

Human COL2A1-directed SV40 T Antigen Expression in Transgenic and Chimeric Mice Results in Abnormal Skeletal Development

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Abstract. The ability of SV40 T antigen to cause abnormalities in cartilage development in transgenic mice and chimeras has been tested. The *cis*-regulatory elements of the COL2A1 gene were used to target expression of SV40 T antigen to differentiating chondrocytes in transgenic mice and chimeras derived from embryonal stem (ES) cells bearing the same transgene. The major phenotypic consequences of transgenic (pAL21) expression are malformed skeleton, disproportionate dwarfism, and perinatal/neonatal death. Expression of T antigen was tissue specific and in the main characteristic of the mouse $\alpha 1(\text{II})$ collagen gene. Chondrocyte densities and levels of $\alpha 1(\text{II})$ collagen mRNAs were reduced in the transgenic mice. Islands of cells which express cartilage characteristic genes

such as type IIB procollagen, long form $\alpha 1(\text{IX})$ collagen, $\alpha 2(\text{XI})$ collagen, and aggrecan were found in the articular and growth cartilages of pAL21 chimeric fetuses and neonates. But these cells, which were expressing T antigen, were not properly organized into columns of proliferating chondrocytes. Levels of $\alpha 1(\text{II})$ collagen mRNA were reduced in these chondrocytes. In addition, these cells did not express type X collagen, a marker for hypertrophic chondrocytes. The skeletal abnormality in pAL21 mice may therefore be due to a retardation of chondrocyte maturation or an impaired ability of chondrocytes to complete terminal differentiation and an associated paucity of some cartilage matrix components.

DURING vertebrate development, the establishment of a cartilage template involves the orderly differentiation of chondrocytes. This is important as the template forms a temporary framework for the formation of endochondral bone (Horton, 1993). Cartilage also is important because it has to withstand high cyclical loads and absorb shocks. This is achieved by the synthesis of a complex extracellular matrix, consisting of hyaluronate-proteoglycan complexes (Rosenberg and Buckwalter, 1986) and at least five different collagens, type II, VI, IX, X, and XI (Mayne and Irwin, 1986), one of the major components of which is type II collagen. There is growing evidence that mutations in the human $\alpha 1(\text{II})$ collagen gene (COL2A1)¹ can cause some inherited chondrodysplasias, disorders which result in skeletal abnormalities characterized by disproportionate

dwarfism (Spranger and Maroteaux, 1990; Horton and Hecht, 1993). Mutations in COL2A1 have been implicated in Stickler syndrome (Francomano et al., 1987; Ahmad et al., 1991, 1993; Brown et al., 1992; Winterpacht et al., 1993), Kniest dysplasia (Winterpacht et al., 1993; Poole et al., 1988), lethal (Langer-Saldino) achondrogenesis (Vissing et al., 1989), spondyloepiphyseal dysplasia (Lee et al., 1989; Tiller et al., 1990; Cole et al., 1993; Vikkula et al., 1993a), Goldblatt syndrome (Bonaventure et al., 1992), hypochondrogenesis (Horton et al., 1992), and also in primary generalized osteoarthritis (Palotie et al., 1989; Vikkula et al., 1993b; Knowlton et al., 1990; Ala-Kokko et al., 1990). Dominant negative mutations in the $\alpha 1(\text{II})$ collagen gene expressed in transgenic mice result in severe chondrodysplasia (Metsäranta et al., 1992; Garofalo et al., 1991; Vandenberg et al., 1991). These studies confirm the association of mutations in COL2A1 with some forms of human chondrodysplasia. However, over 150 different chondrodysplastic conditions have been described in humans varying from being perinatal lethal, to deforming during life, to being so mild that they are difficult to detect (Spranger and Maroteaux, 1990; Horton and Hecht, 1993). The molecular bases underlying these disorders are largely unknown.

SV40 T antigen (Tag) is the major multifunctional regula-

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1. *Abbreviations used in this paper:* COL2A1, $\alpha 1(\text{II})$ collagen gene; d.p.c., days post coitum; ES, embryonal stem cells; Tag, SV40 T antigen.

tory protein of simian virus 40 and has multiple regulatory effects on eukaryotic cells (Stahl and Knippers, 1987; Butel and Jarvis, 1986; Fanning and Knippers, 1992). Expression of Tag in transgenic mice also promotes tumorigenesis (Hanahan, 1988; Adams and Cory, 1991). Tag has been found to stimulate cell proliferation by accelerating the cell cycle and inducing DNA synthesis. Transformation of some types of cells by Tag is also known to result in downregulation of the expression of some extracellular matrix components for example collagenase (Imai and Takano, 1992) and type I collagen (Bornstein and Sage, 1989; Parker et al., 1992). The differentiated phenotype of cultured chondrocytes has been shown to be sensitive to viral transformation and the expression of Tag or other oncogenes, which can result in varying degrees of dedifferentiation and a decrease in type II collagen synthesis (Adams et al., 1982; Benya and Brown, 1986; Thenet et al., 1992; Horton et al., 1988). However recently, Tag has been shown to induce chondrocyte proliferation without loss of the differentiated phenotype (Mallein-Gerin and Olsen, 1993).

Apart from mutations in cartilage matrix genes which result in a structurally defective extracellular matrix, it is possible that inappropriate control of chondrocyte differentiation can cause abnormal skeletogenesis brought about by a perturbation of cartilage development. Alternatively, chondrodysplasia could arise from a perturbation of levels or timing of expression of extracellular matrix components of cartilage and bone. To investigate these possibilities, based on the ability of Tag to alter the proliferative/differentiated state of cultured chondrocytes, we have used Tag as a tool to interfere with skeletogenesis by targeting Tag expression to differentiating chondrocytes in transgenic mice.

DNA sequences sufficient to direct tissue specific expression of the human COL2A1 gene in mice have been previously identified (Lovell-Badge et al., 1987b) and cell transfection studies have shown that the first intron of the rat α 1(II) collagen gene contains a chondrocyte specific enhancer element (Horton et al., 1987). Since the first introns of the rat and human α 1(II) collagen genes show high degree of conservation (Ryan et al., 1990), it was likely that the human intron sequences also contain a similar cartilage specific enhancer element. We therefore constructed a chimeric plasmid containing 4.5 kb of 5' flanking DNA and 1.8 kb from the first intron of the human COL2A1 gene linked to the Tag gene and introduced this construct (pAL21, see Fig. 1) into the mouse germline by transgenesis. This paper reports that the phenotypic consequence of carrying this COL2A1-Tag transgene is major skeletal malformation, disproportionate dwarfism, and perinatal death in the transgenic mice. Expression of Tag was tissue specific and we also show that the likely cause of the skeletal abnormality may be an impaired ability of chondrocytes to differentiate properly.

Materials and Methods

Generation and Identification of Transgenic and Chimeric Mice

Two plasmids were used to generate transgenic mice (see Fig. 1 [Cheah et al., 1985]). DNA inserts of plasmids pAL21 and pAA2 were released using KpnI and NotI and NotI alone, respectively (Fig. 1), and microinjected into the pronuclei of one cell embryos from F1 hybrid mice (C57BL/6 \times CBA/N). Transgenic mice were identified by PCR, Southern blot or dot blot analyses of genomic DNA prepared from the tails of embryos or recently

weaned mice. Identification of pAL21 transgenic mice by PCR was performed using nested oligonucleotide primers to the early region of the Tag gene (GenBank accession No. J02400) as follows: primer 1, 5'-TCAGGG-GGAGGTGTGGGAGG-3' (nt 2704-2723); primer 2, 5'-GGGCACTGG-AGGGGAGTCCA-3' (complementary to nt 3371-3390); primer 3, 5'-GAG-CCTTGGGACTGTGAATC-3' (nt 2808-2828) and primer 4, 5'-CCTAAA-ACACTGCAGGCCAG-3' (complementary to nt 3198-3217). PCR amplifications were for a first round of 20 cycles of denaturation at 94°C 0.1 min, annealing 65°C, 0.5 min and synthesis (2.5 U *Taq* polymerase, Cetus Corp.), 72°C 0.5 min using primers 1 and 2, followed by a second round of 20 cycles using 1 μ l from the first reaction and primers 3 and 4. The transgenic identities of pAL21 mice were confirmed by Southern hybridizations of BamHI digested genomic DNAs using a 2.7-kb BamHI fragment from pAL21 as a probe. pAA2 mice were identified by the PCR using the following oligonucleotide primers for the *E. coli lacZ* gene: 5'-CGTAATAGC-GAAGAGGCCCG-3'; 5'-TCCAACGCAGCACCATCACC-3' (nt 112-130, and complementary to nt 558-577, respectively; translation start = 1).

DNA insert from pAL21 (25 μ g) and 250 ng of BamHI linearized plasmid carrying the neomycin resistance gene, (pGKneo, gift of R. Krumlauf, National Institute for Medical Research, London, UK) was incorporated into the genome of CCE embryonal stem (ES) cells (gift of E. Robertson, Harvard University) by electroporation at 160 V, 960 μ F using a Gene Pulser apparatus (BioRad Labs., Hercules, CA). After growth in medium (Robertson, 1987) containing 0.4 mg/ml G418, resistant ES colonies were selected, and the presence of Tag transgene was confirmed by Southern blot analysis. Mouse chimeras were generated by injecting cells from a CCE line carrying four copies of pAL21 into C57BL/6J blastocysts (Bradley, 1987). Chimerism was determined on the basis of agouti pigmentation in the coat and by determination of copy number of pAL21 in somatic tissues.

