

# Tissue-Specific Targeting of the *Pthrp* Gene: The Generation of Mice with Floxed Alleles\*

BIN HE, RON A. DECKELBAUM<sup>†</sup>, DENGSHUN MIAO<sup>†</sup>, MARK L. LIPMAN<sup>‡</sup>,  
MICHAEL POLLAK, DAVID GOLTZMAN, AND ANDREW C. KARAPLIS<sup>†</sup>

*Divisions of Endocrinology (B.H., R.A.D., A.C.K.) and Nephrology (M.L.L.), Department of Medicine, Department of Oncology (M.P.), and Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montréal, Québec, Canada H3T 1E2; and Calcium Research Laboratory (R.A.D., D.M., D.G.), Department of Medicine, Royal Victoria Hospital, McGill University, Montréal, Québec, Canada H3A 1A1*

## ABSTRACT

PTH-related peptide (PTHrP) has been implicated in a variety of developmental and homeostatic processes. Although mice homozygous for the targeted disruption of the *Pthrp* gene have greatly expanded our capacity to investigate the developmental roles of the protein, the perinatal lethality of these animals has severely hindered the analysis of Pthrp's postnatal physiological effects. To overcome this obstacle, we have generated mice homozygous for a floxed *Pthrp* allele, *i.e.* two *loxP* sites flanking exon 4 of the *Pthrp* gene, which encodes most of the protein, with the aim of accomplishing cell type- and tissue-specific deletion of the gene. The ability of the Cre enzyme

to cause recombination between the *loxP* sites and excision of the intervening DNA sequence was tested *in vivo* by crossing this strain to mice carrying a *cre* transgene under the transcriptional control of the human  $\beta$ -actin promoter. The ubiquitous deletion of the floxed allele in the *cre/loxP* progeny resulted in perinatal lethality as a consequence of aberrant endochondral bone formation, fully recapitulating all the phenotypic abnormalities observed in the conventional *Pthrp* knockout mouse. The availability of the floxed *Pthrp* mice will serve as a valuable tool in genetic experiments that aim to investigate the physiological actions of Pthrp in the postnatal state.

PTH-related peptide (PTHrP) was initially identified as the humoral factor responsible for hypercalcemia in malignancy. It is now recognized that its spectrum of physiological actions encompasses a wide variety of developmental and homeostatic processes during fetal and adult life (1). Mice homozygous for *Pthrp* gene ablation are born alive but die soon after birth because of a multitude of skeletal deformities arising as a consequence of diminished proliferation and accelerated differentiation of chondrocytes in the developing endochondral skeleton (2–4). However, the perinatal lethality of the *Pthrp*-null mice precludes observation of potential postnatal tissue-specific alterations arising in the complete absence of Pthrp. Studies using heterozygous *Pthrp*-null and rescued *Pthrp*-null mice have implicated pivotal roles for the protein in bone formation (5), mammary gland development (6), tooth eruption (7), epidermal differentiation (8), and neuronal protection (9) in the postnatal state. Nevertheless, the complexity of the phenotypic alterations associated with these transgenic models makes the interpretation of these findings rather difficult to consolidate.

Therefore, to circumvent these limitations, it has become desirable to generate a mouse strain missing both alleles of the *Pthrp* gene only in a particular cell type. In this context, chondrocyte proliferation and differentiation would be expected to proceed unaltered. It would be predicted that these mice would be viable and amenable for studying tissue-specific biology in the complete absence of Pthrp.

The technology for producing such a conditional knockout is based on the *cre/loxP* site-specific recombination system of bacteriophage P1 that infects the bacterium *Escherichia coli*. Cre recombinase is an enzyme that catalyzes site-specific recombination between 34-bp sequences of phage DNA, termed *loxP* sites, thereby removing the DNA between them, leaving one *loxP* site behind (10). By combining the *cre/loxP* site-specific recombination system with embryonic stem (ES) cell technology, the capability of achieving conditional gene targeting has greatly expanded. The production of such a conditional knockout requires the generation of two mouse strains. One strain carries the gene of interest flanked by two *loxP* sites (floxed gene). The second is a conventional transgenic strain in which the Cre recombinase enzyme is expressed in a cell type- or developmental stage-specific manner. Appropriate mating between these two strains results in excision of the floxed DNA in a defined spatial or temporal manner.

In this study, we describe the generation of a mouse line in which *loxP* sites were introduced in the genome flanking nearly the entire coding region of the *Pthrp* gene. The capacity to excise the floxed gene *in vivo* was confirmed by crossing these animals to mice carrying the *cre* transgene driven by the human  $\beta$ -actin promoter. Total body Cre-

mediated recombination of the *Pthrp* gene resulted in a form of lethal chondrodysplasia, the characteristics of which faithfully recapitulated those of the conventional *Pthrp* knockout mouse.

## Materials and Methods

### Construction of the targeting vector

For constructing the floxed targeting vector, DNA sequences derived from the ploxPneo-1 plasmid (from A. Nagy, Lunenfeld Institute, Toronto, Canada), the targeting vector pPTHrPTV (2), and pPGKneoNTRtkpA plasmid (from R. Jaenisch, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA) were used. The ploxPneo-1 plasmid was partially restricted with *Xba*I, then completely digested with *Sal*I, and ligated either to the 1.2-kb *Pst*I/*Sac*I fragment of the murine *Pthrp* gene, encompassing exon 4 (plox4), or to the 4.2-kb *Xba*I/*Nsi*I fragment derived from the pPGKneoNTRtkpA vector (ploxPGKneoNTRtkpA), encoding the neomycin resistance (*neo*<sup>r</sup>) and herpes simplex virus-thymidine kinase (*hsv-tk*) selectable genes with the 5' nontranslated region (*nt*r) of encephalomyocarditis virus inserted between them, following treatment of all DNA fragments with Klenow and dNTPs. The plox4 plasmid containing exon 4 was digested with *Kpn*I/*Xho*I and the *Kpn*I/*Xho*I polylinker sequence derived from the pCDNA3 vector was inserted. Following further restriction with *Xho*I, the 4.2-kb *Xho*I fragment obtained from the ploxPGKneoNTRtkpA plasmid was ligated, resulting in plasmid plox4/loxPGKneoNTRtkpA. Restriction of this plasmid with *Not*I provided a DNA fragment encompassing the floxed 1.2-kb *Pst*I/*Sac*I segment of the *Pthrp* gene followed by the floxed PGKneoNTRtkpA cassette.

