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56. The project described here represents the efforts of a relatively small group of faculty, postdoctoral researchers, and graduate students in the Physics Department at Columbia: A. E. Terrano (now at Rutgers University) and K. Barad (at Barnard College), H.-Q. Ding (now at Caltech), and F. P. Butler, H. Chen, Y. F. Deng, M. S. Gao, P. F. Hsieh, H. Shi (in the Electrical Engineering Department), L. I. Unger, and T. J. Woch in addition to the authors. We thank the Intel Corporation and the U.S. Department of Energy for their support.

Research Articles

Activation of Cell-Specific Expression of Rat Growth Hormone and Prolactin Genes by a Common Transcription Factor

CHRISTIAN NELSON, VIVIAN R. ALBERT, HARRY P. ELSHOLTZ, LESLIE I.-W. LU, MICHAEL G. ROSENFELD

In the anterior pituitary gland, there are five phenotypically distinct cell types, including cells that produce either prolactin (lactotrophs) or growth hormone (somatotrophs). Multiple, related cis-active elements that exhibit synergistic interactions appear to be the critical determinants of the transcriptional activation of the rat prolactin and growth hormone genes. A common positive tissue-specific transcription factor, referred to as Pit-1, appears to bind to all the cell-specific elements in each gene and to be required for the activation of both the prolactin and growth hormone genes. The data suggest that, in the course of development, a single tissue-specific factor activates sets of genes that ultimately exhibit restricted cell-specific expression and define cellular phenotype.

EUKARYOTIC GENES ARE TRANSCRIPTIONALLY REGULATED by protein factors that bind cis-acting promoter and enhancer elements (*1*), some of which exert their actions in a tissue-specific manner (*2*). During the developmental program of organogenesis, there is a serial appearance of phenotypically distinct cell types that exhibit selective patterns of gene expression. Understanding the mechanisms determining the sequential activation of these differentiated states requires the elucidation of factors governing the cell type–specific expression of genes. The expression of two evolutionarily related genes, prolactin and growth hormone (GH), in two phenotypically distinct cell types (lactotrophs and somatotrophs, respectively) of the anterior pituitary gland (*3*) provides a model system for the analysis of cell type–specific gene expression within an organ. During pituitary development the appearance of somatotrophs temporally precedes that of lactotrophs (*4*). The transient coexpression of growth hormone in more than 95 percent of prolactin-producing cells before the appearance of mature lactotrophs (*4*) raises the possibility that these two genes may share a common developmental signal for activation. We now provide evidence that a common tissue-specific transcription factor is required for activation of these two genes expressed in phenotypically distinct cell types.

A common cell-specific factor binds to the prolactin and growth hormone enhancer elements. Tissue-specific enhancers in the 5′ flanking regions of both the prolactin and growth hormone genes appear to dictate their pituitary-specific expression (*5*). We have used deletion mapping and protection from digestion by deoxyribonuclease I (DNase 1) by binding of nuclear proteins (DNase I footprinting analysis) to identify prolactin enhancer

The authors are in the Eukaryotic Regulatory Biology Program, Center for Molecular Genetics, University of California, San Diego, School of Medicine, San Diego, CA 92093. In addition, H. P. Elsholtz and M. G. Rosenfeld are in the Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, and C. Nelson is a graduate student in the Department of Biology, University of California, San Diego.

elements critical for cell-specific expression. Prolactin 5′ flanking deletions linked to the luciferase reporter gene were assayed for expression in a rat pituitary cell line (GC); this line expresses both prolactin and growth hormone. Accurate transcriptional initiation from these promoters has been documented (*5*). On the basis of deletional analyses (Fig. 1A), we suggest that cell-specific transcription of the rat prolactin gene depends on a distal enhancer segment (−1830 to −1530), accounting for 99 percent of activity, and a proximal region (−422 to −36), accounting for 1 to 2 percent of maximal activity. This proximal region contains several regulatory sequences (*6*), consistent with evidence that has suggested a transcriptional role for the prolactin proximal region (*5, 7*). Neither of the prolactin enhancer regions were active in HeLa or rat fibroblast cell lines (Fig. 1C) or in a series of other cell lines (*5*), confirming tissue-specific transcriptional function for both elements.

The DNase I footprint analysis of the prolactin enhancer regions with crude or fractionated GC nuclear extracts revealed four protected regions in both the distal enhancer (sites 1D to 4D) and in the proximal region (1P to 4P), none of which were observed with extracts prepared from HeLa or 208F fibroblast cell lines (Fig. 1B). The HeLa and 208F nuclear extracts exhibited the expected pattern of DNase I protection of the SV40 early promotor (*1*). Analysis of the proximal region with concentrated extract suggested an additional protected region having the boundaries −110 to −82, while no further clear footprints were observed in the region extending from −200 to −350 (Fig. 1B). The transcriptional effects of the distal enhancer binding proteins identified by footprint analysis were examined by deletion of each binding site (Fig. 1D). Deletion of site 1D, 2D, or 4D resulted in loss of most of the distal enhancer transcriptional activity, an indication of the importance of interac-

