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

all *p* alleles associated with these additional phenotypes were derived from radiation-induced mutagenesis, alterations in genes adjacent to the *p* gene may be the source of this phenotypic pleiotropy (*4*, *6*, *7*).

Using the genome scanning method, we have identified and cloned DNA fragments from the *p* locus that are duplicated in the genome of the mutant allele pink-eyed unstable, p^{un} (*8*). We used chromosome walking and pulsed-field gel electrophoresis to identify the boundaries of this duplication and to characterize the duplicated segment of ~70 kb that is present in the p^{un} allele (*9*). The structure of the p^{un} duplication, together with genetic observations (*4*), allowed us to predict that the gene encoded by the *p* locus and disrupted by the duplication of genomic DNA extends beyond the duplication on both sides. We isolated overlapping λ phage clones with sequences that spanned the DNA duplication (*9*).

To search for evolutionarily conserved exons (*10*), individual λ clones with sequences from the *p* locus were hybridized to human DNA in the presence of competing total mouse DNA to block hybridization of repetitive DNA. Distinct cross-hybridization signals were detected with phage clone U700, which contained sequences located at a boundary of the p^{un} duplication (*9*). A 400-bp Dra I fragment of unique-sequence DNA was subcloned from this phage insert. This fragment contained an open reading frame and hybridized to the genomic DNA of 18 mammalian species (*11*). Because the action of the *p* gene is autonomous in MCs (*12*), we used the Dra I fragment to screen cDNA libraries prepared from melanoma and MC RNAs (*13*). One cDNA clone, MC2701, obtained from a murine MC library, was selected for further study.

Clone MC2701 contained a sequence of 3127 nucleotides with a long open reading frame of 2499 nucleotides (*13*). If the methionine codon at nucleotide position 131 is the translation initiation site, the polypeptide encoded by MC2701 will have 833 amino acids (91,863 daltons) (Fig. 1A). The predicted protein encodes 12 hydrophobic domains capable of spanning the lipid bilayer (Fig. 1B). Characterizations of melanosome abnormalities in *p* mutants suggest that this protein may be associated with melanosome membrane structures (*3*). MC2701 was not found to be homologous to any known eukaryotic protein. However, we noted amino acid similarities in its hydrophobic domains with a variety of protein receptors, ion transport proteins, and ion channels. These similarities most likely reflect amino acid composition (*14*).

Analysis of RNA from various cells and tissues demonstrated that MC2701 represents the *p* gene and that disruption of this gene in mice bearing mutations at the *p* locus leads to abnormal melanogenesis (Fig. 2). The *p* transcript was most abundant in murine MCs grown in culture. Comparable amounts of transcript were observed in pigmented melanoma cells. The transcript was also present in neonatal and adult eye tissue, presumably as a result of expression of the *p* gene in the retinal pigmented epithelium and the choroid body, known sites of melanogenesis in the eye. Small but detectable amounts of the transcript were also observed in fetal, neonatal, and adult brain RNA, and moderate amounts were detected in adult testis and ovary RNA (Fig. 2A). Because several *p* alleles affect neurological and reproductive function (*2*, *4*), it will be important to determine if expression of the *p* gene is functionally relevant in these tissues to understand the nature of these diverse phenotypes. No detectable hybridization signal was observed in heart, kidney, spleen, liver, or thymus.

The *p* transcript is present in sufficient amounts in the eyes of mice to allow rapid measurement of the relative steady-state amounts of this transcript in various mutant *p* alleles, ten of which were studied (Fig. 2B). In six homozygous mutants characterized by severe hypopigmentation, the wild-type transcript (3.3 kb) was either aberrant in size (p^{un} and p^{J}) or undetectable [p^{6H}, p^{25H}, *p* (the original mutant allele), and p^{cp}], providing strong evidence that disruption of this gene is responsible for the hypopigmentation phenotype. Each of these mutations exhibits large-scale differences in DNA structure at the *p* locus, as compared with wild-type DNA (Fig. 3 and Table 1). In contrast, the wild-type transcript is detected in those homozygous mutants characterized by intermediate pigmentation (p^{bs}, p^{d}, and p^{9J}).

