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**Cloning and Characterization of a Novel DNA-
Binding Protein That Can Mediate the Cellular
Transcriptional Action of Protein Kinase A**

by

MAKIKO SUZUKI FLISS

A dissertation submitted to the Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements for the degree of Doctor of
Philosophy, The City University of New York

1997

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Abstract

Cloning and Characterization of a Novel DNA-Binding Protein That Can Mediate the Cellular Transcriptional Action of Protein Kinase A

by

Makiko Suzuki Fliss

Advisor: Professor Carter Bancroft

A novel transcription factor, *Ickis*, was cloned from a pituitary GC cell expression library, using the prolactin (PRL) gene regulatory-element site1P as a probe. *Ickis* binds specifically to the distal end of site1P, adjacent to the site for the pituitary-specific factor, Pit-1, implying a potential interaction between these proteins. The complete *Ickis* cDNA (1919 bp), obtained using 5'-RACE PCR, encodes a novel protein containing three WD-motifs, but no classical DNA-binding motif. Genomic southern analyses revealed that *Ickis* is a single copy gene that is well-conserved from yeast to human, suggesting an important physiological role for *Ickis*.

Northern analysis showed that *Ickis* mRNA accumulates in GH3 pituitary cells with a size of circa 1.9 kb, corresponding well with the

length of the cloned *lckis* cDNA. Analysis of a human organ blot revealed tissue-specific forms of *lckis* mRNA, which might be generated from alternative splicing and/or promoter usage. RT-PCR analysis of human tumors revealed that *lckis* mRNA is present in many pituitary cell-types but in only a subset of neuronal cells. *lckis* may thus play a regulatory role outside of the pituitary, and exhibit tissue-specific activity via isoforms.

Western analysis using GH3 (pituitary) cells showed nuclear accumulation of *lckis* protein circa 46 KDa in size, which corresponds well with the molecular mass deduced from the *lckis* coding region. Transient co-transfection analysis showed that *lckis* can activate the PRL promoter in heterologous C6 cells. *lckis* but not Pit-1 was further activated by PKA, suggesting that *lckis* may be a downstream effector for PKA regulation of PRL. *lckis* and Pit-1 exhibited additive activity on this promoter. Similar analyses were performed using a GAL4-*lckis* chimera, in order to examine *lckis* action in the homologous GH3 cells. GAL4-*lckis* activated a GAL4 promoter construct, indicating that *lckis* contains a functional transactivation domain. This fusion protein was also able to reconstitute PKA activation in the homologous cellular environment. In summary, *lckis* is a novel transcription factor that can activate the PRL promoter via site1P, and can also mediate transcriptional actions of PKA.

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I would like to express my deepest appreciation to my husband, Al, for his enormous support, critical advice, and his technical contributions throughout this project. Al and Snowey, together always provide me a comfort and a cheerful environment for my life.

I would like to dedicate this entire work to my dearest sister, *Nami*, whose spiritual input to my life should be always remembered and be most highly acknowledged. I believe that my best wishes and love will reach her, and I deeply appreciate that I had many of her precious memories with her. We will always love you.

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CHAPTER I

BACKGROUND AND INTRODUCTION

Background and Introduction

[1] Prolactin and its gene regulation

Prolactin (PRL) is one of the most versatile hormones, and its expression is almost exclusively limited to the lactotrophic cells of the anterior pituitary. PRL-like immunoreactivity is also found in a variety of organs and tissues such as mammary gland, adrenal gland, corpus luteum, prostate, testes, urethral gland, gut, lymphoitic cells, and pancreatic islet (Seo, 1985, and Baulieu *et al.*, 1990). This broad distribution reflects more than 100 different actions elicited by PRL; initiation of lactation, maintenance and regression of corpus luteum, stimulation of steroid biosynthesis in the ovary, immunomodulation in lymphocytes, and osmoregulation in the kidney (DeVlaming, 1979, Nicoll, 1980, for review).

It should be noted that PRL is under general negative regulation by the hypothalamus. Physical separation of the pituitary gland from the hypothalamus results in constitutive PRL production (Baulieu *et al.*, 1990). A number of factors have been identified that inhibit PRL release and/or synthesis in the pituitary, including dopamine, GAP or PIF, and GABA, and in some cases, somatostatin (Baulieu *et al.*, 1990). The following factors are able to remove hypothalamic inhibition; thyrotrophin releasing hormone (TRH), Ca^{2+} , vasoactive intestinal peptide (VIP),

pituitary adenylate cyclase-activating peptide (PACAP) (Chen, 1993), epidermal growth factor (EGF), and, estrogen (Baulieu *et al.*, 1990). These multiple signals are transmitted to the PRL promoter via specific transcription factors in order to maintain homeostasis and/or respond to physiological needs. This is not unexpected since the PRL promoter contains multiple hormone-responsive elements (see diagram below).

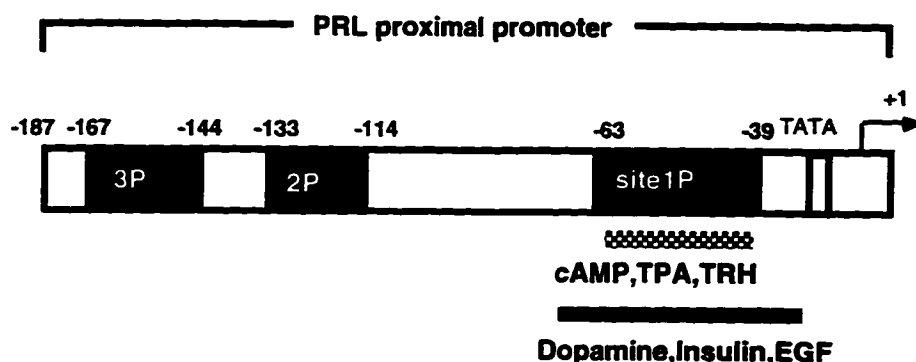


Figure 1. Site1P mediating hormone-dependent regulations

[2] Discovery of Pit-1

Two groups simultaneously cloned and characterized pituitary-specific factor 1, Pit-1 (Ingraham *et al.*, 1988, Bodner *et al.*, 1988). Nelson *et al.* ('88) identified that Pit-1 was able to bind to multiple cis-elements in the PRL promoter including site1P, 2P, 3P, and 4P (see diagram above). The existence of a pituitary-specific factor had been speculated since a

unique DNA foot-printing profile was obtained using a pituitary extract (Nelson *et al.*, 1986). The functional significance of this tissue-specific factor was examined using a cell-free *in vitro* transcription system. Activation of the growth hormone (GH) gene was observed only upon the addition of pituitary extract (Gutierrez-Hartmann *et al.*, 1987). The region of the promoter that is critical for the activation of GH expression mapped precisely to the pituitary-specific DNA foot-printing sites (Ingraham *et al.*, 1988, and Castrillo *et al.*, 1989). These data indicated that pituitary-specific factor(s) can directly bind to cis-elements in the GH promoter and therefore may be able to direct the tissue-specific expression of the GH gene. In addition to GH gene activation, Pit-1 is also able to activate PRL promoter (Ingraham *et al.*, 1988). Since a certain population of cells can express both GH and PRL (Chatelain *et al.*, 1979, Hoeffler *et al.*, 1985), it is possible that a single factor is involved in the activation of these two distinct, but evolutionary related genes (Nelson *et al.*, 1988). Nelson *et al.* (1988) indicated that a common transcription factor could bind to both the PRL and GH promoters and possibly activate both genes. In order to test this hypothesis, DNA footprinting analysis was performed in combination with various competitors. Pit-1 is capable of binding GH, and TSH β sites, but binds to the PRL sites only weakly (Bodner *et al.*, 1988). DNA footprinting analysis strongly suggested that more than one transcription factors is capable of binding the Pit-1 cis-elements (Nelson *et al.*, 1988). If

Pit-1 is the only tissue-specific transcription factor, all Pit-1 cis-elements should be competed by other Pit-1 target sites. Other Pit-1 binding sites were unable to displace binding to site1P. Interestingly, an excess amount of Pit-1 binding elements in the GH promoter was able to displace the proximal half of the site1P. Together, these data strongly suggest that some factor other than Pit-1 can specifically bind to the distal portion of site1P. However, Nelson *et al.* (1988) claimed that only a single factor, Pit-1, binds to all the PRL cis-elements. At the very same time, slightly different observations were made by Bodner *et al.* (1988). They concluded that Pit-1 is GH-promoter-specific. Footprinting data clearly showed that nuclear extract depleted of Pit-1 was able to produce a cell-specific footprint on the PRL promoter. This suggests that some tissue-specific factor other than Pit-1 can bind to the PRL promoter cis-elements. Therefore, Bodner's group considered Pit-1 as a somatotroph-lineage factor. The controversy continues, since Pit-1 can transactivate both the GH and PRL genes (Nelson *et al.*, 1988, Mangalam *et al.*, 1989, Fox *et al.*, 1990, Li *et al.*, 1990, Radovick *et al.*, 1992), leading to the conclusion that Pit-1 is "sufficient" for GH and PRL promoter activation. However, the question still remains whether Pit-1 is "necessary" or "redundant" for the PRL promoter.

[3] Significance of the PRL promoter site1P cis-element

Site1P can mediate a response to c-AMP and protein kinase A (PKA) (Iverson *et al.*, 1990, Elsholtz *et al.*, 1991, Okimura *et al.*, 1994, and Rajnarayan *et al.*, 1995). The activation of PRL promoter activity is greatly reduced when site1P is mutated (Rajnarayan *et al.*, 1995). In addition, tandem copies of site1P cis-elements are sufficient to mediate the PKA-linked signal to the PRL promoter (Okimura *et al.*, 1994). The importance of site1P initially suggested that Pit-1 might act as the ultimate downstream effector of the cAMP/PKA signal transduction pathway. However, PKA-mediated phosphorylation of Pit-1 is not required, since the PRL promoter is still responsive to the activation of cAMP/PKA pathway in a phosphorylation-deficient mutant Pit-1 (Okimura *et al.*, 1994, Fishberg *et al.*, 1995). Moreover, Gellersen *et al.* (1995) provided evidence that the PRL promoter is stimulated by PKA in the heterologous uterine sarcoma cell line (SKUT-1B-20), which does not express Pit-1. Interestingly these non-pituitary cells utilize the same promoter as does the pituitary, again suggesting that the PKA-mediator is not only Pit-1 but also unlikely to be a tissue-specific factor. In similar studies performed using HeLa cells, PKA also activated the PRL promoter via site1P in the absence of Pit-1 (Rajnarayan *et al.*, 1995). Furthermore, a chimeric protein containing DNA-binding domain of GAL4 fused to Pit-1 did not transmit transcriptional activity in response to cAMP, while GAL4-CREB

fusion protein did (Okimura et al., 1994). They concluded that phosphorylation of Pit-1 is not required for cAMP response, and additional transcription factors that interact site1P is the one to play critical role for cAMP-regulated signal mediation. Taken together, these observations strongly imply that a non-tissue specific factor may be the mediator of kinase action on Pit-1 binding sites in the PRL promoter.

These accumulated studies indicate that site1P is at least one of the PKA responsive elements in the PRL promoter. Since Pit-1 is not a downstream effector for PKA, additional factors must mediate this kinase signal via site1P.

[4] Search for a PKA mediator protein that binds to site1P

It should be noted that at least one factor that binds to site1P must be responsible for the action of PKA. This factor may indeed be a known transcription factor, since several transcription factors are known to bind to the site1P element. For instance, Oct-1 (octamer-binding protein) can recognize site1P and form a heterodimer with Pit-1 (Voss *et al.*, 1991). This protein is in the POU homeodomain family along with Pit-1 and is expressed in various tissues. Co-expression of Oct-1 and Pit-1 can synergistically activate the PRL promoter (Voss *et al.*, 1991). However, Oct-1 DNA binding activity is abolished upon phosphorylation (Segil *et*

al., '91), suggesting that this protein is not the PKA effector via site1P. TEF (Thyrotroph Embryonic Factor) is also a site1P-binding protein (Drolett *et al.*, 1991). While its expression is restricted to the developing thyrotrophs, TEF mRNA becomes expressed broadly in a variety of tissues post-development. It would be interesting to determine whether TEF is a functional substrate for PKA. LSF-1 (Lactotroph Specific Factor) could be also a possible candidate for a kinase substrate (Alexander *et al.*, 1990, and Harvey *et al.*, 1991). This factor was termed LSF-1 since it is detected in the pituitary lactotrophs but not in HeLa cells. LSF-1 has been reported to preferentially bind to the proximal region of the Pit-1 binding site (site1P) of the PRL, but is unable to bind to the GH promoter (Harvey *et al.*, 1991). LSF-1 is also reported to be able to bind multiple elements in the TSHB promoter and initiate its transcription *in vitro* (Alexander *et al.*, 1990). However, LSF-1 has yet to be cloned and fully characterized. The final candidate is mLIM-3 which belongs to the class IV homeo gene family (Seidah *et al.*, 1994, and Bach *et al.*, 1995,). Its transcripts are widely expressed in the developing pituitary gland (Seidah *et al.*, 1994, and Bach *et al.*, 1995). Like Oct-1, mLIM-3 can form a heterodimer with Pit-1 and can enhance the ability of Pit-1 to activate the PRL promoter (Bach *et al.*, 1995). However, it remains to be determined whether this protein is phosphorylated.

None of these protein have been demonstrated to be a downstream effector of the action of PKA. I describe in this thesis how I have cloned a novel site1P-binding protein (termed *lckis*) from a pituitary expression library, and characterized both its tissue expression and its ability to act as a kinase-regulated transcription factor.

CHAPTER II

CLONING OF A NOVEL PROTEIN *ICKIS* THAT RECOGNIZES A SPECIFIC CIS-ELEMENT IN THE PROLACTIN PROMOTER

Summary

A novel DNA-binding protein was cloned from a pituitary (GC cell) expression cDNA library using the PRL promoter cis-element site1P as a probe. This site1P-binding protein was termed *lckis* (named after a Japanese legendary character who can transform its appearance upon a particular stimulus). *lckis* binds the most proximal cis-element of the PRL promoter specifically at the distal end of site1P, while Pit-1 (a pituitary-specific transcription factor) binds the proximal portion of site1P. This suggests a potential interaction between *lckis* and Pit-1. *lckis* contains three WD-motifs, which may be involved in protein-protein interactions and often represent regulatory proteins (Van-der-Voorn and Ploegh, 1992, and Neer *et al.*, 1994). *lckis* contains no apparent DNA-binding motif, suggesting that *lckis* may represent a new class of DNA-binding proteins. Southern analysis revealed that *lckis* is a single copy gene and evolutionarily conserved throughout species, suggesting a physiological importance for *lckis*.

Introduction

A number of transcription factors have been isolated using the *in situ* filter binding method (Vinson, *et al.*, 1988, and Singh *et al.*, 1989 for review). This method is an application of the Southwestern binding technique, in which filter-bound proteins are detected using labeled oligonucleotide probes. The major advantage of Southwestern cloning is that it allows one to quickly obtain a cDNA clone encoding a DNA-binding protein without the labor-intensive process of protein purification and microsequencing. Moreover, this technique is advantageous when the target protein is in low abundance. This is in contrast to DNA-affinity chromatography which requires large amounts of starting protein. *In situ* filter binding detection requires only an appropriate expression library, thus facilitating the screening procedure.

There are, however, several concerns when using this method. One might isolate general DNA binding proteins which are not specific for the DNA target sequence. This problem could be overcome by performing gel-shift analysis on putative positive clones, which would confirm their sequence-specificity. Another concern is in the optimization of the binding condition for the target protein. For example, the ability of a given protein to form a stable complex with its DNA target sequence may depend upon its phosphorylation state and/or require interactions with additional proteins (homo- or hetero dimerization, phosphorylation, etc.). In order to

optimize critical parameters such as fidelity and sensitivity, binding conditions were examined using Pit-1 (a site1P-binding factor), and its isoform $\Delta 4$ Pit-1 (a non site1P-binding factor) as positive and negative controls, respectively. These conditions were then employed in the screening of a pituitary cell expression cDNA library using site1P.

Results

[1] Use of denaturation/renaturation cycling along with a multimerized probe yields both specific and high intensity signals

In order to detect true positive clones, the specificity of the probe was compared using the following treatments. Pit-1 and Δ 4Pit-1 were used as positive (PC) and negative controls (NC), respectively. Pit-1 is a well characterized site1P-binding protein and Δ 4Pit-1 is a non-site1P-binding Pit-1 isoform (Voss *et al.*, 1993, Day *et al.*, 1994). Both proteins were overexpressed in the *E. coli* PET expression system (Novagen) and affinity purified using Pit-1 antibody (prepared by Dr. Brian Kloss). Each protein was blotted onto a filter and subsequently treated with (refolded) or without (native) a cycle of denaturation/renaturation. After treatment, the filters were incubated with labeled site1P (see Experimental Procedures for details). As seen in **Figure 1** (left panel), site1P fails to distinguish the positive from the negative control under native condition, whereas, after denaturation/renaturation a signal was seen with Pit-1 but not with Δ 4Pit-1. Thus, it appears that refolding of the protein is necessary to obtain binding fidelity when site1P is used as probe. To attempt to increase the sensitivity of this assay system, the site1P oligonucleotide was multimerized (**Figure 2**) and compared as a probe to the monomer

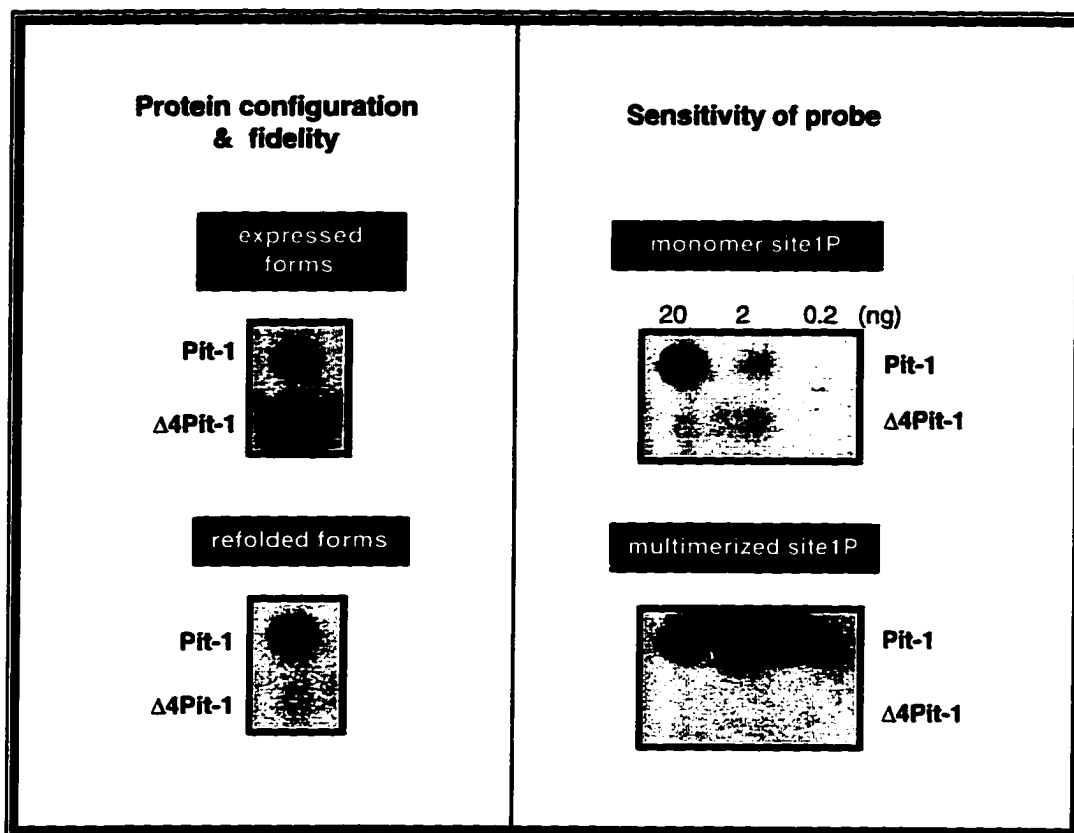


Figure 1. Comparison of sensitivity and fidelity of detection achieved under different binding conditions

Bacterially expressed and purified Pit-1 (site1P-binding protein) and $\Delta 4$ Pit-1 (non-site1P-binding protein) were subjected to filter binding assay under various conditions. Left panel: Labeled site1P was used as probe with both groups of proteins (20 ng) which had been untreated (**expressed forms**) or treated with denaturation and renaturation cycling procedure (**refolded forms**). Right panel: Different concentrations (20, 2, 0.2 ng) of refolded proteins were subjected to filter binding assay using either a monomer or multimer probe.