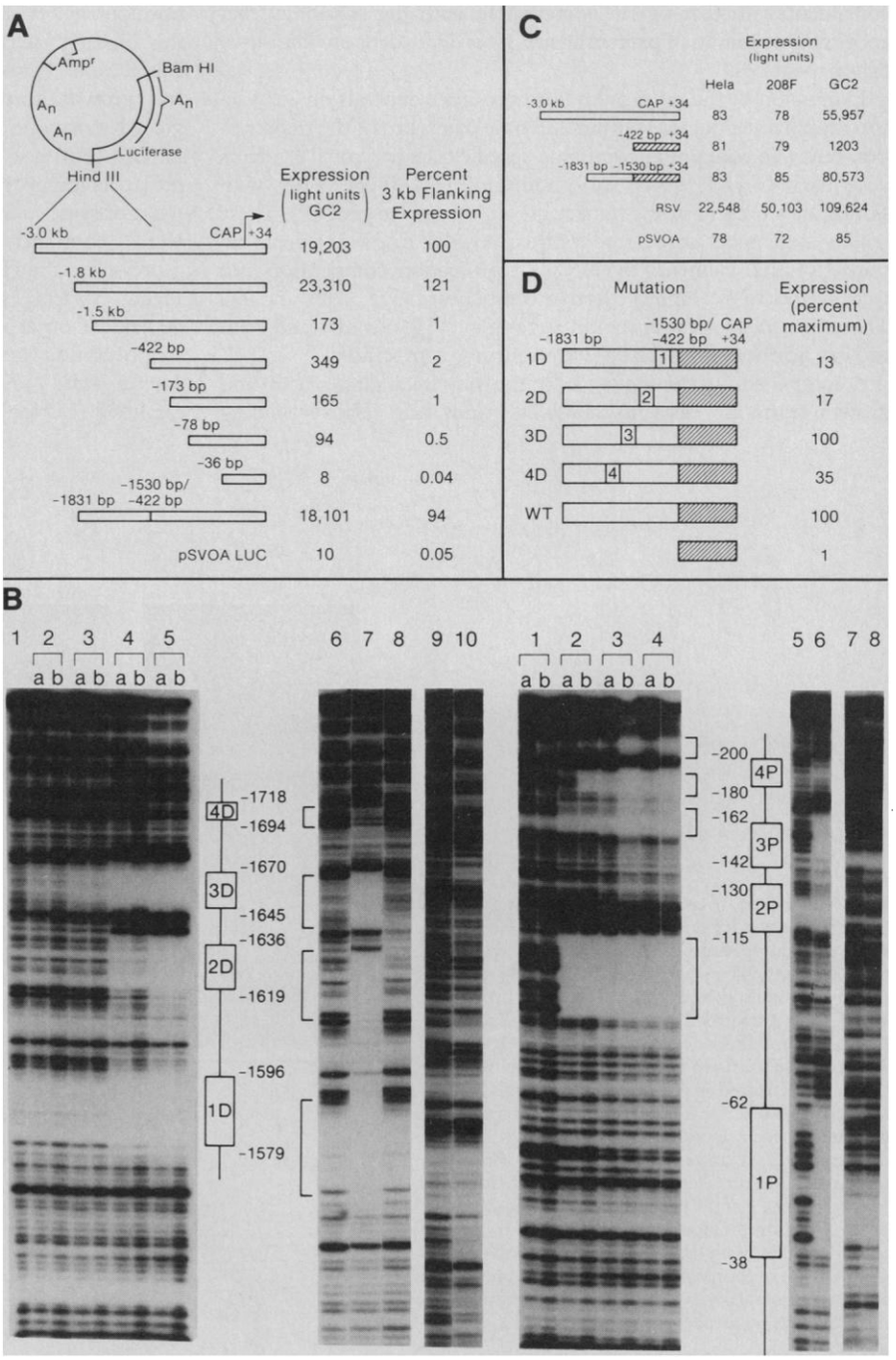

Fig. 1. Identification of cell-specific cis-active transcriptional regulatory elements in the 5′ flanking region of the rat prolactin (Prl) gene. (**A**) Mapping of enhancer elements. Fragments of the 5′ flanking region of the Prl gene were fused to a luciferase gene and transfected into GC cells (*18*). Results are the average of duplicate determinations differing by less than 10 percent minus mock-transfected values. Similar results were obtained in three separate experiments; pSVOA luciferase is identical to the test fusions but lacks a promoter or enhancer 5′ of the luciferase gene (*18*). (**B**) Footprint analysis of the rPRL distal and proximal regulatory domains (*19*). (Left) Analysis of the rPRL distal enhancer showing specific binding activity present in nuclear extracts. (Lanes 1 to 5) Approximately 1 ng of 5′ end-labeled antisense strand probe (*19*) was incubated with (lane 1) 0 μg; (lane 2) 5 μg; (lane 3) 10 μg; (lane 4) 20 μg; or (lane 5) 40 μg of phosphocellulose-fractionated GC extract (0.1 to 0.3*M* KCl fraction) at two different concentrations (a and b) of DNase I. (Lanes 6 to 10) End-labeled antisense strand probe (1 ng) was incubated with nuclear extract prepared from: (lane 7) GC cells (100 μg); (lane 8) HeLa cells (150 μg); or (lane 10) 208F cells (150 μg) and DNase I footprinted. Lanes 6 and 9 contain no protein. (Right) Analysis of the rPRL proximal regulatory region with GC nuclear extracts. (Lanes 1 to 4) 5′ End-labeled antisense strand probe (1 ng) covering the region −248 to +34 bp was incubated with (lanes 1 and 5) 0 μg; (lane 2) 20 μg; (lane 3) 50 μg; or (lanes 4 and 6) 100 μg of phosphocellulose fractionated extract at two different concentrations (a and b) of DNase I; (lanes 7 and 8) 1 ng of 5′ end-labeled sense strand probe covering the region −422 to −173 bp (*19*) incubated in the absence (lane 7) or presence (lane 8) of 100 μg of GC phosphocellulose-fractionated extract. (**C**) Fusion genes transfected into the GC, HeLa, and 208F cell lines. A plasmid containing the RSV promoter and enhancer 5′ of the luciferase reporter molecule was included to verify transfection of all cell lines (*18*). Results shown are the average of duplicate determinations differing by less than 10 percent. Similar results were obtained in two additional experiments. (**D**) Transcriptional effects of distal enhancer element site deletions, created by "loop out" M13 mutagenesis (*20*), and examined for changes in transcriptional enhancement of a 422-bp 5′ flanking Prl DNA luciferase reporter fusion. Values shown are the average of duplicate determinations differing by less than 10 percent, minus mock-transfected values. Similar results were obtained in three separate experiments.

tions between elements. The failure of site 3D to demonstrate any change in transcriptional function (Fig. 1D) may be due to helical position, redundancy of function, or alteration of spacing.

