© 1990 Oxford University Press

Somatotroph- and lactotroph-specific interactions with the homeobox protein binding sites in the rat growth hormone gene promoter

Fred Schaufele, Brian L.West and Tim Reudelhuber
Department of Medicine and Metabolic Research Unit, the University of California, San Francisco, CA 94143, USA

Received March 28, 1990; Revised and Accepted August 1, 1990

ABSTRACT

Nuclear extracts prepared from growth hormone-secreting (GC) and prolactin-secreting (235-1) rat anterior pituitary cell lines were compared for their ability to bind to the DNA sequences conferring tissue-specificity to the expression of the rat growth hormone (rGH) gene promoter. Cell-specific differences in the interaction of Pit-1, a tissue-specific member of the POU-domain transcription factor family, with the pGHF1 binding site were detected by methylation interference experiments; otherwise the Pit-1 proteins present in GC cell and 235-1 cell extracts were similar. Two other protein/DNA complexes, GHF5 and GHF7, were detected by gel mobility shift assays and the binding of both complexes to the rGH promoter depended upon DNA sequences contained within the two binding sites for Pit-1. In contrast to Pit-1 which can bind to either of the two sites independently, a single Pit-1 binding site was insufficient for GHF5 and GHF7 binding; i.e. both Pit-1 binding sites within the rGH promoter were required. Whereas GHF5 was present in nuclear extracts of GC cells and a variety of cells not producing growth hormone, GHF7 binding activity was detected only in the GC cell line (and not in the 235-1 cell line). GHF7 binding activity was therefore more closely correlated with growth hormone gene transcription than was Pit-1. rGH promoters containing mutations which inhibited GHF5, GHF7 and Pit-1 binding were expressed less efficiently than the wild type promoter after transfection into GC cells. One promoter mutation to which the GHF7 complex but not the Pit-1 factor can bind was also transcription deficient demonstrating that Pit-1 binding, independent of GHF7 binding, was nevertheless important to the expression of the rat growth hormone promoter.

INTRODUCTION

How the genome encodes the myriad of different cell types required to complete the body plan remains one of the central questions of modern biology. Studies of the molecular genetics of the fruit fly have demonstrated the importance during development of sequence-specific DNA binding proteins which can affect gene transcription (reviewed in 1,2). A subset of these developmentally important transcription factors contains a common block of 60 amino acids termed the homeodomain (3,4) which has been found to be conserved across broad phylogenetic boundaries (5,6). More recently, a set of cell-type-specific homeodomain-containing proteins was found to contain a second area of homology more amino terminal to the homeodomain. These proteins have been collectively designated the 'POU-domain' proteins (7).

One of the mammalian POU-domain proteins, Pit-1 (also called GHF1) is restricted to the adult anterior pituitary and has been implicated in the expression of the growth hormone (GH) gene (8–15); GH gene expression itself is also restricted to the anterior pituitary gland (16). However, *in situ* hybridizations have detected Pit-1 RNA (17) and protein (14) not only in the subset of cells in the pituitary which produce GH (somatotrophs) but also in the lactotrophs and some thyrotrophs which express prolactin and TSH, respectively but not GH.

In order to study why the growth hormone gene is not expressed in some cell types in which the Pit-1 gene is at least transcribed, we compared nuclear extracts derived from a GH-secreting (GC; Ref.18) and a prolactin-secreting (235-1; Ref.19) rat cell line for proteins binding specifically to sequences within the rat growth hormone (rGH) promoter. We observe that the 235-1 cell line contains a Pit-1 protein with some characteristics very similar to that found in the GC cell line, although a cell-specific difference in the sensivity of the binding of Pit-1 to methylation of the proximal Pit-1 binding site is noticed. *In vitro* transcription and transient expression analyses suggest that the Pit-1 protein derived from 235-1 cells is capable of activating transcription, sharpening further the paradoxical inactivity of the rGH promoter in these cells. We describe two other complexes, GHF5 and GHF7, which require two intact Pit-1 binding sites within the rGH promoter for DNA binding. GHF5 is found in a number of different cell types whereas GHF7 appears to be restricted to the rGH-producing GC cell line. Transient expression assays show that mutant promoters in which the GHF5 and GHF7 (as well as the co-mutated Pit-1) binding sites are destroyed are transcriptionally deficient. However, some transcription from the rGH promoter still occurs in GC cells in the absence of GHF5 or GHF7 binding. Therefore, the restriction of rGH promoter activity to somatotrophic cells is probably not due to GHF7 alone

but may arise from a combination of factors, possibly cell-specific differences in the Pit-1 protein and/or GHF7 distribution.

MATERIALS AND METHODS

Extract preparation and DNA binding assays

Preparation and extraction of liver nuclei was performed essentially as described in Gorski et al. (20) with the further addition of the proteinase inhibitors, antipain, leupeptin, chymostatin and pepstatin A (Sigma) at final concentrations of 5 μg/ml each during the nuclei preparation and extraction steps. Extracts were similarly prepared from GC, 235-1 and HeLa nuclei except the cells were initially homogenized in 0.25 M sucrose-containing buffer and the 'homogenization buffer' contained 1.7M sucrose in the subsequent steps.

DNaseI footprinting (Figure 1A) was performed using fragments labelled with T4 polynucleotide kinase at the sites indicated below. For the gel mobility shift assays (Figures 1B, 5–8), 1 μl (8 μg) of extract was incubated at room temperature for 20 minutes in a 20 μl final volume with (i) 1 μl (ca. 5 fmoles) of radiolabelled DNA in 100 mM KCl, (ii) 1 μl (4 μg) of polydI-dC (Pharmacia), (iii) 8 μl of 30 mM Hepes, pH 7.6 (0°), 120 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA, and 24% glycerol. For the competition studies, each 1 μl (200–500 ng) of the indicated, unlabelled oligonucleotides in 100 mM KCl was substituted for 1 μl of 100 mM KCl added to the uncompeted incubations. Following incubation, the samples were put on ice and loaded directly onto pre-run (2 hrs) 4%, 29:1 (poly-:bis-acrylamide) gels precooled to +4°. Electrophoresis buffer was 6.7 mM Tris, 1 mM EDTA, 3.3 mM $CH_3COO^-Na^+$, adjusted to pH 7.5 (21°) with CH_3COOH. DNaseI footprints of gel-shifted material (Figure 5B) were performed by digesting the complexes formed after 20 minutes of incubation with 50 ng of DNaseI (Worthington) at room temperature for one minute (stopped by the addition of EDTA to 12.5 mM) before loading onto the native polyacrylamide gel. Detection of the GHF5 and GHF7 complexes required the incubation of 8 μg of crude GC cell nuclear extract with radiolabelled −210/−6 (Figure 5) or −210/−51 (Figures 6,7) rGH promoter fragments in the presence of 200 ng each of the following pGHF1 and GHF2 oligonucleotides (positions of the GHF2 and pGHF1 DNaseI-protected regions are underlined):

```
GHF2 : AGCTTGCGATGTGTGGGAGGAGCTTCTAG
              ACGCTACACACCCTCCTCGAAGATCCTAG
pGHF1: AATTCTGGCTCCAGCCATGAATAAATGTATAGGGAAAAAGGCAGACCG
                GACCGAGGTCGGTACTTATTTACATATCCCTTTTTCCGTCTGGCCTAG
```