RNA Probes, RNA Analyses, and In Situ Hybridization

Riboprobe for the detection of mouse α 1(II) collagen mRNAs in RNase protection assays were transcribed in vitro using plasmid pEL111 which contains exon 1 as described previously (Cheah et al., 1991). Probes for *Col2a-1* mRNAs used for in situ hybridization were pSOK10, containing the alternatively spliced exon 2 characteristic of type IIA procollagen mRNA and pEL111 (Cheah et al., 1991; Ng et al., 1993). Tag mRNAs were detected by RNase protection assays using a riboprobe transcribed from pKC32 (Fig. 1 [Hanahan, 1985]).

α 1(IX) short form and long form collagen (Muragaki et al., 1990) probes, pNJ59 and pNJ60 respectively, were made by RNA-PCR. Single-stranded cDNA was transcribed from 40 μ g of 17.5-d fetal total RNA by AMV reverse transcriptase (35 U, Seikagaku America Inc., Rockville, MD) using a primer complementary to exon 8 of the mouse α 1(IX) collagen gene (5'-CCGGAATCCAGGAGGC-3') in 0.05 M Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 125 μ M each of dATP, dCTP, dGTP, and dTTP at 42°C for 30 min. This cDNA was used as template for generating pNJ59 and pNJ60 by the PCR. pNJ59 was made using oligonucleotide primers complementary to exon 1* of mouse α 1(IX) collagen gene (5'-ATGGCC-TGGGTCGCTGGGG-3', as 5' primer and 5'-TTGAGCAGCACACAA-GCGGA-3', as 3' primer). pNJ60 was made using an oligonucleotide primer corresponding to exon 4 ([5'-GTAGACTTCAGGATTC-3'] as 5' primer) and exon 6 (5'-AGCATCCACTGGAGTTCAA-3') as 3' primer. The PCR was carried out in buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 375 μ M each of dATP, dCTP, dGTP, dTTP, 50-ng primers, and 5 U *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT) as follows: 1 min denaturation of DNA, 94°C; 1 min annealing reaction, 65°C; 2 min extension reaction, 72°C for 30 cycles. Both the RNA-PCR products were cloned in pGEM7-Zf(+) (Promega Corp., Madison, WI).

The α 2(XI) collagen probe was pLm3B1 which contained a 900-bp BamHI fragment encoding part of the C-propeptide of the mouse α 2(XI) collagen gene (Lui, C.-Y., L. J. Ng, and K. S. E. Cheah, manuscript in preparation) subcloned in pUBS (gift from G. Murphy, John Innes Institute, Norwich, UK). pNJ62 contained a 800-bp KpnI-BamHI fragment of mouse aggrecan cDNA (generous gift from Y. Yamada, National Institute of Dental Research, Bethesda, MD) subcloned in pGEM7-Zf(+). Mouse type X collagen probe was pRK26, encoding part of the COOH-terminal noncollagenous domain and 3' untranslated region (Kong et al., 1993). Mouse α 1(I) collagen probe contained exon 2 of the gene (Cheah et al., 1991).

Total RNA was prepared from heads, limbs, or ribs of embryos by the lithium chloride-urea method (Lovell-Badge, 1987). RNase protection assays and in situ hybridization were carried out as described (Cheah et al., 1991).

Immunohistochemistry and Western Blot Analyses

Cryostat sections (7 μ m) fixed in acetone were immunostained using a mouse monoclonal antibody (ID9) specific for SV40 large T antigen (gift of David Lane, University of Dundee, Scotland) as described (Cheah et al., 1991). Protein was extracted from mouse fetal limbs or neonate tissues (Fig. 6, see legend) and from a Tag expressing monkey epithelial cell line, 483/SV40 (gift of Beverley Griffin, Royal Postgraduate Medical School, London, UK). Cells/tissues were resuspended in 4% SDS, 20% glycerol, 125 mM Tris-HCl, pH 6.8, 1% 2-mercaptoethanol and disrupted by sonication on ice. Samples were boiled for 5 min, cell debris removed by centrifugation for 10 min at 10,000 g. Proteins were separated by SDS-PAGE and transferred to Hybond C Super membrane (Amersham Corp., Arlington Heights, IL). Membranes were prehybridized for 2 h at 20°C in 5% nonfat dry milk, PBS, 0.3% Tween 20 (PBST), and hybridized with ID9 antibody overnight at 4°C in PBST. After washing in PBST, membranes were incubated for 1 h with peroxidase-conjugated second antibody and the hybridization signal detected using Amersham's ECL system and Fuji RX film.

Results

COL2A1 Regulatory Elements in pAL21 Confer Tissue Specificity of Transgene Expression

The potential of the COL2A1 regulatory elements to direct Tag expression with proper tissue specificity was assessed by injecting a control construct similar to pAL21 except that Tag was replaced by the *lacZ* gene (pAA2, Fig. 1). The pattern of expression of *lacZ* in pAA2 transgenic fetuses obtained from different injection experiments was monitored at 9.5, 12.5, and 13.5 d. Fig. 2 shows that in a 12.5-d pAA2 transgenic embryo, *lacZ* staining was detected in sites expected for chondrogenic expression of $\alpha 1(\text{II})$ collagen mRNA. Low level expression was also seen in brain neuroepithelium. Similar results were obtained for 13.5-d fetuses (not shown). Histological sections of 9.5-d embryos show staining in the paraxial mesenchyme which gives rise to the chon-

drocranium (data not shown). Strong and specific expression in precursors of the cartilaginous primordia of the skeleton such as in vertebrae, limbs, and chondrocranium was also found between 12.5–18.5 d in fetuses from a pAA2 transgenic line of mice (data not shown). Expression of pAA2 in different transgenic fetuses (not shown, Leung, K. K. H., and K. S. E. Cheah, unpublished results) generally correlates with the pattern of expression of the mouse *Col2a-1* gene in chondrogenic and nervous tissues (Wood et al., 1991; Cheah et al., 1991), confirming that Tag expression could be targeted with proper tissue specificity by the COL2A1 regulatory elements in pAL21 mice.

pAL21 Transgenesis Is Associated with Perinatal Lethality and Abnormality, Not Tumorigenesis

Only 15 pAL21 transgenic mice were found among 184 adults, a low rate of 8% compared to a 32% transgenesis rate for adults obtained from injections using pAA2 (Table I). These pAL21 transgenic founders were morphologically normal and none developed tumors (at 2–3 years of age) before death of natural causes. Five founders were sterile and two others failed to transmit the transgene. Of the remaining 8 fertile founders, none of the transgenic offspring developed tumors. Female founder 123 produced a litter of 9 with only one transgenic pup which died at 11 d after birth of unknown cause and necropsy revealed no obvious anatomical abnormality. Founder 123 failed to breed thereafter and died naturally at 2.3 yr. Male founder 124 transmitted the transgene to only one out of 22 offspring. Back-crossing the transgenic offspring to founder 124 gave one normal heterozygous male and 2 abnormally small mice which were homozygous. The abnormal pups were cannibalized shortly afterward, leaving no tissues for biochemical or histological analyses. Two ab-

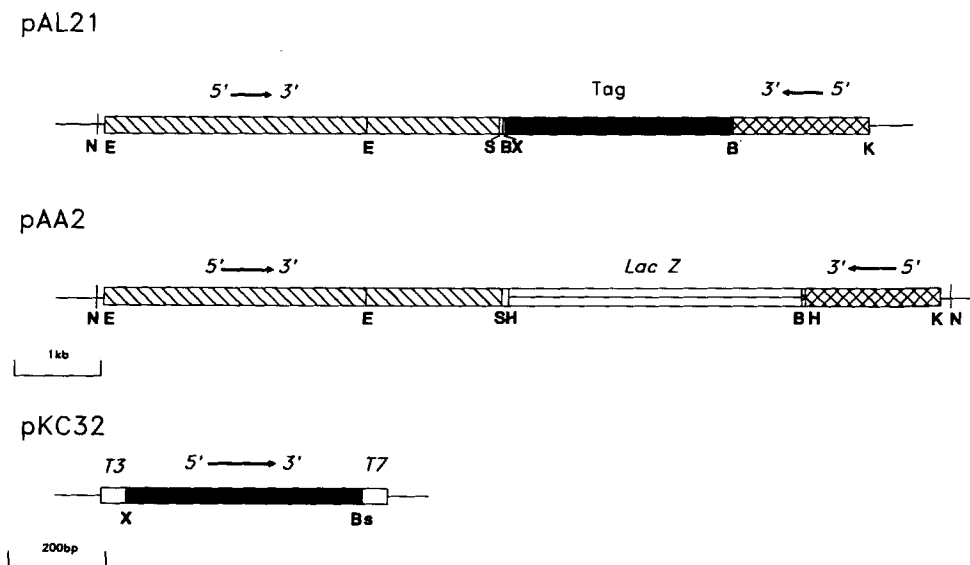


Figure 1. Restriction enzyme maps of plasmids. pAL21 contained 4.5 kb 5' flanking DNA sequence of the COL2A1 gene (Cheah et al., 1985) including the TATA box linked to an XbaI-BamHI fragment from RIPI-Tag (gift of D. Hanahan; [Hanahan, 1985]) containing the Tag gene (including the transcriptional start site) and a 1.8-kb KpnI-BamHI fragment which contains part of the first intron. pAA2 contained the same COL2A1 sequences in pAL21 linked to the *E. coli lacZ* gene. pKC32 contained a 476-bp BstXI-XbaI fragment from the 5' end of the Tag gene yielding a specific 254 base protected fragment in RNase protection assays. *Hatched box*, COL2A1 5' flanking sequences; *cross-hatched box*, COL2A1 first intron sequences; *solid box*, Tag gene; *lined box*, *lacZ* gene.