To construct the remaining part of the targeting vector, ploxPneo-1 was digested with *Xho*I, the ends were blunted, and *Eco*RI linkers were attached. *Kpn*I restriction of the resulting plasmid was followed by ligation of the 3.6-kb *Eco*RI fragment derived from the pPTHrPTV, composed of the 3'-flanking homology sequence, after blunting and addition of *Kpn*I linkers. The 5'-flanking sequence of homology was derived as a 3.4-kb *Xho*I fragment from the pPTHrPTV plasmid and was inserted into the *Bam*HI site of the resulting vector. Restriction of this plasmid with *Eco*RI released a DNA fragment encompassing both homology sequences, which when ligated to the *Not*I fragment derived from plasmid plox4/loxPGKneoNTRtkpA, resulted in the final targeting vector, pPTHrPfloxyTV.

### Generation of the *Pthrp* floxed mice

The pPTHrPfloxyTV plasmid (25  $\mu$ g) was linearized at the unique *Not*I site and electroporated into R1 ES cells. Thirty-six hours later, selection was initiated with 300  $\mu$ g/ml G418, resistant ES cell clones were isolated, and genomic DNA was prepared. Following restriction with *Eco*RI and size fractionation on 0.8% agarose gel, the DNA was transferred onto nitrocellulose filters and hybridized with the 1.1-kb *Bam*HI/*Sac*I genomic fragment containing sequences encoding exon 5 of the *Pthrp* gene. One of the targeted clones underwent a second round of electroporation with 25  $\mu$ g of supercoiled plasmid pBS185 (from A. Nagy, Lunenfeld Institute) containing the *cre* recombinase gene under the control of human cytomegalovirus promoter/enhancer. After selection in medium containing 2  $\mu$ M ganciclovir for 5 days, clones were picked and expanded. Genomic DNA was prepared and examined for type II deletions by Southern blot analysis.

Appropriately targeted ES cells were microinjected into 3.5-day postcoitus BALB/c blastocysts and then transferred into uteri of 2.5-day postcoitus pseudopregnant CD1 mice. Seventeen days later chimeric animals were born. Extensively chimeric males were mated to BALB/c females and, following germ line transmission, animals heterozygous for the floxed allele were crossed to generate mice homozygous for the targeted allele.

### Mouse strains

The Z/AP mice were provided by C. Lobe (Sunnybrook Health Science Center, Toronto, Canada). The human  $\beta$ -actin-*cre* mice were a generous gift from B. Morgan (Harvard University School of Med-

icine, Boston, MA) and G. R. Martin (University of California, San Francisco, CA).

### Histology

All animal studies were conducted in accordance with principles and procedures dictated by the highest standards of humane animal care. Newborn mice were killed, femurs, tibiae, and ribs were removed and fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution) for 24 h at 5 C. Samples were in turn decalcified in EDTA-glycerol solution (14.5 g EDTA, 15 ml glycerol, 85 ml distilled water, and solid sodium hydroxide added until a final pH of 7.3 was reached) for 1–2 days at 5 C. Following dehydration in graded alcohol, tissues were embedded in low-melting-point paraffin, and 5- $\mu$ m sections were cut on a rotary microtome and stained with hematoxylin and eosin (H & E).

### Preembedding lacZ staining

Preembedding lacZ staining was performed as described, with some modifications (11). Samples were fixed with PLP fixative overnight at 5 C, washed three times for 30 min in lacZ wash buffer (2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 in PBS), and stained in 0.5 mg/ml X-gal, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide in lacZ wash buffer at 37 C overnight with shaking while protected from light. Following staining, samples were decalcified, embedded in paraffin, and 5- $\mu$ m sections were cut on a rotary microtome. Tissues were dewaxed, hydrated by passage through graded alcohol series, washed in running water for 3 min, and mounted with Kaiser's glycerol jelly.

### Human placental alkaline phosphatase staining

Tissue staining for human placental alkaline phosphatase activity was performed as previously described (12). Briefly, tissue sections were preincubated in TBS (50 mM Tris-HCl, 150 mM NaCl, 0.01% Tween 20, pH 7.6) at 70–75 C for 30 min to inactivate endogenous alkaline phosphatase activity. Following overnight incubation in 1% MgCl<sub>2</sub> and 100 mM Tris-maleate buffer (pH 9.2), sections were incubated for an additional 2 h at room temperature in a 100-mM Tris-maleate buffer containing naphthol AS-MX phosphate (0.2 mg/ml, Sigma, St. Louis, MO) dissolved in ethylene glycol monomethyl ether as substrate and Fast Red TR (0.4 mg/ml, Sigma) as stain for the reaction product. After washing with distilled water, the sections were counterstained with Vector methyl green nuclear stain (Vector Laboratories, Inc., Ontario, Canada) and mounted with Kaiser's glycerol jelly.

### *Pthrp* immunohistochemistry

Paraffin sections were stained for *Pthrp* using the avidin-biotin-peroxidase complex technique. Sections were first treated with 0.5% bovine testicular hyaluronidase (Sigma) for 30 min at 37 C, to increase antibody penetration and access to epitopes. Rabbit antiserum against *Pthrp* 1–34 peptide was applied to sections overnight at room temperature. As a negative control, the preimmune serum was substituted for the primary antibody. After washing with high salt buffer (50 mM Tris-HCl, 2.5% NaCl, 0.05% Tween 20, pH 7.6) for 10 min at room temperature followed by two 10-min washes with TBS, the sections were incubated with secondary antibody (biotinylated rabbit antigoat IgG; Sigma), washed as before and processed using the Vectastain ABC-AP kit (Vector Laboratories, Inc.). Red pigmentation to demarcate regions of immunostaining was produced by a 10- to 15-min treatment with Fast Red TR/ Naphthol AS-MX phosphate (Sigma, containing 1 mM levamisole as endogenous alkaline phosphatase inhibitor). The sections were then washed with distilled water, counterstained with methyl green, and mounted with Kaiser's glycerol jelly.