The genomic structure of the *p* gene in p^{un} mice confirmed our hypothesis that coding sequences of the *p* gene, disrupted by the 75-kb duplication, flank the duplication in both the 5′ and 3′ directions (*11*). Several genomic fragments hybridizing to the *p* gene are duplicated in p^{un} DNA (Fig. 3), and the *p* transcript is larger in p^{un} mice (4.8 kb) than in wild-type mice (3.3 kb) (Fig. 2B). The p^{un} mutant allele exhibits a high frequency of reversion to wild-type pigmentation in both the eyes and coat (*15*). In p^{un} mice, the genetic reversion event results in the loss of duplicated sequences [probably by unequal crossing-over (*8*, *9*)], which restores the normal linear array of coding information in the *p* gene (Fig. 3). Moreover, the spontaneous in vivo "rescue" of the wild-type phenotype observed in the genetic reversion of p^{un} mutant mice to revertant mice (p^{un+5}) is coupled with the disappearance of the larger (aberrant) form of the transcript and the appearance of the normal-sized transcript (Fig. 2B), providing direct evidence that MC2701 encodes the *p* gene.

We obtained a human counterpart of the murine *p* cDNA by screening a human melanoma cDNA library (*13*) with the 400-bp Dra I fragment of mouse genomic

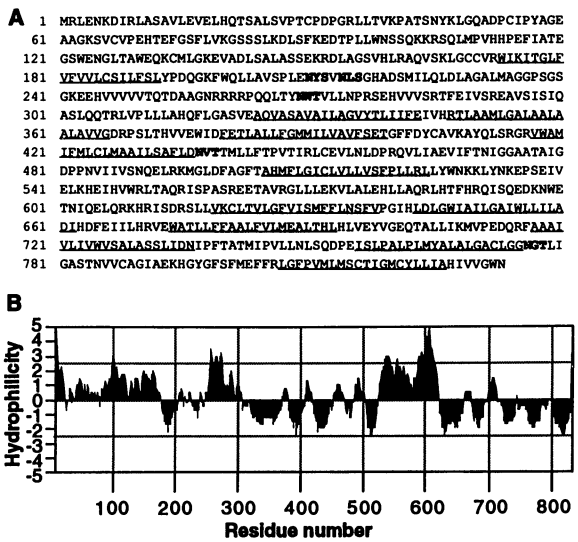

Fig. 1. The predicted mouse *p* gene product. (**A**) Amino acid sequence deduced from the coding region of clone MC2701 (*27*). Amino acids are numbered from the putative initiator methionine (nucleotide 131); however, no Kozak sequence is associated with this methionine (*29*). The potential transmembrane domains are underlined, and potential sites of *N*-linked glycosylation (Asn-X-Ser/Thr) are in bold letters; there is no canonical signal-peptide sequence (*30*). The nucleotide sequence was determined by the chain termination method (*31*) with the use of Sequenase (U.S. Biochemicals) and has been deposited with GenBank (*13*). (**B**) Hydrophilicity plot of the predicted amino acid sequence of the mouse *p* gene. The plot was generated with the MacVector sequence analysis program (International Biotechnologies, New Haven, Connecticut) with a window size of 20 and with the Goldman, Engelman, and Steitz (GES) hydrophilicity scales (*32*). Negative values indicate hydrophobic regions that may be buried in the hydrophobic environment of a lipid bilayer.

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DNA that we used to obtain clone MC2701. Several partial cDNA clones homologous to the mouse *p* gene were isolated; the largest had a 2.2-kb insert (hu-p14). DNA sequence analysis of this clone revealed a high degree of homology between the predicted mouse and human *p* gene amino acid sequences (84% identical from amino acids 283 to 414 of the predicted mouse protein), suggesting that functional determinants in the *p* gene product are conserved in evolution.

