MOLECULAR AND CELLULAR BIOLOGY, Apr. 1990, p. 1452–1460
0270-7306/90/041452-09$02.00/0

Vol. 10, No. 4

Human-Mouse Interspecies Collagen I Heterotrimer Is Functional during Embryonic Development of Mov13 Mutant Mouse Embryos

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Received 29 September 1989/Accepted 14 December 1989

To investigate whether the human proα1(I) collagen chain could form an in vivo functional interspecies heterotrimer with the mouse proα2(I) collagen chain, we introduced the human COL1A1 gene into Mov13 mice which have a functional deletion of the endogenous COL1A1 gene. Transgenic mouse strains (HucI and HucII) carrying the human COL1A1 gene were first generated by microinjecting the COL1A1 gene into wild-type mouse embryos. Genetic evidence indicated that the transgene in the HucI strain was closely linked to the endogenous mouse COL1A1 gene and was X linked in the HucII transgenic strain. Northern (RNA) blot and S1 protection analyses showed that the transgene was expressed in the appropriate tissue-specific manner and as efficiently as the endogenous COL1A1 gene. HucII mice were crossed with Mov13 mice to transfer the human transgene into the mutant strain. Whereas homozygous Mov13 embryos die between days 13 and 14 of gestation, the presence of the transgene permitted apparently normal development of the mutant embryos to birth. This indicated that the mouse-human interspecies collagen I heterotrimer was functional in the animal. The rescue was, however, only partial, as all homozygotes died within 36 h after delivery, with signs of internal bleeding. This could have been due to a functional defect in the interspecies hybrid collagen. Extensive analysis failed to reveal any biochemical or morphological abnormalities of the collagen I molecules in Mov13-HucII embryos. This may indicate either that there was a subtle functional defect of the interspecies hybrid protein which was not revealed by our analysis or that another gene has been mutated by the retroviral insertion in the Mov13 mutant strain.

Type I collagen, a major structural protein of the extracellular matrix, is distributed mainly in the skin, bones, and tendons. Functional type I collagen molecules are triple-helical structures composed of two α1(I) and one α2(I) chains. The two polypeptide chains are encoded at the unlinked COL1A1 and COL1A2 loci, respectively. The polypeptides are synthesized as precursor proα1 or proα2 chains, which assemble within the cell into a triple-helical procollagen molecule. After secretion into the extracellular space, the procollagen propeptide domains at the N and C termini are proteolytically removed to produce functional collagen molecules which undergo fibrillogenesis (7, 21).

The collagen chains are highly conserved in evolution, and the protein sequence of the α chains of type I collagen differs as little as 4% between species as distant as mice and humans (19). This high evolutionary conservation suggests that the primary sequence of the polypeptide chains is important for their biological function. Indeed, a number of point mutations are known which result in a dominant perinatal lethal form of osteogenesis imperfecta (8, 28, 30). In these molecularly well-defined mutations a single amino acid substitution in one of the α chains disturbs proper helix propagation and stability, leading to a severe functional defect of type I collagen. The molecular analysis of collagen defects has been limited by the availability of mutations in human patients.

One experimental approach for studying the physiological effects of mutations on collagen function would be to introduce a gene that encodes one polypeptide component of collagen into animals that express the other component. The transferred gene could be engineered in vitro to carry defined mutations of interest (26). For this approach, the endogenous gene corresponding to the transferred gene must be functionally deleted, with no effect on the expression of the other subunit component. Mov13 mice carry a Moloney leukemia proviral gene in intron 1 of the COL1A1 gene (13). This results in a block of transcription of the gene in most tissues with the exception of odontoblasts (11, 14, 24). Transcription of the α2(I) gene, however, is not affected by this insertional mutation. Therefore, the introduction of wild-type or variant COL1A1 genes into Mov13 cells should allow functional testing of a given mutation on collagen synthesis. To test the feasibility of this approach, we have previously introduced the human proα1(I) collagen gene into proα1(I)-deficient Mov13 mouse cells and demonstrated that the human proα1(I) chain could associate with the endogenous proα2(I) chain to form stable mouse-human interspecies collagen I molecules (23). Because these experiments were performed in tissue culture, the study did not address whether the interspecies collagen I heterotrimer would be functional in vivo.

In the present study we have introduced the human proα1(I) gene into homozygous Mov13 embryos. The human

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transgene was expressed in a tissue-specific manner and at normal levels. Protein analyses demonstrated the formation of stable interspecies type I collagen heterotrimers which formed fibrils of normal morphology. The collagen molecules were functional in vivo, supporting the survival of homozygous Mov13 animals to birth.

