The Pit-1β Domain Dictates Active Repression and Alteration of Histone Acetylation of the Proximal Prolactin Promoter*

Scott E. Diamond‡ and Arthur Gutierrez-Hartmann§¶

From the §Department of Medicine and Department of Biochemistry and Molecular Genetics, Program in Molecular Biology and Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received for publication, July 10, 2000
Published, JBC Papers in Press, July 31, 2000, DOI 10.1074/jbc.M006048200

A critical problem in current molecular biology is to gain a detailed understanding of the molecular mechanisms by which related transcription factor isoforms with identical DNA sequence specificity mediate distinct transcription responses. Pit-1 and Pit-1β constitute such a pair of transcription factor isoforms. Pit-1 enhances the Ras signaling pathway to the prolactin promoter, and Pit-1β represses basal prolactin promoter activity as well as Ras signaling to the prolactin promoter in pituitary cells. We have previously demonstrated that the β-domain amino acid sequence dictates the transcriptional properties of Pit-1β. Here, we show that five hydrophobic β-domain residues are required for Pit-1 isoform-specific repression of Ras signaling, and we demonstrate that sodium butyrate and trichostatin A, pharmacological inhibitors of histone deacetylation, as well as viral Ski protein, a dominant-negative inhibitor of recruitment of N-CoR/mSin3 histone deacetylase complexes, specifically reverse β isoform-specific repression of Ras signaling. Moreover, we directly demonstrate, with a chromatin immunoprecipitation assay, that the Pit-1β isoform alters the histone acetylation state of the proximal prolactin promoter. This differential analysis of Pit-1/Pit-1β isoform function provides significant insights into the structural determinants that govern how different transcription factors with identical DNA sequence specificity can display opposite effects on target gene activity.

Most transcription factors are members of extended families defined by conserved structural motifs, typically in the DNA-binding domain, yet differing in other domains, especially the transactivation domain (TAD). A number of transcription factors are expressed as a set of proteins derived from a single gene via alternative promoter usage or splicing events that result in virtually identical transcription factor isoforms that, nonetheless, can mediate distinct responses (e.g. PR-A versus PR-B; TRβ1 versus TRβ2; Oct 2.1 versus Oct 2.5; Ets-1 versus Ets-1β; and Pit-1 versus Pit-1β) (1–6). The molecular mechanisms, by which related transcription factor isoforms with identical DNA sequence specificity mediate distinct transcriptional responses, remain an area of active investigation.

Pit-1 is a pituitary-specific POU homeodomain transcription factor that governs both anterior pituitary cell identity and hormone gene expression (reviewed in Ref. 7). Pit-1 occurs in vertebrates, including humans, as two principal splice isoforms (Fig. 1). The β isoform arises from an alternative splice-acceptor sequence at the end of the first intron resulting in a 26-amino acid insertion, the β-domain, at position 48 in the TAD, between the first and second exons, TAD1 and TAD2 (Fig. 1) (5, 6). The β isoform differs from Pit-1 only in the TAD and displays identical DNA sequence specificity with respect to the prolactin (PRL) promoter (5) but has dramatically different transcriptional properties than Pit-1, and these differences are dictated by the unique amino acid sequence of the β-domain TAD insertion (8). The β-domain insertion causes Pit-1β to act as a pituitary-specific repressor of both basal transcription of the rat (r) PRL and of Ras signaling to the rPRL promoter gene (reviewed in Ref. 8). Additionally, the β-domain blocks functional interaction with Ets-1 (9) and functional interaction with the thyroid hormone and retinoic acid receptors (10) in nonpituitary cells. Nevertheless, this same 26-amino acid insertion endows Pit-1β with even greater potency with regard to mediation of protein kinase A signaling to the rPRL promoter (8, 11).

In this article, we focus on identifying key β-domain residues that are responsible for the β isoform-specific repression of Ras signaling to the rPRL promoter, as well as identifying a mechanism for this repression. We have utilized an epitope-scanning approach to replace sequentially 6 amino acid blocks of the β-domain in order to identify a limited subset of functionally important residues. Replacement of each of these residues with alanine identified five hydrophobic residues that are required for the β-domain to act as a transcriptional repressor of Ras signaling to the rPRL promoter. Moreover, we demonstrate that the β-domain does not simply disrupt TAD structure but functions as an active repression domain, which modifies the acetylation state of the proximal PRL promoter in a manner dependent upon an N-CoR/mSin3-containing histone deacetylase complex (HDAC). Thus, analysis of the Pit-1/Pit-1β isoform pair provides significant insight into the structural determinants of transcription activation versus repression mediated by two nearly identical transcription factor isoforms.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Monolayer cultures of HeLa human cervical carcinoma cells and GH3/T2 rat pituitary tumor cells (12) were maintained in

* This work was supported in part by National Institutes of Health Grant R01 DK37667 (to A. G.-H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a postdoctoral fellowship from the Lalor Foundation, a University of Colorado Cancer Center research seed grant, and National Institutes of Health Mentored Research Scientist Development Award K01 DK02752-01. Present address: Dept. of Physiology, University of Kentucky College of Medicine, MS-508 Albert B. Chandler Medical Center, 800 Rose St., Lexington, KY 40536-0298.

¶ To whom correspondence should be addressed: Depts. of Medicine and of Biochemistry and Molecular Genetics, Program in Molecular Biology, Colorado Cancer Center, University of Colorado Health Sciences Center, 4200 East Ninth Ave., Box B-151, Denver, CO 80262. Tel.: 303-315-8443; Fax: 303-315-4525; E-mail: a.gutierrez-hartmann@uchsc.edu.

1 The abbreviations used are: TAD, transactivation domain; PRL, prolactin; rPRL, rat PRL; HDAC, histone deacetylase complex; RSV, Rous sarcoma virus; HA, hemagglutinin; PCR, polymerase chain reaction; CMV, cytomegalovirus; EBS, Ets-binding sites; PAGE, polyacrylamide gel electrophoresis; ChIP, chromatin immunoprecipitation; ES, epitope-scanning; AS, alanine-scanning.
Dulbecco's modified Eagle's medium, 15% horse serum, 2.5% fetal bovine serum, and 50 μg/mL 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% confluence using 0.25% trypsin and 0.05% EDTA.

