A 26-Amino Acid Insertion Domain Defines a Functional Transcription Switch Motif in Pit-1β*

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Pit-1, a pituitary-specific member of the POU homeodomain transcription factor, specifies three anterior pituitary lineages; governs growth hormone, prolactin, and thyrotropin gene expression; and mediates basal and Ras-stimulated prolactin promoter activity in GH4 pituitary cells. Alternate splicing of the Pit-1 message produces the Pit-1β isoform, which contains a 26-amino acid insertion, the β-domain, within the amino-terminal transactivation domain. The β-domain functions as a molecular switch, such that Pit-1β blocks both basal and Ras-stimulated prolactin promoter activity in GH4 pituitary cells yet preferentially enhances protein kinase A-stimulated prolactin promoter activity in HeLa reconstitution system. To determine whether the amino acid sequence of the β-domain dictates function, we replaced it with five different 26-amino acid sequences. These mutants fail to block basal or Ras-stimulated rat prolactin promoter activity and fail to optimally enhance the protein kinase A response of prolactin promoter. These data demonstrate that the amino acid sequence of the β-domain specifies its role as a molecular switch. Additionally, the presence of both Pit-1 and Pit-1β in pituitary cells allows diverse incoming signals to utilize structurally different forms of the same gene product, which can interact with distinct co-factors, integrating multiple signaling pathways at the level of the nucleus.

Pit-1 is a pituitary-specific member of the POU homeodomain family of transcription factors, which includes the mammalian transcription factors Oct-1 and Oct-2, the Caenorhabditis elegans factor Unc-86, and at least 20 other transcription factors (1). Expression of Pit-1 is required for the normal growth and development of three anterior pituitary cell types, thyrotrophs, somatotrophs, and lactotrophs (2), as well as the proper expression of the anterior pituitary hormones prolactin (PRL),1 growth hormone (GH), and thyroid-stimulating hormone-β (3, 4). The Pit-1 transcript contains six exons and five introns (5) and encodes a 33-kDa protein containing two regions important for transcriptional regulation of target promoters: an N-terminal transactivation domain (TAD) spanning amino acids 1–80 (6) and a C-terminal DNA binding and dimerization domain consisting of a POU-specific domain (amino acids 128–198) and a POU homeodomain (amino acids 214–273) (7–9) (Fig. 1). Both the POU-specific domain and the POUS homeodomain are necessary for high affinity DNA binding (10), while the transactivation domain is sufficient to activate transcription of a reporter gene when fused to the LexA or c-Jun DNA binding domain (6, 10).

Pit-1β, a splice variant of Pit-1, arises from the use of an alternate 3' splice acceptor at the end of the first intron of the Pit-1 transcript (5, 11, 12) and contains a 26-amino acid (aa) insertion at position 48 in the transactivation domain (Fig. 1). This 26-aa insertion domain endows the Pit-1β isoform with a range of unique negative and positive transcriptional properties. Pit-1β acts as a dominant negative repressor of transcription from the rPRL promoter in pituitary cells, such as GH4 somatolactotrophs and α-thyroid-stimulating hormone thyrotrrophs (5, 11–13), and inhibits the Ras response of the rPRL promoter in GH4 cells (14, 15). Moreover, Pit-1β fails, in nonpituitary cells, to interact functionally with Ets-1, a widely expressed transcription factor required for full reconstitution of rPRL promoter activity.2 Yet, Pit-1β is even more competent to mediate signaling by PKA to the rPRL promoter in a HeLa reconstitution assay than is Pit-1.3 Pit-1β demonstrates its repressive functions in pituitary cells but not in nonpituitary cells, implying that Pit-1β interacts with a cell type-specific factor to repress basal and Ras-activated rPRL expression. Because dimerization between splice variants had been identified as a mode of repression (18, 19), it stood to reason that a pituitary-specific Pit-1/Pit-1β heterodimer might serve as such a repressor.