(This study was performed by H. Wu in partial fulfillment of the requirements for the Ph.D. degree from Harvard Medical School, Cambridge, Mass.)

MATERIALS AND METHODS

Generation of transgenic mice carrying the human proα1(I) collagen gene. Fertilized eggs were obtained from SJL/C57BL F1 mice, and about 100 copies of the linearized human proα1(I) collagen cosmid clone CG103 (2) were microinjected into the male pronucleus. The eggs were incubated overnight, and two cell embryos were transferred to the oviduct of pseudopregnant foster females.

Genotyping of mice. DNA was isolated from placenta or brain following disruption of the tissues in 100 mM NaCl–10 mM Tris (pH 8.0)–25 mM EDTA in a Polytron homogenizer. High-molecular-weight DNAs were isolated following digestion with proteinase K as described previously (11, 12).

DNA was digested with the restriction enzyme *Eco*RI and separated on an 0.8% agarose gel. After transfer to GeneScreen Plus nylon membrane (Du Pont, NEN Research Products), the upper portions of the blots were hybridized with a ^{32}P-labeled mouse-specific 0.65-kilobase (kb) *Bst*EII-*Hpa*I fragment located in intron 1 of the mouse proα1(I) collagen gene, which hybridizes to a 14-kb *Eco*RI fragment in wild-type mice and to a 23-kb *Eco*RI fragment in Mov13 mice. This probe does not hybridize to the human transgene. To determine the sex of the animals, we hybridized the lower portions of the blots to a mouse Y chromosome-specific probe, pY2 (15). To estimate the copy number of the human transgene in animals, we rehybridized the blots to pJB8 (18), which is the parental cosmid of the vector used for cloning the human COL1A1 gene, and estimated the copy number by densitometry of the films.

Northern (RNA) blot analysis. Total RNA was prepared from the limbs of day 18 embryos by the method of Auffray and Rougeon (1). Glyoxal-denatured RNA samples were separated on a 1% (wt/vol) agarose gel and transferred to GeneScreen Plus nylon membrane. Filters were hybridized to the oligo-labeled human proα1(I) collagen cDNA clone pHuc (27) or to the end-labeled human-specific 43-base synthetic oligonucleotide probe which corresponds to nucleotides 180 to 222 of the transcribed region (23). Hybridization was also performed by using the rat α_1-tubulin probe (16), which serves as an internal control.

S1 analysis. S1 analysis was performed to compare the steady-state mRNA levels between the human transgene and mouse endogenous gene in different tissues. Total tissue RNAs were prepared from 4-day-old newborn HucII mice. To measure the mouse α1(I) collagen mRNA, which served as an internal control, we isolated a 1.3-kb *Xba*I fragment from pI (11). To measure the expression levels from the human α1(I) collagen transgene, we isolated a 1.4-kb *Xba*I-*Hin*dIII fragment from pHCH2.3 in which a 2.3-kb *Hin*dIII fragment (2) containing the 5′ end of the human COL1A1 gene was subcloned into pUC19. These two probes were 3′ end labeled with ^{32}P by using Klenow polymerase. RNA (5 μg from each tissue) was ethanol precipitated with a mixture of human and mouse S1 probes which were empirically determined to be in excess. After denaturing at 80°C, half of the hybridization was carried out at 58°C, the optimal hybridization temperature for the mouse probe, and the other half was carried out at 64°C, the optimal hybridization temperature for the human probe, for 16 to 20 h. Digestion was initiated by adding 400 U of S1 nuclease (Bethesda Research Laboratories, Inc.) in 0.45 ml of prechilled 0.25 M NaCl–0.03 M sodium acetate (pH 4.5)–1 mM $ZnCl_2$–5% glycerol and continued at room temperature for 1 h. The digestion products from the two hybridization reactions were combined, ethanol precipitated, and fractionated by electrophoresis on 5% polyacrylamide–8 M urea gels (5). After being fixed and dried, the gels were exposed directly to Kodak X-OMAT AR film and quantitated on a Betagen betascope.

Transformation of primary cells. Embryos were isolated at day 16.5 of gestation, and the cells were dispersed by treatment with trypsin and plated on tissue culture dishes. To derive permanent cells lines, we transformed the cultures with simian virus 40 (SV40). The genotype of the cell lines was determined by Southern analysis as described above.