Plasmids—The rat PRL promoter luciferase expression vector, pβ, PRL luc, contains the firefly luciferase coding region under the control of a 498-bp fragment (−452 to +73) of the PRL promoter downstream of three polyadenylation tail sites in pβ, luc under control of the SV40 early promoter. Plasmids pRSV HA Pit-1 and pRSV HA Pit-1A-ES1, pRSV HA TAD-term, pRSV HA Pit-2A, pRSV HA Pit-1A-ES5, and pRSV HA Pit-1A-ES6, which encode HA-tagged Pit-1 and Pit-1 deleted for its TAD (amino acids 1–80) under the control of the CMV promoter, and the human TAD express N-terminally HA-tagged Pit-1 and Pit-1 deleted for its TAD (amino acids 1–80) under the control of the actin promoter (13), Plasmid pRSV5 v-Ski contains the avian Sloan-Kettering virus ski oncogene under the control of the RSV promoter (14) and was the generous gift of Dr. Edward Stavnezer (Case Western Reserve University, Cleveland, OH). Plasmid DNAs were prepared by Qiagen (Qiagen Inc., Chatsworth, CA) columns and purified by gel electrophoresis.

Mutant Pit-1 Constructs—The vectors pRSV HA Pit-1-ES1, pRSV HA Pit-1-ES2, pRSV HA Pit-1-ES4, pRSV HA Pit-1-ES5, and pRSV HA Pit-1-ES6, which encode HA-tagged Pit-1β5 with different epitope-scanning mutations of the 26-amino acid β-domain, as well as the vectors pRSV HA Pit-1-AS1, pRSV HA Pit-1-AS2, pRSV HA Pit-1-AS5, pRSV HA Pit-1-AS4, pRSV HA Pit-1-AS5, pRSV HA Pit-1-AS6, pRSV HA Pit-1-AS7, pRSV HA Pit-1-AS8, and pRSV HA Pit-1-AS9, which encode HA-tagged Pit-1β5 with different alanine-scanning mutations of the 26 amino acid β-domain, were constructed as follows.

All mutant β-domain constructs were constructed by nested PCR mutagenesis of the Pit-1 transactivation domain as described previously (8, 11). The pRSV HA Pit-1 plasmid was used as a substrate for PCR mutagenesis in which the 26-amino acid β-domain was substituted with six different epitope-scanning sequences (see Table I) or nine different alanine-scanning sequences (Table II), and an HA epitope tag was retained at the N terminus of all of the Pit-1 constructs. Common 5′- and 3′-deoxyoligonucleotides were utilized, as well as mutation-specific mutagenic deoxyoligonucleotides that encode the nucleotide substitutions in the β-domain. Amplified DNA was initially subcloned into pBluescript SK+ plasmid DNA in all assays.

Protein Assays—Luciferase Assays—Transient transfections were performed in triplicate, in at least three separate experiments. After incubation for 24 h, cells were harvested with phosphate-buffered saline containing 3 μg/ml EDTA, pelleted, and resuspended in 100 μM potassium phosphate buffer, pH 7.8, 1 mU dithiothreitol. Cells were lysed by three cycles of freeze-thawing and by vortexing between thaws. Cell debris was pelleted by centrifugation at 10,000 × g at 4 °C, and the supernatant was used for subsequent assays. Luciferase activity in the supernatant was measured in duplicate using a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, San Diego, CA). Relative light units for each transfection were calculated by normalizing for total protein. Protein assays were performed according to the method of Bradford (16) using commercially available reagents (Bio-Rad). Results are expressed as the fold activation of the pRRL promoter = S.E. for at least three experiments, each in triplicate.

Western Blot Analysis of HA-tagged Pit-1 Proteins—Transient transfections were performed as above. Cells were harvested with phosphate-buffered saline containing 3 μg/ml EDTA, pelleted, and resuspended in a triethanolamine/SDS solubilization buffer (50 mM triethanolamine, 115 mM NaCl, 2.2 mM EDTA, and 0.44% SDS) (117). Lysed extracts were passed through a 25-gauge needle seven times. The protein content of each extract was assayed according to the method of Lowry et al. (18), using commercially available reagents (Bio-Rad). Equal amounts (100 μg) of protein from each extract were separated on 15% SDS-polyacrylamide gels and transferred to Immobilon-P (polyvinylidene difluoride) membrane (Millipore, Bedford, MA). The proteins were detected with a monoclonal anti-HA primary antibody (Babco, Richmond, CA), secondary sheep anti-mouse HRP-conjugated antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and ECL media (Amersham Pharmacia Biotech). Dilutions of 1:1,000 of the primary anti-HA monoclonal antibody and 1:10,000 of the secondary sheep anti-mouse antibody preparation were used.
The β-Domain Does Not Simply Disrupt TAD Function to Repress Ras Signaling—We have previously demonstrated that the β-domain insertion converts Pit-1 from a co-activator to a repressor of Ras signaling to the rPRL promoter (9, 20). Moreover, we have shown that the amino acid sequence of the β-domain itself is of the same size and in the same position as the POU-domain insertion in its TAD. Methods were used to identify small regions of the TAD that are important for repression, and co-transfection of the Pit-1 TAD and POU-specific DNA-binding domain; FL represents the 15-amino acid flexible linker between the POU-specific and POU-homeodomain basic domains; α1–4 and α1–3 represent their α-helices; Hinge represents the region between the TAD and the bipartite DNA-binding domain; PB represents the 26-amino acid β-domain insertion in its TAD. Bottom, the location of the ΔTAD mutation is shown, a deletion of amino acids 2–80.