The 26-aa β-domain has been conserved across the vertebrate lineage (5, 11, 12, 20–22) (Tables I and II). The N-terminal 12 amino acids of the β-domain have been especially well conserved among mammals, avians (92%), and teleost fish (67%), whereas the C-terminal 14 amino acids, also found in Pit-1T, a thyrotrroph-specific splice variant of Pit-1 (23), have been poorly conserved. This conservation of structure raises the possibility of conservation of function, such that the amino acid sequence of the β-domain, and not the resultant altered spacing of the TAD generated by the inserted β-domain, confers upon the Pit-1β isoform its unique properties. Thus, the β-domain would not simply disrupt a pre-existing structure but rather would encode an intrinsic functional motif.

To investigate this question, we constructed five mutant Pit-1 expression vectors that contain distinct 26 amino acid...
substitutions for the β-domain at position 48 of the TAD. We found that each of these substitution mutations, which preserve the location and 26-aa spacing of the wild-type β-domain, abrogated all of the unique properties of the Pit-1β isoform. Specifically, the Pit-1β-mediated repression of basal and Ras-stimulated rPRL promoter activity, Pit-1β’s inability to interact functionally with Ets-1, and the Pit-1β-mediated enhancement of the PKA effect are all lost with each of the β-domain mutations. We also tested the hypothesis that Pit-1 and Pit-1β can combine to repress basal rPRL promoter activity and, surprisingly, found that their transactivation properties were synergistic. These studies allow us to conclude that the wild-type amino acid sequence of the β-domain is required for manifestation of the unique properties of Pit-1β, through a β-domain-specific functional interaction with a cell type-specific target distinct from Pit-1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Monolayer cultures of HeLa human cervical carcinoma cells, kindly provided by Dr. Peter Sarnow (University of Colorado Health Sciences Center) and GH4 rat pituitary tumor cells (24) were maintained in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 50 μg/ml of penicillin and streptomycin at 37°C in 5% CO2. The medium was changed 16–18 h before each transfection. Cells used for transfections were harvested at approximately 60–80% confluency using 0.05% trypsin and 0.5 mM EDTA.

**Plasmids**—The rat prolactin and growth hormone (25) promoter luciferase expression vectors, pA_PRL/luc and pA_GH/luc, contain the firefly luciferase coding region under the control of a 498-base pair fragment (−425 to +73) of the rPRL promoter and a 593-base pair fragment (−528 to +65) of the rat GH promoter, respectively. These fragments were subcloned downstream of three polyadenylation termination sites in pA_luc (26). The pRSV-PAK plasmid pRK5-12 was the PKA catalytic subunit derived from Chinese hamster ovary cells and was kindly provided by Dr. R. A. Maurer (University of Oregon Health Science Center, Portland, Oregon). Plasmid pSV Ras (27) contains the T24 bladder carcinoma Harvey Ras valine 12 mutant oncogene (V-12 Ras) under control of the SV40 early promoter. The plasmid pSV5-Ets-1 encodes the p68 chicken Ets-1 (28) under the control of the SV40 early promoter. Plasmids pRSV-Pit-1 and pRSV-Pit-1β were generously provided by Dr. M. Karin (University of California, San Diego) and contain the coding regions for Pit-1 and Pit-1β (6, 29). Plasmid DNAs were prepared by CsCl centrifugation or by Qiagen (Qiagen Inc., Chatsworth, CA) columns and quantified by absorbance at 260 nm or fluorometry and compared with DNA standards on agarose gels (29).

**Hemagglutinin (HA)-tagged Pit-1 Constructs**—The vectors pRSV-HA Pit-1 and pRSV-HA Pit-1β, which encode HA-tagged versions of Pit-1 and Pit-1β, and the vectors pRSV-HA Pit-1-BPV, pRSV-HA Pit-1-AU5, pRSV-HA Pit-1-FLAG, pRSV-HA Pit-1-INV, and pRSV-HA Pit-1-MYC, which encode HA-tagged Pit-1βs with different substitution mutations of the 26-amino acid β-domain, were constructed as follows. All constructs required polymerase chain reaction mutagenesis of the Pit-1 TAD, and in order to minimize the target sequence to be submitted to polymerase chain reaction amplification, a HindIII-PvuII fragment encompassing nucleotides 1−456 of Pit-1 or 1–534 of Pit-1β was subcloned into a derivatized pGem-7Z (Promega) plasmid DNA whose SacI site had been converted to a PvuII site (pGem7P). The resulting Pit-1 or Pit-1β isoform plasmid was subcloned into pCI neo (Promega) or pCI/HA neo (25) to generate the β-domain isofroms. The locations of the trans-activation domain and 26-aa spacing of the wild-type are present in another, thyrotroph-specific, isoform, Pit-1-T.

