

The Turkey Transcription Factor Pit-1/GHF-1 Can Activate the Turkey Prolactin and Growth Hormone Gene Promoters *in Vitro* but Is Not Detectable in Lactotrophs *in Vivo*

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The transcription factor Pit-1/GHF-1 plays an important role in regulating the prolactin (Prl) and growth hormone (GH) genes in mammals. In this study, the role that Pit-1 plays in regulating the prolactin and growth hormone genes in avian species was examined by cotransfection assays and immunofluorescence staining of pituitary sections. In cotransfection assays, turkey Pit-1 activated the turkey Prl, turkey GH, and rat Prl promoters 3.8-, 3.7-, and 12.5-fold, respectively. This activation was comparable to rat Pit-1 activation of these same promoters. A point mutation in the turkey Pit-1 cDNA, which changed leu-219 to ser-219, resulted in a 2-, 2-, and 10-fold reduction in the activation of the turkey Prl, turkey GH, and rat Prl promoters, respectively. Unexpectedly, coexpression of tPit-1 (leu-219) and tPit-1 (ser-219) activated turkey Prl and rat Prl promoters 9.4- and 35.9-fold, respectively, but had no effect on the turkey GH promoter. Dual-label immunofluorescence analysis of turkey pituitary sections revealed that Pit-1 was not detectable in prolactin-staining cells but was detectable in GH-staining cells. Taken together, these data indicate that in the domestic turkey, Pit-1 can activate the turkey Prl promoter *in vitro*, but does not appear to play a role in regulating Prl gene expression *in vivo*. Pit-1, however, still likely plays a role in regulating GH gene expression. © 2001 Academic Press

Key Words: turkey; Pit-1; prolactin; growth hormone; pituitary.

INTRODUCTION

The transcription factor Pit-1/GHF-1 (referred to here as Pit-1) is a member of a large family of POU-domain proteins (named after the proteins Pit-1, Oct-1/Oct-2, and unc86 which share this common domain). In mammals, Pit-1 is a 33-kDa nuclear protein, which contains an amino terminal transactivation domain, a central POU-specific domain, and a carboxy terminal POU homeodomain (Ingraham *et al.*, 1988; Nelson *et al.*, 1988; Theill *et al.*, 1989). The POU homeodomain is required for low-affinity DNA binding, whereas the POU-specific domain is necessary for high-affinity DNA binding and DNA-dependent Pit-1–Pit-1 interactions (Ingraham *et al.*, 1990). The avian and teleostean Pit-1 proteins differ from their mammalian counterparts by the presence of an additional exon located between exons 2 and 3. In turkeys, this exon encodes 38 amino acids (Kurima *et al.*, 1998), whereas in fish it encodes 33 amino acids (Ono and Takayama, 1992).

Pit-1 plays an important role in regulating expression of the pituitary genes, prolactin (Prl), growth hormone (GH), and thyroid-stimulating hormone- β (TSH- β) subunit (Ingraham *et al.*, 1988; Nelson *et al.*, 1988; Mangalam *et al.*, 1989; Fox *et al.*, 1990; Haugen *et al.*, 1994). Pit-1 also autoregulates expression of its own gene in rats and mice (Chen *et al.*, 1990; McCormick *et al.*, 1990; DiMattia *et al.*, 1997). The upstream regions of the Prl and GH genes contain a variety of regulatory elements that bind Pit-1 and various other transcription factors and hormones (Cohen *et al.*, 1996). Mammalian Pit-1 binds to the regulatory region of its target genes through the consensus Pit-1 binding site, 5'-A(^A/_T)(^A/_T)TATNCAT-3' (Nelson *et al.*, 1988). Avian and fish genes have similar but not identical Pit-1 binding sites and thus Ohkubo *et al.* (1996) have proposed a teleost/avian consensus Pit-1 binding site of 5'-(^T/_A)NCTNCAT-3'.

A number of Pit-1 isoforms have been identified, which arise from alternative splicing pathways or alternative transcription initiation sites. The Pit-1 β isoform (alternatively called Pit-1a or GHF-2) contains an extra 26 amino acids in mammals and an extra 28 amino acids in turkey compared to Pit-1 due to alternative splicing at the 3' end of intron 1. Mammalian Pit-1 β activates transcription from the GH promoter but is reduced in its ability to activate transcription from the Prl or Pit-1 promoters (Morris *et al.*, 1992; Konzak and Moore, 1992; Theill *et al.*, 1992). The mammalian Pit-1T isoform contains an extra 14 amino acids as a result of alternative splicing and shows a synergistic stimulatory effect on the TSH- β promoter but not the GH or Prl promoters (Haugen *et al.*, 1994). The mammalian Δ 4Pit-1 isoform lacks most of the POU domain due to a deletion of exon 4 and inhibits Prl promoter activity (Day and Day, 1994; Voss *et al.*, 1994). A unique Pit-1 isoform has been identified in turkeys (tPit-1W*; Kurima *et al.*, 1998) and chickens (cPit-1 γ ; Tanaka *et al.*, 1999), which arises following transcription initiation upstream of the 5' splice site in exon 2.

In the mature rat pituitary gland, Pit-1 mRNA is expressed in all five cell types. Pit-1 protein, however, is detected only in the somatotrophs, lactotrophs, and thyrotrophs, suggesting that Pit-1 expression is under posttranscriptional control (Simmons *et al.*, 1990). In humans, Pit-1 mRNA is detectable in the fetal pituitary at 6 weeks of gestation and precedes the onset of

pituitary hormone immunoreactivity. Pit-1 mRNA and protein are present only in lactotrophs, somatotrophs, and thyrotrophs and are absent in corticotrophs and gonadotrophs of the developing human fetal pituitary (Puy and Asa, 1996).