The relation between the multiple elements critical for the cell-specific expression of the rat prolactin gene was investigated by DNase I footprint competition. Double-stranded oligonucleotides including elements in the prolactin distal enhancer and proximal enhancer were tested for their ability to compete for binding to each prolactin enhancer element in a DNase I protection assay (Fig. 2, A and B). All prolactin sites specifically and successfully competed for binding to prolactin enhancer elements, but with different affinities, suggesting that all sites bound a common factor. Although the two distal elements (2D and 4D) did not compete for binding at the concentrations used in this experiment, specific competition was observed at higher oligonucleotide concentrations (*8*). The failure of site 2D to compete efficiently by itself although its footprint appears coincident with that of 1D is consistent with the possibility that cooperative binding of protein at site 2D is dependent on binding of factor to site 1D.

Expression of the rat growth hormone gene depends on a tissue-specific enhancer located within 235 base pairs (bp) of the transcription start (*5*), containing two tissue-specific transcriptional elements designated GH1 (−99 to −69) and GH2 (−140 to −110), with similar sites present in the human growth hormone gene (*9*). These enhancer elements were tested for their possible relation to prolactin transcriptional elements by DNase I protection competition and were found to be highly effective competitors (Fig. 2, A and B). Thus, two of the sites in the prolactin gene (1P and 3D) and both growth hormone sites 1 and 2 were strong competitors.

A comparison of the sequences of the individual rat prolactin and growth hormone elements (Fig. 2C) and their relative binding affinities suggested a consensus sequence with a core of A $(\frac{A}{T})(\frac{A}{T})$ TATNCAT. To examine the specificity of this sequence, we constructed a mutation that altered 6 bp of the prolactin site 1P element, resulting in a change of four bases of core consensus sequence (Fig. 2C). This oligonucleotide (1P MUT), which retained most of the extensive AT-rich sequence of site 1P, failed to compete with any of the distal site footprint sequences for binding (Fig. 2A). Because the consensus sequence is potentially related to the TATAA sequence, oligonucleotides encompassing the prolactin, growth hormone, and other TATAA regions, as well as the thyroid hormone (T_3) response region of the rat growth hormone gene, were tested. These all failed to compete for binding.

A common cell-specific factor transcriptionally activates prolactin and growth hormone elements in vitro. The above data suggested that the same protein or family of related proteins binds to the critical regulatory elements of both the prolactin and growth hormone genes. To further confirm the transcriptional function of this binding factor, we performed in vitro transcription analyses. Initial experiments established tissue-specific expression of prolactin and growth hormone chimeric genes (Fig. 3A). Prolactin and growth hormone fusion genes demonstrated efficient transcription in GC (but not in HeLa) extracts (Fig. 3A). Deletion of the prolactin and growth hormone cell-specific enhancers abolished this tissue-specific transcription. In contrast, a Rous sarcoma virus (RSV) construct, used as a control, was actually more efficiently expressed in the HeLa than in the GC extracts. Complementation of HeLa extracts established that the cell-specific expression was dependent on a positive, GC transcription factor (*10*). Each transcription unit generated α-amanitin–sensitive transcripts of the correct size, indicating accurate initiation in a polymerase II–catalyzed reaction (Fig. 3A).

