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Mutation of the POU-Specific Domain of Pit-1 and Hypopituitarism Without Pituitary Hypoplasia

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A point mutation in the POU-specific portion of the human gene that encodes the tissue-specific POU-domain transcription factor, Pit-1, results in hypopituitarism, with deficiencies of growth hormone, prolactin, and thyroid-stimulating hormone. In two unrelated Dutch families, a mutation in Pit-1 that altered an alanine in the first putative α helix of the POU-specific domain to proline was observed. This mutation generated a protein capable of binding to DNA response elements but unable to effectively activate its known target genes, growth hormone and prolactin. The phenotype of the affected individuals suggests that the mutant Pit-1 protein is competent to initiate other programs of gene activation required for normal proliferation of somatotrope, lactotrope, and thyrotrope cell types. Thus, a mutation in the POU-specific domain of Pit-1 has a selective effect on a subset of *Pit-1* target genes.

The pituitary transcription factor Pit-1 is part of the large POU-domain gene family defined by a highly conserved, bipartite DNA binding domain that consists of the POU-specific domain (POU-S) and POU-homeodomain (POU-HD) regions (*1*). Biochemical and ontogeny studies have shown that Pit-1 is the critical, cell-specific transcription factor for activating expression of the prolactin (*Prl*) and growth hormone (*GH*) genes in the anterior pituitary gland (*2*, *3*). Additionally, Pit-1 acts to regulate expression of its own encoding gene (*4*). Detailed analyses have suggested that both the POU-S and POU-HD contact DNA, with the POU-S domain required for high-affinity, site-specific binding and DNA-dependent cooperative binding events observed on many response elements (*5*). Deficiencies of GH, Prl, and thyroid-stimulating hormone (TSH) in the Snell dwarf mouse are caused by a point mutation [$Trp^{261} \rightarrow Cys$ (W261C)] in the POU-HD that eliminates binding of the defective protein to the *GH* and *Prl* genes (*6*). Homozygosity for the Snell mutation results in severe hypoplasia of the anterior pituitary gland, with the absence of somatotropes, lactotropes, and thyrotropes (*7*).

There have been several reports of multiple pituitary hormone deficiency involving GH, Prl, and TSH in humans (*8*, *9*). We examined the possibility of *Pit-1* gene (*Pit-1*) mutation in two unrelated Dutch families, each with of two affected and three unaffected siblings (*8*). Studies were conducted with informed consent. The affected children in Family I had severe growth impairment with heights 10.2 standard deviations (SD) below the mean for age before beginning human GH (hGH) treatment. The older affected child in Family II had a height of −8.2 SD at age 3.5, but the younger child's height was only 1.0 SD below the mean before hGH treatment was started at 9 weeks of age. All four failed to increase serum GH and Prl levels above detection limits of 0.5 μg/liter and 50 mU/liter in response to GH-releasing hormone and thyrotrophin-releasing hormone (normal > 10 μg/liter and >500 mU/liter). The affected children in Family I had normal serum T4 amounts before GH treatment, but these amounts decreased to 36 and 45 nmol/liter (normal is 70 to 170 nmol/liter) after 1 year of hGH treatment. The affected children in Family II had more severe, symptomatic central hypothyroidism that was recognized shortly after birth.

The normal human *Pit-1* cDNA sequence was determined to provide a basis for comparison with the sequences in the affected children (*10*). A 2.31-kb isolate was found to contain the entire coding region and all but ten nucleotides of the 5′ untranslated region, as judged by the transcriptional start site of the rat *Pit-1* gene. The 873-bp open reading frame showed 85% DNA homology and greater than 95% amino acid sequence homology with bovine, rat, and mouse sequences. Our results are in agreement with those of Lew and Elsholtz (*11*). There were no differences between the bovine Pit-1 protein in the POU-S domain and a single, conservative Met-for-Leu substitution within the POU-HD. The first 40 amino acids were the most divergent region among the four species, exhibiting nonconservative differences.