## Results

### Targeting the *Pthrp* locus

Our strategy for accomplishing cell-type specific targeting centered on the scheme used to generate *Pthrp*-negative mice

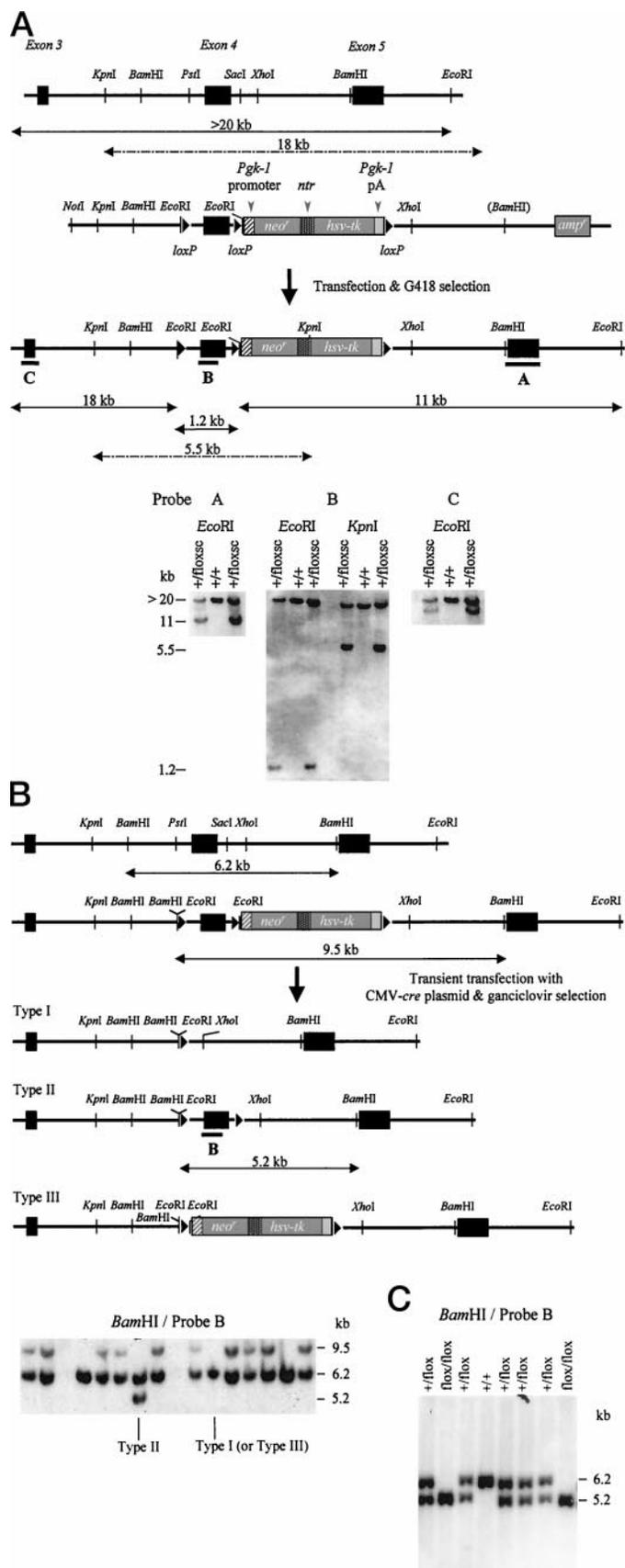


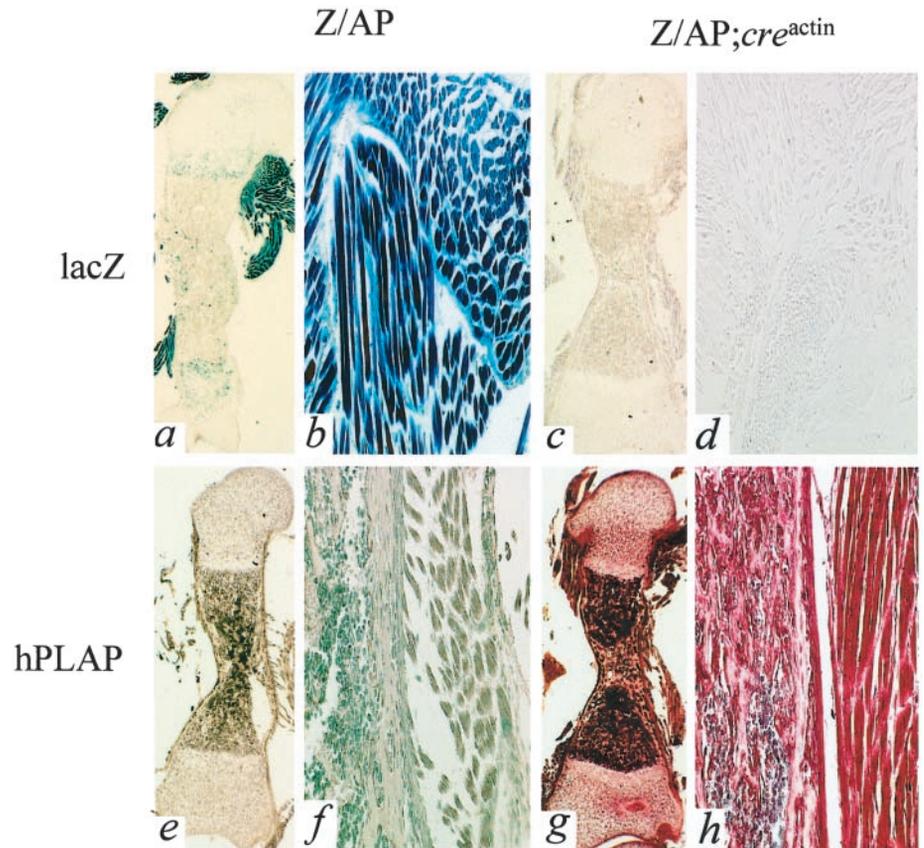
FIG. 1. Targeting the murine *Pthrp* gene in ES cells. A, Introducing the *loxP* sites and selectable marker genes in the *Pthrp* locus. Top, A

(2) in which deletion of 1.2-kb of genomic DNA sequences (*PstI/SacI* fragment) encompassing exon 4 of the murine *Pthrp* gene (13), resulted in a null allele (Fig. 1A). The expectation was that exon 4, when flanked by *loxP* sites, would remain fully functional, but following Cre-mediated excision it would recapitulate the *Pthrp*-null allele. In the design of the targeting vector, the mouse phosphoglycerate kinase promoter was used to drive expression of both the *neo<sup>r</sup>* and *hsv-tk* selectable genes. The *ntr* was inserted between the two genes so that a bicistronic mRNA will be generated. Because the *ntr* sequence includes an internal ribosomal entry site (14), the *hsv-tk* gene would be translated in a cap-independent manner.

