Function of the Homeodomain Protein GHF1 in Pituitary Cell Proliferation

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Mutations that cause pituitary dwarfism in the mouse reside in the gene encoding the transcription factor growth hormone factor 1 (*GHF1* or *pit1*). These dwarf mice (*dw* and *dw^J*) are deficient in growth hormone (GH) and prolactin (PRL) synthesis and exhibit pituitary hypoplasia, suggesting a stem cell defect. With antisense oligonucleotide technology, a cell culture model of this genetic defect was developed. Specific inhibition of GHF1 synthesis by complementary oligonucleotides led to a marked decrease in GH and PRL expression and to a marked decrease in proliferation of somatotrophic cell lines. These results provide direct evidence that the homeodomain protein GHF1 is required not only for the establishment and maintenance of the differentiated phenotype but for cell proliferation as well.

HOMEODOMAIN-CONTAINING PROteins are transcriptional regulators that participate in the determination of a cell's fate (*1–3*). GHF1 (Pit1) is a mammalian homeodomain protein that is a member of the POU subclass, which contains related homeodomain-containing transcription factors (*4, 5*). It is expressed in the anterior pituitary, where it appears to be responsible for activating the GH and PRL genes of the somatotrophic lineage (*6*). Transcription of *GHF1* is positively autoregulated (*7, 8*) and induced by cyclic adenosine monophosphate (cAMP) by means of the cAMP response element–binding (CREB) protein, which binds to the *GHF1* promotor (*7*). The action of the somatotroph-specific mitogen growth hormone–releasing factor (GRF) (*9, 10*) is mediated by the second messenger cAMP. We provide evidence that GHF1 is also required for pituitary cell proliferation.

Two naturally occurring mutations that cause pituitary dwarfism in mice, known as the Snell and Jackson dwarfs (*11*), have been shown to reside in *GHF1* on chromosome 16 (*12, 13*). The more extensively studied Snell dwarf is deficient not only in GH and PRL synthesis (*13, 14*) but also suffers from pituitary hypoplasia (*11*) (Fig. 1, A and C). Sections of normal and dwarf pituitaries were stained with propidium iodide and visualized by fluorescence confocal microscopy. This examination indicates that the total cell number in the pars distalis of the anterior pituitary is markedly reduced in dwarf pituitaries, as compared to the cell number in their normal counterparts. This defect is specific for the pars distalis and does not affect the developmentally related pars intermedia. Therefore, the Snell dwarf phenotype appears to result from more than the lack of GH and PRL expression; the defect may actually affect development of the somatotrophic lineage, which is a major contributor to the anterior pituitary cell mass. Although the *dw* mutation is a missense mutation that results in the substitution of a cysteine for the conserved tryptophan residue in the homeodomain of GHF1, the dwarf pituitary is deficient in GHF1 mRNA (*12*) and protein (Fig. 1, B and D). This is probably due in part to the decreased DNA binding activity of variant GHF1 (*13*), which results in a decrease in transcription of the positively autoregulated *GHF1* (*7, 8*). However, a decrease in GHF1 expression may also result from the failure of somatotrophic precursors to proliferate.

The ontology of GHF1 expression (*15*) and its induction by cAMP (*7*), a mitogenic signal for somatotrophic cells (*10*), suggested that GHF1 may function in proliferation of somatotrophic precursors. This would explain how the Snell dwarf mutation leads to anterior pituitary hypoplasia. The GH- and PRL-producing pituitary tumor cell lines GC and 235 provide convenient models for testing this hypothesis. Despite having unlimited proliferative capacity, these cells express somatotrophic differentiation markers and contain high concentrations of GHF1 (*4, 5*). We used antisense oligonucleotides (*16*) to inhibit GHF1 expression in GC cells. Because of the relative stability and abundance of GHF1 mRNA and protein, we found it necessary to maintain the cells in the presence of oligonucleotides (50 μM) for a minimum of 6 days. This treatment resulted in a reduction of the amount of GHF1 and GH mRNA in GC cells incubated with AS1, an oligonucleotide complementary to positions 118 to 138 of GHF1 mRNA (*17*) (Fig. 2A). Although the amount of GHF1 mRNA was reduced fivefold in antisense-treated cells in comparison to cells treated with a control GHF1 sense oligonucleotide or untreated cells (*13*), a greater reduction was observed in the amount of GHF1 protein; the GHF1 protein was essentially undetectable (Fig. 2B). Incubation with the sense oligonucleotide had no effect on GHF1 levels. Because GC cells do not express PRL, we used 235 cells (*18*) to assess the effect of the GHF1 antisense oligonucleotide on PRL expression. Treatment with GHF1 antisense oligonucleotides led to a marked and specific reduction in the concentrations of both GHF1 mRNA and PRL mRNA (Fig. 2C).

