The pituitary-specific regulatory gene *GHF1* contains a minimal cell type-specific promoter centered around its TATA box

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GHF-1 is a pituitary-specific transcription factor responsible for activation of the growth hormone (GH) gene. The *GHF1* gene is expressed exclusively in cells of the somatotrophic lineage, and its transcription is extinguished in somatic cell hybrids. The minimal sequences required for differential transcription of *GHF1* in GH-expressing and -nonexpressing cell lines and somatic cell hybrids were localized to a 15-bp region surrounding and including its TATA box. This 15-bp fragment acts as a cell type-specific promoter element and is recognized by a transcription factor present in GH-expressing cell lines. Hence, in addition to enhancers and upstream promoter elements, the TATA element (TATA box plus surrounding sequences) can be, in certain cases, an important determinant of cell-type-specific transcription.

[**Key Words:** *GHF1*; pituitary-specific regulatory gene; cell-type-specific promoter; transcription factor; TATA box]

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Exclusive expression of the growth hormone (GH) gene in anterior pituitary somatotrophs is largely determined by binding of transcription factor GHF-1 to two sites within its promoter (Bodner and Karin 1987; for review, see Karin et al. 1990). GHF-1, also known as Pit-1, is a homeo domain protein encoded by a gene whose expression is restricted to cell types that belong to the somatotrophic lineage (Bodner et al. 1988; Ingraham et al. 1988). Expression of GHF-1 transcripts and protein is highly regulated, and the accumulation of GHF-1 protein correlates both temporally and spatially with activation of the *GH* gene (Dollé et al. 1990). The cell-type specificity of *GH* transcription can be explained by the restricted distribution of its activating factor GHF-1 (Karin et al. 1990). The *GHF1* gene is also controlled by a yet-to-be-identified extinguisher responsible for extinction of GH expression in hybrids of pituitary and fibroblast cells (McCormick et al. 1988). Extinction is a poorly understood regulatory mechanism, revealed by studies on somatic cell hybrids (Thompson and Gelehrter 1971; Davidson 1974; Weiss and Chaplain 1971; Killary and Fournier 1984), but it may contribute to the restricted expression of cell-type-specific *trans*-activators seen in vivo (Baumhuerter et al. 1990; Junker et al. 1990). Full understanding of positive and negative controls that es-

tablish cell-type-specific patterns of gene expression requires careful analysis of the mechanisms regulating the activity and expression of the regulators themselves.

Such studies were recently initiated with *GHF1*, whose promoter region was identified and some of the sequences required for its cell-type-specific and regulated expression determined (McCormick et al. 1990). The *GHF1* promoter contains two binding sites for the ubiquitous CREB (cAMP response element-binding) protein, which is responsible for induction of transcription in response to cAMP (Gonzalez et al. 1989). CREB is thought to be involved in stimulating the somatotroph-specific expression of *GHF1* in response to the hypothalamic hormone and growth factor, GH-releasing factor. In addition, *GHF1* is positively autoregulated by GHF-1 itself (McCormick et al. 1990). Positive autoregulation, however, cannot be solely responsible for the pituitary-specific expression of *GHF1*, because during development *GHF1* transcripts appear 48–72 hr before GHF-1 protein (Dollé et al. 1990). Indeed, inactivation of the GHF-1-binding site causes only a partial reduction in *GHF1* promoter activity, without eliminating its pituitary-specific transcription (McCormick et al. 1990). Thus, another pituitary-specific factor appears to be involved in establishment of *GHF1* transcription.

To delineate further the mechanisms responsible for pituitary-specific expression of *GHF1* and its repression in somatic cell hybrids, we determined the minimal se-

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quences sufficient for its differential transcription in extracts of the GH-expressing anterior pituitary cell line GH3, GH-nonexpressing Ltk⁻ cells, and GH3XLtk⁻ somatic cell hybrids in which GH and GHF-1 expression are either extinguished or reactivated (McCormick et al. 1988). We found that a 15-bp region surrounding and including the TATA box of the *GHF1* gene acts as a minimal pituitary-specific promoter recognized by a protein enriched in GH-expressing cells. Mutational and biochemical evidence indicate that this 15-bp sequence functions as a cell-type-specific TATA element.

Results

Extinction of GH occurs at the level of GHF1 transcription

Previous studies on the extinction of GH expression in somatic cell hybrids correlated extinction with disappearance of GHF-1 protein and RNA (McCormick et al. 1988). To determine the level at which GHF-1 expression is regulated in this system, we examined the presence of *GHF1* transcripts in nuclear RNA extracted from the parental cell lines GH3 (GH positive) and Ltk⁻ (GH negative), and their hybrids C2 (GH positive), C6 (GH negative), and C19 (GH negative). Processed and unprocessed *GHF1* transcripts were present only in the GH-positive cell lines GH3 and C2 (data not shown). Southern blotting analysis revealed the presence of the rat *GHF1* gene in all of the hybrids (data not shown). To determine whether extinction is mediated at the level of *GHF1* transcription, whole-cell extracts (WCE) of the various cell lines were used for testing the efficiency of the *GHF1* promoter by in vitro transcription reactions analyzed by primer extension (Bodner and Karin 1987; McCormick et al. 1988). A 115-nucleotide cDNA product corresponding to transcripts initiated at the *GHF1* promoter (McCormick et al. 1990) was detected only in reactions that contained either GH3 or C2 extracts (Fig. 1). No such transcripts were generated upon incubation of *GHF1*-chloramphenicol acetyltransferase (CAT) with either Ltk⁻ or C6 extracts. Mixing experiments indicated that the *GHF1* promoter was active regardless of the order of extract addition. Therefore, lack of *GHF1* transcription in Ltk⁻ cell extracts and probably in the C6 and C19 somatic cell hybrids is unlikely to be caused by dominant repressors that bind tightly to the promoter.