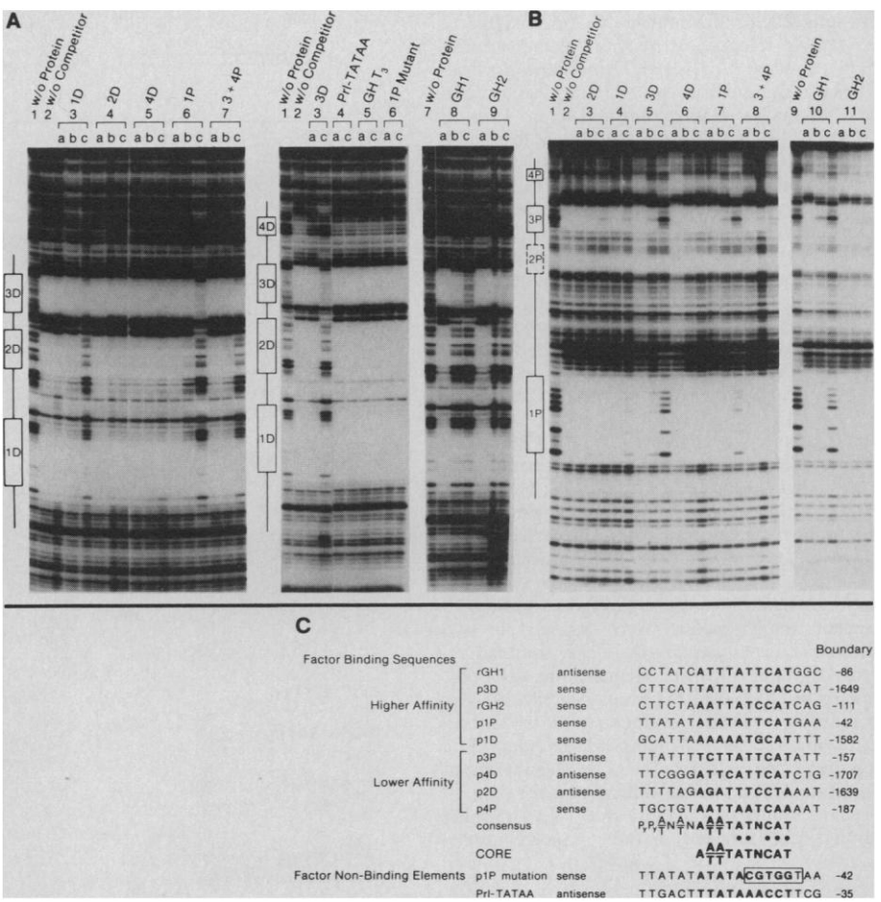

Fig. 2. Evidence of common factor binding sites in the rPRL and rGH enhancers by DNase I competition. (**A**) Competition analysis of the rPRL distal enhancer with oligonucleotides comprising individual factor binding sites (*21*). DNase I protection analysis was performed with 20 μg of a 0.1 to 0.3*M* KCl phosphocellulose fraction of GC nuclear extract. Approximately 1 ng of 5′ end-labeled antisense probe (*19*) was incubated in 20 μg of extract with (a) 50-fold, (b) 250-fold, or (c) 750-fold molar excess of the indicated oligonucleotides, then subjected to DNase I digestion. (**B**) Competition analysis of the rPRL proximal enhancer. Reaction conditions are identical to (A). (Lanes 1 and 9) no protein; (lane 2) 20 μg of protein without competitor; and (lanes 3 to 11) 20 μg of protein with the indicated competitor. (**C**) Comparison of footprinted sites and sequences tested in competition analysis showing the consensus binding sequence. Sequences are listed in order of decreasing affinity.

Further analyses suggested that individual cell-specific prolactin and growth hormone elements were functional in vitro. Thus, serial deletions of both the prolactin proximal (−172, −78, −36) and growth hormone enhancer regions (−235, −180, −110, −39) resulted in a progressive decrease in transcription (Fig. 3B), consistent with removal of cell-specific elements defined by DNase I protection. The distal prolactin enhancer was more effective when placed immediately proximal to the transcription initiation site (at −36 bp) than at −172 bp (Fig. 3B). The distance-dependent activity of the prolactin enhancer is consistent with the attenuation effects seen in vitro when enhancers are placed at increasing distance from the transcription start site (*11*).

We used competition experiments to further test the possibility that a common factor is responsible for the transcriptional activation of both the prolactin and growth hormone genes. For specificity of competition, we evaluated the effects of a single cell-specific element (1P) on the transcription of prolactin and RSV fusion genes. Competition of the 1P oligonucleotide inhibited transcription of a prolactin proximal enhancer–luciferase fusion gene at a tenfold molar excess (Fig. 3C). Transcription from the RSV promoter was unaffected at 100-fold molar excess of the oligonucleotide.