The role that Pit-1 may play in regulating the avian Prl and GH genes in poultry is unclear. It is reported here that turkey Pit-1 can activate the turkey Prl, turkey GH, and rat Prl promoters comparable to that of rat Pit-1 in cotransfection assays. However, immunofluorescence staining of pituitary sections indicate that Pit-1 and Prl are not coexpressed in lactotrophs, whereas Pit-1 and GH are coexpressed in somatotrophs.

MATERIALS AND METHODS

Expression Vector and Reporter Gene Constructs

Turkey Pit-1* (tPit-1*) expression vectors were constructed by insertion of tPit-1 cDNAs into the expression vector pLTR/SV40 by standard procedures. This vector contains the avian sarcoma virus long terminal repeat (ASV-LTR) promoter and the SV40 splice and polyadenylation sites. The asterisk indicates that these tPit-1 constructs contain the 38 amino acids encoded in exon 2a not present in mammalian Pit-1. The tPit-1* cDNA reported in Kurima *et al.* (1998) contained ser-219, which corresponds to amino acid position 176 in mammalian Pit-1. New tPit-1* cDNAs containing leu-219 were synthesized by RT-PCR from total pituitary RNA. Reverse transcriptase was initiated with primer AS6 (5'-AACAGGAACCCACAGCTA-3', nt 247 to 264 in exon 6; Kurima *et al.*, 1998). PCR was performed with primers tPit-S45 (5'-GCTGCCATTAATCGCTCAG-3', nt 56 to 74 in exon 1) and tPit-AS44 (5'-GCTTTACCGGCACTCGTGGTG-3', nt 214 to 234 in exon 6) for tPit-1* and tPit-1 β *. Primer tPit-S5 (5'-GCAGGAGAACTAGATACG-3', nt -47 to -30 in exon 2) was paired with tPit-AS44 to generate tPit-1W*. To generate a comparable rat Pit-1 (rPit-1) expression vector, the rat Pit-1 cDNA was excised from a SV40/rat Pit-1 plasmid (from H. Elsholtz, University of Toronto) and inserted into plasmid pLTR/SV40.

Turkey Prl (tPrl), turkey GH (tGH), and rat Prl (rPrl) reporter vectors were constructed by insertion of the

5' flanking region of each gene upstream of the luciferase gene. Plasmid rPrl-LUC was constructed by cloning of 3.0 kb of the rat Prl promoter (obtained from H. Elsholtz) into the luciferase vector, pGL3-basic (Promega, Madison, WI). Plasmid tPrl-LUC was constructed by cloning of 2.0 kb of the 5' flanking region of the turkey Prl promoter (Kurima *et al.*, 1995) into pGL3-basic. Plasmid tGH-LUC was constructed by cloning of 0.9 kb of the turkey GH 5'-flanking region into pGL3-basic. The tGH 5'-flanking region was isolated with the GenomeWalker Kit from Clontech (Palo Alto, CA) and two exon 2-specific primers based on the sequence of the tGH cDNA (Foster *et al.*, 1990). The two turkey GH-specific primers used for genome walking were tGH1 (5'-CAGCACAGCGTTG-GTGAACAGGTTGGA-3') and tGH2 (5'-CGTGAC-CACAGCAATGAGGAGAGGAGA-3'). Surprisingly, an approximately 1950-bp PCR product containing the tGH 5' flanking region was obtained after first-round PCR with only primer tGH1. This tGH fragment was cloned into plasmid pGEM-T Easy (Promega) for sequencing. As expected, this fragment contained the 5' flanking region, exons 1 and 2, and intron 1 of the turkey GH gene. A fragment containing the 5' flanking region, the transcription initiation site, but lacking the translational start site was amplified with primer tGH3 (5'-CGCTCGAGCCAGGAGAGCT-GCTCGGGTG, nt 23 to 42) and a primer specific for pGEM-T Easy and cloned into plasmid pGL3-basic.

Cell Culture and Transfection

Mouse L and Hela cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotic-antimycotic (90 IU/ml penicillin, 90 μ g/ml streptomycin, 0.9 μ g/ml amphotericin) in a humidified 37° incubator with 5% CO₂. For transfection assays, 2 × 10⁵ mouse L cells were plated in 6 × 35-mm tissue culture plates and incubated for 24 h. Cells were transfected with a DNA:lipofectamine complex containing 25 μ g lipofectamine (Life Technologies, Gaithersburg, MD), 1 μ g Pit-1 expression vector, 5 μ g promoter-luciferase reporter vector, and 0.125 μ g CMV- β -galactosidase plasmid (from R. N. Day, University of Virginia) in OptiMEM medium (Life Technologies). Cell extracts were prepared 42–45 h after transfection. Cells were washed in phosphate-buffered saline (PBS)

and lysed with 200 μ l of reporter lysis buffer (Promega).

Luciferase activity was measured in a Lumat LB9501 luminometer (Berthold, Bad Wilbad, Germany) by mixing of 50 μ l of cell lysate with 300 μ l of ATP buffer (5 mM ATP, 25 mM glycyglycine, pH 7.8, 15 mM MgSO₄) and injection of 100 μ l luciferin (1 mM luciferin, 25 mM glycyglycine, pH 7.8, 15 mM MgSO₄). Light units were measured for 10 s. β -Galactosidase activity was measured spectrophotometrically. Twenty-five microliters of cell lysate were diluted with 25 μ l of reporter lysis buffer and mixed with 250 μ l of β -galactosidase assay buffer (100 mM NaPO₄, pH 7.5, 2.3 mM MgCl₂, 100 mM β -mercaptoethanol, 3.5 mM *O*-nitrophenyl- β -d-galactopyranoside). Reactions were terminated with 500 μ l of 1 M Na₂CO₃ and absorbance was measured at 420 nm. Samples were assayed in duplicate. To correct for variations in transfection efficiencies, luciferase activity was normalized to β -galactosidase activity for each sample.

