

Title : Pitx factors are involved in basal and hormone-regulated activity of the human prolactin promoter.

Short title : Regulation of hPRL gene expression by Pitx factors.

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ABSTRACT

The pituitary-specific POU homeodomain factor Pit-1 likely interacts with other factors for cell-specific expression of prolactin. Here we identify the paired-like-homeobox transcription factors Pitx1 and Pitx2 as factors functionally activating the proximal human prolactin promoter (hPRL-164luc). Using *in vitro* binding assays and a series of site-specific mutations of the proximal hPRL promoter, we mapped the B1 and B2 bicoid sites involved in Pitx mediated transactivation of the hPRL-164luc construct. In somato-lactotroph GH4C1 cells, basal proximal hPRL promoter activity was inhibited by a Pitx2 dominant-negative form in a dose-dependant manner, while binding-disruptive mutations in the Pitx sites significantly reduced basal activity of the promoter. We also show that synergistic activation of hPRL-164luc by Pitx2 and Pit-1 requires the integrity of the B2 Pitx binding site, and at least one of the P1 and P2 Pit-1 response elements. In addition, mutation in the B2 Pitx site results in attenuation of the promoter's responsiveness to FK, TRH and EGF. Conversely, Pitx1 or Pitx2 overexpression in GH4C1 cells leads to an enhancement of the drugs stimulatory effects. Altogether, these results suggest that full responsiveness to several signalling pathways regulating the hPRL promoter requires the B2 Pitx binding site, and that Pitx factors may be part of the proteic complex involved in these regulations. Finally, *in situ* hybridization analysis showing coexpression of the PRL and Pitx2 genes in rat and human lactotroph cells corroborates the physiological relevance of these results.

Introduction :

The specific expression of human prolactin (hPRL) in somatolactotroph and lactotroph cells of the anterior pituitary is under the control of a promoter composed of a superdistal region (-5100/-4430bp), two distal regions (-3474/-2600 ; -1968/ -1064bp) and a proximal promoter (-250/+1bp) (1, 2). Different parts of the hPRL promoter are subjected to regulation by a variety of hormones and neuromediators. Dopamine (DA) is the main negative regulator of PRL expression : its binding to the dopamine type 2 receptor leads to reduction of intracellular cAMP levels and inhibition of Protein Kinase A (PKA) activity, leading to decreased hPRL expression (2). Conversely, hormones such as Vasoactive Intestinal Peptide (VIP) are able to activate PRL expression by increasing the intracellular cAMP concentration (5). Hormones and growth factors such as insulin and Epidermal Growth Factor (EGF) lead to a stimulation of the promoter activity mediated by the trans-membrane tyrosine kinases. Finally, factors such as thyrotropin releasing hormone (TRH) can induce another second messenger, Ca⁺⁺, which can stimulate the PRL promoter activity (5). All these second messengers pathways converge to the nucleus where their effects are ultimately mediated by transcription factors.

The most important and best studied of these transcription factors is Pit-1, a POU homeodomain factor governing temporal and spatial cell-specific expression of PRL, growth hormone (GH) and thyrotropin stimulating hormone β (TSH β) genes in response to diverse signaling cascades (6-12). Two binding sites for Pit-1 are located in the superdistal region of the hPRL promoter, 8 in the distal enhancer and 3 in the proximal promoter (2, 13). Two of the three Pit-1 binding-sites (P1 and P2) located in the proximal part of the promoter are sufficient to confer regulation of the human PRL gene by cAMP and Ca⁺⁺ transducing pathways (14). Two other important transcription factors, jun-D and c-fos interact to form the AP-1 complex, which cooperates with Pit-1 via binding to foot-print P1 to synergistically activate both basal hPRL gene transcription, and in response to activation of the MAPKinase pathway (15, 16). Recent studies report that the coactivator CBP/p300 is necessary for the AP-1/Pit-1 stimulation of the hPRL proximal promoter (16, 17). The third important element in the hPRL proximal promoter is the A sequence (18) which is mainly involved in the cAMP stimulation of

the PRL promoter, and is crucial for regulation the hPRL proximal promoter by different other signal transduction pathways (18, 19). The A sequence, which contains a motif similar to the CRE-binding site overlapping an Ets-binding site, exhibits high affinity binding to ubiquitous and pituitary-specific factors, whose nature and function are not yet fully identified (18, 19).

Interactions of Pit-1 with cell-type specific partners such as the estrogen nuclear receptor (20), with ubiquitous transcriptional factors such as Ets factors (21), or with pan-pituitary transcriptional regulators such as Lhx3 (9), Pitx1 and Pitx2 (7, 8) are required for terminal differentiation of lactotroph cells and direct regulation of the PRL gene. The Pitx family is a class of bicoid homeodomain proteins required for development of several organs (22). Among the three members of this family characterized up to now, Pitx1 and Pitx2 are expressed in the anterior pituitary and in a number of pituitary cell lines (22-24) while Pitx3 is not (25). During mouse development, Pitx1 and Pitx2 gene expressions partially overlap and are for exemple involved in specification of the stomodeum and its epithelial derivatives which include the pituitary anlage, Rathke's pouch. Inactivation of mouse Pitx1 gene (25, 26) and gain of function experiments in chick (27) shows that Pitx1 expression in the pituitary is crucial for gonadotroph, thyrotroph and corticotroph cells differentiation and hormone transcription (26). Pitx1 acts as transcription regulator of rat pituitary POMC, α GSU, β LH, β FSH, and PRL promoters (7, 28), interacting with cell-restricted factors such as SF-1 (7, 29), Egr-1 (30), the heterodimer NeuroD1/Pan 1 (31), Tpit (32), and Pit-1 (7). As shown by gene targeting experiments (33, 35), Pitx2 acts as a global executor of left/right asymmetry (36, 37) and might have a role in early determination of the pituitary, suggested by early arrest of pituitary development at the committed Rathke's pouch in Pitx2^{-/-} mice (32, 35). Pitx1 and Pitx2 as well as their isoforms share the same binding specificities and activate the same rat pituitary promoters (38).

We recently evidenced that several human pituitary gene promoters and particularly hPRL, were targets for Pitx2 (39). Here, we concentrated on the 164 bp fragment of the hPRL proximal promoter, previously shown to be sufficient to drive basal activity in somatolactotroph cells and to mediate the responses to almost all second messengers (13-16, 18-20). We show that Pitx factors participate in the basal activity of the hPRL promoter, as well as in its activation by forskolin, TRH, or

EGF treatments. Finally, the physiological relevance of these results is reinforced by demonstration that PRL and Pitx2 genes are coexpressed not only in rat but also in human lactotroph cells.

Material and methods.

- **Plasmid constructs and mutagenesis**

The reporter plasmid hPRL-164luc was previously described (15). Human Pitx1 was provided by Dr D.A Clayton (Stanford University, California). Human Pitx2 isoform a and Pit-1 full length cDNA coding regions were cloned by PCR using normal pituitary tissues and specific oligonucleotide sequences, and subcloned into the CMV driven eukaryotic expression vector pcDNA3 (Invitrogen). The mutations of the P1 and P2 binding sites in the hPRL-164luc construct were as described elsewhere (15). Mutations of the B1, B2 sites in the hPRL-164luc construct, the R91P and R271W mutations in the pcDNA3hX2 and pcDNA3hPit-1 constructs respectively were generated by PCR using the Quick Change Mutagenesis (Stratagene) and the following commercially synthesized oligonucleotides (Life Technologies, Inc), showing the mutations in bold :

B1mut : 5'-GAAGATATCAAAG**CGGT**TATAAAGCCAATATCTGGGAAAGAG-3'

B2mut : 5'-GAAATTATGGGGGTAC**CGGT**CAATGACGGAAATAGATGACC3'

R91P : 5'-GGT TCA AGA ATC GCC **CGG** CCA AAT-3'

R271W : 5'-GGCAGAGAGAAAAATGGGTGAAAACAAGTC-3'.

Plasmid DNA was purified using the Qiafilter Plasmid Maxi Kit (Qiagen) and all mutations were confirmed by DNA sequencing (ABI Prism BigDye terminator cycle sequencing ready reaction kit, Applied Biosystem).