In Northern (RNA) blot analysis, the human *Pit-1* cDNA hybridized to a single band of approximately 2.4 kb in human pituitary polyadenylated RNA. No Pit-1 message was observed in RNA from human decidua or placenta. This suggests that the *Pit-1* DNA response elements in *Prl* and chorionic somatomammotropin genes do not contribute to their regulation in these tissues (*12*).

Genomic cloning was done to examine regulatory elements and define exon-intron boundaries (*13*). The *Pit-1* autoregulatory binding sites described for the rat and mouse in the 5′ flanking and 5′ untranslated re-

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gions, respectively (PB1 and PB2) (Fig. 1) were completely conserved (*4*). Regions outside of these two binding sites showed scattered areas of homology. Examination of the human gene through position −420 did not disclose sequences that corresponded to the two adenosine 3′,5′-monophosphate (cyclic AMP) response element binding (CREB) sites that are located at positions −200 and −163 in the rat *Pit-1* gene (*4*). This absence implies that cyclic AMP regulation of *Pit-1* gene expression may differ among mammalian species. The human *Pit-1* exon-intron boundaries were identical to those described for mice (*6*).

Reverse transcription and nested polymerase chain reaction (PCR) were used to amplify *Pit-1* cDNA from Epstein-Barr virus–transformed lymphocytic cells from the affected children, siblings, and parents in the two families. A set of two primer pairs was designed to amplify a region that encoded amino acids 120 through 278, which encompassed both the POU-S and POU-HD regions (*14*). The second round of amplification yielded distinct products of 527 and 362 bp. Sequencing of both PCR fragments showed that the larger corresponded to the expected *Pit-1* sequence and that the other reflected an alternatively spliced product that lacked exon 4. An alternatively spliced *Pit-1* gene product has also been observed in rat pituitary cDNA (*15*).

Sequencing of the 527-bp cDNA products from the affected individuals in both families showed a single cytosine for guanine transition that changed amino acid 158 from Ala to Pro (A158P). We confirmed the mutant sequence first detected in cDNA by sequencing a 185-bp fragment of genomic DNA amplified with intron 3 and exon 4 primers. Informative regions for wild-type and A158P mutant specimens are shown in Fig. 2A.

The cytosine for guanine substitution predicts loss of a Bst NI restriction site. Therefore, Bst NI digestion of the 185-bp PCR product was used to distinguish A158P alleles from normal wild-type alleles. As shown in Fig. 2B, the two affected siblings in Family I were homozygous for the A158P allele, having only the 185-bp band and not the 114- and 71-bp bands. Both parents and two unaffected siblings in Family I were heterozygous, and one normal sibling was homozygous for the normal allele. In Family II, the two affected siblings showed only the A158P pattern, and the father was a heterozygote. However, the mother had only the normal, wild-type allele.

These results suggested that the affected siblings in Family II might be compound heterozygotes for the A158P allele, inherited from the father, and a *Pit-1* deletion allele, inherited from the mother. To evaluate this possibility, we performed Southern (DNA) analysis of genomic DNA. No polymorphic restriction fragments were observed after hybridization to three different probes that represented different regions of *Pit-1* cDNA. The intensity of hybridization to a *Pit-1* cDNA probe was estimated by densitometry, with concurrent hybridization to a GH-releasing hormone cDNA probe as an internal control. The ratio of the density of *Pit-1* to that of the GH-releasing hormone bands was 0.52 ± 0.08 (SD; $n = 6$) relative to that of two control specimens in digests prepared with six restriction enzymes. Studies of the entire family showed that the mother, two affect-

Fig. 1. The human *Pit-1* 5′ flanking sequence. The sequence is shown in comparison to the mouse and rat sequences; negative numbering is given relative to the rat *Pit-1* RNA start site indicated by an arrow. Positive numbers indicate the 5′ untranslated region upstream of the initiator methionine codon, which is underlined. Dashes indicate identical bases, and dots represent gaps introduced by the computer alignment program (Gene Works 2.0, IntelliGenetics). The CREB sites in the rat and mouse sequences are in bold italics and underlined, and the Pit-1 binding elements (PB1 and PB2) are bracketed and in bold type. Lowercase letters designate bases preceding the transcript start site, and uppercase letters designate bases after the start site.

