Allosteric Effects of Pit-1 DNA Sites on Long-Term Repression in Cell Type Specification

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Reciprocal gene activation and restriction during cell type differentiation from a common lineage is a hallmark of mammalian organogenesis. A key question, then, is whether a critical transcriptional activator of cell type–specific gene targets can also restrict expression of the same genes in other cell types. Here, we show that whereas the pituitary-specific POU domain factor Pit-1 activates growth hormone gene expression in one cell type, the somatotrope, it restricts its expression from a second cell type, the lactotrope. This distinction depends on a two–base pair spacing in accommodation of the bipartite POU domains on a conserved growth hormone promoter site. The allosteric effect on Pit-1, in combination with other DNA binding factors, results in the recruitment of a corepressor complex, including nuclear receptor corepressor N-CoR, which, unexpectedly, is required for active long-term repression of the growth hormone gene in lactotropes.

Development of the six hormone-secreting cell types in the pituitary gland provides an excellent mammalian model for defining the mechanisms that underlie differentiation of distinct cell types from a common primordium (*1*). Three of the six cell types emerge from the lineage that expresses the tissue-specific POU domain transcription factor Pit-1 (*2*–*4*), which has been genetically established as being required for activation of the growth hormone, prolactin, and thyroid stimulating–β genes in somatotrope, lactotrope, and thyrotrope cell types, respectively (*5*). The cis-acting sequences of the rat growth hormone and prolactin genes necessary to correctly target reporter expression to somatotropes and lactotropes harbor multiple Pit-1 DNA binding sites (*6, 7*). The minimal growth hormone gene information required for selective expression in somatotropes, but not lactotropes, resides in the proximal 320 base pairs (bp) of the promoter, with as few as 181 bp being sufficient to target reporter expression in vivo (*7*). This region contains evolutionarily well conserved sequences, including two Pit-1 binding sites and a thyroid hormone response element (Fig. 1A). Lactotrope-specific prolactin gene expression requires 3 kilobases (kb) of 5′ flanking sequence, which includes an estrogen-regulated Pit-1–dependent enhancer and four additional Pit-1 sites in the promoter (*6*).

Pit-1 DNA sites in cell type–specific restriction. On the basis of the requirement of Pit-1 for activation of both growth hormone and prolactin gene expression, the conservation of its distinct recognition sites, and the flexibility of POU domain proteins in binding to their cognate DNA elements (*8, 9*), we first asked whether the Pit-1 DNA recognition elements themselves might be a critical component of cell type–specific expression. Analysis of the growth hormone promoter in vivo was conducted by generating transgenic mice with a reporter gene under the control of 320 bp of wild-type or mutated rat growth hormone promoter. The role of the two high-affinity Pit-1 binding sites in the growth hormone promoter, GH-1 and GH-2, was assessed by substitution with a site of similar affinity from the prolactin promoter, the conserved Prl-1P site (*2, 4*). Surprisingly, the substitution of the two Pit-1 sites resulted in the reporter being expressed in lactotropes, in addition to somatotropes, as evaluated by double-label immunohistochemistry with antisera directed against pituitary hormone cell type markers and an antibody specific for the reporter protein (*10*). Substitution of the proximal GH-1 site alone resulted in the same outcome, suggesting that somatotrope cell type–specific expression of the growth hormone gene is achieved by actively repressing its expression in lactotropes in a manner dependent on the precise sequence of a single conserved Pit-1 recognition site (Fig. 1B).

Allosteric effects of Pit-1 DNA sites. These data led us to investigate whether Pit-1 bound to the growth hormone GH-1 cognate response element in a structurally distinct fashion from the prolactin Prl-1P site, as a potential explanation underlying the role of the GH-1 site in cell type–specific restriction. Bacterially expressed Pit-1 POU domain was purified and cocrystallized (*11*) with synthetic double-stranded oligonucleotides corresponding to the GH-1 or Prl-1P sequences (Figs. 1A and 2B). The analyses were performed to resolutions of 3.0 Å for the GH-1 complex and 3.05 Å for the Prl-1P complex (*11*). Analysis of the cocrystals revealed a striking structural difference in how the bipartite POU DNA binding domain is accommodated on these two response elements (Fig. 2A). Remarkably, the spacing between the DNA contacts made by the POU-specific domain (POU_S) and the POU homeodomain (POU_H) of each monomer changed from 4 bp on binding to the Prl-1P element to 6 bp on the GH-1 element. Overall, the prolactin complex resembles the previously reported structure of Pit-1 bound to an artificially derived DNA element (*9*) in which the POU_S and POU_H of each monomer were bound to perpendicular faces of the DNA. The subdomains inserted their recognition helices (α3 helices) into adjacent major grooves, giving the appearance of surrounding the DNA. In contrast, in binding to the GH-1 element, the POU_S and POU_H domains of each monomer moved farther apart by an extra 2 bp so that they were accommodated on the same, rather than perpendicular, faces of the DNA (Fig. 2, A and B). The dimerization interface was maintained in the two complexes, whereby the COOH-terminus of the recognition helix of the POU_H domain of one monomer was inserted into a hydrophobic cavity on the surface of the POU_S domain (between helices α3 and α4) of the other monomer. Protein-DNA contacts in the major groove were generally similar between the two complexes, in which residues Val^{47} and Asn^{51} of POU_H domain specified the AT cores and residues Gln^{44}, Thr^{45}, and Arg^{49} of POU_S specified the ATNG/A cores (Fig. 2C). The GH-1 complex, however, lacked minor groove contacts because the POU_H domain NH_2-terminal arm did not penetrate the minor groove to the same extent as in the Prl-1P complex.

