A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the *pit-1* gene

Simon J. Rhodes,^{1,2} Ruoping Chen,^{1–3} Gabriel E. DiMattia,² Kathleen M. Scully,^{2,4} Kristin A. Kalla,^{2,5} Sheng-Cai Lin,^{2,5} Victor C. Yu,^{2,6} and Michael G. Rosenfeld^{2,5}

⁵Howard Hughes Medical Institute, ²Eukaryotic Regulatory Biology Program, ⁴Biomedical Graduate Sciences Program, University of California, San Diego, School and Department of Medicine, La Jolla, California 92093-0648 USA

Pit-1 is a tissue-specific POU domain factor obligatory for the appearance of three cell phenotypes in the anterior pituitary gland. Expression of the *pit-1* gene requires the actions of a cell-specific 390-bp enhancer, located 10 kb 5' of the *pit-1* transcription initiation site, within sequence that proves essential for effective pituitary targeting of transgene expression during murine development. The enhancer requires the concerted actions of a cell-specific *cis*-active element, Pit-1 autoregulatory sites, and atypical morphogen response elements. Pituitary ontogeny in the Pit-1-defective Snell dwarf mouse reveals that *pit-1* autoregulation is not required for initial activation or continued expression during critical phases of Pit-1 target gene activation but, subsequently, is necessary for maintenance of *pit-1* gene expression following birth. A potent 1,25-dihydroxyvitamin D₃-responsive enhancer element defines a physiological site in which a single nucleotide alteration in the sequence of core binding motifs modulates the spacing rules for nuclear receptor response elements. Unexpectedly, the major retinoic acid response element is absolutely dependent on Pit-1 for retinoic acid receptor function. On this DNA element, Pit-1 appears to function as a coregulator of the retinoic acid receptor, suggesting an intriguing linkage between a cell-specific transcription factor and the actions of morphogen receptors that is likely to be prototypic of mechanisms by which other cell-specific transcription factors might confer morphogen receptor responsiveness during mammalian organogenesis.

[**Key Words:** Pit-1; pituitary; enhancer; retinoic acid; vitamin D; morphogen]

Received February 16, 1993; revised version accepted April 7, 1993.

The mammalian anterior pituitary is a critical endocrine gland secreting peptide hormones that regulate adrenal gland function, thyroid activity, sexual development, milk production, and growth. During embryonic development, the anterior pituitary arises from a common ectodermal primordium in Rathke's pouch, ultimately generating five distinct hormone-producing cell types: corticotrophs, secreting adrenocorticotrophic hormone; thyrotrophs, secreting thyroid-stimulating hormone (TSH); gonadotrophs, secreting follicle-stimulating hormone, and luteinizing hormone; lactotrophs, secreting prolactin; and somatotrophs, secreting growth hormone (Schwind 1928; Simmons et al. 1990; Dubois and Hemming 1991; Voss and Rosenfeld 1992).

Analysis of prolactin and growth hormone gene activation revealed that a tissue-specific transcription factor could bind to and *trans*-activate these genes (Nelson et al. 1986, 1988; Bodner and Karin 1987; Cao et al. 1987;

Gutierrez-Hartmann et al. 1987; Lufkin and Bancroft 1987; Ye and Samuels 1987). Identification of the transcription factor Pit-1 (GHF-1) (Bodner et al. 1988; Ingraham et al. 1988) led to confirmation that the *pit-1* gene product could function as a transcriptional activator of the prolactin and growth hormone genes (Ingraham et al. 1988; Mangalam et al. 1989). Pit-1 is a member of a large family of transcription factor developmental regulatory proteins that have been structurally conserved throughout evolution: the POU domain factors (Rosenfeld 1991; Ruvkun and Finney 1991). Pit-1 initially is expressed on embryonic day 14 (e14) in the central caudal region of the mouse pituitary (day 15 in rat), before initial activation of growth hormone and prolactin gene expression (Dolle et al. 1990; Simmons et al. 1990), and, ultimately, Pit-1 is detected in lactotroph, somatotroph, and thyrotroph cell types (Simmons et al. 1990). The pituitaries of dwarf mice [Snell (*dw/dw*) and the Jackson (*dw^J*) allelic mutation] that harbor defective *pit-1* genes, lack thyrotroph, lactotroph, and somatotroph cell types, indicating that functional Pit-1 protein expression is required for the maintenance of these three cell lineages (Li et al. 1990). In addition, mutations within both the POU-specific and

¹The first two authors contributed equally to this study.

Present addresses: ³Clayton Peptide Biology Lab, Salk Institute, San Diego, California 92186-5800 USA; ⁶Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511.

POU homeo domains of Pit-1 are associated with dwarfism and pituitary dysfunction in humans (Pfaffle et al. 1992; Radovik et al. 1992; Tatsumi et al. 1992).

The importance of the Pit-1 protein in the control of pituitary ontogeny indicates that investigation of the mechanisms by which the *pit-1* gene itself is activated and regulated during mammalian development is required to understand the molecular basis of pituitary activity and development. Previous analyses have revealed Pit-1-binding sites in the *pit-1* promoter and have provided evidence of a Pit-1 autoregulatory loop with a positive autoregulatory element located upstream of the transcriptional start site (Chen et al. 1990; McCormick et al. 1990) and an inhibitory element downstream of the cap position (Chen et al. 1990; Pfaffle et al. 1992). The mouse, rat, and human *pit-1* promoters are weakly active in cultured pituitary cell lines, and each contains the two autoregulatory Pit-1-binding sites. It has also been suggested that the *pit-1* promoter contains a TATA region element responsible for tissue-specific expression of the *pit-1* gene (McCormick et al. 1991).

In considering mechanisms of tissue-specific activation of developmentally significant transcription factors, the importance of morphogens must be considered. Significant evidence has suggested that morphogen gradients exert critical roles in limb bud and neural tube development (De Luca 1991; Tabin 1991) and regulate gradients of *Hox* gene expression (Boncinelli et al. 1991), and it has become increasingly likely that morphogens exert additional, pivotal roles in organogenesis. Retinoic acid receptor (RAR), vitamin D receptor (VDR), and thyroid hormone receptor (T3R) are members of the nuclear receptor superfamily of ligand-dependent transcription factors that control critical aspects of development as a consequence of binding to response elements on target genes (Evans 1988; Green and Chambon 1988; O'Malley 1990). All three receptors preferentially bind to their cognate DNA response elements as components of heterodimeric complexes with coregulatory proteins (Forman et al. 1989; Burnside et al. 1990; Glass et al. 1990; Liao et al. 1990; Ozono et al. 1990; Beebe et al. 1991; Darling et al. 1991; O'Donnell et al. 1991). The retinoid X receptors (RXRs) have proved to function as one such coregulator (Yu et al. 1991; Bugge et al. 1992; Klierwer et al. 1992; Leid et al. 1992; Mangelsdorf et al. 1992; Marks et al. 1992; Zhang et al. 1992). Nuclear receptor/RXR heterodimers preferentially bind to and activate transcription from response elements consisting of direct repeat, as well as palindromic, arrangements of a core binding consensus (AGGTCA) sequence (Glass et al. 1988; Umesono et al. 1988; Mangelsdorf et al. 1991). Although no absolute binding preference is observed, preferential binding and transcriptional activation by VDR, T3R, and RAR heterodimers are observed on elements varying in the spacing and orientation of core binding motifs (Näär et al. 1991; Umesono et al. 1991). Direct repeats of core binding motifs, spaced by 3, 4, and 5 bp, have been proposed to be the basis of preferential actions of VDR, T3R, and RAR, respectively (Umesono et al. 1991).

In this paper we report the identification of a potent,

cell-specific enhancer of the mouse *pit-1* gene containing a critical cell-specific element, Pit-1 autoregulatory elements, and sites that confer inducibility by retinoic acid and 1,25-dihydroxyvitamin D₃. Remarkably, the major retinoic acid response element (RARE) reveals a synergistic interaction between Pit-1 and RAR mediating pituitary-specific effects of retinoic acid, whereby Pit-1 appears to act as a coregulator on this structurally atypical response element. The *pit-1* gene enhancer provides an initial example of the ability of a cell-specific transcription factor to confer tissue-specific induction by RAR and indicates that a single nucleotide alteration in the sequence of core binding motifs can serve to alter the preferred spacing rules regulating nuclear receptor action. Analysis of pituitary ontogeny in the Snell dwarf mouse revealed that autoregulatory events are not required for initial *pit-1* gene expression or for expression during Pit-1 target gene activation but are essential late in pituitary development for maintenance of *pit-1* gene expression. These observations are consistent with the hypothesis that enhancer-mediated synergistic interactions between a POU domain developmental regulator, morphogen receptors, and the activities of cell-restricted factors are critical components of the mechanisms governing anterior pituitary development.

Results

Distal pit-1 gene sequences are required for pituitary expression of pit-1 reporter genes in transgenic animals

To test whether the *pit-1* promoter alone was sufficient to target *pit-1* expression in the pituitary, transgenes containing the mouse *pit-1* promoter (−327 to +13 bp) and either the human growth hormone gene (−327 *pit-1*/hGH) or the *Escherichia coli* β-galactosidase gene (−327 *pit-1*/LacZ) were constructed (Fig. 1A) and transgenic animals were generated. Tissue extracts of identified transgenic animals were analyzed for hGH by immunoradiometric assay or for transgene-derived β-galactosidase activity by colorimetric enzyme assay. Pituitary and nonpituitary tissues were assayed for hGH content in at least six animals from each −327 *pit-1*/hGH pedigree. There was no detectable hGH in any of the −327 *pit-1*/hGH tissue extracts (Fig. 1B). β-Galactosidase activity above background levels was not detected in any tested tissues of −327 *pit-1*/LacZ animals (see Fig. 1C for pituitary values). Clearly, with two different transgene reporters, −327 bp of *pit-1* 5'-flanking information is insufficient to confer activation of the transgene in the pituitary.