The mutations shown in Figure 5A were obtained by replacing sequences between the indicated coordinates with sequences derived from pUC polylinkers (see ref. 21 for details). The mutations across the pGHF1 and dGHF1 binding sites (Figure 5B) were obtained by M13 mutagenesis using the appropriate oligonucleotides. The mutations were confirmed by sequencing both in the M13 phage and after subcloning into the rGHCAT vectors.

in vitro transcription and RNA analysis

Each *in vitro* transcription reaction was done in a 20 μl final volume with 175 ng (1 μl) of rGHCAT and 50 ng (1 μl) of RSVCAT DNA, 7 μl (56 μg) of GC or 235-1 cell nuclear extract, 2 μl of 10× transcription buffer (250 mM Hepes, pH 7.9 (21°), 500 mM KCl, 60 mM $MgCl_2$), 1 μl of 6 mM NTPs, 1 μl (30 U) RNasin (Promega Biotec) and 7 μl of 6 parts H_2O/1 part glycerol. Reactions were incubated at 30° for 45 mins. and stopped by the addition of 300 μl of 1% SDS, 300 mM NaCl, 10 mM Tris (7.5), 0.1 mM EDTA and 40 μg of Proteinase K (Boehringer). After 30 mins. of Proteinase K digestion, samples were phenol/chloroform and chloroform extracted then ethanol precipitated. 10% of the RNA collected from each transcription reaction was hybridized overnight with a gel purified rGHCAT RNA probe (containing 255 bp of CAT sequence and rGH promoter sequences from −106 to +8 spanning the transcription start site) or RSVCAT probe (containing the same 255 bp of CAT sequence and RSV promoter sequences from −45 to +50). In this fashion, rGHCAT probes map correctly initiated rGHCAT transcripts and detect the internal control RSVCAT transcripts through cross-hybridization with the CAT sequences common to the rGHCAT and RSVCAT transcripts; the reverse holds when the RSVCAT probe is utilized. Samples were RNase A and RNAse T_1 digested the following day and loaded onto 6% sequencing gels.

DNA transfections

12 μg of the appropriate rGHCAT construct containing rGH promoter sequences from −237 to +8 (8) attached to the CAT coding sequence were cotransfected with 2 μg of RSVCAT by electroporation into GC cells. The RNA was collected by the Guanidium/Cesium Chloride method (22) and mapped with RNA probes as described above.

RESULTS

Pit-1 is present in the lactotrophic 235-1 cell line

It was previously reported that Pit-1 RNA was present in lactotrophic (prolactin-secreting) cells in the native mouse pituitary (17). Rat pituitary cell lines that secrete exclusively either growth hormone or prolactin are available (18,19) and these were used in the present analysis to investigate the restriction of GH expression to the somatotrophic cell type. No endogenous rGH RNA was detected in the 235-1 cell line by Northern analysis (data not shown). Transient transfection of either GC or 235-1 cells with plasmids containing the native rGH promoter (−237 to +8) linked to the CAT structural gene yields transcripts in GC cells, but not 235-1 cells (Figure 4A and ref. 23). These data suggest that the inability of rGH RNA to be detected in the lactotrophic cell line lies at the level of the promoter and is not solely a consequence of some post-transcriptional activity on the rGH RNA.

In order to investigate whether the Pit-1 transcription factor is present in lactotrophic cell types, proteins binding to specific DNA sequences within the rGH promoter were compared in nuclear extracts derived from rat pituitary cell lines expressing either prolactin (235-1) or growth hormone (GC). DNaseI footprinting experiments demonstrate that a factor is present in both extracts which binds to and protects both the proximal and the distal Pit-1 binding sites (pGHF1 and dGHF1) within the rGH promoter sequences against DNaseI digestion (Figure 1A, ref. 24). Gel mobility shift assays also detect a Pit-1-like binding activity present in both GC and 235-1 cell (Figure 1B, lanes 1,2): these complexes are not found in liver, are displaceable by an excess of unlabelled competitor oligonucleotide specific for the pGHF1 binding site (lanes 4–6); and are affected by the same promoter mutations (see Figure 5) which affect the Pit-1-specific