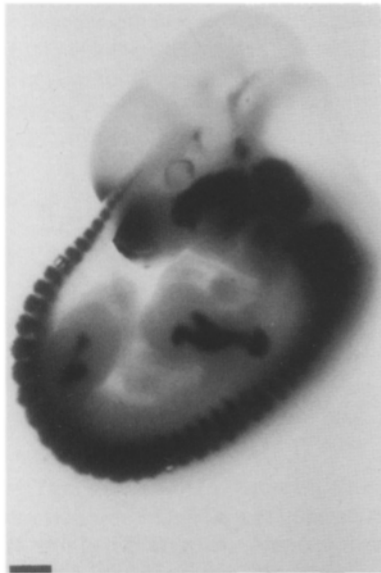


Figure 2. Predominant expression of *lacZ* in cartilaginous and neural tissues of a 12.5-d pAA2 transgenic fetus, reflecting the expression pattern of the endogenous *Col2a-1* gene. pAA2 specimens were fixed in 4% paraformaldehyde for 15–30 min and stained in X-gal reagent overnight and cleared in glycerine for microscopic examination. Approximately 17% of pAA2 transgenic fetuses expressed the transgene. Bar, 0.5 mm.

normally small mice were produced from the heterozygous offspring of mouse 124 and both died of unknown cause near to weaning age. Cannibalization of these mice precluded autopsy.

To determine if the low incidence of pAL21 transgenic mice at weaning age reflected cannibalization at birth and/or lethality in utero, the transgenesis rate and litter sizes were determined at different stages of gestation between 10.5 to 18.5 days post coitum (d.p.c.). The pAL21 transgenesis rate

Table I. Litter Sizes and Transgenesis Frequencies of pAL21 and pAA2 Mice

Age (d.p.c.)	Litter size per 30 embryos transferred (n)		No. of transgenic/No. of animals (%)*	
	pAL21	pAA2	pAL21	pAA2
10.5	21 (2)	6.4 (2)	9/16 (56)	0/10 (0)
11.5	12.5 (2)	na [†]	5/13 (39)	4/9 (44)
12.5	14.0 (6)	7.2 (17)	10/64 (16)	32/119 (27)
13.5	12.3 (3)	9.4 (4)	11/25 (44)	6/27 (22)
14.5	11.1 (13)	na [†]	22/117 (19)	5/9 (56)
15.5	12.5 (3)	ND	20/47 (43)	ND
16.5	9.9 (8)	ND	20/46 (44)	ND
17.5	9.2 (6)	na [†]	9/28 (32)	4/9 (45)
18.5	10.4 (22)	na [†]	61/125 (49)	0/1 (0)
19.5/birth	9.6 (47)	10 (10)	47/272 (17)	75/120 (63)
Weaning	5.9 (32)	10.9 (11)	15/184 (8)	50/157 (32)

Fetuses were harvested at various time points. To take into account the variations in embryo transfer between experiments and to allow inter-litter comparison, litter sizes were normalized on a per 30 embryo-transferred basis.

* Overall incidence of transgenesis for fetal stages: pAL21 = 38.2%; pAA2 = 41.4%.

[†] na, single observation, therefore not analyzed. ND, not determined; n, number of litters analyzed. d.p.c., days post coitum.

was higher for fetuses (overall 38%) than for newborns (17%), but the litter size remained relatively constant up to 18.5 d.p.c. (Table I). There was a high incidence of abnormality and/or stillbirth (37%) in newborn litters obtained from pAL21 injections and the majority (75%) of the abnormal newborns were transgenic. The results suggested that pAL21 transgenic mice were probably being lost during the perinatal period. There was a further loss of pAL21 pups during suckling, resulting in a significant drop in transgenic frequency at weaning age.

Overall 78 (36%) pAL21 transgenic fetuses between 10.5 and 18.5 d were found to display morphological defects compared to a 9% incidence of abnormality among their nontransgenic littermates. Abnormalities associated with pAL21 transgenesis were first identified in 20% of the 11.5-d fetuses and the frequency of recognizable defects increased steadily to 44.3% at 18.5 d.

To test if the neonatal lethality and abnormality was associated with the Tag transgene or was caused by competition between the COL2A1 sequences and the endogenous *Col2a-1* promoter for transcription factors, a comparison of transgenesis rates was made for injections using pAA2. The transgenesis frequencies and litter size at birth for pAA2 fetuses were similar to that of pAL21 up to 19.5 d.p.c. (Table I). A significantly higher transgenic frequency was found for pAA2 at birth and at weaning, and few pups died at birth or showed developmental retardation. Only 1 out of 51 pAA2 embryos and 2 out of 113 nontransgenic littermates at fetal stages displayed developmental retardation (small size) and the frequency of abnormality and lethality for transgenic pAA2 animals at birth was also low (11%). Overall, for experiments using pAA2, 9% of transgenic animals and 6% of nontransgenic littermates were abnormal.

The Major Abnormality in pAL21 Transgenic Mice Is Skeletal Malformation

Characteristic abnormalities in transgenic pAL21 newborn mice were foreshortened limbs with stumpy digits, flattened faces, abnormal neck and shoulder posture, short trunk, subcutaneous edema, and lethargic movement (Fig. 3). Alizarin red–alcian blue staining of skeletons (Fig. 4 A–C [Kessel and Gruss, 1991]) showed (a) retarded ossification of the craniofacial skeleton, (b) inappropriate sizes of skeletal components, (c) poorly developed cervical vertebrae resulting in hyperextension and shortening of the neck, and (d) the lack of chondrification, exemplified by the reduced alcian blue–stained matrix in otic capsules and laryngeal skeleton, and in an extreme case, the absence of frontonasal cartilage.

The types of abnormality associated with pAL21 and pAA2 fetuses are shown in Table II. Among the various types of defects present in the pAL21 fetuses, smaller body size and foreshortened limbs were the most frequently encountered. There was also a specific predominance of defects of the craniofacial, axial, and appendicular skeleton, and the brain in pAL21 fetuses. Skeletal abnormalities included shortening and/or hyperextension of the neck (11% of the transgenic pAL21 fetuses), reduction of limb (22%) and digit lengths (7%), and reduced head size and snout length (10%). Approximately 35% (24/69) of abnormal pAL21 transgenic fetuses displayed multiple skeletal defects. The frequency of multiple skeletal defects in abnormal nontrans-

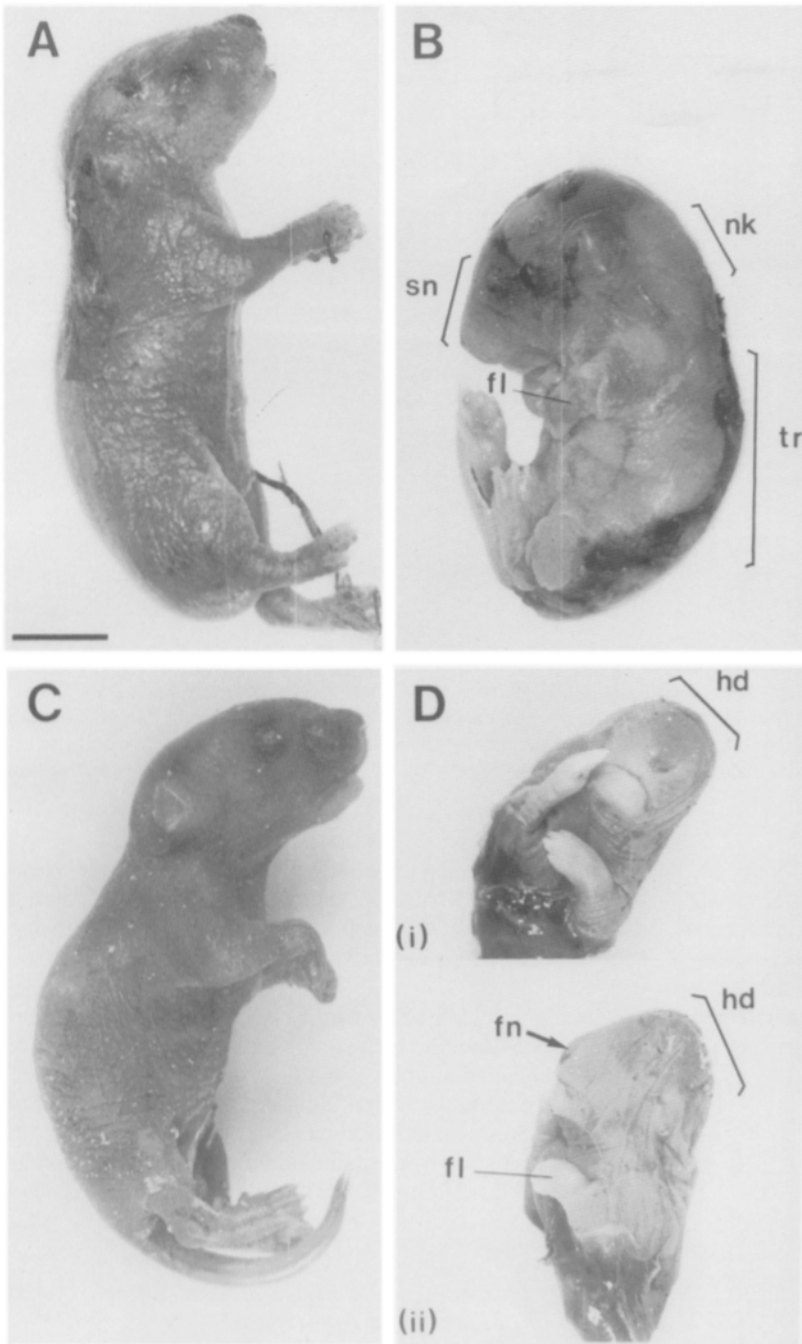


Figure 3. Abnormalities of pAL21 newborn mice. To minimize loss of neonates due to cannibalization at or just after birth, pups were born in rat cages so that they would fall through the larger mesh openings on delivery. (A and C) Nontransgenic pups, (B) transgenic littermate of A showing reduced trunk length (*tr*), foreshortened forelimb (*fl*), poor digit development, shortened neck (*nk*) and snout (*sn*), and (D) the partially cannibalized transgenic littermate of C showing stumpy forelimbs (*fl*), scaphoid and laterally compressed head (*hd*), and flattened face (*fn*). Bar, 5 mm.

genic animals from pAL21 injections was 7.4% (2/27) and none were observed for pAA2. Morphometric analyses of pAL21 transgenic fetuses at 15.5 and 18.5 d revealed a progressive reduction of head size and limb length and also a preferential reduction in the length of distal limb segments (Table III). Histological examination of the craniofacial structures revealed the poor differentiation of nasal septum, deformed and foreshortened cartilage of the base of the neurocranium. This deficiency in the midline chondrogenic tissues resulted in the medial and ventral displacement of craniofacial structures such as the inner ear, jaws, and nasal cavities (Fig. 5). However cleft palate was not observed.