- **Cell culture and transfection :**

GH4C1 somato-lactotroph pituitary cells were grown in HamF10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. Cells were transfected in serum-free medium using the liposome-based transfection kit Transfast (Promega) according to the manufacturer's instructions. Briefly, cells were plated at 200 000 cells/well in 12-well plates 24h prior to transfection, and transfected with 1.5µg of DNA (0.3µg of reporter plasmid, 0.1-1µg of effector plasmid(s), and 0.2µg of Cytomegalo-virus (CMV)-β-Galactosidase or 20ng pTKrenilla luciferase as internal controls for

transfection efficiency. Cells were incubated with the DNA/liposome complexes for one hr, and then supplemented with 1.5ml complete medium. For pharmacological treatments, GH4C1 cells were serum starved 24 hrs after transfection for 8 hrs, and further incubated with either 10mM forskolin, 1mM TRH, or 100 nM EGF for 18 hrs in serum-free medium. African green monkey kidney fibroblast-like CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. CV-1 cells were transfected by the calcium phosphate method with the MBS mammalian transfection kit (Stratagene) according to the manufacturer's instructions. Briefly, cells were plated at 100 000 cells/well in 6-well plates 24h prior to transfection. Transfection were carried out using 3 µg of reporter plasmid, 0.1-1 µg of effector plasmid(s), and 0.3 µg of Cytomegalo-virus (CMV)-β-Galactosidase as internal control for transfection efficiency. Cos-7 cells were grown in DMEM 10% SVF. Transfections were carried out in 100-mm-diameter dishes using the liposome-based transfection kit Polyfect (Qiagen) and 5 µg of Pitx expression vectors. In all transfections, total DNA was kept constant and nonspecific effects of viral promoters were controlled by using the appropriate empty vectors.

- **Luciferase and β-Galactosidase assays**

CV-1 and GH4C1 cells were harvested 48 hrs after transfection and lysed in 200µl Reporter or Passive Lysis Buffer (Promega). After three sequential freeze-thaw cycles, cell debris were pelleted by centrifugation at 10 000xg for 2 min at 4°C and 20µl aliquots of the supernatant were used for subsequent luciferase (Luciferase or Dual Luciferase system, Promega) and β-galactosidase assays. For each control, the total luciferase activity normalized against β-galactosidase activity or renilla luciferase activity was taken as 1, and results were expressed as -fold activation over control. Data are presented as the mean ± SE of three to five independent experiments using different plasmid preparations of each construct. Statistical significance was determined by Wilcoxon non parametric paired test. Significance was declared at $p < 0.05$.

- ***In vitro* transcription/translation :**

TNT T7 Coupled Reticulocyte Lysate System (Promega) was used for *in vitro* transcription/translation. Reactions were carried out in a total volume of 50µl with reticulocyte lysate, 1 µg plasmid DNA, 1mM amino acid mixture, RNasin (GibcoBRL, 40U/ml), T7 RNA polymerase, in the presence, or absence, of ³⁵S-Met (NEN, 10mCi/ml). ³⁵S-Met radiolabeled translation products were separated by SDS-PAGE and exposed to autoradiographic (ARG) film.

- **EMSA :**

Gel shifts were carried out using either the ³²P-labeled double-stranded oligonucleotide 5'-ACCAGGATGCT**AAGCCT**GTGTC-3', containing the CE3 Pitx specific binding site (in bold) of the POMC promoter (28), or oligonucleotide 5'-GCAA**AGGTTT**AATAAGCCAATGC-3' containing the B1 site (in bold), or oligonucleotide 5'-GCATTATGGGG**TAATCT**CAATGC-3') containing the B2 site (in bold). A hundred nanograms of annealed double-stranded DNA was 5'-end labeled in a standard T4 polynucleotide kinase (LifeTechnologies) reaction mixture containing 2µl of 32P-γATP, and purified over a G-25 sepharose column to remove free nucleotides and salt. Variable amounts of *in vitro* translated proteins or Cos-7 cell nuclear protein extracts (39) were incubated on ice for 15 minutes in a 20µl reaction of 1x binding buffer (20mM Hepes, 400mM KCl, 20% glycerol, 2mM DTT, 0,5mM PMSF) containing 1 µg of poly(dI-dC) and 20 000 cpm of the radiolabelled probe. Competition reactions were performed with 100 or 200ng of CE3 itself, native and mutated B1 and B2 and the unrelated SP1-like oligonucleotides. In some cases, 1 µl of Pitx2 polyclonal antibody or preimmune serum was added to the reactions prior to addition of the probe (39). The bound proteins were separated from the free probe on a 8% polyacrylamide gel containing 0,5% TBE by polyacrylamide gel electrophoresis (PAGE) at 180V for 3 hours at 4°C, before exposure to autoradiographic film.

- **In situ hybridization :**

Pituitaries were obtained from male Sprague-Dawley rats (200-250 g b.w., Le Genest Saint-Isle, France). Human normal pituitary tissues were obtained at the time of therapeutic abortion (20-32

weeks gestation), and tumoral pituitary tissues were obtained by transsphenoidal adenomectomies performed on patients who had undergone endocrine preoperative evaluation. Twelve μm cryostat sections were either singled-labelled for Pitx2 or doubled-labelled for Pitx2 and PRL, as previously described (40). The human or rat Pitx2 probe was a 540 bp or a 720 bp fragment located in the 3'UTR of the human or rat cDNA, respectively, subcloned into pPCR/script and labeled with [35S]UTP (PerkinElmer Life Sciences, Paris, France) using T3 or T7 (human and rat antisense probe respectively) or T7 or T3 (human and rat sense probe respectively). The human or rat PRL antisense probe was a 588 bp or a 580 bp fragment of the human or rat cDNA, respectively, subcloned into pPCR/script and labeled with digoxigenin-UTP (RocheMolecular Biology, Meylan, France) using T3 or T7 (human and rat probe respectively). Brightfield or fluorescent images were captured with a color CDD video camera (Coolsnap, Princeton Instruments, France) attached to a Leica microscope equipped with a 100-W mercury-arc lamp and an appropriate filter set. Composites were formed within Adobe Photoshop. Brightness and contrast were altered to generate photographic quality prints.

Results :

The hPRL promoter is a putative target for the Pitx transcription factors.

Within the -164bp part of the hPRL promoter (Fig. 1), foot-print experiments have previously defined two protected regions (P1 and P2) containing Pit-1 binding sites (13). In addition, transfection studies identified a third region, named A sequence, important for both basal and regulated activity of the promoter (18, 19). Sequence analysis of the 164 bp region and comparison with the consensus bicoid-related homeoproteins binding site TAATCC (41) identified two putative bicoid binding sites, named B1 and B2 respectively, and localized at positions -27 (TAAACC, reverse orientation) and -110 (TAATCT) (Fig.1). The ability of the B1 and B2 DNA elements within the hPRL proximal promoter to bind Pitx factors was investigated by gel retardation experiments using B1, B2, or the known bicoid CE3 element of the POMC promoter as probes, and Pitx1 and Pitx2 obtained from *in vitro* translation reactions. In agreement with other studies (38), Pitx1 and Pitx2 were equally efficient at binding the CE3 consensus Pitx site of the POMC promoter (Fig. 2A, left panel), when both factors were present at similar amounts (Fig. 2A, right panel). No complex was obtained when unprogrammed reticulocyte lysate was used (lane 2). Addition of anti-Pitx2 anti-serum prevented the formation of the Pitx2 complex (lane 4), but not that of Pitx1 (lane 7), whereas the preimmune serum had no effect (lanes 5). Analysis of the EMSA performed using B1 or B2 as probes was complicated by the presence of several non specific bands (. 2B). Indeed, for both probes, several bands were observed already in the negative controls (-), i.e when binding was assessed using the products of a translation reaction performed with empty vector (lanes 5 and 10). Nevertheless, as shown in Fig. 2B with Pitx2, when increasing amounts of proteins were used (lanes 6-8, and 11-14), a Pitx2-specific complex that could be discriminated by addition of the anti-Pitx2 anti-serum (lanes 8 and 14) was also detected, although superimposed on one of the non-specific band. This complex ran at a similar position to that obtained with CE3 probe (lane 3).

To obtain more demonstrative data, we then performed experiments in which B1 and B2 oligonucleotides were used as competitors and CE3 as a probe. Fig. 3A shows results obtained with

Pitx1. A specific complex was formed between the Pitx1 protein and the CE3 oligonucleotide (lane 3), as expected, which was competed in the presence of an excess of cold CE3 probe itself (lanes 4, 5). Addition of the unrelated SP1-like oligonucleotide did not either had any effect on the formation of the Pitx1 complex (lanes 14, 15). The B1 (lanes 6, 7) or B2 (lanes 10, 11) probes were also able to compete for Pitx2 binding, while the mutated counterparts B1mut (lanes 8,9) and B2mut (lanes 12, 13), which contain transversions of 3 bp within the bicoid target site, were inactive. Similar competition experiments performed with Pitx2 yielded similar patterns. (Fig. 3B). Finally, DNA-binding assays performed with nuclear extracts from Cos-7 cells transfected with expression vectors for Pitx1 or Pitx2, gave results similar to those obtained with *in vitro* translated proteins (data not shown). Altogether, our results indicated that the B1 and the B2 sites in the hPRL promoter are able to bind Pitx1 and Pitx2 and that both factors share identical DNA-binding properties.