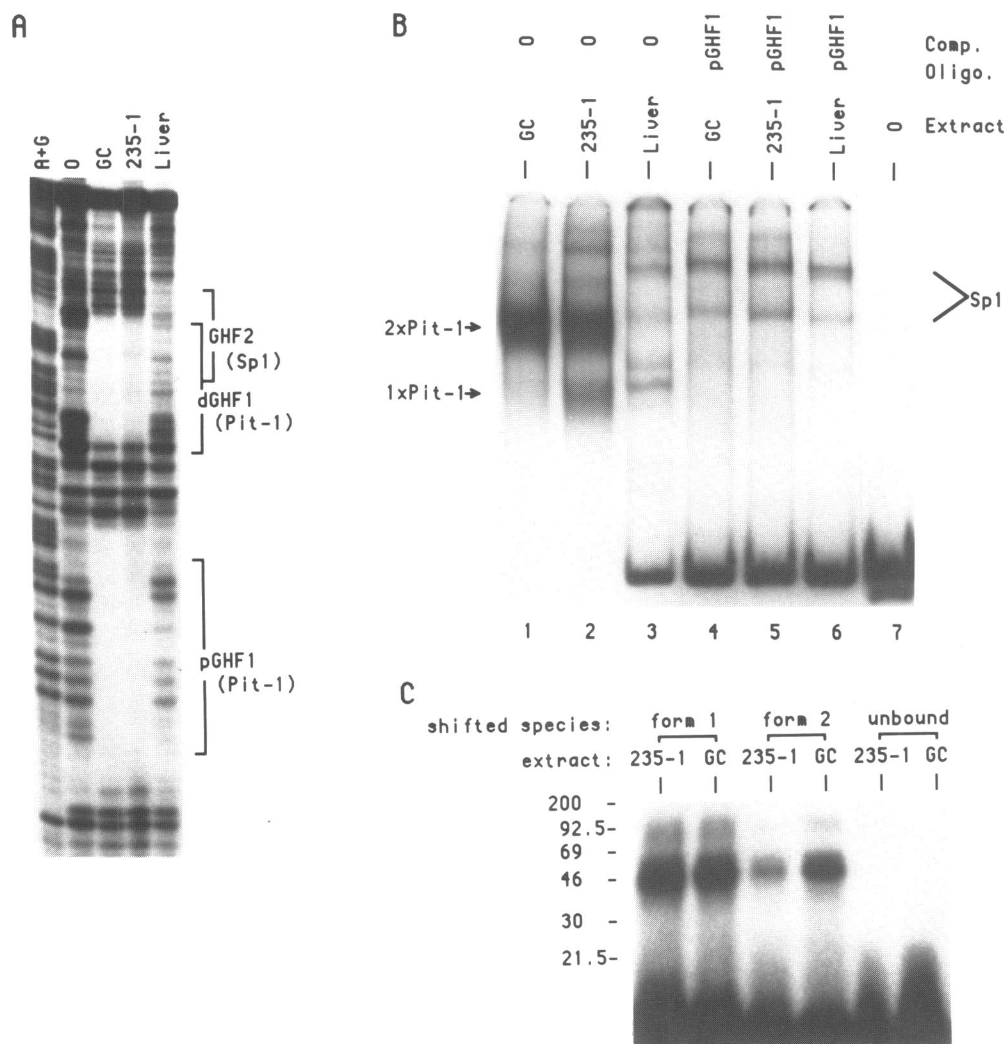

Fig. 1. A, Both the proximal (pGHF1) and the distal (dGHF1) binding sites for the transcription factor, Pit-1, are protected identically against DNaseI digestion when an rGH promoter fragment is pre-incubated with 80 μg of either GC or 235-1 cell nuclear extract. Rat liver extracts protect the GHF2 binding site for the transcription factor Sp1 (21). A+G, Maxam-Gilbert sequencing ladder. **B,** Gel shifts of the −147/−48 fragment with GC, 235-1 or rat liver nuclear extracts. 1× or 2×Pit-1, DNA bound by one or two Pit-1 proteins (see Figure 5A). Sp1, Sp1 specific gel shifts (see ref. 25). **C,** Protein/DNA cross-linking of complexes representing either GC- or 235-1-derived Pit-1 protein bound to a labelled pGHF1 oligonucleotide. Two Pit-1 specific complexes of slightly different mobility (forms 1 and 2) as well as unbound (free) pGHF1 oligonucleotides were isolated from gel mobility shift assays, uv irradiated (see ref. 28) and the proteins cross-linked to the radiolabelled oligonucleotide were detected by SDS gel electrophoresis.

footprints. Furthermore, when the specific Pit-1 protein/radiolabelled pGHF1 DNA complexes visualized by gel mobility shift assays are uv-irradiated and isolated from these gels, the resulting cross-linked protein/DNA complexes migrate identically on denaturing polyacrylamide gels (Figure 1C, forms 1 and 2 refer to two closely migrating, pGHF1-specific, gel shift complexes observed to bind to the pGHF1 oligonucleotide). The size of the cross-linked Pit-1 agrees favourably with the 43 kD doublet reported by Nelson et al. (13) although this is considerably larger (possibly due to the charge and/or mass of the cross-linked DNA) than the 33 kD size suggested by Pit-1 protein purification (24,25) and cDNA sequence analysis. Pit-1 proteins of this size have been detected in both 235-1 and GC cell extracts by southwestern analysis (24).

Thus, DNaseI footprinting (Figure 1A), gel mobility shift assays (Figure 1B) and protein/DNA cross-linking (Figure 1C) all demonstrate that a factor is present in 235-1 cells that binds to the same rGH promoter sequences (Figure 1A, ref. 24) and migrates identically on native (Figure 1B) and denaturing (Figure 1C, ref. 24) gels as the Pit-1 factor derived from GC cells. Both the GC and 235-1 Pit-1 proteins also bind more tightly to the more proximal Pit-1 binding site within the rGH promoter (data not shown). The Pit-1 binding activity also appears to be just as abundant in nuclear extracts prepared from 235-1 or GC cells (data not shown).

The DNA binding characteristics of Pit-1 factors derived from GC and 235-1 cells are not identical

Methylation interference experiments reveal some differences between the Pit-1 proteins derived from 235-1 and GC cells (Figure 2). Whereas the methylation of a guanine residue at position −86 (*) on the growth hormone promoter (+1 = transcription start site) reduces the binding of the 235-1 Pit-1 factor (lane 12), it more severely inhibits the binding of the GC Pit-1 protein (lane 11). Additionally, methylation of a second guanine at position −78 (+) has no effect on binding of the 235-1 Pit-1 but mildly reduces the binding of the GC Pit-1. A similar pattern was observed regardless of whether the rGH fragment

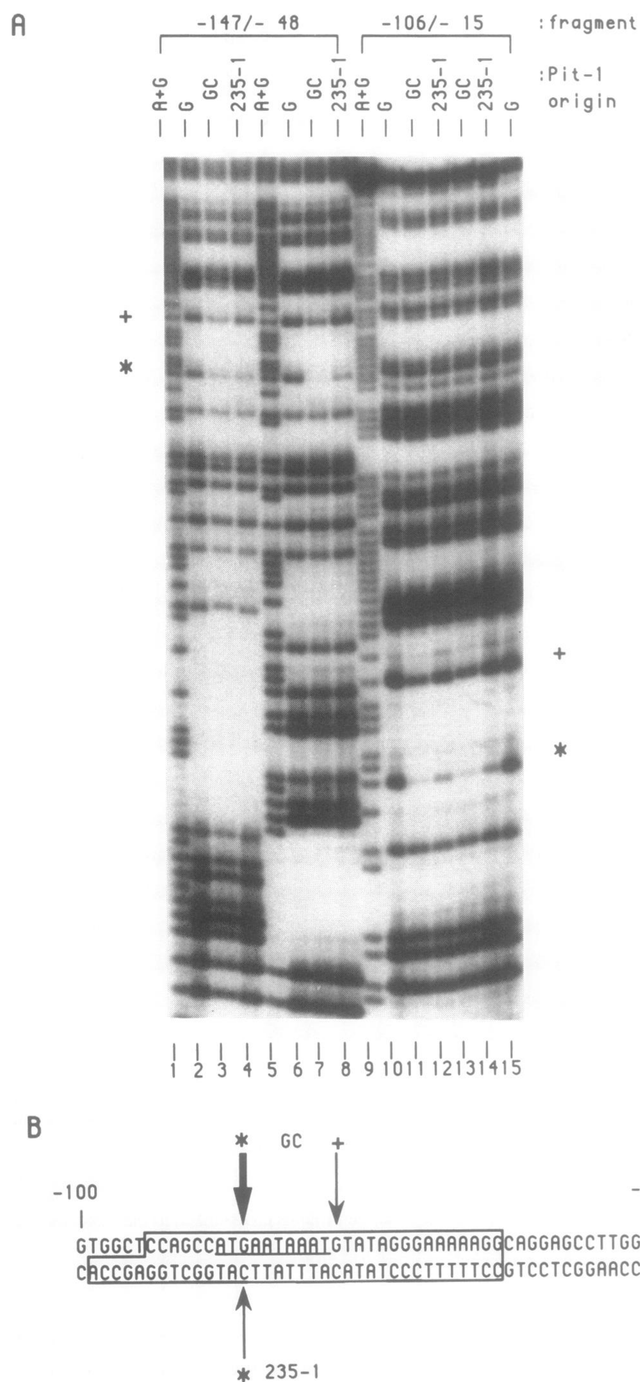

Fig. 2. A, Differential inhibition of the Pit-1 factor derived from GC or 235-1 cells by methylation of guanines at −86 (*) or −78 (+) within the rGH promoter. DNA isolated from GC or 235-1 Pit-1 gel shift complexes formed with randomly methylated rGH promoter fragments (−147/−48 lanes 1−4, −147/−48 containing the −139, −110 mutation (see ref. 21) lanes 5−8, −106/−15 lanes 9−12, or −106/−15 in the presence of a GHF6 competitor oligonucleotide lanes 13−15) was cleaved with piperidine and compared to cleaved unbound DNA (G) on a sequencing gel. **B,** Location of the affected guanines: the proximal Pit-1 footprint, pGHF1, is boxed and the Pit-1 consensus sequence is underlined. Thickness of arrows symbolizes the magnitude of the inhibition of Pit-1 binding by methylation of a particular guanine.

probed covered only the pGHF1 (proximal Pit-1) binding site (lanes 9−12), both the Pit-1 binding sites and the Sp1 binding site (lanes 1−4) or the same fragment as used in lanes 1−4 but with the dGHF1 (distal Pit-1) and the overlapping Sp1 (25) binding sites mutated (lanes 5−8). The difference in the methylation interference pattern also remains when the GC and 235-1 Pit-1 complexes are formed in the presence of an oligonucleotide which competes for the binding of GHF6 (lanes 13−15), a factor which binds immediately downstream of the pGHF1 site and which is more abundant in 235-1 cells than in GC cells (F.S. and T.L.R., unpublished data). The location of the methylated guanine residues which affected Pit-1 binding with respect to the pGHF1 binding site and the Pit-1 consensus sequence (13) is summarized in Figure 2B.