**TABLE I**

| Position | Rat | Human | Turkey | Salmon |
|----------|-----|-------|--------|--------|
| 1        | Val | *     | *      | *      |
| 2        | Pro | *     | *      | *      |
| 3        | Ser | +     | +      | +      |
| 4        | Ile | +     | Val    | Gly    |
| 5        | Leu | +     | *      | *      |
| 6        | Ser | +     | *      | *      |
| 7        | Leu | +     | *      | *      |
| 8        | Ile | +     | *      | Leu    |
| 9        | Gly | +     | *      | +      |
| 10       | Thr | +     | *      | Ser    |
| 11       | Pro | +     | *      | *      |
| 12       | Lys | +     | *      | *      |
| 13       | Cys | +     | *      | Arg    |
| 14       | Leu | +     | Ser    | Ser    |
| 15       | His | +     | *      | *      |
| 16       | Thr | +     | Leu    | Met    |
| 17       | Tyr | +     | His    | His    |
| 18       | Phe | +     | *      | Leu    |
| 19       | Ser | +     | Alα    | *      |
| 20       | Met | +     | Val    | Thr    |
| 21       | Thr | +     | Met    | Ser    |
| 22       | Thr | +     | *      | *      |
| 23       | Met | +     | Leu    | Ser    |
| 24       | Gly | +     | *      | Ser    |
| 25       | Asn | +     | *      | *      |
| 26       | Thr | +     | Val    | Gly    |

* Refers to aa position within the β-domain.

**TABLE II**

| Position | Rat | Human | Turkey | Salmon |
|----------|-----|-------|--------|--------|
| aa 1−26  | 100 | 100   | 92     | 67     |
| aa 13−26 | 70  | 50    | 36     |        |

* Refers to aa position within the β-domain.
integrity of the TAD regions were verified by Sanger sequencing using reagents and protocols obtained from a commercial kit (Sequenase; U.S. Biochemical Corp.) and commercially available T7 and SP6 promoter-specific primers (Promega, Madison, WI). Sequencing was done both in the laboratory and through the University of Colorado Health Sciences Center Cancer Center Core Facility. HA-tagged Pit-1 TAD sequences were excised from pGem-7P by digestion with HindIII and PvuII and ligated to the unique HindIII and PvuII sites of pRSV-Pit-1 to produce pRSV-HA Pit-1, pRSV-HA Pit-1β, pRSV-HA Pit-1-BPV, pRSV-HA Pit-1-AU5, pRSV-HA Pit-1-FLAG, pRSV-HA Pit-1-INV, and pRSV-HA Pit-1-MYC.

Transfections—DNA was introduced into HeLa or GH3 cells by electroporation as follows. Approximately 2–3 × 10^5 enzymatically dispersed cells were mixed with plasmid DNA in a sterile gene-pulse chamber and exposed to a controlled electrical field of 500 microfarads at 220 V, as described previously (32). Cells from individual transfections were then maintained in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 50 μg/ml penicillin/streptomycin at 37 °C. The nonspecific effects of the RSV promoter upon transcription factor availability was controlled for by including amounts of pRSV β-globin plasmid DNA in all assays to render the total pRSV DNA concentration constant.

Luciferase Assays—Transient transfections were performed in triplicate, in at least two separate experiments. After incubation for 24 h, cells were harvested with phosphate-buffered saline containing 3 mM EDTA, pelleted, and resuspended in 100 mM potassium phosphate buffer (pH 7.8), 1 mM dithiothreitol. Cells were lysed by three cycles of freeze-thawing and by vortexing for 1 min between thaws. Cell debris supernatant was assayed as described previously (25). Samples were measured in duplicate using a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, San Diego, CA). Total luciferase units were normalized to total protein present in extract supernatants. Protein content of each extract was measured in duplicate using a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, San Diego, CA). Total luciferase units were normalized to total protein present in extract supernatants. Total luciferase units were normalized to total protein present in extract supernatants. Total luciferase units were normalized to total protein present in extract supernatants.