Expression of tPit-1 isoforms in microinjected cells was performed as described by Storrie *et al.* (1998). Hela cells were plated on glass coverslips and microinjected with tPit-1 expression vectors at a concentration of 300 ng/ μ l. Eight hours after microinjection, cells were washed with PBS, fixed with 3% formaldehyde in PBS for 20 min at room temperature, and then washed three times with PBS containing 50 mM AmCl. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 1–2 min, washed three times with PBS, and incubated with a rabbit anti-rat Pit-1 antibody (No. 1796; diluted 1:1000 in PBS; from S. Rhodes, Indiana University Purdue University-Indianapolis) for 12 h at room temperature. Unreacted primary antibody was removed with three washes of PBS. Secondary antibody (Cy3-conjugated donkey anti-rabbit diluted 1:1000 in PBS; Jackson ImmunoResearch Labs, West Grove, PA) was added for 30 min at room temperature. Coverslips were washed three times with PBS and once with water and then mounted on glass slides in Mowiol 4-88 (Calbiochem, San Diego, CA). Fixed cells were examined with a fluorescence microscope (Zeiss axiovert S100TV, New York, NY) and camera (Dage-MTI Inc., Michigan City, IN). Cells were photographed separately under phase-contrast and then fluorescence. The images were merged with IPLab imaging software (Scanalytics, Fairfax, VA). Im-

munofluorescence was indicated by red pseudocoloring.

Dual-Label Immunofluorescence

Pituitaries collected from the turkey hens ($n = 6$) were utilized for colocalizing Pit-1 with either GH or PRL with a dual immunofluorescent labeling procedure previously described (Ramesh *et al.*, 1998). Briefly, laying hens were anesthetized with sodium pentobarbital and their heads were perfused with physiological saline followed by 4% paraformaldehyde solution. Brains and pituitaries were removed from the cranium and stored in 0.1 M sodium phosphate buffer (pH 7.2) at 4° until processing. Pituitaries were dehydrated, cleared, and embedded in paraffin. Sagittal pituitary sections were cut at 4 μm thickness with a Leica (Leica, Deerfield, IL) rotary microtome and serial sections were mounted on gelatin-coated microscopic slides.

A rabbit polyclonal antibody raised against rat Pit-1 (No. 1796), a mouse monoclonal antibody raised against a synthetic peptide fragment of chicken PRL (Berghman *et al.*, 1992), and a mouse anti-chicken GH antibody (Berghman *et al.*, 1988) were utilized for colocalization of Pit-1 with either PRL or GH. Tissue sections were deparaffinized, hydrated, and incubated for 1 h with 2.5% normal goat serum in 0.01 M Tris-HCl and 0.15 M sodium chloride (pH 7.4; TBS) containing 1% Triton X-100. Slides were then incubated with a mixture of either rabbit anti-Pit-1 antibody (1:500) and mouse anti-PRL antibody (1:5000) or mouse anti-GH antibody (1:3000) for 36 h at 4° in a humid chamber. PRL antibody and GH antibody were preadsorbed with their respective antigen (turkey PRL or turkey GH) for 48 h at 4° and centrifuged at 100,000g for 20 min. The supernatant was used in place of PRL antibody or GH antibody, and addition of Pit-1 antibody was omitted as a control for immunostaining. After a series of washes in TBS, slides were incubated with the biotinylated anti-rabbit IgG (1:400; Vector Lab, Burlingame, CA) for 1 h at room temperature. Slides were then washed in TBS and incubated with a mixture of lissamine conjugated to anti-mouse IgG raised in goats (1:100; Jackson ImmunoResearch Labs) and avidin-fluorescein isothiocyanate (25 $\mu\text{g}/\text{ml}$; FITC; Vector Lab) for 2 h in the dark at room temperature. After washing in TBS, coverslips were

applied with Vectashield (Vector Lab) mounting medium.

The pituicytes showing Pit-1 (yellow-green) in the nucleus and either PRL (red) or GH (red) in the cytoplasm were visualized with a dual-filter set (Zeiss Axioplan microscope) that would permit simultaneous visualization of both red and green fluorescence. Single-exposure photographs of selected microscopic fields were taken with a conventional camera and Fujichrome Slide Film at 1600 ASA speed. The films were routinely processed and 35-mm slides were made.

RESULTS

Cloning of the Turkey Growth Hormone Gene 5' Flanking Region

The tGH 5' flanking region was synthesized by PCR with a GenomeWalker kit and primers specific to tGH exon 2. A 1950-bp PCR product containing the 5' flanking region, exons 1 and 2, and intron 1 of the tGH gene was synthesized. The sequence of 918 bp upstream of the translation start site of the tGH gene is shown in Fig. 1. Alignment of the tGH 5' flanking region with 554 bp of the chicken GH 5' flanking region showed an overall 92% sequence identity. Furthermore, a putative Pit-1 binding site, identified in the chicken GH gene (Tanaka *et al.*, 1992), was 100% conserved in the turkey GH gene.