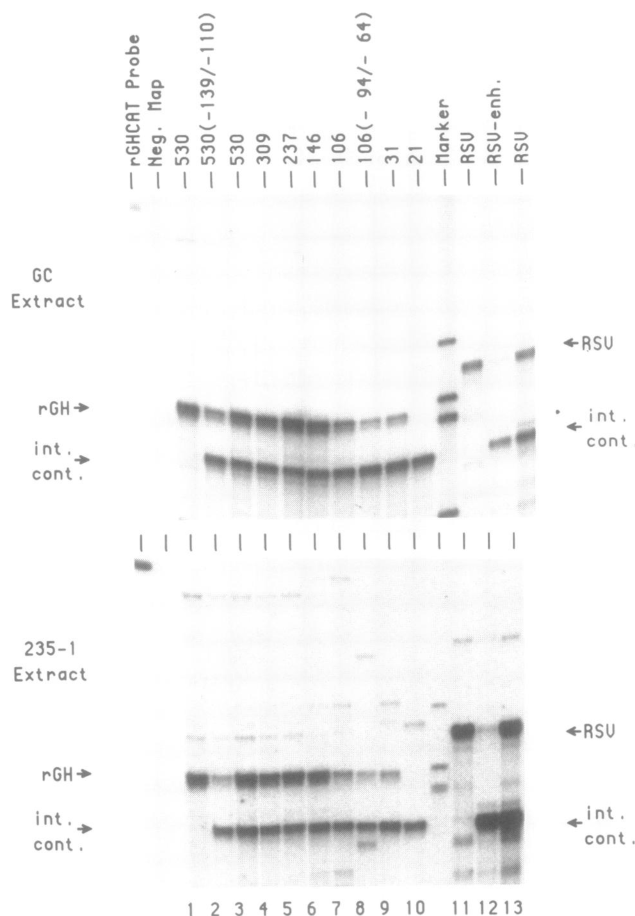

Fig. 3. SP6 mappings of RNAs generated by *in vitro* transcriptions from rGH or RSV promoters in nuclear extracts derived from GC or 235-1 cells. 5′ serial deletions of the rGH promoter to the indicated nucleotides (lanes 1−10) were mapped with an rGHCAT probe. The RSV promoter, with (RSV) or without (RSV-enh) enhancer sequences between −40 and −327 was mapped with an RSVCAT probe (lanes 11−13). int. cont., internal control transcripts generated from rGHCAT (lanes 12,13) or RSVCAT (lanes 2−10). rGHCAT Probe, small aliquot of the RNA probe without RNase treatment. Marker, Hae-III digested ϕX174 DNA (sizes shown in Fig. 8). Neg. Map, negative mapping control where the probe was hybridized without added *in vitro* transcribed RNA then RNase treated.

The 235-1 Pit-1 protein is functional

The presence of a Pit-1 protein in the 235-1 cell extracts does not necessarily imply that it is a transcriptionally active factor. In order to address this question we performed both transient expression assays using artificial constructs containing Pit-1 binding sites and *in vitro* transcriptions using the rGH promoter.

RNase protection experiments performed on RNAs transcribed *in vitro* (Figure 3) shows that constructs containing 530 bp of the rGH promoter attached to the protein-encoding portion of

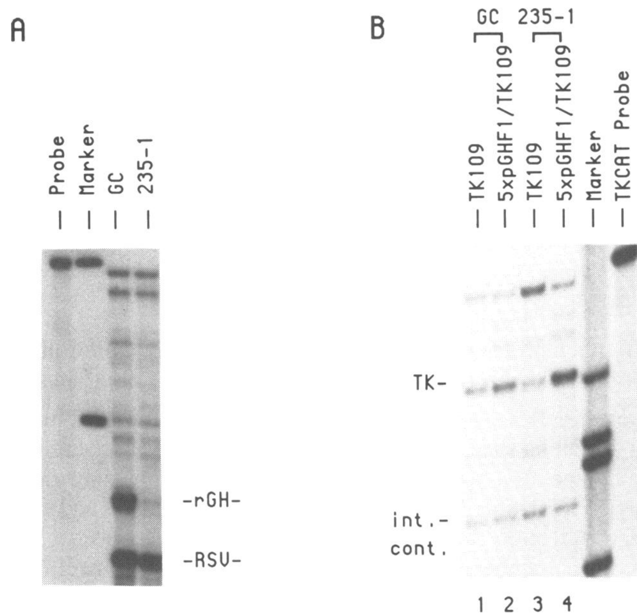

Fig 4. A, RNase protection assays of RNAs expressed from rGHCAT or RSVCAT constructs transfected into 235-1 or GC cells. Marker, HpaII-digested pBR322 DNA. **B,** RNase protection assays of RNAs expressed after transfection of the TK109 or the 5xpGHF1TK109 promoter into GC or 235-1 cells. RSVCAT transcripts serve as internal controls.

the bacterial chloramphenicol acetyl transferase gene (rGHCAT) are efficiently transcribed from the proper rGH transcription initiation site when incubated with NTPs and nuclear extracts prepared from either GC or 235-1 cells (lane 1). Transcription in either extract is unaffected by serial deletions from the 5′ end of the rGH promoter down to −146 (lanes 3−6, RSVCAT transcripts detected by hybridization with the CAT sequences within the rGHCAT probe serve as an internal transcription control). Further deletions eliminating the distal and/or the proximal Pit-1 binding sites (lanes 7,8) reduce transcription down to levels observed from constructs containing the rGH TATA box alone (lane 9). Both GC and 235-1 extacts are also similarly effective in directing upstream sequence-dependent transcription from the RSV promoter (lanes 11−13) demonstrating the validity of comparing the efficiency of the rGH promoters in these extracts.

Therefore, transcription of the rGH promoter in extracts from both the somatotrophic GC and the lactotrophic 235-1 cells requires sequences encompassing the Pit-1 binding sites. This was confirmed using 530 bp long rGH promotors in which mutations selectively introduced into either the proximal or the distal Pit-1 binding sites were less effective than the wild type promoter in promoting *in vitro* transcription (data not shown).

Effective *in vitro* transcription implies only that the 235-1 Pit-1 factor is capable of enhancing transcription from an rGH promoter but reveals little of whether this is so *in vivo*. Indeed the rGHCAT promoter constructs which are very active in GC cells are inactive after transfection into 235-1 cells (23, Figure 4A). In order to investigate whether this difference is due to the Pit-1 factor found in 235-1 cells, we compared the transcriptional activity of 109 bp of the Herpes Simplex Thymidine Kinase promoter (TK109) with that of an artificial promoter in which 5 copies of the proximal Pit-1 binding site (pGHF1) were inserted immediately upstream of the same TK109 promoter. After transfection into either GC or 235-1 cells, the 5XpGHF1/TK109CAT construct was observed to be a more effective promoter (Figure 4B, lanes 2,4) than the parental TK109 promoter (lanes 1,3); transcripts generated from an RSVCAT plasmid serve as an internal control for transfection and RNA recovery. Therefore, a factor seems to be present in 235-1 as well as in GC cells which is capable of binding to multimerized Pit-1 binding sites and enhancing transcription from the TK promoter.