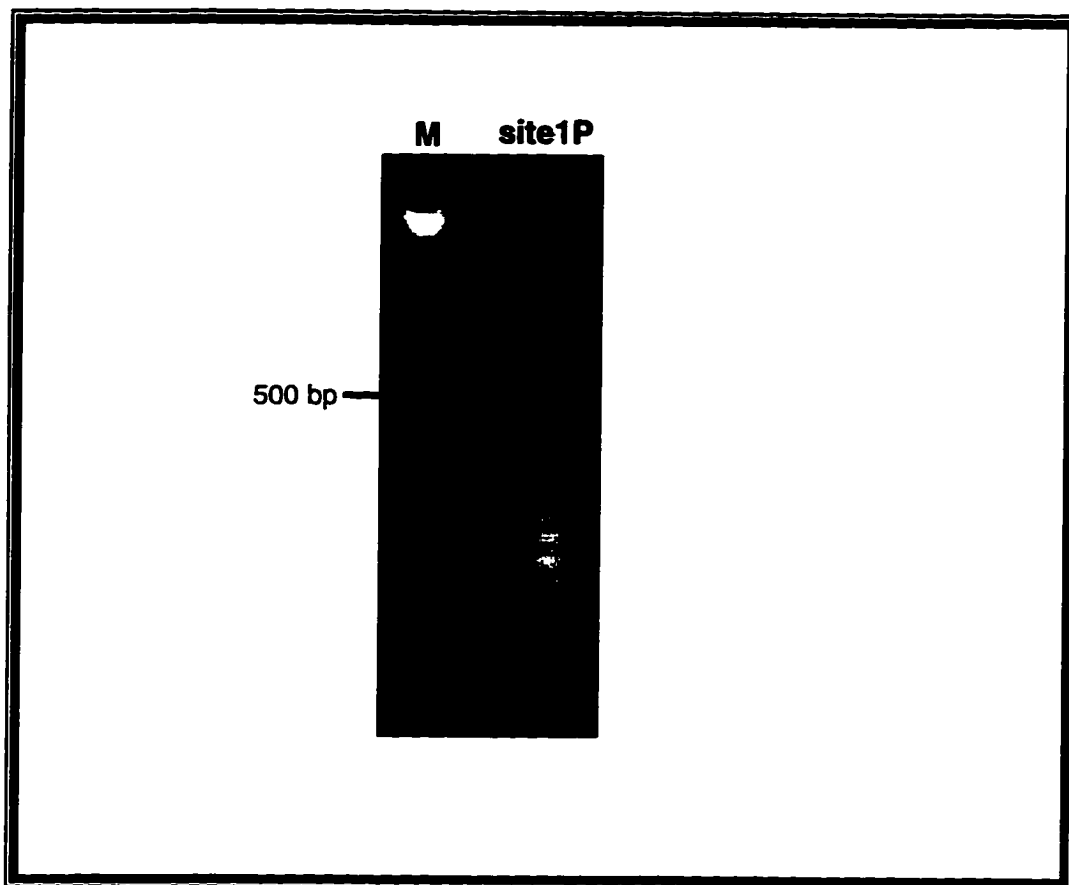


Figure 2. Multimerized site1P

Ligated site1P was analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide. (M: DNA marker, λ HindIII)

form. Results from this assay (**Figure 1**, right panel) show that multimerized site1P gives a better signal to noise ratio than does the monomer form, since the last dilution of Pit-1 (0.2 ng) was still detectable using the multimerized site1P. The denaturation/renaturation cycling procedure along with a multimerized oligonucleotide probe was thus chosen for the following screening for site1P-binding proteins.

[2] Isolation of a protein that specifically recognizes site1P

A cDNA library prepared from a rat pituitary tumor cell line (GC cells) was screened using the in situ filter binding assay, which is illustrated in **Figure 3**. Briefly, the expressed recombinant proteins were transferred onto nitrocellulose, and the filters were subjected to denaturation and renaturation cycles. After blocking with non-fat milk, the filters were incubated with a multimerized site1P probe, washed, and exposed to x-ray film. Secondary and tertiary screens were performed on the positive plaques in order to obtain a single pure plaque. Out of 1.5×10^6 plaques screened, five potential positive clones were obtained, and all of them were subjected to the following screening procedures. Filter binding assays were performed in order to eliminate proteins that non-specifically bind to site1P. Heterologous DNA sequences **CRE** (cAMP Responsive Element), poly[dl-dC] (**dlIdC**), and mutated site1P (***1P***), were used as probes in order to examine the binding specificity of positive clones

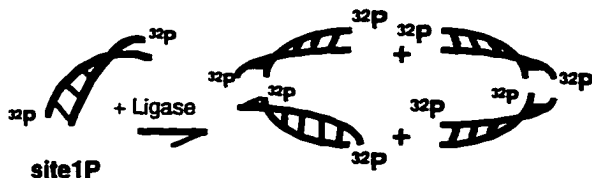
1. Expression of GC pituitary cDNA library



2. Denaturation/renaturation cycling



3. Application of multimerized site1P probe



4. Detection of positives



Rat pituitary library was expressed and transferred onto nitrocellulose filters.

Expressed proteins were fixed by air drying.

Recombinant clones were denatured and slowly renatured.

Filters were blocked with both milk and nonspecific competitor DNA.

Site1P oligonucleotides were end-labeled with γ - ^{32}P -ATP and multimerized by DNA ligase.

Filters were incubated with site1P probe followed by gentle washing.

Expose filters to x-ray film.

Figure 3. Screening procedure for a site1P-binding protein

towards site1P. Only one of the four candidate clones exhibited specific binding to site1P (**Figure 4**). We designated this true positive clone as *lckis*. The four other putative positives displayed non-specific binding to double-stranded oligonucleotides including *1P* and/or CRE (data not shown). These false positive clones were *in vivo* excised, and sequenced. Two out of four false positives have been identified as Nerve Growth Factor-Inducible Protein A (NGFI-A), and Janus Kinase 1 (JAK1). The partial NGFI-A clone contains an in frame DNA-binding domain that is able to recognize GC-rich sequences (Crosby *et al.*, 1991). However, the filter binding experiment showed that this partial NGFI-A can bind to the GC-rich cis-element, Sp1, in addition to site1P, and, *1P* (data not shown). Similar results were obtained from Southwestern analysis (data not shown). Thus, this clone was excluded from further analysis. The JAK1 clone was also omitted from further analysis, since it was out of frame. The two remaining positive clones had an open reading frame less than 14 amino acids in length. These clones exhibited no significant similarity to any other proteins, and failed to exhibit the consistent specificity in binding the site1P probe by the filter binding assay, thus, both clones were also excluded from this study. The true positive clone, *lckis*, was then *in vivo* single strand excised from lambda arm (Stratagene), and transformed into host cells (SOLAR) for the subsequent procedures such as DNA sequencing. The isolated cDNA insert had a 1.2 kb open

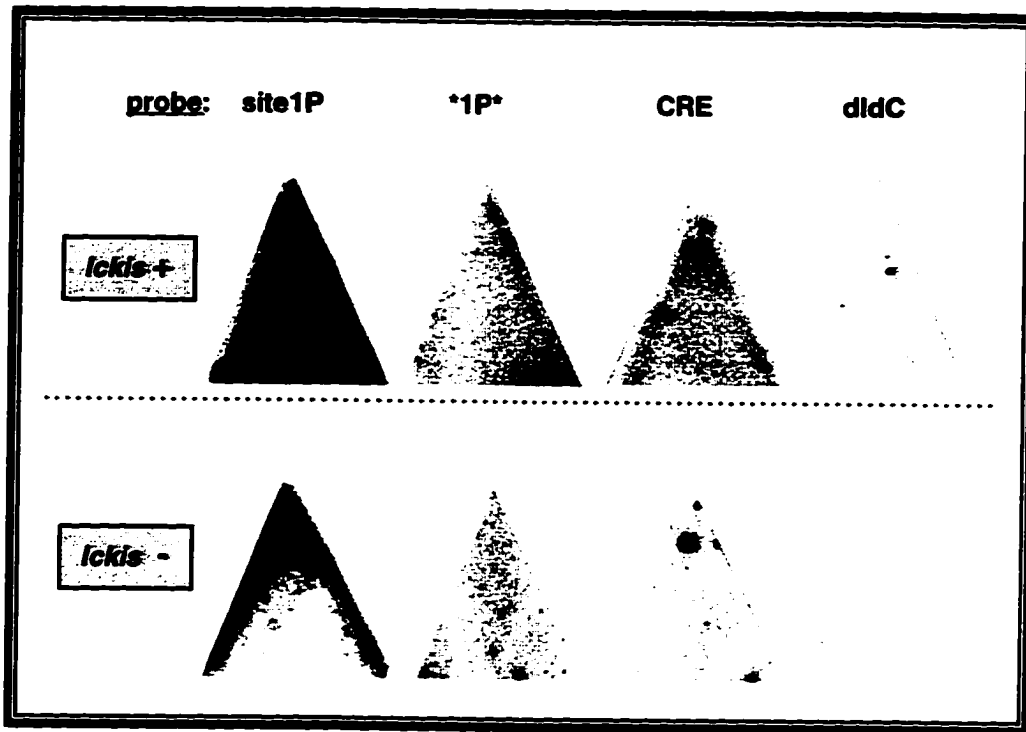


Figure 4. *Ickis* is a site1P-specific binding protein

Filter binding assays were performed using various probes indicated above with [*Ickis*(+)] or without [*Ickis*(-)] the IPTG induction of the *Ickis* protein (Experimental Procedures for details). All filters were subjected to denaturation and renaturation cycling under the same conditions.

reading frame in register with the β -galactosidase gene. *Ickis* is the only candidate that satisfies our initial screening criteria: a protein that recognizes site1P in a DNA sequence-specific fashion (see also below).

[3] DNA-binding specificity of *Ickis* to site1P

In order to further examine the specificity and ability of *Ickis* to interact with the target sequence site1P, the coding region of *Ickis* cDNA was cloned into a bacterial expression vector pET28(+). *Ickis* was produced as a histidine-tagged fusion protein in order to facilitate further purification of the recombinant protein. A time course experiment at various temperatures (40C, 37C, RT) was performed to optimize the production of *Ickis*. As shown in **Figure 5**, the histidine-tag-*Ickis* fusion protein was maximally expressed after 2 hrs of IPTG treatment at 37C. The majority of the fusion-*Ickis* protein was found in the insoluble fraction under all experimental conditions (data not shown). A denaturation and renaturation method was used to recover the protein in a soluble form. Subsequently, a crude bacterial extract containing over-expressed *Ickis* was further purified using nickel affinity chromatography. Renaturation of *Ickis* occurred in the column, and the protein was eluted, subsequently desalted, and concentrated. The last lane (P) in **Figure 5** shows the purified *Ickis*, which was used in subsequent gel shift analysis.

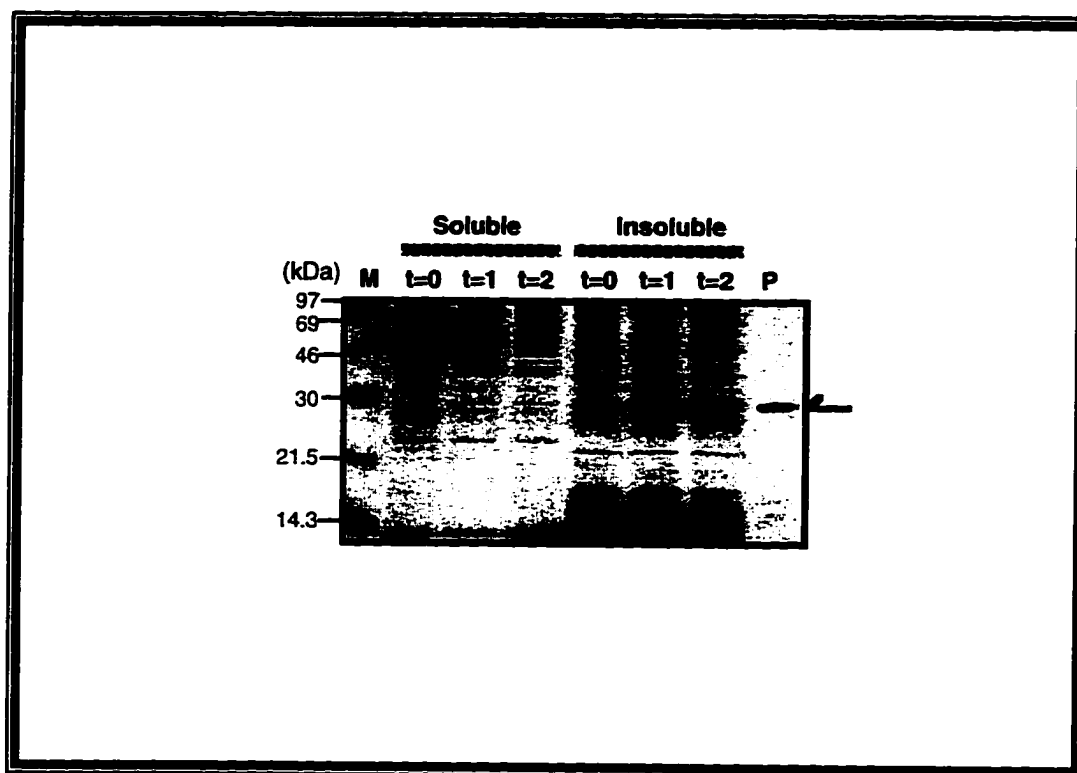


Figure 5. Expression and purification of *Ickis* protein *in vivo*

Bacterial cells containing *Ickis* [pET28b(+)] were induced with IPTG. Aliquots were removed just prior to the addition of IPTG (t=0) and at various time thereafter (t=0, 1, 2 hrs). Cells were processed to separate the soluble from the insoluble fraction. *Ickis* protein in the insoluble fraction was purified on a metal chelation column (P). Samples were analyzed by SDS-PAGE with the indicated molecular weight standards (M).

Figure 6 shows the DNA sequence of the oligonucleotides (cis-elements) used in the gel-shift analysis. These DNAs were used as competitors in order to examine the specific binding of *Ickis* towards site1P (**Figure 7**). Lane 2 demonstrates that recombinant *Ickis* protein binds to site1P. The binding of *Ickis* is diminished by the addition of excess amounts of cold site1P (lanes 3-5), but not by mutated site1P (*1P*) (lanes 6-8). This demonstrates that the *Ickis* protein binds to site1P in a sequence-specific fashion. Bacteria cells containing the empty PET vector were also analyzed as a negative control (**Figure 8**). No complex was detected from this bacterial extract even when a 500-fold excess of protein mixture was added (lanes 2-5).

To examine further the DNA-binding specificity of *Ickis*, competition analyses were performed using both the related and unrelated cis-elements shown in **Figure 6**. As shown in **Figure 9**, none of these cis-elements could compete with the site1P probe, including these that are closely related to 1P (2P, 3P, and, pGHF1). These data strongly suggest that *Ickis* is a specific site1P-binding factor. Moreover, *Ickis* has a binding profile distinct from that of Pit-1, which is known to bind to the 1P, 2P, and pGHF-1 elements in the PRL or GH promoter. Further competition analysis was performed using two mutated site1P oligonucleotides, *1P and 1P* (**Figure 10a**), in order to more precisely identify the target site for *Ickis* within site1P. Mutant *1P contains a mutation in the distal portion

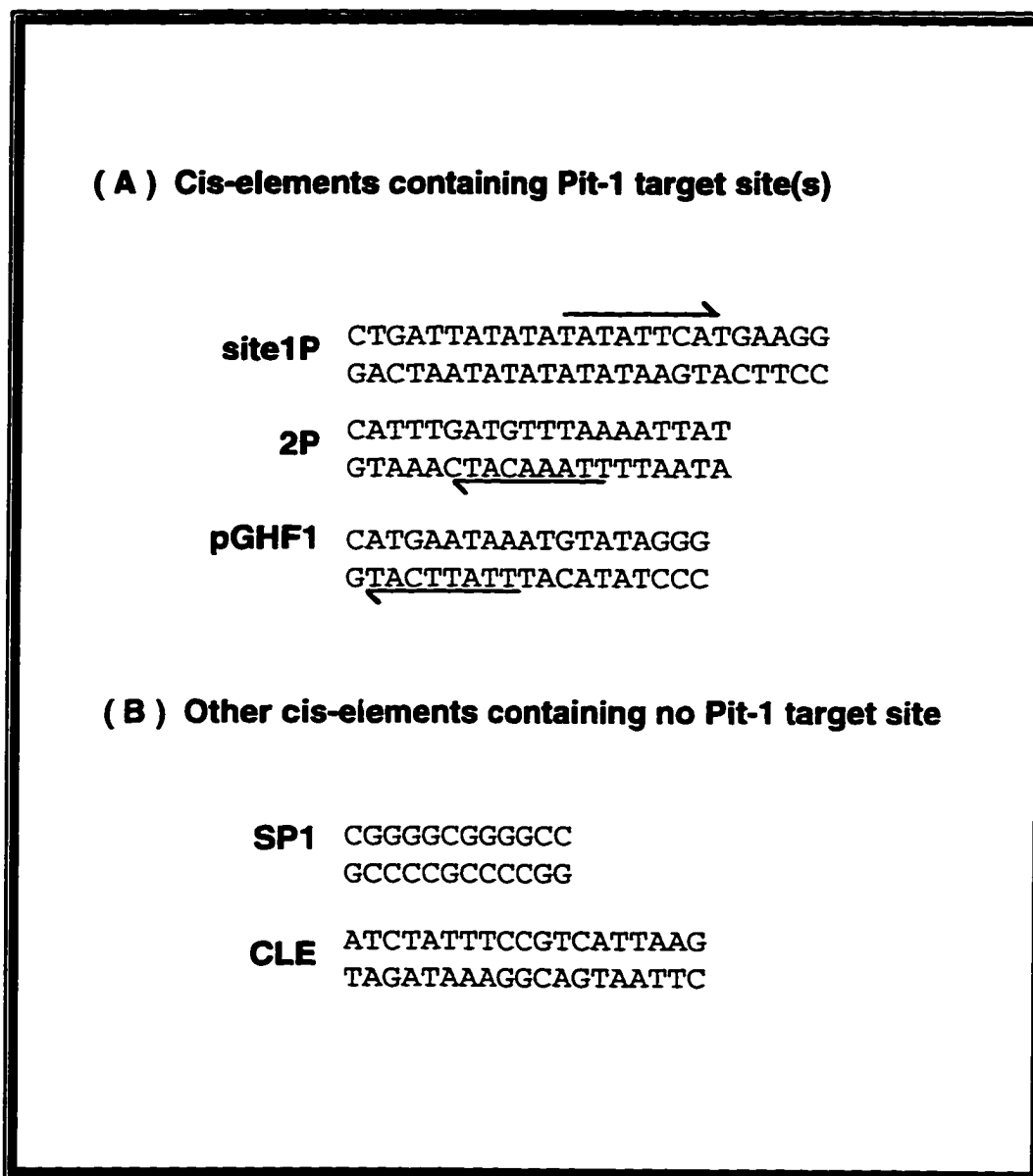


Figure 6. Site1P and other cis-elements used in the gel shift analysis

(A). Each of these cis-elements contains a Pit-1 binding site (indicated by the arrows). Both site1P and 2P are located in the PRL promoter and pGHF-1 is in the GH promoter. (B). Neither cis-element is related to the Pit-1 target sequence. SP1 is a target element for Sp1 transcription factor (Mitchell and Tjian, 1989), and the CLE is a CRE-like element in the PRL promoter.

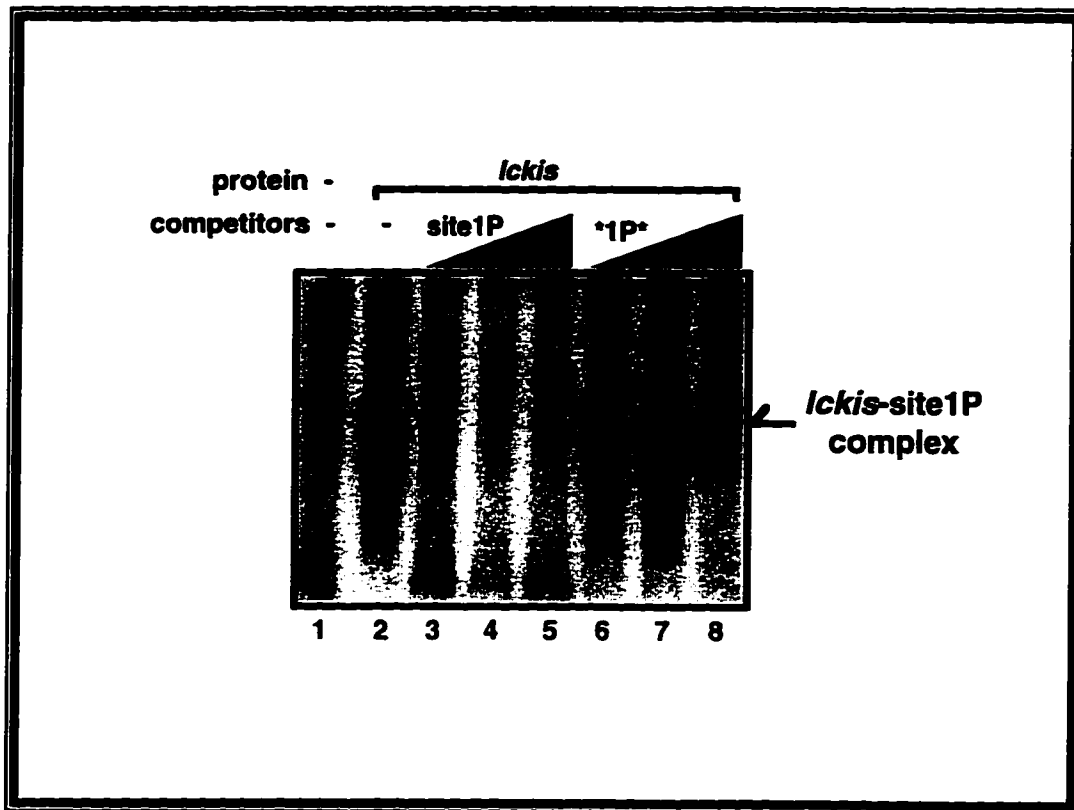


Figure 7. *Ickis* specifically recognizes site1P cis-element

Gel-shift analysis was performed using a his-tagged *Ickis* recombinant protein. Labeled site1P was applied alone (lane1), or incubated with *Ickis* protein, either alone (lane2) or with increasing amounts of the indicated competitors (250-, 500-, and 5000-fold excess), and analyzed on a polyacrylamide gel.