Competition experiments with various prolactin and growth hormone transcription units and oligonucleotides representing the cis-active growth hormone and prolactin elements are shown in Fig. 3D. Transcriptional efficiency of the site 1P containing prolactin promoter was compromised to various extents in the presence of each individual prolactin and growth hormone cell-specific site, with the exception of the low affinity prolactin distal site 2D (Fig. 3D). When the transcriptional effects of distal prolactin enhancer sequences were examined, an identical pattern of competition was observed. When a growth hormone promoter containing a single cell-specific element was used as a template, the oligonucleotides again exhibited efficiencies of transcriptional competition similar to those observed with the prolactin transcription unit (Fig. 3D). The ability of the various sequences to compete in the in vitro transcription assay correlates well with the assignments of relative binding affinity determined by footprint competition analyses and their importance in transfectional analysis (Fig. 4A).

The prolactin and growth hormone TATAA box sequences and the T_3 regulatory region of the growth hormone gene failed to compete in transcription (Fig. 3D). Thus competition is sequence-specific and does not appear to be due to interference with TATAA box binding factors. Most significantly, the cell-specific element mutated in the consensus sequence (1P MUT) failed to compete enhancer-dependent transcription (Fig. 3D).

From these in vitro transcription analyses, we conclude that the protein that binds to the consensus sequence of all tissue-specific elements of the prolactin and growth hormone genes subserves a transcriptional function. To further test this hypothesis, we used sequence-specific affinity chromatography and effected the purification of the protein binding to the high affinity prolactin element 1P by a factor of 10,000 (*12*). Complementation of HeLa extract with affinity-purified material (<1 ng) stimulated transcription specifically from both prolactin and growth hormone fusion genes, whereas no stimulation of the RSV transcription unit was observed (Fig. 4B).

The identity of the protein or proteins specifically binding to the prolactin and growth hormone tissue-specific elements was investigated with ultraviolet cross-linking to a labeled, bromodeoxyuridine-containing oligonucleotide encompassing the high affinity prolactin element 1P. A protein doublet (43.5 and 43 kD) was specifically cross-linked to the consensus region, and binding was effectively competed for by either prolactin or growth hormone cell-specific elements (1P and GH1), but not by an oligonucleotide mutated in the consensus sequence (1P MUT, Fig. 4C). Identical results with protein cross-linking and competition were obtained with the GH1 element. The appearance of the protein as a doublet is likely to result from the cross-linking technique (*13*), but alternative-