**RESULTS**

**Expression of Epitope-scanned Pit-1β Proteins**—It has been previously shown that wild-type Pit-1 and Pit-1β constructs express protein at different levels in transient transfection experiments (6, 8). 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 pSV HA Pit-1 constructs were introduced into HeLa nonpituitary cells and GH4 pituitary cells by electroporation. Extracts from transfected cells were separated by agarose gel electrophoresis, and bands were imaged and quantified on an Alpha Imager 2000 Gel Documentation System (Alpha Innotech, Lake Placid, NY), as modified by Lambert and Nordeen (19). Transient transfections were performed as above. Twenty four hours after transfection, 2 × 10⁶ GH4 cells were cross-linked by addition of formaldehyde into the medium at a final concentration of 1% and incubated for 15 min at room temperature. Cells were washed with ice-cold phosphate-buffered saline and resuspended in 500 μl of ChIP Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCL, pH 8.0, with protease inhibitors). The lysates were sonicated utilizing a Branson Sonifier 450 at power setting 2 with three 10-s pulses at duty cycle 90 and diluted to 3 ml with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl). 1 ml of each sample was precleared by incubating with 80 μl protein A-agarose beads for 30 min at 4°C with rotation. 5 μl of anti-acetyl histone H4 antibody (Upstate Biotechnology, Lake Placid, NY) was added, and immunoprecipitation was done overnight at 4°C with rotation. Immune complexes were collected with 60 μl of protein A-agarose and washed once with 1 ml each of the following buffers in sequence: Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 1500 mM NaCl), LiCl Wash Buffer (250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0); and twice with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Immune complexes were eluted, and cross-links were reversed by heating at 65°C and subjected to proteinase K treatment. DNA was recovered by phenol/ chloroform extraction followed by ethanol precipitation and was used as a template for PCR (25 cycles) using pSH245R primer and TAD promoter-specific commercially synthesized deoxoygulonucleotides (Life Technologies, Inc.) that contain a PRL promoter-specific sequence, GCGTCTTCCTATCTTTTGTC, and a luciferase-specific sequence, GACTCAAGATGTCAGTCAGC. In addition, internal control PCRs were performed with pSV Ras plasmid-specific commercially synthesized deoxoygulonucleotides (Life Technologies, Inc.) that contain an SV40 promoter-specific sequence, GCATCTCAATTGGC, and a luciferase-specific sequence, GACTCAAGATGTCAGTCAGC. Control reactions were performed to ensure that all PCR assays took place in the linear range of response to input DNA. PCR products were separated by agarose gel electrophoresis, and bands were imaged and quantified on an Alpha Imager 2000 Gel Documentation System (Alpha Innotech, San Leandro, CA).

**Chromatin Immunoprecipitation Studies**—Chromatin immunoprecipitation (ChIP) assays were performed according to the protocol for the acetyl-histone H4 ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY), as modified by Lambert and Nordeen (19). Transient transfections were performed as above. Twenty four hours after transfection, 2 × 10⁶ GH4 cells were cross-linked by addition of formaldehyde into the medium at a final concentration of 1% and incubated for 15 min at room temperature. Cells were washed with ice-cold phosphate-buffered saline and resuspended in 500 μl of ChIP Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCL, pH 8.0, with protease inhibitors). The lysates were sonicated utilizing a Branson Sonifier 450 at power setting 2 with three 10-s pulses at duty cycle 90 and diluted to 3 ml with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl). 1 ml of each sample was precleared by incubating with 80 μl protein A-agarose beads for 30 min at 4°C with rotation. 5 μl of anti-acetyl histone H4 antibody (Upstate Biotechnology, Lake Placid, NY) was added, and immunoprecipitation was done overnight at 4°C with rotation. Immune complexes were collected with 60 μl of protein A-agarose and washed once with 1 ml each of the following buffers in sequence: Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 1500 mM NaCl), LiCl Wash Buffer (250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0); and twice with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Immune complexes were eluted, and cross-links were reversed by heating at 65°C and subjected to proteinase K treatment. DNA was recovered by phenol/ chloroform extraction followed by ethanol precipitation and was used as a template for PCR (25 cycles) using pSH245R primer and TAD promoter-specific commercially synthesized deoxoygulonucleotides (Life Technologies, Inc.) that contain a PRL promoter-specific sequence, GCGTCTTCCTATCTTTTGTC, and a luciferase-specific sequence, GACTCAAGATGTCAGTCAGC. In addition, internal control PCRs were performed with pSV Ras plasmid-specific commercially synthesized deoxoygulonucleotides (Life Technologies, Inc.) that contain an SV40 promoter-specific sequence, GCATCTCAATTGGC, and a luciferase-specific sequence, GACTCAAGATGTCAGTCAGC. Control reactions were performed to ensure that all PCR assays took place in the linear range of response to input DNA. PCR products were separated by agarose gel electrophoresis, and bands were imaged and quantified on an Alpha Imager 2000 Gel Documentation System (Alpha Innotech, San Leandro, CA).

**Expression of Epitope-scanned Pit-1β Proteins**—It has been previously shown that wild-type Pit-1 and Pit-1β constructs express protein at different levels in transient transfection experiments (6, 8). 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 pSV HA Pit-1 constructs were introduced into HeLa nonpituitary cells and GH4 pituitary cells by electroporation. Extracts from transfected cells were separated by SDS-PAGE, and Western blot analysis was used to determine the level of Pit-1β protein expression (data not shown). Pit-1β was expressed at lower levels than Pit-1 as observed previously (8); ES1, ES2, and ES5 were expressed at levels similar to but slightly higher than Pit-1β; ES6 was expressed at levels similar to but slightly higher than Pit-1β; ES5 was expressed at levels similar to but slightly higher than Pit-1β; ES6 was
expressed at levels similar to but slightly lower than Pit-1β; whereas ES3 and ES4 were expressed at higher levels than Pit-1β. Relative expression levels of Pit-1β proteins did not differ between cell lines (data not shown). Guided by these results, varying amounts of each of the mutant constructs were then introduced into GH4 pituitary cells by electroporation, and DNA doses that result in similar levels of protein expression were identified as follows: 10 μg of Pit-1β, 25 μg of Pit-1β, 20 μg of ES1, 20 μg of ES2, 10 μg of ES3 and ES4, 20 μg of ES5, and 30 μg of ES6 (Fig. 3A). In the vector-only lane, protein migrating in the Pit-1 range of 30–33 kDa was not detected. Examination of the relative amounts of wild-type versus mutant Pit-1β reveals that the levels of all mutant constructs (lanes 4–9) were roughly equivalent to that of wild-type Pit-1β (lane 3). ES6 (lane 9), however, was expressed at a somewhat lower level than the other constructs. The DNA doses noted above were used for subsequent experiments.