Expression of Pit-1 Proteins—It has been previously shown that wild-type pRSV Pit-1 and pRSV Pit-1β express protein to different levels in transient transfection experiments and that the transcription potency of these two isoforms must be normalized to their levels of expression (11). The mutant Pit-1 proteins presented here might be expressed to levels different from either the wild-type Pit-1 or Pit-1β. In order to exclude the effect of differences in protein expression level on transcription potency, we carried out a series of transfection experiments to find levels of input DNA that would yield similar levels of protein expression from the wild-type and mutant Pit-1 vectors. In a preliminary experiment, 10 μg of each of the pRSV-HA Pit-1 constructs were introduced into HeLa nonpituitary cells by electroporation. Extracts from transfected cells were separated by SDS-PAGE, and Western blot analysis was used to determine the level of Pit-1 protein expression (data not shown). HA Pit-1β was expressed at lower levels than was HA Pit-1, HA Pit-1-FLAG was expressed at a level similar to HA Pit-1, and the other HA Pit-1 constructs were expressed at higher levels than was HA Pit-1. In order to find DNA doses that roughly equalized the levels of Pit-1 expression for each of the constructs, varying amounts of each of the pRSV-HA Pit-1 constructs were introduced into HeLa nonpituitary cells by electroporation in a series of experiments. Having determined the optimal amounts of plasmid DNA for each construct, as described above, we show in Fig. 2 that similar levels of Pit-1 protein expression can be achieved with these plasmid DNA doses. The plasmid amounts transfected were as follows: 10 μg of HA Pit-1, 30 μg of HA Pit-1β, 5 μg of HA Pit-1-BPV, 2 μg of HA Pit-1-AU5, 10 μg of HA Pit-1-FLAG, 5 μg of HA Pit-1-INV, and 5 μg of HA Pit-1-MYC. Equal amounts (100 μg) of total protein from cell lysates of duplicate transfections were analyzed by SDS-polyacrylamide gel electrophoresis, except that lanes 17 and 18 were loaded with the same extract as in lane 16, but with 50 and 200 μg of total protein (Fig. 2). This was done in order to show that we can detect a 2-fold decrease or increase in Pit-1 protein expression relative to that in lane 16. In the vector-only lanes (lanes 1 and 2), the anti-HA antibody does not detect any protein migrating in the Pit-1 range of 30–33 kDa but does detect a nonspecific band of ~50 kDa whose intensity appears to correlate with the amount of total protein loaded. Examination of the relative amounts of HA Pit-1 versus the other Pit-1 constructs reveals that HA Pit-1 (lanes 3 and 4) and HA Pit-1β (lanes 5 and 6) were expressed at

### Table III

| Construct   | Sequence of β-domain* | Origin of β-domain |
|-------------|-----------------------|--------------------|
| HA Pit-1γ   | VPSILSI7QTPC6KHTYF6M5MTGGN | Wild-type Pit-1γ sequence |
| HA Pit-1-BPV | DT6X2IR6PSAT6C4K56NPP16E6Y | Bovine papilloma virus L-1 capsid, aa 415–440 (including AU1 epitope) |
| HA Pit-1-AU5 | TK6X2IR6PSAT6C4K56NPP16E6Y | Bovine papilloma virus L-1 capsid, aa 268–293 (including AU5 epitope) |
| HA Pit-1-FLAG | LIK6X2PSAT6C4K56NPP16E6Y | Human erythrocyte glycopherin, aa 98–112 + FLAG epitope |
| HA Pit-1-INV | SEGW5X2QL6L6KQ65E6H5EP6AED | Sperm whale myoglobin, aa 5–30 + mouse α-involucrin epitope |
| HA Pit-1-MYC | EOKIL5X2EDV6LPSVE6I6NPE6SDQ | Human erythrocyte glycopherin, aa 116–131 + Human c-myc epitope |