Functional analysis of B1 and B2 sites in non-pituitary cell lines :

To assess the relative abilities of Pitx1 and Pitx2 to stimulate the hPRL promoter activity, increasing amounts of CMV-Pitx1 or CMV-Pitx2 expression vectors were cotransfected into CV-1 cells with hPRL-164luc. As shown in Fig. 4A, although Pitx1 and Pitx2 were both able to transactivate the prolactin promoter, they exhibit slightly different patterns of activity : as little as 0.1 μ g of Pitx1 DNA input were sufficient to achieve detectable activation of the promoter, whereas 0.2 μ g were necessary for Pitx2. Furthermore, a clear decrease in activation was observed at the highest dose of Pitx1. This blunting of the response at high doses of Pitx1 was not observed with Pitx2, since increasing Pitx2 DNA inputs resulted in a consistent dose-dependent increase in hPRL promoter activity (Fig. 4A). EMSA performed with nuclear extracts from Pitx1 and Pitx2-transfected cells provided no evidence for a differential expression level of the two factors, as similar binding efficiencies were obtained (Fig. 4B), consistent with the results obtained with *in vitro* translated proteins. Given these observations, we decided for the following transfection experiments to use DNA inputs of 0.5 μ g for both Pitx1 and Pitx2, a dose which resulted in similar transactivation effects for

both factors (5.7 ± 0.5 and 6.4 ± 0.4 respectively). Moreover, under these conditions, none of the factors significantly activated the pTKLuc construct which was used as a negative control (data not shown).

To test the ability of the B1 and B2 sites to drive Pitx-induced activation of the -164hPRL promoter, the two sites were independently or simultaneously disrupted by mutagenesis, generating the B1mutluc, B2mutluc, and B1,2mutluc constructs, respectively. CMV-Pitx expression vectors were cotransfected in CV1 cells together with the various mutant reporter constructs. As shown in Fig. 5A (grey columns) with the CMV-Pitx2 vector, disruption of the B1 and of the B2 bicoid binding sites resulted in a loss of 9 and 42% respectively of the Pitx-induced transactivation, relative to the native hPRL-164luc construct. The Pitx2-induced transcriptional activity of the double B1 and B2 mutant (B1,2mutluc) was only 33% of that of the native construct (Fig. 5A). Superimposable variations in the amplitude of the transactivation effects were observed with a Pitx1 expression vector (data not shown), indicating that the B1 and particularly B2 bicoid sites are able to drive Pitx-induced activation of the hPRL proximal promoter. For comparison, the effects on Pit-1 transactivation were tested for the different Pitx mutant promoter constructs. The capability of Pit-1 to transactivate the hPRL promoter was not impaired when either the B1 or the B2 site was mutated (fig. 5A), but for reasons that remain unexplained, a 20% decrease was repeatedly observed when both B1 and B2 sites were simultaneously disrupted (fig. 5A).

Synergistic activation of the hPRL promoter by Pitx2 and Pit-1 requires the B2 bicoid site and at least one of the two Pit-1 sites.

As already known, the transcription factor Pit-1 is able to activate the hPRL promoter (5.3 ± 0.4 fold induction in our conditions). Fig. 5B shows an analysis performed in CV-1 cells with hPRL promoter constructs in which the P1 and P2 Pit-1 binding sites were independently, or simultaneously mutated. Mutations in the P1, P2, or both sites, reduced the activity of the hPRL promoter in the presence of Pit-1 by about 55%, 20%, and 90% respectively, while they had no effect on the Pitx2-induced transactivation (Fig.5B).

In agreement with previous studies (7, 8), when Pit-1 was tested in combination with either Pitx1 or Pitx2, a synergistic activation of the hPRL-164luc construct was observed with both factors (28 ± 6 ,

and 35 ± 4.1 fold induction, respectively, shown in Fig. 6 for Pitx2), indicating that the hPRL-164luc construct, containing the B1 and B2 Pitx elements, and the P1 and P2 Pit-1 elements, is sufficient for Pitx and Pit-1 transactivation and synergy. We next mapped the *cis* elements required for the Pitx/Pit-1 synergistic activation of the hPRL-164luc construct by testing the effects of site-specific disruption of the Pitx and Pit-1 binding sites. As shown in Fig. 6, while the Pitx2/Pit-1 synergism was conserved on the B1mut/luc construct, it was lost on the B2mutluc and on the B1,2mutluc construct. The synergistic activation by Pit-1/Pitx2 was not affected by disruption of the P1 or P2 Pit-1 sites, but was abolished by the disruption of both. Altogether, these results indicated that the integrity of the B2 site, and of either one of the P1 or P2 sites is required to achieve cooperative activation of the hPRL promoter by Pit-1 and Pitx factors.

Pitx factors are involved in both basal and hormone-regulated activity of hPRL promoter in GH4C1 cells.

Involvement of the B1 and B2 sites in the basal activity of the hPRL promoter was confirmed in pituitary somato-lactotroph cells. GH4C1 cells, which express the endogenous rat PRL gene as well as the Pitx and Pit-1 transcription factors (7, 33, 39), were transfected with the series of reporter constructs mutated in the bicoid-like sites. Disruption of the B1 or B2 site within the hPRL-164luc construct significantly reduced basal activity of the hPRL promoter in GH4C1 cells by 12% and 22% respectively compared the wild type promoter activity, and the B1,2mutluc construct retained only 50% of hPRL-164luc construct (Fig. 7A).

We subsequently used negative-dominant transcription factors constructs which interfere with the action of endogenous factors to further approach the role of Pitx factors in regulating basal hPRL promoter activity. GH4C1 cells were transfected with the hPRL-164luc construct along with R91P, a Pitx2 mutant identified in patients with Rieger syndrome (42). We have previously shown that this point mutation, which replaces a fully conserved Arg of the homeodomain to a Pro, leads to a negative-dominant factor able to counteract both Pitx2 and Pitx1-driven transactivation of several pituitary gene promoters (39). As shown in Fig. 7B, expression of Pitx2-R91P in GH4C1 cells resulted in a dose-dependant inhibition of basal hPRL promoter activity (40% inhibition at maximal doses).

For comparison, and in agreement with the well-known dependence of PRL gene expression on Pit-1, cotransfection of GH4C1 with Pit-1-R271W, a negative-dominant mutant previously identified in patients with combined pituitary hormone deficiency (43) also resulted in a dose-dependent inhibition of the basal activity of the promoter, which was of higher amplitude (65% at maximal doses, Fig. 7B). Control experiments performed on a pTKLuc construct under the same conditions did not reveal any significant inhibitory effect of the Pit-1 nor Pitx2 mutants (data not shown). Thus, inhibitory forms of both Pit-1 and Pitx2 significantly reduced basal hPRL promoter activity in the somato-lactotroph cell line GH4C1, suggesting that in addition to the hPRL promoter major regulator Pit-1, Pitx factors expressed in these cells, such as Pitx1 or Pitx2, may contribute to basal hPRL activity.

As mentioned above, the Pitx B2 element is located within the A fragment (spanning sequence from -115 to -85 in the proximal hPRL promoter, Fig. 1), which was previously shown to be crucial in regulating the hPRL proximal promoter by different signal transduction pathways (14, 18, 19). In our conditions (Fig. 8A), treatment of the cells with 10mM FK, 1mM TRH, and 100 nM EGF significantly activates the hPRL promoter in GH4C1 cells (2-3 fold). Based on these observations, we investigated whether Pitx factors could participate in the activation of the hPRL promoter by forskolin, TRH, and EGF treatments. To this purpose, a CMV-Pitx2 expression vector was transiently transfected in GH4C1 cells along with the hPRL-164luc construct, and cells were treated as above. Cotransfection of Pitx2 increased the activation by FK, TRH and EGF of the hPRL promoter by 150-200% (Fig. 8B), suggesting that Pitx factors might be part of the transcriptional complexes that regulate hPRL gene transcription. Conversely, mutation in the B2 Pitx site in the hPRL promoter resulted in a moderate but significant decrease (17-23%) in the activation of the hPRL promoter induced by FK, TRH and EGF (Fig. 8B).

The Pitx2 and PRL genes are coexpressed in pituitary lactotroph cells.

A number of studies have previously established a ubiquitous pattern of expression for Pitx1 in all cell lineages of the developing and adult pituitary, including somato-lactotroph cell types, in both rat, mouse and human (44, 26, 23). Thus, we focused on the expression of Pitx2 in cells of the lactotroph lineage in human and rat tissues, and we used ISH to assess its coexpression with the PRL gene in

lactotroph cells. As shown in Fig. 9A, the antisense riboprobe for Pitx2 revealed messenger RNAs in most cells of human pituitary tissue, since the microscopic field showed a vast majority of cells covered with silver grains. Sections hybridized with sense riboprobe were completely unlabeled (Fig. 9B). When ISH was performed on rat pituitary tissues, high-level hybridization was seen throughout the anterior and intermediate lobes, but not in the posterior lobe (panel C). Double ISH analysis of Pitx2 and PRL mRNAs was further performed to investigate coexpression of the two genes (Fig. 9D-F). Panel D shows the presence in human normal pituitary of PRL-positive (digoxigenin-UTP labelled) cells also covered with clusters of silver grains, revealing cells positive for both messengers. In one human lactotroph adenoma, which presents as a monomorphous tissue composed of differentiated lactotroph cells, all cells were double-labelled (panel E). Finally, coexpression of PRL and Pitx2 was also observed in rat anterior pituitary (panel F).