Eleven independent transgenic lines carrying 14.8 kb of contiguous mouse *pit-1* upstream sequence were generated: six fused to the hGH reporter (14.8 *pit-1*/hGH) and five to LacZ (14.8 *pit-1*/LacZ). All five 14.8 *pit-1*/LacZ pedigrees displayed very high levels of β-galactosidase activity in the pituitary (Fig. 1C). LacZ transgene activity was not detectable in nonpituitary tissues of any pedigree (Fig. 1E), except in line 9 (β-galactosidase activ-

Figure 1. *pit-1* gene upstream sequences are required to target *pit-1* promoter activity to the pituitary in transgenic animals. (A) Transgenic reporter genes. hGH and β-galactosidase (LacZ) fusion genes were constructed containing either the mouse *pit-1* promoter (-327 *pit-1*/hGH, -327 *pit-1*/LacZ) or containing 14.8 kb of contiguous 5'-flanking sequence (14.8 *pit-1*/hGH, 14.8 *pit-1*/LacZ). Transgenic mice were generated carrying each of these reporter genes. (B) Pituitary extracts from the offspring of identified transgenic pedigrees and control nontransgenic animals were tested for levels of hGH by immunoradiometric assay. Values are means ± S.E.M., where *n* = number of individuals tested. Three representative 14.8 *pit-1*/hGH pedigrees are shown. (C) β-Galactosidase activity of pituitary extracts from identified LacZ transgenic lines was measured by colorimetric enzyme assay using *O*-nitrophenyl-β-D-galactopyranoside as a substrate. (D) hGH levels in tissue extracts from a representative individual of pedigree 14.8 *pit-1*/hGH #15. (Ab. wall) Abdominal wall. (E) β-Galactosidase activities of tissue extracts from 14.8 *pit-1*/LacZ #12. Low levels observed in lung and spleen in this line were not significantly different from background.

ity in the gonad 100-fold lower than in the pituitary). All six 14.8 *pit-1*/hGH pedigrees expressed significant levels of hGH in their pituitaries (Fig. 1B; data not shown). Three pedigrees exhibited pituitary-specific expression of hGH (Fig. 1D). One pedigree (line 8) exhibited ectopic hGH expression in the brain at levels 28- to 226-fold lower than those observed in the pituitaries of the same transgenic pedigree. Ectopic expression of hGH transgenes in the brain has been described previously and appears to be the result of combinations of heterologous promoter/enhancer regions interacting with intronic or 3'-flanking regions of the hGH gene (Lira et al. 1988; Russo et al. 1988; Gunzburg et al. 1991). Two of the pedigrees expressed hGH ectopically in almost all tissues tested (data not shown). Together, these results indicate that regulatory elements upstream of the mouse *pit-1* promoter are critically important in facilitating a high level of pituitary-specific gene expression.

Identification of a potent upstream enhancer of the mouse pit-1 gene

The demonstration that distal sequences within the 5'-flanking region of the *pit-1* gene were required for detectable tissue-specific expression of *pit-1* transgenes prompted us to search for a genomic enhancer that could specifically and effectively regulate the function of the *pit-1* gene. Fragments of mouse genomic DNA, including ~15 kb of upstream sequence, the *pit-1* gene itself (~14 kb), and ~6.5 kb of downstream information, were derived from two overlapping genomic phage clones (Li et al. 1990). These fragments were cloned adjacent to the *pit-1* promoter (Chen et al. 1990) in luciferase reporter genes and were transfected into Pit-1-expressing pituitary GC cells. Intragenic and 3'-flanking regions contained no detectable enhancer activity (data not shown). However, reporter constructs containing either 14.8 or 11.5 kb of the upstream region significantly enhanced *pit-1* promoter activity, whereas those containing 10.2 kb of 5'-flanking sequence did not enhance promoter activity (Fig. 2A). These data indicated that specific enhancing sequences were present between -10.2 and -11.5 kb. To confirm this observation, restriction fragments of the 5'-flanking region were analyzed independently (Fig. 2B). Significant enhancing activity was only

detected in a 5.8-kb *HindIII* fragment that overlapped the -10.2- to -11.5-kb region. Further analysis of the 5.8-kb fragment revealed that a subfragment of ~0.7 kb (HcE0.7) retained strong enhancer function, with further resolution of activity in fragments of 0.5 (EP0.5) and 0.39 kb (EP0.39) (Fig. 2C).

In concert with the properties of eukaryotic enhancers (for review, see Atchison 1988), the 0.7-kb *pit-1* enhancer, in either orientation, significantly enhanced the expression of thymidine kinase promoter reporter genes (Fig. 2D) or SV40 promoter reporters (data not shown) in GC pituitary cells. The enhancer fragment functioned in a cell-specific manner, displaying high activity in pituitary (GC) cells but not in nonpituitary cell types (human cervical carcinoma, HeLa; monkey kidney, CV-1; Fig. 2E). Therefore, a short, highly effective tissue-specific enhancer, located ~10 kb upstream of the mouse *pit-1* gene is able to markedly increase transcription (50- to 100-fold) from the weak *pit-1* promoter in pituitary cells.

Autoregulatory, cell-specific, and control elements within the pit-1 gene enhancer

The nucleotide sequence of the 0.7-kb *pit-1* gene enhancer region is shown in Figure 4A (below). DNase I footprinting experiments using labeled enhancer fragment probes, incubated with either bacterially expressed Pit-1 protein or nuclear extracts from pituitary and heterologous cell lines, demonstrated that the identified enhancer contained five binding sites for Pit-1 (Pit-1 sites a-e, Figs. 3 and 4; data not shown). These sites were protected both by purified Pit-1 protein and by Pit-1-containing GC cell nuclear extracts but not by HeLa nuclear extracts. Of the five Pit-1 sites, four conformed to the previously observed TATNCAT/A consensus (Ingraham et al. 1988; Nelson et al. 1988). In addition, a region of the enhancer was specifically protected by GC pituitary cell nuclear extracts but not by Pit-1 protein or HeLa nuclear extracts. This region was therefore designated the PSE region, for pituitary-specific element. Binding of GC-specific factor(s) to the PSE resulted in both protection from DNase I digestion and the creation of a hypersensitive site for nuclease activity (Fig. 3). The PSE region contained two CANNTG motifs at positions 457 and 471. Such sequences often form the core of rec-

Figure 2. A potent cell-specific enhancer is located upstream of the murine *pit-1* gene. (A) *pit-1* promoter/luciferase reporter genes containing either 14.8 kb of mouse *pit-1* gene 5'-flanking sequence (p14.8), 11.5 kb of 5'-flanking sequence (p11.5), 10.2 kb of 5'-flanking sequence (p10.2), or the *pit-1* promoter alone [with the negative Pit-1 site mutated as described by Chen et al. 1990] were constructed. The activities of each of the fragments was assayed by transient transfection into GC pituitary cells and measurement of luciferase activity after 48 hr. The fold enhancement of promoter activity ($\pm$ S.E.M.) for each fragment is shown. (B) The 5'-flanking region was analyzed further by testing restriction fragments of the 5'-flanking region as described in A. Restriction sites: (H) *HindIII*; (B) *BglIII*; (E) *EcoRI*; (Hc) *HincII*; (P) restriction site introduced by PCR. (C) A 5.8-kb *HindIII* fragment displaying significant enhancer activity was further divided to reveal that enhancer activity was contained within a minimal fragment of 390 bp (EP0.39). Active fragments are denoted by thickened lines. (D) The 700-bp *HincII-EcoRI pit-1* gene enhancer fragment (from HcE0.7) was cloned in both orientations into a Herpes simplex virus thymidine kinase (TK) promoter/luciferase fusion reporter and transfected into GC cells. (E) Reporter genes containing the 700-bp enhancer plus either the heterologous TK or the *pit-1* promoter were transfected into pituitary (GC) and nonpituitary (HeLa and CV-1) cell types. In all assays, values shown are means (or folds) derived from triplicate samples $\pm$ S.E.M. in an experiment representative of at least four independent experiments.

Morphogen inducibility of the *pit-1* gene

Figure 2. (See facing page for legend.)

Figure 3. The *pit-1* enhancer contains *cis*-acting elements for interaction with cell-specific factors. DNase I footprinting analyses of the *pit-1* enhancer are shown. Labeled sense and antisense enhancer fragments were incubated with either bacterially expressed Pit-1 protein (bPit-1), GC pituitary cell nuclear extract (GC), or HeLa cell nuclear extract (HeLa), prior to digestion with DNase I. Pit-1-binding sites c, d, and e (hatched boxes) and the GC-specific PSE motif (open box) are indicated. A hypersensitive site within the PSE region is marked by an asterisk (*).

ognition sites for the helix-loop-helix class of developmental regulatory proteins (Blackwell and Weintraub 1990). In addition, a direct repeat of the sequence AGT-

TCA separated by 4 bp (designated RDE), which is structurally similar to known response elements for the retinoic acid/thyroid hormone/vitamin D class of nuclear receptors, was observed in the antisense strand at position 666, and a degenerate motif with an apparent spacing of 1 bp (TGCTCAaAGTTCA; designated PRE) was noted at position 521.

Three Pit-1 sites (c, d, and e) contribute significantly to enhancer activity, because mutation of each site reduced reporter gene activity by 80–90%, whereas mutation of Pit-1 sites a and b did not significantly alter enhancer activity (Fig. 4B). Cotransfection of *pit-1* 0.5-kb enhancer/promoter reporter constructs and Pit-1 expression vectors in heterologous cells revealed a 50- to 80-fold stimulation of *pit-1* gene expression by Pit-1 (Fig. 9, below). The *pit-1* enhancer therefore contains three functionally important Pit-1-binding sites, indicating that autoregulation of the *pit-1* locus might be a component of its physiological regulation.

We also mutated each of the other elements identified by DNase I footprint analysis and DNA sequencing. Whereas mutation of the AGTTCA direct repeat motif (mut RDE) reduced enhancer/promoter reporter gene activity twofold in GC cultures, mutation of the right-hand AGTTCA motif of the degenerate repeat at position 528 (mut PRE) lowered activity markedly (to ~20%) (Figure 4C). Mutation of the cell-specific PSE sequence also strongly reduced enhancer activity (to ~10%), demonstrating that this region is required for effective enhancer function. Mutation of several other non-Pit-1 binding regions within the enhancer (such as mutation 1 in Fig. 4C) did not alter enhancer activity significantly.

We were unable to recognize any distinct consensus binding sites for known transcription factors within the cell-specific (PSE) region, apart from the two CANNTG motifs at positions 457 and 471. A mutation that altered sequences within both the first CANNTG motif and the region between the motifs (mut PSE#1) affected enhancer activity strikingly (Fig. 4C,D). Individual mutation of the CANNTG motifs (mut PSE#2, mut PSE#3) altered enhancer function only moderately, whereas mutation of the intervening region (mut PSE#4, mut PSE#5) or a combined mutation of both motifs (mut PSE#6) reduced enhancer activity significantly (Fig. 4D). We conclude that the PSE region is a complex element important to *pit-1* gene enhancer function that is likely to contain binding sites for multiple protein complexes.