* Single-letter aa codes are used, and epitopes are underlined.
**FIG. 2. Expression of Pit-1 proteins.** The various pRSV Pit-1 constructs were introduced into HeLa cells by electroporation. In order to achieve equal levels of protein expression for the various HA Pit-1 constructs, varying amounts of each pRSV Pit-1 DNA were introduced, with pRSV levels held constant by the addition of pRSV β-globin. Lanes were loaded as follows: protein molecular weight markers (lane M); no pRSV-HA Pit-1 (lanes 1 and 2); 10 µg of pRSV-HA Pit-1 (lanes 3 and 4); 30 µg of pRSV-HA Pit-1β (lanes 5 and 6); 5 µg of pRSV-HA Pit-1-BPV (lanes 7 and 8); 2 µg of pRSV-HA Pit-1-AU5 (lanes 9 and 10); 10 µg of pRSV-HA Pit-1-FLAG (lanes 11 and 12); 5 µg of pRSV-HA Pit-1-INV (lanes 13 and 14); 5 µg of pRSV-HA Pit-1-MYC (lanes 15–18). After 24 h, cells were harvested and analyzed by SDS-polyacrylamide gel electrophoresis. The blot was probed with a mouse monocolonal anti-HA epitope primary antibody (BABCO). The numbers at the left mark the position of protein molecular weight markers (Life Technologies, Inc.). All lanes were loaded with extract containing 100 µg of total protein, except for lanes 17 and 18, which were loaded with the same extract as lane 16, but with 50 and 200 µg of total protein (i.e. with 0.5 times and 2 times as much total protein loaded).

roughly equal levels and that the levels of all Pit-1 constructs were, with the exception of HA Pit-1-AU5 (lanes 9 and 10) and HA Pit-1-MYC (lanes 15 and 16), within 2-fold the level of HA Pit-1. HA Pit-1-AU5 was expressed at barely detectable levels at this amount of input DNA; however, this mutant Pit-1 protein was detectable at higher levels of input DNA, indicating that the protein can be expressed (data not shown). In contrast, HA Pit-1-MYC was expressed at a level more than 2-fold greater than was HA Pit-1 in one of the duplicates. The relative DNA doses required to generate similar Pit-1 and Pit-1β protein levels are consistent with previous findings and again show that Pit-1β displays some level of intrinsic instability (11). Additionally, our data indicate that alteration of the β-domain reverses this instability, since less input DNA is required for the mutants, thus mapping the source of the Pit-1β isoform instability to the sequence of the β-domain. The DNA doses noted above were used for all further experiments. Experiments carried out in HeLa and GH4 cells in parallel showed that the relative pattern of expression of wild-type and mutant Pit-1 proteins was the same in both cell lines and that there were no cell-specific influences on Pit-1β protein production (data not shown).

Differences in apparent mobility among the Pit-1 constructs were detected. Since sequencing had shown that all constructs contain the same number of nucleotides, and therefore encode the same number of amino acids, two explanations remained: post-translational modification of the substituted sequences or sequence-specific effects on gel mobility of the mutant β-domains. The latter effect has been observed in other systems (16).

**Mutant Pit-1βs Function as Transcription Factors**—This substitution mutagenesis experiment could have induced alterations in the three-dimensional structure of each mutant Pit-1β such that it could no longer activate transcription under any circumstances. Such a result would preclude the examination of the effects of changing the amino acid sequence of the β-domain on the specific aspects of transcriptional activation modulated by the β-domain. To address this problem, we utilized previous findings that in the nonpituitary HeLa, Ltk−, and Rat-6 cell lines, the transcription potency of the Pit-1β isoform, when normalized to its lower protein level, is similar to that of Pit-1 on the rPRL promoter (5, 11). We used the HeLa nonpituitary cell line and our previously optimized Pit-1 protein-expression system to test the transcription potency of each mutant construct.

The HA-tagged wild-type and mutant Pit-1s were introduced into HeLa nonpituitary cells with a rPRL promoter-driven luciferase reporter, and their ability to transactivate target promoter activity was measured. Fig. 3 depicts the results of a representative experiment. HA Pit-1β actually displayed a stronger effect on transcription of the target promoter compared with Pit-1 (29- versus 15-fold, respectively); this difference may be due to the slightly higher levels of Pit-1β expression with these amounts of input DNA (Fig. 2). All of the mutant Pit-1βs were able to transactivate the rPRL promoter in the 15–30-fold range, except for HA Pit-1-AU5. Again, the transcription effect generally correlated with the level of Pit-1 protein expressed, whereas that of Pit-1-INV and Pit-1-MYC would be somewhat lower. Thus, the mutant β-domains have no deleterious effect on target promoter transactivation in vitro, and their transcription potencies in this reconstitution assay generally correlate with their protein expression levels.