Discussion.

There is now a large body of evidence indicating that full activity of the PRL gene promoter, requires contribution from a constellation of regulatory elements that cluster into proximal and distal domains of the 5'-flanking region. There is also substantial evidence indicating that most of these regulatory elements allow their cognate DNA-binding proteins to interact directly and cooperatively with one another. Results presented herein indicate that the hPRL promoter depends upon functional Pitx-regulatory elements residing in the -164bp proximal promoter region, previously shown to be sufficient to drive basal activity in somatolactotroph cells and to mediate the responses to almost all second messengers.

Pitx1 and Pitx2 are the earliest known genetic markers for the nascent Rathke's pouch, the precursor of the anterior and intermediate lobes of the pituitary gland. They are expressed constitutively throughout development and in adult pituitary cell lineages. In the lactotroph lineage, we provide evidence by double in situ hybridization for coexpression of the Pitx2 and the PRL genes

in rat PRL secreting cells, as well as in normal and tumoral human lactotroph cells, while immunolocalisation experiments have previously detected Pitx1 protein in the nuclei of all cells of developing and adult rat pituitary (44). The overlapping patterns of expression of Pitx1 and Pitx2 in the lactotroph lineage, and their highly homologous sequences (97% similarity in homeodomain and 67% identity in the C-terminal putative transactivational domain) are elements in favor of a functional redundancy for the two factors. In agreement with previous studies (38), we show that Pitx1 and Pitx2 share similar *in vitro* DNA-binding specificities when assessed on the bicoid POMC promoter CE3 element, and we extend this observation to the B1 and B2 bicoid elements of the hPRL promoter. However, although Pitx1 and Pitx2 are both able to activate transcription driven by the hPRL-164 promoter, transfection of increasing DNA doses revealed different patterns of activation for the two factors. The reasons for this do not appear to result from differences in the levels of expressed proteins in our conditions, as indicated by DNA-binding assays. Interestingly, the differential abilities of human Pitx1 and Pitx2 to transactivate the hPRL promoter were also observed when the mouse counterparts of these transcription factors were used in transfection experiments (unpublished data). This observation is reminiscent of the discrete variations in the relative abilities of Pitx1 and Pitx2 and their isoforms to activate a set of pituitary hormone gene promoters despite their conserved DNA-binding properties (38). The drop in activation of the hPRL promoter at the high dose of Pitx1 remains to be explained and might reflect squelching. However, the fact that this decrease is observed only with Pitx1 but not with Pitx2 suggests that there is a titrable component interacting with Pitx1 but not with Pitx2, which might correspond to differential interactions with cofactors *in vivo* too. Altogether these data indicate that Pitx1 and Pitx2 transcription factors may be able to recruit different partners to activate transcription, and that the gene dosage for each factor might be crucial for proper and optimal function.

These results are interesting in light of the recent report from Suh et al. (45). Comparison of hypomorphic (reduced function, Pitx2^{neo}) and null alleles in mice revealed that Pitx2 is required in a dose-dependant manner for initiating expansion of Rathke's pouch, and at later stages for specification and expansion of the gonadotropes and Pit-1 lineage within the ventral and caudomedial anterior pituitary. In addition, Suh et al. tested for overlapping functions of Pitx1 and Pitx2 by generating

double mutants carrying Pitx1⁻ and Pitx2^{neo} alleles (45). Analysis of the mutants revealed that during the initial steps of pituitary development, whereas the loss of Pitx1 function in Pitx1^{-/-} mice appears to be fully compensated by Pitx2, Pitx1 is able to compensate a reduction (Pitx2^{neo/neo} mice) but not a total loss (Pitx2^{-/-} mice) in Pitx2 function. Although pituitary development in the double mutants did not progress far enough to assess the effects on individual differentiated cell types, these results indicated that pituitary development relies not only on Pitx2 dosage but also requires the combined dosage of Pitx1 and Pitx2.

The hypothesis that Pitx factors might participate in the basal expression of the PRL gene was supported by experiments performed in the pituitary somato-lactotroph GH4C1 cell line which expresses the endogenous PRL, Pitx and Pit-1 genes. R91P (42), a dominant negative form of Pitx2, has the capability in CV-1 cells to almost completely block the wt-Pitx2-induced activation of its target promoters, to prevent the Pitx2/Pit-1 synergistic activation of the hPRL promoter and to counteract the Pitx1-driven transactivation effects (39). Transfection of increasing amounts of R91P in GH4C1 cells induced a corresponding dose-dependant inhibition of the hPRL-164 promoter construct activity. The decrease, however, was of relatively low amplitude compared to that produced under the same conditions by a dominant negative mutant form of Pit-1. The idea of a functional role for Pitx factors in regulating basal promoter activity was reinforced by the decrease of the hPRL promoter activity observed in pituitary cells when B1 and B2 sites were independently or simultaneously mutated. Altogether, these data may reflect that besides the major homeodomain transcriptional regulator Pit-1, Pitx factors may be recruited in the transcription factors complex required for the basal activity of the hPRL gene promoter in somatolactotroph cells.

Of the two Pitx DNA-binding sites in the hPRL-164bp fragment, the most important in regulation of the promoter appears to be the B2 site, located between Pit-1 binding sites P1 and P2. Indeed, disruption of the B1 site, although it abolishes binding, has only moderate effects on the activation of the promoter measured both in heterologous CV-1 cells and in homologous GH4C1 cells. In contrast, the introduction of a binding-disruptive mutation in the B2 motif lead to a 20% decrease in the basal activity of the promoter in GH4C1 cells, and to more than 40% decrease in the Pitx-induced activation of the promoter in CV-1 cells. In addition, abolition of the B2 binding site specifically

prevented synergistic activation of the hPRL promoter by Pitx factors and Pit-1. This effect was dependent on Pitx-binding since Pit-1-induced transactivation remained unchanged when B2 was disrupted. Functional analysis of a series of constructs containing individual or pairwise mutations in the Pitx and Pit-1 sites indicates that besides binding of Pitx factors to the B2 site, the integrity of either the P1 or P2 site is also required to achieve Pit-1/Pitx2 synergism. These data corroborate the model discussed by Amendt et al. in which binding of Pitx2 to DNA is necessary for proper synergy with Pit-1 (8). The authors proposed that interaction between the C-terminal tail and N-terminal domain of Pitx2 would functionally interfere with DNA binding. When Pitx2 binds its DNA target-site, the interaction between N- and C-terminal domains is disrupted, allowing C-terminal protein-protein interaction with other factors such as Pit-1, and subsequent synergistic activation of the transcription.

In addition to decreasing activation by Pitx factors in CV-1 cells and its basal activity in GH4C1 cells, mutation in the B2 binding site also attenuates responsiveness of the hPRL promoter to FK, EGF and TRH. The B2 element is located within A sequence (-115 to -85) and overlaps in part with a TGACG motif similar to the ATF/CREB-binding site found in many cAMP-regulated promoters. An Ets-binding site partially overlapping the TGACG motif is also identified in this fragment (Fig. 1). EMSA performed herein with a probe centered on B2 revealed in addition to the Pitx-specific complex, several other proteins bound to DNA which were observed with *in vitro* translation TNT reactions or Pitx-transfected Cos-7 cell nuclear extracts. These data are reminiscent of previous South-western and gel-shift studies demonstrating that the A fragment was able to bind numerous proteins from heterologous or pituitary cell extracts. These proteins consist in both ubiquitous factors such as a factor of 100kD whose identity remains to be established, factors of the Ets family (unpublished data), as well as pituitary-specific factors, including Pit-1 (18). Altogether, these data underline the high complexity of the A fragment. Previous studies showed that the A fragment is required together with Pit-1 binding sites P1 and P2 for full cAMP response of the hPRL promoter (14), and that point mutations in the TGACG motif of A sequence strongly reduces cAMP stimulation of the hPRL promoter (18). In addition, although they activate mostly distinct pathways, TRH and EGF were shown to stimulate the hPRL promoter via identical cis elements (19). In this study, we show that a mutation in the B2 bicoid site preventing binding of Pitx1 and Pitx2 results in an

attenuation of the responsiveness of the promoter to both FK, TRH and EGF. On the other hand, we also show that overexpression of Pitx1 or Pitx2 in GH4C1 cells leads to an enhancement of the stimulatory effects of the drugs. Altogether, these results suggest that full responsiveness to several signalling pathways regulating the hPRL promoter requires a functional B2 Pitx binding site, and that Pitx factors may be part of the protein complex involved in these regulations. At that point, however, the precise molecular mechanisms underlying Pitx factors participation in these events remain to be determined. Pitx factors could be direct nuclear targets of signalling pathways, or could rather functionally interact with other transcription factors of the protein complex involved in these regulations, such as the 100kD factor binding to the TGACG motif adjacent to the B2 site. Considering the requirement of the B2 site for full Pitx/Pit-1 synergistic activation of the hPRL promoter, and considering the central role of Pit-1 in both basal and hormone-regulated activity of the hPRL promoter, Pitx factor could also participate in the signalling pathways regulating hPRL gene expression through combinatorial and cooperative interactions with Pit-1 bound to P1 or P2 sites.