```
Human   -393  cacttcttttgtttcatgttttatcgcagatttaatgagaagttgtttaaaaa
Rat     -370  ttt-atcc-ac---ca....a--tcattt-ag.-gtt--c-aaatggt---..
Mouse   -363  cct-atcc-ac---....gga--gtattt-agg-gct--c-aaatgga---..

Human   -340  aatctcatatttatacatttgcaattgtgttagtagcaaaaagatacctacta
Rat     -324  c---------..c--a-ag-tttc--g-a----aaa-c-------...-gc-ta
Mouse   -316  c---------..t--a-ac-tgtc--a-a----..c-c------...-.a-ct

Human   -287  tttttcaattgattgtggcattatttttttgagggacgtgttttcaaaattgt
Rat     -276  t------.....---cca-acc-g-ag-t----aa--actttt--aaag--c-
Mouse   -271  c------.....---cca-ttc-t-ac-c----aa--ac...g--ttga--c-

Human   -234  tgattcctctctaaaaggtctgaactactgaagttgtcaaataaggtttcaga
Rat     -228  -ga.ctctct-tt----...-tcccact-atgacgtcs-----a-----t-t
Mouse   -226  -.....c-ac----tta-catttat-gaagatgcca-----a-----t-t

Human   -181  tttctcccttcccctccatctataccccagaatcagcttccttctctgccatg
Rat     -179  tt-t-g---t-.t-ct--ct-cgtacgtcsc-a-caggtttc--cg--ggcac
Mouse   -181  .c-t-g---g-.t-ct--ct-tgtacgtcst-a-caggcttc--ct--ggcac

Human   -128  gattatgtttggggtaaaaaaggaagacggttcagattgataaagcaatactc
Rat     -127  agat-gta--t------....-a-----a----ggg-ta-c-------c----
Mouse   -130  agac-gta--t------....-t-----a----gag-ca-c-------c----

                                          PB1
Human    -75  ctg.....ggaaaa.gactattaacatgtataaagggatttccttgcagtata
Rat      -78  -------gg----aa----------t---------cag------
Mouse    -81  ---tggggaa----aa----------t---------cac------

                                                    PB2
Human    -28  aatactagc..gcgcaccctcctggtgcctCAGAGCCTTCCTGATGTATATAT
Rat      -30  --------ct--atg----------GC--------------
Mouse    -28  ---------ct--a..------g-----------TGT-------------

Human    100  GCAGGTAGTGAGAATTGAATC.GGCCCTTTGAGACAGTAATATA.ATAAAACT
Rat       90  ---A.--GG---.CG------.-G--.-----T.----------..T---...
Mouse     96  ---AG--CG---AAG------A-C--.-----TA----------ATA---...