Treatment with AS1 also led to a marked decrease in the rate of DNA synthesis in GC and 235 cells (Table 1). A similar level of inhibition was observed in five separate experiments and with a second antisense oligonucleotide (AS3) complementary to positions 99 to 127 of GHF1 mRNA (*17*). Treatments with GHF1 sense oligonucleotide, two nonspecific oligonucleotides, and an antisense oligonucleotide complementary to GH mRNA (*17*) had no effect on GC cell proliferation. The inhibition of GC cell proliferation is probably not due to a cytotoxic effect, as it was reversible. GC cultures were incubated with AS1 for 9 days and divided: one-half of the culture was incubated in the absence of and one-half in the presence of AS1. Measurement of ^{3}H-labeled thymidine incorporation indicated that removal of the

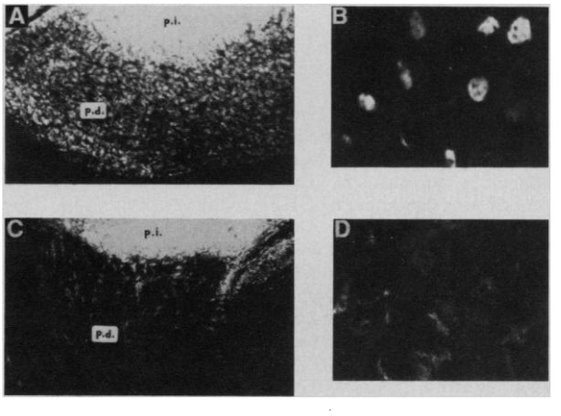

Fig. 1. Histochemical analysis of normal (**A** and **B**) and Snell dwarf (**C** and **D**) anterior pituitaries. (A and C) Sections of normal and dwarf pituitaries were stained with propidium iodide (*28*) and visualized by confocal microscopy (final magnification, ×45.6); p.i., pars intermedia; p.d., pars distalis. (B and D) Sections of normal and dwarf pituitaries stained with antibodies to GHF1 (final magnification, ×228).

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antisense oligonucleotide resulted in an increase in DNA synthesis (Fig. 3A). We obtained similar results by examining the incorporation of bromodeoxyuridine (BrdU) into DNA (Fig. 3B). Cells incubated with AS1 for 9 days exhibited only background fluorescence when examined for either BrdU incorporation or GHF1 expression. Five days after the removal of AS1, the increase in BrdU incorporation paralleled the increase in GHF1 immunoreactivity. In addition, the inhibitory effect of the GHF1 antisense oligonucleotides was specific to GC and 235 cells, as they did not affect the proliferation of Rat2, Rat6, or NIH 3T3 fibroblasts (Table 1).

Our experiments establish a direct correlation between reduced GHF1 expression and decreased GH and PRL expression. In addition, GHF1 expression is correlated with pituitary cell proliferation. These findings, together with the hypoplastic phenotype of the Snell dwarf anterior pituitary, indicate that in addition to determination of cell fate, GHF1 is likely to function in the proliferation of somatotrophic precursors; therefore mutations that inhibit GHF1 activity interfere with the expansion of this cell lineage.

Our conclusions are consistent with what is known about this biological system. In addition to its effects on GH synthesis and secretion, the hypothalamic hormone GRF is also a specific mitogen for somatotrophs. Unlike many other mitogens, GRF uses cAMP as a second messenger (*9, 10*). *GHF1* transcription is also induced by cAMP(7). Overexpression of GRF in transgenic mice leads to specific somatotrophic hyperplasia (*19*), and some human GH-secreting pituitary adenomas (43%) have mutations that constitutively activate the stimulatory G protein that couples the GRF receptor to adenylate cyclase (*20*). These findings underscore the importance of this signaling system for the expansion of the somatotrophic lineage. It has been observed that overexpression of a nonphosphorylated variant CREB protein driven by the GH promoter in transgenic mice leads to pituitary hypoplasia (*21*). On the other hand, expression of a GH-cholera toxin fusion gene leads to pituitary hyperplasia and gigantism in transgenic mice, an effect caused by chronic activation of the adenylate cyclase (*22*).