Epitope-scanning Pit-1β Proteins Retain Variable Transcription Function—Epitope-scanning mutagenesis could have induced alterations in the three-dimensional structure of the mutant Pit-1βs such that they could no longer activate transcription under any circumstances, and such a result would preclude the identification of functionally important β-domain residues. To address this problem, we utilized an isoform-insensitive HeLa transcription reconstitution system, in which the Pit-1β isoform retains basal transcription potency on the rPRL promoter (8).

The wild-type and mutant Pit-1β/Pit-1β constructs were introduced into HeLa nonpituitary cells with an rPRL promoter-driven luciferase reporter, and their ability to transactivate target promoter activity was measured. Pit-1β displayed a stronger effect on transcription of the target promoter compared with Pit-1 (210- versus 102-fold, respectively) (Fig. 3B). All but one (ES2) of the epitope-scanning mutants resulted in diminished basal activity, compared with that of wild-type Pit-1β (Fig. 3B), yet were expressed at levels comparable to the wild-type Pit-1β (Fig. 3A). In particular, the almost 90-fold difference in transcription activation potency between ES2 (347-fold) and ES3 (5-fold) cannot be explained by any difference in protein expression and is likely due to a difference in intrinsic activity of the proteins. These data identify β-domain amino acids altered by the ES3 mutation but not by the ES2 mutation, PKCL, as contributing to the basal transcription potency of Pit-1β. Nevertheless, the key point is that the epitope-scanning β-domain mutants are all capable of transactivating the rPRL promoter, albeit to a varying degree.

Two Regions of the β-Domain Are Required for Repression of Ras-stimulated rPRL Promoter Activity in Pituitary Cells—In HeLa nonpituitary cells, both Pit-1β and Pit-1 activate transcription. However, in GH4 pituitary cells, the two isoforms have opposite transcriptional effects with regard to Ras signaling to the rPRL promoter; Pit-1 enhances and Pit-1β represses the oncogenic RasVal12 response (9, 22). Moreover, we have previously demonstrated that the amino acid sequence of the β-domain dictates this repression (8) (Fig. 2). In order to identify small regions of the β-domain that are functionally important in repressing the Ras response, the Pit-1β mutant epitope-scanning constructs were introduced into GH4 pituitary cells by electroporation in the presence of a rPRL-driven luciferase reporter with and without pSV Ras (Fig. 4).

As documented previously, co-transfection of a Pit-1 construct enhanced the Ras response from 3-fold in its absence to 9-fold in its presence, whereas co-transfection of the Pit-1β isoform not only failed to enhance the Ras response but reduced it to 50% below that achieved by Ras alone. This inhibitory effect of Pit-1β on Ras signaling to the rPRL promoter in pituitary cells defines the stereotypical repressor phenotype of Pit-1β in this assay (8). Therefore, we tested the Pit-1β epitope-scanning mutants for their ability to reproduce the repressor

![Image](image)
phenotype. Three of the epitope-scanning mutations, ES1, ES3 and ES6, repressed the Ras response to the same extent as wild-type Pit-1β (Fig. 4), despite displaying variable basal transcriptional potency in the isoform-insensitive HeLa cells. All of the alanine-scanned Pit-1 proteins retained protein expression; the levels of all proteins did not differ between cell lines (data not shown). These DNA doses were used for all further experiments.

Expression of Alanine-scanning Pit-1β Proteins—Ten μg of RSV HA Pit-1, 25 μg of RSV HA Pit-1β, and 25 μg each of the mutant constructs were introduced by electroporation into HeLa nonpituitary cells (data not shown) and GH4 cells (Fig. 5A) pituitary cells. Extracts from transfected cells were separated by SDS-PAGE, and Western blot analysis was used to determine the level of Pit-1β protein expression; the levels of all mutant constructs were roughly equivalent to that of wild-type Pit-1β. AS3, AS5, and AS6 were expressed at a somewhat higher level than the other constructs, with AS3 displaying a second band of slightly slower mobility. Relative expression levels of Pit-1β proteins did not differ between cell lines (data not shown). These DNA doses were used for all further experiments.

Alanine-scanning Pit-1β Proteins Retain Transcription Function—All of the alanine-scanned Pit-1β proteins retained basal transcriptional potency in the isoform-insensitive HeLa

### Table II

| Construct | Amino acid sequence of β-domain |
|-----------|--------------------------------|
| Pit-1β    | VPSILSLIQTPLKCLHTYFSMTTMGNT    |
| AS1       | VPSILSLAQTPLKCLHTYFSMTTMGNT    |
| AS2       | VPSILSLAQTPLKCLHTYFSMTTMGNT    |
| AS3       | VPSILSLIQTPLKCLHTYFSMTTMGNT    |
| AS4       | VPSILSLIQTPLKCLHTYFSMTTMGNT    |
| AS5       | VPSILSLIQTPLKCLHTYFSMTTMGNT    |
| AS6       | VPSILSLIQTPLKCLHTYFSMTTMGNT    |
| AS7       | VPSILSLIQTPLKCLHTYFSMTTMGNT    |
| AS8       | VPSILSLIQTPLKCLHTYFSMTTMGNT    |
| AS9       | VPSILSLIQTPLKCLHTYFSMTTMGNT    |

**A**  

**B**  

**Fig. 3.** The epitope-scanning Pit-1β proteins retain variable transcription function. A, the various pRSV HA Pit-1 constructs were introduced into GH4 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. After 24 h cells were harvested and analyzed by SDS-PAGE and Western blot (see “Experimental Procedures”). Lanes were loaded as follows: No pRSV HA Pit-1 (lane 1); 10 μg of pRSV HA Pit-1 (lane 2); 25 μg of pRSV HA Pit-1β (lane 3); 20 μg of pRSV HA Pit-1-ES1 (lane 4); 20 μg of pRSV HA Pit-1-ES2 (lane 5); 10 μg of pRSV HA Pit-1-ES3 (lane 6); 10 μg of pRSV HA Pit-1-ES4 (lane 7); 20 μg of pRSV HA Pit-1-ES5 (lane 8); and 30 μg of pRSV HA Pit-1-ES6 (lane 9). B, mutant and wild-type pRSV Pit-1 constructs were introduced into HeLa nonpituitary cells by electroporation with 3 μg of pA3 PRL luc-425. No IV HA Pit-1 plasmid DNA amounts were adjusted for equal protein expression. Total pRSV plasmid amount was maintained constant with pRSV β-globin DNA. After 24 h, cells were harvested and total light units measured (see “Experimental Procedures”).