Functional interactions of Pitx factors with transcription factors have been characterized in the hormonal regulation of other pituitary genes. For example, as shown by several groups in gonadotrope cell lines as well as in transgenic mice, Pitx1 not only activates the LH β promoter in synergy with SF-1, but also confers responsiveness to GnRH by interacting with Egr-1, one of the downstream effectors of this pathway (29, 30, 46). Similarly, our data reinforce the concept that activity of the hPRL promoter is determined through highly cooperative interactions between Pit-1 and other factors, which may include Pitx factors, and indicate that the B2 Pitx element can be added to the list of sites required for defining basal and regulated activity of the hPRL promoter.

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Figures legends :

Fig. 1. Schematic representation of the -164hPRL promoter. Sequences P1 and P2, containing two high affinity Pit-1 binding sites and the proximal AP1 site, and A sequence are underlined. Consensus motifs for Pitx factors, for Ets factors, and the CRE-like motifs are depicted.

Fig. 2. DNA-binding properties of Pitx factors. A) Left panel : EMSA using the consensus CE3 Pitx-binding site from the POMC gene as a probe and 5 μ l of *in vitro* translated Pitx1 or Pitx2. As control, probe alone or a translation reaction in the presence of empty vector were used. The binding of Pitx2 is abolished by addition of the Pitx2 anti-serum (AS), whereas the preimmune serum (PI) has no effect. The AS has no effect on Pitx1 binding. Right panel : SDS-PAGE analysis of ³⁵S-Met-radiolabeled Pitx1 and Pitx2 obtained from *in vitro* TNT reactions. Pitx1 (36kD) and Pitx2 (30kD) proteins are expressed at similar amount. - : empty vector. B) EMSA using either the CE3 (lanes 1-3), B1 (lanes 4-8) or B2 (9-14) Pitx binding-sites as probes and varying amounts of *in vitro* translated Pitx2. Five μ l of *in vitro* translated Pitx2 were used for binding to CE3, 7 and 10 μ l for binding to B1, and 5, 7, and 10 μ l for binding to B2. Formation of the Pitx2-specific complex was challenged by addition of the Pitx2 anti-serum (AS). 0, probe only. -, empty vector. NS, non-specific band.

Fig. 3. Binding of Pitx1 and Pitx2 to the B1 and B2 elements of the hPRL promoter. A) EMSA were performed with a CE3 probe and 5 μ l of *in vitro* translated Pitx1 in the presence or absence of the indicated excesses of unlabeled competitor oligonucleotides CE3 (lanes 4, 5), the intact B1 (lanes 6, 7) and B2 (lanes 10, 11) sites, their mutated counterparts B1mut (lanes 8, 9) and B2mut (lanes 12, 13) containing transversion of 3 bp, and the unrelated Sp1 oligonucleotide (lanes 14, 15). 0, probe only. -, empty vector. NS, nonspecific band. B) Same experiments performed with *in vitro* translated Pitx2. -, empty vector. NS, nonspecific band.

Fig. 4. Differential transcriptional activation of the -164hPRL promoter by Pitx1 and Pitx2. A) Non-pituitary CV1 cells were transfected by the calcium phosphate method using reporter plasmid hPRL-164luc and varying amounts (0, 0.1, 0.2, 0.5 and 1 μ g) of CMV-pcDNA3 expression vectors encoding Pitx1 and Pitx2 full-length cDNAs. A CMV- β -galactosidase plasmid was used as internal control for transfection efficiency. Cells were harvested after 48h and assayed for luciferase. Results were normalized with respect to β -galactosidase activity and are expressed as -fold activation over control. Transfections were performed in triplicate for each condition within a single experiment. Data are represented as the mean \pm SE of three independent experiments. B) Expression of Pitx1 and Pitx2 driven by the CMV-pcDNA3 vectors was monitored by EMSA : nuclear extracts (5 μ g) of Pitx1- and Pitx2- transfected Cos-7 cells were tested for Pitx-binding activity on a CE3 probe. Pitx1 and Pitx2 have similar binding efficiencies.

Fig. 5. Pitx-induced transactivation of -164hPRL promoter is driven by the B1 and B2 binding-sites and independent of the Pit-1 elements. A) Four different hPRL-164luc reporters (wild type, mutated B1 site : B1mutluc, mutated B2 site : B2mut/luc, and mutated B1 and B2 sites : B1B2mut/luc) were transfected in CV1 cells with a CMV-Pitx2 expression vector or empty vector. For comparison, the four constructs were also transfected in CV-1 cells with a CMV-Pit-1 expression vector. B) CV1 cells were transfected with wild type hPRL-164luc construct or mutated in P1, P2, or both binding sites of Pit-1, and a CMV expression vector encoding Pitx2 or Pit-1. Results were normalized with respect to β -galactosidase activity and are expressed as luciferase activity relative to the control wild type hPRL-164luc construct, arbitrarily set to 100%. Transfections were performed in triplicate for each condition within a single experiment. Data are represented as the mean \pm SE of five independent experiments.

Fig. 6. Site requirements for Pitx and Pit-1 synergy. The Pit-1/Pitx2 synergistic activation of the hPRL proximal promoter was tested on the series of reporter constructs with site-specific disruption of the Pitx and Pit-1 binding sites transfected in CV-1 cells together with Pitx2 and Pit-1 expression

vectors. The synergistic activation observed on the wild type construct was lost when B2 was disrupted, or when either of the P1 and P2 binding-sites were simultaneously mutated. Transfections were performed in triplicate for each condition within a single experiment. Data are represented as the mean +/- SE of five independent experiments.

Fig. 7. Pitx factors contribute to basal hPRL activity in GH4C1 cells. A) GH4C1 cells were transfected by the liposome-based method using the hPRL/luc construct and hPRL/luc mutated in B1, B2 or in both B1 and B2. A CMV- β -galactosidase plasmid was used as internal control for transfection efficiency. Cells were harvested after 48h and assayed for luciferase. Results were normalized with respect to β -galactosidase activity and are expressed relative to wild type promoter activity. Transfections were performed in triplicate for each condition within a single experiment. Data are represented as the mean +/- SE of three independent experiments. Statistical significance was determined by Wilcoxon non parametric paired test. *, significantly different from hPRL-164luc construct, $p < 0.05$. B) GH4C1 cells were transfected with the hPRL-164luc reporter construct and increasing amounts of expression vectors encoding dominant-negative mutated forms of Pit-1 (R271W) or Pitx2 (R91P). The total amount of pCMV plasmid amount was maintained constant with empty pCDNA3 vector DNA. A CMV- β -gal plasmid was used as internal control for transfection efficiency. Cells were harvested after 48h and assayed for luciferase. Results were normalized with respect to β -galactosidase activity and are expressed as fold activation over control. Transfections were performed in triplicate for each condition within a single experiment. Data are represented as the mean +/- SE of three independent experiments. Statistical significance was determined by Wilcoxon non parametric paired test. *, significantly different from empty vector, $p < 0.05$.

Fig. 8. Pitx factors and the B2 Pitx binding site are involved in stimulated activity of the hPRL promoter in GH4C1 cells. A) GH4C1 cells were transfected with the hPRL-164luc construct. Twenty-four hours after transfection, the cells were serum starved for 8 hrs, and further incubated over-night in serum-free medium in the absence (control) or presence of 10mM forskolin, 1mM TRH, or 100 nM EGF. Cells

were assayed for luciferase, and luciferase values were normalized to β -galactosidase activity. Results are expressed as fold stimulation over control conditions. Statistical significance was determined by Wilcoxon non parametric paired test. *, significantly different from control conditions, $p < 0.05$. B) GH4C1 cells were transfected with the hPRL-164luc construct alone or in combination with a CMV-Pitx2 expression vector, or with the B2mutluc construct, and cells were treated as in A. Results are expressed as fold stimulation relative to the hPRL/luc construct. Transfections were performed in triplicate for each condition within a single experiment. Data are represented as the mean \pm SE of three independent experiments. Statistical significance was determined by Wilcoxon non parametric paired test. *, significantly different from hPRL-164luc construct, $p < 0.05$.

Fig. 9. Coexpression of Pitx2 and PRL genes in rat and human lactotroph cells. A, B : Brightfield microautoradiographic view of fetal anterior pituitary sections hybridized with a human Pitx2 antisense (A) or sense (B) probe. Hybridization with the sense probe resulted in a lack of signal, demonstrating the specificity of the probe and of the hybridization technique. C : Macroautoradiographic view of a rat pituitary section hybridized with a radiolabeled rat Pitx2 antisense probe (A : anterior lobe, I : intermediate lobe, N : neural lobe). D-F : Double labeling for Pitx2 mRNA (hybridized with a radioactive Pitx2 antisense probe and revealed by microautoradiography ; grains were inverted to white for better visualization) and PRL mRNA (hybridized with a digoxigenin-labeled PRL antisense probe and revealed by a fluorescent technique) in human fetal (D) or adenomatous lactotroph (E) tissues or adult rat anterior pituitary (F). Scale bars equals 25 μ m in A, 1 mm in C and 12.5 μ m in D-F.

