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REGULATION OF GROWTH HORMONE EXPRESSION BY THYROTROPIN-RELEASING HORMONE THROUGH THE PITUITARY-SPECIFIC TRANSCRIPTION FACTOR PIT-1 IN CHICKEN PITUITARY

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Pit-1 is a pituitary-specific POU-domain DNA binding factor, which binds to and trans-activates promoters of growth hormone- (GH), prolactin- (PRL) and thyroid stimulating hormone beta- (TSHβ) encoding genes. Pit-1 has been identified in several mammalian and avian species. Thyrotropin-releasing hormone (TRH) is located in the hypothalamus and it stimulates TSH, GH and PRL release from the pituitary gland. In the present study, we successfully developed a competitive RT-PCR for the detection of Pit-1 expression in the chicken pituitary, that was sensitive enough to detect picogram levels of Pit-1 mRNA. Applying this method, the effect of TRH injections on Pit-1 mRNA expression was determined in the pituitary of chick embryos and growing chicks. In both 18-day-old embryos and 10-day-old male chicks the Pit-1 mRNA expression was significantly increased following TRH injection, thereby indicating that the stimulatory effects of TRH on several pituitary hormones is mediated via its effect on Pit-1 expression. Therefore, a semi-quantitative RT-PCR method was used to detect possible changes in GH levels. TRH affected the GH mRNA levels at both developmental stages. These results, combined with the data on Pit-1 mRNA expression, indicate that Pit-1 has a role in mediating the stimulatory effects of TRH on pituitary hormones like GH.

Key words: Chicken, pituitary, Pit-1, TRH, GH

Pit-1, originally discovered in mammalian pituitary (Bodner et al., 1988), is a pituitary-specific POU-domain DNA binding factor, which binds to and trans-activates promoters of growth hormone- (GH), prolactin- (PRL) and thyroid stimulating hormone-β- (TSHβ) encoding genes (Bodner et al., 1988; Steinfelder et al., 1992). Pit-1 gene expression is autoregulated and its initial activa-

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tion is under the control of the *Prophet of Pit-1 (PROP-1)* gene (Sornson et al., 1996). Although Pit-1 mRNA is found in all five types of hormone-producing anterior pituitary cells, its protein is mainly expressed in the lactotrophs, somatotrophs and thyrotrophs which produce PRL, GH or TSHβ.

The importance of Pit-1 as a regulator of anterior pituitary development has been demonstrated in studies on dwarf mouse strains (Camper et al., 1990) that have hypoplastic anterior pituitaries and a conspicuous absence of three pituitary cell types – lactotrophs, somatotrophs and thyrotrophs. These data indicate that Pit-1 is necessary for the ontogeny of GH-, PRL- and TSHβ-producing cells. Thus the expression of Pit-1 precedes that of GH, PRL and TSHβ during embryogenic development in the rat and mouse, illustrating the importance of Pit-1 in the regulation of the production of GH, PRL and TSHβ.

In non-mammalian species, Pit-1 has already been cloned from chicken pituitary (Van As et al., 2000). In an earlier study, we determined the ontogeny of Pit-1 during embryonic life of the chicken (day 1–day 21 of incubation) (Van As et al., 2000). The mRNA for Pit-1 was first detected in the pituitary on day 5 of embryonic life. The presence of Pit-1 at this early stage of embryonic development suggests that Pit-1 may be physiologically important during embryonic development of birds as it precedes the gene expression and secretion of GH, PRL and TSHβ. It, however, remains to be established whether this early expression of Pit-1 is necessary for the initiation of GH, PRL and TSHβ expression in avian species.

Thyrotropin-releasing hormone (TRH) is produced in the hypothalamus and it stimulates thyrotropin (TSH) release from the pituitary gland. TRH also releases GH in vertebrates including birds (Harvey, 1990). The sensitivity and responsiveness of avian somatotrophs to mammalian TRH stimulation *in vivo* exceeds that to mammalian GHRHs (Harvey and Scanes, 1984). The potent GH-releasing activity of TRH in young chicken, however, is well in contrast with the small or no increase in adult chickens (Harvey et al., 1981). Since TRH directly affects the chicken pituitary, TRH pituitary receptors are likely to mediate the effect of TRH on GH and TSH secretion (Harvey and Baidwan, 1989). TRH also has an effect on the PRL secretion of domestic fowl *in vitro* and *in vivo* (Harvey et al., 1978). All the actions of TRH on PRL (Yan et al., 1991) and TSHβ (Steinfelder et al., 1992) secretion and gene expression are known to be mediated through Pit-1 in mammalian species.