**Fig. 4.** Two small regions of the β-domain mediate repression of Ras-stimulated rPRL promoter activity in pituitary cells. Mutant and wild-type pRSV HA Pit-1 constructs were introduced into GH4 pituitary cells by electroporation with 3 μg of pA3 PRL luc-425 and 5 μg of pSV Ras. Plasmid pRSV HA Pit-1 plasmid DNA amounts were adjusted for equal protein expression (see Fig. 3). Total pRSV plasmid amount was maintained constant with pRSV β-globin DNA. After 24 h, cells were harvested and total light units measured (see “Experimental Procedures”).
reconstitution assay (Fig. 5B). In contrast to the epitope-scanning (ES) proteins, for which transcriptional potency was variable (Fig. 3B), the alanine-scanning (AS) proteins displayed basal activity comparable to wild-type Pit-1β (140-fold) (Fig. 5B). The higher transcription potency of AS6 (478-fold) or the lower transcription activity of AS4 (67-fold) cannot be explained by extreme levels of protein expression, since AS6 and AS4 are expressed at levels equivalent to the other AS mutants and wild-type Pit-1β (Fig. 5A). In addition, AS3, despite the novel slower-migrating band, activated the rPRL promoter to the same extent as did wild-type Pit-1β. Again, the key point is that the alanine-scanning β-domain mutants are all capable of transactivating the rPRL promoter in an isoform-insensitive assay.

**Five Hydrophobic β-Domain Residues Mediate Repression of the rPRL Promoter Ras Response**—In order to identify specific β-domain residues that are required for repression of Ras signaling, the alanine-scanning constructs were assessed for retention of the Pit-1β repressor phenotype in GH4 pituitary cells. Again, Pit-1 enhanced the Ras response from 6-fold without Pit-1 to 13-fold with Pit-1, whereas the Pit-1β isoform inhibited the Ras response by 50% (Fig. 6A). Four of the alanine-scanning proteins, AS3, AS4, AS7, and AS9, acted like wild-type Pit-1β and repressed the Ras response (Fig. 6A). Despite the aberrantly sized bands seen with AS6 (Fig. 5A), it had no effect on repression, and AS3 repressed the Ras response at least as well as Pit-1β (Fig. 6A). Five mutations, AS1, AS2, AS5, AS6, and AS8, abrogated β-domain mediated repression of the Ras response. Two of these mutations, AS5 and AS8, eliminated the β-domain-mediated repression and restored the 6-fold Ras response but did not allow further enhancement of the Ras effect (Fig. 6A). In contrast, three mutations, AS1, AS2, and AS6, switched Pit-1β into an enhancer of Ras signaling, such that they functioned essentially the same as Pit-1 (−13-fold) (Fig. 6A). These data identify five β-domain amino acids (leucine 7, isoleucine 8, tyrosine 17, phenylalanine 18, and methionine 20) (Fig. 6B) that are required for repression of the Ras response of the rPRL promoter.

**Pharmacological Inhibitors of Histone Deacetylation Reverse the β-Domain-dependent Repressor Phenotype**—Two distinct models could explain β-dependent repression of Ras signaling to the rPRL promoter. In one model, the Pit-1β isoform acts as a classic dominant-negative inhibitor of Ras signaling, by binding to the Pit-1 site (FP IV) of the rPRL promoter composite Pit-1/Ets-1 Ras response element required for the Ras effect (9, 22). In the second model, the β-domain imparts the repressor phenotype by modulating functional interaction with, or the function of, an HDAC, and thus altering the acetylation state of target promoters.

To distinguish between these two models for β-domain-dependent repression, we first tested the role of HDACs using sodium butyrate and trichostatin A. Both sodium butyrate and trichostatin A have been widely used as pharmacological inhibitors of histone deacetylase activity (25, 26) and are being considered for clinical use in managing certain cancers (reviewed in Ref. 27). We used EtsZ, a recombinant dominant-negative inhibitor, as a control. EtsZ consists of an Ets DNA-binding domain fused to LacZ and prevents transactivation of target promoters by competitively binding to Ets-binding sites (EBS) on DNA (13). Specifically, EtsZ blocks the Ras response of the rPRL promoter by interfering with endogenous Ets-1 binding to the rPRL promoter composite Pit-1/Ets-1 Ras response element (20, 28). EtsZ was therefore expected to be insensitive to treatment with histone deacetylase inhibitors.

In the absence of histone deacetylase inhibitors, Ras activated rPRL promoter activity by 4-fold, whereas Pit-1β and...
Moreover, similar effects were seen with 50 and 200 ng/ml repression by EtsZ, the competitive inhibitor of EBS binding-mediated repression of the Ras response yet had no effect on 24 h, cells were harvested and total light units measured (see “Experimental Procedures”). B, the five hydrophobic residues leucine 7, isoleucine 8, tyrosine 17, phenylalanine 18, and methionine 20, which are required for β-domain-dependent repression of Ras signaling.