Although this change in spacing of the bipartite POU_S and POU_H domains between the Prl-1P and GH-1 sites was unexpected, it is nonetheless consistent with the notion of flexibility among POU domain proteins that allows them to adopt different configurations on different DNA elements (*8, 9*). In the Oct-1/DNA complex on the octamer site (*8*), the POU_S and POU_H domains are bound to opposite faces of DNA (spacing of 2 bp), and the relative orientation of the POU_S and POU_H is antiparallel versus parallel in the Pit-1/DNA complexes.

The importance of the 2-bp spacing difference observed in the cocrystal structures is further supported by the observation that these

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additional 2 bp (TT residues), between the POU_S and POU_H domains binding sites, are a highly conserved feature of the GH-1 DNA site in different organisms (Fig. 3A). To evaluate whether these additional spacing residues are the basis of the restriction of growth hormone gene expression from lactotropes, we deleted them from the GH-1 site in our growth hormone promoter construct and tested the effect on repression of reporter expression in vivo. Strikingly, analysis in four independent lines of transgenic mice demonstrated that this 2-bp deletion resulted in a failure to effectively restrict reporter gene expression from lactotropes, whereas expression in somatotropes was only slightly lowered (Fig. 3B) (*12*). Therefore, the difference in spacing observed in cocrystal structures between the DNA contacts made by the POU_S and POU_H domains on the GH-1 site versus the Prl-1P site appears to be a critical component of cell type–specific restriction of growth hormone gene expression from lactotropes.

Corepressor requirements for long-term repression. This ability of Pit-1 to participate in both cell type–specific restriction and activation of transcription is consistent with the hypothesis that POU domain factors, like nuclear receptors, can alternatively associate with either corepressors or coactivators (*13, 14*). Using single-cell nuclear microinjection assays, we investigated potential molecular mechanisms for the restriction of growth hormone gene expression from lactotropes based on the recruitment of corepressors to the GH-1 site. We found that Pit-1 or a Gal-4/Pit-1 fusion protein could actively repress transcription of the thymidine kinase (tk) promoter under the control of multimerized GH-1 or upstream activator sequence (UAS) elements, respectively, in Rat-1 fibroblasts and that this repression was blocked by the injection of specific anti–nuclear receptor corepressor (α–N-CoR) immunoglobulin G (IgG) (Fig. 4, A and C) (*15*). On the basis of previous biochemical data (*16*), we next evaluated the potential role of N-CoR (*17–19*) in the observed Pit-1–dependent repression (*16, 19*). Although direct examination of growth hormone gene expression in pituitary glands from N-CoR (–/–) mice was not feasible because N-CoR gene–deleted embryos die by embryonic day 15.5 (*20*), Pit-1 and Gal-4/Pit-1 proved to effectively mediate repression in wild-type (+/+) but not in N-CoR (–/–) mouse embryonic fibroblast (MEF) cultures (Fig. 4B). Repression in the N-CoR (–/–) MEFs was rescued by nuclear microinjection of an N-CoR expression vector. Single-cell nuclear microinjection of specific blocking IgGs suggested that histone deacetylase–1 (HDAC-1) and HDAC-2 were required (Fig. 4C). Therefore, whereas Pit-1 is critical for activation of transcription that is dependent on cofactors such as CREB binding protein (*16*), it also appears capable of a reciprocal role in inhibition of transcription by the recruitment of corepressors. The Pit-1 POU domain, but not the NH_2-terminus, was able to transfer the repression effects of Pit-1 (Fig. 4C).