These conclusions were supported by DNase I footprinting experiments (Fig. 2). A large area of protection (footprint I), determined previously to encompass sequences from -78 to -23 of the *GHF1* promoter (McCormick et al. 1990), is seen upon incubation of the *GHF1* probe with the GH3 or C2 extracts. This footprint is not observed upon incubation with the Ltk⁻, C6, and C19 extracts. On the other hand, these extracts contain factors that generate footprints 1 and 2 covering sequences from -50 to -40 and -80 to -63, respectively. Other areas of protection can be detected upstream to footprint 2. However, because they lie up-

Figure 1. Analysis of *GHF1* promoter activity in parental and hybrid cells. A *GHF1*-CAT ($\Delta 5'$ -2000) plasmid (100 ng) was incubated with WCE (75 μ g) prepared from the indicated cell lines, under standard in vitro transcription reaction conditions (McCormick et al. 1988). After a 60-min reaction, the generated transcripts were quantitated by primer extension. In the mixing experiments, the *GHF1*-CAT template was first incubated with 75 μ g of one extract for 30 min on ice in the absence of ribonucleotide triphosphates (rNTPs), followed by an equal amount of the second extract, addition of rNTPs, and incubation at 30°C for 60 min. The primer extension products were separated on a 10% sequencing gel and visualized by autoradiography for 12 hr.

stream of position -90, the previously described 5' border of the region involved in cell-type-specific expression (McCormick et al. 1990), they were not investigated in further detail. Thus, footprint I correlates with *GHF1* transcription, whereas its absence and the presence of footprints 1 and 2 correlate with lack of promoter activity.

Definition of the minimal GHF1 promoter

To determine the sequence requirements for differential *GHF1* transcription, progressive 5' deletion mutants were constructed and tested for activity in WCE of expressing (GH3, C2) and nonexpressing (Ltk⁻, C6) cells. To control for the activity of the different extracts and efficiency of the reactions, the human α -globin gene was included as an internal control. Transcripts initiated from the α -globin promoter generate a 66-nucleotide primer extension product.

Figure 2. Footprint analysis. *GHF1* probe, end-labeled at position +1, was incubated with 30 μ g of the indicated WCEs, which were partially enriched by heparin-agarose chromatography (Bodner and Karin 1987) or with 30 μ g of BSA. The location of protein-binding sites was determined by DNase I footprinting and is marked by brackets. (I) –78 to –23; (1) –50 to –40; (2) –80 to –63. (A, B) Two different experiments with the same probe.

All of the *GHF1* promoter mutants thus examined exhibited differential transcription (Fig. 3). A small increase in transcription in GH3 and C2 extracts was seen upon deletion of sequences between –2000 and –200, whereas deletion of sequences between –2000 and –90 had no further effect. Deletion of sequences between –90 and –51 led to a two- to threefold decrease in transcription in expressing extracts. This decrease correlates with deletion of a GHF-1-binding site involved in positive autoregulation (McCormick et al. 1990). A further deletion to –41 had no effect on transcription in extracts of expressing cell lines but appeared to result in a slight increase in basal promoter activity in nonexpressing extracts. Hence, sequences located between positions –41 and +1 are sufficient for differential transcription in vitro. Therefore, the factors that bind to sites 1 and 2,

which are located upstream of –41, are unlikely to be responsible for the transcriptional inefficiency of the *GHF1* promoter in nonexpressing cell extracts. On the other hand, a pituitary-specific factor binding to the 3' half of footprint I could be responsible for the increased activity of the truncated *GHF1* promoter in the GH3 and C2 extracts. The 5' half of footprint I was shown to contain a GHF-1-binding site (McCormick et al. 1990).

A 15-bp minimal promoter

Next we tested whether a synthetic oligodeoxynucleotide containing *GHF1* sequences from –39 to –24 can function as a cell-type-specific promoter in vitro. The 3' border of this fragment was chosen according to the 3' border of the pituitary-specific footprint (I in Fig. 2). This synthetic DNA fragment was inserted upstream of position –46 of a truncated *TK* promoter (Fig. 4A). The resultant hybrid promoter directed efficient transcription in GH3 extracts but not in Ltk[–] extracts (Fig. 4B). The transcripts were initiated 29 bp downstream of the first (5'-most) T of the *GHF1* TATA element, and no specific initiation was observed from the *TK* promoter itself. [The primer extension products migrating at the size expected for the *TK*-initiated transcripts (82 nucleotides) are nonspecific products from the α -globin gene. No such products were seen in the absence of the α -globin template]. The truncated *TK* promoter itself appeared inactive in both extract types. The ability of the 15-bp *GHF1* TATA region oligonucleotide to direct initiation of transcription to a site located 25–30 bp downstream is in agreement with the known property of TATA elements (Breathnach and Chambon 1981). However, unlike other TATA elements, the *GHF1*-derived element functions in a cell-type-specific manner. The efficiency of the 15-bp fragment is similar to that of the Δ 5'–41 *GHF1* promoter.

To exclude the possibility that part of the *GHF1* TATA element activity is due to the action of GHF-1 itself, we used an oligodeoxynucleotide representing the high-affinity GHF-1-binding site of the *GH* promoter (Bodner and Karin 1987) to compete for GHF-1 binding. Although a 10^3 -fold molar excess of the GHF-1-binding site led to a significant decrease in transcription from the *GH* promoter, it had only a very small effect on the weaker GFTATA/*TK* hybrid promoter (Fig. 4C). These results are in agreement with previous findings, which indicate that GHF-1 does not bind to this part of the *GHF1* promoter (McCormick et al. 1990).