Procollagen labeling in transformed cell lines. Procollagen was biosynthetically labeled in transformed fibroblasts from homozygous Mov13 embryos carrying the HucII transgene, normal mouse fibroblasts, human dermal fibroblasts (3), and Mov13 cell lines transfected with the mouse COL1A1 gene (23) by using previously described methods (3, 23). In brief, postconfluent cultures were labeled for 18 h in medium containing 10% (vol/vol) fetal calf serum (Flow Laboratories), 0.15 mM sodium ascorbate (Sigma Chemical Co.), and 10 μCi of L-[5-^{3}H]proline per ml (26 Ci/mmol; Amersham Corp.). After labeling, the medium and cell layer fractions were separated for analysis and procollagen was precipitated from both fractions with 25% saturated $(NH_4)_2SO_4$. The precipitates were dissolved in 0.05 M Tris hydrochloride–0.15 M NaCl (pH 7.5). Portions were then analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis on 5% (wt/vol) polyacrylamide gels, as previously described (3, 4), under nonreducing conditions.

Analysis of transgenic-mouse dermal collagens. Circular biopsies (diameter, 4 mm) were taken from day 19 embryo skin, diced, defatted with chloroform-methanol (2:1), and lyophilized to a constant dry weight. Dermal collagens were then serially extracted with 0.05 M Tris hydrochloride–0.15 M NaCl (pH 7.5) and subjected to pepsin digestion (100 μg/ml in 0.5 M acetic acid) (3, 26). Collagens solubilized in each extract were quantitatively loaded onto 5% (wt/vol) polyacrylamide gels for electrophoretic analysis. Collagen bands were stained with Coomassie blue (3, 26) and quantitated by densitometry by comparison with gel tracks loaded with a known amount of a type I collagen standard. Collagens not solubilized by these extractions were recovered from the insoluble residue by digestion with cyanogen bromide, and the resultant peptides were analyzed by electrophoresis on 12.5% (wt/vol) polyacrylamide and densitometry (9).

Analyses of bone collagen. Samples of midshaft femoral and calvareal bone were dissected from term embryos. Bone was dried, weighed, and hydrolyzed, without prior decalcification, in 6 M HCl for 24 h at 108°C. Hydroxyproline and hydroxylysine were quantified by reverse-phase high-pressure liquid chromatography by the method of precolumn derivatization with phenyl isothiocyanate. Hydroxypyridinium cross-linking amino acids were resolved by reverse-phase high-pressure liquid chromatography and quantified by fluorescence by using an established method (10).

TABLE 1. Genetic mapping of *HucII* locus to X chromosome[a]

No. copies of transgene in parent		No. of offspring with following no. of copies of transgene[b]:		
Male	Female	0	1	2
1	0	19 (m), 0 (f)	0 (m), 16 (f)	0 (m), 0 (f)
1	2	0 (m), 0 (f)	11 (m), 0 (f)	0 (m), 13 (f)

[a] The copy number of the human transgene was determined by optical densitometry of DNA Southern blots. Filters were hybridized to pJB8, which is the parental cosmid of the vector used for cloning the human COL1A1 gene.
[b] Abbreviations: m, male; f, female.

Histologic analysis. Newborn embryos were fixed in 10% buffered Formalin, dehydrated in graded alcohols and xylenes, and embedded in Paraplast Plus. Serial 4-μm sections were prepared from each embryo (approximately 200 to 300 per embryos) and stained with Harris hematoxylin and eosin (25).

RESULTS

Derivation of transgenic mice carrying the human gene on an autosome or on the X chromosome. The human proα1(I) collagen cosmid clone CG103, which includes the 18-kb structural region and 1.6 kb of 5′ and 20 kb of 3′ flanking sequences, was microinjected into the pronuclei of SJL/C57BL F1 mouse embryos. Two transgenic mouse strains, HucI and HucII, were derived, as determined by slot blot analysis of tail DNA with the cosmid pJB8 as a probe. Each of these strains carried a single complete copy of the human proα1(I) collagen gene in the germ line. The transgene in HucI mice was autosomally inherited (see below), whereas in HucII mice it was X-linked. This was shown by crossing HucII males carrying a single copy of the human gene to normal females. When the offspring were genotyped all daughters but no sons carried the transgene. Similarly, when hemizygous males and homozygous females were crossed, all sons carried one copy and all daughters carried two copies of the transgene (Table 1). Animals homozygous for the HucII or the HucI gene were normal, indicating that the insertion of the transgene had not caused any detectable recessive mutation.