Binding of GHF5 and GHF7 require sequences contained within two Pit-1 binding sites

If 235-1 cells contain a Pit-1 factor which is functional *in vivo*, then why is the rGH promoter inactive in these cells? The cell type specificity of the rGH promoter is retained utilizing 237 bp of promoter (26,27,Figure 4A). We therefore looked elsewhere within this 237 bp for differences in DNA binding proteins present in extracts of 235-1 and GC cells. Two other previously described transcription factors, GHF3 and Sp1, bind to and positively regulate rGH promoter activity in GC cells (21,28) but both are equally abundant in 235-1 and GC cell extracts (Figure 1B and data not shown) and are therefore unlikely to be responsible for the inactivity of the rGH promoter in the 235-1 cell line.

Other previously undescribed complexes were found to bind to sequences overlapping the two Pit-1 binding sites (Figure 5A); these binding events were initially obscured by the overwhelming abundance of Pit-1. Gel mobility shift assays performed using GC cell nuclear extracts and labelled rGH promoter fragments, including nucleotides from −210 to −6 and spanning the two Pit-1 binding sites, detects a number of complexes representing the binding of one or two Pit-1 factors or Sp1 to this fragment (Figure 5A, lane 2); these assignments were based upon oligonucleotide competitions and comparisons of the effects of mutations on footprinting and gel retardations (Figure 5 and ref. 21) or methylation interference and DNaseI footprinting of the gel shifted material (Figure 2, ref. 21 and data not shown). Competing away Pit-1 binding to both its proximal and distal binding sites within the radiolabelled −210/−6 fragment with a 1000 fold molar excess of an unlabelled oligonucleotide encompassing the proximal Pit-1 binding site (pGHF1, lane 7) leaves a series of bands, most of which represent different forms of Sp1 binding to its site (GHF2) within the growth hormone promoter (21). The GHF2 binding site partially overlaps the distal binding site for Pit-1 (21, see Figure 1A).

Competing away both Pit-1 and Sp1 binding with unlabelled oligonucleotides uncovers yet a further series of complexes (lane 12). The binding of two of these complexes, GHF5 and GHF7, although not eliminated by competition with the pGHF1 oligonucleotide, nevertheless was selectively inhibited by mutations within the radiolabelled fragment which disrupted Pit-1 binding to either the proximal (lane 13) or distal binding sites (lanes 13,14); mutations affecting Sp1 binding did not inhibit GHF5 or GHF7 binding (lane 16). This indicated that the GHF5 and GHF7 binding sites at least partially overlapped with the pGHF1 and dGHF1 binding sites.

To identify the nucleotides required for GHF5 and GHF7 binding more precisely, an extensive series of sequential point mutations across the Sp1 and the two Pit-1 binding sites (Figure 5B) was created. Again, all mutations affecting GHF5 and GHF7 binding also were inhibitory to Pit-1 binding at either the dGHF1 or the pGHF1 binding site (summarized in Figure 5B); no mutations disrupting the Sp1 binding site alone inhibited GHF5

or GHF7 binding. Note that the distribution of mutations affecting GHF5 and GHF7 binding was discontinuous with intermediate mutations (−115 to −90) not inhibiting binding. Thus, the sequences required for GHF5 and GHF7 binding overlap with those required for Pit-1 binding despite the fact that oligonucleotides containing neither the pGHF1 (Figure 5) nor the dGHF1 (data not shown) binding sites compete for GHF5 and GHF7 binding. However, one mutation (−115, −110) was obtained which disrupted Pit-1 binding to the dGHF1 binding site but affected neither GHF5 nor GHF7 binding.

The GHF5 and GHF7 binding sites were also localized by incubating extract with an end-labelled −210/−51 DNA fragment and pGHF1 and GHF2 competitor oligonucleotides followed by a short DNaseI treatment immediately before loading onto a band shift gel, excising gel plugs containing the GHF5 and GHF7 complexes, isolating the DNA (containing nicks introduced by the DNaseI) and then running this DNA on a sequencing gel. The location of the GHF7 footprint thus identified is summarized in Figure 5B (gray box). Note that this footprint is bipartite covering sequences within both the proximal and the distal GHF1 binding sites which is consistent with the bipartite nature of the mutations affecting GHF7 binding. No footprints were observed with the GHF5 complex but three DNaseI hypersensitive sites were noticed.

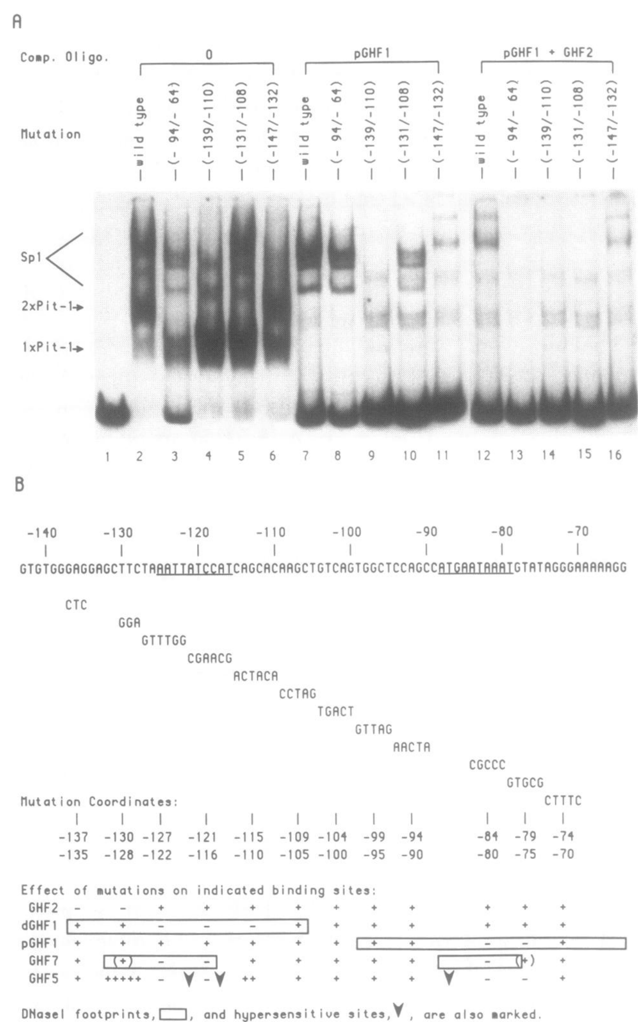

Fig. 5. A, The GHF5 and GHF7 complexes are detected after competing away Pit-1 and Sp1 binding with oligonucleotides spanning the pGHF1 and GHF2 binding sites, respectively (lanes 12−16). Lanes 7−11, competition with an oligonucleotide spanning the pGHF1 binding site. Either wild type (wt) or mutant (see ref. 21) radiolabelled −210/−6 fragments were incubated with GC extract and the indicated competitor oligonucleotides and the resultant complexes were analyzed by gel mobility shift assays. **B,** Summary of the effects of sequential substitution mutations on the binding of GHF5, GHF7, Sp1 or Pit-1 to the GHF5, GHF7, GHF2 or pGHF1/dGHF1 binding sites, respectively. The wild type nucleotides are substituted with the indicated nucleotides. +, ++, (+) or −, summarize whether the indicated factor binds, binds better than the wild type sequence, binds less well than the wild type sequence or does not bind at all, respectively, to promoters containing the indicated mutation. The location of the Pit-1, GHF7 and GHF5 footprints and hypersensitive sites (arrows) are shown. The Pit-1 consensus binding site (13) is underlined.