Figure 4. Sequence and mutational analyses of *pit-1* gene enhancer motifs. (A) Nucleotide sequence of the 0.7-kb mouse *pit-1* enhancer region. Base pairs are numbered from the *HincII* site to the *EcoRI* site (restriction sites shown in italics). The five Pit-1-binding sites revealed by DNase I footprinting are shown in reverse type and labeled Pit-1 a–e. PSE, RDE, and the putative half-sites of the PRE motif also are in reverse type and labeled. Arrows denote the locations of potential nuclear receptor half-site motifs within PRE and RDE. The position of the truncation giving the similarly active 0.5-kb enhancer is shown by an arrow. (B) The Pit-1-binding sites revealed by DNase I footprinting were mutated in either the 0.7- or 0.5-kb enhancer fragment, and the resulting activity was determined by transfection into GC cells using *pit-1* promoter/luciferase reporter genes as described in Fig. 2. The activity of the intact enhancer/promoter reporter gene [wild type (w/t)] was set at 100%. The activity the *pit-1* promoter alone is shown for comparison. (C) The PSE, PRE, or RDE sequence of the 0.5-kb enhancer fragment was mutated, and the activity of the resulting sequence was determined. A similar mutation of another enhancer region (mut#1) did not affect enhancer function. (D) Mutation of the PSE motif. The effect of the indicated mutations on the activity of a *pit-1* enhancer/promoter were assayed as described above. Mutated positions are shown in lowercase type and are indicated by asterisks (*). Activities are given as the percentage of intact enhancer activity ($\pm$ S.E.M.).

Morphogen inducibility of the *pit-1* gene

Figure 4. (See facing page for legend.)

Stimulation of enhancer activity by retinoic acid and 1,25-dihydroxyvitamin D₃

Because the important RDE and PRE *cis*-active sequences corresponded to the known core-binding motifs for morphogen receptors, we investigated the potential regulation of the *pit-1* gene enhancer by retinoic acid, thyroid hormone, and vitamin D. Enhancer-specific induction was observed when the 0.5-kb enhancer reporter was cotransfected with expression vectors for nuclear receptors in pituitary (GC) cultures incubated with either all-*trans* retinoic acid (16-fold) or 1,25-dihydroxyvitamin D₃ (15-fold) but not thyroid hormone (3,3',5-triiodo-L-thyronine) (Fig. 5). Inductions by all-*trans* retinoic acid (6- to 10-fold) and 1,25-dihydroxyvitamin D₃ (15-fold) also were observed in transfections lacking exogenous nuclear receptors (not shown). Treatment of cul-

tures with the retinoic acid derivative 9-*cis* retinoic acid (Heyman et al. 1992; Levin et al. 1992) resulted in induction ratios similar to those observed with all-*trans* retinoic acid (not shown). Furthermore, treatment of cultures with estrogen, glucocorticoids, or agents that increase cellular levels of cAMP (forskolin and dibutyryl cAMP) failed to increase *pit-1* enhancer/promoter reporter gene expression (data not shown) above those inductions observed with the *pit-1* promoter alone.

Mutation of the direct repeat motif (mut RDE) removed nearly all enhancer responsiveness to 1,25-dihydroxyvitamin D₃, but only partially lowered the induction by all-*trans* retinoic acid (Fig. 5). This element is therefore referred to as the RDE (retinoic acid/vitamin D element). The RDE transferred strong responsiveness to 1,25-dihydroxyvitamin D₃ but only minimal response to retinoic acid, when fused to a heterologous promoter, at ambient receptor levels in GC pituitary cells (Fig. 6A,B). Mutation of the element abolished response with either ligand (Fig. 6B). However, in cotransfection of GC cells with vectors expressing either VDR or RAR α and treatment with appropriate ligands, the RDE was able to confer response to both 1,25-dihydroxyvitamin D₃ (~25-fold) and retinoic acid (~25-fold) (data not shown). The RDE/thymidine kinase reporter was not responsive to treatment with thyroid hormone (data not shown). In addition, strong responsiveness to 1,25-dihydroxyvitamin D₃ was observed following transfection of heterologous VDR-deficient CV-1 cells with the RDE reporter plus a VDR expression vector, but not with the mutated RDE (Fig. 6C). Whereas the RDE could transfer effective activation by retinoic acid to the thymidine kinase promoter in CV-1 cells in the presence of cotransfected RAR (Fig. 6C), this effect was not observed in the absence of exogenous RAR (data not shown). It was therefore concluded that the substitution of T for G at the third position of each of the direct repeat motifs caused this DR+4 element to exhibit ligand-dependent activation by VDR, and not T3R, indicating that the precise nature of major groove contacts may alter the spacing rule, in which an AGGTCA DR+4 element has been suggested to serve as a T3R element (Umesono et al. 1991; Näär et al. 1991).

Gel retardation analyses using nuclear receptor proteins demonstrated that RAR, VDR, and T3R alone bound poorly to the RDE element (Fig. 6D). Addition of the nuclear receptor coregulator RXR α , which is expressed widely throughout mammalian tissues (Bugge et al. 1992; Mangelsdorf et al. 1992), however, enabled the formation of RXR α /nuclear receptor heterodimer complexes with the RDE (Fig. 6D). In accord with the transcriptional responses, VDR/RXR heterodimers exhibited the strongest binding to the AGTTCA DR+4 element, with weaker binding by RAR/RXR and T3R/RXR heterodimers (Fig. 6D). The RDE site formed a complex with protein from GC nuclear extracts that migrated at a similar rate to the RXR/VDR and RXR/RAR complexes (Fig. 6D). Probes containing mutated RDE elements (mut RDE) were not bound by any tested nuclear receptor combination (Fig. 6D). Previously characterized response elements for each type of nuclear receptor (the osteocal-

Figure 5. The *pit-1* enhancer confers retinoic acid and 1,25-dihydroxyvitamin D₃ inducibility to the *pit-1* promoter. (A) The *pit-1* 0.5-kb enhancer/promoter luciferase fusion reporter gene [wild type (w/t)] was assayed for the presence of hormone-responsive elements. The effects of the indicated mutations (mut) of the RDE direct repeat element (mut RDE), the PRE (mut PRE), or both elements (mut RDE + mut PRE) was examined. (B) Reporter genes were transiently cotransfected into GC cells with expression vectors for the indicated receptors. Cultures were grown in charcoal-stripped medium and treated with 0.5 μ M all-*trans* retinoic acid (RA), 0.1 μ M 1,25-dihydroxyvitamin D₃ (Vit D), 0.1 μ M 3,3',5-triiodo-L-thyronine (T3), or with solvent alone as a control. The fold induction ($\pm$ S.E.M.) of the activity of cultures treated with the indicated ligand compared with control cultures is shown. Any nonspecific effects observed with control reporters containing the promoter alone were subtracted.

heterodimers indicates that a single nucleotide alteration can dictate alternative core motif spacing preferences for nuclear receptor heterodimers.

The second putative nuclear receptor-binding element (PRE) was next examined. In the context of the 0.5-kb

enhancer, mutation of the right AGTTCA motif of the degenerate repeat (mut PRE) lowered baseline activity significantly (to 10–20%; Fig. 4) and reduced response to retinoic acid markedly, but did not affect induction by 1,25-dihydroxyvitamin D₃ (Fig. 5). Mutation of both the RDE direct repeat element and the PRE AGTTCA motif (mut RDE + mut PRE) abolished enhancer responsiveness to both retinoic acid and to 1,25-dihydroxyvitamin D₃ (Fig. 5). Thus, the PRE appears to be the major element required for retinoic acid modulation of enhancer activity, whereas the RDE appears to primarily mediate regulation by 1,25-dihydroxyvitamin D₃.

Because the retinoic acid responsiveness of the *pit-1* gene enhancer appeared to be primarily mediated by the structurally atypical PRE motif, we examined whether this element also could transfer retinoic acid responsiveness to

a heterologous promoter. Oligonucleotides containing the TGCTCAaAGTTCA sequence and the adjacent Pit-1 site e (designated the PRE oligonucleotide), or mutated variants of this region, were cloned into the thymidine kinase promoter/luciferase reporter (Fig. 7A). Cotransfection of Pit-1-expressing GC cells with these reporters and a RAR expression vector revealed that the element conferred strong inducibility by retinoic acid to the TK promoter (Fig. 7B). Significant responses (6- to 16-fold) to retinoic acid treatment also were observed without cotransfection of receptor (data not shown), indicating that the PRE element is active at the endogenous levels of RAR in GC cells. Mutation of the AGTTCA right half-site of the PRE (mutant 1) or the Pit-1 site (mutant 2) virtually eliminated the observed response to retinoic acid, indicating that both binding motifs were required

Figure 7. The PRE region confers Pit-1-dependent retinoic acid inducibility. (A) Oligonucleotides representing the *pit-1* enhancer region encompassing the degenerate left half-site motif (L), the right half-site (R), and the adjacent Pit-1-binding site e [forming the PRE wild-type (w/t) oligonucleotide], or PRE oligonucleotides mutated at various positions (mutants 1–5) are depicted. The half-sites and the Pit-1 site are denoted by boldface type, and mutated positions are indicated in lowercase type. Oligonucleotides were cloned into thymidine kinase (TK) promoter/luciferase reporter constructs. (B) Wild-type and mutant reporter constructs were cotransfected with a RAR α expression vector into Pit-1-expressing GC cells, and the fold induction of luciferase activity following treatment with 0.5 μ M all-*trans* retinoic acid over control cultures treated with solvent alone was determined. (C) The PRE reporter gene was transfected into CV-1 cells (no endogenous Pit-1) with either an expression vector containing no insert (none), a RAR α expression vector, a Pit-1 expression vector, or both RAR α and Pit-1 vectors. (D) Cotransfection of wild-type and mutant PRE oligonucleotide/TK reporter genes with both RAR α and Pit-1 expression vectors in CV-1 cells. (E) Transfection of CV-1 cells with the PRE wild-type reporter gene plus a Pit-1 expression vector and either expression vectors encoding RAR α , VDR, or T3R and treatment with all-*trans* retinoic acid, 1,25-dihydroxyvitamin D₃, or thyroid hormone. Values are fold induction by hormone over solvent-treated controls ($\pm$ S.E.M.).

for the response (Fig. 7B). The region was therefore designated as PRE (Pit-1-dependent retinoic acid response element). The sequence TGCTCA forms a degenerate left half-site at a spacing of 1 bp upstream of the AGTTC A half-site at position 528. A mutation designed to disrupt this motif and other cryptic repeats at potential spacings of 0–5 bp (mutant 3) significantly lowered response to retinoic acid (Fig. 7B), but ~20% of activity was still observed. Addition of 3 bp between the PRE half-site and the Pit-1 site (mutant 4), resulting in a spacing of 7 rather than 3 bp, also strongly reduced response to retinoic acid, indicating that the distance between the Pit-1 site and the AGTTCA motif is important to function of the PRE element (Fig. 7B). Interestingly, conversion of the degenerate left TGCTCA sequence to a perfect repeat of AGTTCA (mutant 5) did not increase responsiveness to retinoic acid; rather, introduction of this stronger binding site actually decreased response below that in which the TGCTCA sequence was completely disrupted (mutant 3). These results suggest that the PRE is not simply a degenerate DR + 1 repeat.