Introduction of the human COL1A1 gene into the Mov13 mutant rescues the lethal phenotype to birth. To test whether a human-mouse interspecies type I collagen heterotrimer could function in the animal, we derived mice which had no functional mouse proα1(I) collagen gene but carried one or two copies of the human gene. HucI and HucII mice were crossed with Mov13 mice, which have a functional deletion of the proα1(I) collagen gene due to a proviral insertion into intron 1 of the gene and produce normal amounts of proα2(I) RNA. Embryos homozygous at the *Mov13* locus die between days 13 and 14 of gestation due to the lack of type I collagen. Therefore, survival of mutant embryos carrying the human insert beyond day 14 of gestation would indicate that a collagen type I interspecies heterotrimer can function in vivo. Double heterozygous mice (Mov13/wt,wt/HucI mice and Mov13/wt,wt/HucII mice) from each strain were derived and were intercrossed to produce homozygous Mov13 animals carrying either the HucI or the HucII transgene. Embryos were removed at day 19 of gestation and genotyped by Southern analysis, with a 0.65-kb *Bst*EII-*Hpa*I fragment from intron 1 of the mouse COL1A1 gene as a probe (Fig. 1). This probe does not recognize the human transgene. In such an analysis, a 14-kb *Eco*RI fragment is indicative of the endogenous murine COL1A1 gene and a 23-kb fragment of the Mov13 allele which carries the proviral insert. The absence of the 14-kb band (Fig. 1, lanes 4 and 8) therefore distinguishes homozygous Mov13 animals from heterozygous or wild-type littermates. The human COL1A1 transgene was revealed by two approximately 6- to 7-kb *Eco*RI fragments detected by the cosmid vector probe. The sex of each animal was determined by using a Y-specific probe, pY2.

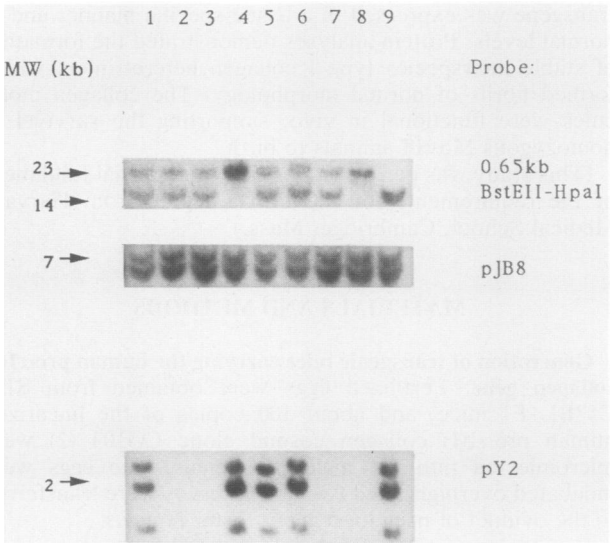

FIG. 1. Southern blot of *Eco*RI-digested placenta DNA from newborn Mov13 mice carrying the *HucII* locus. The upper portion of the filter was hybridized to the mouse-specific collagen probe, *Bst*EII-*Hpa*I fragment. Arrows indicate the 14-kb wild-type *Eco*RI fragment and 23-kb Mov13 *Eco*RI fragment which allow determination of the Mov13 genotype. The filter was rehybridized to cosmid pJB8 which detected two approximately 6- to 7-kb fragments (arrowed) from the cosmid vector. The lower portion of the filter was hybridized to the Y chromosome-specific probe pY2 (15). Genotypes in each lane: 1, M/+, HucII/Y; 2, M/+, HucII/HucII; 3, M/+, HucII/HucII; 4, M/M, HucII/Y; 5, M/+, HucII/Y; 6, M/+, HucII/Y; 7, M/+, HucII/HucII; 8, M/M, HucII/HucII; 9, +/+, HucII/Y, where +/+ denotes wild type, M/+ denotes Mov13, heterozygote and M/M denotes Mov13 homozygote.

The analysis of more than 70 offspring of the (Mov13/wt,wt/HucI) × (Mov13/wt,wt/HucI) cross at day 18 of gestation showed no fetuses which were homozygous at the *Mov13* locus (data not shown). At day 12 or 13 of gestation, the expected fraction of 25% homozygous embryos was found, none of which, however, carried the human transgene. This suggested that the human transgene was linked to the endogenous mouse COL1A1 gene which has been mapped to chromosome 11 (20). To derive a recombinant, Mov13/wt,wt/HucI mice were crossed with wild-type mice and the offspring were genotyped. None of 450 offspring had the predicted recombinant genotype, Mov13/wt,HucI/wt. This indicated that the genetic distance between the exogenous human COL1A1 gene and the mouse COL1A1 gene was less than 0.22 centimorgans (cM). It therefore was not feasible to use the HucI strain for rescuing Mov13 mutant mice.