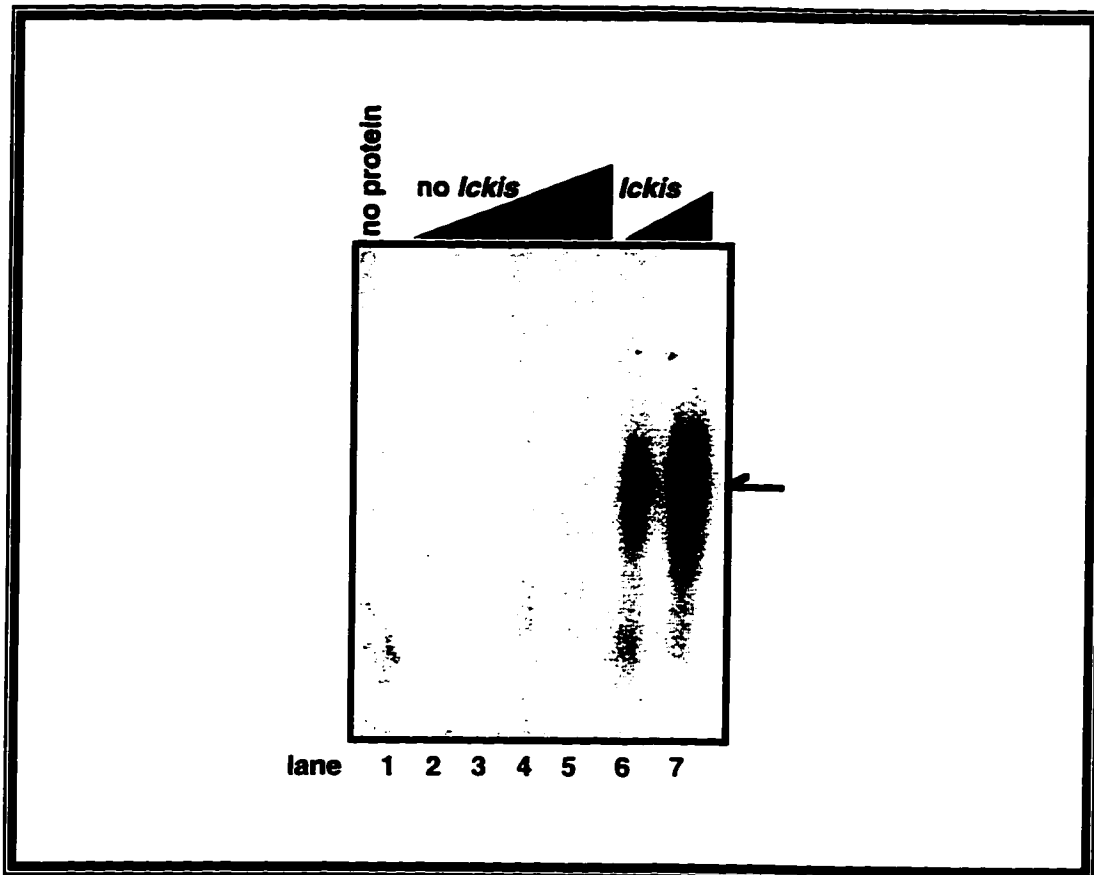


Figure 8. Purified *Ickis* but not bacterial proteins can bind to site1P

Gel-shift analysis was performed using labeled site1P plus increasing concentrations of either the bacterial extracts containing no *Ickis* protein or partially purified *Ickis* protein (*Ickis*) recovered from bacterial cells containing *Ickis* fusion vector. Both proteins were prepared from the insoluble fraction and refolded as described in Experimental Procedures. Lanes received either probe alone (lane 1), or probe plus either 10-, 25-, 50- and 100- fold concentrated bacterial extract (lanes 2-5), or 0.1 and 1 fold excess bacterial extract containing *Ickis* (lanes 6-7). Arrow indicates the *Ickis*-site1P complex.

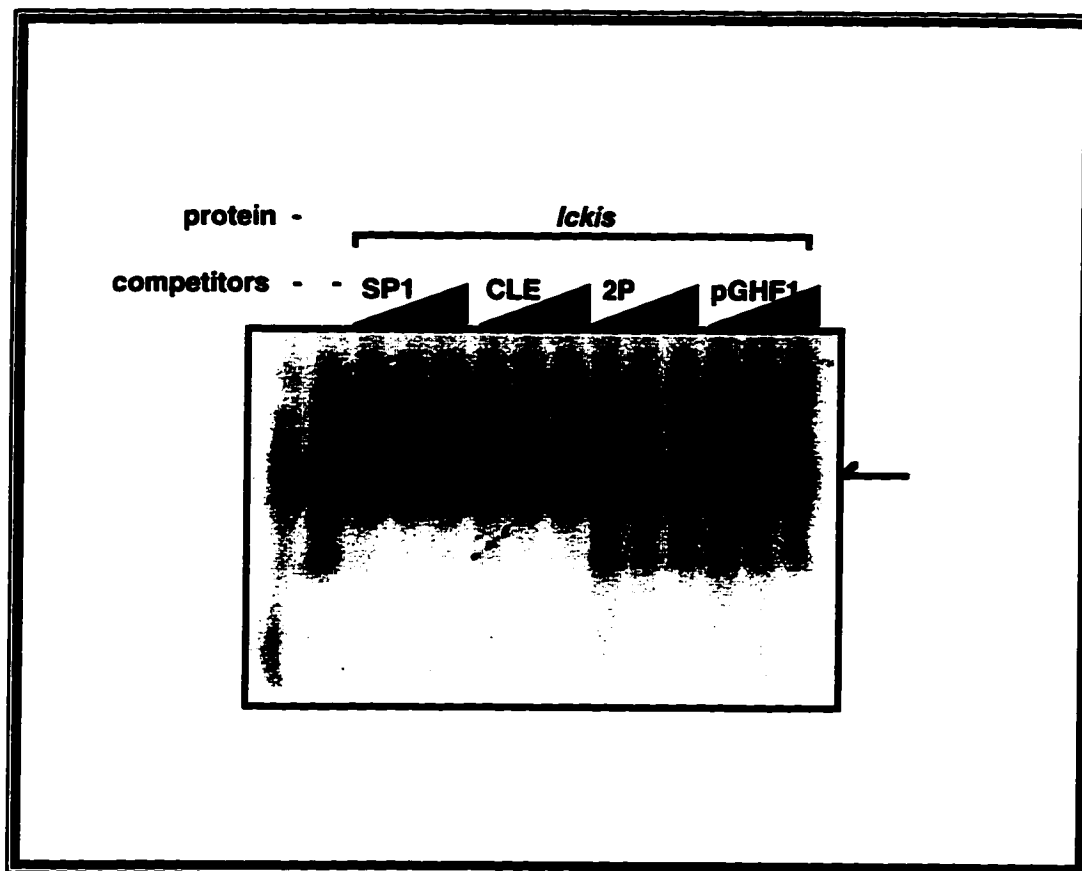
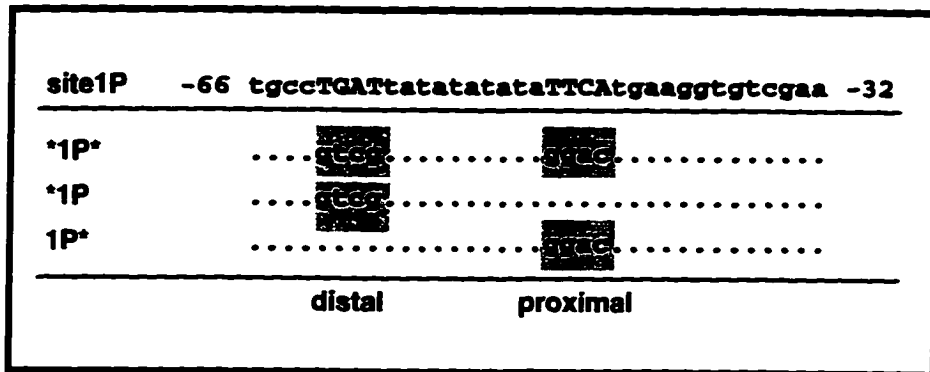


Figure 9. Further analysis of the specificity of *Ickis* toward site1P

Gel-shift competition analysis of *Ickis*. Same as the previous gel shift experiment except for the competitors.

a.



b.

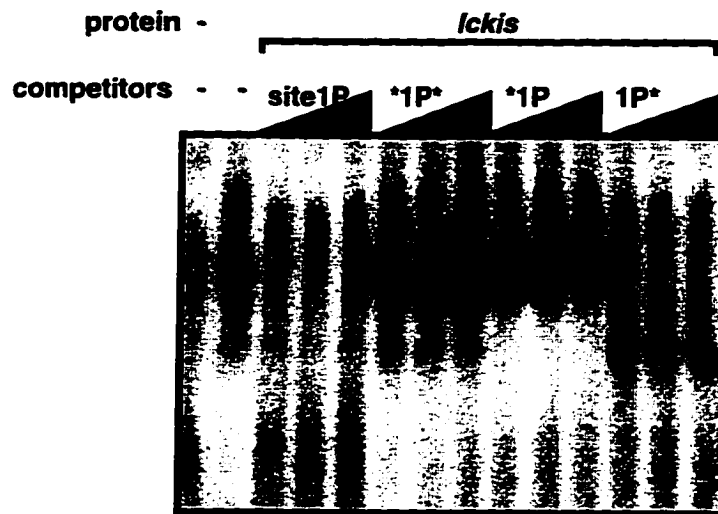


Figure 10. *Ickis* binds to the distal end of site1P

a. Sequences of mutated site1P competitors used in the gel shift analysis. Mutation at the proximal site corresponds to a part of the Pit-1 binding site. b. Gel-shift competition analysis was performed using site1P as a probe. Same procedure as in the previous gel shift was carried out.

of site1P, but the Pit-1 binding site is intact. It has been demonstrated that Pit-1-site1P complexes can be displaced by excess amounts of *1P, but not by 1P* which has a disrupted Pit-1 target element (Yan *et al.*, 1991). Interestingly, the opposite binding profile was shown for *Ickis* (Figure 10), since 1P* was able to displace *Ickis* from site1P, while *1P could not. This suggests that *Ickis* preferentially binds to the distal side of site1P, whereas, Pit-1 binds to the proximal side (Figure 11). Based on these results, the minimal target sequence for *Ickis* was deduced. Figure 12 shows an alignment of cis-elements, with the *Ickis* minimum target sequence on top. Bases identical to the one on top are displayed in bold letters. The region CTGATT of site1P seems to be required for *Ickis* binding. These observations together with the earlier results, strongly suggest that *Ickis* recognizes site1P in a sequence-specific manner, and that its binding site in the distal portion of site1P may not overlap the Pit-1 binding site in 1P. It is thus possible that these two proteins can co-occupy site1P.

[4] Isolation of the full length *Ickis* cDNA

The initial *Ickis* cDNA clone contained no candidate for an initiator methionine. In order to obtain additional 5'-sequence, the original pituitary (GC) cDNA library was rescreened with the coding region of *Ickis*. Several positive clones were isolated, however, none of these clones

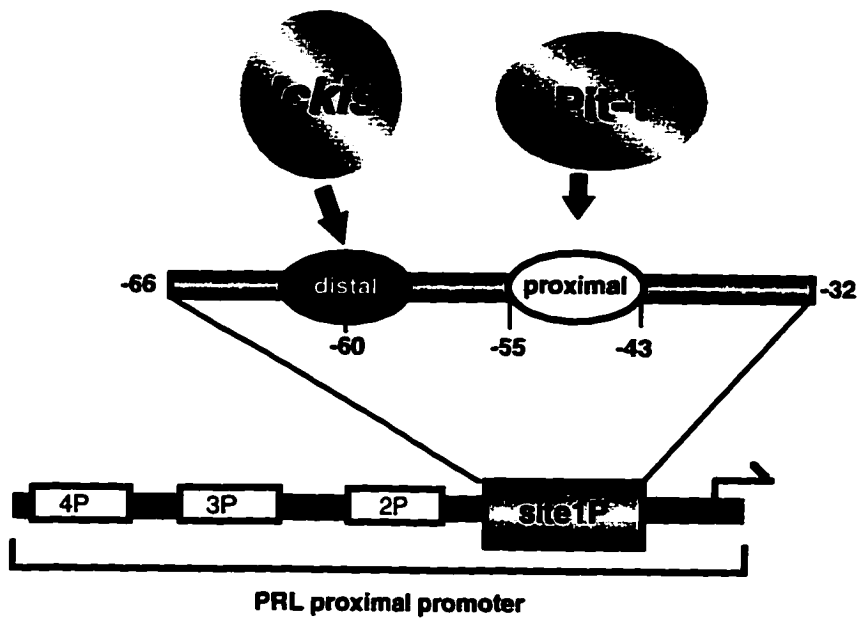


Figure 11. Predicted *Ickis* target site on site1P

Shown are the known binding site for Pit-1 (Jacobson *et al.*, 1997), and the deduced location of the *Ickis* binding site.

		cTGATt		
1P	tgc	ctgatt	ata] <i>Ickis</i> target sequence
1P*	tgc	ctgatt	ata	
*1P	tgc	cgtcgt	ata] <i>Ickis</i> non-target sequences
pGHF1	gcc	atgaat	aaa	
2P	cat	ttgatg	tta	
CLE	tta	atgacg	gaa	
3P^e	ttc	ctgaat	atg	

Figure 12. Predicted minimum target sequence for *Ickis*

@ Cis-element 3P was also incorporated as a *Ickis* non-target sequence since it could not displace *Ickis* from site1P (data not shown)

contained a putative initiator methionine, suggesting that very few clones in this library, if any, contain the complete *Ickis* coding sequence. Thus, 5'-RACE (Rapid Amplification of cDNA 5'-End)-PCR was applied, in order to extend extremely rare RNA species (Ausubel *et al.*, 1995). Using poly(A)-enriched RNA from GH3 cells (2 ug) as a starting material, the gene specific primer RACE1 (**Figure 13**) was used to reverse transcribe mRNA. The resultant cDNA strand was anchored with d(C)_n, and then PCR amplified with RACE1 and oligo-d(G)₁₃ primers. The nested primer RACE2 (**Figure 13**) was then employed to obtain and enrich for the PCR product of interest. A single positive band was detected (data not shown) and subcloned into pBlueScript using blunt-ligation, and was subsequently sequenced. The sequence of three independent clones contained sequence that overlapped with the original *Ickis* sequence in addition to more than a hundred bases extended 5'-end, however, none of these clones contained an in-frame methionine. The 5'-RACE was then repeated using new RACE primers Ext1 and Ext2 (**Figure 13**) along with oligo-d(G) primer. For this second round of RACE, the thermostable polymerase rTth (Perkin Elmer) was used to reverse transcribe *Ickis* mRNA. This polymerase was used in order to minimize the effects of any stable secondary structure formed during first strand synthesis. A single band was obtained from this round of RACE-PCR, extending the original 5'-end by an additional 647 nucleotides. The resulting sequence (**Figure**

-114 ccgagaccctcggcagtcttccggaaaactctagcgccaagttc
ctgagctttcggaggcaggcgcgcatgtcgtgggttccgcggggttgccggtgaacgtgcggcggg

1 atgggtcggcgccgggtgtggagctgtaccgggccccgttcccgttgtagcgcgcttcgg
61 atagacccaagactgggctgctcatcgctgcccggaggaggagctgccaagaccggc
121 ataaagaatggcgtgcattttctgcagctagagctgatcaacgggtgectgagcgcttcc
181 ttgctgcaactctcatgacacgggagacacgggcccaccatgaatttggcgcttgctggtgac
241 attctgcttgcggacaggatgccagtgctcagcttcttcgtttccaggtccatcaacag
301 aagggcagtaaagcggagaagtcaggttccaaggagcaccaggtcgacagagaaagggg
361 gctcctccagcagagaagaatcgggagcacaagttcaccgggaaggggtgaactcaaa

421 gtaaagaatttggagccagtcagacagacttcagcaatgaaccgctgcagaaagtgtg
(Ext1) (Ext2)
481 tgcttcaaccatgataaacaccctgcttgccaccggaggaactgatgggtcatgttcgtgct
Initial 5'-end
541 tggaaggtacctagcctagagaaagtcttgagtttaaagcccacgaaggggagattgga

601 gatttggctttgggtcctgatggcaagttggttactgtgggctgggactttaaggcctcc
(RACE2)
661 gtgtggcagaaggatcaactggtgacacagctacagtggcaagagaatggaccacctct

721 tctaacacaccgtaccgctaccaggcctgcaggtttggccaggttccagatcagcctggt
(RACE1)

781 gggctgcgactcttcacagtgcagataccccacaagcgcctacgacagccccaccctgc
841 tacctcacagcctgggacagttccaccttcttgctcttcggaccaggtcctgtggccat
901 gaagtcatttcctgcctcactgtcagtgaaatcgggtaccttccctagcctaggcacggctc
961 actggctctgtcgccatctacatagctttctctctccagcgctgtattatgtgaaggag
1021 gccatggcattgtgggtgacagatgtgaccttctacctgagaaggggtgcccgtccaaag
1081 ctcttgggccccatgaaacggccctgttctctgtggctgtggatagtcgttggcagttg
1141 cacctgctgccctcacggcggaggttcccgtatggctcctgctcctgctatgtgtggc
1201 cttattatcgtgaccatcctgctgctccagagtgccttcccggggttctttaacatcct
1261 gaccaatgggagtcaccttggacagtactaccttctggagcagagtcattgaggcccat
1321 gactgaagctgcatctgatgaaatggatgggtactgcccgtccctgctaaacgctgcgcc
1381 agtggcctccctatcactctgggtcctgggagccctgcttccactgtggatccatttan
1441 gacagtgtggctgaagctcagggccacactgcctgcctcgtttcctctgcctcccagggc
1501 tccagagccgagctcttccataggaacatgtgaagatgccaaagagcccagagcattgccc
1561 atccttctcgcagagactgttttctcctccccttccagctctgcccacaaggtcctcag
1621 ctttgtcgagacaaagtctgtggaagaggcaaaaggaagaccaggttagcgggtgactctgt
1681 aggtagcaccagccagtcagggccagacgcacagggagttcctgggtgacctactgcagc
1741 ctgaggaaagggaaagtgaacctcagtttattagggaggaagagttgatatttaataaag
1801 aaaga

Figure 13. Primers used in 5'-RACE-PCR and their relative positions

13) would now total 1919 nucleotide in length, which is in close agreement with the 1.9 kb mRNA size determined by Northern analysis (Chapter III). There are two potential initiation codons in the newly obtained 5' sequence, at positions 1 and 93 (Figure 14). The first AUG appears to be the better candidate for the initiation codon, because it predicts an approximately 48.5 kD protein (437 amino acids), which corresponds well to the estimated size of the protein determined by Western analysis (Chapter IV). Taken together, these data suggest that the additional 5' sequence obtained after two round of 5'-RACE likely has provided the complete coding region of *lckis* gene. It is interesting to note that two potential mRNA destabilizing elements (bold) were found at the extreme 3'-end of the *lckis* cDNA along with a consensus sequence for a polyadenylation signal (underlined) (Figure 14).

