A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally

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The anterior pituitary gland provides a model for investigating the molecular basis for the appearance of phenotypically distinct cell types within an organ, a central question in development. The rat prolactin and growth hormone genes are expressed selectively in distinct cell types (lactotrophs and somatotrophs, respectively) of the anterior pituitary gland, reflecting differential mechanisms of gene activation or restriction, as a result of the interactions of multiple factors binding to these genes. We find that when the pituitary-specific 33-kD transcription factor Pit-1, expressed normally in both lactotrophs and somatotrophs, is expressed in either the heterologous HeLa cell line or in bacteria, it binds to and activates transcription from both growth hormone and prolactin promoters in vitro at levels even 10-fold lower than those normally present in pituitary cells. This suggests that a single factor, Pit-1, may be capable of activating the expression of two genes that define different anterior pituitary cell phenotypes. Because a putative lactotroph cell line (235-1) that does not express the growth hormone gene, but only the prolactin gene, appears to contain high levels of functional Pit-1, a mechanism selectively preventing growth hormone gene expression may, in part, account for the lactotroph phenotype.

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The hierarchical activation of regulatory genes appears to be responsible for the temporal and spatial patterns of gene expression required for normal development (Gehring 1987; Scott and Carroll 1987). A number of *Drosophila* genes that regulate such pattern development contain a conserved 60-amino-acid sequence referred to as the homeo domain (McGinnis et al. 1984; Manley and Levine 1985; Gehring 1987; Levine and Hoey 1988), and proteins containing this putative DNA-binding domain also have been identified in yeast, worms, and vertebrates [Carrasco et al. 1984; Shephard et al. 1984; Colberg-Poley et al. 1985; Awgulewitsch et al. 1986]. Following the initial pattern formation that takes place early in embryonic development, it has been proposed that tissue-specific factors activate the characteristic genes that define the cellular phenotype (e.g., Walker et al. 1983; Nelson et al. 1986, 1988; Sen and Baltimore 1986; Staudt et al. 1986; Bodner and Karin 1987; Hammer et al. 1987; Costa et al. 1988).

We use the anterior pituitary gland, derived from the embryonic ectoderm of Rathke's pouch, as a model to study cellular differentiation. This structure differentiates into five distinct cell types, distinguished on the basis of the secreted hormone. The two most prevalent pituitary cell types are the growth hormone-producing

somatotrophs and prolactin-producing lactotrophs. The evolutionary relatedness of the growth hormone (GH) and prolactin (PRL) genes (Cooke et al. 1981) and their transient coexpression during development (Chatelain et al. 1979; Watanabe and Daikoku 1979; Hoeffler et al. 1985) suggest that similar mechanisms might be expected to control activation of the two genes. Both the rat PRL promoter (−422 to +33) and rat GH promoter (−180 to +8) direct cell-specific expression in vitro (Nelson et al. 1986, 1988; Bodner and Karin 1987; Cao et al. 1987; Guitierrez-Hartmann et al. 1987; Lufkin and Bancroft 1987; West et al. 1987; Ye and Samuels 1987) and in transgenic mice (Behringer et al. 1988; Lira et al. 1988; Crenshaw et al. 1989) and therefore possess sufficient information to direct cell specificity.

On the basis of competition experiments involving DNase I footprinting and mutational analyses (Nelson et al. 1988), we proposed that several related sequences in the PRL and GH promoters bound either common or functionally related, pituitary-specific, transcription factors termed Pit-1 (summarized in Fig. 1A). On the basis of the characterization of a candidate protein, we were able to clone a cDNA from a rat pituitary library, as a result of the ability of the encoded 33-kD protein to bind to sequences of both PRL and GH genes. We pro-

posed that this protein represented Pit-1 (Ingraham et al. 1988). This protein was notable not only for its transcriptional effects and pituitary-restricted expression, but also for its structure. At the carboxyl terminus was a stretch of 60 amino acids with homology to the homeo domain present in many *Drosophila* developmental proteins (Gehring 1987). Toward the amino terminus was a 76-amino-acid region that was homologous to three other proteins cloned recently, Oct-1, Oct-2, and *unc-86* (Herr et al. 1988). The human transcription factors Oct-1 (Sive and Roeder 1986; Sturm et al. 1988) and Oct-2 (Clerc et al. 1988; Ko et al. 1988; Muller et al. 1988) bind to the octameric recognition site (ATTTGCAT) of target genes, which differs from that of Pit-1 (A_TATNCAT) by only a single base pair (Nelson et al. 1988). Oct-1 is expressed widely, whereas Oct-2 appears to be more restricted, being expressed in the B lymphocytes and also in certain areas of the brain (He et al. 1989). *unc-86* is a *Caenorhabditis elegans* protein that was detected by the effects of its mutation, which resulted in alterations in neuronal cell development (Chalfie et al. 1981; Finney et al. 1988).