**β-Domain-specific Sequences Mediate Repression of Basal and Ras-stimulated rPRL Promoter Activity in Pituitary Cells**—While in HeLa nonpituitary cells both Pit-1β and Pit-1 are positively acting transcription factors, in GH4 and α-thyroid-stimulating hormone pituitary cells, Pit-1β, but not Pit-1, represses basal expression from the rPRL promoter (5, 11–13). The precise mechanism for this cell type-specific inhibitory effect of Pit-1β remains unclear. The only structural difference between these two isoforms is the β-domain; we therefore tested whether the amino acid sequence of the β-domain imparts the Pit-1β-mediated repression of rPRL promoter activity in pituitary cells. The Pit-1β mutant constructs were introduced into GH4 pituitary cells by electroporation in the presence of a rPRL-driven luciferase reporter (Fig. 4). As shown previously, HA Pit-1 has little effect on rPRL promoter activity in this system (13, 15), whereas HA Pit-1β acted as a dominant negative repressor of rPRL expression, decreasing reporter expression 3-fold from basal levels. Additionally, each of the five β-domain mutants lost the dominant negative effect attributed to the β-isofrom splice variant. These results demonstrate that the wild-type amino acid sequence of the β-domain is necessary for interference with basal rPRL expression in pituitary cells and that the altered spacing of the TAD generated by the β-domain is not sufficient to cause transcriptional repression.

Another attribute of the Pit-1β isoform is its ability to repress the oncogenic V-12 Ras signaling to the rPRL promoter, which normally requires a functional interaction between Pit-1
and Ets-1 (15). Pit-1β repression of the Ras response appears to occur by Pit-1β forming a nonproductive complex with Ets-1. Again, the β-domain could act either by disrupting the structure of the Pit-1 TAD or through properties inherent in its own sequence. In order to test whether repression of Ras signal transduction requires a wild-type β-domain, the mutant and wild-type Pit-1 constructs were introduced into GH4 pituitary cells by electroporation in the presence of the rPRL-driven luciferase reporter and pSV Ras (Fig. 5). As documented previously, co-transfection of a Pit-1 construct enhances the Ras response from 11-fold in its absence to 32-fold in its presence, and co-transfection of the Pit-1β isoform only failed to enhance the Ras response but actually reduced it to one-third the level achieved by Ras alone (Fig. 5). In contrast, each substitution mutation of the β-domain resulted in a switch of the Pit-1β phenotype, such that each no longer repressed the Ras response, but instead enhanced the Ras response of the rPRL promoter as effectively as did Pit-1, from 11-fold up to 24–42-fold (Fig. 5). These data demonstrate that the wild-type sequence of the β-domain is required for repression of the Ras response of the rPRL promoter and, as before, that the altered spacing of the TAD generated by the β-domain is insufficient to cause transcriptional repression.

β-Domain-specific Sequences Interfere with the Functional Interaction between Pit-1β and Ets-1 in Nonpituitary Cells—In addition to the role of Ets-1 in mediating the Ras response of the rPRL promoter, we have recently found that Ets-1 plays a critical role in determining basal rPRL promoter activity as well and that it does so by functionally and physically interacting with Pit-1. A HeLa nonpituitary cell reconstitution system was used to demonstrate that Pit-1 synergizes with Ets-1 to optimally reconstitute rPRL promoter activity, whereas the Pit-1β isoform synergizes poorly, if at all, with Ets-1. To investigate whether β-domain-specific sequences interfere with the ability of Pit-1β to synergize with Ets-1, we assessed the ability of the Pit-1 mutants to interact functionally with Ets-1 in the HeLa reconstitution system. The Pit-1 constructs were introduced into HeLa nonpituitary cells by electroporation in the presence of the rPRL promoter-driven luciferase reporter and Ets-1 (Fig. 5). As documented previously, co-transfection of a Pit-1 construct enhances the Ets-1 response from 14-fold in its absence to 1,261-fold in its presence. Alternatively, co-transfection of the Pit-1β isoform only enhanced the Ets-1 response from 14- to 103-fold (Fig. 6). Despite the fact that each substitution mutation of the β-domain contained a 26-aa insert, each mutant resulted in a striking switch in response such that each β-mutant was now able to functionally interact with Ets-1 in a manner indistinguishable from Pit-1, which is devoid of any insert (Fig. 6). These data once again demonstrate the importance of the wild-type β-domain sequence for the β-specific effect.