During normal mouse development, GHF1 protein and GH transcripts and protein are first detected on embryonic day (ED) 16 (*15*). Although the ontology of GRF expression in the mouse is not known, hypothalamic GRF is first detected on ED 18 in the rat (*23*), a developmental time that is nearly equivalent to ED 16 in the mouse. On ED 16 the anterior pituitaries of Snell dwarfs are similar in size to those of their normal counterparts. Slight differences can be detected during the next several days of fetal development and marked differences in the size and morphology of the anterior pituitaries occur postnatally (*24*). Thus the

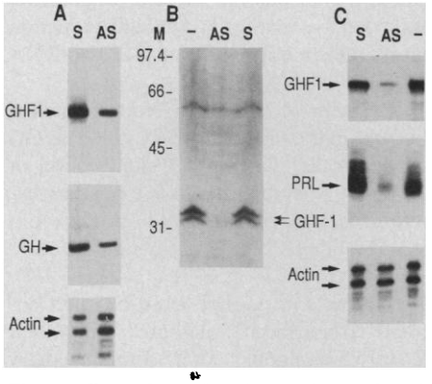

Fig. 2. Effects of GHF1 antisense oligonucleotide on gene expression in pituitary cells. Cells were plated at 10% confluence on 100-mm plates in DMEM that contained horse serum (10%) and fetal calf serum (2.5%). Sense (S) or antisense (AS) GHF1 oligonucleotides (AS1 and S1) were added every 72 hours (*17*). (**A**) To examine GHF1 and GH expression in GC cells, we extracted total cellular RNA after 6 days of treatment with either S or AS oligonucleotides and analyzed the RNA by ribonuclease (RNase) protection assays with probes specific for GHF1, GH, or actin. (**B**) For protein analysis, nuclear extracts were prepared from either untreated GC cells (−) or cells treated for 9 days with S or AS GHF1 oligonucleotides; the extracts were separated by SDS–polyacrylamide gel electrophoresis and transferred to nylon membranes as described (*29*). Protein concentration was determined with the Bio-Rad protein assay kit with bovine serum albumin as the standard. The immunoblot was probed with antibodies to GHF1 and developed with ^{125}I-labeled Protein A (*29*). M, molecular size standards in kilodaltons. (**C**) To examine PRL and GHF1 expression in 235 cells, we extracted total cellular RNA from either untreated cells (−) or cells treated for 6 days with S or AS GHF1 oligonucleotides and analyzed the RNA by RNase protection with probes specific to GHF1, PRL, or actin.

Table 1. Inhibition of DNA synthesis by antisense GHF1 oligonucleotides. The cell lines were plated at 10% confluence in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum that was heat inactivated. Sense and antisense oligonucleotides were added every 72 hours to a final concentration of 50 μM (*17*). Cell proliferation was determined by [^{3}H]thymidine incorporation into trichloroacetic acid–precipitable material after 12 hours of incubation with unlabeled thymidine (3 mM) and 1 μCi of [^{3}H]thymidine (25 Ci/mmol). Values represent the averages ± SEM of three different experiments each done in duplicate. The levels of DNA synthesis are expressed as percent of the level measured in nontreated control cultures.

Cell line	Oligonucleotide	DNA synthesis (percent of control)
GC	AS1	35 ± 5
	AS3	57 ± 7
	Rel2	92 ± 2
	Rel6	100 ± 1
	S1	97 ± 3
	GH1	105 ± 5
235	AS1	24 ± 3
	S1	113 ± 23
Rat2	AS1	98 ± 2
	AS3	96 ± 1
Rat6	AS1	98 ± 9
	S1	100 ± 11
NIH 3T3	AS1	97 ± 1
	AS3	96 ± 1

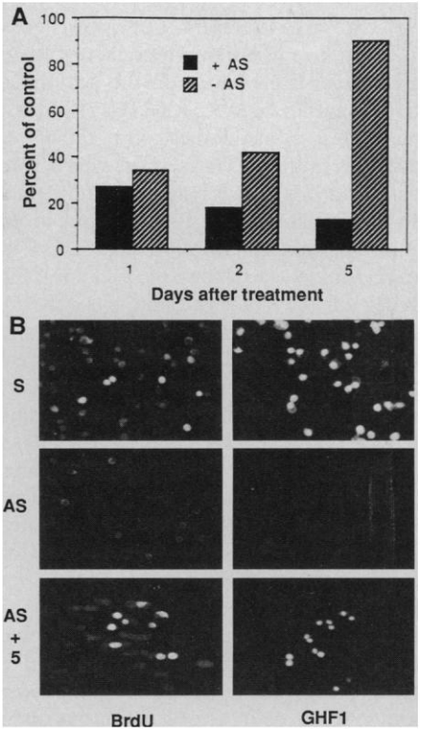

Fig. 3. Reversible inhibition of GC cell proliferation and GHF1 expression by antisense oligonucleotides. (**A**) GC cells were plated on 24-well plates and treated for 9 days with AS1 (50 μM) as described in Table 1. The cells were washed twice and incubated with (+ AS) or without (− AS) AS1 for five additional days. [^{3}H]thymidine incorporation was determined at days 1, 2, or 5. The values represent average relative amounts of [^{3}H]thymidine incorporation in triplicate cultures compared to [^{3}H]thymidine incorporation in duplicate control cultures treated with S1 for 9 days. The SEMs were in all cases less than 10% of the reported values. (**B**) GC cells were grown on cover slides in six-well plates and treated with AS1 as described in (A). We identified the cells carrying out DNA synthesis by BrdU incorporation and by staining the cells with an antibody to BrdU DNA (*30*). The expression of GHF1 was determined in duplicate cultures subjected to the same treatments by indirect immunofluorescence (final magnification, ×70). S, cells treated with S1 for 9 days; AS, cells treated with AS1 for 9 days; AS + 5, cells treated with AS1 for 9 days and then incubated for 5 days in the absence of AS1.