Activation of the Turkey GH, Turkey Prl, and Rat Prl Regulatory Regions by Pit-1

Turkey Pit-1 expression vectors were cotransfected into mouse L cells with a CMV- β -galactosidase plasmid and luciferase reporter constructs containing 2.0 kb of the tPrl 5' flanking region, 0.9 kb of the tGH 5' flanking region, or 3.0 kb of the rat Prl 5' flanking region. The turkey Pit-1* expression vectors contained a single amino acid difference at position 219, which corresponds to amino acid position 176 in mammalian Pit-1. The leu-219 to ser-219 mutation alters one of the invariant amino acids identified in the POU homeodomain (Schöler, 1991). Turkey Pit-1*(leu-219) activated the tPrl, tGH, and rPrl promoters 3.8-fold (bar 1),

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tcagcacagcgttgggtaacaggttggaccacagccatgggggcaaagtgtgtcagtg -812
cctgtgggagtgaaagccctcctgcccgttctccatcccaggttctacctcgtgtggctc -752
agggataggcgtgctgggacttctgctcttccccttcttcttcttcttcttccacacca -692
gttctctgcttaaccagagggcatctctcattctctaccaagcaaaagcagaaagcagctg -632
accgccagtttttttctcattcgtatcaaaacaagagcagaaggctctgggatcactgtga -572
tcgccagaccccaagccttggccaactgaggagctgagaaaccacccacagctctgccg -512
ttgggttctgcagtgggcaggtggatccaagcaaacactgcctgtgaagctcttctgaca -452
gcateccatctgctctaaccctactaaatgggaagccacccacactgctctaagccagaa -392
gtccccctgcactgcctgccagccctgttaactgtggggcagaaaaaccaggcaggaaaa -332
ccagctggattttctacctgtgtgggaaattcccccatgtaagcacagaaacagatttggg -272
gtgggatttccaatggcgataaaacctctggttgcaataaacagcagaatatgaagaaaa -212
agttcggcactaattttatccccaggcaaacatcgtcccacactttccatctccgtata -152
aatgactagaatgaggtagcaccatggccaacacatctgcatttatgcaaggagggatat -92
ggagaggtggcagtgatcgtgaacaccccatccattttaaacagacccccgcgtatataag -32
gggtgtctcactgttaccatcacctggatgAAAGGAGGAAACGCTCAAGCAACACCCGA 29
GCAGCTCTCCTGGCGGGAATG

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FIG. 1. Nucleotide sequence of the turkey growth hormone gene 5' flanking region. The sequence ends with the ATG start codon in exon 1. The nucleotides that are identical to the tGH cDNA (Foster *et al.*, 1990) are shown in capital letters and the nucleotides that correspond to the putative Pit-1 binding site identified in the chicken GH gene (Tanaka *et al.*, 1992) are double underlined. This sequence will appear in the GenBank, EMBL, and DDBJ Nucleotide Sequence Databases under Accession No. AF286532.

3.7-fold (bar 5), and 12.5-fold (bar 9), respectively, in mouse L cells (Fig. 2). These results are comparable to rat Pit-1 activation of the tPrl (5.8-fold, bar 4), tGH (5.0-fold, bar 8), and rPrl (21.5-fold, bar 12) promoters. Turkey Pit-1*(ser-219) showed a 2-, 2-, and 10-fold reduction in activity on the tPrl (bar 2), tGH (bar 6), and rPrl (bar 10) promoters, respectively, compared to tPit-1*(leu-219). Surprisingly, coexpression of tPit-1*(leu-219) and tPit-1*(ser-219) led to a 9.4- and 35.9-fold activation of the tPrl (bar 3) and rPrl (bar 11) promoters, respectively, but had no effect on the tGH promoter (bar 7). The coexpression of tPit-1*(leu-219) and tPit-1*(ser-219) represents a 2.5- and 2.9-fold increase in activation of the tPrl and rPrl promoters, respectively, compared to tPit-1*(leu-219) expression alone.

Immunolocalization of Turkey Pit-1, Prl, and GH in Pituitary Sections

Expression of turkey Pit-1, GH, and Prl was examined by dual-label immunofluorescence in pituitary sections from the cephalic and caudal lobes of laying turkey hens (Figs. 3A and 3B). Pit-1 staining should be confined to the nucleus because Pit-1 is a nuclear transcription factor, whereas GH or Prl staining should be detectable in the cytoplasm. In Fig. 3A,

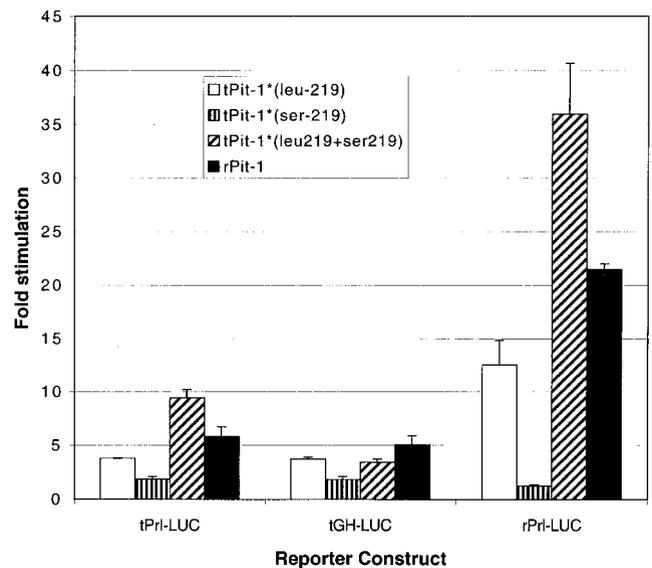


FIG. 2. Activation of the turkey prolactin, turkey growth hormone, and rat prolactin promoters by turkey and rat Pit-1. Two tPit-1* variants (leu-219 and ser-219) and rat Pit-1 were cotransfected with reporter vectors tPrl-LUC (bars 1-4), tGH-LUC (bars 5-8), or rPRL-LUC (bars 9-12). A CMV- β -galactosidase construct was also cotransfected to control for differences in transfection efficiency. Luciferase activity was divided by β -galactosidase activity to normalize the results. Fold stimulation represents corrected luciferase activity relative to luciferase activity from a cotransfection with a tPit-1-less expression vector. Values shown are the average of duplicate samples from two different experiments \pm standard error.