Transfection of heterologous CV-1 cells with expression vectors for RAR or Pit-1 did not result in significant induction of promoters containing the PRE following retinoic acid treatment (Fig. 7C). However, cotransfection of both Pit-1 and RAR expression vectors led to a strong response to treatment with retinoic acid (30- to 90-fold), indicating a synergistic requirement for both Pit-1 and RAR in PRE function (Fig. 7C). Western analysis of nuclear extracts of transfected CV-1 cultures using an anti-Pit-1 antiserum revealed that treatment with retinoic acid did not affect steady-state levels of expressed Pit-1 protein (data not shown). Transfection of CV-1 cells with expression vectors for RAR α and Pit-1 with each of the wild-type and mutant PRE oligonucleotide reporter genes gave similar results to those observed in GC cells (Fig. 7D). To examine whether the isolated PRE region specifically conferred response to retinoic acid treatment, CV-1 cells were transfected with the PRE wild-type reporter plus RAR, VDR, or T3R and were treated with the appropriate ligand. As shown in Figure 7E, the PRE responded strongly in the presence of RAR/RA but was unresponsive to either vitamin D or thyroid hormone. Thus, this atypical element exhibits a restricted pattern of nuclear receptor responsiveness.

Because the Pit-1-dependent action of RAR could be observed in the absence of the degenerate TGCTCA motif, a potential model for the mechanism of this element was that Pit-1 was acting as a coregulator for RAR, even in the absence of potential RXR-binding motifs. Gel retardation analyses using low levels of recombinant Pit-1 and RAR were performed resulting in the formation of several complexes that were not formed in reactions containing each protein individually (Fig. 8A,B). These complexes were selectively removed using anti-Pit-1 antiserum (Fig. 8A,B) and anti-RAR antiserum (data not shown). In contrast, addition of Pit-1 to RXR-binding reactions did not result in the formation of any new complexes (Fig. 8B). The RAR/Pit-1 interaction was examined further in gel retardation assays using mutant PREs.

Mutation of either the Pit-1 site (mutant 2) or the putative RAR-binding AGGTCA right-hand half-site (mutant 1) prevented formation of the extra complexes, whereas PRE oligonucleotides containing a disruption of the degenerate putative RXR half-site permitted formation of the complexes (Fig. 8A). These data are consistent with weak cooperative binding interactions between Pit-1 and RAR on the *pit-1* PRE. Addition of Pit-1 protein to ABCD-binding reactions (Glass et al. 1989) resulted in increased binding of ³⁵S-labeled RAR to biotinylated PRE probes (data not shown).

Gel retardation analyses using recombinant RAR and RXR proteins demonstrated that the PRE oligonucleotide formed very weak complexes with each protein but bound more strongly in reactions containing both proteins, indicating a preference for the RAR/RXR heterodimer (Fig. 8B, lanes 3–5). Comparison with parallel binding reactions using labeled β RARE oligonucleotides revealed that the RAR/RXR interaction with the PRE was considerably weaker (~60-fold) than that with β RARE (data not shown), consistent with the degenerate nature of the putative RXR left half-site. Mutation of either the left TGCTCA sequence (mutant 3) or the right AGTTCA motif (mutant 1) of the PRE abolished detectable binding by RAR, RXR, or RAR/RXR heterodimers (Fig. 8C). However, mutation of the Pit-1 site (mutant 2), did not affect cooperative interactions between RAR and RXR on the PRE (Fig. 8C). Recombinant Pit-1 protein bound to the PRE element, forming three predominant complexes (Fig. 8B, lane 1) in accord with the multiple complexes that have been observed previously in binding reactions containing Pit-1 and a prolactin gene Pit-1-binding site (Prl-1P; Ingraham et al. 1990; Voss et al. 1991). At low concentrations of Pit-1, only the lower monomeric complex was observed (Fig. 8B, lane 2). Binding reactions containing low levels of Pit-1, RAR, and RXR proteins formed two predominant high-molecular-weight complexes (Fig. 8B, lane 9). The lower complex migrated at a rate equivalent to the RAR/RXR heterodimer. This complex was removed by antisera against both RAR and RXR but not by preimmune serum (Fig. 8B; data not shown). The upper band appeared to represent a complex containing RAR, RXR, and Pit-1, because Pit-1 antiserum selectively abolished formation of this complex, whereas RAR antiserum prevented formation of both the upper and lower complexes (Fig. 8B; data not shown). These data suggest that the PRE is a complex element that may permit weak cooperative binding interactions between RAR and Pit-1, as well as those between RAR and RXR, which may be required for physiological function. The contrast between the potent activity of the PRE site in transfection assays and its weak protein-binding properties observed in vitro suggests that additional factors are required to stabilize PRE activity in vivo.

Pit-1 autoregulation is not required for critical early phases of pit-1 gene expression

The presence of multiple complex elements within the

Figure 8. Protein-binding characteristics of the PRE. Gel retardation analysis of the PRE wild-type (w/t) oligonucleotides were incubated with either purified bacterially expressed rat Pit-1 (P), human RAR α , human RXR α , or combinations of these proteins. (A) Anti-Pit-1 antiserum, (aP) anti-human RAR α , (pi) preimmune serum. (A) Interactions between Pit-1 and RAR were examined using wild-type and mutant PRE oligonucleotides. Arrowheads indicate novel Pit-1/RAR complexes. (B) Interactions between RAR, RXR and Pit-1. A higher concentration of Pit-1 was used in lane 1, low concentrations were used in lanes 2–11. (●) The RAR/RXR complex, (*) the RAR/RXR/Pit-1 complex. (C) Cooperative interaction between RAR and RXR was examined with mutant PRE oligonucleotides either disrupting the right half-site (mut#1), the Pit-1 site (mut#2), or the degenerate left half-site (mut#3).

pit-1 gene enhancer in addition to autoregulatory Pit-1 sites raises the question of whether autoregulation actually operates during the developmental expression of *pit-1* in vivo and whether there is a requirement for Pit-1 in the initial phase of expression of the *pit-1* gene. The *pit-1* gene of the Snell dwarf (*dw*) mouse (Snell 1929) (which ultimately lacks thyrotroph, lactotroph, and somatotroph cell types in the mature pituitary gland) encodes a defective form of the Pit-1 protein (Li et al. 1990). The Snell dwarf mutant form of Pit-1 ($W^{261} \rightarrow C$) was tested directly for its ability to *trans*-activate the *pit-1* enhancer in heterologous cells. Cotransfections of CV-1 cells with a *pit-1* enhancer/promoter reporter, plus expression vectors for wild-type rat Pit-1, rat Pit-1 lacking an activation domain ($\Delta 8-80$), or rat Pit-1 containing a point mutant identical to that of the Snell dwarf mouse (tryptophan²⁶¹ $\rightarrow$ cysteine), were performed. Wild-type Pit-1 strongly *trans*-activated the enhancer/promoter reporter (~ 80 -fold), whereas Pit-1 lacking an amino-terminal activation domain had reduced activity and the Snell mutant Pit-1 was inactive and did not *trans*-activate the enhancer (Fig. 9).

The Snell mutation permitted an ontogenic analysis to distinguish between two alternative possibilities. The *pit-1* gene in *dw* mice might be initially activated in the absence of functional Pit-1, suggesting that Pit-1-independent activation mechanisms are effective for initial and early expression, whereas functional Pit-1 protein ultimately might be required for sustained *pit-1* gene expression and maintenance of the lactotroph, somatotroph, and thyrotroph lineages. Alternatively, functional Pit-1 could be required for effective *pit-1* gene ex-

pression, and, therefore, *pit-1* would not be activated in the *dw* mouse pituitary. To examine this issue, in situ hybridization experiments using radiolabeled *pit-1* cRNA probes on wild-type and *dw/dw* embryonic and postnatal mouse sections were performed. In situ hybridization revealed that the *pit-1* gene was normally activated on e14 in the identical central caudal distribution in *dw/dw* mice as was observed in the wild-type mouse anterior pituitary gland (Fig. 10). Pit-1 expression was detectable in *dw/dw* mice until postnatal day 0 (p0)–p5; however, by e18.5, hybridization signals in *dw/dw* mice were clearly significantly less than those in the anterior pituitary glands of wild-type mice (Fig. 10). In contrast, at this time, expression of the anterior pituitary marker α -glycoprotein (α -GSU) gene was equivalent in dwarf and normal pituitaries (Lin et al. 1992). These data suggested that functional Pit-1 protein is not required for the initial activation of *pit-1* gene expression or for its sustained expression for at least 4 days during pituitary ontogeny. However, Pit-1 is subsequently required for sustained *pit-1* gene expression.

Discussion

Determinants of pituitary-specific activation of the Pit-1 commitment factor

In this paper we have demonstrated that 14.8 kb of 5'-flanking *pit-1* genomic sequence is sufficient to target effective pituitary expression of transgenes in 11 distinct pedigrees. Within this regulatory region we have located a tissue-specific enhancer that appears to be required for *pit-1* gene expression. Analysis of the *pit-1* enhancer has provided insights into the potential molecular mechanisms that are utilized to activate and maintain expression of a gene encoding a transcription factor that is integral to the determination of specific cell phenotypes during mammalian organ development.

Characterization of the *pit-1* gene enhancer provided an opportunity to evaluate the role of autoregulatory mechanisms in the maintenance of gene expression of a critical tissue-specific transcription factor. Autoregulation is a highly utilized strategy by which maintenance of memory of transcriptional commitments is achieved. Such a mechanism has been proposed for many regulatory transcription units, such as the *myoD* gene, which encodes a transcription factor important in mammalian skeletal muscle differentiation (Thayer et al. 1989), and the *ftz*, *Dfd*, and *eve* genes, which encode *Drosophila* developmental regulatory proteins (Hiromi and Gehring 1987; Harding et al. 1989; Regulski et al. 1991). In the case of the *pit-1* gene, both the promoter and enhancer contain Pit-1 response elements, but the enhancer, with three Pit-1 *cis*-active elements, accounts for most of the regulation of the *pit-1* gene by Pit-1 protein. The implications of autoregulatory sites in the *pit-1* gene enhancer could be directly tested because of the availability of the Snell mouse genetic model. The Snell dwarf mouse produces a defective Pit-1 protein, and we have established that this defective protein is not capable of binding to, or

Figure 9. The Snell mouse Pit-1 molecule is unable to activate *pit-1* enhancer/promoter reporter genes. CV-1 cells were transiently cotransfected with a reporter construct containing the 0.5-kb *pit-1* enhancer and expression vectors for either wild-type rat Pit-1 (w/t), rat Pit-1 with amino acids 8–80 deleted ($\Delta 8-80$), rat Pit-1 with a point mutation identical to that found in the Snell mouse (W261C), or with the expression vector alone (none). Luciferase activities ($\pm$ S.E.M.) of triplicate assays are shown.