[5] Sequence analysis of the *lckis* cDNA

Figure 14 shows the cDNA sequence and deduced amino acid sequence of *lckis* with the candidate initiation codon, ATG (shown as bold). A search of the GenBank database revealed that *lckis* is a novel protein, but exhibits sequence with limited sequence similarity to a number of expressed tag sequences (EST) (National Center for Biotechnology Information). Two sequences from mouse lymph node (AA111707 and AA184937) matched *lckis* at 80-90% identity over 400

-114 ccgagaccctcggcagctctccggaaaactctagcgccaagttcctgagc
 ttccggaggcgagggcgggcatgctcgtgggttcccggggttggcgggtaacgtgcccggcg

1 **atgggctcggcgccggggtgtggagctgtaccgggccccgttcccgttgtacgcgcttcgg**
 1 **M G R R R G V E L Y R A P F P L Y A L R**
 61 atagaccccaagactgggctgctcatcgctcggggcggaggaggagctgccaagaccggc
 21 **I D P K T G L L I A A G G G G A A K T G**
 121 ataaagaatggcgtgcatcttctgagctagagctgatcaacgggtgctgagcgcttcc
 41 **I K N G V H F L Q L E L I N G C L S A S**
 181 ttgctgactctcatgacacggagacacgggcccaccatgaatttggcgcttgctgggtgac
 61 **L L H S H D T E T R A T M N L A L A G D**
 241 attctgcttggcggacaggatgcccagtgctcagcttcttctgcttccaggtccatcaacag
 81 **I L L A G Q D A Q C Q L L R F Q V H Q Q**
 301 aagggcagtaaacgggagaagtcaggttccaaggagcaccaggtcgacagagaaagggg
 101 **K G S K A E K S G S K E H P G R Q R K G**
 361 gtcctccagcagagaaagaaatcggggagcacaagttcacccggaaggggtgactcaaa
 121 **A P P A E K K S G A Q V H P E G V E L R**
 421 gtaaagaatttggggcagtcacagacagacttcagcaatgaaccgctgcagaaagtgtg
 141 **V K N L E A V Q T D F S H E P L Q K V V**
 481 tgcttcaaccatgataaacacctgcttggcaccggaggaaactgatggtcatgttcggtc
 161 **C F N H D N T L L A T G G T D G H V R V**
 541 tggagggtacctagcctagagaaagtcttggagtttaaagcccacgaaggggagattgga
 181 **W K V P S L E K V L E F K A H E G E I G**
 601 gatctggcttgggctgagtgcaagttggttactgtgggctgggactttaagcctcc
 201 **D L A L G P D G K L V T V G W D F K A S**
 661 gtgtggcagaaggatcaactgggtgacacagctacagtggaagagaatggaccacctt
 221 **V W Q K D Q L V T Q L Q W Q E N G P T S**
 721 tctaacacaccgtaccgctaccaggcctgaggttggggcaggttccagatcagcctggt
 241 **S N T P Y R Y Q A C R F G Q V P D Q P G**
 781 gggctcgcactcttcacagtgagatccccacaagcgcctacgacagccccaccctgc
 261 **G L R L F T V Q I P H K R L R Q P P P C**
 841 tacctcacagcctgggacagttccaccttcttgcctcttcggaccaggtcctgtggccat
 281 **Y L T A W D S E T F L P L R T R S C G H**
 901 gaagtcatttctgctcactgtcagtgatcgggtaccttccctagggcctagggcaggtc
 301 **R V I S C L T V S E S G T F L G L G T V**
 961 actggctctgtcgccatctacatagcttctctctccagcgcctgtattatgtgaaggag
 321 **T G S V A I Y I A F S L O R L Y Y V K E**
 1021 gcccattggcattgtggtgacagatgtgaccttctacctgagaaggggttgcgggtccaaag
 341 **A H G I V V T D V T F L P E K G C G P K**
 1081 ctccttgggccccatgaaacggccctgttctctgtggctgtggatagtcgttggcagttg
 361 **L L G P H E T A L F S V A V D S R C Q L**
 1141 cacctgctgcccctcagggcggaggttcccgtatggctcctgctcctgctatgtgtggc
 381 **H L L P S R R S V P V W L L L L C V G**
 1201 cttattatcgtgacctcctgctgctccagagtgccctcccggttctttaaactcct
 401 **L I I V T I L L L Q S A F P G F L ***
 1261 gaccaatgggagtcaccttggacagtaacttctggagcagagtcattgaggcccat
 1321 gactgaagctgcatctgatgaaatggatgggtactgcccgtcctgctaaacgctgccc
 1381 agtggcctccctatcactctgggtcttgggagccctgcttccacctgtggatccat~~ttta~~
 1441 gacagtggtgtcgaagctcagggccacactgctgctctgcttctctgctcccagggc
 1501 tccagagccgagctctcctaggaacatgtgaagatgccaaagagcccagagggcattgccc
 1561 atccttctcgcagagactgttttctctctcccctccagtcctgcgcacaaggtcctcag
 1621 ctttctcgcagacaaagctctgtggaagaggcaaaaggaagaccaggtagcgggtgatctgt
 1681 aggtgacaccagccagtcaggccagacgacagggagttcctgggtgacctactgcagc
 1741 ctgaggaagggaaagtgaacctcagttatttaggcaggaagagttgat~~ttta~~aaag
 1801 aaaga

Figure 14. *Ickis* nucleotide and predicted amino acid sequences

Three potential WD-motifs are underlined. There are consensus sequences for mRNA destabilizing elements (bold) and polyadenylation signals (underline) in the 3'-untranslated region.

bases (**Figure 15**). High scores were also seen for EST cDNA sequences derived from human infant brain (R13035, R1274), and human fetal lung (W04666) (data not shown). No further information is available about these EST's. These data suggest the potential distribution of *Ickis* transcripts in tissues other than pituitary gland.

Further analysis of the *Ickis* sequence at the amino acid level revealed that *Ickis* is a novel protein containing three motifs related to the WD consensus (**Figure 16**). *Ickis* contains no other commonly recognizable regulatory motifs such as leucine zipper, zinc finger, helix-turn-helix, etc. when it is analyzed using the TBLAST, FASTA, MOTIF, and PROFILESCAN. Hence, *Ickis* may contain a novel DNA-binding domain.

[6] *Ickis* is a single copy gene that is evolutionarily conserved

Genomic southern analysis was carried out in order to determine whether *Ickis* is a unique gene. The results shown in **Figure 17** imply that *Ickis* is a single copy gene, since a single band is obtained for EcoRI and HindII digests of rat (GH3 cell) genomic DNA, as well as for a series of digestions of human genomic DNA. The observation in rat DNA of two bands with XhoI and PvuI implies that each enzyme may cut within the intron region(s) since neither recognition site is found in the *Ickis* cDNA.

***Ickis* x AA111707**

```

1001 ggctcctgtggccatgaaagtcattctcctgcctcactgtcagtgaaatcgggt
1 .....cccccaatccacaagccaaagccctgtatgcaactgacg
1051 acctcctcctaggccttaggcacgggtcactggctctgttgcacatctacatagc
37 Aaccctgttccct.....caacgttagcttccaaactagccttctctctacncc
1101 tctctctctctccagggcctgtcattatctgaaaggggcccattggcattctgtg
83 tttctc.ccccaaccctgataatgatacgaagaccctt.ccaattgttc
1151 tgacagatgtgacccctctcctcctgagaagggcttgcctccaaagctcctt
131 taacagatgtgacccctctcctcctgagaagggcttgcctccaaagctcctt
1201 gggcccaatgaaacggcctgctctctctgtgctgtgatagtctgttgcac
181 gggcccaatgaaacggcctgctctctctgtgctgtgatagtctgttgcac
1251 gcttgcactgtctgcccctcaccggcggagctctcccgtaagctctctgctcc
331 gttcactgtctgcccctcaccggcggagctctcccgtaagctctctgctcc
1301 tctctatgttctggccttatctatcctgaccatcctgctctccagagtgcc
281 tctctatgttctggccttatctatcctgaccatcctgctctccagagtgcc
1351 tctcccggttctctctaaacatcctgaccatgaggctcactccttggacag
331 tctcccggttctctctaaacatcctgaccatgaggctcactccttggacag
1401 tactaccctctctgagcaggtcctatgaggcccctgactgaaagctgcactc
381 tactaccctctctgagcaggtcctatgaggcccctgactgaaagctgcactc
1451 gatgaaatggaatggttactgcccgtctcctgctaaacgctgcggcagtgcc
431 gatgaaatggaatggttactgcccgtctcctgctaaacgctgcggcagtgcc
1501 ctccctaccctctctggtctctgggagccctgcttccactgtggatccat
480 ctctccatccctctggt.....

```

***Ickis* x AA184937**

```

1301 tgcctatgtgctggccttatttatcgtgaccatcctgctgctccagagtgcc
1 .....cattcttctctccagactgcc
1351 tctcccggttctctctaaacatcctgaccatgaggctcactccttggacag
23 tctcccggttctctctaaacatcctgaccatgaggctcactccttggacag
1401 tactaccctctctgagcaggtcctatgaggcccctgactgaaagctgcactc
73 tactaccctctctgagcaggtcctatgaggcccctgactgaaagctgcactc
1451 gatgaaatggaatggttactgcccgtctcctgctaaacgctgcggcagtgcc
123 gatgaaatggaatggttactgcccgtctcctgctaaacgctgcggcagtgcc
1501 ctccctaccctctctggtctctgggagc.cttgcctctcagctgtggaatcca
172 ctctccatccctctctggtctctgggagc.cttgcctctcagctgtggaatcca
1550 tctcagacagctgtggtctctgaggctcagggtcaccctgctgctctctctct
222 cttaaacatctgtgtctcgaagctcagggtcaccctgctgctctctctctct
1600 E....ctgctctccagggctccagagctcagctctctc....ctaggaaac
272 tcagccctccctcccaagccctccagagctcagctctctc....ctaggaaac
1641 atgtgaaagtgcccaagagcccccagagcacttgcctcctctctcagagaga
322 atgtgaaagtgcccaagagcccccagagcacttgcctcctctctcagagaaa
1691 ctgtctctctctctctctctctctctctctctctctctctctctctctctct
372 ctgtctctctctctctctctctctctctctctctctctctctctctctctct
1741 cggagcacaagctctgt.....ggagagggcaaaa
421 tcgacacagaccctgtctctgagcacttctctttagccttgcgaagaccacaa
1769 gggagcccaagctgagctgtgctctgtaggtagcaccagccagctcagggc
471 agagagcccaagctgagctgtgctctgtaggtagcaccagccagctcagggc

```

Figure 15. DNA sequence comparisons between *Ickis* and EST's

Nucleotide sequence similarity between *Ickis* and cDNAs (accession number indicated at top) derived from mouse lymph node.

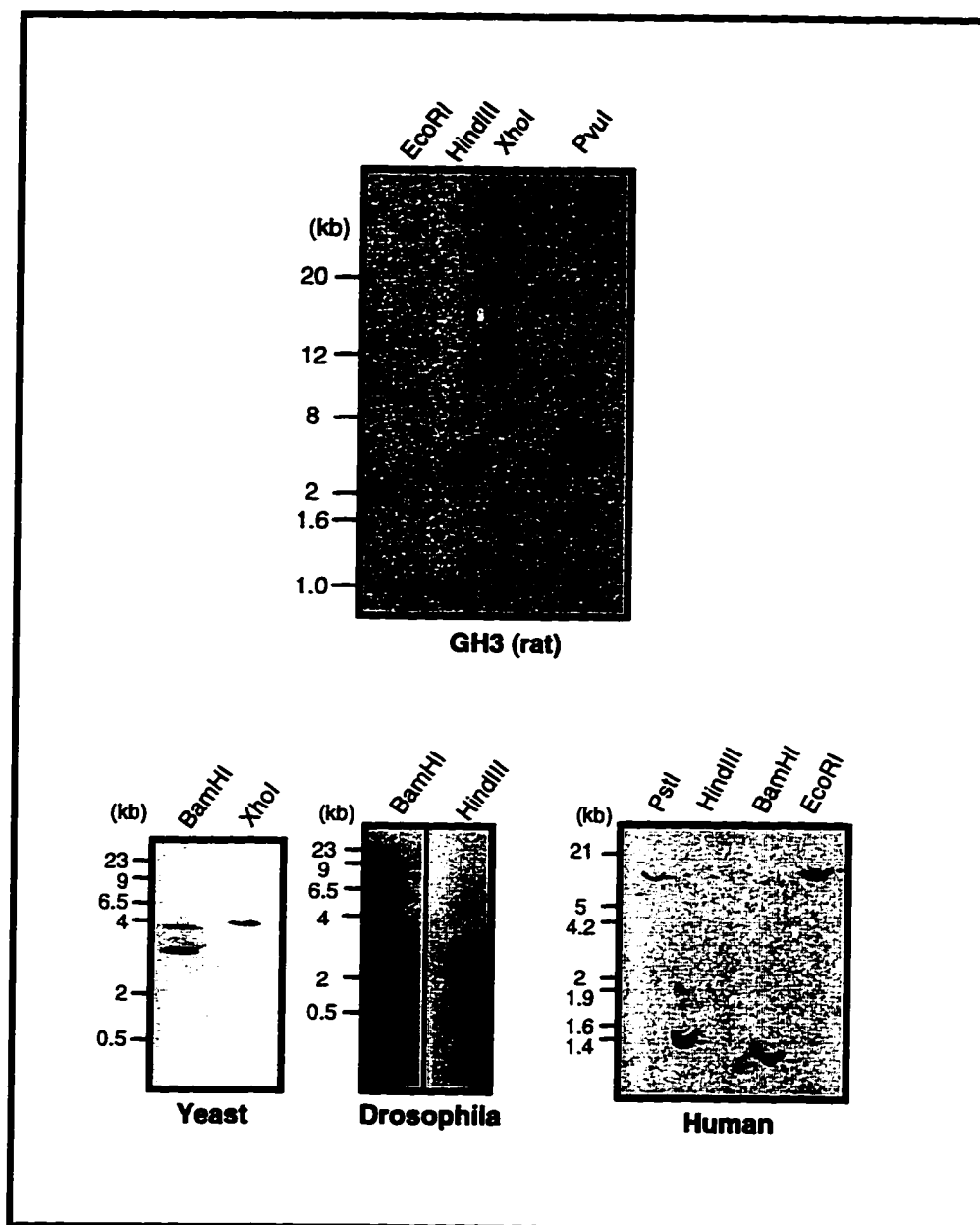


Figure 17. Genomic Southern blot analysis of *Ickis*

Genomic DNA was isolated and digested with the restriction enzymes indicated at the top of each filter. The entire *Ickis* cDNA was used as a probe and stringency of hybridization was adjusted for each species (see Experimental Procedures for detail).

Drosophila and *S. cerevisiae* genomic DNAs were also analyzed in order to determine whether *lckis* is evolutionarily conserved. In yeast, single and double bands were obtained from a XhoI and BamHI digest, respectively. In *Drosophila*, both BamHI and HindIII restriction enzymes digestions yielded single bands. Taken together, these results suggest that *lckis* is a single copy gene and has been highly conserved through evolution. This implies that *lckis* serves an important physiological function.

Discussion

Cloning of *Ickis* using a DNA recognition site probe

A novel DNA-binding protein was isolated using the filter binding detection technique. This method has been used to clone other sequence-specific-binding proteins including POU-domain containing transcription factors, Pit-1 (Ingraham *et al.*, 1988), Oct-2 (Clerc *et al.*, 1988), and CREB (Hoeffler *et al.*, 1988). The cDNA for Pit-1 was cloned from the rat pituitary expression library using a synthetic site1P as a probe (Ingraham *et al.*, 1988). Interestingly in that study, the distal half of site1P was modified in order to maximize the Pit-1 binding activity, suggesting that Pit-1 was unable to bind site1P under these screening conditions. It should also be noted that TEF was cloned using a "natural" site1P, however, Pit-1 was not isolated (Drolet *et al.*, 1991). Thus, it is perhaps not surprising that Pit-1 was not isolated using the present screening protocol.

***Ickis* specifically binds to the distal portion of the site1P**

Because site1P covers a relatively large area compared to the other cell-specific cis-elements (Nelson *et al.*, 1988), it is likely that site1P is the target site for multiple transcription factors. This has led to the isolation of another protein that recognizes site1P, TEF, which has not been identified

as a regulator of PRL gene expression. An interesting observation made by d'Emden *et al.* (1992), indicated that site1P plays a greater role in PRL gene regulation when compared to the other Pit-1 cis-elements. d'Emden *et al.* (1992) found that if site1P is disrupted along with either 2P or 4P, most of the promoter activity is abolished. On the other hand, promoter activity was maintained when 2P, 3P, or 4P were disrupted in combination. If Pit-1 is the main and only factor binding to these element, any of the combinations of the Pit-1 sites should have similar effects. This may imply that site1P is the target site for the transcription factor(s) other than Pit-1. Alternatively, the positioning of Pit-1 on site1P might have spatially critical parameter determining the strength of interactions, or to enable with other factors to have more stable physical contact to the basal machinery. The deleterious effect of site1P deletion becomes more apparent when an additional cis-element is combined, possibly implying that a higher ordered complex might be built on site1P cis-element as a starting base. Finally, x-ray crystallography studies showed that Pit-1 contacts the proximal site1P region, suggesting the presence of an additional factor which binds to the distal region. With these assumption, however, this study can be simply suggesting that potent role of site1P in the PRL gene regulation.

***Ickis* is a WD-motif containing protein**

Analysis of the deduced amino acid sequence of *Ickis* demonstrated the presence of three WD-motifs. The WD-motif often, but not always, ends in the dipeptide tryptophan (W) and aspartate (D), and is often tandemly repeated (Van-der-Voorn and Ploegh, 1992, and Neer *et al.*, 1994). The structure of the WD-motif was first demonstrated in the β -subunit guanine nucleotide-binding protein (Lambright *et al.*, 1996), but it has subsequently been found in more than 30 other proteins. This growing family includes the Tup1 transcription factor (Keleher *et al.*, 1992), PRP4 RNA splicing factor (Dalrymple *et al.*, 1989), Groucho Enhancer of split, which involves in neurogenesis (Hartley *et al.*, 1988), and many other proteins in different classes. Interestingly, some WD-motif containing proteins serve regulatory functions, and are involved in signal transduction (Neer *et al.*, 1994). WD repeats are also thought to play important roles in protein-protein interaction. For example, WD-repeats in FAN (Factor Associated with Neutral sphingomyelinase activation) associate directly with the TNF-R (Tumor Necrosis Factor Receptor) (Adam-Klages *et al.*, 1996). Similar observations were made in other WD-motif containing proteins, such as dTAFII80 (tightly associated factors) (Dymlacht *et al.*, 1993) and Tup1 (Keleher *et al.*, 1992). Therefore, one

could hypothesize that *Ickis* may interact with other classes of nuclear factors via its WD-motifs.

Other elements in *Ickis* cDNA

As shown in **Figure 14**, *Ickis* cDNA contains both putative polyadenylation sites as well as an mRNA destabilizing element (Malter, 1989, Haile *et al.*, 1989, and Chen *et al.*, 1994) in the 3'-untranslated region. Both of these elements can provide additional control for the activity of a protein, since they can regulate the half-life of its message, and thus the amount of the protein. It is thus possible to speculate that expression of *Ickis* is tightly regulated at post-transcriptional level.

Conclusion

Ickis binds specifically to the site1P element in the PRL promoter.

This DNA-binding protein can recognize the distal portion of site1P, which is located adjacent to the Pit-1 target site. *Ickis* belongs to the family of WD-motif-containing proteins that in most cases play regulatory roles. Because the WD-motif is often involved in protein-protein interaction, it is possible that *Ickis* interacts with other nuclear factors to modulate the activity of its target promoter. *Ickis* is encoded by a single copy gene that has been evolutionarily conserved. These results suggest that *Ickis* is a transcription factor that recognizes the cis-element site1P. I have tested this hypothesis as described in **Chapter IV**.

CHAPTER III

CHARACTERIZATION OF *ICKIS* GENE EXPRESSION

Summary

Ickis mRNA was found in rat GH3 pituitary cells. The size of *Ickis* mRNA was 1.9 kb as determined by Northern blot analysis. Analysis of a human organ blot revealed three mRNA species closely related to the *Ickis* gene. One species is specific to the brain, placenta, and lung, whereas, another is detected only in the heart, skeletal muscle, and pancreas. This suggests that the *Ickis* gene might be modified in a tissue-specific fashion via alternative splicing and/or differential promoter usage. RT-PCR analysis demonstrated the presence of *Ickis* transcripts in the normal human pituitary as well as in various human pituitary tumors. These data suggest that *Ickis* may play a regulatory role outside of the pituitary.

Introduction

Tissue-specific regulation of gene expression is controlled by transcription factors. Some transcription factors are expressed in certain cell-types, allowing for the activation of a target promoter in a cell-type-specific manner. Tissue-specific regulation is also possible for a ubiquitous transcription factor especially when its isoforms are expressed in a spatio-specific fashion. For example, **CREM** (cAMP Responsive Element Modulator) can exert tissue-specific activity despite its widespread expression (Lalli *et al.*, 1996, and Lamas *et al.*, 1996), since it can exist as various isoforms created by alternative exon shuffling. Differential regulation by isoforms is also seen with the tissue-specific factor, Pit-1 (Konzak *et al.*, 1992, Morris *et al.*, 1992, Voss *et al.*, 1993, and Day and Day, 1994) and other transcription factors (Rio, 1993, for review). Most of these isoforms exhibit modulated activity because a critical domain is modified or shuffled by alternative splicing. Dominant-negative isoforms can be generated by exclusively producing a particular domain such as a DNA-binding domain or dimerization domain. This is accomplished by alternative promoter usage. Thus alternate splicing and/or differential promoter usage can be used to generate proteins possessing tissue-specific activity. This indicates that further analysis of isoforms may give some insight into the structure and function of domains and element(s) responsible for the differential activity.

In this chapter, the expression profile of *lckis* mRNA was examined in order to identify the tissues in which it might function. In contrast to the

pituitary-specific transcription factor, Pit-1, the *Lckis* gene is widely expressed. Thus *Lckis* might play a general role even though it specifically recognizes site1P, a tissue-specific PRL cis-element. However, the presence of distinct *Lckis* mRNA sizes in different tissues suggests that *Lckis* may play specific roles in certain tissues. Interestingly, distinct species of *Lckis* are detected in certain tissues including brain, lung, and placenta. Finally, analysis of the expression profile of the *Lckis* transcript throughout embryogenesis (work conducted by Dr. Thomas Lufkin), reveals a potential role for *Lckis* in the developing pituitary and in several other distinct tissues.