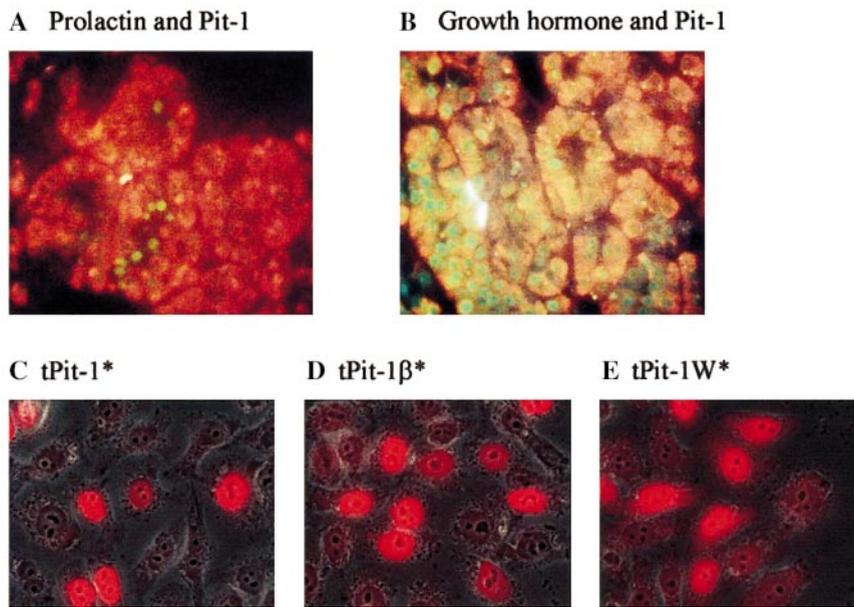


FIG. 3. Immunofluorescent detection of Pit-1 and/or prolactin and growth hormone in pituitary sections of laying hens and microinjected HeLa cells. (A) Dual-label immunofluorescence for tPit-1 (yellow-green) and prolactin (red) in the cephalic lobe of laying hens. (B) Dual-label immunofluorescence for tPit-1 (yellow-green) and GH (red) in the caudal lobe of laying hens. Original magnification is $\times 630$. Phase-contrast and fluorescent imaging of tPit-1* (C), tPit-1 β * (D), and tPit-1W* (E) expression in microinjected HeLa cells. Fixed cells were first photographed under phase-contrast and then photographed by fluorescence microscopy. The images were merged. Fluorescence is represented by red pseudocoloring. Original magnification is $\times 1500$.

intense staining for Prl (red) is observed in the cytoplasm of cells from the cephalic lobe, whereas the nuclei are not stained for Pit-1 (yellow-green). A few cells show Pit-1 staining in the absence of costaining for Prl and are presumed to represent nonlactotroph cells. In Fig. 3B, the majority of the cells in the caudal lobe costain for GH (red) and Pit-1 (yellow-green), demonstrating that Pit-1 is present in GH-expressing cells.

To determine whether the rabbit anti-rat Pit-1 antibody recognizes all three tPit-1 isoforms, expression vectors containing tPit-1*, tPit-1 β *, and tPit-1W* cDNAs were microinjected into HeLa cells. Expression of all tPit-1 isoforms is clearly detectable by immunofluorescence in the nuclei (Figs. 3C–3E). There was no detectable fluorescence in the nuclei of HeLa cells microinjected with the vector lacking tPit-1 cDNAs (data not shown). These results demonstrate that the rabbit anti-rat Pit-1 antibody can recognize all turkey Pit-1 isoforms and that the lack of Pit-1 staining in the nuclei of lactotrophs is due to the absence of Pit-1 and not the inability of the antibody to detect turkey Pit-1.

Together, the cotransfection and immunofluorescence data indicate that in the domestic turkey, Pit-1 can activate the turkey Prl promoter *in vitro*, but does not appear to play a role in regulating Prl gene expression *in vivo* because Pit-1 is not detectable in the nuclei of lactotrophs. Pit-1, however, still likely plays a role in regulating GH gene expression.

DISCUSSION

The amino acid sequence of Pit-1 is highly conserved in mammals, birds, and fish. One notable exception is the presence of a 38- or 33-amino acid-encoding exon between exons 2 and 3 of turkeys and fish, respectively. These additional amino acids are inserted between amino acids 72 and 73 of mammalian Pit-1, which lies on the border of the mapped transcriptional activation domain. Using deletion analysis, Theill *et al.* (1989) mapped the transactivation domain to amino acids 1–73 and Ingraham *et al.* (1990)

mapped the transactivation domain to amino acids 8–80. Our results showed that activation of the tPrl, tGH, and rPrl promoters by tPit-1* and rPit-1 was comparable *in vitro*, demonstrating that the presence of the additional 38 amino acids does not significantly affect Pit-1 activity *in vitro*.

A comparison of Pit-1 from a number of species has identified a set of invariant amino acids in the POU-specific domain (Schöler, 1991). This domain plays an important role in increasing the affinity and specificity of binding of Pit-1 to DNA, and thus mutations in this domain may affect Pit-1 function (Ingraham *et al.*, 1990). Our previously reported cDNA sequence for tPit-1 contained a serine at position 219 (Kurima *et al.*, 1998). The mRNA for the original cDNA library was extracted from a turkey hen from the University of Minnesota flock. This amino acid corresponds to amino acid 176 (leu) in mammalian Pit-1, which is amino acid 59 in subdomain B of the POU-specific domain (Schöler, 1991). The leucine at this position was found to be invariant among several POU proteins. A new pituitary RT-PCR product, containing leu-219, was synthesized from turkeys obtained from Nicholas Turkey Breeding Farms. Currently, it is not known whether the tPit-1*(ser-219) variant represents a cloning artifact or a natural mutation in a hen in the University of Minnesota flock in 1988, when the cDNA library was constructed.