Fig. 1

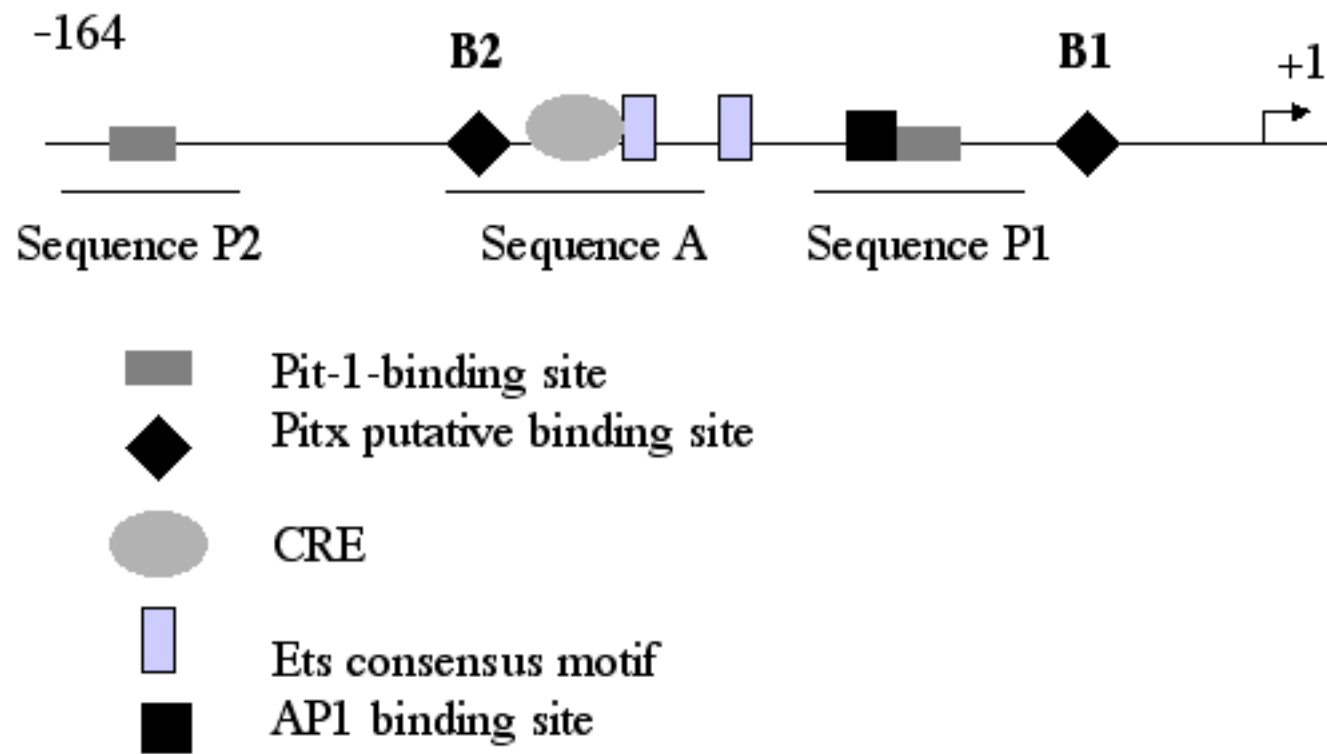


Fig. 2

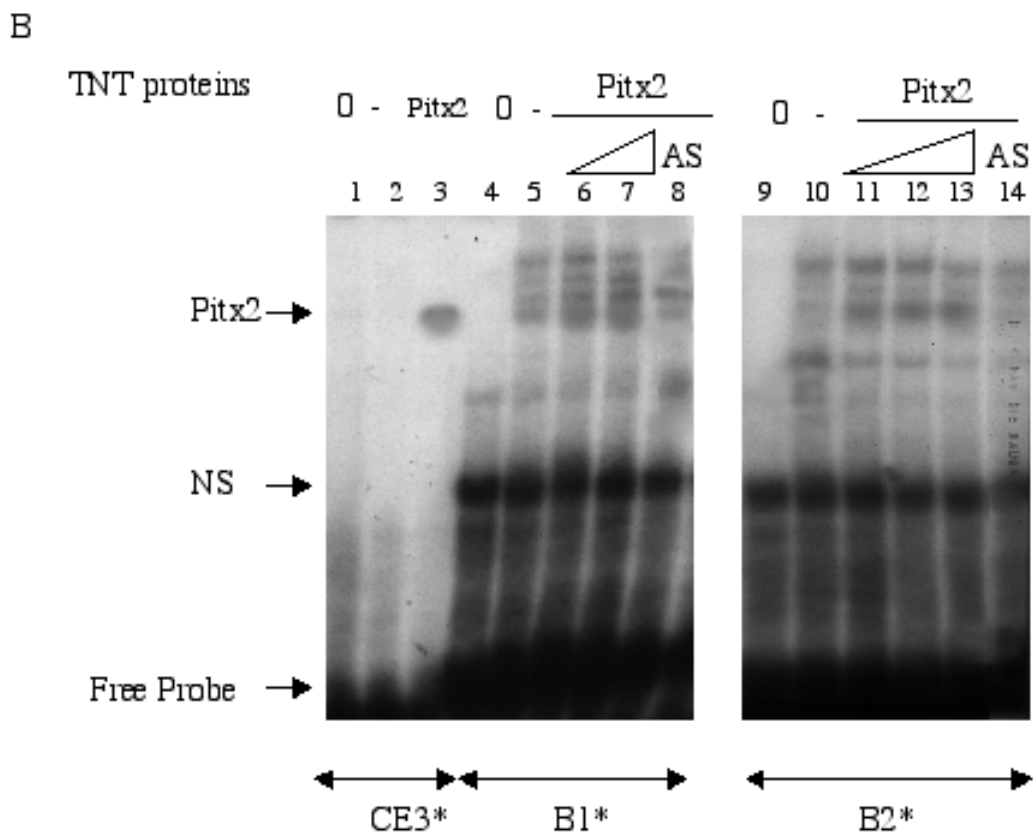
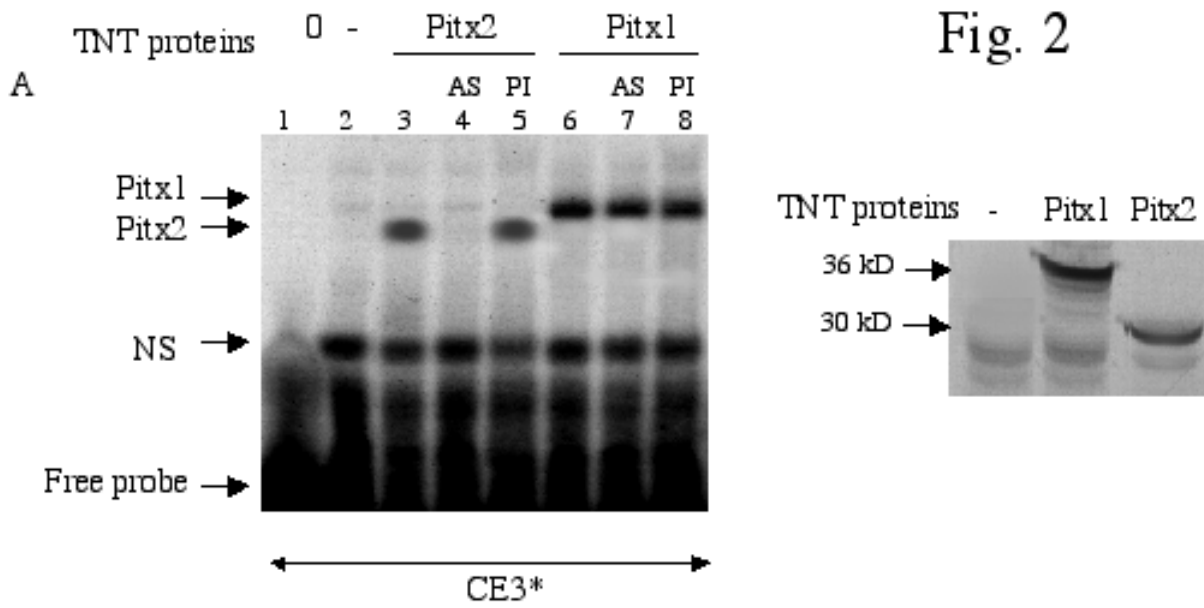