EtsZ repressed the Ras response by 50 and 66%, respectively (Fig. 7). However, in the presence of 5 mM sodium butyrate, Pit-1β enhanced the 5-fold activation by Ras to 16-fold, whereas EtsZ-mediated repression was unchanged. Addition of 100 ng/ml trichostatin A also switched Pit-1β into an enhancer of Ras signaling. Trichostatin A and Pit-1β increased the 7-fold activation by Ras alone to 28-fold but had no effect on EtsZ, which continued to strongly repress the Ras response. Thus, both sodium butyrate and trichostatin A reversed β-domain-mediated repression of the Ras response yet had no effect on repression by EtsZ, the competitive inhibitor of EBS binding. Moreover, similar effects were seen with 50 and 200 ng/ml trichostatin A (data not shown). These results implicate histone deacetylation in the mechanism of β-domain-dependent repression of Ras signaling and demonstrate that the mechanisms by which the β-domain and the simple dominant-negative inhibitor, EtsZ, repress Ras signaling are distinct.

v-Ski Protein Reverses β-Domain-dependent Repression—Pit-1 has recently been shown to interact with both a co-repressor complex that contains an N-CoR/mSin3 HDAC and a signal-dependent co-activator complex (29). The Sloan-Kettering virus Ski oncogene, v-ski (30), is a dominant-negative viral form of mammalian c-Ski protein and a specific inhibitor of transcriptional repression mediated by N-CoR/mSin3-containing HDACs. We examined the effect of v-Ski on Pit-1β-mediated repression of the Ras response, in order to determine whether the Pit-1β repressor phenotype is mediated by an N-CoR/mSin3-containing HDAC.

In the absence of v-Ski, Ras activated rPRL promoter activity by 3-fold, whereas Pit-1β and EtsZ reduced it (Fig. 8). However, co-transfection of pRSV t3 v-Ski (5 μg) switched Pit-1β from a repressor to an enhancer of the Ras response, increasing the 4-fold activation by Ras to 9-fold. EtsZ-mediated repression was unchanged. In addition, 2- and 10-μg doses of pRSV t3 v-Ski reversed the Pit-1β repressor phenotype (data not shown) without affecting the EtsZ-mediated inhibition. Thus, v-Ski, like the pharmacological histone deacetylation inhibitors, sodium butyrate and trichostatin A, blocks the repressive effect of the β-domain, yet has no effect on repression by EtsZ.

\( \text{v-Ski}\) Alters the Acetylation State of the Proximal PRL Promoter—In order to test directly the hypothesis that the Pit-1 β-domain represses PRL promoter expression by altering the level of histone deacetylation of the proximal PRL promoter, we performed a ChIP assay. The ChIP assay has been used by a number of laboratories to probe the effects of hormones, DNA-binding factors, and transcription cofactors on the acetylation state of chromatin (reviewed in Ref. 19). In the assay, total cellular extracts are reversibly cross-linked, and DNA bound to acetylated histone proteins is immunoprecipitated with antibodies directed against acetylated histones. The cross-links are reversed and DNA-precipitated, and DNA sequences of interest are amplified by PCR. Changes in the amounts of specific PCR products reflect changes in the amount of acetylated histone bound to the DNA sequence.

We examined the effect of Pit-1 and Pit-1β on the acetylation state of the proximal PRL promoter in two independent ChIP assays with internal control experiments to verify that the biological effect in question, repression of Ras activation of PRL promoter activity, had occurred (see “Experimental Procedures”). In addition we utilized the SV40 promoter of the pSV Ras plasmid as a control promoter to demonstrate that Pit-1β-dependent changes in histone deacetylation were specific for the proximal PRL promoter and not global changes in histone deacetylation.

In two independent ChIP assays, Pit-1 increased the amount of proximal PRL promoter associated with acetylated histone H4 by 2–3-fold, whereas Pit-1β decreased the amount of proximal PRL promoter associated with acetylated histone H4 by 60% (Fig. 9). Neither Pit-1 nor Pit-1β had an appreciable effect on the amount of SV40 internal control promoter associated with acetylated histone H4. In parallel control experiments to verify Pit-1β repression of Ras signaling, Pit-1 enhanced the Ras response from 2- to 4-fold, whereas the Pit-1β isoform inhibited the Ras response by 80% (data not shown). Thus, the presence of the β-domain endows Pit-1β with the ability to increase histone deacetylation in a target promoter-dependent manner.

**DISCUSSION**

Although a number of functionally distinct transcription factor isoforms with identical DNA specificities have been identified (1–6, 31), the molecular mechanisms by which structural alterations outside of DNA- or ligand-binding domains specify altered transcriptional function remains a relatively unstudied subject. The work presented here shows that the naturally occurring β-domain insertion converts the Pit-1β isoform into a transcriptional repressor by modulating functional interaction with histone deacetylases.

Only two other transcription factor splice isoforms, both nuclear receptors, are known to alter distinct interactions with
co-activators or co-repressors. Alternate exon usage in the TRβ2 isoform of TRβ introduces a novel N terminus that reverses a functional interaction with N-CoR, thus altering the mechanism of ligand-independent repression (32). The HNF4α2 splice isoform of nuclear receptor hepatocyte nuclear factor 4α (HNF4α) contains a 10-amino acid insertion in the inhibitory F-domain, which abrogates an F-domain block of interaction with the GRIP1 co-activator (33). Thus, the Pit-1/Pit-1β isoform pair represents the first example of differential interaction with either co-repressors or co-activators by transcription factor splice isoforms outside of the nuclear receptor family.