β-Domain-specific Sequences Confer upon Pit-1β an Enhanced Ability to Respond to PKA in Nonpituitary Cells—We have previously shown that Pit-1 serves to significantly en-
The observation, both here and previously, that Pit-1β acts in pituitary cells as a dominant negative effector (Fig. 4) (5, 11–13), yet acts in nonpituitary cells as a positive effector (Figs. 3 and 7) (5, 11), argues that a cell type-specific factor is required for the cell-specific, dominant negative effects of the Pit-1β isoform. To confirm that the differential effects of the two Pit-1 isoforms in GH4 pituitary cells occur at various DNA doses with respect to the rPRL promoter, increasing amounts of each Pit-1 expression vector were transfected separately into GH4 cells, and PRL-luciferase reporter activity was measured. Fig. 8A shows that, in agreement with previous results of a single-dose study (Fig. 4), increasing doses of HA Pit-1 had little effect on rPRL promoter activity, while increasing doses of HA Pit-1β repress basal rPRL activity in GH4 pituitary cells to levels one-third those in the absence of Pit-1β. Since Pit-1 is a pituitary-specific transcription factor that is required for PRL gene expression, it seemed a possible candidate target for Pit-1β, via the formation of a nonproductive Pit-1-Pit-1β heterodimer. To directly test this hypothesis, we examined the effect of forming Pit-1-Pit-1β heterodimers in HeLa nonpituitary cells, by introducing increasing DNA doses of Pit-1β together with a constant amount (10 μg) of Pit-1 expression vector. Fig. 8B shows that co-transfecting increasing DNA doses of Pit-1β resulted in a significant and Pit-1β dose-dependent enhancement of the Pit-1 effect, increasing the effect from 7-fold, in the absence of Pit-1β, to 1,337-fold, in the presence of the highest dose of Pit-1β (30 μg). Indeed, the effects of these two Pit-1 isoforms were more than additive (i.e. 10 μg of Pit-1 alone was 7-fold, and 30 μg of Pit-1β alone was 28-fold, but together the effect was 1,337-fold) and thus synergistic. Clearly, Pit-1 is not the cell type-specific target mediating the inhibitory effect of Pit-1β. Together, our data suggest...
that another cell type-specific factor mediates the repressor function of Pit-1β in GH4 cells and that it is the presence and precise amino acid sequence of the β-domain that imparts these selective functions to the Pit-1β isoform.

**DISCUSSION**

Because Pit-1 is expressed at levels that are 7–8-fold greater than Pit-1β, it has been assumed that Pit-1 is the dominant functional isoform with respect to pituitary-specific gene expression and cell function. However, the level of Pit-1 expression is very high (0.5% of total protein) (8, 17), and thus the actual amount of Pit-1β expression is itself quite high for a transcription factor (11). Moreover, Pit-1β is the only isoform found in salmon and turkey, and the amino acid sequence of the β-domain, particularly the first 12 amino acids, is highly conserved, from teleosts to primates (Tables I and II), suggesting that the Pit-1β isoform may preserve the ancestral gene structure. In this paper, we have specifically addressed the potential functions of the Pit-1β isoform compared with Pit-1 and addressed the contributions of the β-domain structure to its various functions. Here we show, controlling for equivalent levels of protein expression, that the Pit-1β domain functions as a sequence-specific molecular switch and that the amino acid sequence of the β-domain confers upon the Pit-1 β isoform unique transcriptional properties. The specific amino acid sequence of the β-domain is required to enhance the responsiveness of the Pit-1β isoform to PKA-mediated signaling and to block its ability to mediate basal and Ras-activated rPRL gene transcription.