Results

[1] *Ickis* is a relatively abundant mRNA expressed in GH3 cells

I first investigated expression of the *Ickis* transcript in GH3 cells. Northern blot analysis demonstrated the presence of a single 1.9 kb transcript in GH3 (somatomammotrophic) cells (**Figure 18 left**). The size of the mRNA correlates well with the length of the *Ickis* cDNA described in Chapter II. In order to estimate the relative abundance of the *Ickis* transcript, Northern blots were probed with Pit-1 cDNA under the same experimental conditions (**Figure 18 right**). To control for RNA quantity, duplicate RNA samples were visualized using ethidium bromide staining (**Figure 18 bottom**). *Ickis* mRNA levels were comparable to that of Pit-1, suggesting that *Ickis* is relatively abundant in GH3 pituitary cells.

[2] *Ickis* mRNA is widely expressed, but exhibits a tissue-specific pattern of expression.

In order to examine the expression pattern of the *Ickis* gene in adult animals, Northern analysis was performed using a human RNA organ blot (Clontech) (**Figure 19 upper panel**). An identical Northern blot was also probed with a β -actin cDNA in order to assure mRNA integrity (**Figure 18 lower panel**). The results demonstrate the presence of three distinct *Ickis* transcripts which are differentially expressed in an

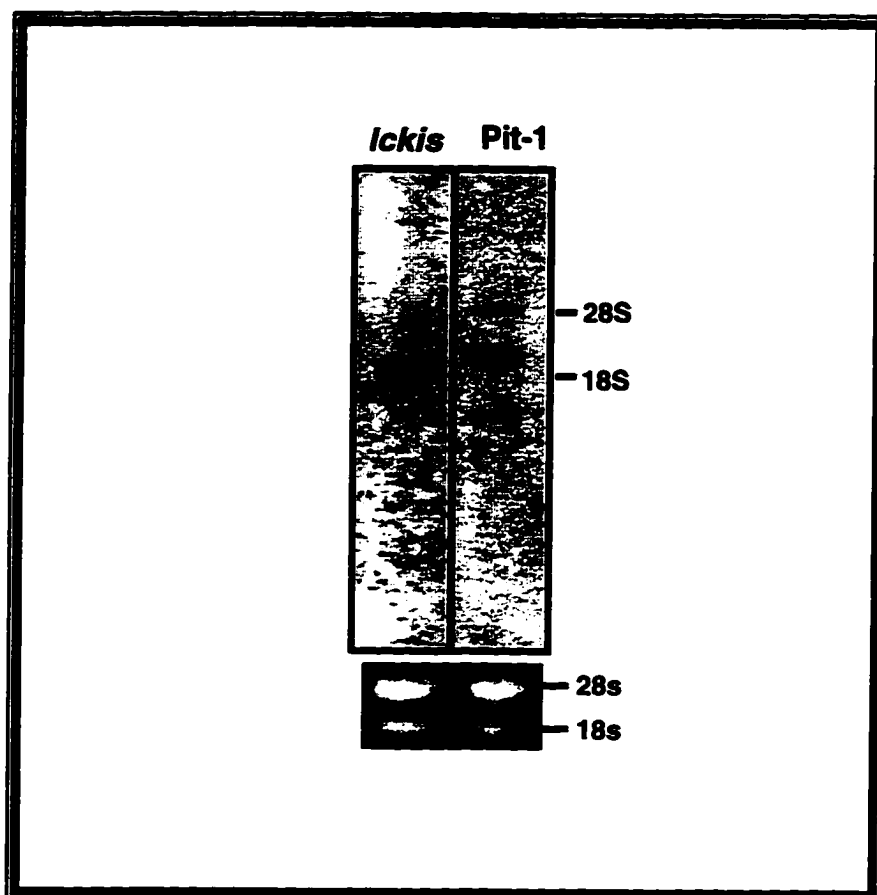


Figure 18. Northern blot analysis of *Ickis* mRNA in GH3 cells.

Aliquots (15 ug) of RNAs were subjected to Northern blot analysis using identical experimental conditions. To allow for the comparison of signal intensities, the filters were incubated with cDNA probes for either *Ickis* or Pit-1cDNA. Both probes were labeled to equivalent specific activities, and the same amount of each probe was employed.

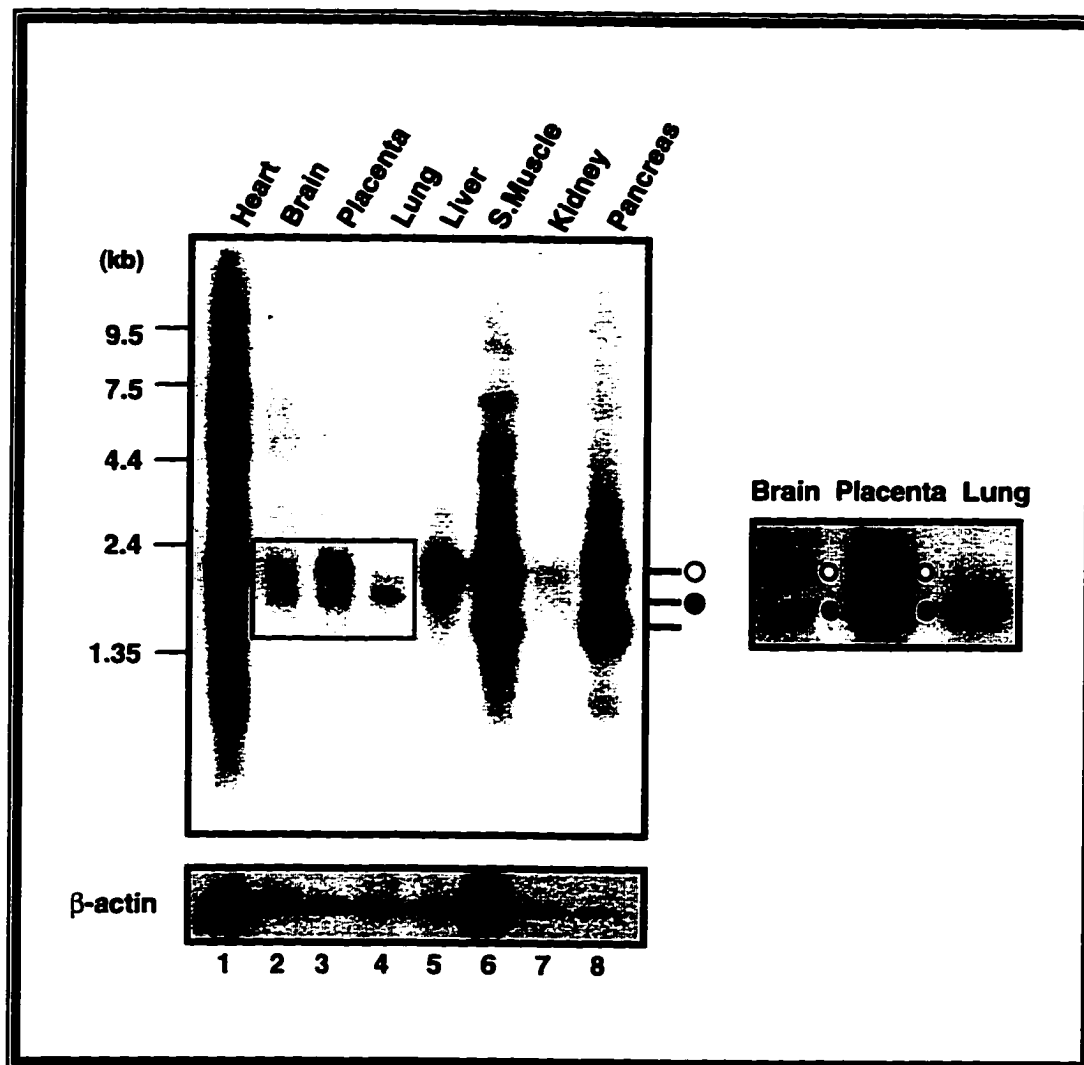


Figure 19. Three distinct *Ickis* transcripts are differentially expressed in an organ-specific fashion

Northern analysis of *Ickis* was performed using a human multiple organ blot (Clontech). Poly(A)-enriched (2ug) samples were probed with either the *Ickis* coding region (upper panel), or β -actin (lower panel). The squared area inside of the blot is enlarged to demonstrate the specific transcripts present in the brain, placenta, and lung (filled circle) along with the non-tissue-specific form (open circle).

organ-specific manner. A 2.2 kb transcript (open circle) is detected in all tissues examined except lung (the kidney band is detectable upon longer exposures). By contrast, smaller isoforms migrating at either 1.9 (filled circle) or 1.5 kb (no mark) are expressed in a tissue-specific manner. The 1.5 kb-transcript was detected in heart, skeletal muscle, and pancreas tissues (lanes 1, 6, and 8), while the 1.9 kb-transcript was found only in the brain, placenta, and lung (lanes 2-4, see left panel for detail).

[3] *Ickis* is expressed in a number of different pituitary cell-types

Northern blot and RT-PCR analyses was performed on various pituitary cell types in order to determine the distribution of *Ickis* mRNA in the pituitary. It is seen in **Figure 20** that *Ickis* mRNA was detected in GH-, PRL-, ACTH-, and TSH-secreting human tumors (**a. upper panel**) as well as normal pituitary tissue (lower panel) (kindly provided by Dr. Michael Woloschak). Since most pituitary tumors are monoclonal in origin, it is likely that each tumor arises from a particular pituitary cell-type. *Ickis* mRNA was also detected in pure PRL-secreting mammotrophic (235-1) and ACTH-secreting corticotrophic (AtT-20) cell lines (**Figure 20 b**). These results, which are summarized in **Table 1**, suggests that the expression of the *Ickis* gene in the pituitary is not restricted to PRL-secreting (GH3) cells.

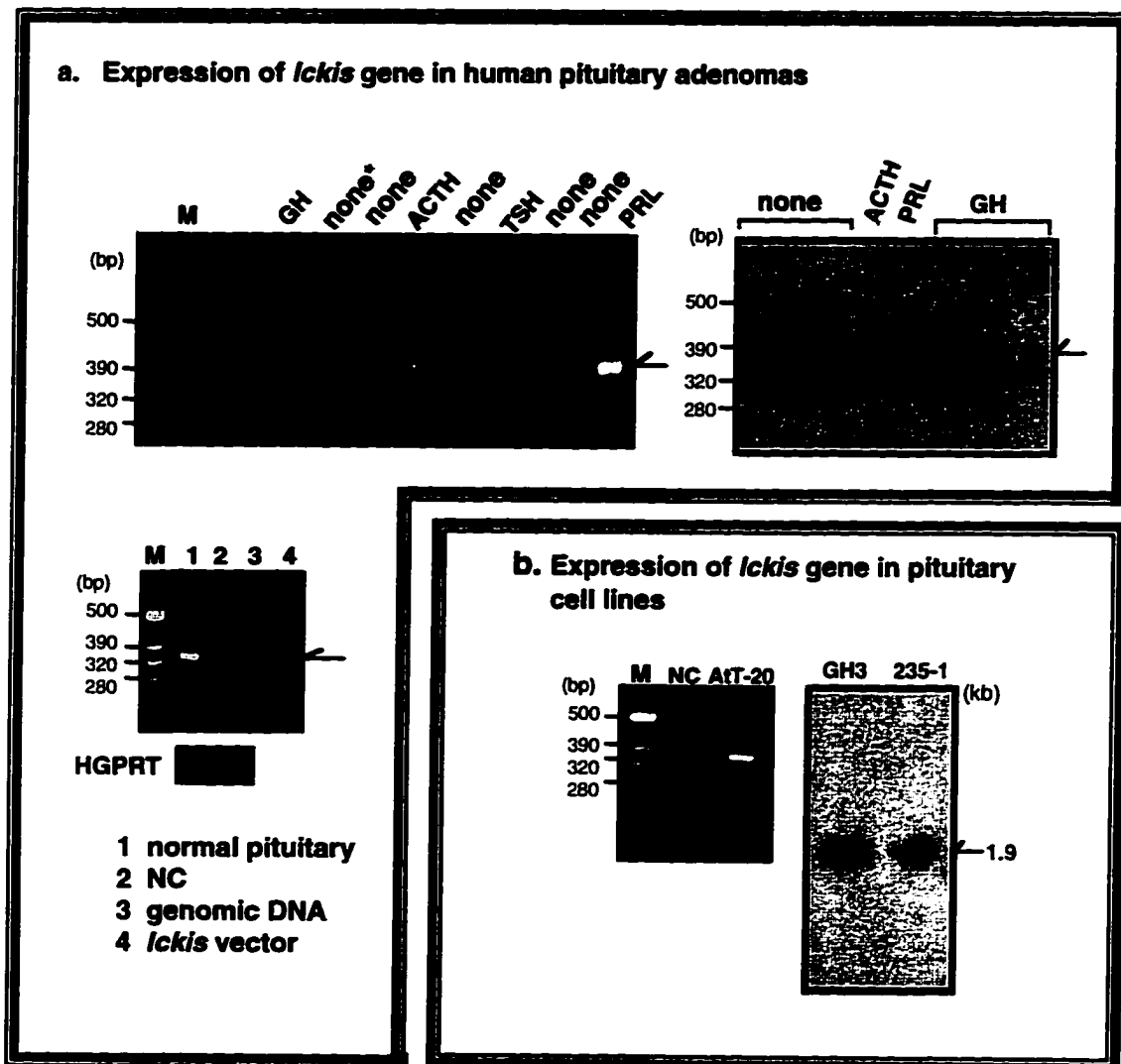


Figure 20. Expression of *lckis* transcripts within the pituitary.

a. The cDNA samples from either human tumors (top) or normal pituitary (bottom) were subjected to either nested-PCR (left panel) or PCR-Southern analysis (right panel) (see Experimental Procedures for detail). none*: no-hormone-secreting tumor. **b.** RT-PCR and Northern analysis of *lckis* in using pituitary cell lines as sources. Total RNA (1ug) prepared from various cell lines were subjected to either RT-PCR (AtT-20 cells) or Northern blot analysis (GH3 and 235-1 cells). Arrows indicate the predicted size of either the PCR product or *lckis* transcript.

Type of pituitary	<i>lckis</i> (+)	<i>lckis</i> (-)	Total (+)/(-)
Non-secreting	4	4	4/4
GH-secreting (somatotroph)	3*	2	3/2
PRL-secreting (lactotroph/mammotroph)	2*	1	2/1
ACTH-secreting (corticotroph)	2*	1	2/1
TSH-secreting (tyrotroph)	1	0	1/0

Table 1. Expression of *lckis* transcripts in pituitary tumors and cell lines

* Results from pituitary cell lines, both GH3 and AtT-20 cells are included as GH-/PRL- and ACTH-secreting cells, respectively.

[4] Expression of *Ickis* in neuronal tissues

Analyses of *Ickis* transcripts using *in situ* hybridization

demonstrated the presence of *Ickis* mRNA in developing dorsal root ganglia (DRG) and in the brain of the mature mouse (data provided by Dr. Thomas Lufkin and Dr. Blum at Mount Sinai, respectively). In order to further characterize the expression of *Ickis* mRNA in neuronally derived cells, PCR and Northern analyses was performed with various neuronal cell lines and tumors (RT products were kindly provided by Dr. Andrew Chan at Mount Sinai) (**Figure 21a**). In order to confirm the identity of the PCR product, the second round of PCR was performed using the internal primer set (see Experimental Procedures for detail). **HGPRT** (Hypoxanthine-guanine phosphoribosyl transferase) transcripts were also amplified (primers generously provided by Dr. James Bieker) as a control to assess the integrity of the RNA (**Figure a lower panel**). *Ickis* transcripts were detected in a brain tumor (HA115) and in two astrocytomas (HA1394A, HA690), but were not amplified in any other neuronal tissues examined including neuroepithelioma (SK.N.MC), and two glioma samples (HA1781, HA207). The lack of detectable *Ickis* mRNA in glioma tissues suggested that the rat glial C6 cells might represent a useful *Ickis*-negative cell line for functional studies of *Ickis*. Northern analysis (**Figure 21 panel b**) showed that *Ickis* mRNA is not detectable in the C6 cell, indicating that this is correct. Taken together, these results show that the *Ickis* gene is expressed in a region-specific manner in neuronal tissues.

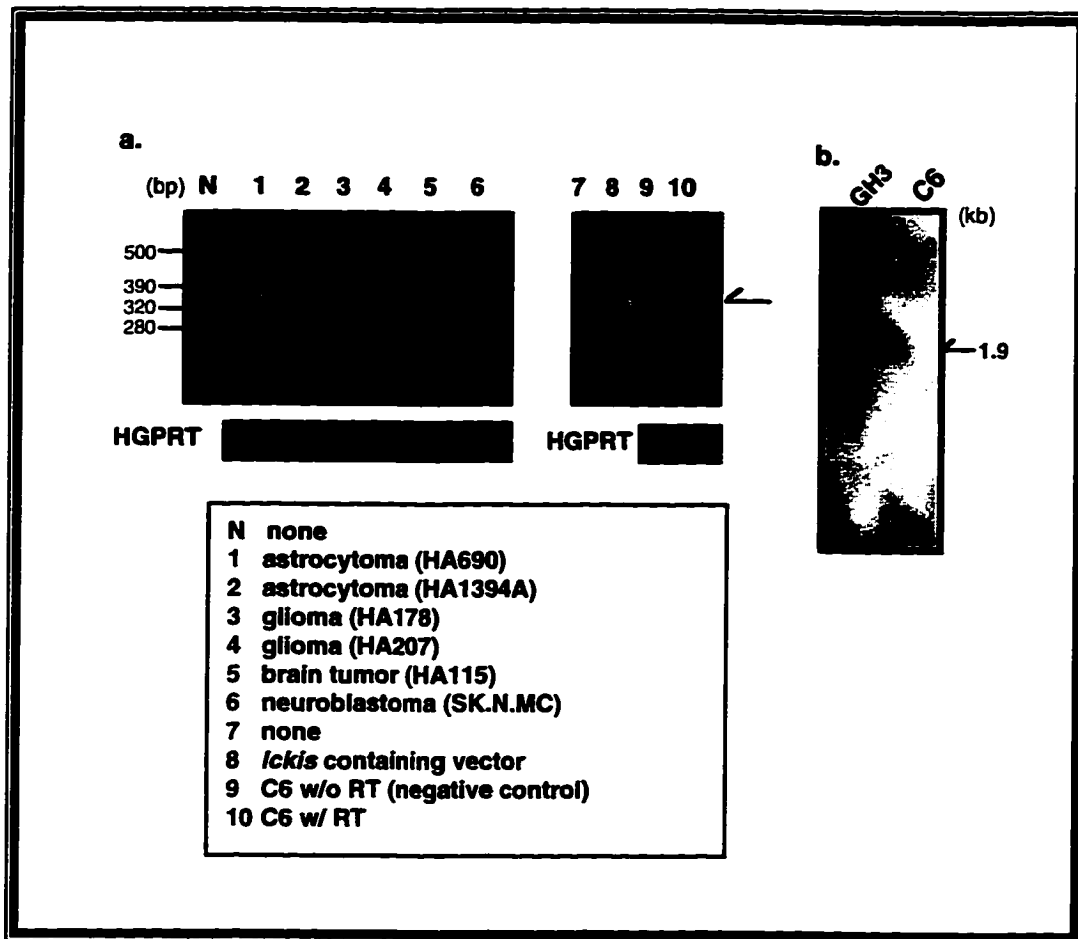


Figure 21. Distribution of *Ickis* transcripts among neuronal cells.

a. Aliquots of cDNA (2ul) from the indicated human tumors were amplified using gene specific primer sets for either *Ickis* (top) or HGPRT (bottom). Lane 2 sample was not available for HGPRT analysis. For analysis of C6 cells, a polyA-enriched sample (100ng) was used as starting material. **b.** PolyA-enriched RNA (1 ug) prepared from either GH3 or C6 cells were subjected to Northern blot analysis. An overexposed filter is shown.

Discussion

[1] Alternative splicing may yield organ-specific *Ickis* isoforms possessing different activities.

The results from the human organ blot demonstrated the presence of distinct species of *Ickis* mRNA, two of which exhibited restricted tissue distribution. Since *Ickis* is a single copy gene, these different forms may be generated via alternative splicing and/or differential promoter usage. As seen with CREM, a single copy gene can generate different isoforms. These isoforms can exhibit either agonistic or antagonistic activity depending on how the exons are arranged (Stehle *et al.*, 1993, Lalli *et al.*, 1996, and Lamas *et al.*, 1996). Thus, CREM activity can be greatly modified via these isoforms which are spatio-specifically expressed. It would be interesting to determine whether *Ickis* utilizes a similar strategy to that of CREM. Alternatively, the additional bands seen in this blot may represent hybridization to *Ickis*-related genes. Subsequent identification of these tissue-specific *Ickis* isoforms or related genes can greatly contribute to the understanding of the functional role of *Ickis* in each tissue.