Human     49  CTGATTTGGGGAGCAGCGGTTCTCCTTATTT.TTCTACTCTCTTGTGGGAATG
Rat       46  ...GCGGACT-GCA-----TGCGT----G--.C------TCT----------
Mouse     47  ...GTGAACT-GCA---A-CGGGT----G--TT------TTC----------
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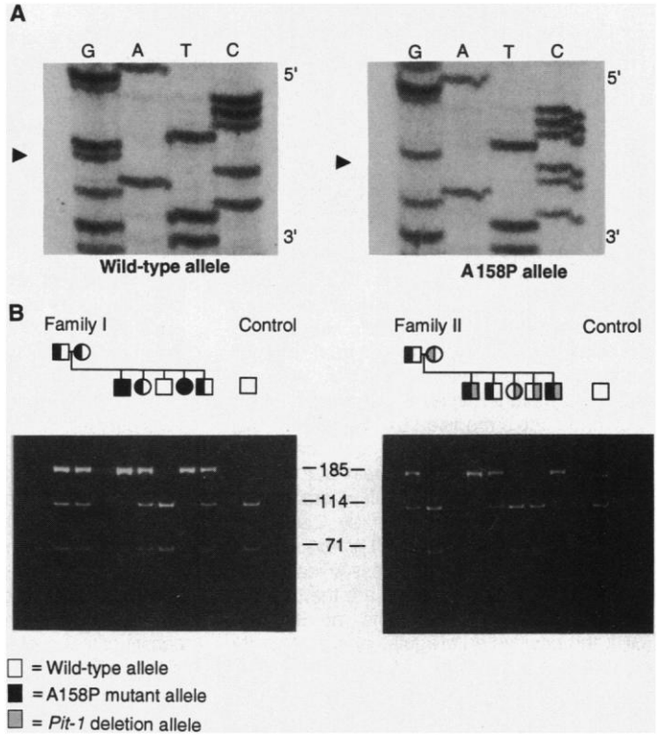

Fig. 2. (**A**) Genomic sequencing of wild-type and A158P mutant human *Pit-1* alleles. Codon 158 is GCA, specifying Ala, in PCR products from a normal control DNA and CCA, specifying Pro, in PCR products from the older affected sibling in Family II. (**B**) Bst NI digestion of genomic PCR products that contained exon 4 of Pit-1. PCR products of 185 bp were obtained by 30 cycles of amplification with an intron 3 sense primer (CTGGAAAGT-TGGAGCTGATG) and an exon 4 antisense primer (CCAGCCATT-TGGATAATATT). The 185-bp products were digested with Bst NI (recognition sequence CCNGG), electrophoresed on a Nusieve: agarose (3:1) gel (FMC BioProducts, Rockland, Maine), and stained with ethidium bromide. Clear symbols indicate wild-type alleles (114- and 71-bp fragments); filled symbols indicate A158P alleles (181-bp fragments); and hatched symbols indicate *Pit-1* deletion alleles. Molecular size markers are indicated in the middle in base pairs. Circles, females; squares, males.

ed siblings, and two of three unaffected siblings carried single copies of the *Pit-1* gene. We concluded that the disease allele transmitted by the mother in Family II involved deletion of the entire coding sequence of the *Pit-1* gene.

To analyze the functional effects of the A158P point mutation, we made an identical substitution in the normal human *Pit-1* cDNA by site-directed mutagenesis. Studies with bacterial cell lysates that expressed wild-type and A158P Pit-1 protein showed that the A158P substitution had only a minimal effect on DNA binding function (Fig. 3A). The most pronounced effect was a threefold reduction in binding to the proximal Pit-1 response element of the rat growth hormone gene (GH site). There were smaller reductions in binding to the positive autoregulatory site (PB1) in the *Pit-1* gene and to the *Prl* response element. In contrast, substitution of two Pro residues for Glu at amino acid 155 (E155P) and Ala at amino acid 159 (A159P) completely abolished binding, as previously shown (*5*).

Qualitative differences between the binding of wild-type and the A158P protein were observed at limiting protein concentrations. Wild-type Pit-1 formed three complexes with either the *GH-1* or *Prl-1P* DNA binding sequences (C2A, C2B, and C1) (Fig. 3B). Both C2 species represent dimers, whereas C1 is a monomer (*5*). Similar binding studies performed with the A158P mutant showed selective loss of the dimeric species C2B. The functional significance of this difference is not known at present.