The nucleotides required for GHF5 and GHF7 binding are common to the proximal and distal Pit-1 binding sites

Interestingly, although being affected by mutations within the Pit-1 binding sites, GHF5 and GHF7 binding was not eliminated by competition with an oligonucleotide encompassing only a single Pit-1 binding site (Figure 6, lane 2, both pGHF1 and GHF2 oligonucleotide competitors are present). However, rGH promoter fragments spanning two Pit-1 binding sites are effective competitors for GHF5 and GHF7 binding (lanes 3,7). Furthermore, whereas mutation of either the proximal (pGHF1) or the distal (dGHF1) Pit-1 binding site still allows Pit-1 binding to the other Pit-1 binding site (8,10,21; see also Figure 5), any binding of either GHF5 or GHF7 requires that the sequences within both Pit-1 binding sites be present. Thus, at least two tandemly arranged Pit-1 binding sites are both required for GHF5 and GHF7 binding which is consistent with the inability of the pGHF1 oligonucleotide (a single Pit-1 binding site) to compete for GHF5 and GHF7 binding.

In order to assess whether the sequences required for GHF5

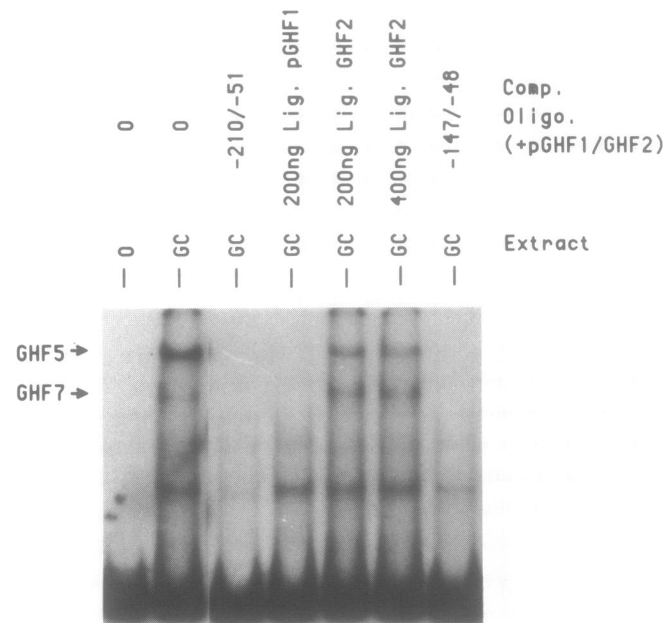

Fig. 6. GHF5 and GHF7 complexes are competed when 200 ng of ligated pGHF1 oligonucleotide is substituted for 200 ng of unligated pGHF1 oligonucleotide. 200 and even 400 ng of ligated GHF2 oligonucleotide do not compete. −210/−51, −147/−48 competitions with fragments of the rGH promoter spanning the indicated sequences encompassing the two Pit-1 binding sites. (+pGHF1/GHF2), each assay contains 200 ng each of pGHF1 and GHF2 oligonucleotide except when one of the monomeric oligonucleotides is substituted by its ligated counterpart.

and GHF7 binding are merely fortuitously associated with the pGHF1 and dGHF1 binding sites or whether they may be a more integral part of the Pit-1 binding sites *per se*, the proximal Pit-1 binding site oligonucleotide (pGHF1) was ligated and tested as a competitor for GHF5 and GHF7 binding. Since a multimerized pGHF1 oligonucleotide (Figure 6, lane 4) competed effectively for GHF5 and GHF7 binding whereas neither a monomeric pGHF1 oligonucleotide nor a monomeric GHF2 oligonucleotide (lane 2) nor a ligated GHF2 oligonucleotide (lanes 5,6) competed, it would seem that the nucleotides required in the dGHF1 binding site for GHF5 and GHF7 binding can be compensated for by nucleotides present within the pGHF1 binding site; this implies that GHF5 and GHF7 binding apparently requires two sites that are of similar sequence. Since the sequences required for GHF5 and GHF7 binding must be contained both in the pGHF1 and dGHF1 binding sites and since the sequences of these two Pit-1 binding sites are not very similar except for those nucleotides which are included in the Pit-1 consensus sequence (13, underlined in Figure 5B), it may be logical to assume that it is at least portions of the Pit-1 binding sites themselves to which GHF5 and GHF7 are binding.

GHF7 binding activity is restricted to the GH-producing cell type

Thus, two binding activities, GHF5 and GHF7, were described which bind to regions of the rat growth hormone promoter previously described as being important for full promoter activity (8,10,11). In order to determine whether these factors may be implicated in the tissue-specificity of rGH promoter activity, we assayed for their presence in a number of cell types by gel mobility shift assays (Figure 7). The GHF5 complex was observed in a number of cell types, including GC, 235-1, HeLa and CHO cells whereas GHF7 binding activity was restricted to the GH-producing GC cell type. Neither GHF5 nor GHF7 was present in rat liver. All extracts tested contained Sp1 and GHF3 binding activity as a control for extract preparation (Figure 1B and data not shown). Therefore, GHF7 binding activity is very tightly correlated with rGH promoter activity, being present in only the GH-producing GC cell line and not the prolactin-expressing 235-1 cell line which does however contain Pit-1.

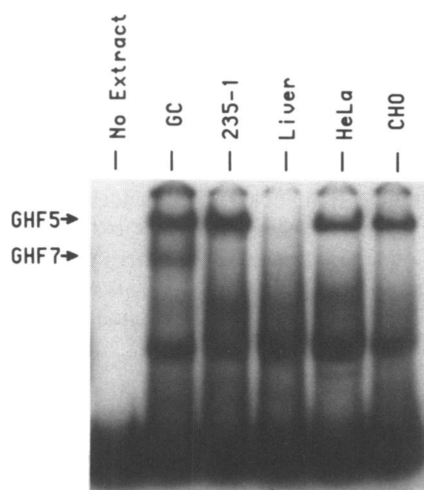

Fig. 7. Tissue distribution of GHF5 and GHF7. Labelled −210/−51 fragments were incubated in the presence of both pGHF1 and GHF2 oligonucleotide competitors with nuclear extracts from the indicated cell lines.