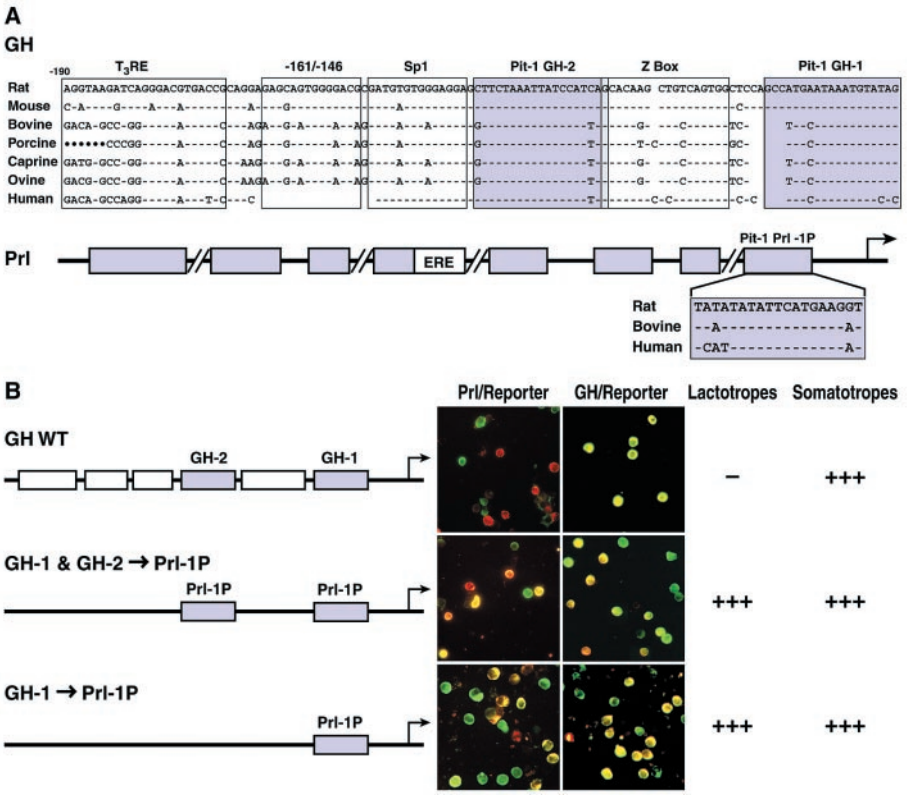

Fig. 1. Conserved Pit-1 sites (blue boxes) in the growth hormone promoter restrict expression of a linked reporter gene from lactotropes. (**A**) Conserved regulatory regions of the growth hormone gene promoter include a positive thyroid hormone response element (T_3RE), –161/–146, an Sp1 response element, the Z box (*27*), and the Pit-1 GH-1 and GH-2 binding sites. Boxes enclose sequences examined by mutational analysis in transgenic mice. Schematic diagram of the rat prolactin gene regulatory regions including an estrogen response element (ERE) and a conserved Pit-1 Prl-1P site. (**B**) Transgenic mice expressing the hGH reporter gene under the control of 320 bp of wild-type rat growth hormone promoter or recombinant promoters containing 19-bp substitutions of GH-1 and GH-2, or GH-1 alone, with Prl-1P (GH-1 & GH-2 → Prl-1P and GH-1 → Prl-1P, respectively). Dispersed pituitary cells from multiple 1- to 2-month-old mice were analyzed for cell type expression of the reporter gene by double-label immunohistochemistry. α-Prl or α-GH antisera and a rhodamine-coupled secondary antibody (red) were used in combination with α-hGH antibody and a fluorescein-coupled secondary antibody (green). Cells with overlapping expression of endogenous hormone and reporter protein appear yellow. Three independent lines were analyzed for GH wild type (WT) and GH-1 & GH-2 → Prl-1P, and 10 were analyzed for GH-1 → Prl-1P. Symbols denote the average of penetrance of reporter gene expression with (–) 0 to 5%, (+) 5 to 25%, (++) 25 to 75%, and (+++) 75 to 100%.

