergo differentiation. The pituitary tumors were cultured to isolate immortalized cells that exhibit many of the expected properties of the somatotropic progenitor. Such established cell lines (GHFT1) provided a model system for analyzing the initial activation of *GHF-1* expression during embryonic development.

Several criteria indicate that the pituitary tumors induced by the −15*GHF*–Tag transgene are attributable to transformation of a cell type with properties very similar to the somatotropic progenitor. First, unlike other pituitary tumors of somatotropic origin that overexpress *GH* and/or *PRL* (Tashjian et al. 1968; Bancroft 1981), the −15*GHF*–Tag-induced tumors do not express either hormone. Like the tumor from which they were derived, GHFT1 cells are negative for both *GH* and *PRL* expression. During development there is a delay of at least 2 days between the appearance of cells expressing *GHF-1* transcripts and cells that express either *GH* or *PRL* (Dollé et al. 1990; Simmons et al. 1990). Second, the GHFT1 cell line and the original tumors express approximately the same level of *GHF-1* mRNA as GC cells do, a cell line with somatotropic characteristics. In contrast,

GHFT1 cells express less *GHF-1* protein than GC cells. This may support a role for translational control or differential protein stability in the regulation of *GHF-1* expression. Although immunohistochemical analysis of sectioned whole mouse embryos characterized the presumptive somatotropic progenitors as having undetectable expression of *GHF-1* protein (Dollé et al. 1990), this conclusion was drawn by use of a method less sensitive than analysis of homogeneous tumor material and cell lines. On the basis of these criteria, we conclude that GHFT1 cells represent the presumptive somatotropic progenitor.

Transfection of reporter genes containing the promoters of both the *GH* and *PRL* genes into GHFT1 cells indicated that in contrast to committed somatotropes (GC cells) and lactotropes (235 cells), the somatotropic progenitors (GHFT1 cells) are incapable of activating these promoters. These results provide an explanation for the dwarf phenotype of the −15*GHF*–Tag transgenic mice and the lack of *GH* and *PRL* expression by the tumors. Whereas the lower level of *GHF-1* protein expression in GHFT1 cells may provide one explanation for the lack of *GH* and *PRL* promoter activity, it is perhaps probable that *GHF-1* is not the only determinant necessary for activation of the *GH* and *PRL* genes and that these cells lack additional transcription factors or post-translational processes required for full activation of these genes that are present later in development. The existence of progenitor cells (GHFT1) provides an avenue for comparing the complement of regulatory factors present in early versus later developmental stages, as exemplified by GC, GH_3, or 235 cells and for determining whether and which additional activators may be required for *GH* and *PRL* expression.

Interestingly, GHFT1 cells also exhibit inefficient activation of the minimal *GHF-1* promoter (−200 bp), even after treatment with forskolin, which induces this promoter (McCormick et al. 1990), indicating that the positive autoregulatory mechanism active in differentiated somatotropes (Chen et al. 1990; McCormick et al. 1990, 1991) is not yet functional in the progenitor cells (GHFT1). These results are intriguing because GHFT1 cells express nearly the same level of *GHF-1* mRNA as do GC cells, in which the minimal *GHF-1* promoter is fully active. Transfection experiments indicate that in GHFT1 cells the *GHF-1* promoter is subject to different control than in GC cells. An important regulatory element active in the progenitor cell line but not in more committed somatotropic derivatives is an enhancer element located between −3.1 and −5.3 kb upstream of the start of *GHF-1* transcription. This enhancer is likely to serve as a target for a transcriptional regulator that is active in the somatotropic progenitor but not in its more differentiated derivatives. Because the −2.5*GHF*–Tag transgene failed to direct expression to the anterior pituitary while the −15*GHF*–Tag transgene conferred targeted expression, it appears that this enhancer element is also an important control element in vivo.