Whether there is a similar regulatory mechanism in birds remains to be established. To find out if Pit-1 is involved in the transcriptional activation of several pituitary hormones by TRH in chicken, we decided to study the expression of Pit-1 and GH after TRH-administration in developing and posthatch chicks. In order to study Pit-1 expression we developed a very sensitive and quantitative competitive RT-PCR, that would allow the detection of pg levels of Pit-1 mRNA.

Materials and methods

Experimental animals

First experiment. Fertilised eggs of Ross chickens (Avibel, Zoersel, Belgium) were incubated at 37.8 ± 0.2 °C and 60% relative humidity. At day 18 after setting, 140 embryos were injected with TRH [3.15 µg/kg body weight (Geris et al., 1998)] (Neosystem, Strasbourg, France), or with a saline solution as a control (n = 60 for TRH and n = 60 for control) or no injection (n = 20). All injections were given intravenously, in an allantoic blood vessel situated near the egg shell membrane. Before injection embryos were killed to obtain pituitaries from untreated animals. At several time intervals after the injection of TRH or saline (5, 15 and 30 min), embryos (n = 20 per time interval and per injection) were killed and pituitaries were removed and pooled in 4 groups of 5 embryos/time interval for the extraction of total RNA to determine the effect of TRH on the expression of Pit-1 and GH.

Second experiment. Two-hundred and twenty 10-day-old male Ross chicks were injected with TRH (3.15 µg/kg body weight) (Neosystem) (n = 100) or a saline solution (as control) (n = 100) or no injection (n = 20). Before injection and at several time intervals after the injection of TRH or saline (5, 10, 15, 30 and 60 min) (n = 20 per time interval and per injection), the chicks were killed and pituitaries were removed and pooled in 4 groups of 5 embryos/time interval for the extraction of total RNA to determine the effect of TRH on Pit-1 and GH expression.

The ethical committee for animal experiments of the K. U. Leuven approved all of the experimental protocols.

Primer sequences

The oligonucleotide primers used for RT-PCR of Pit-1 were selected by a computer program (*DNA*MAN for Windows, Lynnon Biosoft, USA) and synthesised for our use by Invitrogen (Merelbeke, Belgium).

The sense and anti-sense primers for Pit-1 were chosen at an almost equal distance from a unique restriction site for *Nsi*I. The sense sequence of the primer used for Pit-1 was 5'-GCT GCT GTG CAC GGC TCT GAA-3' and the anti-sense sequence was 5'-CTG CGC TTC CTC TTC CGC TC-3' and were both based on the ggPit-1 sequence reported (Van As et al., 2000). The sequence predicts a native PCR product of 182 base pairs (bp) corresponding to bases 601–782. The unique restriction site for *Nsi*I is located at base 678.

The sense sequence for the primer used for GH was 5'-CAT CCA GTC CTG GCT GAC TCC CGT GCA-3' and the anti-sense sequence was 5'-GCG TCC TCG TTG CGC AGG TGG ATG T-3' based on the sequence reported pre-

viously (Lamb et al., 1988). The sequence predicts a native PCR product of 216 bp corresponding to bases 363–578.

The sense sequence for the primer used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was 5'-TTG GCC GTA TTG GCC GCC TG-3' and the anti-sense sequence was 5'- TTG CTG GGG TCA CGC TCC TG-3' based on the sequence reported (Panabieres et al., 1984). The sequence predicts a native PCR product of 220 bp corresponding to bases 25–244.

Reverse transcription

Total RNA was extracted from chicken pituitaries using the Trizol® reagent (Invitrogen). Complementary DNA was synthesised by the extension of the anti-sense primer. Nine microlitres of RT-mix for each sample (1 µg total RNA) was prepared by using single-strength first-strand buffer (Invitrogen), 5 U RNasin (Promega, Leiden, The Netherlands), 10 U Moloney murine leukaemia virus reverse transcriptase (Invitrogen), 0.5 mM PCR nucleotide mix (Promega), 5 mM DTT (Invitrogen), and 1 µM of anti-sense primer. The RT reaction was as follows: 37 °C for 45 min followed by 5 min at 95 °C.