Mutational analysis of the *GHF1* TATA element

These results suggested that the TATA box of *GHF1*, in combination with its flanking sequences, functions as a cell-type-specific minimal promoter. To investigate this possibility further, the *GHF1* TATA box region (from –39 to –24) was replaced with the corresponding regions of the herpes simplex virus (HSV)-*TK* and human α -globin promoters to generate the GT and GG mutant promoters (Fig. 5A). We also substituted the corresponding region of the HSV-*TK* promoter with the *GHF1*

Figure 3. Analysis of 5'-deletion mutants. The transcriptional efficiency of progressive 5' deletions of the *GHF1*-CAT fusion gene was determined by in vitro transcription in GH3, Ltk⁻, C6, and C2 extracts. The end point of each deletion mutant is indicated in the upper panel. Transcription reactions contained 100 ng of each of the *GHF1*-CAT constructs, 200 ng of α -globin, serving as an internal control, and 75 μ g of protein extract.

TATA region, to generate the TG mutant promoter. Activities of all promoters were tested in GH3 and Ltk⁻ extracts. Although the efficiency of the TG promoter is lower than that of the wild-type *GHF1* promoter, the results shown in Figure 5B indicate that both of these promoters, which contain the *GHF1* TATA region, were preferentially transcribed in the GH3 extract. The differential efficiency of the *GHF1* and TG promoters is probably due to the different strengths of their upstream elements. Because the upstream elements of the *GHF1* promoter are inactive in Ltk⁻ cells, it is difficult to derive any conclusions from the lack of activity of the GT and GG promoters in this extract. However, the results obtained with the GH3 extract demonstrate that substitution of the TATA element of the weak *TK* promoter with the TATA element of the *GHF1* promoter converts the *TK* promoter to a pituitary-specific promoter in the absence of pituitary-specific upstream elements. Furthermore, substitution of the *GHF1* TATA element with the corresponding elements of the *TK* or α -globin genes abolished its GH3-specific activity despite the presence of pituitary-specific upstream elements.

To delineate further the sequence requirements for the pituitary-specific TATA function, single and clustered point mutations were introduced into the *GHF1* TATA box and its immediate surroundings (Fig. 6A). These changes were introduced into the $\Delta 5'-200$ *GHF1* promoter so that binding sites for the upstream activators GHF-1 and CREB (McCormick et al. 1990) were retained. Replacement of the first T of the TATAA sequence with a G (T1) had the most severe effect on transcription in GH3 extracts (11-fold reduction). Replacement of the G preceding the TATAA sequence with a T (T2) also led to decreased promoter activity in the GH3 extract, although the effect was not as large as that of the T1 mutation. Another point mutation (T3) replacing an A downstream of the TATAA sequence with a C also decreased activity, but the effect was less than twofold (Table 1). Two clustered point mutations affecting sequences either downstream (T4) or upstream (T5) of the TATA box also decreased transcriptional efficiency, although the downstream mutations were more influen-

tial. With the exception of T1, none of these mutations had a significant effect on the low basal transcription of *GHF1* in the HeLa cell extracts; and even for T1 the effect on promoter activity in this heterologous extract (twofold reduction) was significantly smaller than its effect on *GHF1* promoter activity in GH3 extracts (Table 1). Longer exposure of the autoradiogram shown in Figure 6B revealed similar effects on the even lower basal transcription in the Ltk⁻ extract.

These results indicate that sequences immediately flanking the *GHF1* TATA box are mostly important for promoter function in GH3 extracts but are not very critical for low basal activity in heterologous extracts. We also examined whether these sequences are important for *GHF1* promoter activity in vivo. Wild-type and mutant *GHF1*-CAT constructs were transfected into GH3 cells incubated in the absence or presence of forskolin, an activator of adenylate cyclase. Previous work indicated that *GHF1* is a cAMP-inducible gene, and the use of forskolin increased the signal-to-noise ratio of such transfection experiments (McCormick et al. 1990). As documented in Table 2, all of the mutations were found to decrease *GHF1* promoter activity in vivo. Thus, the *GHF1* TATA region plays an important role both in vitro and in vivo. Unfortunately, because of the very low activity obtained upon transfection of *GHF1*-CAT into other cell types (McCormick et al. 1990) it was impossible to evaluate the effects of these mutations on *GHF1* promoter activity in Ltk⁻ or HeLa cells.

Different requirements for GHF1 and α -globin transcription

Although the minimal *GHF1* promoter described above is centered around the TATA box, it acts in a cell-type-specific manner and is therefore expected to interact with a unique factor that does not recognize a promoter, such as α -globin, which does not display the same specificity. The experiments shown in Figure 7 support this notion. Preincubation of a GH3 extract with a 50-fold molar excess of a 15-bp *GHF1* TATA element oligonucleotide decreased the transcriptional activity of the