It is of great interest to determine the binding and *trans*-activation proclivities of Pit-1, as it is one of the first described mammalian POU-homeo domain proteins expressed in an organ that contained distinct cell types. Given our previous data about its binding preferences and the evidence that Pit-1 is expressed in both lactotrophs and somatotrophs (Bodner et al. 1988; Ingraham et al. 1988), we predicted that Pit-1 would be involved in the transcriptional activation of the similarly restricted GH and PRL genes. This supposition was supported initially by transient cotransfection studies in HeLa cells, indicating that the Pit-1 cDNA gene product could increase the expression of reporter genes directed by the promoters of either the PRL or GH genes. However, on the basis of experiments using a bacterial fusion protein that contained a portion of the cloned gene product GHF-1 (identical to Pit-1) and partially purified GHF-1 from pituitary cells, it has been suggested that GHF-1 binds to and activates exclusively the GH, but not the PRL, promoter (Bodner et al. 1988; Castrillo et al. 1989). This proposed selectivity of target gene would be in contrast to the actions of many of the *Drosophila* homeo box proteins, which appear to activate sets of genes combinatorially to achieve their dramatic developmental effects (Gehring 1987; Desplan et al. 1988; Hoey and Levine 1988; Levine and Hoey 1988). The critical issue, then, is whether Pit-1 can or cannot activate PRL or GH gene expression at physiological levels of expression. In this paper we report that when the entire Pit-1 protein is expressed in both eukaryotic and bacterial systems, it binds effectively to both the GH and PRL genes and indeed preferentially activates transcription of the PRL promoter at physiological concentrations. We observed this in both *in vitro* systems and in a heterologous cell line. We present evidence for an additional mechanism required to establish the mature lactotroph and somatotroph phenotypes.

Results

To initiate studies regarding PRL and GH transcriptional activation, native Pit-1 from GC cell extracts was purified by combining conventional separation techniques with DNA-affinity chromatography (Kadonaga and Tjian 1986). Activity was assessed by DNase I footprint (Galas and Schmitz 1978), Southwestern blot (Bowen et al. 1980; Jack et al. 1982), and *in vitro* transcription assays (Nelson et al. 1988), using both the PRL and GH promoters. Phosphocellulose chromatography effectively separated Pit-1 activity from a second factor (Prll) that bound to the PRL promoter at -106 to -83 [data not shown]. Following hydroxyl-apatite column fractionation (see Methods), the Pit-1 activity was applied to a DNA affinity column made from multimers of the rat GH promoter element GH1 (see Fig. 1A). The column was developed with a linear KCl gradient; Pit-1 activity eluted between 250 and 450 mM. Size separation of gradient fractions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970), followed by silver stain (Merril et al. 1984), revealed a prominent 33 + 31-kD doublet, as well as an additional 47-kD protein (Fig. 1B). The lower band of the doublet could formally be a different gene product, but is more likely to represent a modified form of the 33-kD protein. Similar doublets have been observed using extracts from pituitary cell lines, with slight variations in molecular weights (Bodner et al. 1988; Cao et al. 1988; Ingraham et al. 1988).

When affinity-purified material was evaluated by a Southwestern blot, specific DNA binding was restricted exclusively to either the 33-kD protein or to the 33 + 31-kD doublet (Fig. 1C). Similarly, the purified protein was able to stimulate transcription from a series of both PRL and GH promoter constructs, when added to an otherwise incompetent HeLa cell nuclear extract (Fig. 1D), and the peak of transcriptional activity also fractionated with the 33-kD protein (data not shown). Affinity column fractions were analyzed in DNase I footprinting reactions with both the PRL and the GH promoters. On both, each of the putative Pit-1-binding sites was protected by the affinity column-purified Pit-1 protein (Fig. 2A,B). The final purification of Pit-1 was judged to be at least 4000-fold, on the basis of the amount of protein required to protect the binding sequence from digestion with DNase I.

Because transient transfection analyses could generate misleading results on the basis of overexpression of the encoded *trans*-acting factor, the Pit-1 cDNA was incorporated into an SV40-based expression vector (Fig. 3A; Lin et al. 1987; Nelson et al. 1988) and transfected stably into HeLa cells. These cells (HeLa/Pit1⁺) expressed a 33-kD protein that comigrated with the phosphocellulose- and affinity-purified protein from GC extracts, but at levels <10% that in GC cells, as assayed by Southwestern blot (Fig. 2C) or gel-shift (Fried and Crothers 1983; data not shown), and permitted evaluation of whether Pit-1 would activate GH or PRL gene expression at these low levels. Transfection of these HeLa/