Following electroporation of the targeting vector and G418 selection, a homologous recombination event introduced the *neo<sup>r</sup>-ntr-hsv-tk* genes cassette and three *loxP* sites into the *Pthrp* locus, as schematically depicted in Fig. 1A. G418-resistant ES cell clones (276 clones) were isolated and genomic DNA was prepared and examined by Southern blot analysis following restriction with *EcoRI* and hybridization with probe A, a 1.1-kb *BamHI/SacI* genomic fragment 3' to the targeted homologous region and encompassing exon 5 of the murine *Pthrp* locus (2). In the case of a targeted recombination event, the wild-type allele would be expected to yield a more than 20-kb fragment, whereas the floxed allele would result in an 11-kb fragment. Two clones having undergone the expected homologous recombination event were identified and subjected to further restriction mapping and Southern blot analysis to confirm the fidelity of the targeting event using either an internal probe (probe B) corresponding to a 382-bp *AvrII/SmaI* genomic fragment encoding for part of exon 4 (13) or an approximately 600-bp PCR-amplified fragment encompassing exon 3 of the *Pthrp* gene (probe C), located 5' to the targeted homologous region (Fig. 1A). In addition, the blots were stripped and rehybridized with the

schematic representation of the genomic organization of part of the murine *Pthrp* gene and below that of the linearized targeting vector. Following transfection and selection with 300  $\mu$ g/ml G418, 2 of 276 clones were shown to have undergone the expected targeting event, as indicated. Shown at the bottom is a Southern blot analysis of genomic DNA samples from the 2 targeted clones (+/floxed; +, wild-type allele; floxed, floxed allele with the selection genes cassette) following digestion with *EcoRI* (solid lines) or *KpnI* (dashed lines) and probing with three different probes (A, B, C). Genomic DNA from wild-type (+/+) ES cells is shown as control. Probes A and C are exonic sequences flanking regions of homology in the targeting vector, used to verify the fidelity of the recombination event at the 3' and 5' ends of the *Pthrp* locus, respectively. The double arrowhead lines indicate the expected restriction fragments and numbers above indicate their anticipated size.  $\blacktriangleright$ , *loxP* sites; *ntr*, the 5' ntr of encephalomyocarditis virus; *Pgk-1*, the mouse phosphoglycerate kinase promoter and polyadenylation signal (*pA*). B, Cre-mediated excision of the selectable marker genes cassette. Transient transfection of one targeted ES clone with plasmid expressing Cre under the control of the *CMV* promoter. Below, three potential recombination events (types I, II, and III) are illustrated but only clones with type I and II deletions are expected to survive ganciclovir (2  $\mu$ M) selection. Genomic DNA from several surviving clones was restricted with *BamHI* and resulting fragments (double arrowhead lines) probed with probe B. C, ES cells with type II deletion were used to generate the *Pthrp*<sup>flox/flox</sup> mice using standard protocols. Shown in this study, is a Southern blot of tail tip genomic DNA following digestion with *BamHI* and hybridization with probe B from wild-type (+/+) mice and litter mates heterozygous (+/floxed) and homozygous (floxed/floxed) for the floxed *Pthrp* allele.

FIG. 2. Cre expression driven by the human  $\beta$ -actin promoter. Following matings between Z/AP and  $cre^{actin}$  mice, offspring of the genotypes Z/AP (A, B, E, and F) and Z/AP; $cre^{actin}$  (C, D, G, and H) were analyzed for *lacZ* (A–D) and hPLAP expression (E–H). A peculiarity of the Z/AP transgene is that it does not express *lacZ* in chondrocytes and adipocytes (as shown), as well as erythrocytes. The presence of Cre resulted in complete excision of the *lacZ* gene and the concurrent expression of alkaline phosphatase activity in all tissues examined, including cartilage and adipose tissue (G and H). Magnification:  $\times 25$  (A, C, E, and G) and  $\times 200$  (B, D, F, and H).



*neo<sup>r</sup>* probe, to verify that only a single copy homologous integration event had occurred with no evidence of random integration (data not shown).

One of the two targeted clones was expanded and the ES cells underwent a second round of electroporation with 25  $\mu$ g supercoiled pBS185 plasmid containing the *cre* recombinase gene under the control of human *CMV* promoter/enhancer. The loss of the *neo<sup>r</sup>-ntr-hsv-tk* genes cassette following excision by Cre recombinase activity, was expected to make the ES cells ganciclovir resistant. After selection in medium containing ganciclovir, 135 surviving clones were picked and expanded. Genomic DNA was again prepared and examined for type II deletions (Fig. 1B; floxed exon 4 of *Pthrp* gene) by Southern blot analysis following digestion with *Bam*HI and hybridization with probe B. The presence of a 5.2-kb band (type II recombination) in conjunction with a 6.2-kb fragment (wild-type allele) in 5 of these clones confirmed the successful removal of the selection genes cassette, while leaving the floxed exon 4 of *Pthrp* intact. The presence of the 9.5-kb mutant band along with the 6.2-kb wild-type band in several clones indicated that selection with ganciclovir was not particularly effective, perhaps due to low or no expression of the *hsv-tk* gene.