Figure 4. Construction and analysis of the minimal *GHF1* promoter. (A) Construction of the minimal *GHF1* promoter. A synthetic double-stranded oligodeoxynucleotide corresponding to *GHF1* sequences from -39 to -24 (see Fig. 5A) was inserted upstream to the truncated HSV-TK ($\Delta 5'-46$) promoter. The length of primer extension products that correspond to transcripts initiated 30 bp downstream of the *GHF1* TATA element and from the authentic *TK* promoter is indicated. (B) In vitro transcription of GFTATA/TK (lanes 1 and 3) and TK $\Delta 5'-46$ (lanes 2 and 4) in GH3 and Ltk⁻ extracts. Reactions contained 100 ng of the various test templates and 200 ng of the α -globin internal control template. (C) *GHF1* minimal promoter activity is not conferred by GHF-1. GH3 extracts were incubated with up to 10³-fold molar excess (oligodeoxynucleotide : template) of a double-stranded oligodeoxynucleotide representing the high-affinity GHF-1 site of the *GH* gene for 15 min on ice. GFTATA/TK and *GH*-CAT templates (100 ng each) were added together with the α -globin control template (200 ng), and in vitro transcription reactions were performed and analyzed by primer extension. The location of extension products corresponding to transcripts initiated at the minimal *GHF1* promoter and the *GH* and α -globin promoters is indicated.

GHF1 promoter in comparison to its activity either in the absence of any competitor or in the presence of 50-fold molar excess of a 15-bp oligonucleotide spanning the α -globin TATA region (Fig. 7A). On the other hand, the *GHF1* TATA element oligonucleotide did not cause a meaningful decrease in α -globin promoter activity in comparison to the activity observed in the absence of any competing oligonucleotide. The α -globin TATA element oligonucleotide led to a nonspecific increase in the activity of both promoters, probably by titrating a general inhibitor of transcription present in these extracts. These results suggest that the *GHF1* TATA element interacts with a factor that is not involved in transcription of the α -globin promoter.

To compare the transcriptional requirements of the *GHF1* and α -globin promoters further, we examined their sensitivity to mild heat treatment, which is thought to inactivate the TATA recognition factor TFIID (Nakajima et al. 1988). Treatment of the GH3 extract at

47°C decreased transcription of the α -globin and *GH* promoters while having no effect on the truncated *GHF1* promoter (Fig. 7B). Similar results were found with the adenovirus major late promoter as an internal control (data not shown). These and other results (see Fig. 10, below) suggest that transcription of the *GHF1* promoter in GH3 extracts does not depend on the heat-sensitive form of the TFIID complex. On the other hand, the low basal transcription of *GHF1* in Ltk⁻ extracts appears to be TFIID-dependent as it is heat sensitive (data not shown). Inhibition of *GHF1* transcription by 1 μ g/ml of α -amanitin (Fig. 7A) indicates that it is nonetheless transcribed by RNA polymerase II.

The GHF1 TATA element interacts with a cell-type-specific factor

Further evidence for a pituitary-specific factor interacting with the minimal *GHF1* promoter was provided by

Figure 5. The *GHF1* TATA element is responsible for pituitary-specific transcription. (A) The sequence of the *GHF1* TATA box region is indicated (WT), as well as the nucleotide changes used to convert it to the corresponding sequences of the *TK* (GT) and α -globin (GG) promoters. Also indicated is the sequence of the TATA box region of the HSV-*TK* gene (TK) and the nucleotide substitutions used to convert it to the *GHF1* TATA sequence (TG). The arrows indicate a palindromic sequence. (B) Transcriptional analysis of the *GHF1*-WT(Δ 5'-200), GT(Δ 5'-200), GG(Δ 5'-200), TK(Δ 5'-109) and TG(Δ 5'-109) promoters in GH3 and Ltk⁻ extracts. Primer extension products corresponding to transcripts initiated at the *GHF1*, *TK* and α -globin (internal control) promoters are indicated.

cross-linking experiments. Incubation of a labeled *GHF1* TATA element oligonucleotide with GH3 extracts and irradiation with UV generated several protein-DNA adducts, the most abundant of which migrated on SDS-polyacrylamide gels with apparent molecular masses of

40 and 45 kD (Fig. 8). The 45-kD complex may represent the cross-linking of two DNA strands to the same polypeptide, whereas the 40-kD complex could be attributed to cross-linking of a single DNA strand. The lower abundance complexes migrating at ~80 and 90 kD may rep-

Figure 6. Mutational analysis of the *GHF1* TATA element. (A) The sequence of the wild-type *GHF1* TATA element (-39 to -24) is indicated (WT), as well as the sequences altered by the various point mutations (T1-T5). (B) Transcriptional analysis of the wild-type and mutant (T1-T5) *GHF1* (Δ 5'-200) promoters in GH3 and Ltk⁻ extracts. Reactions included 100 ng of each test template; autoradiography was for 12 hr.

Table 1. Activity of GHF1 TATA element mutants in GH3 and HeLa cells

Construct	GH3	HeLa
WT	1.00	1.00
T1	0.09	0.52
T2	0.50	0.78
T3	0.70	1.00
T4	0.40	1.13
T5	0.70	1.25

The activities of the various *GHF1* TATA element mutants are shown relative to that of the wild-type (WT) promoter. These values were derived by Cerenkov counting of the GH3 lanes in Fig. 6B and densitometric analysis of the HeLa lanes. All values were normalized to the internal control provided by the α -globin promoter. Please note that in both extract types the wild-type *GHF1* promoter was given an arbitrary value of 1.00 even though the *GHF1* promoter was 11-fold more active in the GH3 extract.

resent dimeric forms of the 40- and 45-kD complexes. In support of this notion, we found that the yield of these complexes was reduced upon shorter cross-linking times (cf. Fig. 8A with B and C). These complexes were specific, as they were competed by the wild-type *GHF1* TATA region oligonucleotide but not by the T1 mutant oligonucleotide (Fig. 8A), a high-affinity GHF-1 binding site (Fig. 8B), or an α -globin TATA element oligonucleotide (Fig. 8C). In addition, the mutant T1 oligonucleotide bound to this polypeptide with much lower efficiency than the wild-type oligonucleotide (Fig. 8A). As expected, incubation of the GHF-1-binding site oligonucleotide with GH3 extracts led to formation of two specific complexes (Fig. 8B) with molecular masses consistent with those of GHF-1 polypeptides complexed with DNA (Castrillo et al. 1989).