Retarded brain development was observed in some transgenic fetuses (18%). There was poor differentiation of neu-

ronal layers of the brain, disproportionate growth of the mid-brain and forebrain, retarded development and inadequate foliation of the cerebellum, and a general reduction of brain size, which may be secondary to poor skull development (Fig. 5).

Tissue Pattern of Expression of Tag in pAL21 Mice Is Characteristic of Type II Collagen

The tissue pattern of expression of the COL2A1-Tag transgene was assessed in fetuses between 12.5 d and 18.5 d and in newborns using RNase protection assays, Western analyses and immunohistochemistry. RNase protection assays yielded protected fragments of expected size, suggesting cor-

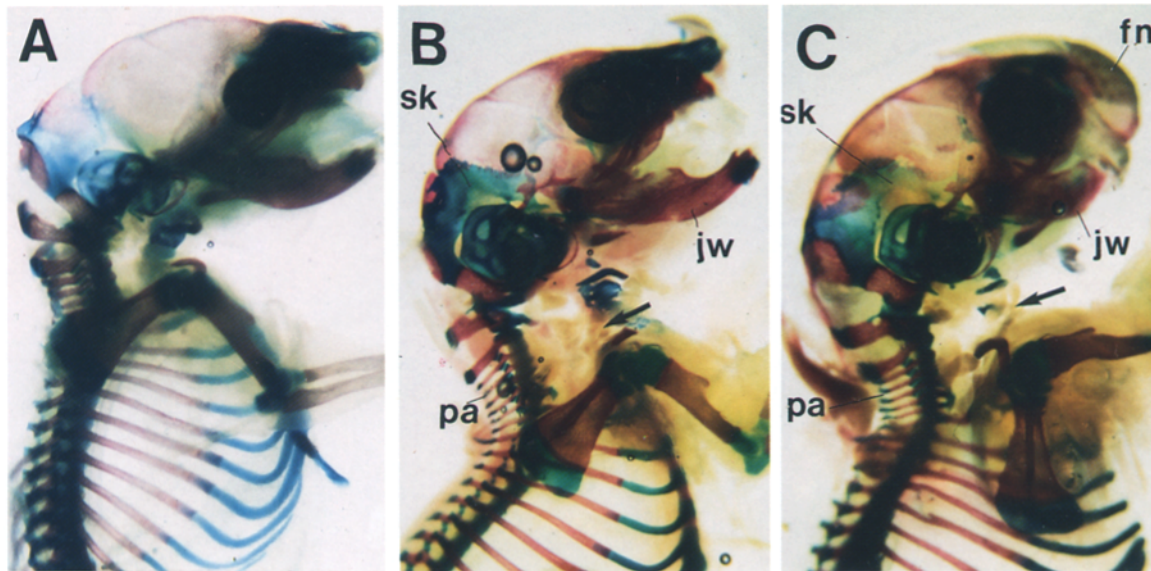


Figure 4. Alizarin-alcian blue skeletal preparations of (A) normal and (B and C) abnormal transgenic pAL21 newborn. Newborn mice were eviscerated and the skeletons were stained with alizarin red S and alcian blue (Kessel and Gruss, 1991). The transgenic pups display hyperextended neck due to the thinner posterior arches (pa) of cervical vertebrae and the congested intervertebral space. Chondrification is diminished in the sutural bone such as the temporal and supra-occipital components of the skull (sk), and in the larynx and trachea (arrow). The orbital cavity is oversized, but mandible and maxilla (jw) are underdeveloped. Pup (C) in addition shows a severely deficient frontonasal cartilage (fn). Bar, 5 mm.

rect initiation of transcription. Tag-specific antibody detected a 97-kD polypeptide expected for Tag in Western analyses, indicating that the transgene mRNA was properly translated (Fig. 6). Not all transgenic animals expressed Tag but a significant proportion did. Including fetuses and newborns, the total number where Tag mRNA could be detected was 22 animals out of 59 tested. Of 26 pAL21 transgenic

Table II. Incidence of Defects Observed in pAA2 and pAL21 Fetuses

Phenotype	pAA2		Frequency (% of all fetuses)* pAL21	
	NT	T	NT	T (% incidence)†
Resorbed/dead	ND	ND	0.3	10.8 (21.7)
Small size	5.8	9.4	4.4	13.5 (27.2)
Short limb	0	0	2.0	13.5 (21.7)
Short snout/small head	0	0	0.3	9.9 (20.0)
Short/hyperextended neck	0	0	0	5.4 (10.9)
Deformed or shortened skull base	0	0	0	0.9 (1.8)
Short trunk	0	0	0.6	1.8 (3.6)
Poor digit formation	0	0	0.6	7.2 (14.5)
Poor cartilage differentiation	0	0	0	3.6 (7.3)
Deformed brain	0	0	0	1.8 (3.6)
Retarded brain development	0	0	0	9.0 (18.2)

Fetuses from pAL21 and pAA2 experiments collected at various times of gestation (Table I) were first examined macroscopically for morphological assessment and processed histologically for determination of tissue abnormalities.

* Overall incidence of abnormal transgenic fetuses: pAL21 = 36.4% (78/214); pAA2 = 6.3% (8/126).

† Incidence of defect type in pAL21 transgenic fetuses.

NT, non-transgenic.
T, transgenic.
ND, not determined.

newborns tested, Tag expression was detected by both Western analysis and RNase protection assays in 10, of which 8 displayed abnormalities such as reduction in limb length and short snouts. A representative selection of analyses is shown in Fig. 6.

Immunohistology performed on 44 pAL21 transgenic fetuses revealed specific localization of Tag in chondrocytes in 48% of abnormal animals (Fig. 7 A), and, in some of these, a low level expression in neural tissues (data not shown). Tag expression was also detected by RNase protection in 12.5 d whole embryos and by immunostaining in the cartilage of fetuses at 14.5 d (data not shown). Nine of 14 abnormal new-

Table III. Morphometric Analyses of Size Differences between pAL21 and Wild-type Fetuses

	% Reduction in size for pAL21 fetuses	
	15.5 d.p.c. (5 pAL21; 10 wt)	18.5 d.p.c. (10 pAL21; 24 wt)
Head size	5.1	10.1 ($p < 0.07$)
Crown-rump	0.2	0.3
Arm length	4.9	5.3
Leg length	7.2	5.7 ($p < 0.05$)
Finger length	21.5 ($p < 0.05$)	15.3 ($p < 0.05$)
Toe length	18.3 ($p < 0.05$)	23.4 ($p < 0.05$)

Camera lucida drawings of the six body parts were made using a projection attachment on a Wild-dissecting microscope. Measurements of length were done by nonlinear tracing using a computer-assisted digitizer driven by a stereometry program. Head size was computed by multiplying the maximum length along the vertex-jaw and rostral-occipital axes. Data were tested for significant difference by non-parametric Mann-Whitney U test.
wt, wild-type.
d.p.c., days post coitum.

