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.

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).
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′-AACAGGAAACCACAGCTA-3′, nt 247 to 264 in exon 6; Kurima et al., 1998). PCR was performed with primers tPit-S45 (5′-GCTGCCATTAATCGCT-3′, nt 56 to 74 in exon 1) and tPit-AS44 (5′-GCTTACCGCAGCTGTGCTG-3′, nt 214 to 234 in exon 6) for tPit-1* and tPit-1β*. Primer tPit-S55 (5′-GCGAGGAAGTATAGAGCC-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
Cells were washed in phosphate-buffered saline (PBS) extracts were prepared 42–45 h after transfection. Cells were cultured and transfected with a DNA:lipofectamine complex containing 25 μg lipofectamine (Life Technologies, Gaithersburg, MD), 1 μg Pit-1 expression vector, 5 μg promoter–luciferase reporter vector, and 0.125 μg 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 μl of reporter lysis buffer (Promega).

Luciferase activity was measured in a Lumat LB9501 luminometer (Berthold, Bad Wilbad, Germany) by mixing of 50 μl of cell lysate with 300 μl of ATP buffer (5 mM ATP, 25 mM glycylglycine, pH 7.8, 15 mM MgSO₄) and injection of 100 μl luciferin (1 mM luciferin, 25 mM glycylglycine, 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 μl of reporter lysis buffer and mixed with 250 μl 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 μl 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 micro-injected with tPit-1 expression vectors at a concentration of 300 ng/μl. 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 co-localization 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 μg/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(hee-219) activated the tPrl, tGH, and rPrl promoters 3.8-fold (bar 1),
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 tPit1*(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, 

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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.

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**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 ± standard error.
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-1β*, 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 to tPit-1* alone, but had no effect on the tPrl 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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