Incubation of the *GHF1* TATA element oligonucleotide with Ltk⁻ extracts led to formation of a major complex with a different mobility from those formed by the GH3 extract (Fig. 8B). These complexes were not specific as they were not competed by an excess of unlabeled oligonucleotide (data not shown). Complexes of identical mobilities were also formed by incubation of the GHF-1-binding site oligonucleotide with the Ltk⁻ extract, providing further evidence for their lack of specificity (Fig. 8B). A very low level of the 45-kD protein-*GHF1* TATA element adduct was formed by the C6 extract, whereas the C2 extract was similar in its activity to the GH3 extract (data not shown). Hence, the *GHF1* TATA element interacts with a specific binding protein that is present in considerably higher levels in GH3 and C2 cells in comparison to Ltk⁻ and C6 cells and is different from GHF-1. We named this complex, protein-pituitary transcription factor (PTF).

The pituitary-specific factor stimulates transcription of the GHF1 promoter

For further characterization PTF was partially purified from GC cell extracts (GC is a GH-expressing cell line

similar to GH3). Because the footprint pattern of the *GHF1* promoter is rather complex (see Fig. 2) we used the UV cross-linking assay to identify PTF activity. After initial fractionation by (NH₄)₂SO₄ precipitation, a GC whole-cell extract was applied to a heparin-agarose column and eluted by a stepwise KCl gradient. PTF activity eluted at 0.4 M KCl; these fractions were pooled and applied to a Sephadex S-300 gel filtration column. As shown in Figure 9A, PTF-binding activity detected by UV cross-linking eluted from this column with an apparent molecular mass of 40 kD (on the basis of the profiles of globular protein size markers). The same fractions also contained an activity that protected sequences between positions -39 and -22 of the *GHF1* promoter from DNase I digestion (Fig. 9B). Appearance of this footprint was associated with protection of a second region located immediately upstream of the *GHF1* TATA element, within plasmid sequences. A strong hypersensitive site was located between the two footprints. Most importantly, the same partially purified fractions were capable of stimulating *GHF1* minimal promoter activity when added to an Ltk⁻ extract (Fig. 9C). The transcriptional stimulatory activity coincided with the *GHF1* TATA element-binding activity.

The S-300 fractions that contained PTF transcriptional activity were concentrated by application to a CM-Sephadex column eluted with a 0.1–1.0 M KCl gradient. PTF-containing fractions were identified by the *in vitro* transcription assay. To compare the effect of PTF on the *GHF1* and α -globin promoters we added this partially purified preparation to normal and heat-treated GC nuclear extracts. Previous experiments indicated that GC nuclear extracts are partially deficient in PTF activity in comparison to GH3 whole-cell extracts and therefore serve as suitable recipients for testing PTF activity (H.

Table 2. Activity of GHF1 TATA element mutants *in vitro* and *in vivo*

Construct	In vitro	In vivo
WT	1.00	1.00
T1	0.09	0.07
T2	0.50	0.19
T3	0.70	0.30
T4	0.40	0.30
T5	0.70	0.28
GG	0.03	0.20
GT	0.08	0.35

The relative activities of the wild-type (WT) *GHF1* promoter and the various mutants were determined *in vitro* as described in Table 1 and *in vivo* by transfection into GH3 cells (McCormick et al. 1990). GH3 cells were transfected with 10 μ g of the various $\Delta 5' - 200$ *GHF1*-CAT constructs (wild-type and TATA element mutants), together with 2.5 μ g of RSV-luciferase. Forty hours later, forskolin was added to 10 μ M, and cells were harvested after 5 hr of incubation. The amounts of extract used to determine CAT activity were normalized according to luciferase activity. The numbers correspond to the relative levels of CAT activity as determined by AMBIS counting of thin-layer plates.

Figure 7. (A) Inhibition of *GHF1* transcription by excess TATA element. GH3 extracts were incubated with 0- to 50-fold molar excess (oligodeoxynucleotide : template) of oligodeoxynucleotides corresponding to the *GHF1* (GF-TATA) or α -globin (GL-TATA) TATA box regions (for sequences, see Fig. 5A). After 15 min incubation on ice the *GHF1*-CAT and α -globin templates were added and transcription reactions were carried out as described above. The reactions displayed in lanes α included 1 μ g/ml of α -amanitin in addition to 50-fold molar excess of the competing oligodeoxynucleotides. Reactions included 100 ng of each template. The ratio of *GHF1* to α -globin transcription is indicated at the bottom of each lane. (B) *GHF1* transcription is insensitive to mild heat treatment. The $\Delta 5'-41$ *GHF1*-CAT, GH-CAT, and α -globin templates (100 ng of each) were transcribed in either untreated GH3 nuclear extracts or in extracts that were preincubated at 47°C for 2 or 5 min.

Brady, unpubl.). PTF stimulated *GHF1* transcription in both extract types (Fig. 10). As shown above (Fig. 7B), preincubation of the GC extract at 47°C for 10 min did not decrease transcription from the *GHF1* promoter while it severely reduced α -globin transcription. PTF had no effect on α -globin transcription either in the non-treated or the heat-treated GC extract.