Transcriptional repressors can act through either passive or active mechanisms (reviewed in Refs. 34 and 35). Passive transcriptional repressors act by blocking such functions as nuclear localization or DNA binding that are necessary for transcriptional activation but that are not directly involved in nuclear signal transduction. Such repressors as Drosophila knirps protein (36), mammalian ATF-2 (37), and Drosophila I-POU protein (38) can function as passive repressors. EtsZ, the recombinant dominant-negative construct used in this article, also acts as a passive binding site competitor in the context of the rPRL FPIV composite Ras response element (28). Active repressors interfere with the molecular mechanism of signal transduction in an activator-specific manner by blocking activation functions of specific transcription factors, recruiting co-repressors, or by sequestering co-activators. Examples of active
repressors include YY1 repression of CREB-mediated transcription (39), KRAB-KAP-1 repression (40), the t(8;21) AML-1/ETO fusion protein (41), Ume 6 (42), TR (43), PLZF protein (44), and Lax3/BCL6 oncoprotein (45). The data presented here (Figs. 7–9) clearly demonstrate that the β-domain endows the Pit-1β isoform with the properties of an active repressor. Pit-1β blocks Ras signaling through a mechanism quite distinct from that of EtsZ, the artificially constructed passive binding site competitor. Moreover, by four separate methodological approaches (site-specific mutagenesis, pharmacological inhibitors, co-expression of a dominant-negative effector, and chromatin immunoprecipitation assay), we show that five hydrophobic residues present as a bipartite element within the β-domain and that the β-domain mediates repression of the Ras response by modulating the acetylation state of the proximal PRL promoter in a manner dependent upon an N-CoR/mSin3-HDAC with Pit-1. The ability of two pharmacological inhibitors of histone deacetylase activity, trichostatin A and sodium butyrate, to reverse β-domain repression of Ras signaling to the rPRL promoter is consistent with such a mechanism for β-domain repression. In fact, sodium butyrate and trichostatin A appear to phenocopy some of the epitope- and alanine-scanning mutations of the β-domain and convert Pit-1β from a repressor to an enhancer of Ras signaling. By contrast, neither of these inhibitors affected the dominant-negative EtsZ inhibitory response (Fig. 7). This specificity of the pharmacological inhibitors for the β-domain-dependent repressor phenotype supports the validity of this approach and our interpretation of the data.

The use of v-Ski as a molecular tool allowed us to address the following two concerns raised by the pharmacological inhibitor experiments used here: 1) the possibility that these inhibitors had nonspecific effects, and 2) that the pharmacological approach cannot distinguish between different HDAC subtypes. The c-Ski protein is a normal component of an HDAC subtype, and the pharmacological approach cannot distinguish between different HDAC subtypes. The β-domain yet retains basal transcription function in the HeLa reconstitution assay (Figs. 3A and 5A), and complete substitution mutations of the β-domain also retain basal transcription activity (11). The ES and AS mutations have differential effects on the basal transcriptional potency of Pit-1β (Figs. 3A and 5A), despite the similarity of their overall levels of protein expression (Figs. 3A and 5A). It is possible that the ES mutations that resulted in decreased basal transcriptional activity of Pit-1β may function by modulating the ability of either a single component of the Pit-1-Ets1 complex or a novel interaction surface generated by the complex to interact with co-repressor(s).

The role of the β-domain in basal transcriptional activity of Pit-1β is unclear at this time. For example, Pit-1 lacks the β-domain yet retains basal transcription function in the HeLa reconstitution assay (Figs. 3A and 5A), and complete substitution mutations of the β-domain also retain basal transcription activity (11). The ES and AS mutations have differential effects on the basal transcriptional potency of Pit-1β (Figs. 3A and 5A), despite the similarity of their overall levels of protein expression (Figs. 3A and 5A). It is possible that the ES mutations that resulted in decreased basal transcriptional activity of Pit-1β may function by modulating the ability of either a single component of the Pit-1-Ets1 complex or a novel interaction surface generated by the complex to interact with co-repressor(s).

The data presented in this report advance our understanding of the repressor function of Pit-1β, showing that the β-domain represses Ras signaling by modulating the acetylation state of the proximal prolactin promoter in a manner dependent upon the function of an N-CoR/mSin3-containing HDAC. Taken together, our results suggest that the β-domain may modulate changes in either or both of the Pit-1 or Ets-1 three-dimensional structures that alter the balance of POU-domain interactions with co-activators (CPB) and co-repressors (N-CoR/mSin3-containing HDACs), in a manner reminiscent of the HNF4α2 isoform (33). However, an interaction with the N-CoR/mSin3 complex containing HDAC, through a signal-specific cofactor, cannot be ruled out.

Acknowledgments—We thank Connie Hoon-Mastalir, Kelley Brodsky, Kristin Eckel, and Duane Mata for technical assistance, and members of the Gutierrez-Hartmann laboratory for their helpful suggestions and comments. We thank Drs. David Gordon and Edward Stavnezer for providing crucial reagents; Drs. Steven Nordeen and James Lambert for providing advice and assistance with the ChIP assays; and Drs. Dawn Duval, David Gordon, and William Wood and Dana Manning and Connie Hoon-Mastalir for critical reading of this manuscript. Tissue
β-Domain Modulates Interaction with Histone Deacetylase

culture media and DNA sequencing were provided by the Tissue Culture Core and DNA Sequencing Core Facilities of the Colorado Comprehensive Cancer Center (supported by National Institutes of Health Grant R30 CA46934).