Mutation of this amino acid had a marked effect on the activity of Pit-1 on its target promoters. Turkey Pit-1*(ser-219) showed a 2-, 2-, and 10-fold reduction in activity compared with tPit-1*(leu-219) when tested with the tPrl, tGH, and rPrl promoters, respectively. These results demonstrate the importance of leucine at position 59 in subdomain B of the POU-specific domain. The molecular basis for this change in activity is not known. Jacobson *et al.* (1997) have shown by high-resolution X-ray analysis that leucine in this position does not directly contact DNA. Surprisingly, coexpression of tPit-1*(ser-219) with tPit-1*(leu-219) resulted in a 2.5-fold increase in activation of the tPrl promoter and a 2.9-fold increase in activation of the rat Prl promoter compared to tPit-1*(leu-219) alone, but had no effect on the tGH promoter. This enhanced activity is presumably due to heterodimer formation between tPit-1*(ser-219) and tPit-1*(leu-219), but it is not known why the stimulatory effect is observed with

both the turkey and the rat Prl promoters but not with the turkey GH promoter.

Activation of the rPrl promoter was consistently greater than that of the tPrl promoter. Ohkubo *et al.* (2000) have similarly shown that the chicken Prl promoter is activated only three- to ninefold in rat pituitary GH₃ cells. This finding could be related to the number of Pit-1 binding sites in the avian and mammalian Prl genes. The rat Prl gene contains eight Pit-1 binding sites (Nelson *et al.*, 1988) in the 5' flanking region, whereas the turkey Prl (Kurima *et al.*, 1995) and chicken Prl (Ohkubo *et al.*, 2000) genes contain only two putative Pit-1 binding sites. It is possible that activation of the Prl promoter is regulated in part by the number of Pit-1 molecules bound. Consistent with this hypothesis, Nelson *et al.* (1988) and Mangalam *et al.* (1989) have shown that deletion or mutation of individual Pit-1 binding sites results in a decrease in promoter activation by Pit-1 in cotransfection assays.

The lack of Pit-1 nuclear costaining in turkey lactotrophs was an unexpected finding. In rats and humans both GH- and Prl-expressing cells costain for Pit-1 (Bodner *et al.*, 1988; Puy and Asa, 1996). These results differ from those reported here. In a laying turkey hen, the cephalic lobe of the pituitary is normally heavily populated with Prl-expressing lactotrophs, whereas the caudal lobe contains GH-expressing somatotrophs (Ramesh *et al.*, 1996). Immunostaining of the cephalic lobe revealed that Prl-expressing cells (lactotrophs) did not show nuclear costaining for Pit-1. In contrast, GH-expressing cells (somatotrophs) in the caudal lobe showed nuclear costaining for Pit-1. The lack of Pit-1 staining in lactotrophs is not due to the inability of the rabbit anti-rat Pit-1 antibody to detect the tPit-1*, tPit-1β*, and tPit-1W* isoforms. It is possible that this antibody does not recognize a tPit-1 isoform that has not yet been identified. This possibility, however, seems unlikely since this anti-rat Pit-1 antiserum is polyclonal and can detect at least two tPit-1 isoforms on Western blots (Kurima *et al.*, 1998). Furthermore, in chickens only cPit-1 and the cPit-1γ isoform, which is equivalent to tPit-1W*, have been identified (Tanaka *et al.*, 1999).

Ohkubo *et al.* (2000) reported that Pit-1 could bind to and activate the chicken Prl promoter, suggesting that Pit-1 plays a role in regulating the chicken Prl gene. This group used transfection of cPrl-luciferase reporter constructs into rat pituitary GH₃ cells and gel

shift/supershift assays using nuclear extracts prepared from chicken pituitaries or GH₃ cells. The interaction of Pit-1 with the chicken Prl promoter seen in the gel shift assays may be an anomaly of this *in vitro* system. Because both turkey and chicken pituitaries contain many cell types, nuclear extracts prepared from whole pituitaries would contain a mixture of nuclear proteins from all pituitary cells. If the pattern of Pit-1 expression in turkey pituitaries is similar to that in chickens, then the Pit-1 in the nuclear extract is likely from nonlactotroph cells, such as somatotrophs. Thus, the interaction of Pit-1 with the chicken prolactin gene observed in the gel shift assay would not be expected to occur *in vivo*.

The present results suggest that the mechanism for regulating Prl gene expression in birds is different from that in mammals. In mammals, Prl secretion is under tonic inhibitory control by dopamine. Studies with primary pituitary cultures have shown that dopamine suppresses transcription of the Prl gene (Maurer, 1981). Elsholtz *et al.* (1991) have further shown that a minimal rat Prl or Pit-1 promoter region, containing a single Pit-1 binding site, was sufficient to confer dopamine inhibition. These data suggest that dopamine may regulate the DNA binding or transactivating functions of Pit-1. In contrast, prolactin secretion and expression in birds is mainly under the control of the positive-acting, hypothalamic Prl-releasing factor, vasoactive intestinal polypeptide (VIP). VIP has been shown to increase Prl mRNA abundance in perfused chicken cephalic lobes (Kansaku *et al.*, 1995) and turkey anterior pituitary cell cultures (Xu *et al.*, 1996). Dopamine plays a different role in regulating Prl gene expression in birds (Youngren *et al.*, 1996). Xu *et al.* (1996) have shown that a dopamine D2 receptor agonist blocked the VIP-stimulated increase in Prl mRNA levels in turkey pituitary cells. Thus, in mammals a positive-acting factor such as Pit-1 is necessary for activation of Prl gene expression, whereas in birds Pit-1 may be redundant because of the positive action of VIP.