PCR amplification

PCR was performed by adding the following components to the reaction mixture: 10 × REDTaq DNA Polymerase buffer (Sigma, Saint Louis, USA), 0.25 mM PCR nucleotide mix (Promega), 0.5 µM of each primer and 7.5 U REDTaq™ DNA polymerase (Sigma) in a total volume of 40 µl. The PCR reaction for Pit-1 was performed as follows: 95 °C for 3 min; 30 cycles at 93 °C for 45 sec, 56 °C for 1 min, and 72 °C for 15 sec; and a final extension for 5 min at 72 °C. The PCR reaction for GH was performed as follows: 95 °C for 3 min; 30 cycles at 94 °C for 45 sec, 55 °C for 45 sec, and 72 °C for 25 sec; and a final extension for 5 min at 72 °C. The same PCR reaction for GH was used for GAPDH. PCR products were size separated by electrophoresis on 2% agarose gel (Invitrogen) containing 1% ethidium bromide. A 100-bp DNA ladder (Eurogentec, Herstal, Belgium) was used as molecular weight standard. After electrophoresis, the gel was scanned using a densitometer (Image Master VDS, Pharmacia Biotech). Negative control RT-PCR with diethyl pyrocarbonate-treated water was included in all experiments.

Preparation of internal standard

The PCR-Script™ Amp Cloning Kit (Strategene GmBH, Heidelberg, Germany) was used for the cloning of a Pit-1 cDNA fragment. This clone was then used for the construction of the internal standard (IS) (or mutant Pit-1 cDNA fragment). The Pit-1 cDNA fragment contained a unique restriction site for *Nsi*I. For the Pit-1 internal standard, this unique restriction site for *Nsi*I was

replaced by a unique restriction site for *Xba*I. The construction of the IS is illustrated in Fig. 1. Both primers used contained a specific tail of an adaptor sequence (5'-*TATCAA*-3') and the *Xba*I recognition sequence (5'-**TCTAGA**-3'). The 20 bp at the 3' end of the primers were complementary to the 20 bp that surrounded either site of the original *Nsi*I restriction site present in the cloned Pit-1 cDNA fragment. The sense sequence for this new primer used was 5'-*TAT CAA* **TCT AGA** CAT GAA ACT GAA ATC AAT AC-3' and the anti-sense sequence was 5'-*TAT CAA* **TCT AGA** CAT TCT TAA AGC TCA GCT-3'. PCR with those primers was performed by adding the same components to the reaction mix as mentioned in *'PCR amplification'*. The PCR reaction was performed as follows: 95 °C for 3 min; 30 cycles at 93 °C for 45 sec, 44 °C for 1 min, and 72 °C for 3.5 min; and a final extension for 5 min at 72 °C. The obtained pBluescript® II SK plasmid contained a Pit-1 cDNA fragment with a unique restriction site for *Xba*I instead of *Nsi*I.

In vitro *transcription of mutant RNA*

This mutant Pit-1 clone was then used as target DNA for the *in vitro* transcription of mutant RNA as described before (Stofflet et al., 1988). DNA was first linearised with restriction enzyme *Sac*I (Invitrogen). The enzyme was then removed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was desalted over a Sephadex G50 column (Pharmacia). The transcription reaction mixture (20 µl), containing 5 × transcription buffer, 0.5 mM of each NTP (Roche), 1.6 U RNase inhibitor (Roche), 40 U T7 RNA polymerase (Roche), 10 mM DTT (Invitrogen) and 4 µg of target DNA, was prepared. The mixture was incubated at 37 °C for 30 min and subsequently treated with 34 U RNase-free DNase (Roche) to fragment DNA. The reaction was stopped by the addition of EDTA to the final concentration of 5 mM.

Competitive RT-PCR

Three validating trials were conducted before conditions were established for ideal quantitative analysis. The amounts of IS, the starting amount of total RNA, and the serial dilutions were refined to permit adequate quantification of mRNA. Serial dilutions of competitor Pit-1 mutant RNA (10, 5, 2.5, 1.25, 0.625 and 0.3125 ng) were established. One µg total RNA from pituitary tissue was required for adequate quantification. Total RNA was mixed with an aliquot of a given dilution of competitor Pit-1 mutant RNA. Mixtures were transcribed into cDNA and co-amplified. Digestion of PCR products by *Xba*I selectively cuts DNA fragments derived from the IS. Samples were run on 3% agarose gel containing ethidium bromide. The relative amount of DNA in each band was measured by densitometry (Image Master VDS, Pharmacia). Using the Image Master densitometer software, the percentage digested PCR product relative to the total

product (100%) was calculated as a function of the initially added concentration of mutant RNA. A sigmoidal curve was fitted through the plot using the SlideWrite Plus program (Advanced Graphics Software Inc., Carlsbad, CA). From the 50% level of PCR product originating from each initial RNA, the initial concentration of mutant RNA was calculated. Since both RNAs amplify with the same efficiency, at the 50% level, the initial amount of the native mRNA is equal to the initial amount of added mutant RNA.