REFERENCES
1. Kastner, P., Krust, A., Turoeite, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990) EMBO J. 9, 1603–1614
2. Hodin, R. A., Lazar, M. A., Winstead, B. I., Darling, D. S., Koenig, R. J., Larsen, P. R., Moore, D. D., and Chin, W. W. (1989) Science 244, 76–79
3. Wirth, T., Pries, A., Anwender, A., Zwilling, S., and Oeler, B. (1991) Nucleic Acids Res. 19, 43–51
4. Koizumi, S., Fisher, R. J., Fujiwara, S., Jorcyk, C., Bhat, N. K., Seth, A., and Koizumi, M. (1992) Oncogene 7, 675–681
5. Theill, L. E., Hattori, K., Domenico, D., Castrillo, J. L., and Karin, M. (1992) EMBO J. 11, 2291–2299
6. Konzak, K. E., and Moore, D. D. (1992) Mol. Endocrinol. 6, 241–247
7. Pickett, C. A., and Gutierrez-Hartmann, A. (1997) in Diseases of the Pituitary: Diagnosis and Treatment (Wierman, M. E., ed) Vol. 3, pp. 1–31, Humana Press Inc., Totowa, NJ
8. Diamond, S. E., and Gutierrez-Hartmann, A. (1996) J. Biol. Chem. 271, 28925–28932
9. Bradford, A. P., Conrad, K. E., Wasylko, C., Wasylko, B., and Gutierrez-Hartmann, A. (1995) Mol. Cell. Biol. 15, 2849–2857
10. Sanchez-Pacheco, A., Pena, P., Palomino, T., Guell, A., Castrillo, J. L., and Aranda, A. (1996) FEBS Lett. 422, 153–157
11. Diamond, S. E., Chiono, M., and Gutierrez-Hartmann, A. (1999) Mol. Endocrinol. 13, 228–238
12. Conrad, K. E., and Gutierrez-Hartmann, A. (1999) Oncogene 7, 1279–1286
13. Langer, S. J., Bortner, D. M., Roussel, M. F., Sherr, C. J., and Ostrowski, M. C. (1992) Mol. Cell. Biol. 12, 5355–5362
14. Tokitou, F., Nomura, T., Khan, M. M., Kaul, S. C., Wadhwa, R., Yasukawa, T., Kohno, I., and Ishii, S. (1999) J. Biol. Chem. 274, 4485–4488
15. Keesh, C. A., and Gutierrez-Hartmann, A. (1998) Mol. Endocrinol. 3, 832–839
16. Bradford, M. (1976) Anal. Biochem. 72, 248–254
17. Ottaviano, Y., and Gerace, L. (1985) J. Biol. Chem. 260, 624–632
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19. Lewis, J. R., and Nordeen, S. K. (2000) in Methods in Steroid Receptor Molecular Biology (Lieberman, B., ed) Humana Press Inc., Totowa, NJ
20. Conrad, K. E., Oberwetter, J. M., Valliancourt, R., Johnson, G. L., and Gutierrez-Hartmann, A. (1994) Mol. Cell. Biol. 14, 1553–1565
21. Lin, P., Jensen, L., Cowart, Y., Nakai, L., Lim, X., and Sundberg, J. (1999) J. Infect. Dis. 179, 1263–1269
22. Bradford, A., Conrad, K., Tran, P., Ostrowski, M., and Gutierrez-Hartmann, A. (1996) J. Biol. Chem. 271, 24639–24648
23. Wertman, K. F., Drubin, D. G., and Botstein, D. (1992) Genetics 132, 337–350
24. Diamond, S. E., and Kirkegaard, K. (1994) J. Virol. 68, 863–876
25. Cander, E. P., Reeves, R., and Dave, J. R. (1978) Cell 14, 105–113
26. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1999) J. Biol. Chem. 274, 17174–17179
27. Saunders, N., Dicker, A., Popa, C., Jones, S., and Dahler, A. (1999) Cancer Res. 59, 399–404
28. Bradford, A., Wasylko, C., Wasylko, B., and Gutierrez-Hartmann, A. (1997) Mol. Cell. Biol. 17, 1065–1074
29. Xu, L., Lavinsky, R. M., Dassen, J. S., Flynn, S. E., Mclnerny, E. M., Mullen, T. M., Heinz, T., Szeto, D., Korzus, E., Kurokawa, K., Aggarwal, A. K., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1998) Nature 395, 301–306
30. Li, T., Mruck, C. M., Teumer, J. K., and Stavnezer, E. (1986) J. Virol. 57, 1065–1072
31. Maki, Y., Bos, T., Davis, C., Starbuck, M., and Vogt, P. K. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2848–2852
32. Hillesen, A. H., Monden, T., Madura, J. P., Lee, K., and Wondisford, F. E. (1996) J. Biol. Chem. 271, 28516–28520
33. Sladek, F. M., Ruse, M. D., Jr., Nemecuyen, L., Huang, S. M., and Stallcup, M. R. (1999) Mol. Cell. Biol. 19, 6509–6522
34. Hanna-Rose, W., and Hansen, U. (1999) Trends. Genet. 15, 229–234
35. Herschbach, B. M., and Johnson, A. D. (1993) Annu. Rev. Cell Biol. 9, 479–509
36. Hoch, M., Gerwin, N., Taubert, H., and Jackle, H. (1992) Science 256, 94–97
37. Choi, C. Y., Choi, B. H., Park, G. T., and Rho, H. M. (1997) J. Biol. Chem. 272, 16934–16939
38. Treacy, M. N., Neilson, L. I., Turner, E. E., He, X., and Rosenfeld, M. G. (1992) Cell 68, 491–505
39. Galvin, K. M., and Shi, Y. (1997) Mol. Cell. Biol. 17, 3723–3732
40. Agata, Y., Matsuda, E., and Shimizu, A. (1999) J. Biol. Chem. 274, 16412–16422
41. Lutterbach, B., Westendorf, J. J., Linggi, B., Patten, A., Moniwa, M., Dave, J. R., Huyh, K. D., Barbwett, V. J., Lavinsky, R. M., Rosenfeld, M. G., Glass, C. E., and Seto, E. (1998) Mol. Cell. Biol. 18, 7176–7184
42. Kadosh, D., and Struhl, K. (1997) Cell 89, 365–371
43. Koenig, R. J. (1998) Thyroid 8, 763–713
44. David, G., Alland, L., Hong, S. H., Wong, C. W., DePinho, R. A., and Dejean, A. (1998) Oncogene 16, 2549–2556
45. Dhdair, P., Albaghi, O., Lin, R. J., Anisie, S., Quest, S., Leutz, A., Kerckhoff, J. P., Evans, R. M., and Leprince, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10762–10767
46. Nomura, T., Khan, M. M., Kaul, S. C., Dong, H. D., Wadhwa, R., Colmenares, C., Kohno, I., and Ishii, S. (1999) Genes Dev. 13, 412–423
47. Yang, B. S., Hauser, C. A., Henkel, G., Colman, M. S., Van Beveren, C., Stacey, K. J., Hume, D. A., Maki, R. A., and Ostrowski, M. C. (1996) Mol. Cell. Biol. 16, 538–547
48. Wasylko, C., Bradford, A. P., Gutierrez-Hartmann, A., and Wasylko, B. (1997) Oncogene 14, 899–913
49. Toulon, R. M., Castillo, A. I., and Aranda, A. (1998) J. Biol. Chem. 273, 26652–26661
50. Bradford, A., Brodskey, S. E., Kuhn, L., Liu, Y., and Gutierrez-Hartmann, A. (2000) J. Biol. Chem. 275, 3100–3106