Because of the ability of Pit-1 to act as an N-CoR–dependent repressor, we performed chromatin immunoprecipitation (ChIP) analysis (*20*) to evaluate the association of Pit-1 and N-CoR with the growth hormone promoter in vivo. In the absence of pure somatotrope or lactotrope cell populations, we used the presence or absence of RNA polymerase II (Pol II) as the criterion for separating actively transcribed genes from nontranscribed genes (*21*). Following formaldehyde cross-linking, DNA chromatin preparations from adult mouse pituitary glands were subjected to a two-step ChIP analysis. After a first round of IP with anti–Pol II (α–Pol II) IgG, the bound (+) and unbound (–) fractions were subjected to a second round of ChIP with either α–N-CoR or α–Pit-1 IgGs. Polymerase chain reaction amplification with primers specific for the mouse growth hormone gene promoter detected occupancy by Pit-1, but not by N-CoR, in the Pol II (+) fraction. In the Pol II (–) fraction, both Pit-1 and N-CoR were associated with the promoter (Fig. 4D). These data suggest that Pit-1 occupies the growth hormone gene promoter in cell types that do and do not transcribe the growth hormone gene; however, these data also suggest that N-CoR is selectively recruited only in cells in which the gene is not transcribed.

To exclude the possibility that contamination of the Pol II (–) fraction by Pol II–bearing chromatin fragments might have influenced the result of the first ChIP analysis, we employed an alternative strategy in which a first round of ChIP analysis was performed with α–N-CoR IgG to select nontranscribed genes, followed by a second round with α–Pol-II, α–Pit-1, or α–thyroid hormone receptor β ($T_3R\beta$) IgGs. Although Pol II, as expected, was not associat-

ed with growth hormone gene promoters immunoprecipitated with α–N-CoR, occupancy by both Pit-1 and $T_3R\beta$ was detected. In contrast, in the N-CoR (–) fraction, Pol II, as well as Pit-1 and $T_3R\beta$, were detected, which is consistent with active transcription in the absence of recruitment of the N-CoR complex (Fig. 4E). Together, these data suggest that Pit-1 and $T_3R\beta$ are present on the growth hormone gene promoter when it is transcribed in somatotropes and when it is nontranscribed, as in lactotropes. N-CoR, however, is associated only with the nontranscribed growth hormone gene promoter.

To further evaluate the function of N-CoR in the restriction of growth hormone gene expression from lactotropes, we generated four independent lines of transgenic mice using 3 kb of 5′ rat prolactin sequences to direct expression of a hemagglutinin (HA)–tagged COOH-terminal domain (amino acids 2053 to 2300) of N-CoR exclusively to lactotropes (Prl/N-$CoR_{2053–2300}$) (*22*). This region of N-CoR interacts with unliganded nuclear receptors (*23–25*) and with Pit-1 (*16*) but lacks all transferable repression domains and may, therefore, function as a dominant-negative. Pituitary sections from control and transgenic mice were double-labeled with α-GH and α-Prl antisera and imaged using deconvolution microscopy to determine the proportion of lactotropes that coexpressed growth hormone. In the line of mice with the highest expression of the Prl/N-$CoR_{2053–2300}$ transgene, >50% of prolactin-positive cells (lactotropes) coexpressed growth hormone, whereas in age- and gender-matched control mice, coexpression occurred in 1 to 4% of prolactin-positive cells (Fig. 4F). Double-labeling with α-HA antibody and α-GH antisera to examine growth hormone expression specifically in those lactotropes that expressed detectable levels of the transgene also re-