Fig. 3

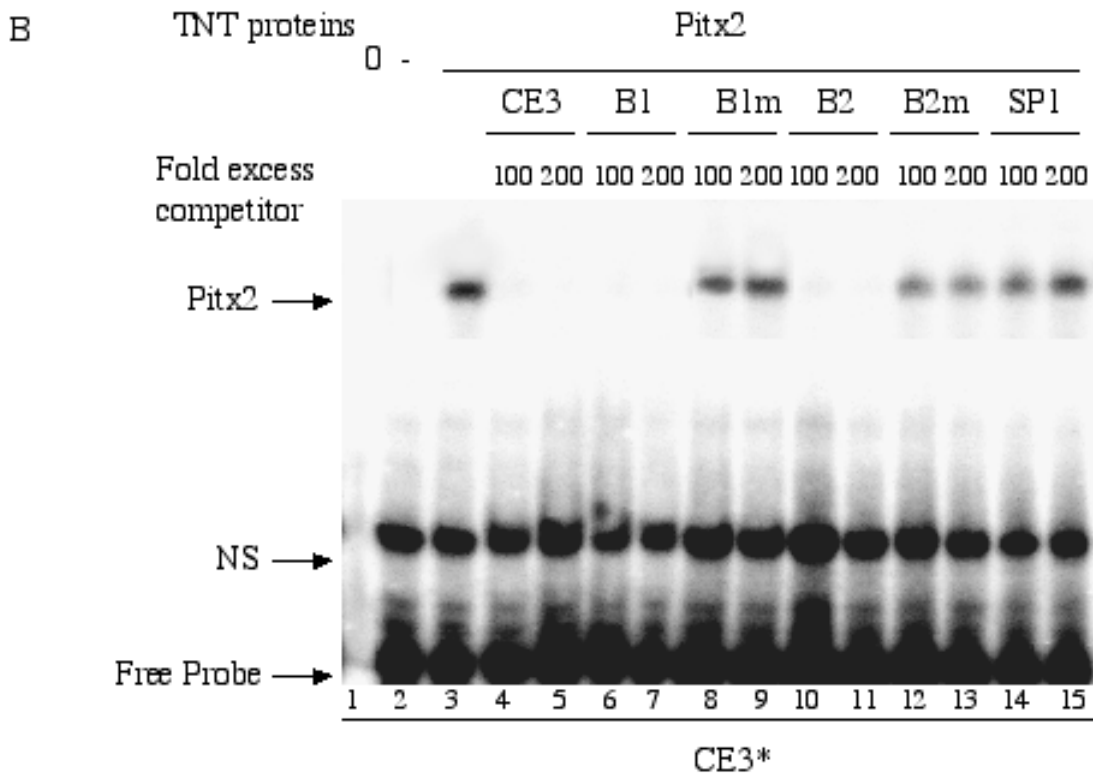
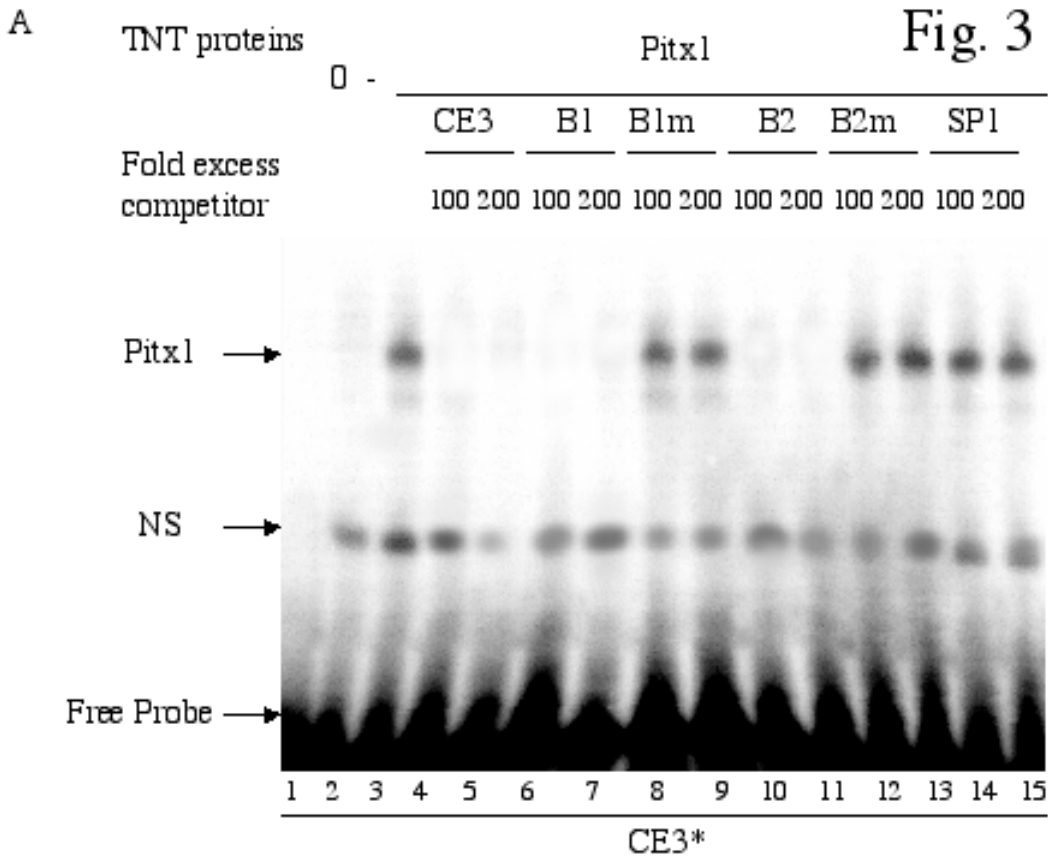


Fig. 4

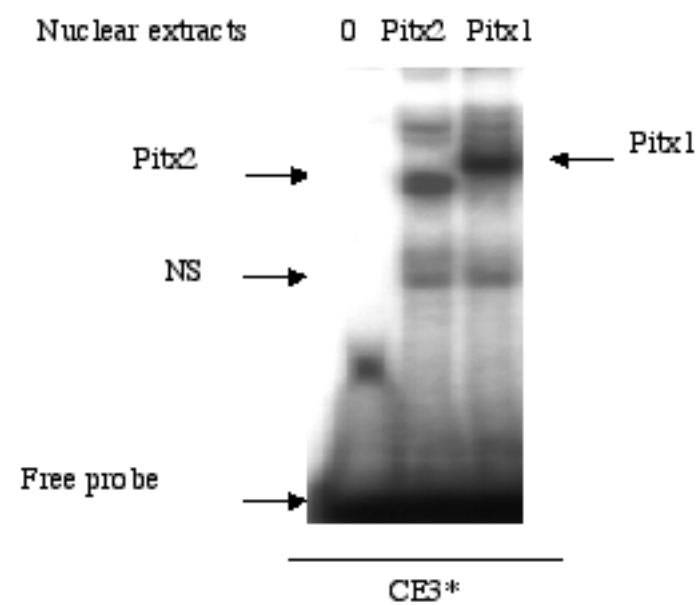
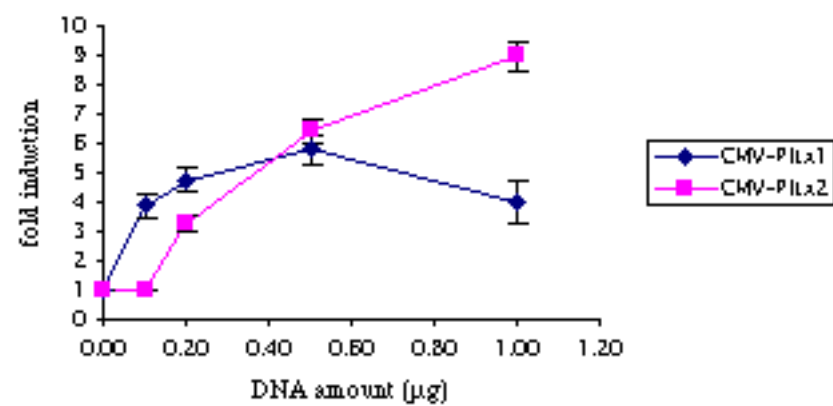