Figure 10. *pit-1* gene expression during development in Snell mice harboring defective *pit-1* genes. Fixed sagittal sections of normal mouse (wt) or Snell dwarf (dw) embryo pituitaries were hybridized to ^{35}S -labeled Pit-1 cRNA probes in situ and visualized by dark-field microscopy. Pit-1 expression is observed in the central caudal portion of the anterior pituitary. Sections shown are from e14.5, e16.5, e18.5 and from p5. (P) The posterior pituitary; (A) anterior pituitary; (r) the rostral tip of the anterior pituitary.

trans-activating, the *pit-1* gene enhancer or promoter, consistent with its failure to *trans*-activate the growth hormone and prolactin promoters. The demonstration of an effective, initial activation of *pit-1* gene expression at e14.5 and maintenance of expression for >4 days in the Snell mouse reveals that Pit-1 autoregulation is not critically required for either initial activation or early maintenance events. However, the progressive loss of *pit-1* gene expression from e18.5 to p0, preceding any apparent alterations in cell populations in the Snell dwarf, indicates that Pit-1 autoregulation plays a role in later maintenance of *pit-1* gene expression. Therefore, Pit-1-independent mechanisms must be sufficient to initiate the temporally and spatially specific patterns of *pit-1* gene activation. We speculate that this later requirement for Pit-1 may reflect the loss or functional modification of a transiently expressed essential factor involved in early activation, such that autoregulation is subsequently required to maintain significant *pit-1* gene expression. This positive autoregulatory loop would presumably ensure that once the *pit-1* gene has fulfilled its early developmental roles, its expression would be stably maintained, serving a memory function required for maintenance of the lactotroph, thyrotroph, and somatotroph cell phenotypes in the anterior pituitary gland (Li et al. 1990).

The importance of the *pit-1* enhancer in appropriate targeting of *pit-1* promoter activity in transgenic animals and the potent induction of *pit-1* and heterologous promoter activities by the enhancer in transfection assays are consistent with the model that *cis*-active elements within the enhancer are required for initial activation of the *pit-1* gene. A detailed analysis of the *pit-1* gene enhancer has suggested that synergistic interactions between transcription factors are required. Initial activation of the *pit-1* gene may involve cell-specific factors, such as those interacting with the cell-specific *cis*-active (PSE) sequence, acting in concert with other, nonorgan-specific factors. Interplay between retinoic acid and its receptor is a potentially important aspect of anterior pituitary gland development, and the synergistic interaction between Pit-1 and RAR has revealed several novel features that may underlie cell-specific morphogen actions. Considerable evidence suggests that retinoic acid exerts strong and varied effects on mammalian development and differentiation and has been implicated in the regulation of the *Hox* genes which, like the *pit-1* gene, encode developmentally important homeo domain factors (Boncinelli et al. 1991; De Luca 1991; Tabin 1991). We suggest that combined actions of cell-restricted factors and retinoic acid-mediated effects are required for effective, sustained activation of the *pit-1* gene. Because

the *pit-1* enhancer does not require synergistic interactions with any specific promoter elements for effective function, it is likely that the enhancer contains sufficient information to alone direct pituitary-specific gene expression. In this regard, the *pit-1* gene enhancer apparently differs from the enhancer of the prolactin gene, which is also regulated by Pit-1 but which synergizes with the prolactin gene promoter for optimal gene expression [Nelson et al. 1986; Crenshaw et al. 1989; Iverson et al. 1990].

Core DNA-binding motifs alter preferences for nuclear receptor heterodimers

The identification of a structurally distinct vitamin D response element in the *pit-1* gene enhancer has several implications for nuclear receptor-mediated gene regulation. In higher animals, vitamin D is known to play essential roles in maintaining serum calcium levels and in bone development [De Luca et al. 1990; Holick 1991]. However, many genes have recently been described to be either positively or negatively regulated by vitamin D (for review, see Holick 1991; Pike 1991; Walters 1992), suggesting that vitamin D may play a wide role in mammalian homeostasis and development. In the anterior pituitary gland, vitamin D metabolites modulate the transcription and secretion of both TSH and prolactin in pituitary cells [Murdoch and Rosenfeld 1981; Wark and Gurtler 1988; Törnquist et al. 1990]. Vitamin D and its receptor are found in pituitary cell lines [Haussler et al. 1982; Stumpf et al. 1987], and VDR mRNAs are present in the embryonic rat pituitary at early stages of development [S. Rhodes and M. Rosenfeld, unpubl.].

Our findings that *pit-1* enhancer contains a vitamin D response element is consistent with a direct transcriptional effect of vitamin D and its receptor in pituitary regulation. The *pit-1* vitamin D response element (RDE) consists of a direct repeat motif (AGTTCA) spaced by 4 nucleotides. Näär et al. (1991) and Umesono et al. (1991) have proposed that nuclear receptors prefer specific response elements by virtue of the distinct spacing between the core binding motifs and a specific spacing code, the "3,4,5 rule" has been proposed, linking spacing of direct motifs to the actions of vitamin D, thyroid hormone, and retinoic acid, respectively [Umesono et al. 1991]. Further studies have suggested that spacings of 1 and 2 bp may also serve as RXR/RAR and RXR/RXR motifs [Mangelsdorf et al. 1991; Durand et al. 1992], consistent with a relaxed spacing rule for RAR. In contrast, the T3R receptor has clear preference for 4-bp spacing between repeats. The *pit-1* gene RDE appears to provide a corollary of these rules for vitamin D effects. We have observed only minimal responses to thyroid hormone using both RDE and intact *pit-1* enhancer reporter genes. Thus, whereas RXR/RAR and RXR/VDR heterodimers prefer AGGTCA sites with spacing of 1/2/5 and 3 bp, respectively, the AGTTCA core motif clearly modulates the nature of preferred spacing by VDR/RXR heterodimers to a DR + 4 site.

Pit-1 and RAR synergy as a model for cell-specific retinoic acid effects

RAR has been shown to require other factors, initially referred to as coregulators, for effective function, and RXRs were demonstrated by several independent approaches to function as effective coregulators of the RAR. The *pit-1* enhancer contains a strong, highly unusual RARE. Quite unexpectedly, this motif referred to as PRE requires both Pit-1 and RAR for induction by retinoic acid, providing an initial example of a cell-specific transcription factor-dependent RARE. Analysis of this element has provided evidence that factors other than RXRs can physiologically serve as RAR coregulators. This element contains an AGTTCA half-site 3 bp 5' of a Pit-1-binding site, which appears to be the site of binding of the RAR, because mutation of this sequence abolishes PRE function and binding by RAR. On the basis of the demonstration of polarity of RXR/RAR heterodimers on their sites [Kurokawa et al. 1993]. The divergent upstream motif TGCTCA probably serves as a low-affinity RXR-binding site. This motif is spaced by 1 nucleotide from the RAR AGTTCA core site oriented as a direct repeat. Although the divergent TGCTCA half-site is required for detectable binding of RAR/RXR heterodimers, significant retinoic acid response (20–30% wild-type) remains with mutation of the TGCTCA motif to sequences that abolish RAR/RXR heterodimeric binding. Unexpectedly, we found that on the PRE Pit-1 may permit weak cooperative binding of RAR even in the absence of the RXR-binding element. Therefore, in this case, Pit-1 can be considered to act as a coregulator of RAR, serving a role in *trans*-activation similar to that which previously served to define the RXR as an effective tissue-independent RAR coregulator. Mutation of the putative RXR-binding TGCTCA site to a consensus AGTTCA core binding motif, thereby generating a perfect DR + 1 site, markedly increased the efficacy of RAR/RXR heterodimer and RXR homodimer binding [S. Rhodes and M. Rosenfeld, unpubl.], but significantly decreased the efficacy of the sequence as a RARE. Such elements were actually less effective than mutant sites that entirely eliminated the RXR-binding motif. Thus, the Pit-1-dependent retinoic acid response element is distinct from the DR + 1 motif identified by Durand et al. (1992) in the mouse CRABP II gene that can serve as a RARE. The *pit-1* enhancer has provided an initial, physiological example of a highly atypical RAR element, which is dependent on a tissue-specific factor to enable function of the RAR, presumably in concert with RXR and other, as yet unidentified, factors.

Atypical RAREs are likely to be of widespread biological significance. For example, a RARE located within the complex transcriptional control region of the phosphoenolpyruvate carboxykinase (PEPCK) gene, in which there are no distinct direct repeat or palindromic sequences [Hall et al. 1992], is located within a domain that enables function of adjacent glucocorticoid response elements [for review, see Granner et al. 1991]. In contrast to the *pit-1* PRE that absolutely requires Pit-1 for func-

tion, the PEPCK RARE confers retinoic acid responsiveness when transferred without neighboring transcription factor recognition elements (Lucas et al. 1991). While the Pit-1 dependence of RAR on the *pit-1* PRE is an intriguing mechanism, other distinct molecular mechanisms for synergistic interactions between Pit-1 and other nuclear receptors are likely to operate on other transcription units. Synergistic interactions between two nuclear receptors and between nuclear receptors and other transcription factors have been described for the T3R, estrogen receptor (ER), and the glucocorticoid/progesterone class of receptors (e.g., Schüle et al. 1988). Pit-1 acts synergistically with both ER and T3R on the rat prolactin gene distal enhancer (Day et al. 1990; Simmons et al. 1990), the growth hormone promoter (Schaufele et al. 1992), and placental lactogen gene promoter (Voz et al. 1992), respectively.

The complex regulatory motifs within the *pit-1* enhancer indicate that transcription of the *pit-1* locus is subject to multiple mechanisms of control. The observation that Pit-1 is not required for the early phase of *pit-1* gene activation provides an experimental basis for exploring the mechanisms required for the initial activation of the *pit-1* gene. The linkage of morphogen receptor actions to *pit-1* gene expression provides further evidence for the potential widespread role of morphogen receptors in mammalian organogenesis. The synergistic interactions between RAR and a tissue-specific transcription factor in the *pit-1* enhancer suggests that comparable elements may function in coordinating the effects of morphogen gradients in many organs, dependent on the presence of temporally and spatially restricted transcription factors.