The difference in Pit-1β protein expression relative to Pit-1 appears to be intrinsic to the Pit-1β coding sequences rather than a pituitary-specific regulation of splicing (5, 11, 12). Additionally, the Pit-1β and Pit-1 expression constructs utilized here are driven from identical Rous sarcoma viral promoters yet exhibit the relative overexpression of Pit-1 versus Pit-1β in HeLa nonpituitary cells (Fig. 2). These expression levels must be due to either differences in translational efficiency or in mRNA and/or protein stability that are independent of the pituitary cell type. The latter seems most likely, since these constructs lack any differences in 5'- or 3'-untranslated sequences that might regulate translational efficiency. Indeed, from these studies, it is clear that the β-domain is the intrinsic structure that governs Pit-1β protein expression level, since substitution mutagenesis of the β-domain usually increases the relative expression level of the Pit-1β isoform (Fig. 2) and because Pit-1 and Pit-1β only differ by the β-domain. To circumvent problems in interpretation of the data that might be due to differential protein expression levels, we carefully adjusted the plasmid DNA concentrations of the various β-domain mutants that were transfected to achieve equivalent levels of Pit-1 and Pit-1β protein production (Fig. 2). In so doing, we have directly demonstrated that Pit-1β is as efficient a transactivator of rPRL promoter activity as is Pit-1 (Fig. 3), a result previously suggested by mathematical normalization (11), and that the β-domain sequence can be altered without significantly diminishing its transcription potency for the rPRL promoter.

The development of any mechanistic model that explains the differential effects of Pit-1 and Pit-1β must take into account (i) the cell-specific behavior of the β-domain (Figs. 3–5); (ii) the optimal enhancement of the PKA response by Pit-1β (Fig. 7); and (iii) the marked transcription synergy of Pit-1 and Pit-1β (Fig. 8B). The model that we propose is that combinatorial interactions of Pit-1 isoforms with other transcription factors control rPRL promoter activity and that the precise combination of factors dictates ultimate effects. For example, we have previously shown that Pit-1 and Ets-1 interact functionally and physically to allow both basal and Ras-activated transcription from the rPRL promoter and that Pit-1β fails to interact functionally with Ets-1 in a reconstitution of basal rPRL promoter activity, although it retains its ability to interact physically with Ets-1 (15).6 Since Ets-1 is expressed in GH4 pituitary cells but not in HeLa cells,6 we utilized the HeLa cell system to show that Pit-1 interferes with the transcriptional potency of Ets-1 in a β-domain sequence-specific manner (Fig. 6). Moreover, mutation of the β-domain reverses the inhibitory effects of Pit-1β in GH4 pituitary cells (Figs. 4 and 5), which contain Ets-1, whereas the β-domain, or mutations thereof, do not decrease the transcriptional potency of Pit-1β in HeLa nonpituitary cells (Fig. 3), which lack Ets-1. These data indicate that a Pit-1/Ets-1 functional interaction is productive, while the Pit-1β/Ets-1 interaction is actually inhibitory.

In keeping with our hypothesis, we propose that the differential effects of the β-domain on the PKA response are due to a functional interaction of Pit-1β with an as yet unidentified
transcription factor (Factor X) that is a target of the PKA signaling pathway, and that the Pit-1β-Factor X functional interaction is more productive than the Pit-1/factor X functional interaction. Moreover, the Pit-1·Pit-1β combination appears to be more potent than either the Pit-1·Pit-1 or Pit-1β·Pit-1β combinations (Fig. 8B), further corroborating our hypothesis.

In summary, these data indicate that the β-domain encodes a transcription switch motif that selectively impairs the functional interaction with Ets-1 yet enhances the functional interaction with Pit-1 and with a component of the PKA pathway. The ability to generate an array of Pit-1 isoforms with altered transcriptional properties in a pituitary cell nucleus might allow different signaling intermediates to “select” specific Pit-1 isoforms with which to interact and thus to regulate pituitary hormone production. Such differential interactions might allow for an enhanced repertoire of signal integration for the highly regulated pituitary hormones.

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