This study suggests the following program of developmental regulation of *GHF-1* gene expression. Transcription of the *GHF-1* gene is initially activated through the stage-specific enhancer found upstream of −3 kb. The initial activation of *GHF-1* is likely to depend on at least one cell type-specific activator other than *GHF-1* itself. This activation, which is likely to occur at or before e13, may signal the divergence of the somatotropic progenitor from cells destined to express glycoprotein hormones. The progenitor of the gonadotrope and thyrotrope can be detected as early as e11 (Simmons et al. 1990) by the expression of α-subunit transcripts (the somatotropic progenitor GHFT1 cells and tumors are negative for α-subunit gene expression). Within the next 2 days, (between e13 and e15), the number of *GHF-1*-expressing cells increases and more GHF-1 protein is produced (Dollé et al. 1990). Once the amount of GHF-1 protein has reached a critical threshold, *GHF-1* transcription is likely to be maintained by positive autoregulation, as observed in the committed somatotropic cell lines GC and GH_3 (Chen et al. 1990; McCormick et al. 1990). When positive autoregulation has been achieved, the upstream enhancer element is dispensable for maintaining *GHF-1* transcription. Interestingly, the factor, or factors, that activates this enhancer is either no longer expressed or is rendered inactive in the more differentiated somatotropic cells, as indicated by the lack of enhancer activity after transfection into 235, GC, or GH_3 cells. Activation of the *GH* and *PRL* genes may be attributable to increased levels of *GHF-1* protein that are maintained by positive autoregulation and/or may also require the activity of an additional transcriptional regulator.

Positive autoregulation ensures that *GHF-1* expression will be maintained above a certain threshold and will not be affected by fluctuations in the level or activity of the earlier activator(s). This two-step regulatory strategy is similar to that used by bacteriophage λ (Herskowitz and Hagen 1980), in which the initial decision between lytic and lysogenic growth phases occurs through activation of the cI repressor gene by the cII activator. cI subsequently controls its own transcription in a positive autoregulatory manner. Although our studies are perhaps the first demonstration of a temporally controlled regulatory cascade involved in mammalian organogenesis, such regulatory cascades are known to occur during *Drosophila* development (Akam 1987; Ingham 1988). Genetic analysis indicates the existence of regulatory cascades in which one group of transcriptional regulators present early in development initiate the expression of other transcriptional regulators acting at later developmental stages (Akam 1987; Ingham 1988). For example, the striped pattern of *even-skipped* homeo box gene expression is initiated by the action of the gap genes. Subsequently, *even-skipped* autoregulation refines the striped pattern and maintains expression in the absence of the gap gene proteins (Goto et al. 1989; Jiang et al. 1991). Such two-step mechanisms in which the expression of an important regulatory gene is ultimately stabilized by positive autoregulation protects the maintenance of a differentiated phenotype from fluctuations in the level of an initial or transient activator.

Targeted immortalization of progenitor cell types us-

ing transgenes consisting of an oncogene fused to the regulatory regions of genes involved in cell type determination should prove powerful for the study of other regulatory hierarchies involved in mammalian organogenesis. The availability of immortalized progenitor cell lines should facilitate the biochemical characterization of transcriptional regulators that act during development but are otherwise available in prohibitively small quantities. In many cases, as illustrated here, such transcriptional regulators may no longer be present, or active, in more readily available differentiated cell types or tissues derived from adult animals, preventing investigation of such mechanisms. The stage-specific immortalization of cells for creation of cultured cell lines is therefore an invaluable tool for analysis of transcriptional regulatory cascades during mammalian development.

Materials and methods

Construction of transgenes

Transgene −2.5*GHF*–Tag was constructed by ligating a restriction fragment from an artificially generated *Bam*HI site at +1 of the rat *GHF-1* gene (McCormick et al. 1990) to the *Xba*I site at −2.5 kb to the SV40 early region. Transgene −15*GHF*–Tag was constructed by ligating additional fragments of the *GHF-1* control region up to the *Sal*I site at −15 kb to −2.5*GHF*–Tag (Fig. 1). The SV40 Tag gene includes the protein-coding region for large T and small t antigens, with the translation initiation and transcription termination sites, but lacks the SV40 early promoter/enhancer (Hanahan 1985).

Creation of transgenic mice

The −2.5*GHF*–Tag transgene was excised by digesting the plasmid with *Not*I and *Sal*I, and the −15*GHF*–Tag transgene was excised by *Sal*I digestion (Fig. 1). DNA fragments were purified by agarose gel electrophoresis and binding to glass beads (Geneclean, BIO101, Inc.). Approximately 1–2 pl of a solution of DNA at a concentration of 2 μg/ml was microinjected into the pronuclei of fertilized one-cell mouse embryos (Hogan et al. 1986). The F_2 embryos were derived from matings of CB6F1/J (C57Bl/6J × BALB/cJ) males and 7- to 10-week-old CB6F1/J females (Harlan Sprague-Dawley). Injected embryos were reimplanted into ICR pseudopregnant female mice (Hogan et al. 1986). The presence of the transgene in the resulting mice was determined by preparing genomic DNA from a small piece of tail and assaying by Southern blot analysis (Meinkoth and Wahl 1984).