[2] Expression of *Ickis* gene within pituitary and neuronal cells

Ickis mRNA is widely expressed in tumors arising from different pituitary cell-types. This suggests that *Ickis* may regulate genes other

than PRL, since the expression of the PRL gene is limited to the lactotrophic cells of the pituitary. For example, pituitary-specific Pit-1 has multiple target promoters including PRL, GH, and TSH β , suggesting the presence of related cis-elements in these promoters. Similarly, TEF (thyrotroph embryonic factor) also recognizes site1P (Drolet *et al.*, 1991), which overlaps with the *lckis* target sequence. TEF binds to multiple sites in the TSH β promoter, suggesting that this promoter may also be a potential target site for *lckis*. Similar to *lckis*, TEF exhibits a widespread distribution in adult animals despite its restricted expression during development (Drolet *et al.*, 1991).

lckis mRNA is expressed in a relatively region-specific manner in neuronal cells, since tumors arising from only a subset of neuronal cell-types contain detectable *lckis* transcripts. However, it is somewhat surprising that *lckis* mRNA is present in astrocytoma and not in glioma, since these cell types are so closely related. Finally, a glial cell line (C6) did not express detectable levels of either *lckis* mRNA (**Figure 21**) or protein by Western blot analysis (data not shown). I therefore used these cells for functional studies of *lckis*, as described in **Chapter IV**.

[3] Spatio-temporal patterns of *Ickis* expression during development (Experiments performed by Dr. Thomas Lufkin at Mount Sinai)

In order to gain insight into a potential role for *Ickis* during embryogenesis, *in situ* hybridizations were performed by Dr. Thomas Lufkin. Spatio-temporal expression of *Ickis* was detected with a labeled RNA probe corresponding to 900 bases in the 3' end of the *Ickis* coding region (cDNA provided by the author). *Ickis* was expressed developmentally in several tissues of neural crest origin, including dorsal root ganglia (DRG), the future pituitary (Rathke's pouch), and cartilaginous tissues of the cranio-facial areas. *Ickis* expression was observed during the embryonic days 10.5 (E10.5) to 14.5 (E14.5). These results suggest that *Ickis* is expressed in a highly dynamic and spatio-temporal manner during development.

The temporal expression (on day E12.5 only) of *Ickis* in Rathke's pouch is of particular interest, suggesting the potential involvement of *Ickis* during pituitary differentiation. Several homeodomain transcription factors are also expressed in the developing Rathke's pouch (Seidah *et al.*, 1994, Bach *et al.*, 1995, Gage *et al.*, 1996, and Szeto *et al.*, 1996). Interestingly most of these transcription factors do not play an active role in cell-type specification, but rather are thought to be responsible for the organ-specification of the pituitary. The presence of *Ickis* transcripts in differentiated somatotrophic, lactotrophic, corticotrophic, and thyrotrophic

tumors does not exclude a potential role of *Ickis* as an organ-specific factor.

It is also interesting to note that *Ickis* contains potential mRNA destabilization elements in the 3'-untranslated region. As is the case of Fos and CREM transcription factors, elements found in the 3'-untranslated region can regulate the half-life of mRNA. It is possible that the expression of the *Ickis* gene during development might be also regulated at the post-transcriptional level.

CHAPTER IV

CHARACTERIZATION OF *ICKIS*
AS A TRANSCRIPTION FACTOR

Summary

A novel protein, *Ickis* was functionally characterized as a transcription factor. Accumulation of *Ickis* protein was found predominantly in the nucleus of GH3 rat pituitary cells by Western blot analysis, using antibody directed against *Ickis*. This analysis suggests that *Ickis* (or an immunologically related protein) has a molecular mass of approximately 46 KDa, which corresponds well with the deduced size of the *Ickis* coding region (Chapter II). Transient co-transfection analyses showed that *Ickis* activates the PRL promoter in heterologous C6 cells. *Ickis* but not Pit-1 action is further stimulated by PKA, suggesting that *Ickis* may be one of a downstream effectors for transcriptional activator of PKA. Moreover, *Ickis* and Pit-1 are additive in transactivating the PRL promoter, likely through binding to adjacent sequences in a common cis-element (site1P). Furthermore, similar observations were obtained in the homologous GH3 cells using a GAL4-*Ickis* chimera and a 5 x GAL4 target promoter. This *Ickis* chimeric protein is sufficient to activate GAL4 promoter and its action is also stimulated upon the expression of PKA. Taken together, these results imply that *Ickis* is a novel transcription factor that can activate the PRL promoter, and whose action is potentiated by PKA.

Introduction

The experiments described in **Chapter II** demonstrated that *Ickis* is a sequence-specific DNA-binding protein. In this Chapter, I have characterized *Ickis* as a transcription factor. The action of transcription factors is commonly assessed by using transient co-transfection assays. In these assays, both the putative transcription factor and the target promoter constructs are transfected into cells. Typically, the promoter construct is fused to a reporter gene and transactivation can be indirectly measured via activity of the reporter gene product.

Previous studies demonstrated that a site 1P-binding protein other than Pit-1 must transmit the cAMP/PKA signal to the PRL gene. Deletion and mutational analysis of site1P suggested that site1P is an important cAMP responsive element (Keech *et al.*, 1992, and Rajnarayan *et al.*, 1995). Initially Pit-1 was thought to be responsible for transmitting the cAMP/PKA signal, since Pit-1 is phosphorylated by PKA *in vivo* and *in vitro* (Kapiloff *et al.*, 1991). However, further studies demonstrated that the phosphorylation of Pit-1 does not play a functional role in PKA signaling, since a phosphorylation-deficient Pit-1 can stimulate PRL gene expression as effectively as wild-type Pit-1 (Fischberg *et al.*, 1994, and Rajnarayan *et al.*, 1995). Moreover, a GAL4-Pit-1 chimera also failed to respond to cAMP stimulation (Rajnarayan *et al.*, 1995). This study clearly demonstrated that transcriptional activation in response to cAMP could take place in the absence of the phosphorylation of Pit-1. Taken together, these findings provide compelling evidence that Pit-1 is not a

functional substrate for PKA in the PRL promoter. This leads one to postulate that some other site1P-binding factor(s) must play a role in this signaling pathway. The experiments described in this Chapter imply that *Ickis* is a PKA-regulated transcription factor. Thus *Ickis* may represent the protein that mediates PKA action on the PRL promoter via site1P.

Results

[1] *Ickis* localizes to the nucleus of GH3 cells

To investigate whether GH3 rat pituitary cells express *Ickis*, and where it is located, nuclear and cytoplasmic fractions were prepared, and subjected to Western blot analyses with antibody specific for *Ickis* (Figure 22). *Ickis* is detected mainly in the nucleus, and migrated at a molecular weight of approximately 46 kDa. The size of the *Ickis* polypeptide correlates well with the expected size deduced from the cDNA sequence (Chapter II). The fact that the majority of cellular *Ickis* is localized to the nucleus further supports the idea that *Ickis* is a transcription factor.

[2] *Ickis* transcriptionally activates the PRL promoter in a heterologous system

Transient co-transfection assays were performed in order to identify *Ickis* as a transcription factor. C6 (rat glial) cells were selected as a heterologous system to analyze the action of *Ickis* on the PRL promoter, since, as described in Chapter III, these cells contain neither protein nor mRNA for *Ickis*. The open reading frame coding for *Ickis* was placed under the control of the constitutively active RSV promoter (effector construct), and the (-113) PRL-CAT construct (Figure 23 top) was used as a reporter construct. This PRL promoter construct was used because it contains a restricted number of cis-element(s), including

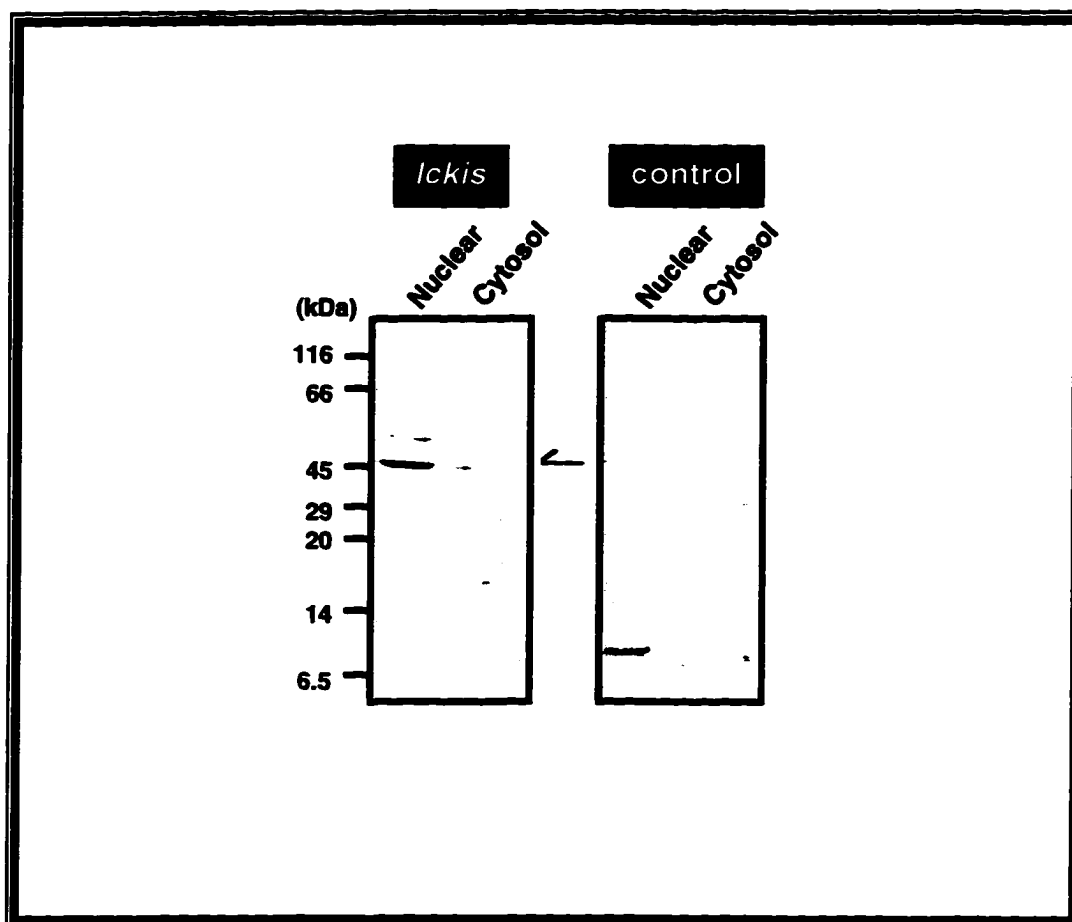


Figure 22. Nuclear accumulation of *Ickis* in GH3 cells

Nuclear and cytosolic fractions were prepared from GH3 cells and subjected to Western blot analysis. Each fraction contains the equal number of cells and duplicated samples were applied to the polyacrylamide gel, transferred onto the nitrocellulose, and incubated with the either *Ickis* antibody (*Ickis*) or preimmune serum (control). Arrow indicates the *Ickis* protein.

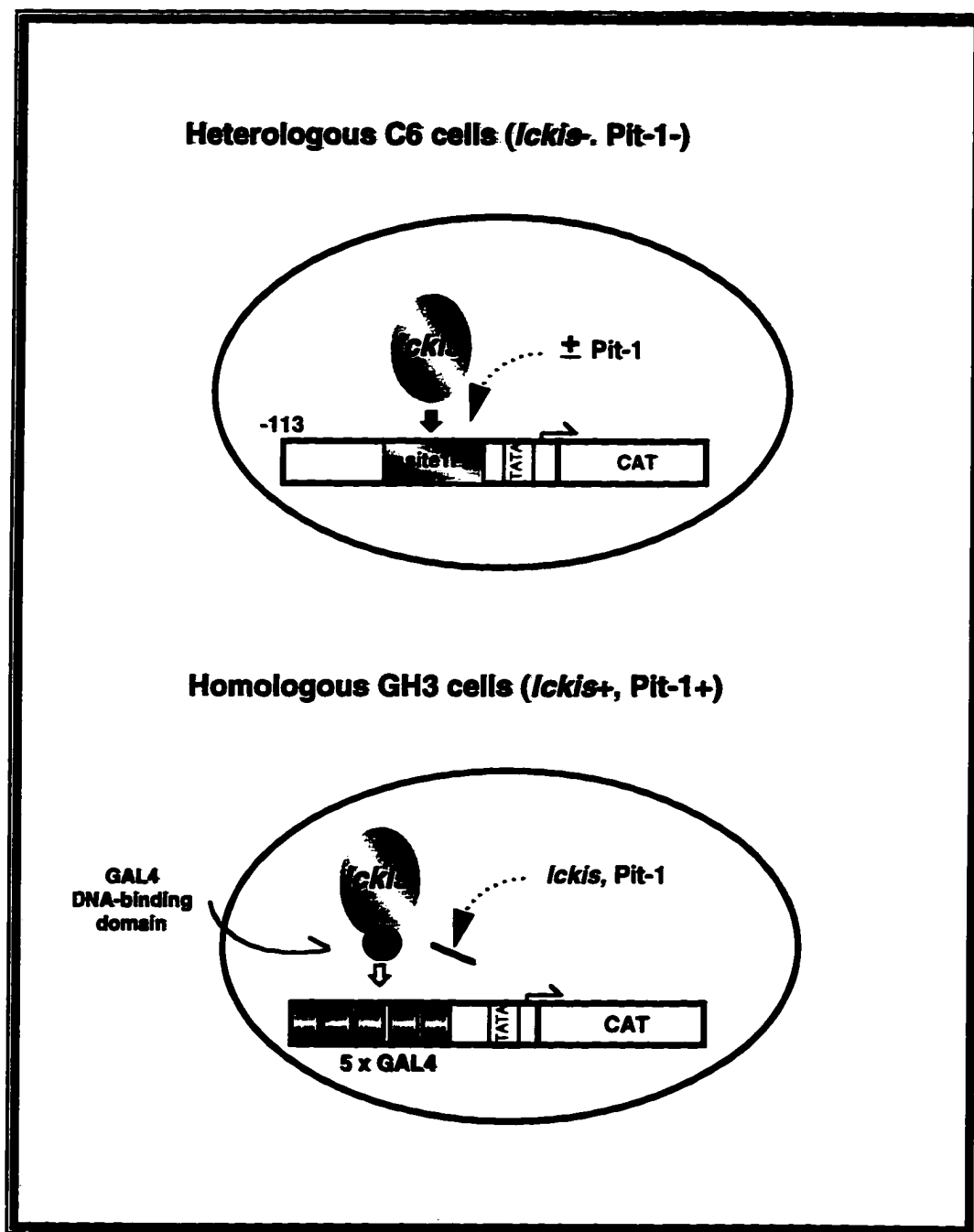


Figure 23. Heterologous vs. homologous system

site1P, and because maximal transactivation by both Pit-1 and *Ickis* was seen with the (-113) PRL promoter construct when compared to other promoters including (-207)PRL, (-187)PRL, or, (1P)²(-39)mMT CAT (data not shown). *Ickis* was observed to transactivate the PRL promoter, to a level similar to that observed for Pit-1 (**Figure 24**). This result implies that *Ickis* is a transcription factor. In order to compare more precisely the relative activities of *Ickis* and Pit-1, C6 cells were transfected with various amounts of RSV-*Ickis* and RSV-Pit-1 constructs (**Figure 25**). Linear dose-dependent activation was observed for *Ickis*, equivalent to the profile seen with RSV-Pit-1 (**Figure 25**). Based on these results, it was concluded that one to five ug of either effector was sufficient for activation of the (-113) PRL reporter construct. Significantly, these results imply that *Ickis* is a transcriptional activator of the PRL promoter, and that its activity is of a similar potency to that of Pit-1.

[3] *Ickis* and Pit-1 are additive in their action on the PRL promoter

In order to explore a possible interaction between *Ickis* and Pit-1 on site1P, each construct was transfected along with the (-113) PRL-CAT reporter gene. A series of transfectional analyses showed an additive effect on transcriptional activity, when both factors were introduced into cells (**Figure 26**). As before, they exhibited equivalent activities when present alone. When both were present, their effect was approximately additive.

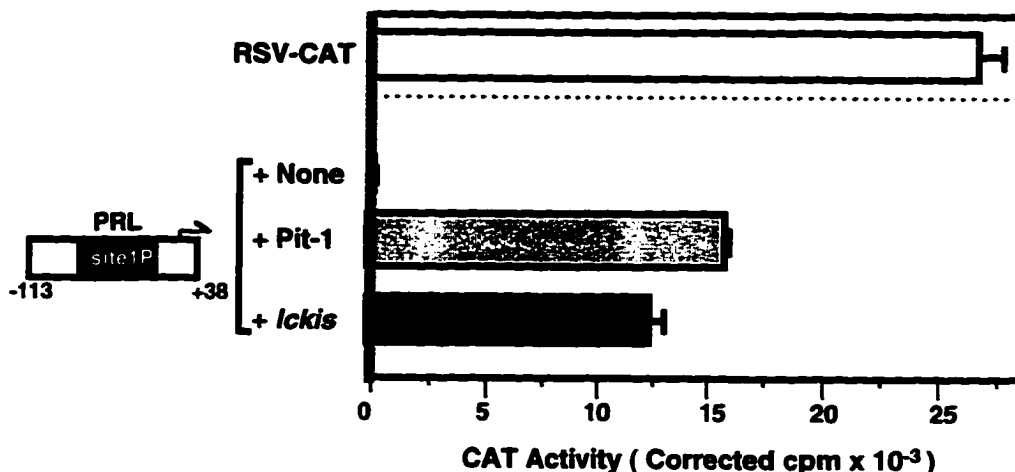


Figure 24. *Ickis* can transactivate PRL promoter activity in heterologous (C6) cells.

C6 cells (1.5×10^6 /tube) were electroporated at 300V (960uF) in D10 media with 10 ug of the (-113) PRL-CAT and 2ug of RSV- β gal, plus either 1ug of RSV-CAT (positive control), or 5 ug of the following: control construct (RcRSV; **None**), or RSV-Pit1 (**Pit-1**) or, RSV-*Ickis* (***Ickis***). Following transfection, cells were divided into three 60 mm dishes, and incubated for 48 hrs. Cells were then collected, lysed, incubated for 3 hours with substrate, and assayed for CAT enzymatic activity using phase extraction. The β -galactosidase activity was determined by using *o*-nitrophenyl β -D-galactopyranoside as a colorimetric substrate, and used to correct CAT activity for transfection efficiency. Shown are the mean plus/minus SE of results with triplicate dishes. Similar results were obtained in two additional experiments.

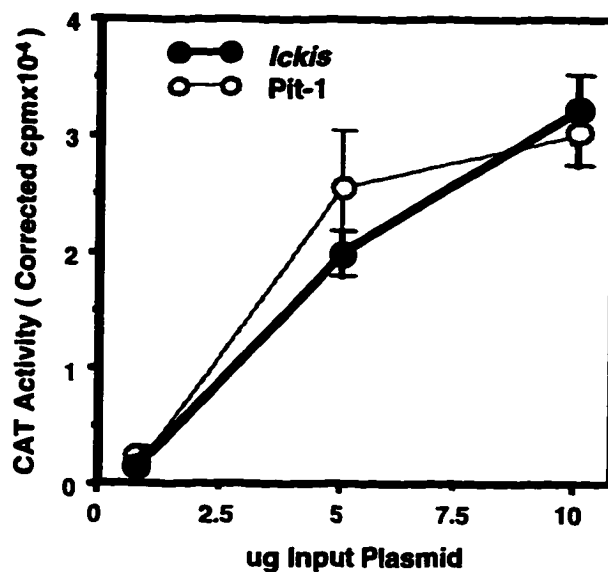


Figure 25. Dose-response of *Ickis* on (-113) PRL-CAT in C6 cells

C6 cells (1.5×10^6 /tube) were electroporated at 300V (960uF) in D10 media with 10 ug of the indicated PRL-CAT and 2ug of RSV- β gal, plus either the indicated amounts of RSV-*Ickis* (*Ickis*) or RSV-Pit1 (*Pit-1*). Following transfection, cells were divided into three 60 mm dishes, and incubated for 48 hrs. Cells were then collected, lysed, incubated for 3 hours with substrate, and assayed for CAT enzymatic activity using phase extraction. The β -galactosidase activity was determined by using *o*-nitrophenyl β -D-galactopyranoside as a colorimetric substrate, and used to correct CAT activity for transfection efficiency. Shown are the mean plus/minus SE of results with triplicate dishes. Similar results were obtained in two additional experiments.