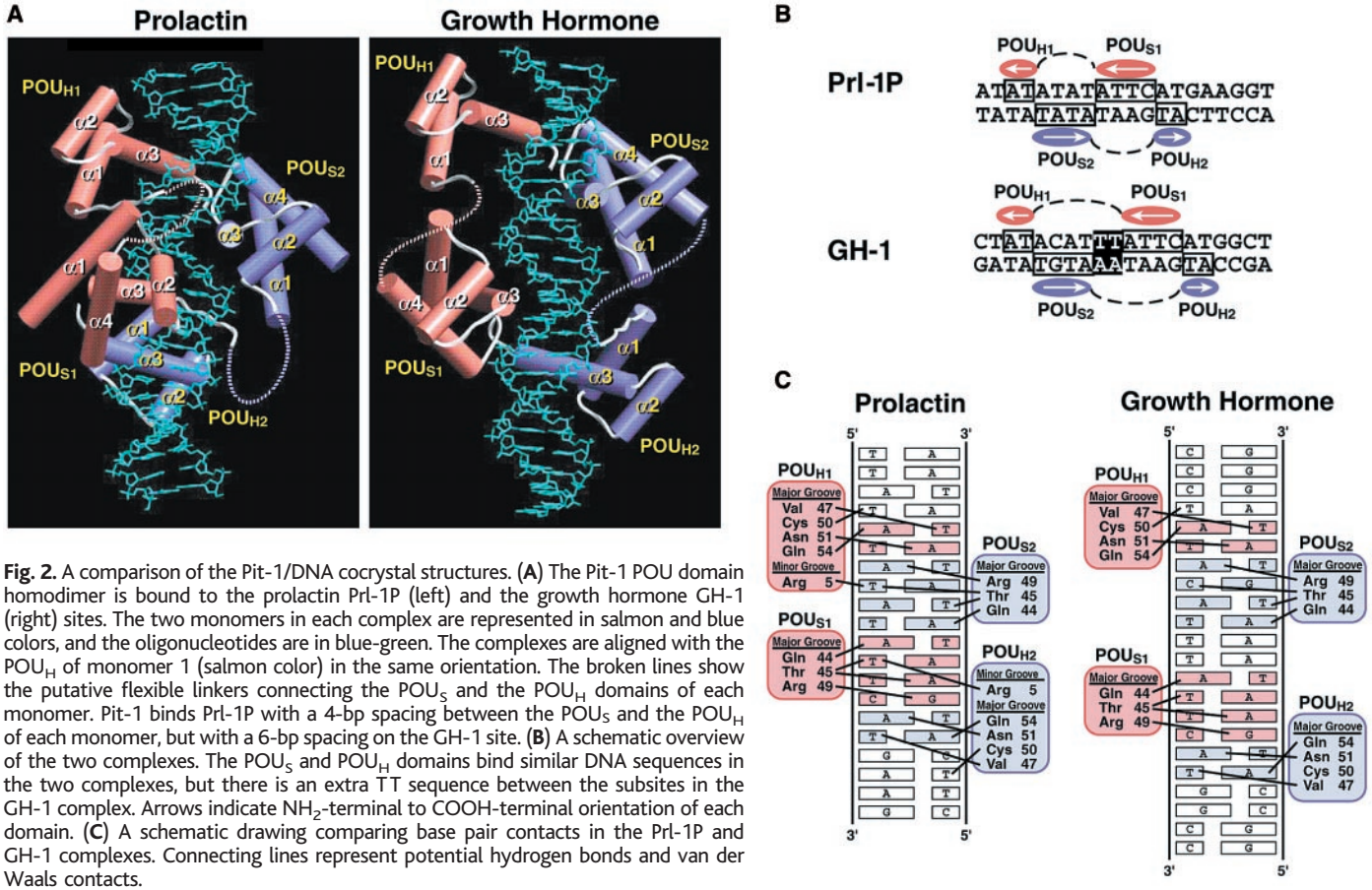

Fig. 2. A comparison of the Pit-1/DNA cocrystal structures. (**A**) The Pit-1 POU domain homodimer is bound to the prolactin Prl-1P (left) and the growth hormone GH-1 (right) sites. The two monomers in each complex are represented in salmon and blue colors, and the oligonucleotides are in blue-green. The complexes are aligned with the POU_H of monomer 1 (salmon color) in the same orientation. The broken lines show the putative flexible linkers connecting the POU_S and the POU_H domains of each monomer. Pit-1 binds Prl-1P with a 4-bp spacing between the POU_S and the POU_H of each monomer, but with a 6-bp spacing on the GH-1 site. (**B**) A schematic overview of the two complexes. The POU_S and POU_H domains bind similar DNA sequences in the two complexes, but there is an extra TT sequence between the subsites in the GH-1 complex. Arrows indicate NH_2-terminal to COOH-terminal orientation of each domain. (**C**) A schematic drawing comparing base pair contacts in the Prl-1P and GH-1 complexes. Connecting lines represent potential hydrogen bonds and van der Waals contacts.