Furthermore, activation of the mammalian Prl gene requires both Pit-1 and the estrogen receptor. The distal enhancer element (–1550 to –1718) of the rat Prl gene contains four Pit-1 binding sites adjacent to an estrogen response element (Nelson *et al.*, 1988). Day *et al.* (1990) have shown that a cooperative interaction between Pit-1 and the estrogen receptor is required for

estrogen responsiveness of the rat Prl gene. In contrast, computer analysis of the turkey Prl gene revealed neither distal Pit-1 binding sites nor an estrogen response element (Kurima *et al.*, 1995). The lack of an estrogen response element is consistent with the findings that cultured turkey pituitary cells did not secrete Prl in response to estrogen (Knapp *et al.*, 1988). Because estrogen and the estrogen receptor do not appear to play a role in regulating the turkey Prl gene, the presence of Pit-1 as an interacting protein is also likely not necessary.

In summary, the immunofluorescence and cotransfection data together indicate that Pit-1 can activate the tPrl and tGH genes *in vitro*, but Pit-1 may not play a role in regulating Prl gene expression *in vivo*. Pit-1, however, likely still plays an important role in regulating turkey GH gene expression *in vivo*. The results for activation of the turkey Prl promoter are different from that of mammals, whereas results for GH activation are similar. Thus, although much can be learned from mammalian systems, caution must always be used when this knowledge is applied to avian systems.

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REFERENCES

- Berghman, L. R., van Beeumen, J., Decuyper, E., Kuhn, E. F., and Vandesande, F. (1988). One-step purification of chicken growth hormone from a crude pituitary extract by use of a monoclonal immunoabsorbent. *J. Endocrinol.* **118**, 381–387.
- Berghman, L. R., Grilles, L., Vanhamme, L., Proudman, J. A., Foidart, A., Balthazard, J., and Vandesande, F. (1992). Immunocytochemistry and immunoblotting of avian prolactin using polyclonal and monoclonal antibodies toward a synthetic fragment of chicken prolactin. *Gen. Comp. Endocrinol.* **85**, 346–357.