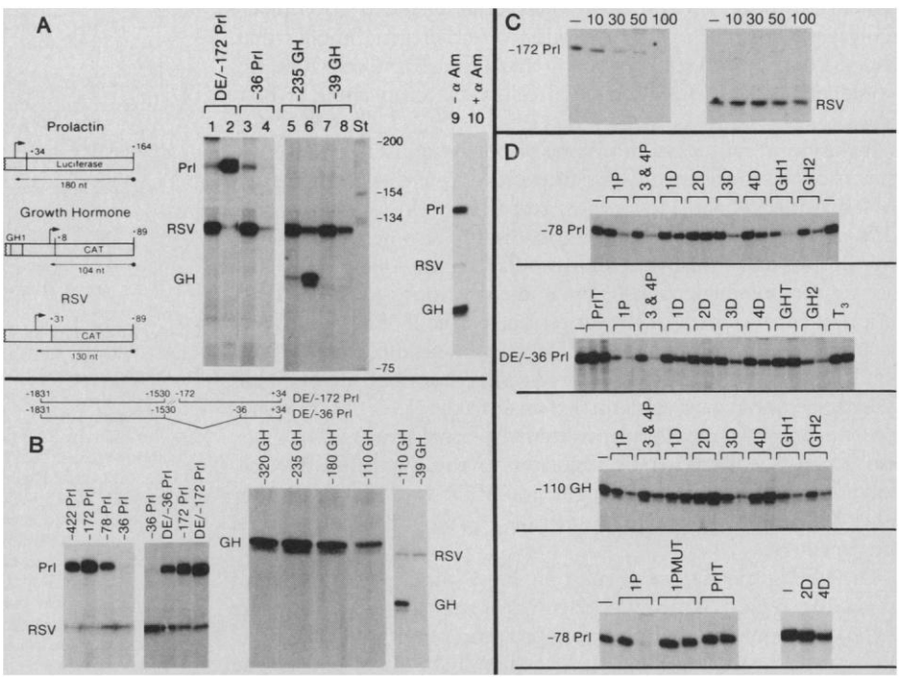

Fig. 3. Enhancer-dependent tissue-specific transcription of the rat prolactin and growth hormone promoters in vitro. (**A**) Autoradiographs showing primer extension analysis of RNA synthesized in vitro (*22, 23*). The map indicates the location of the predicted primer extension products for the three fusion genes used for in vitro transcription analyses. Plasmid names indicated above the lanes refer to the amount of 5′ flanking information present (for example, −36 Prl). Reactions in lanes 1, 3, 5, and 7 contained 20 μl (200 μg of protein) of HeLa nuclear extract. Reactions in lanes 2, 4, 6, and 8 contained 20 μl (200 μg of protein) of GC nuclear extract. Primer extension products of transcription from DE/−172 Prl and −235 GH in GC nuclear extract in the absence (lane 9), and presence (lane 10), of α-amanitin (αAm at 1 μg/ml). Molecular size markers (nucleotides) are a Hinf I digest of pBR322. (**B**) Prolactin and growth hormone enhancer elements are functional in vitro. Transcriptional analyses of the prolactin distal enhancer (DE) (−1833 to −1530) adjacent to either 172 or 36 bases of prolactin 5′ flanking DNA and serial deletions of the prolactin proximal enhancer (−422 to −36) and the growth hormone enhancer (−320 to −39). (**C**) Promoter-specific competition of transcription from prolactin (−172 Prl) and RSV (RSV-CAT) promoters in the presence of increasing amounts (10- to 100-fold molar excess) of a double-stranded oligonucleotide corresponding to site 1P. Dash represents no competitor. (**D**) Competition of transcription of the prolactin and growth hormone transcription units, −78 Prl, DE/−36 Prl, and −110 GH, as indicated. The oligonucleotides used for competition (*21*) are indicated above the lanes and are present at 30- and 100-fold molar excess over plasmid DNA (left and right lanes of each doublet, respectively). In the lowest panel, oligonucleotides corresponding to sites 2D and 4D were present at a 200-fold molar excess, and 1P MUT refers to the 6-bp mutation of site 1P (Fig. 1C). PrlT and GHT contain the prolactin and GH TATAA boxes and T_3 the GH T3 response element.

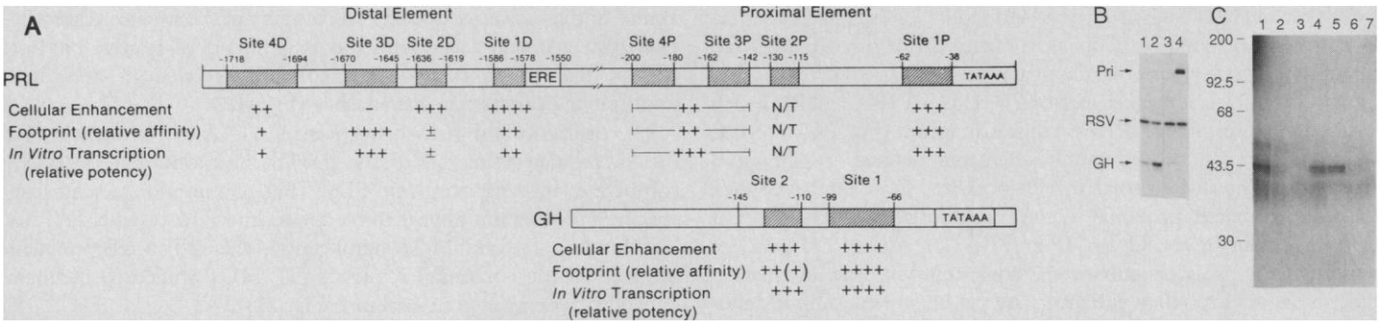

Fig. 4. (**A**) Summary of transcriptional effects and binding affinity of individual prolactin and growth hormone cis-active elements. N/T, not tested; − indicates no competition; ++++ indicates maximal effects. (**B**) Complementation of HeLa nuclear extract with affinity-purified factor. Transcription from −180 GH (lane 1) and −172 Prl (lane 3) in 10 μl of HeLa crude nuclear extract. Lanes 2 and 4 show transcription of the same plasmids in 10 μl of HeLa nuclear extract to which has been added <1 ng of affinity-purified protein. (**C**) Identification of the factor binding to the cell-specific elements of the rat Prl and GH genes after ultraviolet cross-linking of the partially purified factor to BrdU, ^{32}P-labeled product 1P element alone (*13*) (lane 1), or in the presence of 10- (lanes 2, 4, and 6) or 200-fold (lanes 3, 5, and 7) molar excess of the prolactin 1P element, the mutant 1P element (1P MUT) (lanes 4 and 5) or GH site 1 (lanes 6 and 7) oligonucleotides. The autoradiograph of the SDS-polyacrylamide gel reveals a doublet of 43,500 and 43,000 daltons, as indicated. Migration of protein standards are indicated. Cross-linking data obtained with labeled GH site 1 as probe displayed an identical pattern of bound protein and probe competition.

ly could represent two forms of a single gene product, or even different proteins. A far less prominent doublet (approximately 55 kD) was observed with the prolactin 1P element, but was competed for by the oligonucleotide (1P MUT) mutated in the core consensus sequence, suggesting that this protein or proteins bound to another portion of the sequence. This doublet is unlikely to have critical transcriptional function because the mutated 1P element (1P MUT) binds this doublet but fails to compete in transcription in vitro.