Quantification of Pit-1 mRNA in the pituitary of 18-day-old embryos and 10-day-old male Ross chicks

Decreasing amounts of IS were added to 1 µg of total RNA isolated from pituitary tissue. RT-PCR products were digested with *Xba*I and size separated by electrophoresis. The band size and intensity were determined: the upper band corresponding to the PCR product derived from the native RNA and the lower band from the digested IS. The amount of mRNA was calculated. The competitive RT-PCR was performed on the four different RNA pools of 5 embryos/chicks for each treatment.

Relative quantification of GH mRNA in the pituitary of 18-day-old embryos and 10-day-old male Ross chicks

The expression of the mRNA for GH was determined in the pituitary. The levels of expression were compared between the controls and treated samples by a semi-quantitative analysis method using the housekeeping gene GAPDH. After electrophoresis, gels were scanned and the intensities of the different bands were measured on a densitometer (Image Master VDS, Pharmacia Biotech, The Netherlands) as previously described. Data were normalised to GAPDH mRNA abundance and expressed as a relative amount.

Data analysis

Data on the amount of Pit-1 mRNA are presented as ng mRNA/µg total RNA. Those for GH mRNA are presented as the ratio GH:GAPDH. Statistical analyses were performed using the statistical package GLM (SAS Institute). The non-parametric Kruskal-Wallis test was performed to calculate differences between treatments and between times. P values of < 0.05 were considered statistically significant.