Figure 1. (A) The rat GH and PRL promoters are shown to scale. Regions protected from DNase I digestion following incubation with GC nuclear extracts are hatched. Asterisks indicate DNase I hypersensitive sites in the presence of nuclear extract. The similarly hatched boxes (GH1, GH2, P1–P4) designate those elements proposed to bind Pit-1. [Site Prl1 is referred to as 2P by Bodner et al. (1988) and Castrillo et al. (1989), who combine the P2 and P3 sites to a single site referred to as 3P '▷' = TATNCAT, the Pit-1 consensus binding sequence.] T₃ on the GH promoter is a reported thyroid hormone response element (Glass et al. 1987). The element labeled Prl1 has not been characterized extensively, but appears to be PRL-specific. (B) Protein (1.8 mg) from the flowthrough of the hydroxyl-apatite column was applied to 0.9 ml of affinity column resin [multimers of the GH1 site]. After two 1-ml washes, the bound protein was eluted with a linear gradient of KCl to 700 mM in PC buffer (top). (Bottom) 100 μ l of each fraction was concentrated by acetone precipitation, analyzed by SDS–PAGE, and silver-stained (Merril et al. 1984). Lanes: (Applied) 1 μ g of the hydroxyl-apatite flowthrough; (Flow Thru) 1 μ g of the affinity-column flowthrough; (Wash 1 and Wash 2) 100 μ l of the respective washes, processed as per the fractions. (C) The indicated fractions were concentrated by acetone precipitation, size-fractionated by SDS–PAGE, transferred to nitrocellulose, and incubated with radiolabeled $-173..+33$ PRL promoter and 3 μ g/ml of salmon sperm DNA as a nonspecific competitor in binding buffer (see Methods). Lanes: (Markers) Protein standards; (Applied) 2 μ l of the hydroxyl-apatite column flowthrough; (Flow Thru) 10 μ l of the affinity-column flowthrough; (Wash 1 and Wash 2) 5 μ l of the 150 mM KCl column washes; (Fraction #s) 5 μ l of each fraction. (D) In vitro transcription with affinity column-purified Pit-1. Transcription from PRL and GH promoter–luciferase (De Wet et al. 1987) fusion genes is shown, as indicated, using 10 μ l (100 μ g) of HeLa cell nuclear extract alone (–) or supplemented with 5 μ l of affinity column fraction 21 (+). Correctly initiated transcripts were visualized by primer extension using 32 P-labeled luciferase primers.

Pit1⁺ cells with GH fusion genes that contained 180 or 110 bp of 5'-flanking information, or even three copies of the GH1 element fused to a minimal promoter ($-36..+33$ PRL) resulted in levels of expression 20- to 100-fold greater than those observed when the same constructs were transfected into the parental cell line (Fig.

3B). Activation of transfected PRL promoter fusion genes in the HeLa/Pit1⁺ cells was consistently more dramatic, reaching levels >200-fold higher than those observed in untransfected HeLa cells (Fig. 3B). In contrast, a series of other promoters [e.g., the Rous sarcoma virus (RSV) promoter (Gorman et al. 1982b) and the glucocorticoid (Ya-

Figure 2. (A,B) The DNase I footprints generated by crude nuclear extracts, as well as PC and affinity column-purified fractions, are compared to those generated by the extracts from the HeLa/Pit1⁺ cells. Lanes [for both the ^{-312..+8}GH (labeled at +8; A) and the ^{-422..+33}PRL (labeled at +33; B) promoters]: (Markers) 'G' reactions; (No Extract) control ladder; (GC Crude) 40 µg of GC crude nuclear extract; (P. Cell) 5 µg of phosphocellulose column fraction 28; (ACE) 150 ng of affinity column, fraction 21; (HeLa/Pit1⁺) 120 µg of HeLa/Pit1⁺ crude nuclear extract; (HeLa) 120 µg of HeLa cell crude nuclear extract; (235-1) 50 µg of 235-1 lactotroph crude nuclear extract. (C) The indicated samples were treated as in Fig. 1C and probed with ³²P-labeled ligated multimers of PRL P1 site in the presence of 3 µg of salmon sperm DNA. Lanes: (P. Cell) 5 µg of a PC column fraction that contained the peak of Pit-1 activity; (ACE) 100 ng of affinity column eluate, fraction 21; (235-1) 100 µg of 235-1 (lactotroph) cell crude nuclear extract; (HeLa/Pit1⁺) 200 µg of HeLa/Pit1⁺ crude nuclear extract; (HeLa) 200 µg of HeLa cell crude nuclear extract. (D,E) Transcription from templates that contained PRL (D) and GH (E) promoter constructs in 20 µl (200 µg) of HeLa cell nuclear extract or 40 µl (200 µg) of HeLa/Pit1⁺ nuclear extract as indicated. Transcription units are designated by the base to which the promoter extends at the 5' border.

mamoto 1985) or cAMP response elements (Montminy et al. 1986) linked to the ^{-36..+33}PRL minimal promoter] exhibited less than a twofold increase in expression in HeLa/Pit1⁺ cell lines (data not shown). Figure 2A shows

that the HeLa/Pit1⁺ nuclear extract generated footprints on the high-affinity GH1 and GH2 sites of the GH promoter that were indistinguishable from those made by GC extracts. When the HeLa/Pit1⁺ extract was incu-