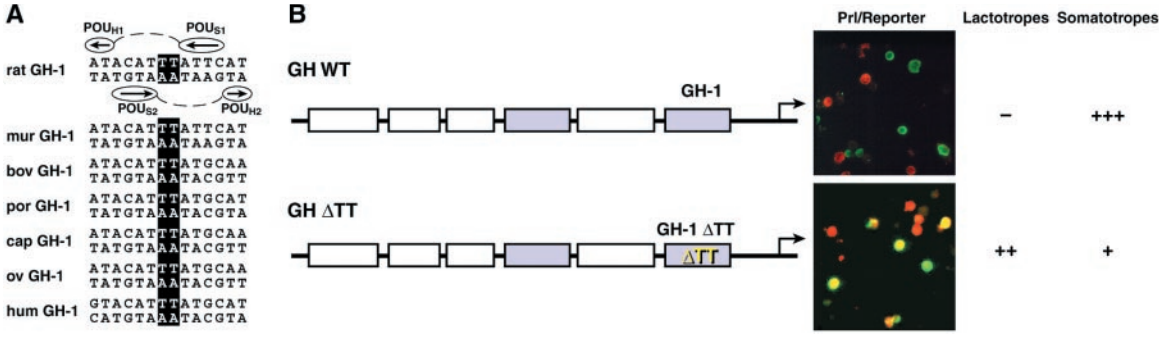

Fig. 3. The role of Pit-1 GH-1 site 2-bp spacing in cell type–specific restriction of growth hormone gene expression. (**A**) Evolutionary conservation of the TT residues in the Pit-1 GH-1 binding site sequence (mur, murine; bov, bovine; por, porcine; cap, caprine; ov, ovine; and hum, human) (**B**) Deletion of the conserved TT residues (ΔTT) results in expression of a linked reporter gene in lactotropes of transgenic mice. Analysis was carried out as in Fig. 1B on four independent lines of mice. Immunostaining is visualized with rhodamine (red) for prolactin and fluorescein (green) for hGH reporter.

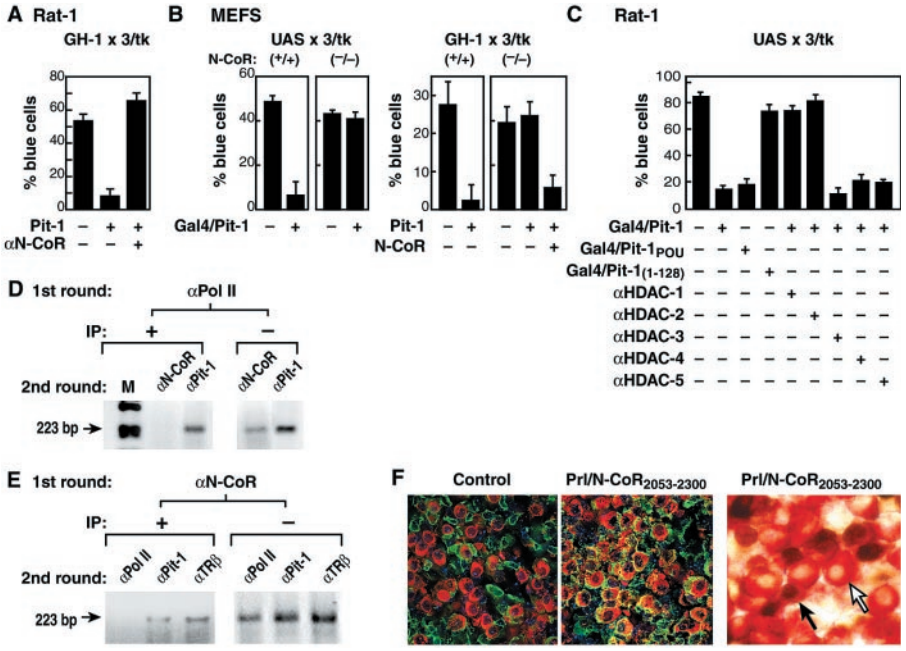

Fig. 4. The role of N-CoR in cell type–specific restriction of growth hormone gene expression. (**A**) Single-cell nuclear microinjection assays in Rat-1 fibroblasts using a GH-1 × 3/tk LacZ reporter, a cytomegalovirus (CMV) Pit-1 expression plasmid, and α–N-CoR IgGs. Results are the mean of three experiments (>300 cells injected per experiment) ± SEM. (**B**) Microinjection assays in MEFs from wild-type (+/+) or N-CoR (−/−) littermates, using either a UAS × 3/tk LacZ reporter and a CMV Gal-4/Pit-1 expression plasmid or a GH-1 × 3/tk LacZ reporter and a CMV Pit-1 or N-CoR expression plasmid. (**C**) In Rat-1 cells, N-CoR–mediated repression by Pit-1 mapped to the POU domain and was blocked by α–HDAC-1 and α–HDAC-2 IgGs. (**D**) Two-step ChIP assay of growth hormone promoter–associated factors. Immununoprecipitation with α–Pol II IgG to fractionate transcribing from nontranscribing chromatin was followed by a second round of immunoprecipitation with α–N-CoR or α–Pit-1 IgGs. (**E**) Two-step ChIP assay using the presence of N-CoR to select nontranscribed chromatin. Pit-1 and $T_3R\beta$, but not Pol II, were associated with the nontranscribed growth hormone promoter. (**F**) Adult pituitary sections from mice expressing HA-tagged Prl/N-$CoR_{2053–2300}$ in lactotropes. The effect on repression of the growth hormone gene in lactotropes was analyzed by double-labeling with α–Prl and fluorescein-coupled secondary antibody (green) and with α-GH and rhodamine-coupled secondary antibody (red). Staining was analyzed with deconvolution microscopy. Representative optical sections are shown (left and middle). Double-labeled cells exhibit central red and peripheral green cytoplasmic staining (yellow at overlap), reflecting an independent sorting of growth hormone and prolactin in distinct granule populations. α-HA and peroxidase-coupled secondary antibody (brown nuclear staining) and α-GH and alkaline phosphatase–coupled secondary antibody (red cytoplasmic staining) are also shown (right). The solid arrow indicates a double-labeled lactotrope, and the open arrow indicates a somatotrope.