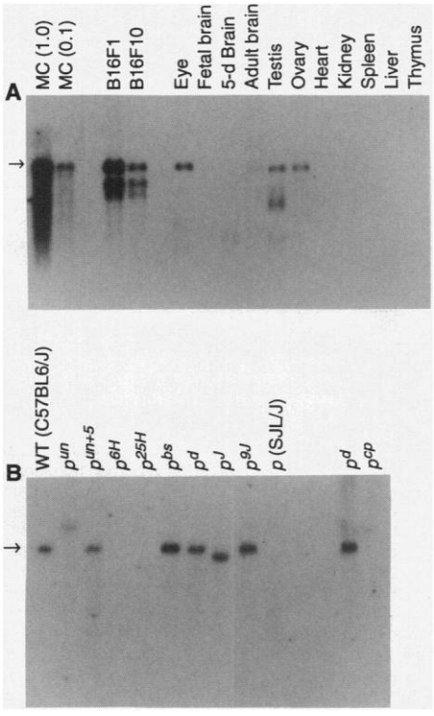


Fig. 2. Northern (RNA) analysis (*33*) with the use of the 2.1-kb Sst I fragment from clone MC2701 as a probe. (**A**) Polyadenylated RNA isolated from the indicated tissues of adult C57BL/6J mice, RNA from MCs (0.1 or 1.0 μg) grown in culture (*26*), or B16F1 and B16F10 murine melanoma cell lines (*25*). All lanes contained 1 μg of RNA (except the second lane). Melanoma cells and MCs both expressed a *p* transcript of 3.3 kb (indicated by the arrow). Melanoma cells expressed additional forms of the transcript (2.6 and 2.2 kb). The size of the transcript detected in the eye, brain (5-d, postnatal day 5), testis, and ovary is apparently identical to the major MC transcript. An additional species (1.9 kb) is also detected in testis RNA. (**B**) Total RNA (5 μg) from eye tissue of mice homozygous for the indicated alleles. The original mutant *p* allele is represented by SJL/J. All samples were isolated from adult mice except for the two rightmost lanes, which came from newborn littermates. Transcript sizes were determined from the positions of 18*S* and 28*S* RNA and RNA markers (BRL). We rescreened the filters for ribosomal protein L32 mRNA (*34*) to ensure that each lane contained the same quantity and integrity of RNA (*11*). The arrow indicates the position of the 3.3-kb transcript.

A syntenic relationship has been demonstrated between gene markers present on human chromosome 15 and mouse chromosome 7. In particular, several of these human chromosome 15 markers have been mapped in mouse and are closely linked to the *p* locus (*7*, *16*). During the course of experiments to establish the long-range restriction maps of the *p* locus in man and mouse by pulsed-field gel electrophoresis (*11*), we noticed that probes derived from the *p* gene gave identical patterns of hybridization to those observed with the human genomic probe IR-10, designated as the *D15S12* locus on human chromosome 15 (*17*). IR-10 contains an exon corresponding to a 3.4-kb cDNA (DN-10) isolated from a human fetal brain cDNA library (*18*). Comparison of the DNA sequence of DN-10 with that of human *p* cDNA clones isolated from a melanoma cDNA library (*13*) indicates that DN-10 is a human homolog of the mouse *p* gene.

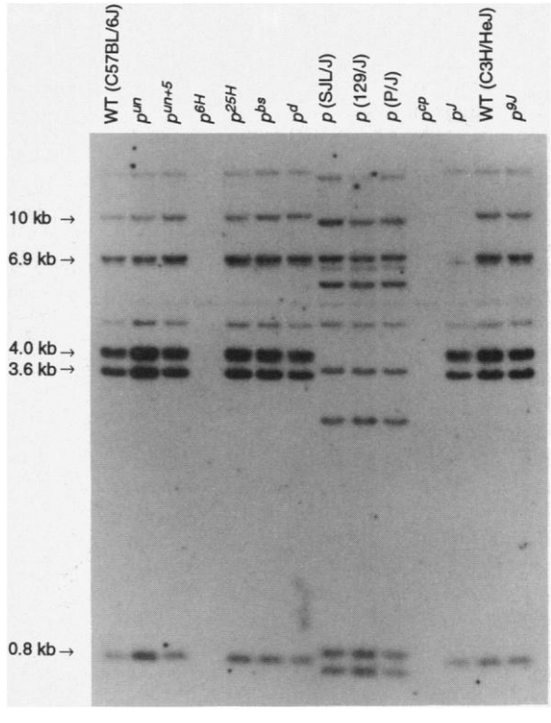
The finding that the human correlate of the mouse *p* gene is the same as the gene represented by IR-10 identifies the corresponding human locus as *D15S12*. This locus has been mapped to chromosome 15q11.2-q12 (*17*, *19*, *20*), in the region associated with Prader-Willi syndrome (PWS) and Angelman syndrome (AS). Failure to inherit the paternal 15q11-q13 region results in PWS, whereas lack of a maternal contribution of this region results

Table 1. Molecular analysis of *p* alleles with MC2701.

Allele*	Origin†	Pigmentation‡	Transcript§	Genomic DNA‖
Wild-type		+	3.3 kb	Normal
p	Spontaneous	−	None	Multiple polymorphisms
p^J	Spontaneous	−	3.0 kb	Deletion
p^{9J}	Spontaneous	+/−	3.3 kb	Unknown defect
p^{un}	Spontaneous	−	4.8 kb	Internal duplication
p^{un+5}	Spontaneous from p^{un}	+	3.3 kb	Normal
p^d	Radiation	±	3.3 kb	Unknown defect
p^{bs}	Radiation	+/−	3.3 kb	5′ Rearrangement
p^{cp}	Radiation	−	None	Deletion
p^{6H}	Radiation	−	None	Deletion
p^{25H}	Radiation	−	None	5′ Rearrangement

*Mice homozygous for the indicated alleles were analyzed. †Referenced in (*28*). ‡Symbols: +, pigmented as intensely as wild-type; −, severe hypopigmentation; +/−, dark eyes and intermediate coat color; and ±, intermediate eyes and coat color. Other phenotypes are associated with some *p* alleles (*2*, *4*, *7*). §Detected in total RNA from the eye (Fig. 2). ‖Detected by Southern analysis [Fig. 3 and (*11*)].