Figure 3. (A) The cDNA encoding Pit-1 is shown incorporated into the expression vector, which uses the SV40 enhancer/promoter and polyadenylation and splice site (*top*); the 291-amino-acid translation product, showing the positions of the POU-specific domain and the POU-homeo domain (*bottom*). (B) HeLa cells transfected stably with the Pit-1 expression vector (HeLa/Pit1⁺) were transfected transiently with 10 μ g of plasmid that contained either the PRL or the GH promoter variants (*left*) fused to the firefly luciferase gene. Luciferase activity in 50 μ g of the cell lysate was measured and compared to the value obtained when the same plasmid was transfected into the parental HeLa cell line (which lacked Pit-1). Values shown represent the mean, determined from five (PRL constructs) or four (GH constructs) experiments.

bated with the PRL promoter (Fig. 2B), the higher-affinity sites P1 and P3 were protected (P3 incompletely), but the lower-affinity sites P2 and P4 were not. The failure to protect the low-affinity sites apparently reflects the low levels of expression of Pit-1 in the HeLa/Pit1⁺ cells (Fig. 2C) and is consistent with a higher affinity of Pit-1 for the P1 and GH1 and GH2 sites. Simi-

larly, nuclear extracts from these cells stimulated both GH and PRL transcription to levels comparable to those from crude GC nuclear extracts and HeLa cell extracts supplemented with purified Pit-1 (Fig. 2D,E).

In the absence of Pit-1, the GH promoter variants are somewhat 'leaky' in this assay, probably as a result of other factors that can activate the promoter (consensus

AP2-, AP3-, and T3-binding sites are located within the part of the promoter that we used). However, competition with oligonucleotides encoding a Pit-1-binding site (GH1) demonstrated that the activation of the GH promoter in these extracts was due to binding of Pit-1 to the cell-specific, *cis*-active elements (Fig. 5D). Therefore, despite slight differences in affinity for Pit-1, both genes were activated by Pit-1 levels well below those present in pituitary cell lines.

To establish the relative threshold of PRL and GH gene activation, Pit-1 cDNA was placed into a transcription unit that permitted effective expression in bacteria (Studier et al. 1986; Rosenberg et al. 1987). Bacterially expressed Pit-1 (Pit-1^{bac}) was able to protect the same sites on the GH and PRL genes as Pit-1 isolated from GC cells (Fig. 4A–F), with a slightly higher affinity for the GH sequences than for those of PRL. In contrast, a bacterial trpE-fusion protein that contained a partial GHF-1 coding sequence was reported to bind solely to the GH but not the PRL promoter sequences (Bodner et al. 1988); however, it did not contain the POU-specific region required for high-affinity, specific DNA binding (Sturm and Herr 1988; H.A. Ingraham and M.G. Rosenfeld, unpubl.). By Southwestern blot analysis (Fig. 6C), Pit-1^{bac} migrated as a single band of ~33 kD, equal to the size of the upper band of the doublet seen in GC and 235-1 cells. As shown in Figure 5, A, B, and C, bacterially expressed Pit-1 increased transcription of both PRL and GH fusion genes greatly when added to HeLa and other heterologous cell nuclear extracts. Titration curves show similar half-maximal levels of Pit-1 for activation of PRL and GH promoters; a series of other promoters are inactive at these levels of Pit-1, including RSV and the minimal PRL promoter (–36 to +33), alone or in combination with oligonucleotides that contained the thyroid hormone response element (Glass et al. 1987; data not shown) or the cAMP response element (Montminy et al. 1986) and a mutation of PRL site P1 that does not bind Pit-1 well (P1 μ ; Fig. 5A). Also, Pit-1^{bac} stimulated the transcription of the PRL distal enhancer *in vitro* when placed 5' of the minimal PRL promoter (–36 to +33) (Fig. 5C). Only when Pit-1^{bac} was added at extremely high concentrations were small element-independent increases in transcription from some of these constructs, typically from the RSV and the P1 μ constructs (Fig. 5A), observed.

This parallel activation of both the PRL and GH genes is somewhat paradoxical, because these two genes are normally expressed in different cell types. The 235-1 lactotroph cell line, which expresses the endogenous PRL but not the GH gene, offers an *in vitro* model in which to explore this conundrum. Transient transfection of a $-235\ldots+8$ GH–luciferase (De Wet et al. 1987) fusion gene into 235-1 cells did not result in luciferase ex-

pression at a detectable level, but a $-422\ldots+33$ PRL–luciferase fusion gene was expressed at high levels (Fig. 6A) and primer extension analysis has confirmed correct cap site utilization from this construct (Nelson et al. 1988). Because of this differential gene activation, the presence of Pit-1 in 235-1 cells was evaluated by DNase I footprinting and binding analyses. As shown in Figures 2, A and B, and 4, A–F, 235-1 crude nuclear extracts protected the same sites on GH and PRL genes as did Pit-1 isolated from GC cells or Pit-1^{bac}. RNase protection assays confirmed the presence of high levels of Pit-1 mRNA in 235-1 cells (Fig. 6B) and Southwestern blot analysis also confirmed the presence of the 33 + 31-kD doublet in 235-1 cells at approximately the same levels found in GC cells (Fig. 6C). Specific binding of an antibody against the carboxyl terminus of Pit-1 to a Western blot also confirmed the expression of Pit-1 in 235-1 nuclear extracts (data not shown). Although 235-1 cells were unable to express GH fusion genes despite the presence of Pit-1, *in vitro* transcription assays were performed using 235-1 nuclear extracts. Surprisingly, a series of GH fusion genes were transcribed effectively in 235-1 nuclear extracts (Fig. 6D) at levels comparable to those observed with GC nuclear extracts. This transcription was decreased by competing oligonucleotides that contained the GH1 site (Fig. 5D), indicating that transcription was Pit-1 dependent.