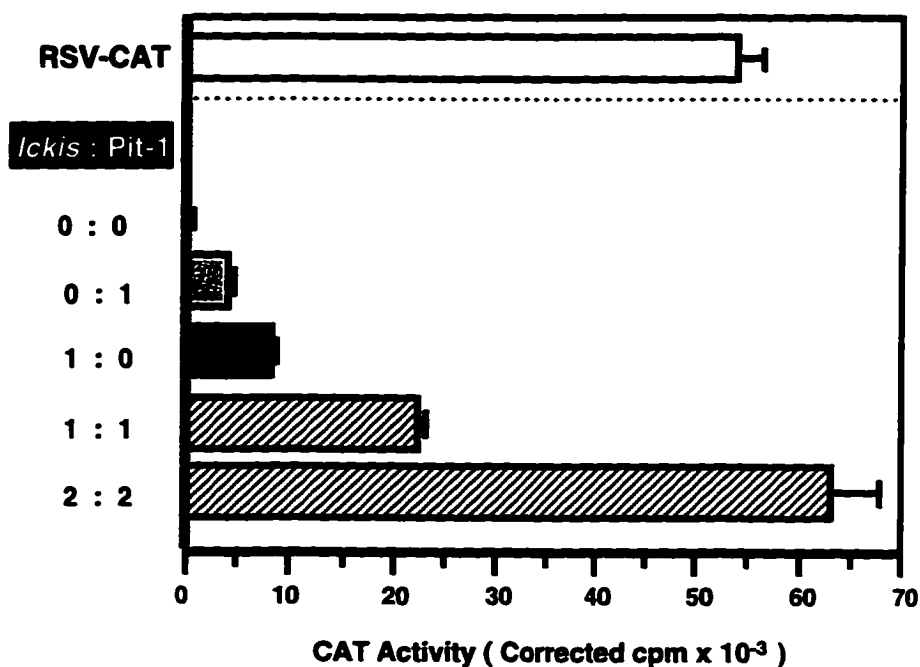


Figure 26. *Ickis* and *Pit-1* are additive on (-113) PRL-CAT in C6 cells.

C6 cells (1.5×10^6 /tube) were electroporated at 300V (960 μ F) in D10 media with 10 μ g of (-113) PRL-CAT and 2 μ g of RSV β gal, plus either 1 μ g of RSV-CAT (positive control), or 10 μ g of control construct (RcRSV; 0:0), or 2.5 μ g of either RSV-*Ickis* (*Ickis*; 1:0) or RSV-*Pit-1* (*Pit-1* ; 0:1), or 2.5 μ g (1:1) or 5 μ g (2:2) of each plasmid. Following transfection, cells were divided into three 60 mm dishes, and incubated for 48 hrs. Cells were then collected, lysed, incubated for 3 hours with substrate, and assayed for CAT enzymatic activity using the phase extraction. The β -galactosidase activity was determined by using *o*-nitrophenyl β -D-galactopyranoside as a colorimetric substrate, and used to correct CAT activity for transfection efficiency. Shown are the mean plus/minus SE of results with triplicate dishes. Similar results were obtained in two additional experiments.

[4] *Ickis* can activate transcription in homologous GH3 cells

The ability of *Ickis* to trans-activate a target promoter was also assessed in a homologous system (GH3 cells). In order to avoid any problems with endogenous factors present in GH3 cells, *Ickis* was fused in frame with the GAL4 DNA-binding domain. As illustrated in **Figure 23**, this allows the chimeric protein to target to a GAL4-promoter indicator construct. Significant activation of the GAL4 indicator was detected upon introduction of the *Ickis*-GAL4 chimera, but not with the GAL4 DNA-binding domain alone (**Figure 27**). These results show that *Ickis* contains a functional transcriptional activation domain, which can confer transcriptional activation upon a heterologous DNA-binding domain. In the future, a transcription factor that is lacking its activation domain can be attached to GAL4-DNA binding domain to compare its activity to that of GAL4-*Ickis* chimera for the better control in this study.

[5] The transcriptional action of *Ickis* is potentiated by PKA.

Since site1P is responsive to a cAMP-mediated signal, and since Pit-1 alone cannot complete this signal transduction pathway, the possible involvement of *Ickis* in cAMP/PKA signaling was explored. GH3 cells were transfected with both the GAL4-*Ickis* chimera and its GAL4 reporter construct, with or without an RSV-PKA expression vector. Co-expression of RSV-PKA yielded a three-fold induction in transcriptional activation (**Figure 28**). These results show that *Ickis* can mediate a PKA-generated signal.

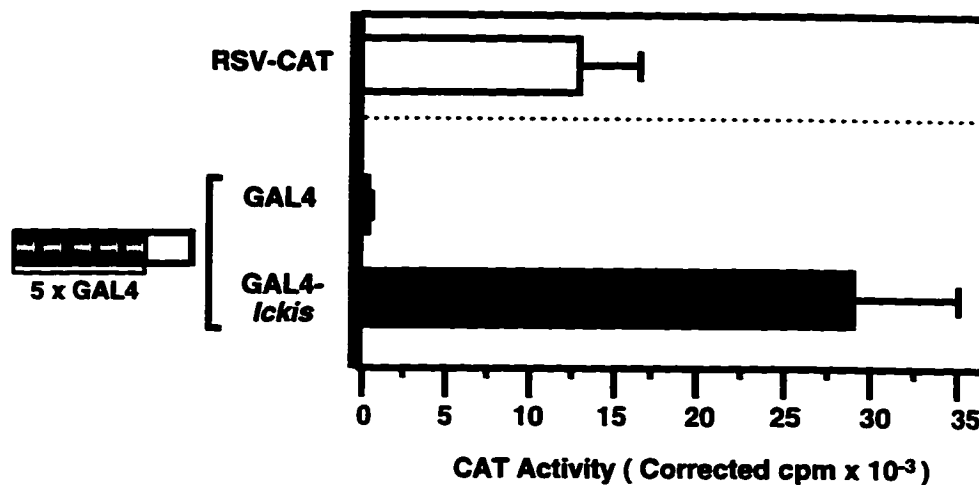


Figure 27. A GAL4-Ickis fusion protein can transactivate activity of a GAL4 promoter in homologous (GH3) cells.

GH3 cells (0.5×10^7 /tube) were electroporated at 240V (960 μ F) in D10 media with 10 μ g of the reporter construct containing 5 GAL 4 binding sites fused to CAT gene (5 x GAL) and 2 μ g of RSV- β gal, plus either 1 μ g of RSV-CAT (positive control), or 5 μ g of either DNA-binding domain of GAL4 (1-147) (GAL4), or 5 μ g of GAL4-Ickis chimera (GAL4-Ickis). Following transfection, cells were divided into three 60 mm dishes, and incubated for 48 hrs. Cells were then collected, lysed, incubated for 2.5 hrs with substrate, and assayed for CAT enzymatic activity using the phase extraction. The β -galactosidase activity was determined by using *o*-nitrophenyl β -D-galactopyranoside as a colorimetric substrate, and used to correct CAT activity for transfection efficiency. Shown are the mean plus/minus SE of results with triplicate dishes. Similar results were obtained in three additional experiments.

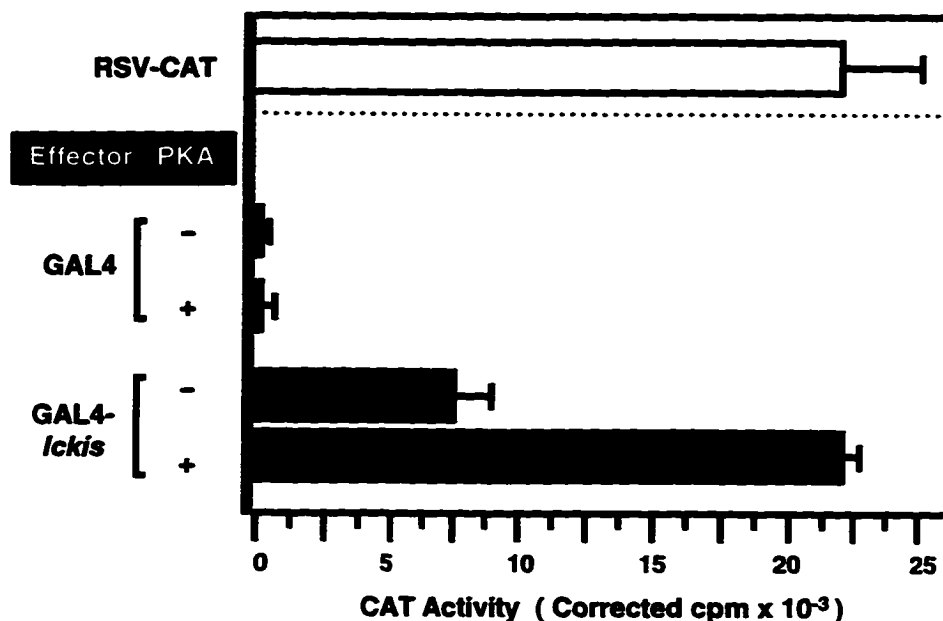


Figure 28. A GAL4-*Ickis* chimera can support PKA-mediated transcriptional activation in GH3 cells

GH3 cells (0.5×10^7 /tube) were electroporated at 240V (960uF) in D10 media with 10 ug of the reporter construct containing 5 GAL 4 binding sites fused to CAT gene (5 x GAL) and 2 ug of RSV- β gal, plus either 1 ug of RSV-CAT (positive control), or 5ug of either DNA-binding domain of GAL4 (1-14) (GAL4), or 5ug of GAL4-*Ickis* chimera (GAL4-*Ickis*) in combination with either 5ug of RcRSV [PKA(-)] or RSV-PKA [PKA(+)]. Following transfection, cells were divided into three 60 mm dishes, and incubated for 48 hrs. Cells were then collected, lysed, incubated for 2.5 hrs with substrate, and assayed for CAT enzymatic activity using the phase extraction. The β -galactosidase activity was determined by using *o*-nitrophenyl β -D-galactopyranoside as a colorimetric substrate, and used to correct CAT activity for transfection efficiency. Shown are the mean plus/minus SE of results with triplicate dishes. Similar results were obtained in two additional experiments.

I then used C6 cells to investigate whether *Ickis* could mediate a PKA regulation of the PRL promoters (**Figure 29**). *Ickis*, but not Pit-1, yielded approximately 8-fold stimulation by co-expression of RSV-PKA. These results imply that *Ickis* is a PKA-regulated transcription factor, and may represent the protein in pituitary cells that mediates PKA action on the PRL promoter via site1P.

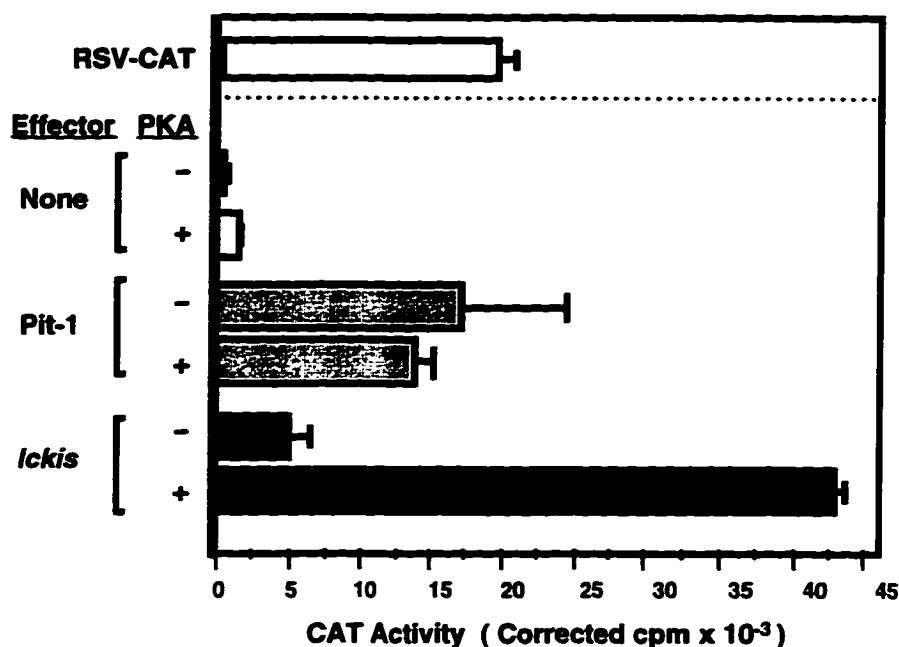


Figure 29. *Ickis* can mediate PKA regulation of PRL promoter activity in C6 cells

C6 cells (1.5×10^6 /tube) were electroporated at 300V (960uF) in D10 media with 10 ug of (-113) PRL-CAT and 2 ug of RSV β gal constructs, plus either 1 ug of RSV-CAT (positive control), or 2.5 ug of either RcRSV (**None**), or RSV-Pit1 (**Pit-1**) or RSV-*Ickis* (***Ickis***), with either 5ug of RcRSV [**PKA(-)**] or RSV-PKA [**PKA(+)**]. Following transfection, cells were divided into three 60 mm dishes, and incubated for 48 hrs. Cells were then collected, lysed, incubated for 3 hrs with substrate, and assayed for CAT enzymatic activity using the phase extraction. The β -galactosidase activity was determined by using *o*-nitrophenyl β -D-galactopyranoside as a colorimetric substrate, and used to correct CAT activity for transfection efficiency. Shown are the mean plus/minus SE of results with triplicate dishes. Similar results were obtained in two additional experiments.

Discussion

***Ickis* is a novel transcription factor**

Ickis can function as a transcription factor based on three pieces of evidence: (1) *Ickis* specifically binds DNA , (2) *Ickis* is primarily localized in the nucleus of GH3 cells, and most importantly, (3) *Ickis* is able to transactivate target genes. In general, a transcription factor has certain features that affect its activity, creating a pattern of constitutive, inducible, or tissue-and/or temporal-specific gene expression. These regulatory roles of a transcription factor are often the result of its ability to precisely recognize specific DNA sequences. Upon sequence-specific binding, transcription factors can interact with other nuclear factors, resulting in either a positive or negative effect on transcriptional activity. Future studies will reveal whether this is the case for *Ickis*.

Absence of an obvious DNA-binding domain in *Ickis*

Ickis currently fall into a group of transcription factors that contain “unknown DNA-binding motifs”, since no obvious DNA-binding motif can be detected in its primary sequence. Extensive structural studies have been performed to understand how a transcription factor can recognize a certain segment of DNA. These analyses often facilitate the identification of DNA-binding motifs such as helix-turn-helix, zinc-finger, and, leucine-zipper basic domains (Mitchell and Tjian, 1989). *Ickis* contains an as yet uncharacterized DNA-binding motif. It is therefore possible that *Ickis* may

prove to contain a novel DNA-binding domain that will also be found in the future in other transcription factors.

Transcriptional activation domain

Distinct activation domains are determined using deletion analysis, and are classified based on their amino acid sequences. Four classic activation domains are known including (1) acidic, (2) proline, (3) glutamine, and/or (4) serine-threonine-rich domain(s) (Mitchell and Tjian, 1989). Additional domains have been subsequently identified, including the proline/glutamine-rich regulatory domain seen in WT-1 (Madden *et al.*, 1991) and YY1 (Bushmeyer *et al.*, 1995). However, the prediction of these domains from the primary or even from the 2^o structure information may be considered significant only when it correlates with the experimental data. This is partially because the activation domains often do not possess well-defined structure, which correlates well with the fact that activation domains are in general insensitive to amino acid substitutions and/or small deletions (Hope and Struhl, 1986, Gill and Ptashne, 1987, and Hope *et al.*, 1988). Thus, one could only speculate whether *Ickis* contains functional activation domains in the absence of experimental proof.

***Ickis* and Pit-1 can act additively on site1P**

Transient transfection analysis strongly suggests that the transcriptional effects of *Ickis* and Pit-1 are additive. It is assumed that Pit-

1 and *Ickis* are in close physical proximity on site1P. It is known that most sequence-specific binding factors must bind to their target DNA site in order to influence transcriptional activity (Mitchell and Tjian, 1989).

Therefore, one can propose an independent binding by these two factors to site1P, resulting in an additive effect on PRL promoter activation.

Alternatively, the actions of these two factors might be mutually exclusive.

If only one factor can access site1P at a given time point, the activation of PRL promoter would equal the sum of both *Ickis* and Pit-1 activity, rather than resulting in a synergy between the two factors. Further studies will be necessary in order to differentiate between these mechanisms.

***Ickis* may represent a novel transcriptional mediator of PKA action**

The observation that *Ickis*, but not Pit-1, can mediate PKA action on PRL promoter site1P suggests that *Ickis* is the pituitary cell protein that mediates PKA action on PRL promoter site1P. The best-known PKA transcriptional mediator is CREB, which is activated by cAMP-mediated phosphorylation (Montminy *et al.*, 1986). There are several putative PKA target sites in *Ickis*. The question of whether *Ickis* is phosphorylated by PKA is being studied by Robert Ashton (a graduate student in Dr. Carter Bancroft Laboratory). In a single preliminary experiment *in vitro*, he has found that *Ickis* is phosphorylated by PKA, implying that *Ickis* may be a PKA substrate (personal communication). While Pit-1 was clearly demonstrated to be a substrate for PKA *in vivo* (Kapiloff *et al.*, 1991), the

effect of Pit-1 phosphorylation was not functionally significant to PRL gene expression. Further studies will be required to determine whether PKA activation of *Ickis* involves phosphorylation of this protein at specific residues.

Potential role of *Ickis* in other organs/tissues

Considering the expression profile of *Ickis* gene, it is hard to believe that the PRL promoter is the only target for *Ickis*. Also, it is not unusual for transcription factors to use alternative splicing as an additional regulatory mechanism. Moreover, tissue-specific splicing can lead to a distinct role for *Ickis* isoforms in individual tissues. *Ickis* may therefore have a more general role, in the form of differentially expressed isoforms, similar to what is seen for the CREM family of transcription factors.

CHAPTER V

**IMPLICATIONS AND
FUTURE DIRECTIONS**

Implications and future directions

***Ickis* may be one of the downstream effectors of PKA action on the PRL promoter.**

I have described in this thesis the isolation and characterization of a novel transcription factor, *Ickis*. This protein was isolated based on its physical interaction with the PRL cis-element, site1P. Because site1P is a critical element that mediates PKA-linked signals, a transcription factor such as *Ickis* might function as a downstream transcriptional effector of PKA action. *Ickis* has the appropriate properties, because it binds specifically to site1P and its transcriptional action is potentiated by PKA. The wide distribution of *Ickis* transcripts further supports earlier speculation that cAMP-PKA-mediated regulation of the PRL promoter may be conferred via a ubiquitous transcription factor (Gellersen *et al.*, 1995 and Rajanarayan *et al.*, 1995). This concept was primarily based on the observation that the PRL promoter is further activated by cAMP and/or PKA in heterologous systems including HeLa and uterine sarcoma cell lines. PRL gene expression is controlled by hormones, neurotransmitters, and growth factors. Both the inhibitor, dopamine, and the stimulator, PACAP, utilize the cAMP-signal transduction pathway to modulate the expression of PRL. Response to these signals have been mapped to site1P and its neighboring sequences. *Ickis* may thus serve as an ultimate downstream effector for these important external stimuli.

Potential role of *Ickis* as a brain transcription factor

Expression of *Ickis* transcripts in the brain and in the neuronal cells suggest the potential physiological significance of *Ickis* in the brain. It is very exciting and interesting to investigate this possibility since the biochemical and genetic analysis of a particular transcription factor in the brain has just begun to reveal its biological significance and the potential involvement of the transcription factor in the function of the brain (Yin *et al.*, 1994, Frank and Greenberg, 1994 and, Kogan *et al.*, 1997). In other words, we have limited knowledge about the brain at the gene level. Very few brain- or neuronal-specific transcription factors are known. Most known transcription factors in neuronal tissues have been discovered because they (or other members of the same family) were initially isolated from other organs or tissues. This is partially because of the heterogeneous nature of the brain, thus, making it more difficult to isolate cell-type-specific transcription factors. Additionally, since we have a limited understanding and knowledge about how the brain functions, it is especially challenging to explore the physiological relevance of brain transcription factors. It is interesting that so many cell-type-specific proteins have been discovered on the cell surface, however, only a small number of the "specific" transcription factors have been identified (Mandel and McKinnon, 1993). It is not impossible to imagine that specialized, cell-type-specific receptors in the brain can activate rather ubiquitous transcription factors. However, this still poses the question of how these

rather general transcription factors can play a specific role in the brain. Alternatively, one can hypothesize that there might be additional families of undiscovered transcription factors that play important roles in the brain. *Ickis* might be a candidate for such a factor, since its unequal distribution among neuronal cells and its potential tissue-specific isoform found in the brain could result in a cell-type-, or region-specific response. Thus, it is of great interest for us to investigate the role of *Ickis* in the brain.

It should also be noted that the pituitary and brain often share common or related families of transcription factors. For instance, Pit-1 is exclusively found in the pituitary, while a closely related family of transcription factors (Bm-family) are distributed throughout the brain. A similar example is the case of TEF, which is also expressed in certain regions of the brain. Interestingly, both of these transcription factors were originally isolated on the basis of their ability to bind to site1P or its modified version. Because it is difficult to identify target gene(s) in the brain, the nature and behavior of brain transcription factors can be approached via conjugate studies of the pituitary. For example, Pit-1 is responsible for pituitary organogenesis and cell survival, and members of the Bm-family have also proven to be involved in cell differentiation and specification in the brain (He et al., 1989). Therefore, an understanding of the role of *Ickis* in the pituitary may help to shed light on the role of *Ickis* in the brain.

Alternative splicing and/or differential promoter usage might be utilized and could result in the generation of functionally different *Ickis* isoforms

It is interesting to note that *Ickis* transcripts exhibit tissue-specific patterns. Transcription factors often occur as isoforms or are encoded by closely related genes. This may explain how a limited array of transcription factors can specifically modulate expression of a large number of genes, and this may also help explain how transcription factors can adjust their action in response to specific upstream signals. An example of the latter is found in CREM, one of whose isoforms is rapidly induced by c-AMP and can act as an antagonist of the cAMP response (Lalli *et al.*, 1996, Lamas *et al.*, 1996). It would be interesting to investigate whether *Ickis* also possess a similar type of isoform.