Expression of Pit-1 and pituitary phenotypic development. The ontogeny of the anterior pituitary presents a developmental model system in which to understand the pathway of commitment to tissue and cellular phenotype. On the basis of our analyses, we propose that the prolactin and growth hormone genes require a common pituitary transcriptional activator, which we term pituitary transcriptional activator–1 (Pit-1) (*14*). The ability of individual growth hormone and prolactin cell-specific elements to compete equivalently for in vitro transcription of either gene implies that, even if Pit-1 were to represent two related transcription factors, this would not account for differential cell-specific activation of both genes.

Inclusion of rat growth hormone or prolactin distal enhancers in chimeric transcription units targets expression of these fusion genes to somatotrophs and lactotrophs, respectively, in transgenic mice (*15*). However, these chimeras also display ectopic expression in thyrotrophs that express thyroid-stimulating hormone (TSH), suggesting the possible action of Pit-1 in thyrotrophs (*15, 16*). Transcription enhancement of the rat prolactin gene by Pit-1 appears to be positively modulated by plasma membrane–mediated peptide regulators including thyrotropin releasing hormone (TRH) and growth factors such as epidermal growth factor (EGF) (*17*). Therefore, the proposed developmental transcriptional activator Pit-1 may also serve as a homeostatic regulator in the mature lactotroph. Potential positive or negative regulation of Pit-1 activity by these or other factors during ontogeny may have profound developmental consequences.

The synergistic actions of multiple Pit-1 binding sites dispersed over a 2-kb region of the rat prolactin gene may be a required aspect of the developmental code dictating prolactin gene expression. If the prolactin, growth hormone, and possibly TSH genes require the same factor, Pit-1, for their activation, then restrictive mechanisms, whether repression or a requirement for additional interactive factors augmenting transcription, must account for their differential expression in mature lactotrophs, somatotrophs, and thyrotrophs. The expression of a cloned gene encoding Pit-1 will be necessary to permit direct assessment of positive and negative developmental regulation of the rat prolactin and growth hormone genes.

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13. Oligonucleotides for prolactin 1P and GH1 sites were labeled in the consensus binding sequence with [γ-^{32}P]ATP, bromodeoxyuridine (BrdU), and oligonucleotide primers. Ultraviolet cross-linking to partially purified GC nuclear extracts was performed for 4 to 10 minutes with a 310-nm emission wavelength at a distance of <1 cm essentially as described [L. A. Chodosh, R. W. Carthew, P. A. Sharp, *Mol. Cell. Biol.* **6**, 4723 (1986); R. Treisman, *EMBO J.* **6**, 2711 (1987)]. The proteins were separated by electrophoresis on an SDS, 10 percent polyacrylamide gel, followed by autoradiography for 12 to 40 hours.
14. Pit-1 appears to represent the protein referred to as PUF-1 by Cao *et al.*, in (*7*).
15. E. B. Crenshaw III *et al.*, in preparation; S. A. Lira *et al.*, in preparation.
16. Pit-1 binding site homologies in the TSH-β gene [F. E. Carr, L. R. Need, W. W.

Chin, *J. Biol. Chem.* **262**, 981 (1987)] were identified extending from −124 to −103 and −78 to −58. Double-stranded oligonucleotides encompassing these sequences (−200 to −90 and −78 to −47) were found to be efficient competitors of prolactin distal enhancer footprints in DNase I analyses. These data suggest that a third gene expressed in discrete cells in the anterior pituitary may be transcriptionally enhanced by a common factor that activates the prolactin and growth hormone genes.