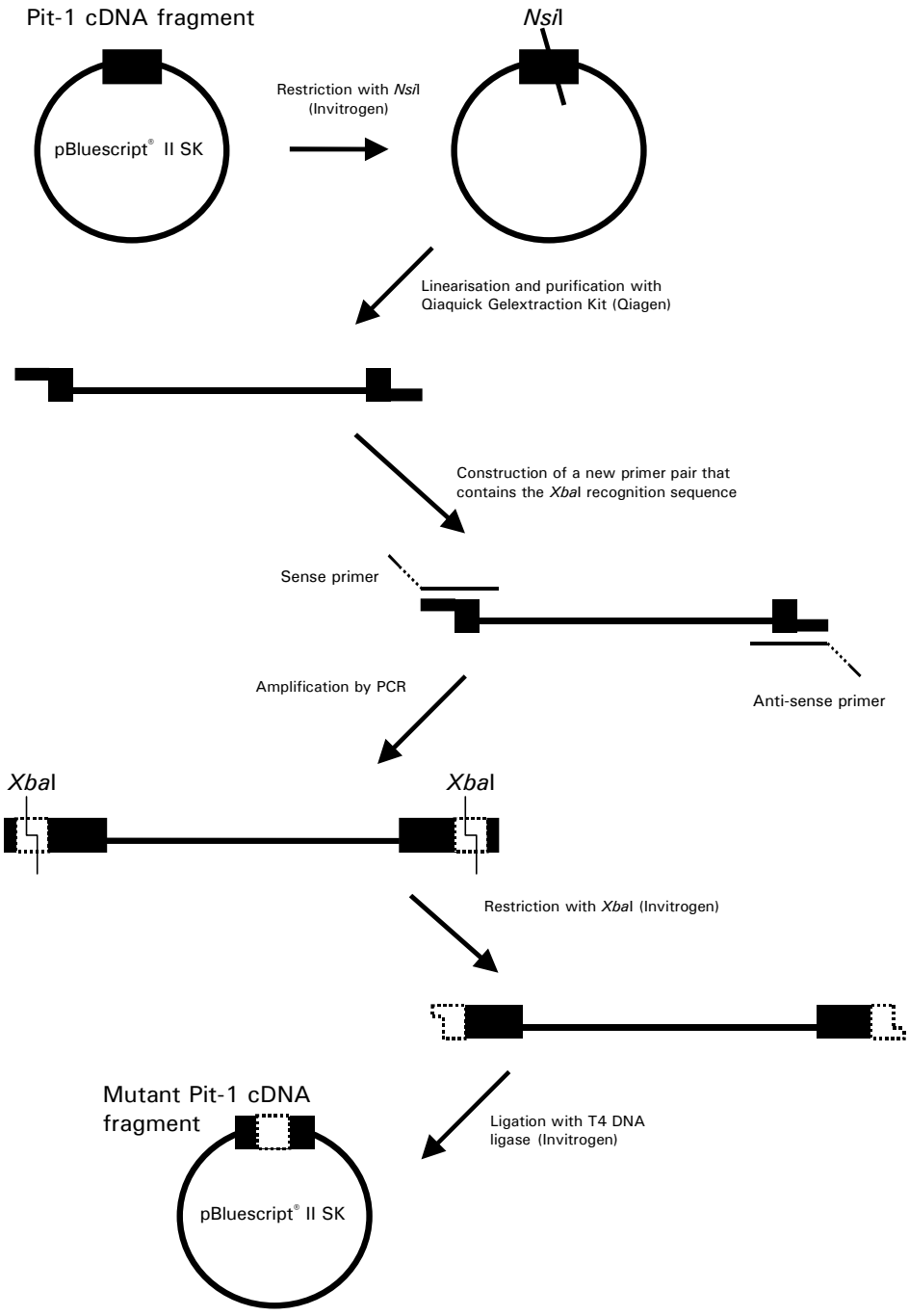

Fig. 1. Preparation of the internal standard for the Pit-1 competitive RT-PCR. The dotted lines represent the recognition sequence for *Xba*I

Results

Competitive RT-PCR

For Pit-1, the primers used for cDNA synthesis and for PCR defined a 182-bp fragment as a product from pituitary tissue RNA and two fragments (after digestion by *Xba*I) of 108-bp and 80-bp as a product from the mutant RNA (Fig. 2). Scans showed that digestion by *Xba*I selectively cuts DNA fragments derived from the mutant RNA (Fig. 3) into two subfragments, resulting in a series of double bands. The upper band of 182 bp on the gel represents the nondigested DNA fragment originating from the native RNA, while the lower two bands of 108 bp and 80 bp correspond to the digested DNA fragment originating from the mutant RNA. The comparison of the band intensities of the three bands, with the known concentration of the mutant RNA, gives a quantitative estimation of the Pit-1 mRNA concentration. The % non-digested PCR product compared to the total amount of RT-PCR product was plotted as a function of the initially added amount of mutant RNA (range 0–10 ng) and a standard curve was fitted.

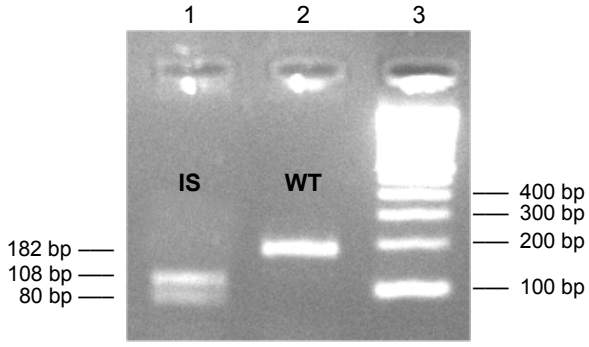

Fig. 2. Agarose gel electrophoresis (2%) of the Pit-1 RT-PCR products (after restriction with *Xba*I) obtained from lane 1: 10 ng mutant RNA (IS); lane 2: 1 µg total pituitary RNA (Wild Type: WT); lane 3: 100 bp DNA molecular weight marker

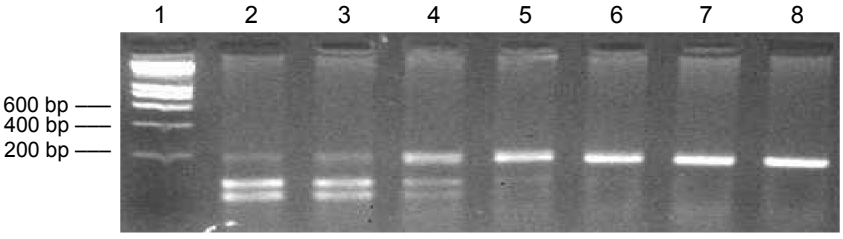

Fig. 3. Agarose gel electrophoresis (3%) of the Pit-1 competitive RT-PCR products from lane 1: 200 bp DNA molecular weight marker; lane 2: 1 µg total RNA + 10 ng IS; lane 3: 1 µg total RNA + 5 ng IS; lane 4: 1 µg total RNA + 2.5 ng IS; lane 5: 1 µg total RNA + 1.25 ng IS; lane 6: 1 µg total RNA + 0.625 ng IS; lane 7: 1 µg total RNA + 0.3125 ng IS; lane 8: 1 µg total RNA + 0 ng IS