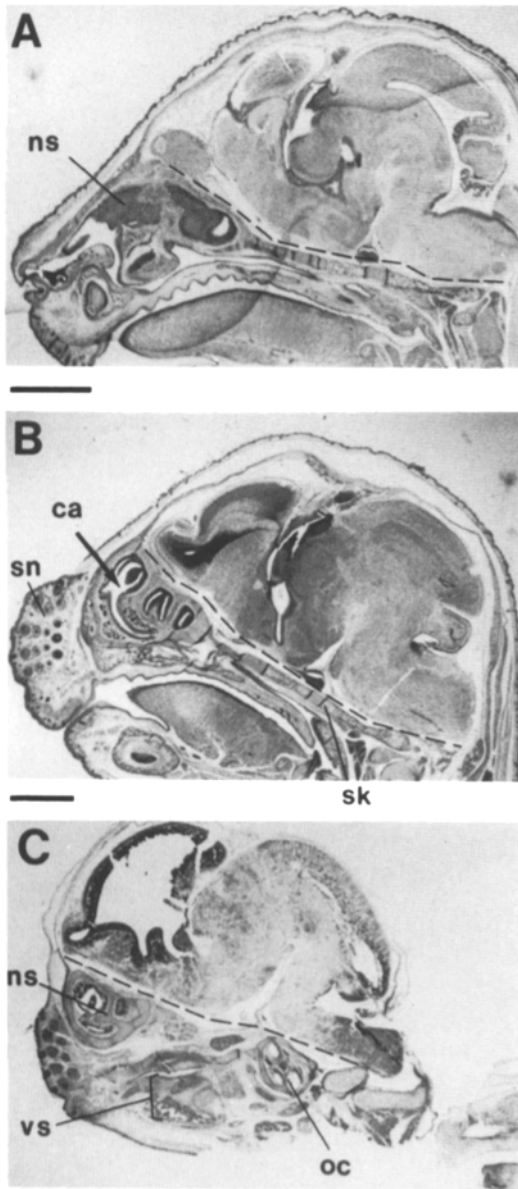
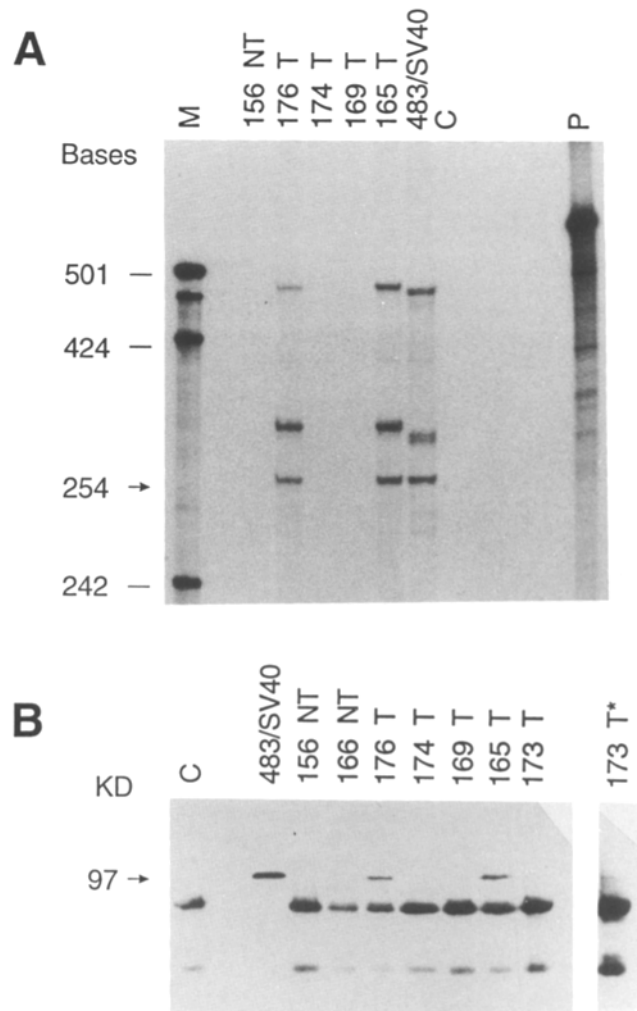


Figure 5. Histological sections of craniofacial regions of 17.5-d (A) nontransgenic fetus and (B) pAL21 transgenic littermate and (C) 15.5-d pAL21 transgenic fetus. Serial wax sections were stained with Harris haematoxylin and eosin. In the transgenic fetuses, the midline skeletal structures such as the nasal septum (*ns*), the visceral skeleton (*vs*), and the skeletal elements in the floor of the neurocranium (*sk*) are poorly developed, resulting in the medial and ventral displacement of the snout (*sn*), the nasal cavities (*ca*), and the otocyst (*oc*). The brain is deformed as a result of the reduction in the size of the cranium, particularly the fore-shortened skull floor (dashed lines). Bar, 1 mm.

Figure 6. Expression of Tag in pAL21 newborn mice. (A) RNase protection assay and (B) Western blot showing Tag expression in some of the transgenic newborns. RNA was prepared from the limbs or part of the head of newborn mice and analyzed for expres-



sion of Tag by RNase protection assays using pKC32 riboprobe (Fig. 1). Protein was prepared from approximately twenty 7- μ m cryostat sections from the same neonates (Nos. 173, 166: head and upper body; 165, 176, 169: head and neck; 156, 174: body) and the presence of Tag assayed by Western blot hybridization using ID9 monoclonal antibody. pAL21 transgenic mice Nos. 173, 174, and 176 were stillborn and abnormal; Nos. 165 and 169 were born alive with normal appearance; Nos. 156 and 166 were nontransgenic littermate. *T*, transgenic; *NT*, nontransgenic. (A) Presence of Tag mRNAs are shown by the expected sized 254 base protected fragment for Tag mRNA. Other higher molecular mass fragments represent partially digested products of RNase digestions. *M*, DNA molecular mass markers, HpaII digests of pGEM2; *C*, tRNA control reaction using yeast tRNA; *483/SV40*, RNA from monkey kidney cell line expressing Tag; *P*, [32 P]UTP-labeled riboprobe. (B) A distinct 97-kD protein was detected by ID9 antibody in the control 483/SV40 cells. *C*, control incubation with primary Tag antibody omitted, showing that the bands of lower molecular mass than 97 kD protein band represent nonspecific binding of the secondary antibody to mouse proteins. Tag expression was detected by both methods in Nos. 165 and 176. Mouse No. 173 expressed Tag at very low levels, with a signal appearing on the Western blot after prolonged exposure (*173T**). These findings were corroborated by immunohistology of sections using ID9 to stain cryostat sections (data not shown). Although transgenic mouse 174 showed no expression by RNase protection or Western, a low level of Tag staining which was patchy could be detected by immunostaining suggesting that it could be mosaic for the transgene. Mouse 169 was normal and did not express Tag.