Materials and methods

Transgene construction

A 376-bp mouse *pit-1* promoter DNA fragment was generated by PCR using the sense primer 5'-GTATTTAAGGAGCTAGCAAATGG-3' and the antisense primer 5'-GCCGCTCGAGCAGGACAGCTCTG-3'. The antisense primer extends from +1 to +13 of the mouse *pit-1* gene [assuming the transcription start site of the rat gene (Chen et al. 1990)] and contains an *XhoI* site at the 3' end. To construct -327 *pit-1*/LacZ, *XhoI* linkers were added to the *lacZ* gene (from pSDKLacZpA; kind gift of Drs. J. Rossant and S. Darling, Mt. Sinai Research Institute, Toronto, Canada). This *lacZ* gene lacks the first eight nonessential amino acids and instead contains a eukaryotic translation consensus sequence and the SV40 small t intron and polyadenylation signal at its 3' end. The *lacZ* gene was then ligated into the *XhoI* site at the 3' end of the 376-bp mouse *pit-1* promoter fragment plasmid. To create -327 *pit-1*/hGH, the *lacZ* gene was exchanged with the complete hGH gene from pØGH (Selden et al. 1986).

The 14.8-kb fragment of mouse *pit-1* 5' sequence was prepared from two subcloned fragments of λ clone 61 (Li et al. 1990). A 6-kb *BamHI* fragment, containing 3.5 kb of 5' DNA/exon I and 2.5-kb intron A, was digested at a unique *NheI* site at -327 and at an *XhoI* site in the 3' vector polylinker. The -327 bp promoter *NheI*-*XhoI* fragment described above was ligated into this plasmid. A -3.5-kb to +13-bp fragment was released

with *BamHI*-*XhoI* and ligated to the additional 11 kb of 5' sequence (in pBSKII) cleaved with *BamHI* at -3.2 kb and *XhoI* in the polylinker. The *lacZ* or hGH gene then was cloned into the unique *XhoI* site to generate 14.8 *pit-1*/LacZ and 14.8 *pit-1*/hGH. Transgenes were released for injection by digestion with *NotI*-*ApaI*.

Production and analysis of transgenic animals

Transgenes were separated from vector by electrophoresis and purified using Elutip D columns (Schleicher & Schuell). Pure fragments were injected into the male pronucleus of oocytes collected from mating C57BL/6J $\times$ DBA/2J F₁ as described by Crenshaw et al. (1987). Oocytes then were transferred to the oviducts of pseudopregnant (C57BL/6J $\times$ DBA/2J) F₁ mice. Transgenic mice were identified by hybridization (Palmiter et al. 1982) to a ³²P-labeled 1050-bp internal *PvuII* fragment of the hGH gene or a 5' 880-bp, *NcoI*-*ClaI* fragment of the *lacZ* gene from pSDKLacZpA. Founder mice then were outbred to B₆D₂ to produce F₁ mice necessary to establish a pedigree and maintain the transgenic line by littermate breeding. Expression of hGH in transgenic animals was analyzed using a highly specific hGH immunoradiometric assay (IRMA) (Nichols Institute, San Juan Capistrano, CA). Pituitary and other tissue extracts were prepared as described by Crenshaw et al. (1989). Tissues were dispersed in cold 0.1 M KPO₄ (pH 7.8) using a 3- to 5-sec burst with a polytron. Extracts were kept on ice and subjected to three freeze-thaw cycles, using a dry ice-ethanol bath, and centrifuged. Protein concentrations were determined by the method of Bradford (Bio-Rad). Pituitary hGH assays were performed in duplicate at dilutions from 1:5 to 1:1500 in equine serum matrix (Nichols Institute). hGH in other tissues was assayed at 1:5 dilutions (typically 20 μ l of extract was assayed by IRMA). Four to eleven mice, selected at random from F₁ and F₂ transgenic animals of each pedigree, were tested. Interassay variation of the IRMA is <7%. *lacZ* transgene expression was determined by spectrophotometric measurement of β -D-galactosidase activity using *O*-nitrophenyl- β -D-galactopyranoside (ONPG) as the substrate (Sambrook et al. 1989). Extrapituitary tissue extracts were made as for hGH assay except that the homogenization buffer was 0.25 M Tris (pH 8). Duplicate 80- μ g protein samples were assayed with 2.9 mM ONPG for 30 min at 37°C. Five micrograms or less of pituitary extract (from lines expressing *lacZ* at high levels) was assayed because greater quantities contained β -galactosidase activity beyond the linear range of the standard curve. Nontransgenic littermate pituitary extracts and -327 *pit-1*/LacZ mice were assayed using 30 μ g or more of total protein. The β -galactosidase standard curve was linear between 0.009 and 0.00075 units (1 unit is the amount of enzyme required to hydrolyze 1 μ mole of ONPG in 1 min at 37°C).

Plasmid construction/DNA sequencing/site-directed mutagenesis

Mapped restriction fragments of λ mouse genomic DNA clones 32 and 61 (Li et al. 1990) either were subcloned into pBSKII(-) for further mapping or sequencing for were directly cloned adjacent to the -210 to +34 rat *pit-1* gene mPitB2 promoter/luciferase reporter plasmid (Chen et al. 1990). Enhancer fragments for constructs EP0.5, EP0.39, and EP0.26 were generated using PCR and primers designed to create *HindIII* restriction sites at the 5' end of each fragment. Contiguous mouse *pit-1* gene 5' genomic fragments (14.8, 11.5, and 10.2 kb) were cloned adjacent to the mouse *pit-1* promoter (-327 to +13) fused to the

luciferase reporter. Enhancer fragments also were cloned into the *Bam*HI site of the -109 to +54 herpes simplex virus thymidine kinase promoter/luciferase reporter (pTKSluc; Glass et al. 1989) following ligation to *Bam*HI linkers if required. Synthetic oligonucleotides were made with *Bam*HI- and *Bgl*II-compatible overhangs for cloning and gel retardation and were as follows: RDE sense (5'-gacCCCATGAACCTCTCATGAACCTCTA-3'); mut RDE sense (5'-gacCCCATAGATTCTCGATATCTGTTA-3'); and PRE wild-type sense (5'-gacTAATATGTGCTCAAAGTTCAGGTATGAATATAAATG-3'). The PRE mutant oligonucleotides were identical in sequence to the wild-type sequence, except for the changes given in Fig. 7. Other response elements were as in Yu et al. (1991). Test oligonucleotides were cloned upstream of pTKSluc. Construct integrity was confirmed by restriction analysis and DNA sequencing. Oligonucleotides for mutagenesis were as follows: mut Pit-1 a (5'-cagttttgatattgggtgaccgtgtgtgtttat-3'); mut Pit-1 b (5'-ctaagtcagtcggtgaccattttgagttatagt-3'); mut Pit-1 c (5'-ggacatgccttggtctctgttgagatccagttcgtttgcaa-3'); mut Pit-1 d (5'-ccttatgcgcctcatgcatcaggtgaccgtatgtgtcctaaagtca-3'); mut Pit-1 e (5'-caagtcaggtggtgacctaataagtggt-3'); mut PSE#1 (5'-tgaaccccttaactatagattgtgtgatatcagatgattttc-3'); mut PSE#2 (5'-tcaactgtttgaagaattcatgtttttcgtccta-3'); mut PSE#3 (5'-gaaccccttaactatgattctgtttgaacagatg-3'); mut PSE#4 (5'-taaacctcaactgtgatcatcagatgattttc-3'); mut PSE#5 (5'-ccttaaacctcaactgtctcagacagatgattttc-3'); mut PSE#6 (5'-aaccttaaacctgtactgtttgaacagatcgtttttcgtcc-3'); 0.5 mut#1 (at position 300) (5'-ttttctttta-caagatattccatcatgtttttagg-3'); mut RDE (5'-tattacaaaaatccccatcgttatcgatatctgtttttgtact-3'); mut PRE (5'-aaatgtgtctcaagatagcgtatgaatataat-3'); and W²⁶¹ → C Snell *dw* rat Pit-1 (5'-gtataggtgtcgttttgaac-3'). Mutations were confirmed by sequencing.

Cell culture, DNA transfection, and luciferase assays

In vitro culture and transient transfection of cells was as described previously (Chen et al. 1990; Ingraham et al. 1990b). Typically, 2 μ g of reporter plasmid and 1 μ g of effector plasmid (if any) were added per 60-mm plate. All transfection groups received equal final DNA concentrations [balanced with appropriate vector plasmid or Rous sarcoma virus (RSV)-neo]. Expression vectors have been described previously: RSV-driven human RAR α and RSV-human T3R β (Glass et al. 1989), RSV-human VDR (Liao et al. 1990), and cytomegalovirus (CMV) promoter-driven rat Pit-1 (Chen et al. 1990; Ingraham et al. 1990b). The Δ 8-80 Pit-1 expression vector has been described previously (Ingraham et al. 1990). The CMV-driven W261C Snell rat Pit-1 expression plasmid was constructed by mutagenesis of rat Pit-1 followed by cloning into the pCMV1 (Ingraham et al. 1990) expression vector. Exogenous compounds were added (in charcoal-stripped medium when required) to the following final concentrations: 1,25-dihydroxyvitamin D₃ (0.1 μ M), all-*trans* retinoic acid (Sigma, 0.5 μ M), 9-*cis* retinoic acid (kind gifts from Hoffman LaRoche, Inc., and Ligand, Inc., 0.1 μ M), thyroid hormone (3,3',5-triiodo-L-thyronine, 0.1 μ M), β -estradiol (0.1 μ M), dexamethasone (0.5 μ M), forskolin (20 μ M), and dibutyryl cAMP (1 mM). Control cultures received equal volumes of solvent (either ethanol or water) alone. After 48 hr, cultures were rinsed with ice-cold PBS, lysed directly on the plate in 50 mM Mes, 50 mM Tris (pH 7.8), 1 mM dithiothreitol, and 0.1% Triton X-100. Lysate supernatants were assayed for luciferase activity using a Monolight 2010 luminometer and luciferin substrate in 60 mM Mes/60 mM Tris (pH 7.8), 1.25 mg/ml ATP, and 7.5 mM MgCl₂.

DNA-binding studies

DNase footprinting was performed essentially as described by

Mangalam et al. (1989) and Drolet et al. (1991). Enhancer fragments were generated by PCR using specific oligonucleotide primers, one of which was labeled with ³²P using T4 polynucleotide kinase and [γ -³²P]ATP. PCR products were gel purified by acrylamide gel electrophoresis. Crude nuclear extracts (30 μ g) or bacterially expressed Pit-1 were incubated with 50,000 cpm of DNA probe. Nuclear extracts were prepared from GC and HeLa cell cultures as described by Mangalam et al. (1989). Bacterially expressed Pit-1 protein was prepared as described by Ingraham et al. (1990). Sequencing ladders were generated from each end-labeled probe by the chemical cleavage method (Sambrook et al. 1989).