Discussion

This work was undertaken to define the minimal sequence requirements for differential transcription of the *GHF1* gene between GH-expressing and nonexpressing cell lines. To our surprise, a *cis* element composed of no more than 15 bp of DNA centered around the TATA box of the *GHF1* gene is sufficient for dictating its cell-type-specific transcription in vitro. As will be discussed below, this element functions as a cell-type-specific TATA element. Such findings are interesting in light of previous work indicating that TATA elements are recognized by TFIID, a protein that is thought to be present in all cell types and is functionally conserved between mammals and yeast (Buratowski et al. 1988, 1989; Cavallini et al. 1988; Nakajima et al. 1988; Hahn et al. 1989; Schmidt et al. 1989; Hoffmann et al. 1990; for review, see Lewin 1990). Therefore, a TATA element is expected to display similar activity in all cell types.

A cell-type-specific TATA element?

Cis elements that affect the efficiency of transcription initiation by RNA polymerase II are usually classified as enhancers, upstream promoter elements, and initiator elements (Breathnach and Chambon 1981; Serfling et al. 1985; Struhl 1989). Although used most commonly, the

TATA element is only one of several types of initiator elements (Smale and Baltimore 1989; Nakatani et al. 1990). The TATA element is characterized by its AT-rich sequence, whose minimal consensus is ATA, and its ability to direct the initiation of transcription from a site located 25–30 bp downstream (Breathnach and Chambon 1981). Although there are ample examples of cell-type-specific enhancers and upstream promoter elements (for review, see Maniatis et al. 1987; Renkawitz 1989), the minimal promoter of the *GHF1* gene appears to be the first example of a cell-type-specific TATA element. To prevent confusion, we wish to make a functional distinction in this discussion between the TATA element and the TATA box. Although the sequence of the *GHF1* TATA box is not considerably different from other TATA boxes of genes that are not pituitary-specific, the sequences that flank it appear to be unique. Analysis of other mammalian genes failed to identify a TATA box whose flanking sequences were similar to those present in *GHF1* (A. McCormick, unpubl.). Hence, the cell-type specificity of the minimal *GHF1* promoter is most likely dependent on flanking sequences but not on the TATA box itself. Indeed, mutations of these sequences have an adverse effect on *GHF1* promoter activity in GH3 but not in Ltk⁻ or HeLa extracts. On the basis of these findings we refer to the region encompassing the minimal *GHF1* promoter that includes the TATA box and its flanking sequences as the TATA element. It may be of interest to note that the *GHF1* TATA element contains an extensive palindrome (Fig. 5A). The significance of this palindrome remains to be determined.

Several criteria indicate that this part of the *GHF1* promoter functions as a bona fide TATA element and not as a conventional upstream promoter element. First, its sequence is AT rich and conforms to the TATA box

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Figure 8. UV-cross-linking analysis of protein binding to the *GHF1* TATA element. (A) Labeled wild-type *GHF1* TATA element oligonucleotide (GF-TATA) and a labeled oligonucleotide containing the T1 mutation (T1) were incubated with a 0.4 M heparin-agarose fraction of a GH3 WCE in the absence or presence of unlabeled GF-TATA (WT) and T1 competitors, prior to UV cross-linking for 15 min. (B) Labeled GF-TATA and *GHF1* oligonucleotides were incubated with either GH3 (GH) or Ltk⁻ (L) WCE in the absence or presence of GFTATA and GHF1 competitors prior to UV cross-linking for 5 min. (C) Labeled *GHF1* (GF) and α -globin (GL) TATA element oligonucleotides (for sequence, see Fig. 5A) were incubated with GH3 extract in the absence and presence of the various competitors as indicated prior to UV cross-linking for 5 min. Protein-DNA complexes were analyzed by electrophoresis on SDS-polyacrylamide gels and visualized by autoradiography for 36 hr. The migration positions of molecular weight standards are indicated; the 40- and 45-kD protein-DNA complexes are marked by arrowheads.

consensus. Substitution of G for the first T within this box leads to a marked decrease in transcriptional efficiency. Second, placing the 15-bp *GHF1* TATA element upstream of the TATA box of the HSV-TK gene does not augment transcription from the TK start site. Instead, transcription is initiated from a site located 30 bp down-

stream of the *GHF1* TATA. The proper selection of the initiation site does not depend on sequences outside of the 15-bp *GHF1* TATA element because it is functional when inserted either within plasmid sequences (Fig. 4) or within the TK promoter (Fig. 5).

Unlike conventional TATA elements, the *GHF1*

Figure 9. Gel filtration analysis of PTF. A heparin-agarose fraction of a GC extract that contained PTF-binding activity was chromatographed on a Sepharose S-300 column. Column fractions were examined for PTF-binding activity with either the UV cross-linking assay (5 min cross-linking time) with the WT (W) and T1 (T) *GHF1* TATA probes (A) or a DNase I footprinting assay with an end-labeled probe derived from the $\Delta 5'$ -41 *GHF1*-CAT construct (B). The elution volumes of molecular weight markers are indicated in A. The region of protection between positions -23 and -38 of the *GHF1* promoter, centered around the TATA box, is indicated at right. The solid line denotes *GHF1* sequences; the broken line denotes plasmid sequences. The same fractions were also examined for their ability to stimulate transcription of the GFTATA/TK hybrid promoter (C). Twelve microliters of each fraction was added to 100 μ g of an Ltk⁻ WCE, and the reactions contained 200 ng of each template.