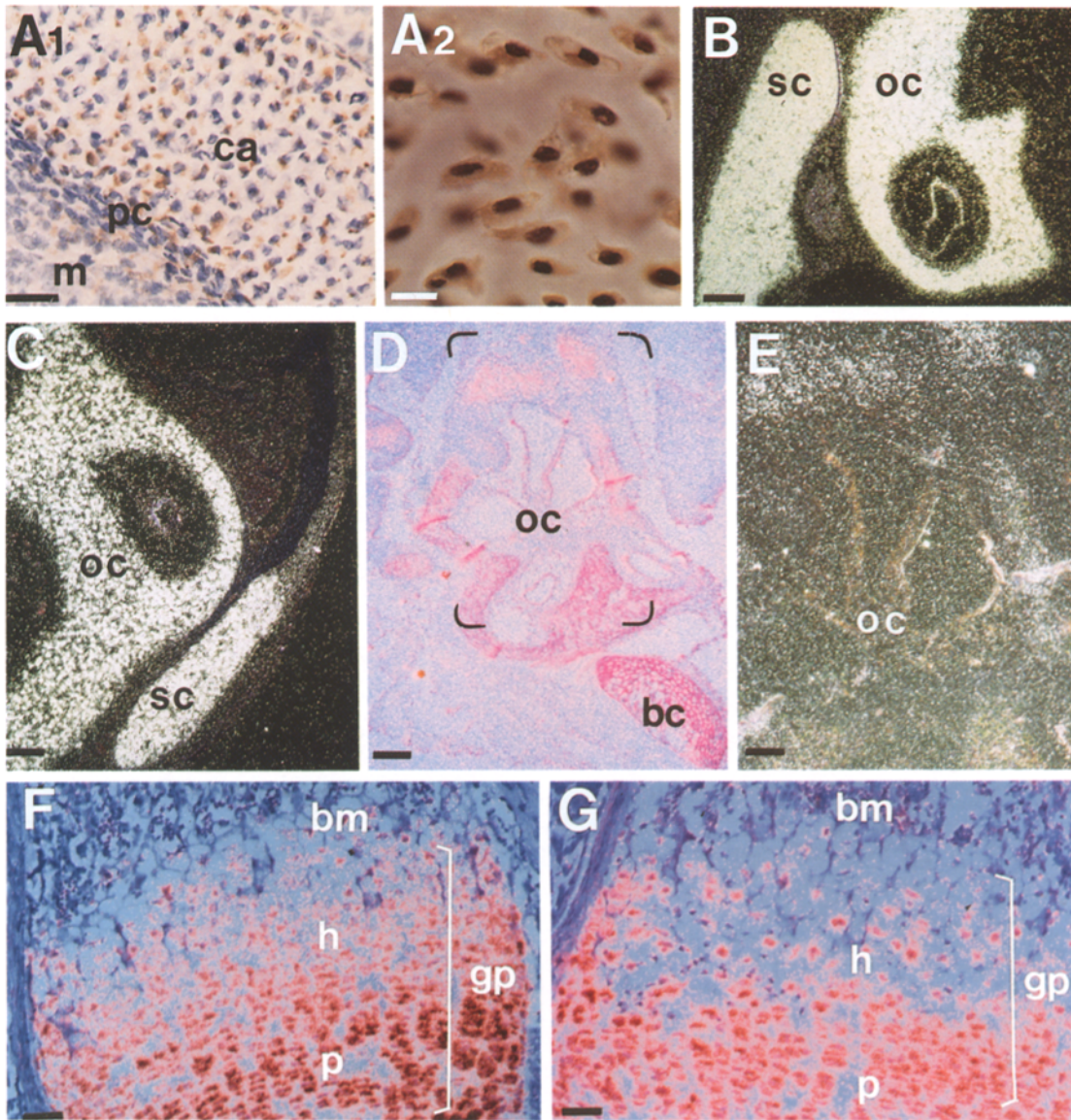


Figure 7. Expression of $\alpha 1(\text{II})$ collagen and Tag in chondrogenic tissues of pAL21 transgenic mice. Sections were counterstained with either 1% toluidine blue (*D* and *E*) or with Harris hematoxylin-eosin (*F* and *G*) after in situ hybridization. *B*, *C*, and *E* are pictures taken under dark-field illumination where silver grains appear white. *D*, *F*, and *G* are double exposures with a combination of bright-field with a blue filter and dark-field illumination under a red filter, to produce superimposed images where silver grains appear pinkish red. (*A*) Immunostaining of Tag (*I*) in the rib cartilage (*ca*) of a 14.5-d pAL21 transgenic fetus and (2) in the cytoplasm and nuclei of chondrocytes in the supra-occipital cartilage of an abnormal pAL21 transgenic newborn which died at birth. Sections were counterstained with haematoxylin. Note tissue-specific staining of Tag in chondrocytes and not in the supporting mesenchyme (*m*) or perichondrium (*pc*). Tag mRNA was also detected by RNase protection in the same newborn (not shown). Levels of expression of Tag varied between individual transgenic founders are expected because of differences in transgene copy number and integration site. (*B*) Strong in situ hybridization to *Col2a-1* mRNA displayed by chondrocytes in the otic capsule (*oc*) and adjacent supra-occipital cartilage (*sc*) of the nontransgenic newborn shown in Fig. 3 *A*. (*C*) Diminished intensity of hybridization to *Col2a-1* transcript in otic capsule (*oc*) and supra-occipital cartilage (*sc*) of a pAL21 transgenic littermate of *B* shown in Fig. 3 *B*. (*D*) Very weak hybridization to *Col2a-1* mRNA in the otic capsule (*oc*) of the 15.5-d pAL21 transgenic fetus is shown in Fig. 5 *C*. The otic capsule has a corrugated instead of a normal spherical shape. Low level hybridization is also seen in the basi-occipital cartilage (*bc*). The region shown at higher magnification in *E* is marked by the corners. (*E*) Higher magnification of region marked in *D*. (*F*) The growth plate (*gp*) of the humerus of a nontransgenic newborn (same as *C* above) showing expression of the *Col2a-1* gene in the proliferating (*p*) and hypertrophic chondrocytes (*h*). *bm*, bone marrow. (*G*) Weaker expression of *Col2a-1* mRNA in the growth plate (*gp*) of the humerus of a pAL21 transgenic littermate of *B* and *C* which had shorter limbs. Note a lower level of hybridization over the proliferating chondrocytes compared to the nontransgenic littermate in *F*. Abbreviations as for *F*. Bars: (*A1*) 20 μm ; (*A2*) 10 μm ; (*B*, *C*, and *D*) 100 μm ; (*E-G*) 50 μm .

borns were positive for Tag immunostaining of chondrocytes. Bone, mesenchyme, muscle, and skin were not stained which is consistent with the absence of type II collagen in these tissues (Fig. 7 A1 and data not shown). Consistent with the known nuclear localization of Tag in SV40-infected cells, expression was detected in the nucleus (Fig. 7 A2). Levels of Tag expression as assessed by the intensity of immunostaining varied between transgenic mice, and there was no direct correlation with degree of abnormality. In 3 out of 10 pAL21 newborns of normal morphology, Tag staining in cartilage was patchy and at very low levels indicating these animals were probably mosaic for the transgene. In two of those animals (such as No. 174, Fig. 6), Tag expression could not be confirmed by RNase protection assays or Western blotting.

Tag expression was found in offspring from only two of the pAL21 transgenic founders which survived to adulthood: 123 and 124, both of which produced abnormal offspring. The single transgenic offspring of 123 was expressing Tag in rib, limb, and calvaria at the time of death. Expression of Tag in line 124 was found at 14.5 d.p.c. but not in newborns. No expression of Tag was found at fetal or adult stages for the other 6 fertile transgenic lines which probably accounts for their normal phenotype and postnatal survival.

Chondrocyte Densities Are Reduced in pAL21 Transgenic Mice

A significant reduction (20–30%) of the cell population was found for the proliferative and prehypertrophic chondrocytes in different cartilaginous organs, including otic capsule, nasal septum, base of skull, vertebrae, occipital bone, digit, and humerus of the transgenic pAL21 fetuses, particularly at late gestation (Table IV). The decreased number of chondrocytes cannot be attributed to an increase in matrix because to the contrary, amounts were reduced. In situ hybridization of tissue sections of pAL21 fetuses at 15.5, 17.5, 18.5 d and in newborns using a *Col2a-1* gene probe showed reduced levels of hybridization (Fig. 7, B–G). The density of

silver grains over the five cartilaginous tissues from a range of pAL21 transgenic and nontransgenic fetuses was examined and compared in experiments using the same riboprobe and identical exposure times. Levels of hybridization in chondrogenic tissues were very high for nontransgenic fetuses. Low levels of hybridization were found in ~20% of the pAL21 fetuses and were associated with severe abnormalities in cartilaginous structures (Fig. 7, D and E). The remaining pAL21 fetuses displayed moderate to strong levels of hybridization (Fig. 7 C). Transgenic fetuses showing strong levels of hybridization were morphologically normal. In the growth plate shown in Fig. 7 G, fewer cells were positive for $\alpha 1(X)$ collagen, a marker for hypertrophic chondrocytes (data not shown).

It was not possible in every case to determine if the lower levels of hybridization seen reflected a decrease in chondrocyte numbers alone or included reduced levels of *Col2a-1* mRNAs on a per cell basis. However, the lower level or absence of hybridization in hypertrophic chondrocytes seen in several pAL21 animals compared to wild-type (Fig. 7, F and G) indicates that cellular levels of *Col2a-1* mRNAs were reduced. In two fetuses hybridization was high in the morphologically normal vertebrae but was low in the under developed nasal septum. In two neonates with craniofacial abnormalities, reduced hybridization signal was found in the otic capsule and growth plate of the humerus (Fig. 7, D and G) but not in proliferating chondrocytes of vertebrae. Lowered hybridization levels found only in abnormal cartilage but not in morphologically normal cartilaginous structures in a single animal suggests a direct association between cartilage abnormality and a reduction in cell density and/or type II collagen gene expression.

pAL21 Chimeras Show Skeletal Malformation and Abnormal Chondrocyte Differentiation

Due to the dominant perinatal lethal effect of the Tag transgene in pAL21 mice, and because each individual transgenic newborn or embryo is the product of unique integrations

Table IV. Comparison of Chondrocyte Densities in Cartilaginous Tissues of pAL21 and Wild-type Fetuses

Cartilaginous structures	Chondrocyte density (No./mm ²)					
	Wild type, n = 5			pAL21 (% reduction in density) n = 5		
	15.5 d.p.c.	17.5 d.p.c.	18.5 d.p.c.	15.5 d.p.c.	17.5 d.p.c.	18.5 d.p.c.
Otic capsule	9.85 ± 0.70	8.03 ± 0.38	9.29 ± 0.80	6.88 ± 0.89 (30.1)	5.65 ± 0.45‡ (29.6)	6.10 ± 0.91* (34.3)
Base of skull	8.20 ± 0.45	8.61 ± 0.36	ND	5.45 ± 0.21‡ (33.5)	6.88 ± 0.27‡ (20.1)	ND
Occipital bone	9.75 ± 0.71	7.90 ± 0.27	ND	7.06 ± 0.54* (27.6)	5.25 ± 0.38‡ (33.5)	ND
Vertebrae	9.80 ± 0.68	7.91 ± 0.77	ND	7.51 ± 0.37* (23.4)	5.48 ± 0.20* (30.7)	ND
Digit	ND	ND	8.72 ± 1.12	ND	ND	6.99 ± 0.79‡ (19.8)
Forelimb humerus	ND	ND	6.74 ± 0.70	ND	ND	5.11 ± 0.62‡ (24.2)
Nasal septum	9.35 ± 0.41	8.51 ± 0.59	8.34 ± 0.37	6.32 ± 0.44‡ (32.4)	6.59 ± 0.43* (22.6)	6.23 ± 0.51‡ (21.0)

Chondrocyte density was estimated on stained sections of pAL21 transgenic and wild-type 15.5-, 17.5-, and 18.5-d fetuses. The total number of chondrocytes (proliferating and prehypertrophic) was scored in a defined area of the cartilage. Scoring was done at 20 magnification and three separate counts were obtained from each section.

* $p < 0.05$.

‡ $p < 0.01$.

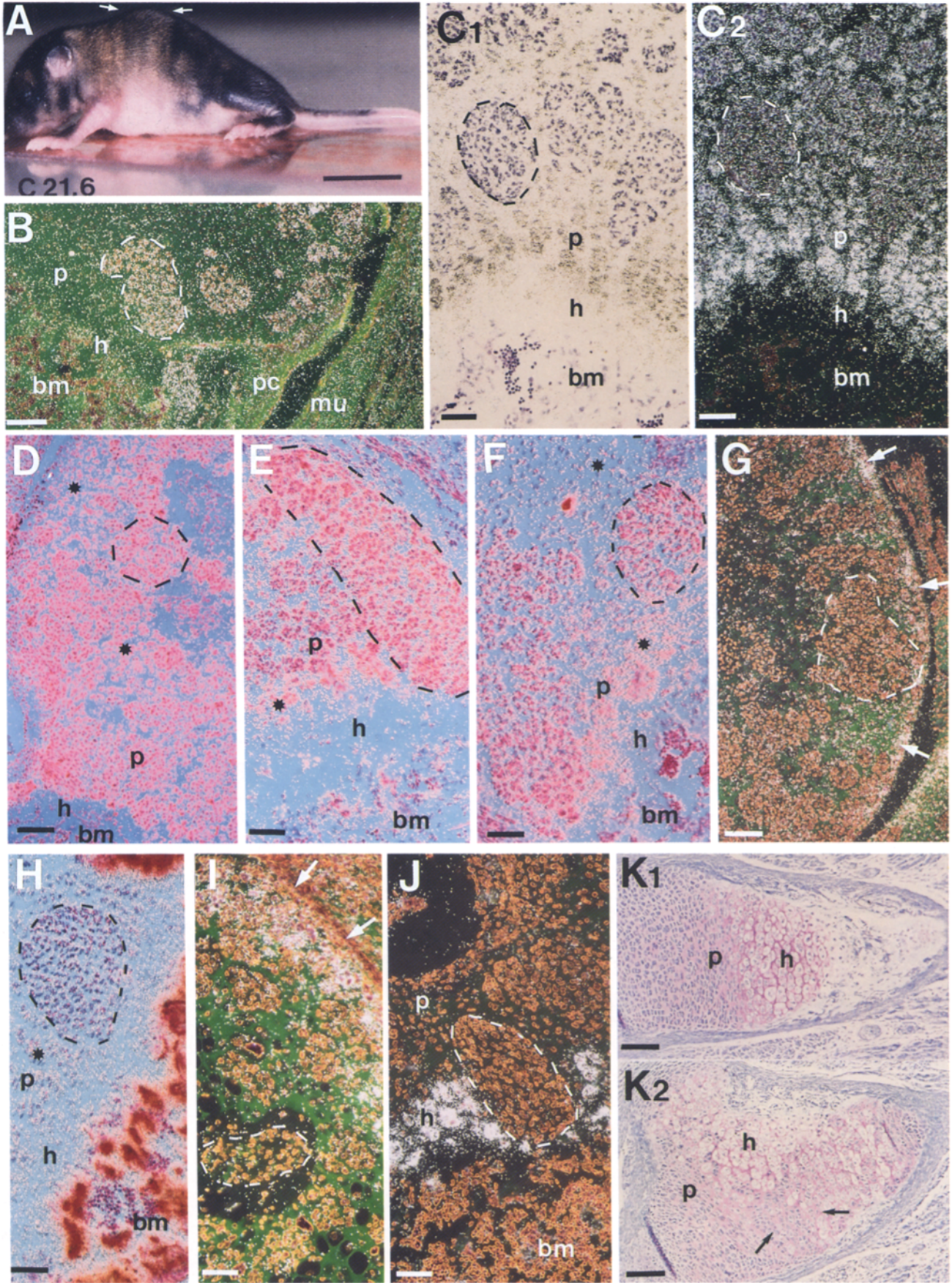
§ $p < 0.001$.