Discussion

The combinatorial actions of transcription factors in determining gene expression are established clearly for both regulatory and developmental events. Therefore, it might be expected that the cell type-specific expression of genes in the anterior pituitary could involve the actions of multiple factors, some of which might actually act to enhance transcription of genes that are ultimately expressed in distinct cell types. In this context, the critical definition of a gene-specific positive transcription factor is its ability to bind to specific *cis*-acting elements and to activate gene transcription as a consequence of this binding. The concentration of a factor, the number of its binding sites, its relative affinity for the sites, and the topology of the sites relative to the sites of other factors will dictate the resultant pattern of gene activation.

On the basis of studies that were confined to footprinting analysis, Castrillo et al. (1989) concluded that Pit-1 (GHF-1) activates GH gene transcription selectively. On the basis of studies that were confined to footprinting analysis, with an incomplete bacterial fusion protein or a partially purified preparation, Castrillo et al. (1989) concluded that GHF-1 (Pit-1) activates GH

Figure 4. (See p. 952.) DNase I footprinting analysis of the GH promoter (end followed by asterisk is labeled; A,D), the PRL promoter (B,E), and the PRL distal enhancer (C,F), with increasing amounts of Pit-1-containing extracts. Lanes: (235-1) Crude nuclear extract from 235-1 lactotrophs; (GC-PC) phosphocellulose-purified GC nuclear extract; (Bact-PC) phosphocellulose-purified Pit-1 expressed in bacteria. Numbers indicate the amount of protein added in micrograms. The sites are the same as those in Fig. 1A. A, B, and C show titration curves with higher protein concentrations than those in D, E, and F.

Figure 4. (See previous page for legend.)

Figure 5. (A) In vitro transcription using bacterially expressed Pit-1 (Pit-1^{bac}). Five microliters of the indicated fraction was added to transcription reactions with constructs that contained the $-173..+33$ PRL promoter (PRL-173), the $-235..+8$ GH promoter (GH-235), or a mutation of the PRL site P1, which reduced binding of Pit-1 by >100-fold (PRL P1 μ). The fractions are from a parallel PC column purification of extracts from bacteria either carrying (+) or not carrying (-) the Pit-1 expression vector. (B) In vitro transcription response with increasing amounts of Pit-1^{bac}. The indicated microliters of Pit-1^{bac} from A, were added to 10 μ l (100 μ g) of HeLa cell nuclear extract with a luciferase fusion gene that contained an oligonucleotide of either PRL site P1 or GH site GH1 fused 5' to the $-36..+33$ PRL promoter. An RSV long terminal repeat (LTR) fusion gene also was included in each reaction as an internal control. (C) In vitro transcription of three constructs that contained Pit-1-binding sites [GH-235 and PRL-173 (Fig. 5A) and the $-1954..-1532$ PRL distal enhancer fused to the $-36..+33$ PRL promoter [DE + PRL-36]], as well as a construct that lacks one [the cAMP response element (Montminy et al. 1986) fused to the $-36..+33$ PRL promoter [CRE + PRL-36]]. The reactions were carried out either without (-) or with (+) the addition of 3 μ l of Pit-1^{bac} PC fraction 10 (see A) to 10 μ l S194 lymphocyte nuclear extract. The RSV LTR control is included also. (D) Pit-1 dependence of transcription from the GH promoter in HeLa, HeLa/Pit1⁺, and 235-1 cell extracts. Lanes for each extract: (-Comp) The transcription from the $-235..+8$ GH-chloramphenicol acetyltransferase (CAT) plasmid in the absence of competing DNA; (+30 $\times$ GH1) transcription in the presence of double-stranded oligonucleotides corresponding to the GH GH1 site at 30 times the molar amount of template; (+100 $\times$ GH1) transcription corresponding to GH with GH1 at 100 times the amount of template.

gene transcription selectively. However, analysis of permanent transfectants expressing Pit-1 at a level 10-fold lower than physiological, both PRL and GH promoters were activated. Unexpectedly, fusion genes that contained the PRL promoter were activated much more effectively than those that contained the GH promoter under these conditions. In concert with these observations, we document that bacterially expressed intact Pit-1 binds specific *cis*-acting elements effectively in the rat PRL promoter. Thus, even though the two GH *cis*-acting binding elements are among the highest Pit-1 affinity sites, our data argue that the PRL proximal promoter sites can be occupied effectively at physiological concentrations of Pit-1. Both Castrillo et al. (1989) and Mangalam et al. (see Pr11, Fig. 1A) find evidence of additional proteins that can bind to PRL promoter *cis*-acting

elements; however, these proteins do not overlap totally in their footprinting patterns. These or additional proteins are likely to exert important functions in PRL gene expression.