TATA element exhibits pronounced cell-type-specificity. Mutagenesis experiments indicate that the TATA element is the major contributor to the cell-type-specific transcription of *GHF1* (Figs. 5 and 6; Table 1). Evidence

that sequences at the vicinity of the TATA box and the start site of transcription are involved in species-specific transcription was presented previously for the *Bombyx mori* fibroin gene, whose core promoter is located be-

Figure 10. Stimulation of *GHF1* transcription by PTF. CM-Sepharose-concentrated PTF was added as indicated (7 μ l/20 μ l reaction) to nuclear extract of GC that was either untreated or preincubated at 47°C for 15 min. Control reactions were supplemented with an equal volume of buffer. All reactions included 100 ng of Δ 5'-41*GHF1*-CAT and 200 ng of the α -globin templates. The location of primer extension products corresponding to properly initiated transcripts from the *GHF1* and α -globin promoters is indicated. The dot indicates the location of prematurely terminated primer extension product of the *GHF1*-CAT transcripts.

tween positions -37 and +10 (Takiya et al. 1990). In addition, transient transfection experiments suggest that the minimal promoters of the chicken ovalbumin and conalbumin genes are located between positions -56 to -1 and -44 to +62, respectively (Dierich et al. 1987). However, only in the case of the *GHF1* promoter was such an activity localized to a short 15-bp fragment centered around the TATA box itself.

A pituitary-specific transcription factor controlling GHF1

The differential activity of the *GHF1* TATA element implies that it must interact with a transcription factor that is either more abundant or more active in GH3 cells than in Ltk⁻ cells. A candidate protein responsible for this differential promoter activity was identified by UV cross-linking experiments. This protein, PTF, is more abundant in extracts of GH- and GHF-1-expressing cells and it interacts with the *GHF1* TATA element but not with the α -globin TATA element. This preference in binding is also reflected in the transcriptional activity of PTF: It stimulates transcription from the *GHF1* but not the α -globin promoter. Stimulation of *GHF1* promoter activity by PTF is observed either after addition to Ltk⁻ extracts, in which it is either absent or present in very

low concentration, or after addition to nuclear extracts of GC cells. The discrimination by PTF between the *GHF1* and α -globin promoters is consistent with the results of the competition experiments which show that the *GHF1* TATA element inhibits transcription from the *GHF1* but not the α -globin promoter. On the basis of these findings, PTF is not likely to be a general transcription factor that affects transcription in a nondiscriminate manner.

Several experiments indicate that the target for PTF is the *GHF1* TATA element. The presence of the TATA box within this element raises questions regarding the relationship between PTF and TFIID. Although the relative insensitivity of the *GHF1* promoter to incubation of the pituitary cell extracts at 47°C, a treatment known to inactivate TFIID (Nakajima et al. 1988), may suggest that its transcription in these cells is TFIID independent, further and thorough fractionation of the basic transcriptional machinery is required to determine this point. It is possible that PTF allows heat-inactivated TFIID to interact with the *GHF1* and not the α -globin promoter by stabilizing its interaction with the *GHF1* TATA element. Although it is possible that PTF is identical to TFIID and therefore provides the same function, several experimental results argue against this. First, PTF is much more abundant in GH3 than in Ltk⁻ cells, whereas the level of TFIID antigenic activity (determined by immunoblotting with anti-TFIID antibodies obtained from Dr. A. Berk) is identical in both cell types. Second, these antibodies do not react with partially purified PTF (H. Brady and J. Fukushima, unpubl.). Third, PTF elutes from the gel filtration column as a 40-kD protein while TFIID fractionates on such columns as a 120-kD complex (Reinberg et al. 1987). However, it is still possible that PTF is a modified form of TFIID that behaves differently on gel filtration columns and has lost the ability to interact with anti-TFIID antibodies raised against an amino-terminal peptide of human TFIID. Only after its primary structure is determined can the relationship between PTF and TFIID be fully assessed.

Because TFIID is currently thought to be required for transcription of all TATA box-containing promoters and possibly even for transcription of "TATA-less" RNA polymerase II promoters (for review, see Lewin 1990), we favor the view that PTF acts in conjunction with TFIID on the *GHF1* TATA element to confer upon it cell type specificity.

Implications for cell-type-specific gene expression

Although the *GHF1* TATA element is distinct in its cell-type-specific activity, it is not the first example of a functionally distinct TATA element. Simon and co-workers (Simon et al. 1988; 1990) have shown that certain TATA elements differ from others in their ability to respond to the viral *trans*-activator E1A. Although E1A responsiveness appears to be associated with the presence of a specific sequence (TATAA), it is not known whether E1A-responsive TATA elements are recognized by a protein distinct from the one recognizing nonresponsive TATA

elements. Functionally distinct TATA elements were also described in the late genes of HSV-1 (Homa et al. 1988). It remains to be determined, however, whether these elements are recognized by proteins distinct from those recognizing the TATA elements of immediate-early and early HSV genes. It is likely that these apparently unique classes of TATA elements are all recognized by the general factor TFIID, which was recently shown to be a target for viral *trans*-activators (Abmayr et al. 1988; Leong et al. 1988; Wu and Berk 1988).

The finding of a cell-type-specific TATA element within the *GHF1* promoter and not in other genes is quite intriguing. For example, no such elements were detected in either the *GH* or prolactin promoters, both of which exhibit similar cell-type specificity (Bodner and Karin 1987; Gutteriez-Hartman et al. 1987; McCormick et al. 1988; Nelson et al. 1988). It seems reasonable that primary control of cell-type-specific regulatory genes will be exerted at the most basic level—that of the TATA element. Such a mechanism may ensure that expression of these genes is tightly controlled to prevent low levels of expression in inappropriate cell types. In the case of *GHF1*, a low level of leaky expression may present a considerable risk, because once activated it is positively autoregulated (McCormick et al. 1990). It remains to be seen, however, whether this regulatory mechanism is generally applied for controlling the expression of cell-type-specific *trans*-activators. It would also be of interest to determine whether negative regulation of PTF activity or expression is responsible for extinction of *GHF1* expression in somatic cell hybrids.