First experiment

Effect of TRH injection on Pit-1 expression in the pituitary of 18-day-old chicken embryos. Before injection (0 min), the concentration of Pit-1 mRNA per µg total RNA was 1.20 ± 0.09 ng (Fig. 4A). This concentration decreased significantly within 5 min after injection of TRH or saline. Fifteen min after injection, the concentration of Pit-1 mRNA in the saline-treated animals was restored to the original level, but treatment with TRH caused a significant increase up to 2 ng Pit-1 mRNA per µg total RNA. A significant decrease compared to the observations at 15 min, from 2 to 0.6 ng Pit-1 mRNA in the TRH-treated animals and from 1.2 to 1 ng Pit-1 mRNA in the saline-treated animals, was observed 30 min after injection. The TRH-treated animals showed a significantly higher expression of Pit-1 mRNA compared to the controls at 15 min after the injection. However, the TRH-treated animals showed a significantly lower expression of Pit-1 mRNA compared to the controls at 30 min after the injection.

Effect of TRH injection on GH expression in the pituitary of 18-day-old chicken embryos. Before injection (0 min), the relative amount of GH mRNA was 2.21 ± 0.21 (Fig. 4B). A significant increase in the relative amount of GH mRNA (2.59 ± 0.31) in response to TRH was detected 15 min after the injection. This was followed by a further significant increase at 30 min (4.56 ± 0.39). The expression of GH mRNA in the saline-treated animals did not change throughout the sampling period and was similar to that observed in the uninjected controls.

Second experiment

Effect of TRH injection on Pit-1 expression in the pituitary of 10-day-old chicks. Before injection (0 min), the concentration of Pit-1 mRNA per µg total RNA was 0.68 ± 0.04 ng (Fig. 5A). This concentration increased significantly within 10 min of TRH administration. At 15 min, the concentration of Pit-1 mRNA further increased significantly above that recorded at 10 min. This was followed by a significant decrease at 30 min after injection. At 60 min, the Pit-1 mRNA concentration continued to decrease but levels remained significantly higher than those in uninjected controls. Throughout the sampling period, Pit-1 levels did not alter in response to saline injection.

Effect of TRH injection on GH expression in the pituitary of 10-day-old chicks. No significant changes in GH mRNA levels were detected until 15 min after injection with TRH (Fig. 5B). Peak levels were reached 30 min after injection. A significant decrease followed at 60 min after the injection although this level was still significantly higher then basal levels and the level reached after 15 min. No changes in GH mRNA levels in response to saline injection were detected.