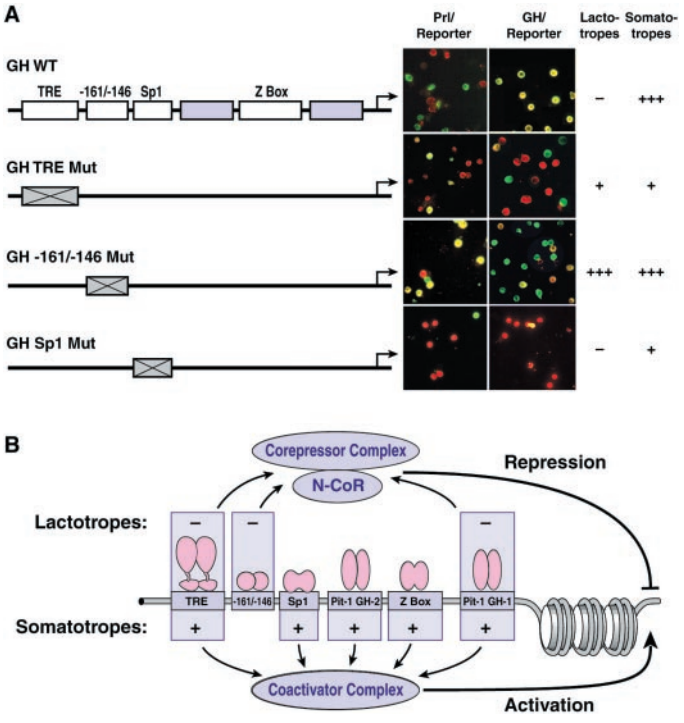

Fig. 5. Multiple cis elements are required for repression of the growth hormone gene in lactotropes. (**A**) The role of conserved elements in the growth hormone gene promoter in expression of a linked reporter in vivo. Independent lines of transgenic mice (three per construct) expressing the hGH reporter gene under the control of recombinant 320-bp rat growth hormone promoters containing substitutions of the thyroid hormone response element (GH TRE Mut), the −161/−146 region (GH –161/–146 Mut), or the Sp1 response element (GH Sp1 Mut) were established (*13*). Exact boundaries are given in Fig. 1A. Mice from each line were analyzed for lactotrope and somatotrope cell type expression of the reporter gene as described in Fig. 1B. The GH TRE Mut and GH –161/–146 Mut resulted in expression in lactotropes. The GH TRE Mut and Sp1 Mut exhibited decreased expression in somatotropes. (**B**) Model of cell type–specific restriction of growth hormone gene expression. The allosteric effects on Pit-1 imposed by the GH-1 site, in combination with T_3R and a third factor, restrict expression of the growth hormone gene from the lactotropes.

vealed that >50% of cells expressing N-$CoR_{2053–2300}$ expressed growth hormone. Together, these data suggest that expression of the Prl/N-$CoR_{2053–2300}$ transgene interfered with lactotrope-specific repression of the endogenous mouse growth hormone gene in vivo.

Multifactorial "repressosome" in cell type–specific restriction. The presence of additional conserved elements in the growth hormone gene promoter led us to investigate the potential participation of additional promoter-binding factors in the mediation of activation and/or repression leading to cell type–specific expression. Each of these conserved promoter elements was, therefore, independently replaced with a neutral sequence in the growth hormone reporter construct, and the effects were analyzed in a series of transgenic lines (*10*). Mutation of the thyroid hormone response element (T_3RE) (*26*) revealed that it is not only required for growth hormone gene expression in somatotropes, but is also required for its restriction from lactotropes. In addition to the known requirement for the Z box, which binds the zinc finger protein Zn-15 and mediates activation (*27*), mutation of the Sp1 binding element revealed a similar role for it in vivo (*28*). Mutation of the conserved element at −161/−146 (relative to the transcription start site) revealed its requirement for the restriction of expression from lactotropes but had no effect on expression in somatotropes (Fig. 5A). Interestingly, the human growth hormone (hGH) gene, which exists in a gene cluster instead of as a single gene in rodents, lacks this –161/–146 element, and its restricted expression has been observed to require a Pit-1–dependent locus control region (*29–31*). These data suggest a combinatorial requirement for DNA binding factors to effectively restrict growth hormone gene expression from the lactotrope cell type.