Fig. 3. Southern blot analysis of Sst I–digested DNA from homozygous wild-type (C57BL/6J or C3H/HeJ) and *p* mutant mice. The original mutant *p* allele is represented by SJL/J, 129/J, and P/J. Digests were subjected to electrophoresis through a 0.8% agarose gel and transferred to Genescreen Plus (DuPont), as described (*8*). The blot was probed with a 1.0-kb Dra III fragment derived from the MC2701 cDNA. Selected size markers at left indicate the position of exon-bearing fragments missing from p^J DNA (10.0 and 6.9 kb) and duplicated in p^{un} DNA (4.0, 3.6, and 0.8 kb). All fragments are deleted in p^{6H} and p^{cp} DNA. A minor 6.2-kb band detected by this probe was present in all DNA and presumably represents cross-hybridizing sequences present at another locus.



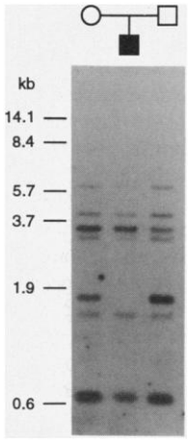


Fig. 4. Molecular analysis of a PWS pedigree. DNA from a hypopigmented PWS proband and his parents were digested with Taq I, subjected to electrophoresis through a 0.8% agarose gel, transferred to Hybond N+ (Amersham), and hybridized to a 1.1-kb Hind III–Eco RI fragment from clone hu-p14. The proband has failed to inherit the 1.8-kb allele for which his father is homozygous, demonstrating the lack of paternal inheritance. Other data (*11*) demonstrate the inheritance of a maternal allele in the proband. Size markers are shown at left.

in AS. However, the critical regions responsible for these two syndromes may be different (*21*). Although PWS and AS are both associated with imprinting of 15q11-q13, they are characterized by distinct phenotypes that perturb the nervous system, cranial facial development, growth and stature, fertility (PWS only), and pigmentation (*20–23*). Disorders involving these same systems, which most likely involve several genes, are also apparent in mice with certain radiation-induced mutations of the *p* locus (*7*). However, none of these mutations in mice appear to be affected by imprinting. Mice heterozygous for mutant *p* alleles are fully pigmented, regardless of the parental origin of the mutant allele.

In man, deletions of *D15S12* are correlated with hypopigmentation. In a comprehensive study of 50 individuals with PWS (*21*), 24 out of 31 (77.4%) individuals with IR-10 deletions were hypopigmented. In contrast, only 2 of 19 (10.5%) individuals with normal amounts of IR-10 were hypopigmented. It is unlikely that *D15S12* mutations are dominant because mutant alleles of the mouse *p* locus are recessive. However, hypopigmentation may be manifest in individuals hemizygous for *D15S12* as a result of the imprinting effects associated with 15q11-q13. The infrequent individuals who are not hypopigmented, but who have deletions of IR-10, may represent variations in imprinting. Because IR-10 represents only a small fraction of the genomic DNA of the *D15S12* locus, some hypopigmented individuals with apparently normal amounts of IR-10 sequences may have genetic lesions in other parts of *D15S12*. Southern blot analysis with a cDNA probe could be used to reveal lesions in *D15S12* not detected by IR-10. The hu-p14 cDNA probe demonstrated the lack of paternal inheritance of this gene in a hypopigmented PWS individual (Fig. 4).

With the identification of the mouse *p* locus gene and its human correlate, we can begin to understand how this gene affects pigmentation, how it is imprinted in humans, and why it is apparently not imprinted in mice. Moreover, on the basis of the phenotype of *p* locus mutations in mice and the association of tyrosinase-positive occulocutaneous albinism (OCA type II) in some individuals with PWS and AS (*22*, *24*), a subset of OCA type II may be caused by mutations in the *D15S12* locus. Thus, mutations of the mouse *p* locus offer models for studying specific aspects of PWS, AS, and potentially OCA type II.

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