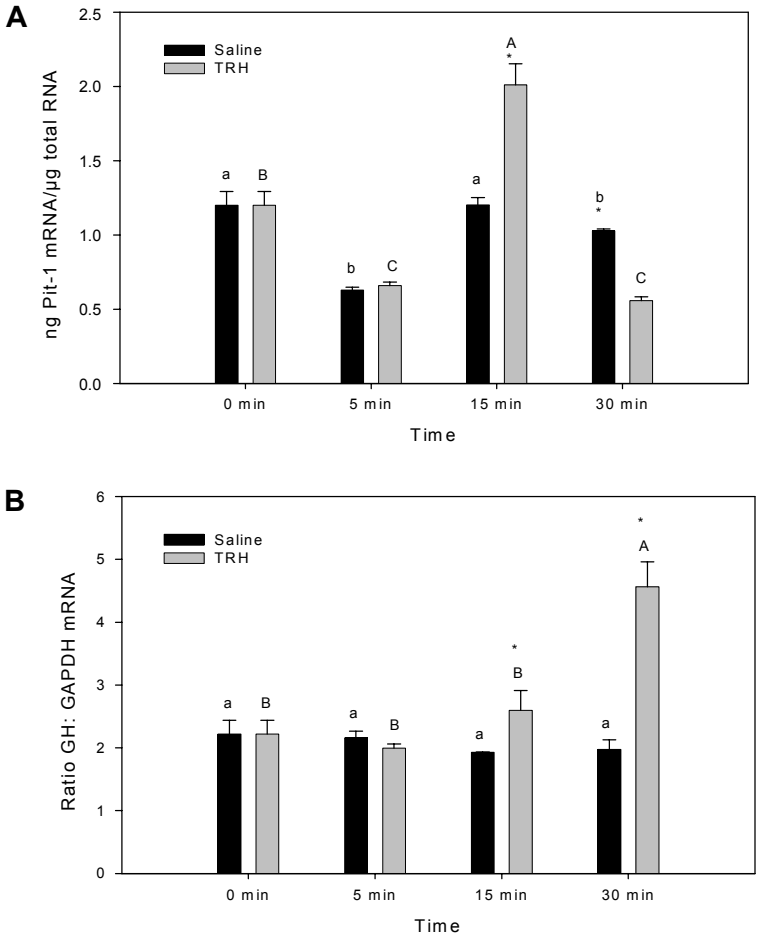

Fig. 4. **A.** Pit-1 mRNA concentration in the pituitary of 18-day-old chicken embryos: before injection (0 min) and 5, 15 and 30 min after the injection of TRH and saline. *: differences at one time point between treatments ($P < 0.05$); a, b, A, B, C: means with no common letter within one treatment are significantly different ($P<0.05$). **B.** Relative amount of GH mRNA in the pituitary of 18-day-old chicken embryos: before injection (0 min) and 5, 15 and 30 min after the injection of TRH and saline. *: differences at one time point between treatments ($P < 0.05$); a, b, A, B: means with no common letter within one treatment are significantly different ($P < 0.05$)

Discussion

To adequately quantify Pit-1 mRNA expression, we have developed a competitive RT-PCR in which specific internal standard molecules (Pit-1 mutant), differing in one restriction site in the amplified portions of the specific target molecules, are amplified simultaneously with the target samples. This method sets up a competition between the target molecule and the IS within the same

PCR reaction. Extracted pituitary RNA reverse transcribed, co-amplified in the presence of varying amounts of the developed IS, and digested with the appropriate enzyme adequately produced for Pit-1 a 182-bp band corresponding to the predicted target cDNA and two bands of 80 and 108 bp corresponding to the restricted IS cDNA. The data show that this method was able to measure mRNA levels for Pit-1 to picogram levels.