Our data argue in favor of the assembly of a "repressosome" complex that is dependent on both the configuration of Pit-1 on a specific cognate site and the actions of other DNA binding factors, which together dictate cell type–specific activation or repression of growth hormone gene expression. The allosteric effects of the high-affinity growth hormone promoter binding element on the configuration of Pit-1 appear to serve as one of the critical determinants (along with thyroid hormone receptor and a –161/–146 binding factor) of interaction with components of corepressor machinery in the appropriate cellular context (Fig. 5B). Allosteric effects of DNA binding sites have been suggested to mediate alternative activation or repression by other classes of transcription factors (*32*). Cofactor-dependent regulation is observed in the activities of Oct-1 on a TAATGARAT element where HCF and VP16 are recruited (*33*) and in the activities of Oct-1/Oct-2 on octamer elements where OCA-B/Bob1/OBF-1 is recruited, dependent on interactions with the POU_S and POU_H domains, as well as with specific nucleotides in the site (*34*–*38*). OCA-B/Bob1/OBF-1 is required for the activation of a subset of Oct-1/Oct-2–dependent genes in B cells, and it is tempting to speculate that, in its absence, a corepressor complex might, in some cases, be associated with Oct-1/Oct-2 on these sites.

In conclusion, we suggest that the selective patterns of hormone-encoding gene expression that define the three cell types of the Pit-1 lineage reflect, in part, differential association of distinct classes of cofactors, including N-CoR, with Pit-1, to mediate activation or repression. This strategy is likely to be prototypical of other cell type specification events in mammalian organogenesis and poses the challenge to now define other factors and/or signals that prevent recruitment of the repressor complex to the growth hormone promoter in somatotropes.

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11. The Prl-1P cocrystals belonged to space group *P*1 with unit cell dimensions of $a = 43.2$ Å, $b = 55.1$ Å, $c = 57.0$ Å, $\alpha = 89.5°$, $\beta = 71.6°$, and $\gamma = 78.0°$. The GH-1 cocrystals belonged to space group *C*2 with unit cell dimensions of $a = 113.0$ Å, $b = 47.9$ Å, $c = 107.5$ Å, $\alpha = 90°$, $\beta = 117.1°$, and $\gamma = 90°$. The structures of both complexes were solved by molecular replacement methods. The final R_{factor} for the Prl-1P complex is 0.239 (R_{free} of 0.312) for all data between 10 and 3.05 Å, with root mean square deviations (rmsd's) on bond lengths and bond angles of 0.008 Å and 1.7°, respectively. The final R_{factor} for the GH-1 complex is 0.258 (R_{free} of 0.334) for all data between 10 and 3 Å, with rmsd's on bond lengths and bond angles of 0.021 Å and 2.5°, respectively. For additional information, see supplementary Web material, available at www.sciencemag.org/feature/data/1055900.shl.
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Spin-Dependent Tunneling in Self-Assembled Cobalt-Nanocrystal Superlattices

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Self-assembled devices composed of periodic arrays of 10-nanometer-diameter cobalt nanocrystals display spin-dependent electron transport. Current-voltage characteristics are well described by single-electron tunneling in a uniform array. At temperatures below 20 kelvin, device magnetoresistance ratios are on the order of 10%, approaching the maximum predicted for ensembles of cobalt islands with randomly oriented preferred magnetic axes. Low-energy spin-flip scattering suppresses magnetoresistance with increasing temperature and bias-voltage.

Increasing density requirements in the microelectronics and magnetic-storage industries continue to motivate the production of devices that function reproducibly at ever smaller dimensions. Nanometer-scale control of material properties has already enabled technologies that exploit electron spin and the discreteness of electronic charge. For example, modern magnetic disc drives employ ultrasensitive readheads based on the giant magnetoresistance (GMR) response of nanometer-thick metal multilayers (*1*). Also, two-dimensional (2D) arrays of spin-dependent tunnel junctions show promise for nonvolatile memory applications and will require reproducible tunnel barriers only 1 nm thick (*2*, *3*). We have combined conventional lithography, chemical synthesis, and self-assembly to produce sub–100-nm, spin-dependent electronic devices with nanometer-scale control of material properties in all dimensions.

Self-assembly is an attractive nanofabrication technique because it provides the means to

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