Analysis of the putative 235-1 lactotroph cell line suggests that Pit-1 alone cannot be responsible for the restricted pattern of PRL and GH expression in mature lactotrophs and somatotrophs. Our data are consistent with either an inhibitory mechanism that prevents the actions of Pit-1 on the GH gene or the possibility that cell-specific expression of GH requires an additional positive activity that is absent in the 235-1 cells. It is likely that similar events restrict PRL gene expression in somatotrophs. Both mechanisms could involve the interaction of Pit-1 with additional proteins or simply the modification of Pit-1 (Jackson and Tjian 1988; Santoro et

Figure 6. (A) Transient transfection of 235-1 lactotroph cells. Plasmids that contained either the GH or PRL promoters fused to the luciferase gene were transfected transiently into both GC and 235-1 cells, using the DEAE-dextran method (Gorman et al. 1982a). Fifty micrograms of cell lysate was analyzed for luciferase expression. (Left) $-312..+8$ GH fusion gene expression (rGH-312) relative to that for the $-422..+33$ PRL construct (rPRL-422) in GC cells; (right) in 235-1 cells, the two values represent the effect of transfecting the reporter plasmid alone or cotransfecting with the Pit-1 expression vector (see Fig. 3A). (□) Reporter gene only; (▨) reporter gene plus Pit-1 expression vector. (We were not able to detect a difference between the transfected Pit-1 and the native Pit-1; the two values may be considered replicate estimates of the same value.) (B) RNase protection assay showing the relative amount of Pit-1 mRNA from GC cells to that from 235-1 lactotroph cells. An antisense probe that incorporated the amino-terminal and POU-specific domain coding information of Pit-1 was transcribed with T7 RNA polymerase and produced a probe of 538 nucleotides, of which 355 nucleotides should be protected by mature transcript. Lanes: (Probe) Input probe; (GC) protected probe using RNA from GC cells; (235-1) protected probe using RNA from 235-1 cells; (Markers) the BRL 1-kb ladder. (C) A comparison by southwestern assay of GC and 235-1 cell nuclear extracts versus Pit-1^{bac}. (Left) Probed with the $-312..+8$ GH promoter fragment; (right) probed with the $-173..+33$ PRL promoter fragment (the PRL probe was $\sim 2 \times$ sp. act. of the GH probe). Lanes: (Markers) Protein standards; (GC-Crude) 70 μ g of GC crude nuclear extract; (GC-PC) 3 μ g of PC-fractionated GC nuclear extract; (235-1-Crude) 70 μ g of 235-1 crude nuclear extract; (Bact-PC) 6 μ g of PC-fractionated lysate from bacteria carrying a Pit-1 expression vector; (HeLa) 70 μ g of HeLa cell crude nuclear extract. (D) In vitro transcription analysis of 235-1 nuclear extracts. The indicated PRL and GH promoter constructs were used as templates for in vitro transcription reactions containing 20 μ l of crude 235-1 nuclear extract.

al. 1988; Yamamoto et al. 1988), which could result in changes in DNA site preference or in affinities for other proteins with which it interacts potentially to activate

or 'squench' transcription (Ptashne 1988). Exploration of the mechanisms responsible for the restriction of the action of Pit-1 in the intact 235-1 cells, including the mod-

ification of Pit-1 or the actions of a second, labile inhibitor, are under investigation.

Lufkin and Bancroft (1987) have shown that fusion of GH3 rat pituitary cells with C127 mouse fibroblast cells stably transfected with constructions that contained PRL 5' sequences allowed the previously quiescent PRL constructs to be expressed. This was not the case with constructs that contained GH 5' sequences, suggesting either a factor that stimulates only PRL or a mechanism preventing GH expression. In similar experiments, when pituitary (GH3) and nonpituitary (mouse L-cell fibroblast) cells were fused to create permanent lines, GH gene expression was inhibited (Strobl et al. 1982). These fusion cell lines have been reported to have no immunologically detectable levels of GHF-1 (McCormick et al. 1988); it would be revealing to examine PRL gene expression in these cells and to ascertain more accurately that GHF-1 is actually absent in these cells.

On the basis of the analysis of GH and PRL gene expression in pituitary cells, it appears that the concept of an interconnected network of genes, described most convincingly in *Drosophila* and *C. elegans* (Scott and Carroll 1987), also appears to operate in pituitary tissue development. The data presented here argue strongly that Pit-1 can regulate the transcription of both the GH and PRL genes, and we predict that the failure to express Pit-1 might result in nonexpression of both GH and PRL in the pituitary gland. The slight differences in affinity between GH and PRL, as determined by footprinting and other binding studies, are obviously not a barrier to transcriptional activation at physiological levels of Pit-1, but activation might be regulated by other factors that modulate the Pit-1 effect. This type of interaction could operate in the inactivation of GH in the 235-1 lactotroph cell line and is in accord with similar events during *Drosophila* development.