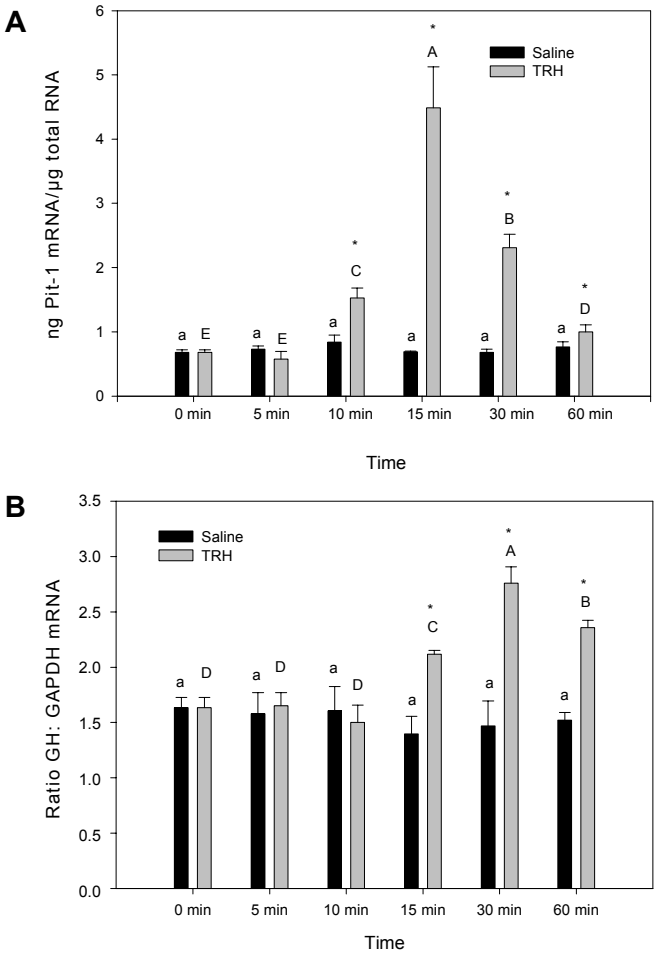

Fig. 5. **A.** Pit-1 mRNA concentration in the pituitary of 10-day-old male chicks: before (0 min) and 5, 10, 15, 30 and 60 min after the injection of TRH and saline. *: differences at one time point between treatments ($P < 0.05$); a, A, B, C, D, E: means with no common letter within one treatment are significantly different ($P < 0.05$). **B.** Relative amount of GH mRNA in the pituitary of 10-day-old male chicks: before (0 min) and 5, 10, 15, 30 and 60 min after the injection of TRH and saline. *: differences at one time point between treatments ($P < 0.05$); a, A, B, C, D: means with no common letter within one treatment are significantly different ($P < 0.05$)

This method was used to determine the absolute level of expression of Pit-1 in 18-day-old chicken embryos and 10-day-old male chicken after injection with TRH. The results clearly show that the injection of TRH increased Pit-1 mRNA levels at both developmental ages and this increase was then followed by a decrease. The data also suggest that the magnitude and the length of the response of Pit-1 expression to TRH increases with age, but that basal levels are higher in embryos than in growing chicks.

It is worth noting that the transient depression in Pit-1 levels in the embryo 5 min after injection was probably due to the stress of handling and injection as the effect was observed in both the control and TRH-injected animals. The response to TRH consisted of an increase (15 min) followed by a decrease (30 min) compared to controls. In 10-day-old chicks, animals showed no signs of injection-stress and Pit-1 levels in response to TRH increased with time of sampling to peak at 15 min, followed by a stepwise decrease to pre-injection levels at 60 min. Thus peak levels were reached in both the embryo and the 10-day-old chick at similar times after injection but the magnitude of response in the 10-day-old chick was a 5-fold increase while this was only 1.6-fold in the embryo. The effect of TRH on the Pit-1 mRNA expression in 10-day-old chicks is higher than in embryos and the effect lasted longer. These differential effects at different stages of development may suggest that the role of Pit-1, possibly in maintaining GH, TSHβ or PRL production, in the pituitary becomes more important with the age of the chicken. The effects on Pit-1 mRNA seen in embryos suggests a dual role of upregulation and downregulation by TRH that is only present in the embryo and disappears after hatching. Pit-1 is known to autoregulate its own expression. Two Pit-1 binding sites, Pit-1B1 and Pit-1B2, are present in the promoter of the Pit-1 gene. Binding of Pit-1 to Pit-1B1 will upregulate the expression but binding to Pit-1B2 will downregulate its expression. The affinity of Pit-1 for the Pit-1B1 binding sites is higher than for the Pit-1B2 binding sites. A downregulation of Pit-1 expression has been explained to be due to the saturation of Pit-1B1 binding sites which will lead to the binding of Pit-1 to the Pit-1B2 binding sites which incidentally can act to downregulate Pit-1 production (Chen et al., 1990).

The data also show that the injection of TRH altered the mRNA level for GH at both developmental ages. At both developmental stages, GH levels increased significantly in response to TRH 15 min after the injection and reaching their peak at 30 min. The magnitude of the response in the embryo was 2-fold higher compared to the controls whereas this was 1.5-fold in the 10-day-old chicks. It can be concluded that TRH has a more pronounced effect on GH stimulation in embryos than in chicks. This is in accordance with previous results of Harvey and Baidwan (1989) and of Kühn et al. (1993). TRH is known as the major GH-releasing factor in the chicken. In mammals, GHRH is the major GH-releasing factor. The significant increase in Pit-1 expression precedes the in-

crease in GH mRNA expression in growing chicks. This is an indication of a role for Pit-1 in the TRH stimulation of GH expression. This is not the case in embryos but again it cannot be excluded that Pit-1 was not already on the rise before the sampling at 15 min with a peak between 5 and 15 min. It is known that the GH promoter contains binding sequences for Pit-1. Binding of Pit-1 to the GH promoter is therefore necessary for GH expression (Nelson et al., 1988).

From our results we can conclude that in birds TRH acts on the gene expression of Pit-1 in embryos and growing chicks. TRH also affected the GH mRNA levels in embryos and 10-day-old chicks. These results indicate that Pit-1 has a role in mediating the stimulatory effects of TRH on pituitary hormones like GH. To our knowledge this is the first article where the mRNA levels of Pit-1 have been quantified.

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