Determination of the physiological role of *Ickis*, and of its mode of action

Is the activity of *Ickis* completely dependent on PKA ? Since there is endogenous PKA present in C6 and GH3 cells, it would be worthwhile to examine the role of *Ickis* in a cell line lacking PKA (e.g., F9 cells), or by using co-transfection analysis of C6 or GH3 cells with a dominant-negative inhibitor of PKA. If the action of *Ickis* absolutely requires PKA, as is the case for CREB (Montminy *et al.*, 1986), it would be interesting to further analyze the relationship between *Ickis* and the CREB family.

An ultimate goal of future studies would be to identify the physiological role of *Ickis*. This will be one of the most difficult questions to answer because of the novel nature of this gene. Some clues can be obtained by determining the chromosome location of the *Ickis* gene, in an attempt to find a phenotypic marker. Ultimately, gene disruption or transfer must be carried out in an intact animal, in order to get a conclusive answer about the role of *Ickis*. Since *Ickis* is expressed in various tissues, gene knock-out experiments might be aided by the use of an inducible promoter. Alternatively, gene transfer experiments with a construct containing a tissue-specific promoter fused to a dominant-negative form of *Ickis* might greatly facilitate the determination of the role of *Ickis* in its target tissues. In either case, further structural and functional analysis of *Ickis* is needed. Functional domains such as DNA-binding and transcriptional activation domains should be identified. This information could be used to construct dominant-negative or constitutively active forms of *Ickis*, and in the identification/isolation of as yet undiscovered *Ickis*-related transcription factors.

CHAPTER VI

EXPERIMENTAL PROCEDURES

Experimental Procedures

[A] Southwestern screening

(1) *E.coli* strains and bacteriophage

The λ ZAPII™ GC expression library (Stratagene) and XL1-blue MRF' bacterial strain were used. Excision of the inserts from the lambda arms was performed by the addition of ExAssist helper phage according to the manufacturer's instruction. Either SOLAR or VCM13 host cells were used to maintain cDNA or DNA sequencing.

(2) DNA probes and labeling

A site1P DNA which corresponds to the PRL promoter (-63/-39) was used as a probe. As shown below, this double-stranded site1P contains Sall sites at both ends to permit concatenation of probe. Ligation of multiple site1P was performed after end-labeling by T4 polynucleotide kinase in the presence of [γ -³²P] ATP (3000 Ci/mmol, Dupont NEN), followed by an addition of DNA ligase (Boehringer Mannheim) for 30 min at room temperature (RT). The ligated DNA obtained was approximately 200 bp in mean length (see **Figure 2**), with a specific activity of 1.5-2.0 x 10⁸ cpm/ug. No more than twenty 132 mm nitrocellulose filters were probed with this amount of probe (0.5-1.0x10⁷ cpm/filter).

5' -TCGACTGCCTGATTATATATATATATTCATGAAGGTGTCGAAC-3'
 3' -GACGGACTAATATATATATAAGTACTTCCACAGCTTGAGCT-5'

(3) Filter preparation and binding condition

Each plate (150 mm) containing approximately 5×10^4 pfu was placed at 42 C, overlaid with a 10 mM isopropyl β -D-thiogalactopyranoside (IPTG)-impregnated nitrocellulose filter (Schleicher and Schuell), and incubated for an additional 6-7 hrs at 37 C or for 15 hrs at RT. Duplicate filter were used except for incubation for 2 hrs at 37 C. After removal from plates, filter were air-dried for 10 min at RT and then subjected to the denaturation/renaturation cycling procedure (Vinson et al., 1988, Singh et al., 1989). All subsequent procedures were carried out at 4C. Briefly, filters were treated twice with the binding buffer [25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM Dithiotheitol (DTT), and 25 mM HEPES (pH 7.9)] containing guanidine hydrochloride (Gn-HCl) for 10 min. The denaturant was gradually removed by sequentially replacing half volume of binding buffer with fresh buffer containing no Gn-HCl. This procedure was repeated three times and filters were placed in fresh binding buffer for 5 min. After repeating this washing step, filters were submerged in binding buffer with 5 % Carnation nonfat dry milk for 60 min, rinsed twice with unsupplemented binding buffer for 5 min, and, exposed to the probe using 5 ml of binding buffer containing salmon sperm DNA (5 ug per filter). The binding reaction used no more than 10 filters in a glass baking dish with gentle shaking for 6-15 hrs at 4 C. Filters were washed three time for 10

min with binding buffer, blotted on 3MM paper, and, exposed to Kodak X-Omat AR film overnight at -70C with intensifying screens.

(4) Isolation and purification of presumptive positive clones

Potentially positive clones were isolated according to the method described in Maniatis et al. (1982). The plaques which showed a diffuse halo like and comet-like appearance were picked with or without alignment with a duplicate filter. These plaques were then subjected to secondary and tertiary screening until a pure positive plaque was obtained.

[B] Selection of true positive clones

(1) Filter binding assay

Individual recombinant bacteriophage was infected into a XL1-blue host and plated as described above with or without treatment with IPTG. After recombinant protein was expressed, it was transferred onto the filters and exposed to either site1P probe, or other unrelated cis-elements: *1P*, CLE, or, non-specific DNA competitor poly(dI-dC). Filters were washed and exposed to x-ray film for detection of signals.

(2) Preparation of his-tagged fusion protein

The coding region of *Ickis* was subcloned in frame into the pET28b(+) expression vector (Novagen) that contains an inducible T7 promoter with six consecutive histidines (His-Tag). Following overnight culture, host cells [BL21(DE3)] containing the either empty or *Ickis* cDNA vector were diluted to 100-fold in rich media (TB broth) with kanamycin (40 mg/ml), and grown at various temperatures (RT, 37 C, and 42 C). A fraction equivalent to the value of $OD_{600}=1$ of cell extract was collected at the sequential time periods (t=0, 1, 2, 2.5, 3, 4 hrs) after the addition of 0.4 mM IPTG, and immediately pelleted to remove the supernatant. All the following procedures were carried out at 4 C. The pellet was resuspended, and insoluble materials were obtained after centrifugation. Both soluble and insoluble fractions were applied to a 12 % polyacrylamide gel in order to analyze the expression profiles of *Ickis* protein. Since most of the *Ickis* protein was in the insoluble fraction (inclusion body), proteins were partially purified by washing the pellet with 0.1 M Tris-Cl (pH 8.5) containing 6.5 M urea. The remaining pellet was suspended in Binding buffer [5 mM imidazole, 0.8 M NaCl, 20 mM Tris-Cl (pH 7.9)] with a supplement of 8 M urea, and passed through a nickel metal chelation affinity column. After several washings, the bound proteins were gradually refolded in the column, and eluted in buffer containing 1 M imidazole. Partially purified *Ickis* protein was then desalted and concentrated by Amicon spin column. All procedures were performed at 4C in the presence of protease inhibitors.

(3) Gel-shift analysis

Affinity purified *Ickis* from above was incubated in 9 ul reaction containing 10 mM Tris-Cl (pH 7.9), 60 mM KCl, 1 mM EDTA, 0.03 % NP40, 4 % Ficoll, 1 mM DTT, 5 ug poly(dI-dC), and 1 ug BSA with or without non-labeled competitors. After 10 min incubation at RT, 1 ng of end-labeled site1P was added for 10 min, and the resulting complexes were analyzed on a 5% non-denatured polyacrylamide gel in 0.25x TBE at 4 C. Gels were dried and subjected to autoradiography for 1-3 hrs at -70 C with intensifying screens.

(4) DNA sequencing

All potentially positive clones were sequenced using Sanger chain termination dideoxysequencing (DNA sequencing kit, USB). Vectors (pBlueScript) containing the cDNA inserts were *in vivo* excised from lambda arms using the procedures suggested by the manufacturer (Stratagene).

(5) 5'-RACE (Rapid Amplification of cDNA End) PCR

Both polyA-enriched GH3 cells and the GC library were used as the template for 5'-RACE. A primer RACE1 (5'-tctggaacctgcccacaaacc-3'), located 249 bases away from the 5'-end of *Ickis* was selected, and the first strand synthesis from RNA was performed by incubation with reverse transcriptase (SuperSCRIPT™II, GibcoBRL), 50 min at 42 C. For DNA

template (GC library as a source), the same primer was used to synthesize the first strand with Taq polymerase (Perkin Elmer) using cycling condition (1 min at 94 C, 1 min at 67 C, and 1 min at 72 C). Prepared cDNA from both sources were pooled, extracted and precipitated by EtOH in the presence of NaOAc (0.3 M final) and tRNA (10 ug). Poly(dC)-tail was added to the recovered sample with 25 units of terminal transferase (Boehringer Mannheim) in the presence of CoCl₂ (1 mM) for 30 min at 37C. Tailed cDNA was extracted and precipitated as described before. One tenth of the reaction mixture was applied to amplify the 5'-ends by using oligo(dG)₁₃ 5' primer and the internal 3'-primer ending at the 83rd base from the *lck* start sites (RACE2; 5'-gccatcaggacccaaagcc-3'). Reaction was carried out 45 cycles (94 C for 1 min, 56 C for 1 min, 72C for 1 min) with AmpliTaq (Perkin Elmer) in the presence of control tube which had no addition of reverse transcriptase at the initial step. The amplified products were analyzed by agarose electrophoresis, cloned into pBlueScriptSKII(+), and sequenced. The second cycle of 5'-RACE was performed using the Ext1 and Ext2 as a extension and PCR primers, respectively (see **Figure 14**). All procedure was as described above except for reverse transcriptase was replaced with the rTth polymerase (Perkin Elmer) for 15 min at 65 C in the presence of Mn⁺.

[C] Preparation and analysis of genomic DNAs

Rat genomic DNA was prepared from the GH3 cells using the basic protocol (Ausubel *et al.*, 1995). Briefly, approximately 1×10^8 cells in suspension were collected and resuspended in 1 ml digestion buffer [100 mM NaCl, 10 mM Tris-Cl (pH 8), 25 mM EDTA, 0.5 % SDS, 0.1 mg proteinase K]. Following overnight incubation at 50 C, DNA was extracted gently with phenol/chloroform/isoamyl alcohol, precipitated with EtOH in the presence of ammonium acetate, washed with 70 % EtOH, and air dried. Pellet was resuspended with TE at 55 C for 1 hr, and subjected to restriction enzyme analysis. Prior to loading samples onto agarose gel, DNase-free RNase was added to increase the resolution of the signal. Aliquots of digested samples were analyzed in parallel in order to visualize the digested DNA with ethidium bromide. Genomic DNAs from human and *Drosophila* were kindly provided by Dr. David Bishop (Mount Sinai). *Drosophilas* were provided by Dr. Manfred Frasch (Mount Sinai), and twenty female and well-fed flies were collected. Briefly, lysis buffer (250 ul per 20 flies) [0.1 M Tris-Cl (pH 9), 0.1 M EDTA, 1 % SDS] was added to the sample, homogenized well, and placed at 70 C for 30 min. Upon the addition of 35 ul 8 M KOAc, tube was placed on ice for 30 min, centrifuged for 15 min at 4 C, and supernatant was saved for precipitation using 150 ul 2-propanol. After 10 min on ice, precipitants was collected, resuspended in 100 ul TE, and extracted by phenol:chloroform twice following precipitation with EtOH. Pellet was washed with 70 % EtOH, air

dried, resuspended in 50 ul TE. Yeast genomic DNA was prepared according to the manufacture's instruction (PureGene, GENTRA).

Southern blot analysis was performed using nitrocellulose filter with high-salt buffer (Ausubel *et al.*, 1995). Briefly, after the denaturation and neutralization of the agarose gel, DNA was capillary transferred onto nitrocellulose membrane and immobilized by baking in a vacuum oven at 80 C for 2 hrs. Filters were prehybridized at 42 C for rat , 39 C for all other genomic DNA samples (human, *Drosophila*, and yeast), followed by the addition of labeled *Ickis* DNA. After overnight incubation, the filter was washed until background level receded, and exposed to x-ray film for 3 hrs to overnight. The *Ickis* coding region was labeled by either PCR or random labeling using the standard procedure (Ausubel *et al.*, 1995).

[D] Nuclear extract preparation

Nuclear extracts were prepared as described by Lee *et al.*, (1988). All buffers except for PBS contained DTT (0.5 mM), PMSF (0.5 mM), and *p*-hydroxymercuribenzoic acid (1 mM), and all reaction were performed at 4C. Cells were subconfluent grown in monolayer or to 1×10^6 cells/ml in suspension, and harvested using a rubber policeman and centrifugation (1000g for 10 min), respectively. Pellets were washed once with PBS and repelleted as before. Cells were resuspended in 1 packed cell volume of Buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl]. After incubation for 15 min, cells were lysed using a 1 ml syringe with a 25G

(5/8") needle for times. Post-nuclear supernatant was then removed by aspiration, and the crude nuclear pellet was resuspended in 2/3 of the packed cell volume of Buffer C [20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl] followed by incubation on a rocking platform. After removal of nuclear debris by centrifugation in a microfuge for 5 min, the supernatant was dialyzed against buffer D [20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20 % glycerol] for 2 hrs, and samples were stored at -80C.

[E] Distribution profile of *Ickis* message

(1) Northern blot analysis

Total and polyA-enriched RNA were prepared from various cell lines and tissues using Oligotex™ column affinity purification system (Qiagen) without and with addition of oligo-d(T)-linked beads, and subjected to separation by 1% formaldehyde agarose gel electrophoresis (Maniatis *et al.*, 1989). Size separated RNA was transferred onto nitrocellulose (Schleicher and Schull) by blotting, and fixed by vacuum oven for 2 hrs at 80C. Filter was then emersed into a prehybridization buffer for at least 4 hrs, and then a gene specific probe prepared by either PCR labeling, or, random labeling method was applied for 15 hrs (Ausubel *et al.*, 1995). Membranes were exposed to x-ray film (Kodak AR) for 1-3 days at 70 C with intensifying screens.

(2) RT-PCR analysis

One to five ug of RNA or 50 to 100 ng of polyA-enriched RNA was annealed with either oligo(dT) or gene specific primer after template RNAs were denatured at 70C for 5 min, and subjected to first strand synthesis in the presence of 1 ul (200 units) reverse transcriptase (SuperSCRIPT™II). After 50 min incubation at 42C, the cDNA was amplified by a set of gene specific primers by Taq polymerase (AmpliTaq™) for 35-40 cycling. Cycling conditions for each set of primers were determined empirically in addition to assistance from the software program (Oligo 4.0).

Nested-PCR was performed using combinations of PCR primers; F2 (position; 1082-1097) & R2 (1776-1761) pair, F4 (1300-1318) & R6 (1654-1636) pair as first and second rounds primer set.

[F] Detection and functional analysis of *Ickis* protein

(1) Preparation of *Ickis* antibody

Four liters of the bacterial culture containing overexpressed *Ickis* protein was extracted to obtain bacterial inclusion bodies. Briefly, pelleted cells were lysed by lysozyme and detergent (deoxycholic acid) in the presence of 50 mM PMSF. Lysate was treated with DNAase I (1 mg/ml) at RT for 15-30 min until it was no longer viscous. After centrifugation, the

pellet was suspended, and washed with 6.5 M urea containing buffer to eliminate as much protein as possible that is component of inclusion body. Subsequently, *Ickis* was extracted from the pellet with elution buffer containing 8 M urea, and sized by the SDS-polyacrylamide gel. The major *Ickis* band was excised, frozen powdered with liquid nitrogen, and further refined by mortar and pestle. This was then employed for the following procedures for the production of antibodies by Cocalico Biologicals, and serum was collected until an appropriate titer was achieved.

(2) Western blot analysis

Both nuclear and cytosolic extracts were prepared in the presence of protease cocktail at 4°C and the integrity of each samples was examined by SDS-polyacrylamide gel electrophoresis. No gross degradation of proteins was detected, and much higher content of proteins were present in the cytosolic fraction. The amounts of each sample was adjusted to represent equal number of cells. Samples were separated by polyacrylamide gel electrophoresis (Laemmli, 1970), and transferred onto nitrocellulose using a semi-dry transfer apparatus (Hoeffer Scientific) for 30-60 min at 100 mA in Towbin buffer (25 mM Tris-base, 192 mM Glycine, 0.0372 % SDS, 20% Methanol). Filters were blocked with 5% Carnation non-fat dry milk in TBST [10 mM Tris (pH 8.0) 150 mM NaCl, 0.05 % Tween 20] for 1 hr, and incubated with *Ickis* antibody (1:250) followed by washing with TBST. Prior to use, this antibody was absorbed with the

bacterial extracts for the enrichment of the antibodies directed against *Ickis* (see below). The secondary antibody conjugated horseradish peroxidase was used at a 1:5000 dilution in TBST with 3 % BSA, and incubated for 30-60 min. After washing with TBST (4 x 10 min), immunoreactive proteins were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's directions (Amersham).

(3) Absorption of *Ickis* antibody with bacterial extracts

An equal volume of 2 x sample buffer was added to a pelleted 50 ml bacterial culture containing no *Ickis* expression vector, mixed thoroughly, and immediately placed at 65-70 C for 10 min. Denatured bacterial extract was separated on a 4.5 % SDS-polyacrylamide gel using the 1.5 mm preparative comb, and transferred to nitrocellulose as described above. After soaking with the 5 % Carnation non-fat milk, the filter was incubated with diluted *Ickis* antibody in TBST containing 3% BSA on the rocking platform for at least one hour or overnight at 4 C. After removal of filter, this primary antibody was employed for western analysis of *Ickis*.

(4) Transient co-transfection assay

Approximately 0.5×10^8 of GH3 or 2×10^6 of C6 cells per each treatment group were subjected to electroporation with the indicated constructs. Cells were collected and resuspended in Dubecco's modified

Eagle's medium (DMEM) containing 10 % fetal bovine serum (appropriate number of cells per 0.75 ml). Suspended cells containing the indicated constructs were placed in a cuvette for electrophoration at the value indicated in each legend, and immediately plated onto three 60 mm plates. After 48 hrs of incubation, cells were harvested using a rubber policeman, lysed by sonication (2 min x 4) in the presence of CAT buffer [0.25 M Tris-Cl (pH 7.8), 10 mM EDTA]. Cell extracts were heated at 65 C for 5 min to inactivate endogenous deacetylases, and subjected to the either CAT enzymatic, or β -galactosidase (LacZ) assay as indicated in each legend. For each reaction, half of the cell extracts was incubated with [3 H] chloramphenicol (0.01 uCi/ul) and substrate butyryl CoA (5 mg/ml) for 4 hrs in order to assay the enzymatic activity in the linear range.

Different conditions were tested using a known positive effector, Pit-1, to maximize the transfection efficiency, which includes set point of voltage, capacitance, the number of cells, and the status of cells. These optimized parameters were fixed throughout the experiments unless the cell line was changed. All experiments were performed independently at least three times. A fixed amount of RSV-CAT construct was employed as a positive indicator throughout the transfection experiments. RSV-directed β -galactosidase expression was measured to equalize the transfectional efficiency among each group, and total input of DNA was adjusted with the RcRSV plasmid. After a day of transfection, each plate was briefly examined under microscopy to look for the gross differences in

cell survival rate among the experimental groups. The data were analyzed only when all the conditions summarized above were properly measured and/or an in acceptable range.

[G] Cell lines and tissue cultures

GH3 rat pituitary cells (Bancroft, 1981) were grown in suspension culture in Minimum Essential Medium (Joklik modified) with 10 mM HEPES (pH 7.4), 25 mM NaHCO₃, 100 mg/ml streptomycin, 100 U/ml penicillin, 2.5 % fetal calf serum, and 15 % horse serum. After transfection, cells were placed onto plates with Dubecco's modified Eagle's medium (DMEM) with 10 % fetal calf serum and maintained at 37 C, 10 % CO₂. C6 rat glial cells (Benda et al., 1969) were maintained in monolayer culture with DMEM containing 5 % fetal calf serum, 100 mg/ml streptomycin and 100 U/ml penicillin. C6 cells were split 1:5 ratio every other day and incubated at 37 C with 10 % CO₂.

[H] Constructs used in the transfection assay

The complete *lckis* cDNA was subcloned into Rc/RSV (Invitrogen) eukaryotic expression vector (RSV-*lckis*). Plasmid RSV-Pit-1 was obtained from Dr. Samuels (New York University) and has been described (Fox et al., 1990). The PRL promoter-CAT reporter construct (-113)PRL-CAT (Lufkin et al., 1989) and RSV-CAT plasmid have been described previously (Jackson and Bancroft, 1988). Both constructs GAL4-DNA binding domain (pSGS424) and GAL4-CAT construct (pG₅EC)

were kindly provided by Dr. Mark Ptashne (Harvard University). Reporter construct pG₅EC contains five consecutive GAL4 binding sites in the upstream of TATA box (Carey *et al.*, 1990). The complete *Ickis* coding region was fused to the GAL4-DNA binding domain (Sadowski and Ptashne, 1989) in register and referred as GAL4-*Ickis* in the text.

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