17. H. P. Elsholtz *et al.*, *Science* **234**, 1552 (1986).
18. The rat PRL-luciferase fusion genes were constructed by ligation of prolactin fragments (*5*) into the Bam HI and Hind III sites of the pSV2ALΔ5′ luciferase [J. R. deWet, K. V. Wood, M. Deluca, D. R. Helinski, S. Subramani, *Mol. Cell. Biol.* **7**, 725 (1987)]. Plasmids were purified by two sequential cesium chloride bandings, and transfections were performed with 10 μg of plasmid per plate. The DEAE–dextran sulfate method of DNA transfer (10 μg per plate) was used as described [C. Gorman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982)]. For 208F and HeLa cells, calcium phosphate coprecipitation was used [F. L. Graham and A. J. Van der Eb, *Virology* **52**, 456 (1973)]. Transfections were performed as described (*5*). GC cells were lysed in buffer containing 100 m*M* K_2HPO_4 (*p*H 7.8) and 1 m*M* dithiothreitol (DTT). Luciferase activity was measured by addition of 50 μg of protein extract to 200 μl (final volume) of a buffer [100 m*M* K_2HPO_4 (*p*H 7.8), 5 m*M* ATP, and 10 m*M* $MgCl_2$]. Light activity was then determined on a luminometer (Analytical Luminescence, Monolight 2001), which automatically injected 50 μl of a 1 m*M* luciferin solution into each reaction. Values were recorded for a 20-second integration of luciferase activity. Values for mock-transfection (with salmon sperm DNA) were typically 40 to 50 light units.
19. Antisense strand 5′ end-labeled probes of the prolactin distal enhancer (PDE) were prepared by addition of [γ-^{32}P]ATP with polynucleotide kinase to a phosphatase-treated Xho I–digested plasmid Prl_5 CAT (*5*) and digestion with Bam HI; the probes were then purified by polyacrylamide gel electrophoresis (10^5 cpm ng^{-1}). The 5′ antisense probe corresponding to nucleotides −248 to +34 was prepared similarly by digestion of the plasmid Prl-422 CAT with Hind III and Hinf I; a sense strand labeled probe was prepared similarly by digestion with Xho I and Hae III (nucleotides −422 to −173). Approximately 1 ng of end-labeled probe was incubated for 20 minutes at 25°C with up to 150 μg of nuclear extract [J. D. Dignam, R. M. Lebovitz, R. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)] in a total volume of 95 μl containing 75 m*M* NaCl, 15 m*M* tris-HCl (*p*H 7.8), 5 percent glycerol, 0.5 m*M* DTT, 2 m*M* Na_2HPO_4, 1 μg of random pentanucleotides, 5 μg of yeast RNA, and 5 μg of poly(dI,dC); DNase digestion was allowed to proceed for 60 seconds after addition of 5 μl of 100 m*M* $MgCl_2$ and 1 to 3 μl of freshly diluted DNase I solution (2 mg/ml). Samples were then extracted with phenol and chloroform (1:1), precipitated with ethanol, and analyzed on an 8*M* urea–6 percent polyacrylamide gel.
20. M13 "loop out" mutagenesis [T. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985)] of the PDE caused deletion of the following sequences: site 1D, −1600 to −1584; site 2D, −1638 to −1622; site 3D, −1674 to −1648; site 4D, −1719 to −1693. A 6-bp Sal I site was introduced into all deletions, which resulted in deletion of an integral number of helical turns.
21. Complementary oligonucleotides used for competition were synthesized, purified by polyacrylamide gel electrophoresis, annealed, filled by Klenow fragment of DNA Pol I, and verified for length by analytical polyacrylamide gel electrophoresis. Concentrations of purified oligonucleotide fragments were obtained by fluorometric analysis. Oligonucleotides corresponding to the following sequences (in nucleotides) were tested: site 1P (−79 to −30); site 1P MUT (−79 to −30) with a six-nucleotide mutation at nucleotides −49 to −45 (Fig. 2C); 3P + 4P (−206 to −133); site 1D (−1607 to −1571); site 2D (−1650 to −1612); site 3D (−1677 to −1644); site 4D (−1727 to −1685); Prl TATAA sequence (−46 to +34); GH TATAA (−39 to +8); GH site 1 (−97 to −62); GH site 2 (−143 to −105); and a sequence (−186 to −145) containing the GH T_3 element [C. Glass *et al.*, *Nature (London)* **329**, 738 (1987)].
22. HeLa nuclear extracts were prepared according to the procedure of Dignam *et al.* [J. D. Dignam, R. M. Lebovitz, R. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)]. GC nuclear extracts were prepared according to the same procedure except that 0.2 percent NP-40 was added to the cell lysis buffer, and nuclei were extracted with a final concentration of 0.3*M* KCl. The concentration of protein in both nuclear extracts was between 6 and 10 mg/ml. The −39 GH-CAT plasmid was constructed by excising a fragment containing all growth hormone 5′ flanking sequences from −235 GH-CAT and replacing it with a double-stranded oligonucleotide corresponding to bp −39 to +8 of the growth hormone sequence.
23. In vitro transcription assays were carried out [K. A. Jones, K. R. Yamamoto, R. Tjian, *Cell* **42**, 559 (1985)] with 275 to 375 ng of supercoiled prolactin or growth hormone plasmid template and 125 ng of RSV-CAT [C. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982)] plasmid. Reactions were initiated by the addition of 20 μl of nuclear extract unless otherwise indicated. For all CAT fusion plasmids, the oligonucleotide primer was a 33-base sequence complementary to nucleotides 29 to 61 of the CAT coding sequence (*5*). For luciferase constructs, the primer was an oligonucleotide consisting of 30 bases complementary to nucleotides 133 to 164 of the luciferase coding sequence [J. R. de Wet, K. V. Wood, M. Deluca, D. R. Helinski, S. Subramani, *Mol. Cell. Biol.* **7**, 725 (1987)]. All competition experiments contained 375 ng of plasmid and 20 μl of GC nuclear extract. Both plasmid and competitor DNA's were present before the addition of nuclear extract.
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