#### Generating the floxed *Pthrp* mice

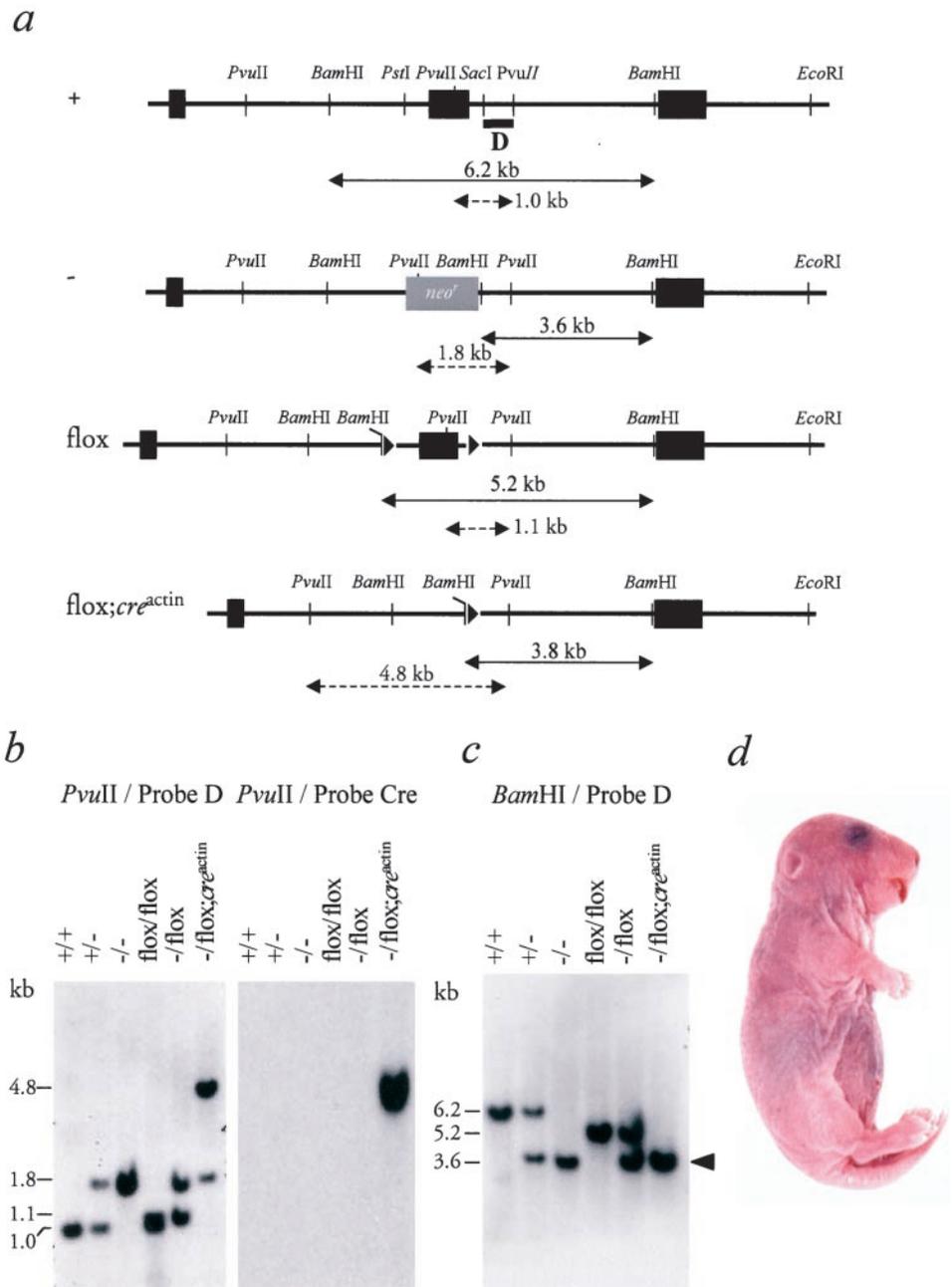
ES cells from one of these appropriately targeted clones were microinjected into 3.5-day BALB/c blastocysts and chimeric male mice were used to generate animals heterozygous for the floxed *Pthrp* gene (*Pthrp<sup>+/flox</sup>*; + and flox signify the

presence of the wild-type and floxed alleles, respectively). Offspring with this genotype were identified by Southern blot analysis of tail genomic DNA and intercrossed to obtain mice homozygous for the altered allele (*Pthrp<sup>flox/flox</sup>*; Fig. 1C). As expected, these animals were viable, fertile, and their overall development appeared normal, indicative that the introduction of *loxP* sites does not interfere with or alter to any significant extent *Pthrp* gene expression *in vivo*.

#### Analysis of Cre function in $\beta$ -actin-cre mice

Next, we wanted to determine whether Cre-mediated excision of the floxed *Pthrp* allele could be successfully accomplished *in vivo*. Total-body deletion of the *Pthrp* gene was to be achieved using a mouse strain carrying a transgene where *cre* was placed under the transcriptional control of regulatory elements from the human  $\beta$ -actin gene (*cre<sup>actin</sup>*), including the promoter, 5' enhancer and intron, 3'-flanking untranslated region, and polyadenylation sequences (15). The *cre<sup>actin</sup>* transgene was expected to be expressed in all cell lineages of the early embryo. As a first step, we set out to verify the *in vivo* efficacy of the  $\beta$ -actin promoter to drive the ubiquitous and functional expression of Cre by crossing the *cre<sup>actin</sup>* mice to the double reporter transgenic line Z/AP mice (11). The latter strain expresses ubiquitously the *lacZ* reporter gene under control of the *CMV* enhancer and chicken  $\beta$ -actin promoter before Cre-mediated excision takes place. However, when it does occur, the *lacZ* gene that is floxed is removed, permitting expression of the second reporter, the human placental alkaline phosphatase gene (hPLAP), only in tissues