Our data are consistent with the possibility that Pit-1 is involved in the activation of the developmental programs that generate mature lactotroph and somatotroph phenotypes.

Methods

Nuclear extract preparation

GC2 cells were grown in spinner flasks to 8×10^4 cells/ml and were concentrated by centrifugation. The cells were washed in ice-cold phosphate buffered saline, pelleted at 1600g, resuspended in four original cell volumes (OCVs) of buffer A [10 mM HEPES (pH 7.9 at 1 M), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)], and swelled for 10 min on ice; they were then centrifuged (1600g, 10 min, 4°C) and resuspended in 2 OCVs of buffer A plus 0.2% NP-40 and protease inhibitor mix (PIM: 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml leupeptin, 4 mM benzamidine, 5 mM antipain). The cells were allowed to swell for an additional 5 min and were then disrupted with an iced Dounce B-size glass homogenizer to prepare the nuclei. After 15 strokes, the preparation was examined microscopically for the presence of nuclei, and more strokes were applied until 70–80% of the preparation was nuclei. The preparation was centrifuged (800g, 10 min, 4°C), and the supernatant was re-

moved carefully. The nuclei were resuspended by vortex in 1.2 ml of buffer C plus PIM/ml of OCV. The total volume at this stage was measured and 3 M KCl was added dropwise to a final concentration of 400 mM KCl, while the preparation was stirred slowly in an ice bath. The extraction continued with slow stirring for 40 min, after which time the preparation was centrifuged (23,000g, 20 min, 4°C) to pellet the nuclei, DNA, and cytoplasmic debris. The supernatant was dialyzed against two changes of 20 mM HEPES (pH 7.9 at 1 M), 20% glycerol, 50 mM KCl, and 0.2 mM EDTA for 3–4 hr per change, and precipitate was removed by centrifugation (15,000g, 15 min, 4°C). The protein concentration was determined as per Bradford (1976), and the extract was frozen at -80°C in 30-ml vials.

Southwestern protocol

The Southwestern protocol was modified from Bowen et al. (1980) and Jack et al. (1982).

Crude nuclear extracts or column fractions were concentrated by acetone precipitation (2 volumes at -20°C , 30 min on ice), followed by centrifugation (15,000g, 7 min, 4°C). The protein pellets were resuspended in SDS-loading dye and boiled for 3 min before loading on an SDS-polyacrylamide discontinuous gel system (Laemmli 1970). The samples were electrophoresed until the buffer boundary had reached the bottom of the gel. The separated proteins were transferred to nitrocellulose (NC) in transfer buffer (TB: 40 mM glycine, 50 mM Tris (pH not adjusted), 0.04% SDS, 20% methanol), using an LKB Electrophor electrophoresis apparatus at 1 mA/cm² NC for 1 hr. After the transfer was complete, the NC filter was soaked (1) in 6 M guanidine for 5 min to denature the proteins completely, (2) in binding buffer (BB: 10% glycerol, 20 mM HEPES (pH 7.9 at 1 M), 50 mM KCl, 1 mM DTT, 0.01% NP-40) for 5 min to renature the protein, and (3) in 5% Carnation nonfat dry milk in BB for 5 min to block the NC. The excess milk was rinsed-off in BB, and the NC filter was inserted into a polyethylene 'Seal-a-meal' bag with 1 ml/10 cm² probing solution that contained 5×10^5 cpm/ml ³²P-labeled probe DNA, either in the presence (3–5 μ g/ml) or absence of nonspecific [poly(dI/dC) or salmon sperm] DNA. After probing for at least 3 hr at 4°C on a rotating wheel, the filter was removed from the bag, washed in several changes of BB for up to 3 hr, blotted dry, covered with Saran Wrap, and put under film with an intensifying screen.

Purification of Pit-1

All procedures were carried out in a 4°C cold room. Nuclear extracts of GC2 cells (see above), were applied to a phosphocellulose (PC; Whatman P11) column equilibrated in 50 mM KCl PC buffer [10% glycerol, 20 mM HEPES (pH 7.9 at 1 M), 1 mM DTT, 2 mM EDTA, 2 mM PMSF, 0.1% NP-40]. After washing the resin with 2 column volumes of 200 mM KCl PC buffer, the column was developed with a linear gradient to 700 mM KCl, in a total of 6 column volumes, adjusting the fraction size to yield ~30 fractions. The active fractions that eluted between 250 and 400 mM KCl were pooled. $MgCl_2$ was added to 2 mM to titrate the EDTA, and the pooled eluate was applied to a column of hydroxyl apatite (Bio-Gel-HTP, Bio-Rad). Most of the Pit-1 activity flowed through the column, was pooled with 3 column volumes of PC buffer wash, and was diluted sixfold in PC buffer minus KCl and applied directly (without addition of nonspecific DNA) to the DNA-affinity column made from multimers of the rat GH proximal Pit-1 site (GH1 in Fig. 1A) attached covalently to Sepharose CL6B (Pharmacia), as described by Kadonaga and Tjian (1986). After washing with 2 column volumes of 150 mM KCl, the activity was eluted with a linear gradient

to 700 mM KCl in PC buffer. In two instances, the active fractions were pooled and reapplied to the affinity column and eluted as described, but as this did not yield a substantially greater purification, it was not repeated.