RNA analysis

Total RNA from tissues and cells was extracted (Chirgwin et al. 1979) and analyzed by Northern blotting (Sambrook et al. 1989), using GeneScreen hybridization transfer membrane (NEN Research Products) and a Posiblotter pressure blotter (Stratagene). Hybridizations were carried out in aqueous solution at 65°C. The probes were generated from plasmids or restriction fragments encoding the cDNAs for the indicated genes (as described in the figure legends) by random oligonucleotide-primed synthesis (Feinberg and Vogelstein 1983). Where indicated, the nylon membranes were washed twice (5 min/wash) in 0.1% SDS, 18 mM NaCl, 1.0 mM NaH_2PO_4, and 0.1 mM EDTA at 100°C, for rehybridization to a second probe.

Cell culture

The GHFT1 cells were established using methods described previously for α-Tag pituitary tumor cell lines (Windle et al. 1990). Clonal cell lines and cell populations were maintained in DME with 5% fetal calf serum, 5% equine serum, 4.5 mg/ml of glucose, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin (Sigma).

Immunocytochemistry and Western blotting

Excised tissues were fixed with 4% paraformaldehyde in PBS for 2 hr at room temperature. After several washes in PBS with 5 mM glycine, the tissue was cryoprotected with 30% sucrose in PBS overnight at 4°C, embedded (Oct: Aqua 1.1), and cut into 15- to 20-μm slices on a cryostat. For immunocytochemistry, all manipulations were carried out at room temperature according to standard procedures (Harlow and Lane 1988). Fixed cells (in 4% paraformaldehyde) or tissue sections were washed in PBS with 5 mM glycine, permeabilized in PBS with 0.3% Triton X-100 for 8 min, and saturated in PBS with 2% goat whole serum and 1% BSA for 15 min. Binding of the primary antibody was for 1 hr using the following dilutions: α-rGH, 1 : 400; α-rPrl, 1 : 1000 (both antibodies were obtained from the National Pituitary Service); α-r*GHF-1*, 1 : 200 (Bodner et al. 1988); and α-Tag, 1 : 20 (mouse monoclonal antibody KT-3). After four washes with PBS, the fluorescent-conjugated secondary antibody was applied for 30 min followed by several washes with PBS and nuclear staining with 0.0001% DAPI for 10 min. The stained tissues or cells were mounted in Mowiol mounting solution. Western blotting was performed as described previously (Castrillo et al. 1991).

DNase I footprinting

A 312-bp rat *GH* promoter fragment (*Kpn*I–*Hin*dIII from rGH–Luc) was labeled at the *Hin*dIII site (+11). An amount of 10,000 cpm of this DNA probe was incubated with 2.5 μg of nuclear extract (Hattori et al. 1990) from the indicated cell type or BSA for 20 min on ice in binding buffer (10 mM HEPES at pH 7.8, 80 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 50 μg/ml of poly-[d(I-C)], 300 μg/ml of BSA). Reactions were shifted to room temperature for 2 min and after the addition of 50 μl of 5 mM $CaCl_2$, 1.5 mM EDTA, and were incubated for 90 sec with 10 ng of DNase I (15 U/μg). The digestion was stopped by the addition of 200 μl of 0.5% SDS, 10 mM EDTA, 100 mM NaCl, and 50 μg/ml of yeast tRNA, the DNA was isolated, and the digestion products were analyzed on a 6% sequencing gel.

Transfections

Cells on 10-cm plates were transfected with 10 μg of the indicated CAT or Luc reporters, as described previously (Angel et al. 1987; McCormick et al. 1990). For transfections with −200*GHF1*–CAT, 10 μM forskolin was added 38–40 hr post-transfection to increase expression from the −200*GHF1* promoter (McCormick et al. 1990). Cells were harvested 5–8 hr later. Although the −200*GHF1* promoter is responsive to forskolin, the enhancer element is not. CAT assay experiments were performed in triplicate and corrected for transfection efficiency by normalizing to an internal Rous sarcoma virus (RSV)–β-gal standard (2 μg per plate). Plasmids containing 312 bp of the *GH* promoter, or 422 bp of the *PRL* promoter driving a Luc reporter gene (Nelson et al. 1988), were utilized for determining promoter activity by Luc assays (de Wet et al. 1987). RSV–Tag was provided by T. Deng.

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GHF-1-promoter-targeted immortalization of a somatotropic progenitor cell results in dwarfism in transgenic mice.

D Lew, H Brady, K Klausing, et al.

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