**FIG. 3.** Strategy for total body Cre-mediated excision of the *Pthrp* gene. *Cre<sup>actin</sup>* mice were mated with animals with the *Pthrp<sup>+/-</sup>* genotype (-, the null allele where exon 4 of *Pthrp* has been replaced with the *neo<sup>r</sup>* gene cassette). Offspring heterozygous for the null allele and carrying the *cre<sup>actin</sup>* transgene (*Pthrp<sup>+/-</sup>;cre<sup>actin</sup>*) were crossed to *Pthrp<sup>flox/flox</sup>* mice to obtain animals with the *Pthrp<sup>-flox</sup>;cre<sup>actin</sup>* genotype. A, Shown are schematic representations of the genomic organization of wild-type and various mutant *Pthrp* alleles as well as changes in the restriction enzyme pattern anticipated. B and C, Southern blot analysis following digestion of tail tip genomic DNA with *Pvu*II (B; dashed lines) or *Bam*HI (C, solid lines) and hybridization with probe D, a 0.65-kb *Sac*I/*Xho*I genomic DNA fragment. B, The right panel shows the same blot as in the left panel after having been stripped and reprobed with a 356-bp PCR-amplified, *cre*-derived fragment using plasmid pBS185 as template DNA (5'-ATGTCCAATTTACTGACCCCTAC-3' and 5'-CGCCGCATAACCACTGAAAC-3' as the forward and reverse primers, respectively). The arrowhead indicates the expected doublet of 3.6- and 3.8-kb fragments. D, Total body Cre-mediated excision of the *Pthrp* gene results in a perinatal lethal form of chondrodysplasia characterized by a domed skull, short mandible leading to tongue protrusion, and short-limb dwarfism.



that express Cre recombinase. In this study, we sought to obtain offspring of the genotype *Z/AP;cre<sup>actin</sup>*, as determined by lack of *lacZ* but presence of hPLAP staining of tail tips. Figure 2 shows stained sections from soft tissues and humeri from a *Z/AP* mouse as well as from a *Z/AP;cre<sup>actin</sup>* littermate. In the absence of *cre<sup>actin</sup>*, tissues failed to stain for alkaline phosphatase but stained intensely for *lacZ* activity, except for chondrocytes and adipocytes, as was reported initially (11). However, in the presence of the transgene, tissues stained intensely for alkaline phosphatase activity, including chondrocytes and adipocytes, consistent with the ubiquitous and complete excision of the floxed *lacZ* gene. Therefore, we concluded that the *cre* gene in the *cre<sup>actin</sup>* mouse line functions efficiently to excise floxed DNA segments in all cell lineages.

#### Generation of a *Pthrp*-null allele by Cre-mediated recombination

Initially, we chose to target the floxed *Pthrp* gene in mice of the genotype *Pthrp<sup>-flox</sup>* (- indicates the null allele where exon 4 of *Pthrp* has been replaced with the *neo<sup>r</sup>* cassette gene). This was chosen in an attempt to facilitate the excisional activity of Cre, because in this mouse line every single cell would have only one functional floxed *Pthrp* allele, the removal of which would result in compound heterozygosity for the null allele (Fig. 3A). It was anticipated that these animals would recapitulate the phenotypic changes observed in the conventional knockout mouse (2). Therefore, we crossed mice carrying the *cre<sup>actin</sup>* transgene to *Pthrp<sup>+/-</sup>*

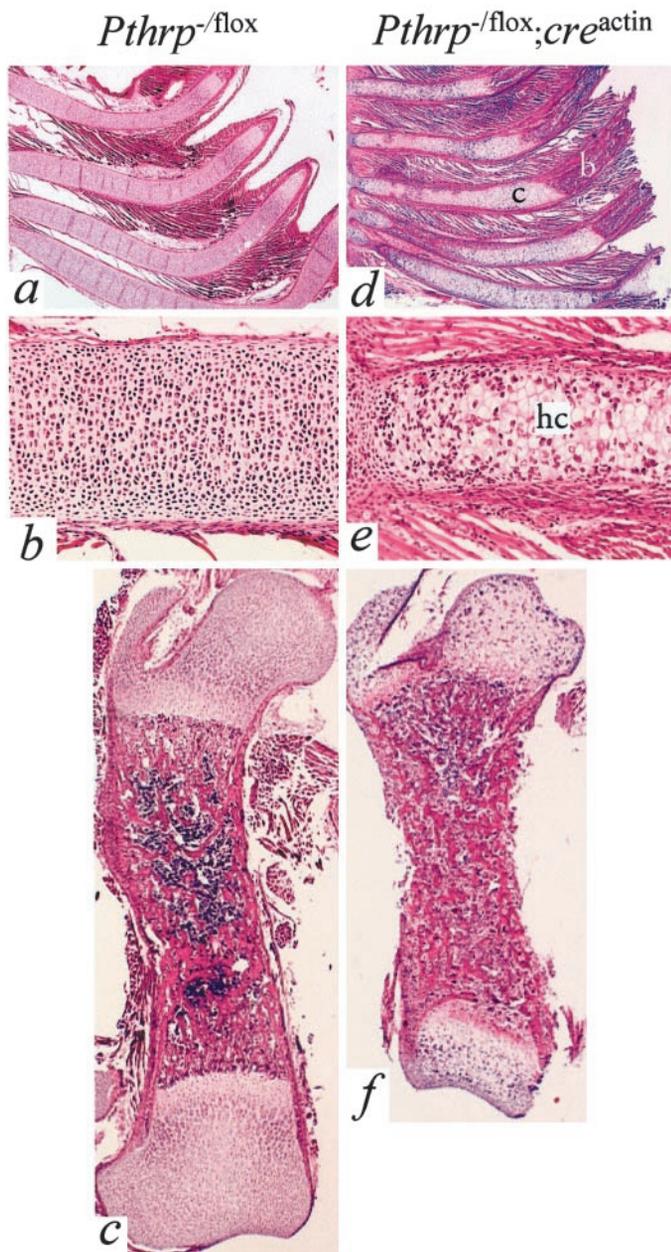


FIG. 4. Histological examination of skeletal preparations from  $Pthrp^{-/lox};cre^{actin}$  mutant mice. Cre-mediated excision of the floxed  $Pthrp$  gene leads to morphological alterations resembling those observed in the  $Pthrp$ -null phenotype. Compared with  $Pthrp^{-/lox}$  mice (A–C), removal of the floxed allele results in inappropriate chondrocyte differentiation and bone formation in the cartilaginous component of the ribs (D and E) and shortening of the long bones (F). b, Bone; c, cartilage; hc, hypertrophic chondrocytes. H & E staining. Magnification:  $\times 25$  (A and D),  $\times 200$  (B and E), and  $\times 25$  (C and F).