- Bodner, M., Castrillo, J.-L., Theill, L. E., Deerinck, T., Ellisman, M., and Karin M. (1988). The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* **55**, 505–518.
- Chen, R., Ingraham, H. A., Treacy, M. N., Albert, V. R., Wilson, L., and Rosenfeld, M. G. (1990). Autoregulation of Pit-1 gene expression is mediated by two *cis*-active elements. *Nature* **346**, 583–586.
- Cohen, L. E., Wondisford, F. E., and Radovick, S. (1996). Role of Pit-1 in the gene expression of growth hormone, prolactin, and thyrotropin. *Endocrinol. Metab. Clin. North. Am.* **25**, 523–540.
- Day, R. N., and Day, K. H. (1994). An alternatively spliced form of Pit-1 represses prolactin gene expression. *Mol. Endocrinol.* **8**, 374–381.
- Day, R. N., Koike, S., Sakai, M., Muramatsu, M., and Maurer, R. A. (1990). Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. *Mol. Endocrinol.* **4**, 1964–1971.
- DiMattia, G. E., Rhodes, S. J., Krones, A., Carriere, C., O'Connell, S., Kalla, K., Arias, C., Sawchenko, P., and Rosenfeld, M. G. (1997). The Pit-1 gene is regulated by distinct early and late pituitary-specific enhancers. *Dev. Biol.* **182**, 180–190.
- Elsholtz, H. P., Lew, A. M., Albert, P. R., and Sundmark, V. C. (1991). Inhibitory control of prolactin and Pit-1 gene promoters by dopamine. *J. Biol. Chem.* **266**, 2919–2925.
- Foster, D. N., Kim, S. U., Enyeart, J. J., and Foster, L. K. (1990). Nucleotide sequence of the complementary DNA for turkey growth hormone. *Biochem. Biophys. Res. Commun.* **173**, 967–975.
- Fox, S. R., Jong, M. T. C., Casanova, J., Ye, Z.-S., Stanley, F., and Samuels, H. H. (1990). The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell-specific elements of both the growth hormone and prolactin gene promoters. *Mol. Endocrinol.* **4**, 1069–1080.
- Haugen, B. R., Gordon, D. F., Nelson, A. R., Wood, W. M., and Ridgway, E. C. (1994). The combination of Pit-1 and Pit-1T have a synergistic stimulatory effect on the thyrotropin β -subunit promoter but not the growth hormone or prolactin promoters. *Mol. Endocrinol.* **8**, 1574–1582.
- Ingraham, H. A., Chen, R., Mangalam, H. J., Elsholtz, H. P., Flynn, S. E., Lin, C. R., Simmons, D. M., Swanson, L., and Rosenfeld, M. G. (1988). A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* **55**, 519–529.
- Ingraham, H. A., Albert, V. R., Chen, R., Crenshaw, E. B., III, Elsholtz, H. P., He, X., Kapiloff, M. S., Mangalam, H. J., Swanson, L. W., Treacy, M. N., and Rosenfeld, M. G. (1990). A family of POU-domain and Pit-1 tissue-specific transcription factors in pituitary and neuroendocrine development. *Annu. Rev. Physiol.* **52**, 773–791.
- Jacobson, E. M., Li, P., Leon-del-Rio, A., Rosenfeld, M. G., and Aggarwal, A. K. (1997). Structure of Pit-1 POU domain bound to DNA as a dimer: Unexpected arrangement and flexibility. *Genes Dev.* **11**, 198–212.
- Kansaku, N., Shimada, K., and Saito, N. (1995). Regionalized gene expression of prolactin and growth hormone in the chicken anterior pituitary gland. *Gen. Comp. Endocrinol.* **99**, 60–68.
- Knapp, T. R., Fehrer, S. C., Silsby, J. L., Porter, T. E., Behnke, E. J., and El Halawani, M. E. (1988). Gonadal steroid modulation of basal and vasoactive intestinal polypeptide-stimulated prolactin release by turkey anterior pituitary cells. *Gen. Comp. Endocrinol.* **72**, 226–236.
- Konzak, K. E., and Moore, D. D. (1992). Functional isoforms of Pit-1 generated by alternative messenger RNA splicing. *Mol. Endocrinol.* **6**, 241–247.
- Kurima, K., Proudman, J. A., El Halawani, M. E., and Wong, E. A. (1995). The turkey prolactin-encoding gene and its regulatory region. *Gene* **156**, 309–310.
- Kurima, K., Weatherly, K. L., Sharova, L., and Wong, E. A. (1998). Synthesis of turkey Pit-1 mRNA variants by alternative splicing and transcription initiation. *DNA Cell Biol.* **13**, 93–103.
- Mangalam, H. J., Albert, V. R., Ingraham, H. A., Kapiloff, M., Wilson, L., Nelson, C., Elsholtz, H., and Rosenfeld, M. G. (1989). A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. *Genes Dev.* **3**, 946–958.
- Maurer, R. A. (1981). Transcriptional regulation of the prolactin gene by ergocryptine and cyclic AMP. *Nature* **294**, 94–97.
- McCormick, A., Brady, H., Theill, L. E., and Karin, M. (1990). Regulation of the pituitary-specific homeobox gene *GHF1* by cell-autonomous and environmental cues. *Nature* **345**, 829–832.
- Morris, A. E., Kloss, B., McChesney, R. E., Bancroft, C., and Chasin, L. A. (1992). An alternatively spliced Pit-1 isoform altered in its ability to trans-activate. *Nucleic Acids Res.* **20**, 1355–1361.
- Nelson, C., Albert, V. R., Elsholtz, H. P., Lu, L. I.-W., and Rosenfeld, M. G. (1988). Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. *Science* **239**, 1400–1405.
- Ohkubo, T., Araki, M., Tanaka, M., Sudo, S., and Nakashima, K. (1996). Molecular cloning and characterization of the yellowtail GH gene and its promoter: A consensus sequence for teleost and avian Pit-1/GHF-1 binding sites. *J. Mol. Endocrinol.* **16**, 63–72.
- Ohkubo, T., Tanaka, M., and Nakashima, K. (2000). Molecular cloning of the chicken prolactin gene and activation by Pit-1 and cAMP-induced factor in GH3 cells. *Gen. Comp. Endocrinol.* **119**, 208–216.
- Ono, M., and Takayama, Y. (1992). Structures of cDNAs encoding chum salmon pituitary-specific transcription factor, Pit-1/GHF-1. *Gene* **226**, 275–279.
- Puy, L. A., and Asa, S. L. (1996). The ontogeny of Pit-1 expression in the human fetal pituitary gland. *Neuroendocrinology* **63**, 349–355.
- Ramesh, R., Proudman, J. A., and Kuenzel, W. J. (1996). Changes in pituitary somatotroph and lactotroph distribution in laying and incubating turkey hens. *Gen. Comp. Endocrinol.* **104**, 67–75.
- Ramesh, R., Solow, R., Proudman, J. A., and Kuenzel, W. J. (1998). Identification of mammosomatotrophs in the turkey pituitary: Increased abundance during hyperprolactinemia. *Endocrinology* **139**, 781–786.
- Schöler, H. R. (1991). Octamania: The POU factors in murine development. *Trends Genet.* **7**, 323–329.
- Simmons, D. M., Voss, J. W., Ingraham, H. A., Holloway, J. M., Broide, R. S., Rosenfeld, M. G., and Swanson, L. W. (1990). Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev.* **4**, 695–711.

- Storrie, B., White, J., Rottger, S., Stelzer, E. H., Suganuma, T., and Nilsson, T. (1998). Recycling of golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. *J. Cell Biol.* **143**, 1505–1521.
- Tanaka, M., Hosokawa, Y., Watahiki, M., and Nakashima, K. (1992). Structure of the chicken growth hormone-encoding gene and its promoter region. *Gene* **112**, 235–239.
- Tanaka, M., Yamamoto, I., Ohkubo, T., Wakita, M., Hoshino, S., and Nakashima, K. (1999). cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. *Gen. Comp. Endocrinol.* **114**, 441–448.
- Theill, L. E., Castrillo, J. L., Wu, D., and Karin, M. (1989). Dissection of functional domains of the pituitary-specific transcription factor GHF-1. *Nature* **342**, 945–948.
- Theill, L. E., Hattori, K., Lazzaro, D., Castrillo, J.-L., and Karin, M. (1992). Differential splicing of the GHF-1 primary transcript gives rise to two functionally distinct homeodomain proteins. *EMBO J.* **11**, 2261–2269.
- Voss, J. W., Wilson, L., Rhodes, S. J., and Rosenfeld, M. G. (1994). An alternative Pit-1 RNA splicing product reveals modular binding and nonmodular transcriptional activities of the POU-specific domain. *Mol. Endocrinol.* **7**, 1551–1560.
- Xu, M., Proudman, J. A., Pitts, G. R., Wong, E. A., Foster, D. N., and El Halawani, M. E. (1996). Vasoactive intestinal peptide stimulates prolactin mRNA expression in turkey pituitary cells: Effects of dopaminergic drugs. *Proc. Soc. Exp. Biol. Med.* **212**, 52–62.
- Youngren, O. M., Pitts, G. R., Phillips, R. E., and El Halawani, M. E. (1996). Dopaminergic control of prolactin secretion in the turkey. *Gen. Comp. Endocrinol.* **104**, 225–230.