DNase I footprinting

Crude nuclear extract or purified fractions were incubated with a double-stranded DNA probe (10–30 fmoles/reaction; single end-labeled on the 5' end with [γ - 32 P]ATP or on the 3' end with [α - 32 P]dCTP) for 15–30 min at room temperature in an 80- μ l reaction that contained 20 mM HEPES (pH 7.9 at 1 M), 2 mM DTT, 2 mM EDTA, and 5% glycerol. DNase I (Worthington, grade DP) was added to a final concentration of 0.1–5.0 μ g/ml in a buffer that contained 70 mM MgCl₂ and was stopped after 2 min by the addition of 70 μ l of 1% SDS, 50 mM EDTA, and 200 μ g/ml yeast tRNA. The sample was extracted with phenol/chloroform, ethanol-precipitated, centrifuged (15,000g, 10 min), washed twice with 70% ETOH, dried over 80°C water, resuspended in 95% formamide loading dyes, and boiled for 3 min. The samples were electrophoresed at 1000–1300 volts in 1/2 $\times$ TBE, on a 0.4-mm thick, 7% polyacrylamide, 8 M urea sequencing gel for 2–4 hr (depending on the probe used), dried, and exposed to film at –70°C.

Constructions and tissue culture

The detailed structure of the SV40/DHFR-based expression vector is described in Lin et al. (1987) and Nelson et al. (1988). The Pit-1 expression vector was constructed from the above vector as described in Ingraham et al. (1988). The HeLa/Pit1⁺ stable cell lines were generated by a modification of the classical method (Southern and Berg 1982) of transfecting 10⁶ HeLa cells with 10 μ g of the SV40-based Pit-1 expression plasmid via the CaPO₄ coprecipitation method (Chen and Okayama 1987). After 2 days, the cells were put under methotrexate selection (800 nM) and amplified by increasing methotrexate concentrations in stages of 5 days (800, 1200, 2500, 5000 nM). Resistant colonies were cloned and maintained under methotrexate selection (400 nM) in Dulbecco's modified Eagle's medium (DMEM).

Transfection of PRL–luciferase and GH–luciferase plasmids (Nelson et al. 1988) into the HeLa/Pit1⁺ cells also was effected by the CaPO₄ coprecipitation method, using 10 μ g plasmid/10⁶ cells in an 8-cm plate. Forty-eight hours after transfection, the cells were washed with 3 ml of Tris-buffered saline and harvested by scraping in 1 ml of 50 mM HEPES (pH 7.9 at 1 M), 15 mM Mg acetate, and 1 mM DTT. The cells were centrifuged at 500g for 2 min, and all but 200 μ l of the supernatant was aspirated, after which the cell pellets were dispersed by Vortex agitation and the cells were lysed by three cycles of freezing (powdered dry ice) and thawing (37°C). The particulate debris was pelleted (13000g, 5 min) and 50 μ l of the supernatant was assayed for luciferase activity by adding 150 μ l of freshly prepared 50 mM HEPES (pH 7.9 at 1 M), 15 mM Mg acetate, and 4 mM ATP and reading the light emitted in a Monolight model 2001, which added automatically 100 μ l of 2 mM luciferin (Analytical Luminescence Laboratory) and integrated the light peak over 10 sec.

Transfection of GC2 cells and 235-1 cells was performed as a modification of Gorman et al. (1982a). Briefly, 2 $\times$ 10⁶ cells/8-cm plate were given 7 ml of fresh DMEM (plus serum) 5 hr before the transfection. The media was aspirated, and 10 μ g of plasmid in 2 ml of media was added to the plate. Immediately afterward, 400 μ g/ml of DEAE–dextran in another 2 ml of media was added dropwise to the plate as it was rocked gently.

The transfected cells were returned to the incubator for 2 hr, the plasmid/DEAE–dextran media was aspirated and 8 ml of media (plus serum) was added and the cells returned to the incubator. After 48 hr, the cells were harvested and assayed as described for the HeLa cells, above.

RNase protection assays

RNA was isolated, poly(A)⁺-selected from 235-1 cells as described previously (Ingraham et al. 1988), and subjected to RNase protection analysis as described (He et al. 1989). Briefly, a clone that contained Pit-1 cDNA extending 3' to a portion of the POU domain in pBKS (Stratagene) was used to generate labeled antisense probe, using T7 RNA polymerase in the presence of [32 P]UTP (>800 Ci/mmol), that generated an RNA transcript of 538 nucleotides. RNA from 235-1 cells (20 μ g) was hybridized to 5 $\times$ 10⁵ cpm antisense probe (heated to 85°C and annealed for 12 hr at 45°C) and digested with RNase A (30 μ g/ml) at 30°C for 45 min prior to electrophoresis on a sequencing gel. Markers used were the BRL 1-kb standard ladder.

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