¶ $p < 0.0556$.

† $p < 0.0079$: significant difference in chondrocyte density between pAL21 fetuses and nontransgenic littermates.

ND, not determined.

d.p.c., days post coitum.



leading to variable severity of phenotype, a developmental analysis of the effect of COL2A1 targeted Tag expression on chondrocyte differentiation and on postnatal skeletal development was not possible. To overcome this difficulty, embryonic stem (ES) cells which carry integrated copies of pAL21 were used to produce mouse chimeras. Four of these chimeras have survived to neonatal stages but were typically smaller than normal non-chimeric mice and displayed severe kyphosis (Fig. 8 A) and retarded ossification of the skeleton. Severity of phenotype correlated well with the degree of chimerism (data not shown). Analyses of two such chimeric neonates, revealed the presence of islands of cells within articular cartilage with densely packed cells (Fig. 8) and reduced intercellular toluidine blue staining indicating reduced amounts of matrix (not shown). Similar islands of cells were found in the epiphyseal growth plate of lone bones but they were not organized into columns of proliferating chondrocytes. These islands of cells could also be found in a chimeric fetus at 15.5 d (Fig. 8 K), which also showed disorganization of other cartilages such as otic capsule and carpals of the foot plate (data not shown). Tissue specific expression of Tag in the chimeric neonates could be demonstrated by RNA and protein analyses. In situ hybridization showed that the cells within these islands expressed Tag mRNAs, which were absent in normally organized chondrocytes, perichondrium, skin, and muscle (Fig. 8 B). Expression of Tag in cartilaginous tissues was also confirmed by Western analyses of protein extracted from chimeric tissues (data not shown).

Chondrogenesis is a multistep process whereby mesenchymal cells differentiate into chondrocytes which mature, proliferate, and finally become hypertrophic. These stages of differentiation are marked by the expression of characteristic mRNAs. Type II procollagen mRNAs are alternatively spliced to give a type IIA form which contains an extra exon encoding a cysteine-rich domain or type IIB form without this exon (Ryan and Sandell, 1990). The IIA form is preferentially expressed by prechondrocytes and proliferating

chondrocytes but not by mature or hypertrophic chondrocytes (Sandell et al., 1991; Ng et al., 1993). Differentiated chondrocytes (mature, proliferating, and hypertrophic) express the type IIB form. There are two alternative transcripts for the $\alpha 1(\text{IX})$ collagen gene, one encoding a long form α chain with a 266-amino acid globular domain, and the other, a short form α chain without. In chick and mouse, maturing and proliferating chondrocytes express long form $\alpha 1(\text{IX})$ collagen mRNA whereas the short form is restricted to prechondrocytes and non-chondrogenic tissues (Swiderski and Solursh, 1992a,b; Liu et al., 1993; Ng, L. J., P. P. L. Tam, and K. S. E. Cheah manuscript in preparation). Type X collagen is only expressed by hypertrophic chondrocytes (Schmid and Linsenmayer, 1987). Chondrocytes do not express type I collagen (Cheah et al., 1991) and its synthesis is a sign of dedifferentiation (Benya and Brown, 1986).

The identity of the cells within the islands and their state of differentiation was assessed by the expression of genes which are characteristic markers for chondrocytes. These cells hybridized with the exon 1 probe of *Col2a-1* gene albeit at a lower level compared to their surrounding normal counterparts (Fig. 8 C). The absence of hybridization to the type IIA-specific probe shows the islands of cells were expressing type IIB procollagen (Fig. 8, C1 and G). These cells also expressed mRNAs for $\alpha 2(\text{XI})$ collagen, the long form of $\alpha 1(\text{IX})$ collagen, and aggrecan (Fig. 8, D-F). Levels of expression of other cartilage characteristic mRNAs, such as aggrecan and $\alpha 2(\text{XI})$ collagen mRNAs were not affected in the disorganized chondrocytes (Fig. 8, D-F). There was no expression of mRNAs for $\alpha 1(\text{I})$ collagen, the short form of $\alpha 1(\text{IX})$ collagen, and type X collagen (Fig. 8, H-J).

Discussion

The skeletal abnormalities displayed by pAL21 mice resemble the disproportionate dwarfism seen in human chondrodysplasia and in those transgenic mice carrying mutant *Col2a-1* alleles (Metsäranta et al., 1992; Garofalo et al.,

Figure 8. Expression of Tag and gene markers on chondrocyte differentiation in articular cartilage and growth plate of pAL21 chimeric mice. In situ hybridization study of genes expressed in the epiphyses of pAL21 chimeric mice. Sections (B-J) were counterstained with Harris haematoxylin-eosin after in situ hybridization. B, C2, G, I, and J are pictures taken under dark-field illumination. D-F and H are double exposures as described for Fig. 7. C1 is a bright-field picture of C2. Sections K1 and K2 were counterstained 1% toluidine blue. The greenish color seen in the dark-field pictures G and I is due to histological staining of the emulsion. (A) An 8-d-old chimeric pup (C21.6) produced using ES cells carrying four copies of pAL21 showing foreshortened snout, stumpy limbs, and a hunched back (arrows) caused by severe kyphosis. Degree of chimerism as determined by estimation of pAL21 copy number in seven different tissues from C21.6 with reference to an endogenous gene (type X collagen) varied from 18–59% (data not shown). (B) Dark-field picture of in situ hybridization showing signal for Tag mRNA in the islands (dashed outlines) of disorganized chondrocytes in the limb articular cartilage and growth plate of C21.6. Hybridization signal was at background levels in normal proliferating (p) and hypertrophic (h) chondrocytes, perichondrium (pc), muscle (mu), and bone marrow cells (bm). (C) In situ hybridization showing strong signal for $\alpha 1(\text{II})$ collagen mRNA in normal proliferating chondrocytes (p) and reduced levels in the islands (dashed outlines) of disorientated chondrocytes in the limb growth cartilage of C21.6. Transcripts correspond to the IIB form of $\alpha 1(\text{II})$ collagen mRNA because IIA is not expressed (see G). Hybridization signal was absent in bone marrow cells (bm). (D-F) In situ hybridization showing strong signals for mRNAs for long form $\alpha 1(\text{IX})$ (D), collagen $\alpha 2(\text{XI})$ (E), aggrecan (F) in the islands of cells (dashed outlines) and the surrounding normally organized chondrocytes (representative regions marked by *). Abbreviations as for B. (G-J) In situ hybridization showing absence of expression of transcripts for the type IIA form of the $\alpha 1(\text{II})$ collagen gene (G), $\alpha 1(\text{I})$ collagen (H), short form $\alpha 1(\text{IX})$ collagen (I), and type X collagen (J) in the islands of cells (dashed outlines). Silver grains over the islands of cells were at background level, comparable to the surrounding normal chondrocytes. Note expression of type X collagen mRNA by hypertrophic chondrocytes (h) surrounding the island of cells. Type IIA and short form $\alpha 1(\text{IX})$ mRNAs were restricted to prechondrocytes (arrows). Type I collagen mRNAs were restricted to the perichondrium (not shown) and trabeculae. G, I, and J, dark-field picture; H, double exposure. Abbreviations as for B. (K) Sections of the long bone cartilage of 15.5 d.p.c. non-chimeric (K1) and chimeric (K2) fetal littermates. Note presence of islands of cells interrupting the hypertrophic zone in K2 (black arrows). Abbreviations as for B. Bars: (A) 1 cm; (B, D, G, and K) 100 μm ; (C-F, H-J) 50 μm .