mice to obtain progeny of the genotype  $Pthrp^{+/-};cre^{actin}$ . Matings of these animals with mice homozygous for the floxed allele ( $Pthrp^{lox/lox}$ ) resulted in the generation of progeny with the desired genotype,  $Pthrp^{-/lox};cre^{actin}$  (Fig. 3, B and C). These animals died in the perinatal period from respiratory failure and exhibited all the phenotypic abnormalities observed in the original  $Pthrp$ -null mice. As depicted in Fig. 3D,  $Pthrp^{-/lox};cre^{actin}$  mice had the characteristic chondrodystrophic features of domed skull, shortened man-

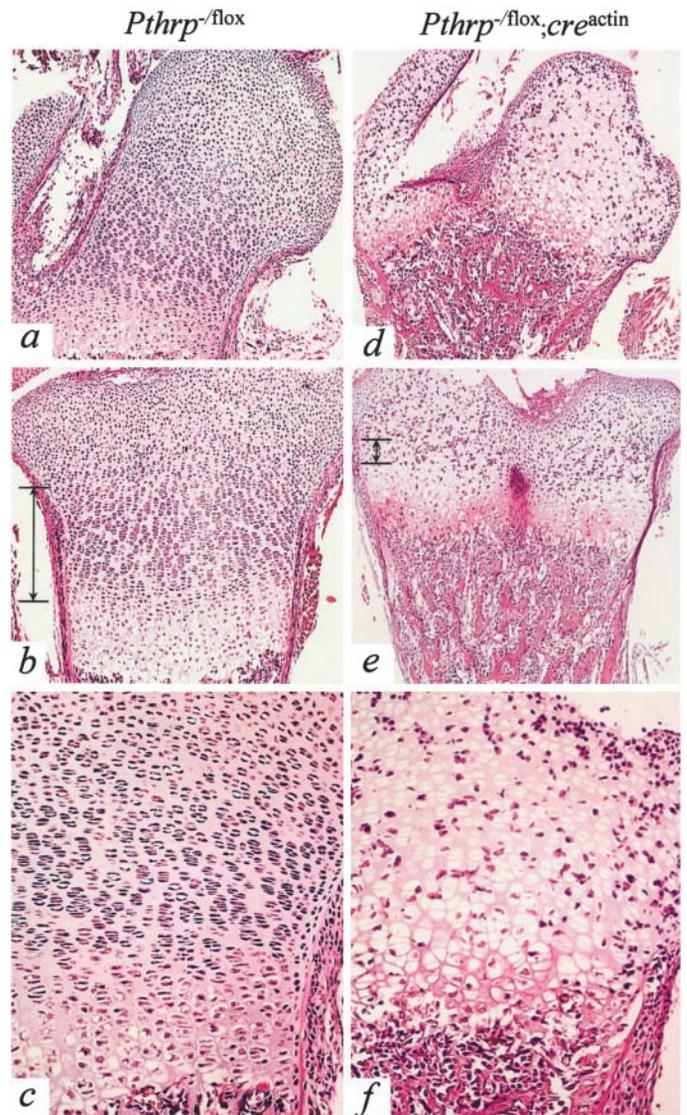
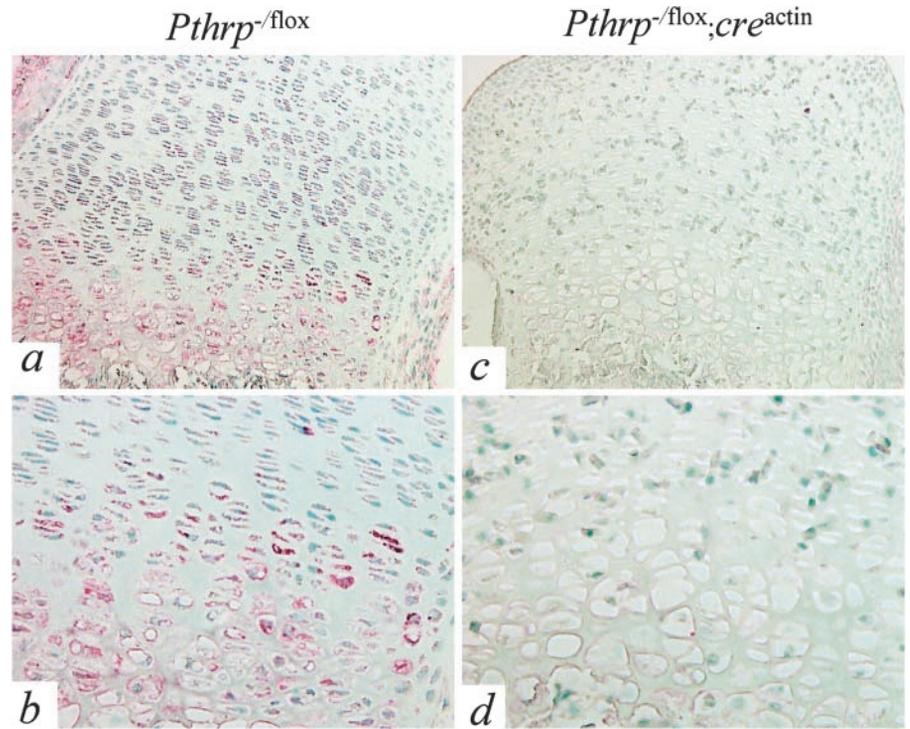


FIG. 5. Growth plate abnormalities at birth arising from Cre-mediated excision of  $Pthrp$ . Histological preparations from proximal femur (A and D) and tibial (B, C, E, and F) epiphyseal growth plate cartilage from  $Pthrp^{-/lox};cre^{actin}$  mice (D and E) show decreased length (arrows) and complete loss of the columnar organization in the proliferating chondrocyte zone compared with  $Pthrp^{-/lox}$  littermates (A and B). Higher power magnification of the mutant growth plate (F) compared with the growth plate of a control littermate (C), demonstrating the premature differentiation of chondrocytes to the hypertrophic state. H & E staining. Magnification:  $\times 100$  (A, B, D, and E) and  $\times 200$  (C and F).