Further analysis of the A158P mutation involved transient cotransfection experiments. Wild-type and mutant *Pit-1* cDNAs were introduced into a eukaryotic expression vector and cotransfected in HeLa cells with fusion genes under control of the promoters of known *Pit-1* target genes. As shown in Fig. 4, the mutant Pit-1 was incapable of increasing transcription from a fusion gene construct that contained a minimal promoter with three rat GH Pit-1 sites (GH-1x3). The A158P protein retained approximately 5% of normal transcriptional activation when tested with constructs that contained a Pit-1 autoregulatory site (mPit B2), the distal enhancer and proximal promoter sites for *Prl* (PRL DE/P), or 320 bp of the 5′ *GH* promoter sequence (GH 320). The mutant Pit-1 protein in transfected cells appeared stable and exhibited proper nuclear localization, as judged by protein immunoblot analysis (*16*).

This report links the phenotype of GH, Prl, and TSH deficiency to a point mutation in the COOH-terminus of the first α helix in the POU-HD domain of Pit-1. The molecular basis of this human disease differs from the defect in Snell mice, in which a point mutation (W261C) in the third DNA recognition helix of the POU-HD eliminates DNA binding and thereby abolishes transcriptional activation. It also differs from the human Pit-1 mutation described by Tatsumi *et al.* (*17*), which introduces a translational stop signal after codon 171 and thus abolishes POU-HD.

Although the A158P Pit-1 protein does not transactivate target genes that encode the hormones GH and Prl, it is involved in pituitary cell development. Magnetic resonance imaging demonstrated normal anterior pituitary size in the two affected indi-

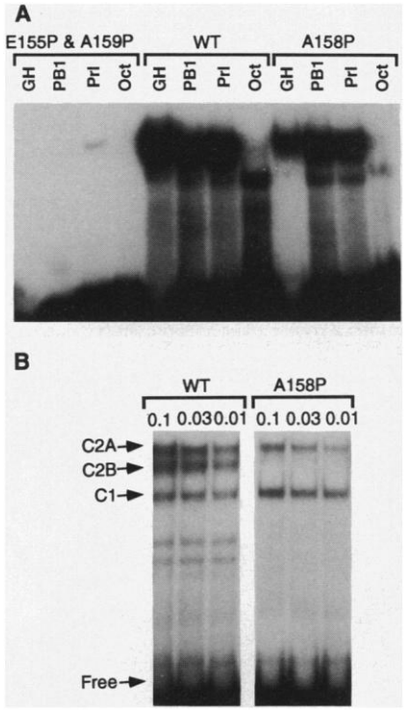

Fig. 3. (**A**) Binding of wild-type and A158P mutant Pit-1 protein to promoter elements. Amino acid residue 158 was changed from Ala to Pro by site-directed mutagenesis with a single-stranded template generated from the full-length wild-type human *Pit-1* cDNA. Wild-type (WT) and mutant human *Pit-1* fragments were subcloned into a modified bacterial expression vector, PHB-H1, at the Nde I and Xba I sites, introduced in the bacterial strain BL21 (DE3), and partially purified. Equivalent amounts of full-length protein, as judged by Coomassie blue staining, were used for all binding studies under previously described assay conditions (*5*). The promoter sequences are the proximal Pit-1 response element of the rat *GH* gene (GH), the positive autoregulatory site (*4*) in the rat *Pit-1* gene (PB1), the *Prl* response element (Prl), and an Oct-1 response element (Oct). (**B**) Binding of wild-type (WT) and A158P Pit-1 proteins to *GH* promoter elements. Binding studies were conducted at limiting protein concentrations, and electrophoretic conditions were chosen to maximize the resolution of differing protein-DNA complexes (*5*).