Materials and Methods

In vitro transcription

Cells used for extract preparation were grown on 150-mm plates as described (McCormick et al. 1988). WCEs were prepared from 3 to 5 ml of packed cells (Bodner and Karin 1987; McCormick et al. 1988). Unless specified otherwise, *in vitro* transcription reactions contained 75–150 µg of extract protein, 200 ng of the control α -globin template, and 100 ng of the specific test template added as supercoiled plasmids. In a standard reaction, WCE in 20 µl of buffer (Bodner and Karin 1987) was mixed with the templates in 5 µl of H₂O and incubated for 15 min on ice before the addition of 20 µl of NTP mixture (1 mM each) and 5 µl of 5% polyvinyl alcohol. RNA synthesis was carried out at 30°C for 60 min. Reactions were terminated, and RNA was extracted and analyzed by primer extension as described previously (Bodner and Karin 1987).

Plasmids

The human α -globin gene in pBR322 (Liebhaber et al. 1980) was used as an internal control. *GHF1*-CAT was described previously (McCormick et al. 1990). The -200, -90, -50 and -41 deletion mutants were generated by PCR amplification (Mullis and Faloona 1987) with primers complementary to the various deletion end points and a second primer complementary to *GHF1* sequences from -18 to +2. The amplified products were cloned in front of the CAT structural sequence in the vector pUCAT- (M. Karin, unpubl.), and their sequence was confirmed by dideoxy sequencing (Hattori and Sakaki 1986). The

GHF1/TK constructs were generated by subcloning *Hind*III-*Sal*I fragments containing *GHF1* promoter sequences derived from the appropriate 5'-deletion mutants, in front of HSV-TK Δ 5'-46 (McKnight et al. 1981). The GFTATA/TK construct was generated by subcloning of the *GHF1* TATA oligonucleotide (see below) upstream of the truncated HSV-TK Δ 5'-46 promoter. The *Hind*III-*Sal*I restriction fragment containing the -200 *GHF1* promoter was subcloned into M13mp19 and single-stranded DNA was used as a template for site-directed mutagenesis (McCormick et al. 1990). The mutagenic oligonucleotides 5'-AG-GAGGGTCG AGGCTCACCT TAATATGCGA AGGAAATCCA TTTATA-3' and 5'-AGGAGGGTCG AGGCTAGGGT TTATGCTTGG GGGAAATCCA TTTATA-3' were used to convert the *GHF1* TATA element to those of the HSV-TK and α -globin genes, respectively (see Fig. 5A). The T1-T5 mutants were generated using the same single-stranded template and appropriate mutagenic oligonucleotides spanning the *GHF1* TATA. The TK TATA element was converted to that of *GHF1* by mutagenizing a *Bam*HI-*Kpn*I fragment containing TK sequences from -109 to +635 subcloned into M13mp19 with the primer 5'-CGAGGCCACA CGCGTAGTAT TTATACTCTG AGTGGACCCCT CGGACCGC -3'. The sequences of all mutants were directly confirmed by dideoxy sequencing.

UV cross-linking

The following oligonucleotides were used as competitors and probes for UV cross-linking experiments:

GHF1-TATA: 5'-AGCTTCAGAGTATAAAATACG
AGTCTCATATTTATGCAGCT-5'

α -globin TATA: 5'-AGCTTCCCAAGCATAAACCCG
AGGGTTCGTATTTGGGCAGCT-5'

GHF1-binding site: 5'-GATCCCATGCATAAATGTACACAG
GGTACGTATTTACATGTGTCTTAA-5'

The 20-µl reaction contained 10 µg of protein extract (either WCE or 0.4 M heparin-agarose fraction), 0.3 ng of end-labeled oligonucleotide probe, 1 µg of poly[d(I-C)], 10 mM HEPES (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 1 mM DDT, and 10% glycerol and was incubated for 20 min on ice. For competition experiments, 25 ng of competitor oligonucleotides was used. UV cross-linking was performed with a Stratagene Stratalinker 254-nm bulb for 5–15 min at a distance of 12 cm. SDS gel sample buffer (20 µl) was added, and samples were heated at 100°C for 3 min and analyzed on 10% polyacrylamide gels containing SDS.

Partial purification of PTF

WCE from 20 g of GC cells was fractionated by (NH₄)₂SO₄ precipitation. The 10–65% saturated (NH₄)₂SO₄ fraction was dissolved in 10 ml of buffer A (20 mM HEPES-KOH (pH 7.6), 100 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.1 mM PMSF and dialyzed against 1 liter of buffer A containing 0.1 mM PMSF at 4°C. After clearing (15 min at 10,000 rpm), the supernatant was applied to a 25-ml heparin-agarose column equilibrated with buffer A that was eluted by a stepwise KCl gradient (0.1–1.0 M) in buffer A. PTF activity eluted at 0.4 M KCl and these fractions were pooled and applied to a 900-ml Sephadex S-300 gel filtration column eluted with buffer A. PTF-containing fractions were pooled and applied to 4 ml CM-Sephacrose CL-6B column eluted with a linear 0.1–1.0 M KCl gradient in buffer A.

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The pituitary-specific regulatory gene GHF1 contains a minimal cell type-specific promoter centered around its TATA box.

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