Gel retardation analysis was as described by Yu et al. (1991). Human RAR α and human RXR α were expressed as glutathione S-transferase fusion proteins in the vector pGEX-KG as described (Guan and Dixon 1991), and were used either as intact fusion proteins or cleaved using thrombin. Oligonucleotides were end labeled with [γ -³²P]ATP using T4 polynucleotide kinase, filled with dNTPs using Klenow fragment, annealed, and gel-purified. Pit-1 antiserum has been described previously (Simmons et al. 1990; Voss et al. 1991). Polyclonal antisera against human RAR α and human RXR α were the kind gifts of Dr. C. Glass (University of California, San Diego).

In situ hybridization

In situ hybridizations were performed essentially as described previously (Simmons et al. 1990; Lin et al. 1992). Briefly, embryos from timed pregnancies of wild-type or Snell mutant mice in ice-cold PBS were fixed in a 4% paraformaldehyde solution and embedded in OCT. Embryos were serially sectioned at 10- μ m intervals in a sagittal orientation and hybridized to ³⁵S-labeled Pit-1 cRNA probes (Simmons et al. 1990). After exposure to emulsion (NTB-2), slides were developed and stained with the fluorescent dye bis-benzimide (Sigma). Dark-field photomicroscopy was performed with an ultraviolet light using a Nikon microscope.

Acknowledgments

We are grateful to C. Nelson for expert cell culture. We thank Drs. S. Li, B. Andersen, J. DiRenzo, D. Drolet, S. Flynn, C. Glass, H. Mangalam, J. Rossant, J. Voss, and M. Wegner for reagents and useful discussion. We thank Susan Martin for valuable help in preparation of this paper. S.J.R. is a postdoctoral fellow of the American Cancer Society, G.E.D. is supported by a fellowship from the Medical Research Council of Canada, V.C.Y. is a Leukemia Society of America Postdoctoral Fellow. M.G.R. is an Investigator with the Howard Hughes Medical Institute. This work was supported in part by National Institutes of Health grant DK18477-18 to M.G.R.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Atchison, M.L. 1988. Enhancers: mechanisms of action and cell specificity. *Annu. Rev. Cell Biol.* 4: 127-153.
- Beebe, J.S., D.S. Darling, and W.W. Chin. 1991. 3,5,3'-triiodothyronine receptor auxiliary protein (TRAP) enhances receptor binding by interactions within the thyroid hormone response element. *Mol. Endocrinol.* 5: 85-93.
- Blackwell, T.K. and H. Weintraub. 1990. Differences and simi-

- larities in DNA-binding preferences of Myod and E2A protein complexes revealed by binding site selection. *Science* **250**: 1104–1110.
- Bodner, M. and M. Karin. 1987. A pituitary-specific *trans*-acting factor can stimulate transcription from the growth hormone promoter in extracts of non-expressing cells. *Cell* **50**: 267–275.
- Bodner, M., J.-L. Castrillo, L.E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* **55**: 505–518.
- Boncinelli, E., A. Simeone, D. Acampora, and F. Mavilio. 1991. HOX gene activation by retinoic acid. *Trends Genet.* **7**: 218–223.
- Bugge, T.H., J. Pohl, O. Lonnoy, and H.G. Stunnenberg. 1992. RXR α , a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J.* **11**: 1409–1418.
- Burnside, J., D.S. Darling, and W.W. Chin. 1990. A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. *J. Biol. Chem.* **265**: 2500–2504.
- Cao, Z., E.A. Barron, A. Carillo, and Z.D. Sharp. 1987. Reconstitution of cell-type-specific transcription of the rat prolactin gene in vitro. *Mol. Cell. Biol.* **7**: 3402–3408.
- Chen, R., H.A. Ingraham, M.N. Treacy, V.R. Albert, L. Wilson, and M.G. Rosenfeld. 1990. Autoregulation of *pit-1* gene expression mediated by two *cis*-active promoter elements. *Nature* **346**: 583–586.
- Crenshaw, E.B. III, A.F. Russo, L.M. Swanson, and M.G. Rosenfeld. 1987. Neuron-specific alternative RNA processing in transgenic mice expressing a metallothionein-calcitonin fusion gene. *Cell* **49**: 389–398.
- Crenshaw, E.B. III, K. Kalla, D.M. Simmons, L.W. Swanson, and M.G. Rosenfeld. 1989. Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements. *Genes & Dev.* **3**: 959–972.
- Darling, D.S., J.S. Beebe, J. Burnside, E.R. Winslow, and W.W. Chin. 1991. 3,5,3'-triiodothyronine (thyroid hormone receptor-auxiliary protein (TRAP) binds DNA and forms heterodimers with the thyroid hormone receptor. *Mol. Endocrinol.* **5**: 73–84.
- Day, R.N., S. Koike, M. Sakai, M. Muramatsu, and R.A. Maurer. 1990. Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. *Mol. Endocrinol.* **90**: 1964–1971.
- De Luca, H.F., J. Krisinger, and H. Darwish. 1990. The vitamin D system: 1990. *Kidney Int.* **38**: S2.
- De Luca, L.M. 1991. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J.* **5**: 2924–2933.
- Dolle, P., J.-L. Castrillo, L.E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1990. Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. *Cell* **60**: 809–820.
- Drolet, D., K. Scully, D.M. Simmons, L.W. Swanson, and M.G. Rosenfeld. 1991. TEF, a transcription factor expressed specifically in the anterior pituitary during embryogenesis, defines a new class of leucine zipper proteins. *Genes & Dev.* **5**: 1739–1753.
- Dubois, P.M. and F.J. Hemming. 1991. Fetal development and regulation of pituitary cell types. *J. Electron Microsc. Techniques* **19**: 2–20.
- Durand, B., M. Saunders, P. Leroy, M. Leid, and P. Chambon. 1992. All-trans and 9-cis retinoic acid induction of CRABP II transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. *Cell* **71**: 73–85.
- Evans, R.M. 1988. Steroid and thyroid hormone receptors as transcriptional regulators of development and physiology. *Science* **240**: 889–895.
- Forman, B.M., C. Yang, M. Au, J. Casanova, J. Ghysdael, and H.H. Samuels. 1989. A domain containing leucine-zipper-like motifs mediates novel *in vivo* interactions between the thyroid hormone and retinoic acid receptors. *Mol. Endocrinol.* **3**: 1610–1626.
- Glass, C.K., J.M. Holloway, O.V. Devary, and M.G. Rosenfeld. 1988. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell* **54**: 313–323.
- Glass, C.K., S.M. Lipkin, O.V. Devary, and M.G. Rosenfeld. 1989. Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. *Cell* **59**: 697–708.
- Glass, C.K., O.V. Devary, and M.G. Rosenfeld. 1990. Multiple cell type-specific proteins differentially regulate target sequence recognition by the α retinoic acid receptor. *Cell* **63**: 729–738.
- Granner, D., R. O'Brien, E. Imai, C. Forest, J. Mitchell, and P. Lucas. 1991. Complex hormone response unit regulating transcription of the phosphoenolpyruvate carboxykinase gene: From metabolic pathways to molecular biology. *Recent Prog. Horm. Res.* **47**: 319–348.
- Green, S. and P. Chambon. 1988. Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet.* **4**: 309–314.
- Guan, K.-L. and J.E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**: 262–267.
- Gunzburg, W.H., B. Salmons, B. Zimmerman, M. Muller, V. Erfle, and G. Brem. 1991. A mammary-specific promoter directs expression of growth hormone not only to the mammary gland, but also to Bergman glia cells in transgenic mice. *Mol. Endocrinol.* **5**: 123–133.
- Gutierrez-Hartmann, A., S. Siddiqui, and S. Loukin. 1987. Selective transcription and DNase I protection of the rat prolactin gene by GH₃ pituitary cell-free extracts. *Proc. Natl. Acad. Sci.* **84**: 5211–5215.
- Hall, R.K., D.K. Scott, E.L. Nousin, P.C. Lucas, and D.K. Granner. 1992. Activation of the phosphoenolpyruvate carboxykinase gene retinoic acid element is dependent on a retinoic acid receptor/coregulator complex. *Mol. Cell. Biol.* **12**: 5527–5535.
- Harding, K., T. Hoey, R. Warrior, and M. Levine. 1989. Autoregulatory and gap gene response elements of the *even-skipped* promoter of *Drosophila*. *EMBO J.* **8**: 1205–1212.
- Haussler, M.R., S.C. Manolagas, and L.J. Deftos. 1982. Receptor for 1,25-dihydroxyvitamin D₃ in GH₃ pituitary cells. *J. Steroid Biochem.* **16**: 15–19.
- Heyman, R.A., D.J. Mangelsdorf, J.A. Dyck, R.B. Stein, G. Eichele, R.M. Evans, and C. Thaller. 1992. 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**: 397–406.
- Hiromi, Y. and W.J. Gehring. 1987. Regulation and function of the *Drosophila* segmentation gene *fushi tarazu*. *Cell* **50**: 963–974.
- Holick, M.F. 1991. Photosynthesis, metabolism, and biologic actions of vitamin D. In "Rickets," Nestlé Nutrition Workshop Series (ed. F.H. Glorieux), vol. 21. Vevey/Raven Press Ltd., New York.
- Ingraham, H.A., R. Chen, H.J. Mangalam, H.P. Elsholtz, S.E. Flynn, C.R. Lin, D.M. Simmons, L.W. Swanson, and M.G.