Selective mutation of a Pit-1 binding site confirms the involvement of Pit-1 in GH expression

The identification of a factor whose cell-type distribution is more tightly associated with GH expression than Pit-1 and whose binding to the rGH promoter is affected by mutations in the same sequences previously used to identify Pit-1 binding as functionally important to GH promoter expression (8,10,11) may call into question the functional significance of Pit-1 binding to the cell-type-specific expression of the rGH promoter. Point mutations through the Pit-1 binding sites identified one mutation in which Pit-1 binding to the dGHF1 binding site was inhibited without apparent effect to GHF5 or GHF7 binding (Figure 5B, −115/−110). The relative abilities of the wild type or the mutant promoters to function was assessed by RNase protection assays on transcripts derived from these promoters after electroporation into GC cells (Figure 8). 237 bp of rGH promoter attached to the bacterial chloramphenicol acetyl transferase structural gene (rGHCAT) were cotransfected with an RSV promoter attached to the same CAT sequences (RSVCAT). Mapping RNA hybridized with an rGHCAT probe identifies transcripts correctly initiated from the rGH promoter start site (Figure 8, rGH) as well as detects RSVCAT transcripts which protect the CAT sequences within the rGHCAT probe against RNase digestion (int. cont.). Mutations which did not affect the binding of Pit-1, GHF5 or GHF7 binding (Figure 5B) had no effect on the efficiency of the rGH promoter (Figure 8, lanes 5,8). All promoters containing mutations which concomitantly eliminated GHF5, GHF7 and Pit-1 binding activity were significantly impaired in their promoter activity (lanes 2,3,6,7, compare to wild type promoter, lane 1). A promoter containing a mutation

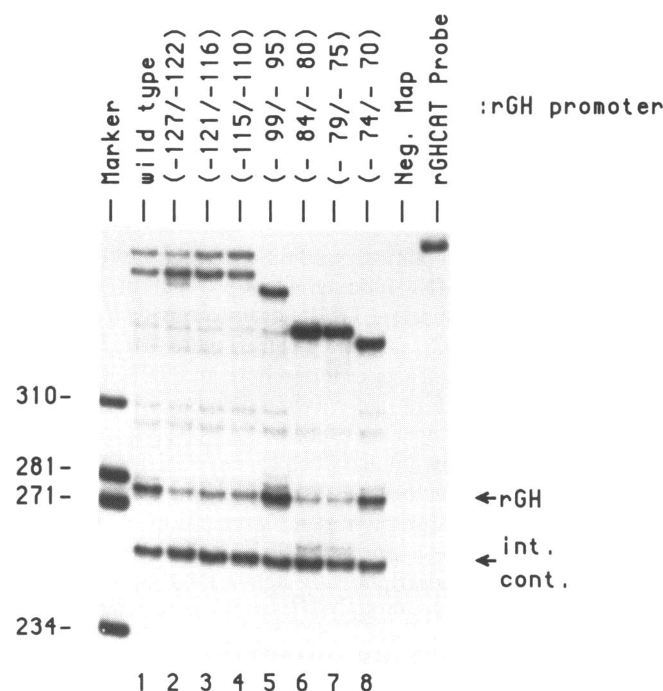

Fig. 8. SP6 mappings of RNAs from GC cells transfected with wild type (wt) or mutant (see Fig. 5B) rGH promoters. RSVCAT transcripts serve as internal controls for all transfections. The protected fragments nearer to the origin of the gel represent transcripts initiated further upstream, within the vector, and mapping to the end-point of homology with the rGHCAT probe (containing wild type sequences to position −106). This end-point is variable for the different transfections owing to the location of the introduced mutations.

(−115,-110) disturbing Pit-1 but neither GHF5 nor GHF7 binding was also observed to be significantly less transcriptionally competent than the wild type promoter demonstrating that Pit-1 binding is important to full rGH promoter activity independent of GHF5 or GHF7 binding.

DISCUSSION

A transcriptionally active Pit-1 factor is present in a cell line not expressing GH

The expression of the GH gene is highly cell-type-specific being restricted to only a subpopulation of cells within the anterior pituitary gland (16,29). A pituitary-specific transcription factor, Pit-1 (also called GHF1) which binds to two functionally important sites within the growth hormone gene promoter (8−11) has been cloned (14,15) and represents a candidate factor responsible for the restriction of GH expression to pituitary cell lineages. Indeed, the expression of a marker gene driven by an otherwise silent rGH promoter transfected into HeLa cells is rescued by co-expressing the cloned Pit-1 cDNA (15,30).

However, some pituitary cell types which do not express GH do contain Pit-1 mRNA (17). This includes the lactotrophic (prolactin-secreting) cell type. We probed nuclear extracts of a lactotrophic cell line, 235-1, for Pit-1 binding activity. A footprinting activity in 235-1 extracts that is identical to the footprints detected with extracts from the GH-secreting GC cell line was observed (Figure 1A, ref. 24). Nucleoprotein complexes formed with rGH promoter fragments and 235-1 or GC Pit-1 proteins also migrate similarly in non-denaturing gels (Figure 1B) and the two binding activities are identical in size on an SDS PAGE when detected by Southwestern blotting (24) or when cross-linked to the proximal Pit-1 binding site (Figure 1C).

The 235-1, like the GC Pit-1 factor, seemed to be capable of activating transcription from the rGH promoter *in vitro* (Figure 3) and even from an artificial construct *in vivo* (Figure 4B). We note that the latter finding was different from that observed by Ingraham et al. (15) who found that the insertion of three contiguous pGHF1 binding sites directly upstream of a prolactin promoter deleted to −36 so as to contain little more than the TATA box enhanced the expression of that construct in GC but not 235-1 cells. In contrast, we found that the insertion of five copies of the pGHF1 binding site upstream of the already transcriptionally competent TK109 promoter made that promoter more active in both GC and 235-1 cells (Figure 4B). The different behavior of the constructs reported here and those of Ingraham et al. (15) are therefore likely to be related to differences in the promoters used. There is a precedent for the overall promoter structure affecting the pituitary cell type specificity of promoters containing Pit-1 binding sites: since the Pit-1 factor has been implicated in the expression of the prolactin promoter (13,24) and since prolactin is expressed in 235-1 cells it would seem that there must be a transcriptionally active Pit-1 factor present in 235-1 cells, even though the rGH promoter is inactive in these cells.

Can subtle differences in the GC and 235-1 Pit-1 account for the disparities in GH expression?

The growth hormone promoter is inactive in the 235-1 lactotrophic cell line despite the presence of an apparently functional Pit-1 transcription factor. This inactivity does not seem to be associated with anything as simple as chromatin structure since the transfected rGH promoter is still inactive in 235-1 cells (23, Figure 4A). As well, the thyroid hormone receptor, which contributes significantly to the expression of the rat growth hormone promoter (31−33) is less abundant in 235-1 than in GC cells (34) and may therefore contribute to the inactivity of the rGH promoter in the 235-1 cell type. However, ectopic expression of the either the chicken alpha or human beta forms of the thyroid hormone receptor did not rescue rGH promoter activity in the 235-1 cell type (34) although other members of the thyroid hormone receptor family, including a pituitary-specific isoform (35), have yet to be tested.

Furthermore, a difference in the methylation interference pattern of the Pit-1 factors derived from the GC and 235-1 cells was noted (Figure 2) suggesting that the Pit-1 proteins in the two cell types are not absolutely identical and that the restriction of GH expression to the GC cell type may be in some way related to this difference. Such a hypothesis would necessitate that the two different Pit-1 isoforms are differentially active as a result of their overall promoter context; i.e. one isoform would prefer the configuration of the GH promoter and the other would prefer the prolactin promoter. Note that both the 235-1 and the GC Pit-1 factors seemed to be active on the rGH promoter *in vitro* (Figure 3) but that this is not true *in vivo* (13,15, Figure 4A).

Cell type specificity of the GHF7 complex

Two other factors, GHF5 and GHF7 were characterized here. The binding of each was dependent upon the structural integrity of sequences within at least two Pit-1 binding sites; single Pit-1 binding sites were insufficient either for direct binding (Figure 5) or as binding competitors (Figures 5−7). Pit-1 binding sites *per se* and not just some other spuriously associated sequences seemed to have been involved in GHF5 and GHF7 binding since contiguous pGHF1 (proximal Pit-1) binding sites were also effective in binding site competitions (Figure 6). Nevertheless, transient expression of one promoter mutation in which the distal Pit-1 binding site is eliminated without affecting GHF5 or GHF7 binding suggests that Pit-1 contributes to the activity of the rGH promoter independently of GHF5 or GHF7 binding (Figure 8).