onset of GHF1 and GRF expression is correlated with the time at which expansion of the somatotrophic lineage is initiated. Defects in GHF1 expression and function prevent the growth and differentiation of these cells, leading to a complete absence of mature somatotrophs and an almost complete absence of mature lactotrophs. Because the Snell dwarf also has a decreased number of thyrotrophs (*14, 24*), it is possible that, in contrast to previous assumptions, this cell type may also be derived from the somatotrophic lineage. However, it is also possible that thyrotroph differentiation or survival may be dependent on the presence of intact somatotrophs or lactotrophs.

Although proper development requires proliferation of stem cells, as well as various determined progenitors, it is not clear how proliferation and differentiation are coupled during development. Genetic and biochemical evidence indicates that homeodomain proteins are involved in the determination of a cell's fate (*1–3*). Homeodomain genes may regulate the expression of genes that control the *Drosophila* cell cycle (*25*). In addition, another POU protein, the ubiquitously expressed Oct1, is important for viral DNA replication (*26*). Our study provides evidence that a homeodomain protein is required for cell proliferation, in addition to its role in cell fate determination. The regulation of expression of homeodomain proteins by lineage-specific growth factors (*27*) may provide an efficient way of coupling proliferation and differentiation.

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17. The oligonucleotides were added every 72 hours for 6 to 9 days to a final concentration of 50 μM. The medium was changed at days 3 and 6. AS1 is complementary to nucleotide positions 118 to 138 (5′-GAAAGGTTGGCAACTCATTCCCAC-3′), AS3 is complementary to positions 99 to 127 (5′-GGCAACTCATTCCCACAAGAGAGTAGAG-3′), and S1 is identical to positions 1 to 25 (5′-CTCAGAGCCGCCCTGATGTATATATG-3′) of GHF1 mRNA. GH1 is complementary to positions 35 to 59 of GH mRNA (5′-CCAAGCAGTGATCTGTCCACAGGAAC-3′. Rel2 and Rel6 are nonspecific control oligonucleotides.
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28. Saggital sections (200 μm) of the entire pituitary were cut as described (*4*). The sections were stained with propidium iodide (20 μg/ml), and the nuclear fluorescence signal of propidium iodide (Molecular Probes) was examined with an MRC-600 Confocal Imaging system (Bio-Rad, Richmond, CA) on a Zeiss microscope. Immunostaining with antibodies to GHF1 was as described (*4*).
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MHC Class I Deficiency: Susceptibility to Natural Killer (NK) Cells and Impaired NK Activity

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The role of major histocompatibility complex (MHC) class I expression in natural killer (NK) cell target recognition is controversial. Normal T cell blasts from MHC class I–deficient mutant mice were found to serve as target cells for NK cells in vitro, which suggests that MHC class I molecules are directly involved in NK cell recognition. Spleen cells from the mutant mice were deficient in their ability to lyse MHC class I–deficient target cells or NK-susceptible tumor targets, and mutant mice could not reject allogeneic bone marrow. Thus, class I molecules may participate in the positive selection or tolerance induction of NK cells.

NEITHER THE ANTIGENS RECOGnized by NK cells nor their antigen receptors have been identified, though their capacity to destroy various tumor cell lines is well documented. The MHC class I molecules may be involved in recognition by NK cells. Tumor cell variants that are deficient for MHC class I expression are often more susceptible to lysis by NK cells than are MHC class I^+ tumor cells (*1–5*). Because susceptible tumor cells may express tumor antigens recognized by NK cells, it is unclear from these experiments whether the MHC class I deficiency enhances recognition of these antigens or is itself sufficient to render the cells sensitive to NK cells. With the use of cells from mice mutant for β_2-microglobulin (β_2M) (*6–8*) and hence deficient for cell surface class I expression, we directly addressed the role of MHC class I expression in the susceptibility of otherwise normal cells to NK cell lysis in vitro and investigated if MHC class I participates in the development of NK activity in vivo.

Spleen cells from C57BL/6 (B6) mice that had been injected 1 day before with polyinosinic:polycytidylic acid (poly I:C), an agent that induces NK cell activity (*9, 10*), lysed both NK-sensitive YAC-1 tumor cells and concanavalin A–induced T cell blasts (Con A blasts) from (B6 × 129)F_3 mice (H-2^b) homozygous for a mutant β_2M allele (−/− mice) (Fig. 1A). Similar results

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