1991; Vandenberg et al., 1991; Spranger and Maroteaux, 1990; Horton and Hecht, 1993) where the mutant phenotype can be attributed to the presence of aberrant type II collagen molecules in the matrix. But the pAL21 phenotype is not caused by a mutation in the *Col2a-1* gene. Several lines of evidence show that the skeletal abnormalities seen in the COL2A1-Tag transgenic mice could be specifically attributed to the transgene. First, the common skeletal abnormality found in large numbers of different pAL21 mice excludes the possibility of an integration site-specific effect. Second, pAA2 transgenic mice showed a lower frequency of unrelated abnormality suggesting that the skeletal abnormalities in pAL21 mice were not due to a competition for *Col2a-1* transcription factors caused by the integration of the COL2A1 sequences.

Third, the detection of Tag in some 12.5-d embryos in advance of chondrogenesis, the ability of the COL2A1 sequences in pAL21 and pAA2 to direct expression of the transgenes to the major sites of expression of *Col2a-1*, and the correlation between the type of affected tissues in pAL21 transgenic fetuses and the known sites of expression of the *Col2a-1* gene during embryogenesis (Table III and [Cheah et al., 1991]) implicate Tag as the cause of the skeletal malformations. Levels of expression of Tag varied in different transgenics since each of these differ in copy number and integration site. Tag may also be inappropriately expressed in some cases because of the influence of genomic sequences at the site of transgene integration. This is normal for transgenic experiments. It is likely that the presence of Tag precedes the appearance of abnormality and although Tag could not be demonstrated in all abnormal pAL21 mice when they were assayed, previous Tag expression cannot be excluded. Tag found in some mice with no clear abnormality could represent a combination of inappropriate timing, low level of expression of the transgene and/or mosaic expression since Tag levels were very low and immunostaining was patchy.

Several observations suggest that the chondrodysplastic phenotype in pAL21 transgenic mice may be caused by an impaired or retarded ability of chondrocytes to differentiate fully. First, there was a correlation between reduction in chondrocyte densities and abnormalities in cartilaginous tissues of some pAL21 fetuses (Figs. 5 and 7). Second, in five abnormal pAL21 18.5-d fetuses showing disproportionate limb reduction, there was reduced chondrocyte density in the growth plate of the humerus (Table IV). Third, in some abnormal pAL21 transgenic neonates there was reduced hypertrophy of chondrocytes and a paucity of matrix (Figs. 4 and 7). Endochondral ossification was also less advanced compared to nontransgenic littermates (Fig. 5). These phenotypic deviations are consistent with an immature state of differentiation (Horton, 1993). Fourth, islands of cells which expressed Tag and were improperly organized were found in the articular and growth plate cartilage of 15.5-d chimeric fetuses and in chondrodysplastic pAL21 chimeric neonates. Because Tag expression could be detected in some transgenic embryos as early as at 12.5 d and reduced chondrocyte densities were found at 15.5 d, an effect of Tag on proliferation cannot be excluded. However such an effect would have to be tested for using specific assays such as thymidine uptake studies.

In the chimeras, the expression of cartilage characteristic genes (type IIB, long form $\alpha 1$ (IX), $\alpha 2$ (XI) collagens and

aggrecan) and the absence of type I collagen mRNA by the disorganized cells within the islands, indicate that they were chondrocytes. The presence of disorganized chondrocytes at fetal as well as neonatal stages and the absence of expression of type X, type IIA, and short form type IX collagens by these cells (Fig. 8), suggest that in the chimeras, pAL21-derived cells can undergo chondrogenesis but there is retardation or failure of progression to the final stages of proliferation, terminal differentiation, and hypertrophy. The absence of type X collagen expression in the islands of cells in the chimeras also suggest that the chondrodysplastic phenotype in Tag mice is not simply a consequence of lowered levels of type II collagen expression. Because the ES-derived chondrocytes fail to participate properly with the host cells in columnar organization, this disrupts the growth plate. The reduction in proliferating and hypertrophic chondrocytes found for pAL21 transgenic mice correlates well with these findings and suggests that retarded differentiation is the more likely. In transgenics there was no disorganization probably because all the cells were behaving similarly. Instead poorer or retarded differentiation and/or proliferation would result in fewer numbers of chondrocytes and a reduction of cartilage size. This defect in differentiation was probably not restricted to the growth plate since other cartilages, such as nasal septum, base of skull, vertebrae, and otic capsule were malformed in some transgenics and chimeras.

It is worth noting that disorganized and densely packed immature chondrocytes have been observed in some examples of human chondrodysplasias where the molecular basis of the disease is unknown (Horton and Hecht, 1993). An example is thanatophoric dysplasia, which is characterized by a lack of organized chondrocytes and a reduction in numbers of proliferative and hypertrophic chondrocytes suggesting a defect in differentiation/proliferation. Reduced chondrocyte proliferation has recently been reported in a short rib syndrome type Beemer patient (Brenner et al., 1993). In addition lethal skeletal dysplasia in mice lacking parathyroid hormone-related peptide has been shown to be associated with premature maturation of chondrocytes (Karaplis et al., 1994).

Cultured SV40 transformed chondrocytes dedifferentiate and downregulate type II collagen gene expression (Thenet et al., 1992), although an exception has been reported recently (Mallein-Gerin and Olsen, 1993). Those cultured SV40 transformed mouse chondrocytes which maintained phenotype were, however, unable to undergo hypertrophy (Mallein-Gerin and Olsen, 1993). It is of note that there was a reduction in the number of hypertrophic chondrocytes in some of the pAL21 transgenic mice and an absence of type X collagen expression in pAL21-ES cell-derived disorganized chondrocytes in the chimeras. Levels of expression of $\alpha 1$ (II) collagen mRNAs were reduced in abnormal transgenics and for those disorganized groups of chondrocytes seen in the chimeras. Restriction of these changes to the islands of cells suggest the effect of Tag is cell autonomous. One way Tag is thought to cause changes in transcriptional activity is by mediating the modification or induction of cellular DNA-binding factors (Stahl and Knippers, 1987; Gallo et al., 1990). An examination of the 5' flanking region and first intron of the human and mouse $\alpha 1$ (II) collagen genes (Vikkula et al., 1992) reveals the presence of several se-

quence motifs known to bind Tag (DeLucia et al., 1983). Tag has also been shown to alter the cell-cycle duration of exponentially growing cells (Sladek and Jacobberger, 1992). Therefore, Tag could have interfered with the expression of the *Col2a-1* gene and the terminal differentiation of chondrocytes in the pAL21 transgenic mice.

To determine the precise cause of the chondrodysplasia in pAL21 mice, it would be important to distinguish if abnormal chondrocyte differentiation was the primary consequence of Tag expression or was secondary, as a response to Tag-induced downregulation of type II collagen expression. A developmental study of Tag expression and the onset of abnormal chondrocyte differentiation in the chimeras as well as identification of the gene targets for Tag action, will help address this question. Since levels of type II collagen expression were reduced not ablated, an additional approach could be to determine the consequence of introducing pAL21 into the germline of phenotypically normal transgenic mice which overexpress type II collagen such as those described by Garofalo et al. (1993). One could also determine if there were abnormalities in chondrocyte differentiation where *Col2a-1* is downregulated, such as may be found for mice heterozygous for a null mutation in the gene created by gene targeting.

The lack of hyperplasia or tumor formation in pAL21 mice is intriguing. In the majority of Tag transgenesis studies, the gene has been controlled by viral promoters, or regulatory elements of genes which are expressed either widely or in specific tissues with expression extending into adulthood. Targeted expression of Tag or integration of the early region of SV40 virus in mice usually promotes tumorigenesis in specific tissues after birth (Hanahan, 1988; Adams and Cory, 1991). The phenotypic consequence of tissue specific and restricted expression of Tag during embryogenesis is poorly understood. We have found previously that expression of the $\alpha 1(\text{II})$ collagen gene declines after birth with little expression once growth ceases (Lovell-Badge et al., 1987). Perhaps this additional imposition of a developmentally regulated schedule which restricts Tag expression to embryonic and growth phases of development is the reason for the absence of tumors in pAL21 mice. Tumor promotion may require sustained postnatal expression of Tag to enable synergistic factors to act. Whether some cell types may be refractory to Tag action with respect to tumor promotion is controversial. In a recent report, SV40 transformed murine chondrocytes which retain their differentiated phenotype failed to produce tumors in mice, suggesting that in chondrocytes, Tag expression alone is not sufficient for tumor promotion (Mallein-Gerin and Olsen, 1993). Targeted expression of Tag via the opsin gene promoter to the retina of transgenic mice results in selective proliferation followed by the degeneration of rod photoreceptor cells (Al-Ubaidi et al., 1992). Transgenic mice expressing Tag under the control of the myelin basic protein promoter displayed hypomyelination and neurological disturbances such as convulsions and hindlimb ataxia (Jensen et al., 1993). These findings indicate that hyperplasia or a predisposition to tumor formation, although common, is not the only consequence of expression of Tag in transgenic mice.

Despite the apparent similarity of the phenotype displayed by pAL21 transgenic mice and other mice expressing mutant *Col2a-1* alleles (Metsäranta et al., 1992; Garofalo et al., 1991; Vandenberg et al., 1991), the chondrodysplasia of

pAL21 mice has not been caused by a structural alteration of a matrix molecule but may be due to a poorer differentiation of chondrocytes. The recent reports of abnormalities in chondrocyte proliferation and maturation in examples of skeletal dysplasia in human and mouse (Brenner et al., 1993; Karaplis et al., 1994) support the possibility that multiple causative factors apart from impaired integrity of matrix components may also result in chondrodysplasia. The COL2A1-Tag transgene provides a tool for investigating this possibility and for analyzing the complex relationships between quality of cartilage matrix, chondrocyte differentiation, and phenotype during skeletogenesis and the pathogenesis of chondrodysplasia. The use of ES cells and chimeras also provides a useful approach for overcoming the problem of perinatal lethality when transgenes produce dominant mutations in matrix genes.

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