The GHF7 complex has thus far been detected only in extracts from the GH-secreting GC cell line and not in extracts from the 235-1 cell line (Figure 7) making it a candidate factor controlling the cell-specific expression of the rGH promoter within the anterior pituitary gland. rGH promoters in which mutations simultaneously destroy the GHF5, GHF7 and either of the two Pit-1 binding sites are less effective than the wild type promoter. However, the fact that these mutant promoters are still capable of directing some transcription suggests that GHF7 alone cannot be responsible for the restriction of rGH expression to the somatotrophic cell lineage. Rather it is possible that GHF7, in conjunction with a number of other factors including cell-type restricted Pit-1 isoforms (Figure 2), factors binding to other sites within the rGH promoter (36,37) or other epigenetic influences, possibly including methylation (38,39), is acting to control the cell-specific expression of the GH promoter.

ACKNOWLEDGEMENTS

We wish to thank Dr. Chris Nelson for critical reading of the manuscript. This work was supported by grants from the American Cancer Society (JFRA-123) and the United States Public Health Service (DK-35283 and AM-19994) and the UCSF Academic Senate Committee on Research to T.L.R.. F.S. was supported by consecutive fellowships from the Swiss National Science Foundation and the Medical Research Council of Canada.

REFERENCES

1. Serfling, E. (1989) Trends Genet. 5, 131–133.
2. Akam, M. (1989) Nature 341, 282–283.
3. McGinnis, W., Levine, M. S., Hafen, E. Kuroiwa, A. and Gehring, W. J. (1984) Nature 308, 428–433.
4. Scott, M. P. and Weiner, A. J. (1984) Proc. Natl. Acad. Sci. USA 81, 4115–4119.
5. McGinnis, W., Garber, R. L., Wirz, J., Kurowa, A. and Gehring, W. J. (1984) Cell 37, 403–408.
6. Gehring, W. J. (1987) Science 236, 1245–1252.
7. Herr, W., Strum, R. A., Clerc, R. G., Corcoran, L. M., Baltimore, D., Sharp, P. A., Ingraham, H. A., Rosenfeld, M. G., Finney, M., Ruvkun, G. and Horvitz, H. R. (1988) Genes Dev. 2, 1513–1516.
8. West, B. L., Catanzaro, D. F., Mellon, S. A., Cattini, P. A., Baxter, J. D. and Reudelhuber, T. L. (1987) Mol. Cell. Biol. 7, 1193–1197.
9. Ye, Z.-S. and Samuels, H. H. (1987) J. Biol. Chem. 262, 6313–6317.
10. Catanzaro, D. F., West, B. L., Baxter, J. D. and Reudelhuber, T. L. (1987) Mol. Endo. 1, 90–96.
11. Lefevre, C., Imagawa, M., Dana, S., Grindlay, J., Bodner, M. and Karin, M. (1987) EMBO J. 6, 971–981.
12. Bodner, M. and Karin, M. (1987) Cell 50, 267–275.
13. Nelson, C., Alberst, V. R., Elsholtz, H. P., Lu, L. I.-W. and Rosenfeld, M.G. (1988) Science 239, 1400–1405.
14. Bodner, M., Castrillo, J.-L., Theill, L. E., Deerinck, T., Ellisman, M. and Karin, M. (1988) Cell 55, 505–518.
15. Ingraham, H. A., Chen, R., Mangalam, H, Elsholtz, H. P., Flynn, S., Lin, C. R., Simmons, D. M., Swanson, L. and Rosenfeld, M. G. (1988) Cell 55, 519–529.
16. Ivarie, R. B., Schacter, B. S. and O'Farrell, P. (1983) Mol. Cell. Biol. 3, 1460–1467.
17. Crenshaw III, E. B., Kalla, K., Simmons, D. M., Swanson, L. W. and Rosenfeld, M. G. (1989) Genes Dev. 3, 959–972.
18. Tashjian, A. Jr., Yasamura, Y., Levine, L., Sato, G. and Parker, M. L. (1968) Endocrinology 82, 342–352.
19. Reymond, M. J., Nansel, D. D., Burrows, G. H., Neaves, W. B. and Porter, J. C. (1984) Acta Endocrinologica 106, 459–470.
20. Gorski, K., Carneiro, M., and Schibler, U. (1986) Cell 47, 767–776.
21. Schaufele, F., West, B. L. and Reudelhuber, T. L. (1990) J. Biol. Chem., in press.
22. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Cold Spring Harbor Laboratory. U.S.A.
23. Nelson, C., Crenshaw III, E. B., Franco, R., Lira, S., Albert, V. R., Evans, R. M. and Rosenfeld, M. G. (1986) Nature 322, 557–562.
24. Mangalam, H. J., Albert, V. R., Ingraham, H. A., Kapiloff, M., Wilson, L., Nelson, C., Elsholtz, H. and Rosenfeld, M. G. (1989) Genes Dev. 3, 946–958.
25. Castrillo, J.-L., Bodner, M. and Karin, M. (1989) Science 243, 814–817.
26. Behringer, R. R., Mathews, L. S., Palmiter, R. D. and Brinster, R. L. (1988) Genes Dev. 2, 453–461.
27. Lira, S. A., Crenshaw III, E. B., Glass C. K., Swanson, L. W. and Rosenfeld, M. G. (1988) Proc. Natl. Acad. Sci. USA 85, 4755–4759.
28. Schaufele, F., West, B. L. and Reudelhuber, T. L. (1990) J. Biol. Chem., in press.
29. Frawley, L. S., Boockfor, F. R. and Hoeffler, J. P. (1985) Endocrinology 116, 734–737.
30. Theill, L. E., Castrillo, J.-L., Wu, D. and Karin, M. (1989) Nature 342, 945–948.
31. Crew, M. D. and Spindler, S. R. (1986). J. Biol. Chem. 261, 5018–5022.
32. Flug, F. Copp, R. P., Casanova, J., Horowitz, Z., Janocko, L., Plotnick, M. and Samuels, H. H. (1987) J. Biol. Chem. 262, 6373–6382.
33. Norman, M. F., Lavin, T. N., Baxter, J. D. and West, B. L. (1989) J. Biol. Chem. 264, 12063–12073.
34. Forman, B. M., Yang, C.-r., Stanley, F., Casanova, J. and Samuels, H. H. (1988) Mol Endorinol. 2, 902–911.
35. Hodin, R. A., Lazar, M. A., Wintman, B. F., Darling, D. S., Koenig, R. J., Larsen, P. R., Moore, D. D. and Chin, W. W. (1989) Science 244, 76–79.
36. Larsen, P. R., Harney, J. W. and Moore, D. D. (1986) Proc. Natl. Acad. Sci. USA 83, 8283–8287.
37. Pan, W. T., Liu, Q. and Bancroft, C. (1990) J. Biol. Chem. 265, 7022–7028.
38. Lan, N. (1984) J. Biol. Chem. 259, 11601–11606.
39. LaVerriere, J.-N., Muller, M., Buisson, N., Tougard, C., Tixier-Vidal, A., Martial, A. and Gourdji, D. (1986) Endocrinology 118, 198–206.