- Rosenfeld. 1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* 55: 519–529.
- Ingraham, H.A., S.E. Flynn, J.W. Voss, V.R. Albert, M.S. Kapiloff, L. Wilson, and M.G. Rosenfeld. 1990. The POU-specific domain of Pit-1 is essential for sequence-specific, high-affinity DNA-binding and DNA-independent Pit-1-Pit-1 interactions. *Cell* 61: 1021–1033.
- Iverson, R.A., K.H. Day, M. d'Emden, R.N. Day, and R.A. Maurer. 1990. Clustered point mutational analysis of the rat prolactin promoter. *Mol. Endocrinol.* 4: 1564–1571.
- Kliwer, S.A., K. Umeson, D.J. Mangelsdorf, and R.M. Evans. 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D₃ signalling. *Nature* 355: 446–449.
- Kurokawa, R., V. Yu, A. Näär, S. Kyakumoto, Z. Han, S. Silverman, M.G. Rosenfeld, and C.K. Glass. 1993. Differential orientations of the DNA-binding domain and carboxy-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. *Genes & Dev.* (in press).
- Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon. 1992. Purification, cloning and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 68: 377–395.
- Levin, A.A., L.J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovey, and J.F. Grippo. 1992. 9-*cis* retinoic acid stereoisomer binds and activates the nuclear receptor RXR α . *Nature* 355: 359–361.
- Li, S., E.B. Crenshaw III, E.J. Rawson, D.M. Simmons, L.W. Swanson, and M.G. Rosenfeld. 1990. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene *pit-1*. *Nature* 347: 528–533.
- Liao, J., K. Ozono, T. Sone, D.P. MacDonnell, and J.W. Pike. 1990. Vitamin D receptor interaction with specific DNA requires a nuclear protein and 1,25-dihydroxyvitamin D₃. *Proc. Natl. Acad. Sci.* 87: 9781–9788.
- Lin, C., S.-C. Lin, C.-P. Chang, and M.G. Rosenfeld. 1992. Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. *Nature* 360: 765–768.
- Lira, S.A., E.B. Crenshaw III, C.K. Glass, L.W. Swanson, and M.G. Rosenfeld. 1988. Identification of rat growth hormone genomic sequences targeting pituitary expression in transgenic mice. *Proc. Natl. Acad. Sci.* 85: 4755–4759.
- Lucas, P.C., R.M. O'Brien, J.A. Mitchell, C.M. Davis, E. Imai, B.M. Forman, H.H. Samuels, and D.K. Granner. 1991. A retinoic acid response element is part of a pleiotropic domain in the phosphoenolpyruvate carboxykinase gene. *Proc. Natl. Acad. Sci.* 88: 2184–2188.
- Lufkin, T. and C. Bancroft. 1987. Identification by cell fusion of gene sequences that interact with positive *trans*-acting factors. *Science* 237: 283–286.
- Mangalam, H.J., V.R. Albert, H.A. Ingraham, M.S. Kapiloff, L. Wilson, C. Nelson, H. Elsholtz, and M.G. Rosenfeld. 1989. A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. *Genes & Dev.* 3: 946–958.
- Mangelsdorf, D.J., K. Umeson, S.A. Kliwer, U. Borgmeyer, E.S. Ong, and R.M. Evans. 1991. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* 66: 555–561.
- Mangelsdorf, D.J., U. Borgmeyer, R.A. Heyman, J. Yang Zhou, E.S. Ong, A.E. Oro, A. Kakizuka, and R.M. Evans. 1992. Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes & Dev.* 6: 329–344.
- Marks, M.S., P.L. Hallenbeck, T. Nagata, J.H. Segars, E. Appella, V.M. Nikodem, and K. Ozato. 1992. H-2RIIBP (RXR β) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. *EMBO J.* 11: 1419–1435.
- McCormick, A., H. Brady, L.E. Theill, and M. Karin. 1990. Regulation of the pituitary-specific homeobox gene *GHF1* by cell-autonomous and environmental cues. *Nature* 345: 829–832.
- McCormick, A., H. Brady, J. Fukushima, and M. Karin. 1991. The pituitary regulatory gene *GHF1* contains a minimal cell-type promoter centered around its TATA box. *Genes & Dev.* 5: 1490–1503.
- Murdoch, G.H. and M.G. Rosenfeld. 1981. Regulation of pituitary function and prolactin production in the GH₄ cell line by vitamin D. *J. Biol. Chem.* 256: 4050–4055.
- Näär, A., J.M. Boutin, S.M. Lipkin, V.C. Yu, J.M. Holloway, C.K. Glass, and M.G. Rosenfeld. 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* 65: 1267–1279.
- Nelson, C., E.B. Crenshaw III, R. Franco, S.A. Lira, V.R. Albert, R.M. Evans, and M.G. Rosenfeld. 1986. Discrete *cis*-acting genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone genes. *Nature* 322: 557–562.
- Nelson, C., V.R. Albert, H.P. Elsholtz, L.I.-W. Lu, and M.G. Rosenfeld. 1988. Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. *Science* 239: 1400–1405.
- O'Donnell, A.L., E.D. Rosen, D.S. Darling, and R.J. Koenig. 1991. Thyroid hormone receptor mutations that interfere with transcriptional activation also interfere with receptor interaction with a nuclear protein. *Mol. Endocrinol.* 5: 94–99.
- O'Malley, B. 1990. The steroid receptor superfamily: More excitement predicted for the future. *Mol. Endocrinol.* 4: 363–369.
- Ozono, K., J. Liao, S.A. Lerner, R.A. Scott, and J.W. Pike. 1990. The vitamin D responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer. *J. Biol. Chem.* 265: 21881–21888.
- Palmiter, R.D., H.Y. Chen, and R.L. Brinster. 1982. Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. *Cell* 29: 701–710.
- Pike, J.W. 1991. Vitamin D₃ receptors: Structure and function in transcription. *Annu. Rev. Nutr.* 11: 189–216.
- Pfaffle, R.W., G.E. DiMattia, J.S. Parks, M.R. Brown, J.M. Wit, J. Jansen, H. Van der Nat, J.L. Van den Brande, M.G. Rosenfeld, and H.A. Ingraham. 1992. Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia. *Science* 257: 1118–1121.
- Radovick, S., M. Nations, Y. Du, L.A. Berg, B. Weintraub, and F.E. Wondisford. 1992. A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. *Science* 257: 1115–1118.
- Regulski, M., S. Dessain, N. McGinnis, and W. McGinnis. 1991. High-affinity binding sites for the Deformed protein are required for the function of an autoregulatory enhancer of the *Deformed* gene. *Genes & Dev.* 5: 278–286.
- Rosenfeld, M.G. 1991. POU-domain transcription factors: powerful developmental regulators. *Genes & Dev.* 5: 897–907.
- Russo, A.F., E.B. Crenshaw III, S.A. Lira, D.M. Simmons, L.W.

- Swanson, and M.G. Rosenfeld. 1988. Neuronal expression of chimeric genes in transgenic mice. *Neuron* **1**: 311–320.
- Ruvkun, G. and M. Finney. 1991. Regulation of transcription and cell identity by POU domain proteins. *Cell* **64**: 475–478.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Schaufele, F., B.L. West, and J.D. Baxter. 1992. Synergistic activation of the rat growth hormone promoter by Pit-1 and the thyroid hormone receptor. *Mol. Endocrinol.* **6**: 656–665.
- Schüle, R., M. Muller, C. Kaltschmidt, and R. Renkawitz. 1988. Many transcription factors interact synergistically with steroid receptors. *Science* **242**: 1418–1420.
- Schwind, J.L. 1928. The development of the hypophysis cerebri of the albino rat. *Am. J. Anat.* **41**: 295–319.
- Selden, R.F., K.B. Howie, M.E. Rowe, H.M. Goodman, D.D. Moore. 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. *Mol. Cell. Biol.* **6**: 3171–3179.
- Simmons, D.M., J.W. Voss, H.A. Ingraham, J.M. Holloway, R.S. Broide, M.G. Rosenfeld, and L.W. Swanson. 1990. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other transcription factors. *Genes & Dev.* **4**: 695–711.
- Snell, G.D. 1929. Dwarf, a new mendelian recessive character of the house mouse. *Proc. Natl. Acad. Sci.* **15**: 733–734.
- Stumpf, W.E., M. Sar, and L.P. O'Brien. 1987. Vitamin D sites of action in the pituitary studied by combined autoradiography-immunochemistry. *Histochemistry* **88**: 11–16.
- Tabin, C.J. 1991. Retinoids, homeoboxes, and growth factors: Toward molecular models for limb development. *Cell* **66**: 199–217.
- Tatsumi, K., K. Miyai, T. Notomi, K. Kaibe, N. Amino, Y. Mizuno, and H. Kohno. 1992. Cretinism with combined hormone deficiency caused by a mutation in the *PIT1* gene. *Nat. Genet.* **1**: 56–58.
- Thayer, M.J., S.J. Tapscott, R.L. Davis, W.E. Wright, A.B. Lassar, and H. Weintraub. 1989. Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58**: 241–248.
- Törnquist, K., L. Forss, and Lamberg-Allardt. 1990. Effects of vitamin D₃ metabolites on thyrotropin secretion from rat pituitary cells in culture. *J. Endocrinol. Invest.* **13**: 391–395.
- Umesono, K., V. Giguere, C.K. Glass, M.G. Rosenfeld, and R.M. Evans. 1988. Retinoic acid and thyroid hormone induce gene expression through a common response element. *Nature* **336**: 262–265.
- Umesono, K., K.K. Murakami, C.C. Thompson, and R.M. Evans. 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D receptors. *Cell* **65**: 1255–1266.
- Voss, J.W. and M.G. Rosenfeld. 1992. Anterior pituitary development: short tales from dwarf mice. *Cell* **70**: 527–530.
- Voss, J.W., L. Wilson, and M.G. Rosenfeld. 1991. POU-domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter. *Genes & Dev.* **5**: 1309–1320.
- Voz, M.L., B. Peers, M.J. Weidig, P. Jacquemin, A. Belayew, and J.A. Martial. 1992. Transcriptional regulation by triiodothyronine requires synergistic action of the thyroid receptor with another *trans*-acting factor. *Mol. Cell. Biol.* **12**: 3991–3997.
- Walters, M.R. 1992. Newly identified actions of the vitamin D endocrine system. *Endocr. Rev.* **13**: 719–761.
- Wark, J.D. and V. Gurtler. 1988. Vitamin D-induction of secretory responses in rat pituitary tumour (GH₄C₁) cells. *J. Endocrinol.* **117**: 293–298.
- Ye, Z.S. and H.H. Samuels. 1987. Cell- and sequence-specific binding of nuclear proteins to 5'-flanking DNA of the rat growth hormone gene. *J. Biol. Chem.* **262**: 6313–6317.
- Yu, V.C., C. Delsert, B. Anderson, J.M. Holloway, O.V. Devary, A.M. Näär, S.Y. Kim, J.M. Boutin, C.K. Glass, and M.G. Rosenfeld. 1991. RXR β : A coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements. *Cell* **67**: 1251–1266.
- Zhang, X.-K., B. Hoffman, P.B.-V. Tran, G. Graupner, and M. Pfahl. 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* **355**: 441–445.

A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the pit-1 gene.

S J Rhodes, R Chen, G E DiMattia, et al.

Genes Dev. 1993, **7**:

Access the most recent version at doi:[10.1101/gad.7.6.913](https://doi.org/10.1101/gad.7.6.913)

References

This article cites 86 articles, 27 of which can be accessed free at:
<http://genesdev.cshlp.org/content/7/6/913.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).