dible resulting in protrusion of the tongue, narrow thorax, protuberant abdomen, and shortened long bones. Skeletal preparations stained with alcian blue (cartilage) and alizarin red S (calcified tissue) confirmed the inappropriate and premature ossification throughout the endochondral skeleton, and the anticipated deformities that arise as a consequence of the complete absence of  $Pthrp$  (data not shown). Histologic examination of long bones from these animals validated the anticipated growth plate abnormalities such as decreased size in the zone of proliferation, disorganization of chondrocyte columns, and premature differentiation of chondro-

FIG. 6. Pthrp immunohistochemistry in growth plate chondrocytes. Histological sections from proximal femur epiphyseal growth plate cartilage from *Pthrp*<sup>-flox</sup>; *cre*<sup>actin</sup> mice (C and D) show complete absence of Pthrp immunostaining in proliferating and prehypertrophic chondrocytes, compared with *Pthrp*<sup>-flox</sup> littermates (A and B). Methyl green counterstain. Magnification: ×200 (A and C) and ×400 (B and D).



cytes to the hypertrophic state (Figs. 4, A–F, and 5, A–F). Moreover, immunohistochemical staining of mutant growth plates for Pthrp failed to detect expression of the protein in chondrocytes (Fig. 6, A–D). These findings established that the floxed *Pthrp* allele was accessible to Cre-mediated excision *in vivo*. Subsequently, these studies were repeated with mice of the *Pthrp*<sup>flox/flox</sup>; *cre*<sup>actin</sup> genotype, and identical results were obtained, indicating that Cre-mediated excision was equally effective in removing two copies of the floxed allele.

### Discussion

In this study, we describe the generation of a mouse line that carries two *Pthrp* alleles flanked by *loxP* sites. The demonstration that the floxed sequence can be effectively excised *in vivo* and thereby reproduce the phenotypic alterations described in the original *Pthrp*-null fetuses, makes this animal model a valuable resource for now examining tissue-specific *Pthrp* gene function in the postnatal state. Such studies would otherwise not be possible in view of the perinatal lethality of the conventional null mutant homozygotes.

The advent of gene targeting in ES cells has, over the last decade, revolutionized the *in vivo* study of gene function and has contributed enormously to our understanding of factors that modulate an ever-increasing number of physiological processes. Yet, as is often the case, early lethality of the mutant strain, an outcome exemplified by the *Pthrp* knockout mouse, or complex phenotypes preclude such studies at later stages of life or in specific tissues. These limitations have led to the development of a new generation of tools for controlling gene expression *in vivo* that aim to circumvent such problems associated with the conventional knockout technology. By combining the *cre/loxP* site-specific recombination with the homologous recombination technology, it has be-

come possible to introduce genomic alterations that are restricting both spatially and temporally (16, 17).

Despite these advances, a number of potential drawbacks have become apparent with the advent of this novel technique. First, the ability to achieve tissue-specific gene deletion depends on the availability of *cre* transgenic mice that possess an exquisite degree of specificity and fidelity and achieve high levels in *cre* expression. Satisfactory tissue-specific restriction is achieved when Cre expression in these transgenic lines is under the transcriptional regulation of the appropriate tissue-specific promoter. On the other hand, nonspecific or low expression can severely confound interpretation of the resultant phenotype. Sorting out promoter specificity and activity has necessitated the generation of reporter mouse lines, like the Z/AP mouse, that provide a precise and accurate assay for Cre-mediated excisional activity at the cellular level (11, 18, 19). Although the Z/AP transgene was shown to be widely expressed in our studies, no *lacZ* expression was observed in chondrocytes and adipocytes, as originally reported (11). However, after Cre excision, both cell types stained intensely for hPLAP activity, suggesting that this discrepancy arises likely from possible sensitivity differences between the *lacZ* and the hPLAP reporters. In support of this conclusion is the profound alterations in the chondrocyte differentiation program observed in our *loxP/cre* progeny, confirming that Cre excisional activity had indeed taken place in these cells. Hence, the binary reporter system of the Z/AP transgene helps discriminate between lack of reporter expression and a lack of Cre excision.

The *cre*<sup>actin</sup> mice used in the present study were particularly ideal for driving expression of the Cre protein ubiquitously, as required for achieving a total-body deletion of the *Pthrp* gene. In crosses using these mice, recombination of the

target gene in the *loxP/cre* progeny has been reported to occur in every cell by the 64-cell stage of embryogenesis (15). The fact that Cre is expressed very early in development, leads to complete recombination in all cells of the embryo, as demonstrated in our studies following crosses of the *cre<sup>actin</sup>* mice to the Z/AP reporter strain. This makes the *cre<sup>actin</sup>* mice a very efficient line for generating progeny that carry the recombined form of the floxed allele.

Second, the resultant phenotype may be rather complex if deletion of the floxed gene is incomplete. This was a problem associated mainly with earlier studies that made use of the wild-type *cre* gene of P1 phage (20). The Cre recombinase expressed from the human  $\beta$ -*actin-cre* transgene used in this study was exceptionally efficient in excising the floxed allele. This degree of efficiency arose from two modifications introduced in the *cre* gene: first, the sequences surrounding the ATG translation initiation codon matched those reported to be optimal for translation initiation in eukaryotic cells (21), and second, the coding sequence for the seven-amino acid nuclear localization signal of the large T antigen of SV40 had been introduced into the amino-terminal region of the *cre* open reading frame (22).

These potential limitations notwithstanding, studies are now underway aiming to target the floxed *Pthrp* allele in a tissue-specific fashion. Crossing the *Pthrp<sup>flox/flox</sup>* strain to mice exclusively expressing Cre in a variety of cell types will facilitate the functional analysis of *Pthrp*'s role in the postnatal state. Findings arising from such studies will undoubtedly provide us with otherwise unobtainable information about potential actions of Pthrp in adult bone homeostasis, mammary gland, prostate and pancreatic islet cell function, blood pressure control and vascular responsiveness, and neuronal protection from apoptotic cell death.

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