Fig. 5

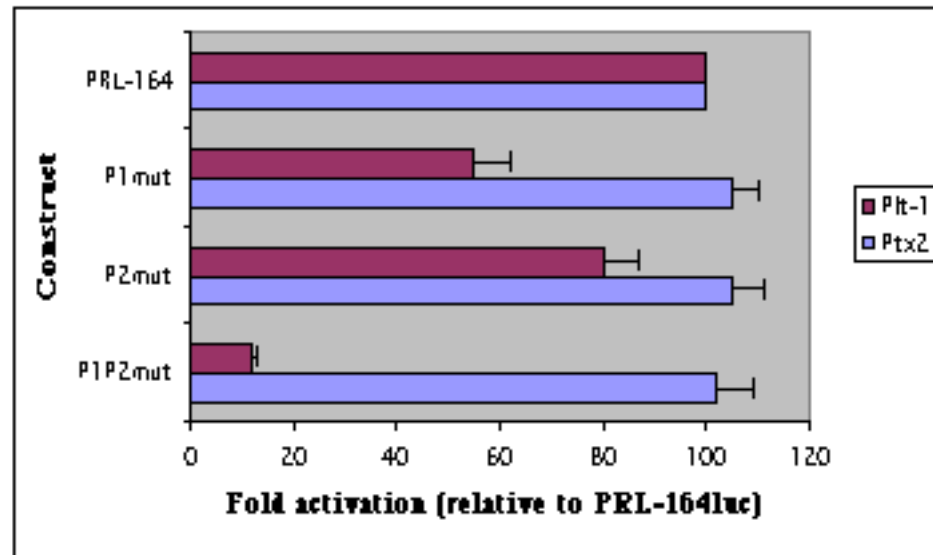
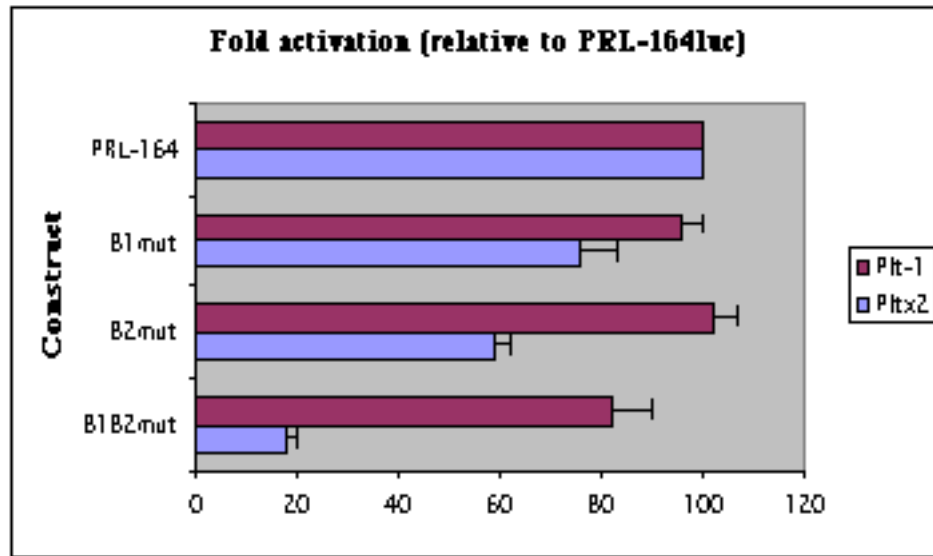


Fig. 6

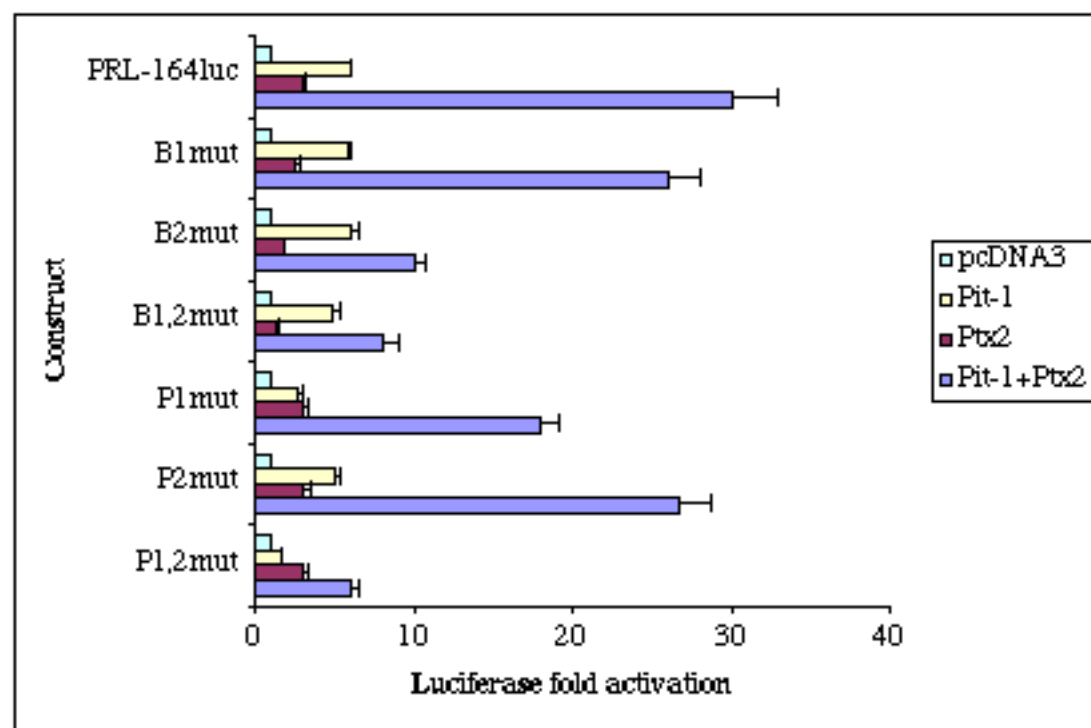
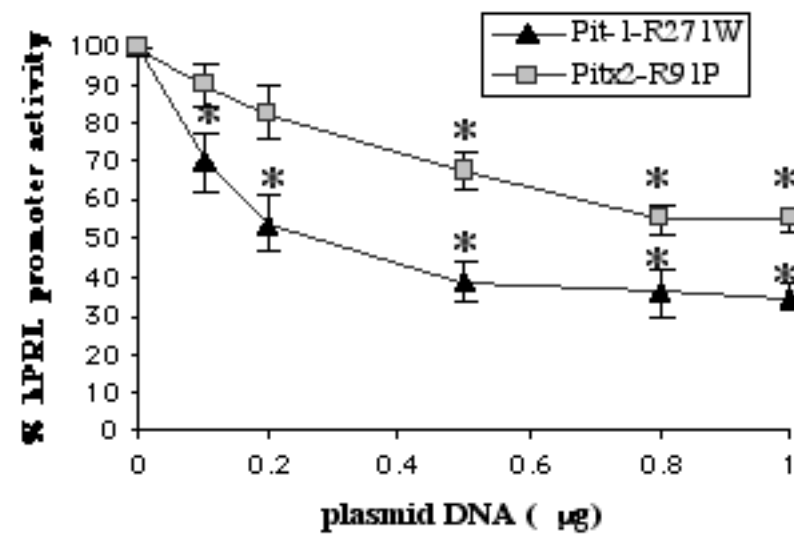
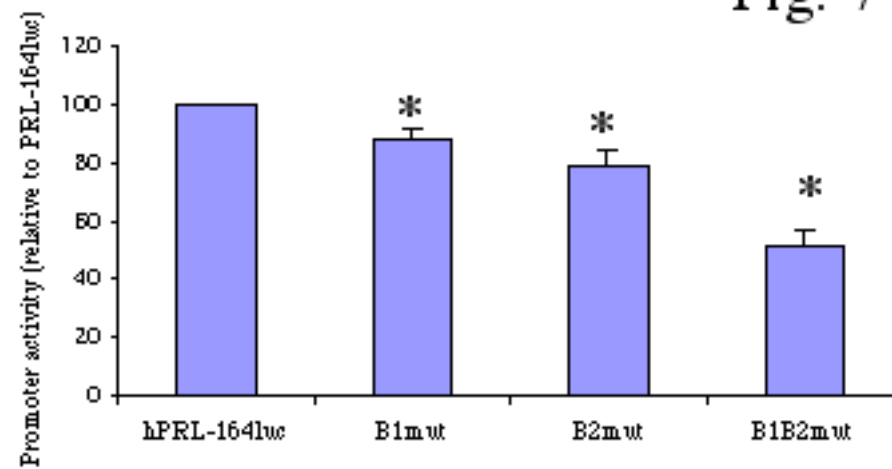


Fig. 7



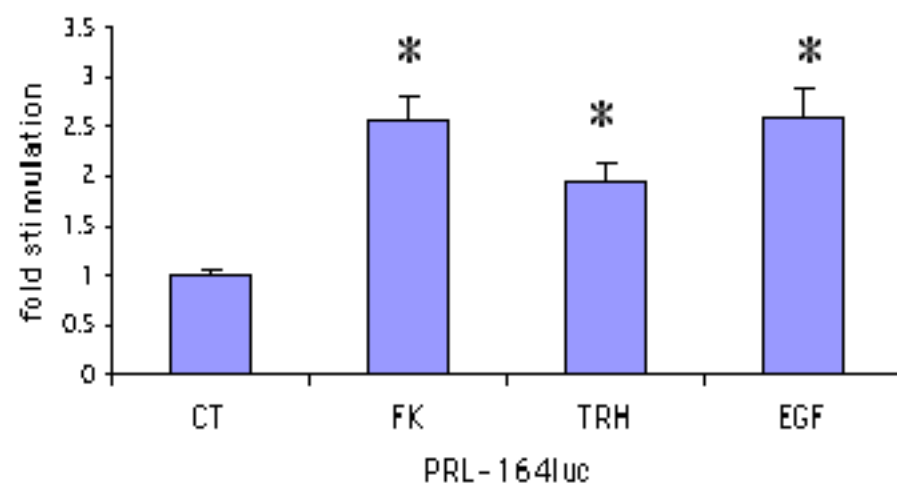


Fig. 8