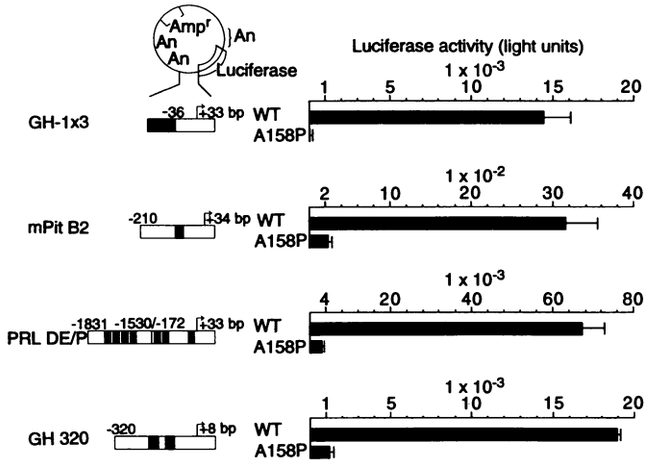

Fig. 4. Transcriptional activation by wild-type and A158P mutant Pit-1 proteins. Amp^r, ampicillin resistance; An, polyadenosine sequences. Effector plasmids that contained the wild-type (WT) or A158P human Pit-1 cDNAs were constructed in a pCMV expression vector as previously described (*4*). Human HeLa cells were plated on 60-mm plates at a density of 5×10^5 cells per plate and transfected with 3.3 μg of effector plasmid and 1 μg of reporter plasmid by calcium phosphate coprecipitation. The various reporter constructs that contained Pit-1 binding sites (shaded boxes) within the context of different gene regulatory regions are shown at left. Each was fused to a firefly luciferase gene. The reporter constructs were three rat GH elements (−62 to +88) fused to the rat Prl TATA box region (−36 to +8), designated GH-1x3; the mouse *Pit-1* 5′ flanking sequence from −210 to +34 with the negative autoregulatory Pit-1 box mutated to eliminate Pit-1 binding (mPit B2); a construct that contained the distal enhancer (−1831 to −1530) and promoter (−422 to +33) elements of the rat Prl gene (PRL DE/P); and the promoter sequence (−320 to +8) of the rat growth hormone gene (GH 320). Cotransfection of the Pit-1 expression plasmids that contained wild-type or A158P mutant sequences was performed with HeLa cells (*4*). We calculated the Pit-1–dependent expression by subtracting the activity of reporter plasmids cotransfected with the control effector plasmid pCMV. Data, shown as absolute light units, represent the average of three replicate dishes per determination, with error bars indicating the SEM. Two independent WT and A158P cDNA constructs were evaluated. The optimal quantity of transfected effector to be used was determined in a titration experiment in which the quantity of the reporter plasmid was held constant at 1 μg per plate.

viduals in Family I. Their hormonal phenotype of severe GH and Prl deficiency coupled with mild central hypothyroidism differs from that of Snell dwarf mice. These mice lack GH, Prl, and TSH and have no recognizable somatotropes, lactotropes, or thyrotropes in their hypoplastic anterior pituitary glands. These differences suggest that the A158P mutant protein remains capable of directing embryonic differentiation and proliferation of somatotropes and lactotropes as well as survival of thyrotropes. The appearance of central hypothyroidism during GH treatment is consistent with the observation that Pit-1 participates in the hormonal modulation of β TSH gene transcription (*18*). Transcriptional activation of other target genes that influence cellular proliferation and survival events may have a less stringent requirement for an intact POU-S domain. Alternatively, Pit-1 may exert a direct effect on DNA replication such as that proposed for Oct-1 activation of viral DNA replication (*19*).

Comparison of the differing phenotypes expressed in the two Dutch families suggests a gene dosage effect. The two affected individuals in Family II, who were compound heterozygotes for the A158P allele and a *Pit-1* deletion allele, had more severe central hypothyroidism than the A158P homozygotes in Family I. The younger heterozygote's anterior pituitary gland was abnormally small. The impact of a reduction in gene dosage may be multiplied because the A158P mutant protein is a poor activator of transcription of the *Pit-1* gene itself. Adult stature in family members with heterozygosity for either the A158P or the deletion allele did not differ from adult stature in family members with two normal *Pit-1* alleles.

Disruption of the predicted α-helical structure in the POU-S domain severely compromises transactivation of a subset of *Pit-1* target genes. Because the A158P mutant protein retains high-affinity binding for its DNA response elements, the resulting human genetic disease suggests that a transcriptional activation function of the POU-S domain of Pit-1 provides a critical contribution to only a portion of the program of developmental gene activation in the human pituitary gland.

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10. We isolated three recombinants from a human pituitary λGT10 cDNA library after probing with the 450-bp Eco RI fragment of a rat cDNA clone (*2*) and washed the recombinants at low stringency (55°C with ×2 saline sodium citrate). Positive clones were further isolated to homogeneity, and DNA was isolated and restricted with Eco RI to excise the insert. A 2.31-kb clone that contained the entire coding 5′ and 3′ untranslated regions was subcloned into PBKSII+ and sequenced according to a standard deaza–guanosine 5′-triphosphate dideoxy protocol with Taq polymerase (Promega) and T3 and T7 promoter primers.
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13. A human genomic library from Clontech (Palo Alto, CA) (HL1067J) was screened under high-stringency conditions with human *Pit-1* cDNA fragments. Four overlapping clones contained the entire protein coding portion of the gene as well as approximately 13 kb of the 5′ and 6.5 kb of the 3′ flanking DNA sequence. We determined exon-intron boundaries by sequencing cloned Eco RI subfragments of the various genomic clones with exon-specific oligonucleotide primers.
14. The *Pit-1* primers used for reverse transcription and PCR were GCAGGAACTCAGGCGGAAAAG, ATACAATAGAAAATCTTATCTGCACTC, CGGAAAAGTAAATTGGTGGAG, and AGATGTTCCTTAGAAATAGAAAATAAAC. Approximately 10 μg of total RNA was incubated with 40 pmol of primer 2 in the presence of 10 mM tris (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 1 mM deoxynucleotide triphosphates, RNasin (10 U), and avian myeloblastosis virus–reverse transcriptase (25 U) (Promega) in a total volume of 20 μl at 42°C for 60 min. We performed the first round of PCR amplification by adding 60 pmol of primer 2, 100 pmol of primer 1, and Taq DNA polymerase (5 U) and increasing the concentration of $MgCl_2$ to 1.2 mM. The temperature profile of 95°C for 1 min, 50°C for 45 s, and 72°C for 40 s was repeated for 30 cycles. The PCR product from the first round was purified by phenol-chloroform extraction and centrifugation over an Ultrafree 30000 MW (Millipore) filter and was used as a template for a second-round PCR amplification with the use of the same cycling program and 100 pmol of the nested primers 3 and 4.
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The Mouse Pink-Eyed Dilution Gene: Association with Human Prader-Willi and Angelman Syndromes

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Complementary DNA clones from the pink-eyed dilution (*p*) locus of mouse chromosome 7 were isolated from murine melanoma and melanocyte libraries. The transcript from this gene is missing or altered in six independent mutant alleles of the *p* locus, suggesting that disruption of this gene results in the hypopigmentation phenotype that defines mutant *p* alleles. Characterization of the human homolog revealed that it is localized to human chromosome 15 at q11.2-q12, a region associated with Prader-Willi and Angelman syndromes, suggesting that altered expression of this gene may be responsible for the hypopigmentation phenotype exhibited by certain individuals with these disorders.

Mutations in mice have been instrumental in the identification of a number of structural and regulatory genes involved in various aspects of development, including pigmentation (*1*). At least 13 alleles of the pink-eyed dilution (*p*) locus on mouse chromosome 7 are defined by reduced pigmentation of both coat and eyes as compared with the wild-type allele (*2*). It has been proposed that the *p* locus encodes a structural protein present in the melanosome organelles of melanocytes (MCs) because mutant *p* melanosomes exhibit structural abnormalities and have a reduced capacity to bind or accumulate melanin (*3*). Furthermore, as some mutant *p* alleles are associated with additional phenotypic features, such as neurological deficits, male sterility, reduced female fertility, and cleft palate (*2*, *4*), the gene product of the *p* locus may also affect the development of other organ systems (*5*). However, because