A different result was obtained when the HucII gene was introduced into the Mov13 mutant strain. The offspring of parents hemizygous or homozygous for the HucII gene and heterozygous for the Mov13 mutation [(Mov13/wt,HucII/HucII) × (Mov13/wt,HucII/Y)] were delivered by cesarean

TABLE 2. Genotype of live offspring from (Mov13/wt,HucII/Y × Mov13/wt,HucII/HucII) parents[a]

Age	Mov13 locus[b]	No. of mice[b]	Total (% of all mice of this age)
Newborn	wt/wt	24 (f), 32 (m)	56 (21.4%)
	M/wt	75 (f), 69 (m)	144 (54.2%)
	M/M	34 (f), 31 (m)	65 (24.4%)
Adult[c]	wt/wt	14 (f), 20 (m)	34 (26.3%)
	M/wt	51 (f), 44 (m)	95 (73.6%)
	M/M	0 (f), 0 (m)	0 (0%)

[a] All females carried two copies of the HucII locus, and all males carried one copy of the HucII locus.
[b] Abbreviations: f, female; m, male; M, Mov13 positive.
[c] Adult mice were 6 weeks of age.

section at day 19 of gestation and genotyped as described above. DNA was extracted, and Southern blots were hybridized to the mouse collagen-specific probe 0.65-kb *Bst*EII-*Hpa*I fragment to determine the Mov13 genotype and with the Y-specific probe pY2 (15) to determine the sex of each fetus (Fig. 1). The results in Fig. 1 show that fetuses which were homozygous at the Mov13 locus were readily identified (see lanes 4 and 8.). As expected, all male offspring carried one copy and all females carried two copies of the human transgene, confirming X linkage of the HucII gene (Table 1). Importantly, 25% of the fetuses were homozygous at the Mov13 locus, indicating that the human COL1A1 gene can compensate for collagen I deficiency in Mov13/Mov13 embryos and prevent fetal death. The rescue was only partial, however, as no homozygous animals survived to weaning (Table 2) but died within 36 h after birth. Soon after delivery, the majority of the homozygous animals developed signs of developmental retardation, failure to feed, weakness, and bleeding into the head, chest cavity, or abdomen. The most consistent pathologic finding in newborn homozygous animals was hemorrhage. Histologic analyses of serial sections revealed recent bleeding in the subcutaneous tissue near the salivary glands, as well as in the thorax and abdomen (Fig. 2). In contrast to the hemopericardium observed in Mov13 homozygous embryos (19), the pericardium in these embryos was without any evidence of bleeding. Evidence of more remote bleeding was seen in the abdominal cavity, manifested as clotted blood.

The results described above indicated that a mouse-human interspecies collagen I heterotrimer can function in the animal and can promote the development of mutant embryos to birth. The failure of the human gene to rescue homozygous Mov13 animals beyond birth could be due to insufficient or inappropriate expression of the transgene or to structural alterations of the interspecies heterotrimer, resulting in functional impairment of the collagen fibrils. Therefore, collagen RNA and proteins were analyzed in transgenic fetuses.

Steady-state mRNA of the human proα1(I) collagen gene in wild-type and Mov13 mice. A human-specific 43-base synthetic oligonucleotide probe was used to distinguish between human and mouse proα1(I) collagen RNA, as described previously (23). Figure 3 shows a Northern analysis of RNA isolated from day 18 Mov13 fetuses carrying the HucII transgene. The Mov13 genotype, the number of human transgenes carried by each fetus, and the sex were determined as described in the legend to Fig. 1 and Materials and Methods and are indicated at the top of the figure. The upper panel shows hybridization to a human proα1(I) collagen cDNA clones, pHuc (13), which detects mouse as well as human proα1(I) collagen RNA. To standardize the amount of RNA loaded on the gel, we also hybridized filters with the rat α_1-tubulin probe (15). The typical collagen RNA species of 4.8 and 5.8 kb were present in all samples but not in a fibroblast cell line derived from a homozygous Mov13 embryo (lane 8) (23). The lower panel shows the same filter hybridized to the human-specific 43-base synthetic oligonucleotide probe. As expected, this probe detected collagen transcripts in RNA from a human fibroblast line (lane 9) as well as the collagen transcripts in RNA from all embryos carrying the human transgene (lower panel, lanes 1, 2, 3, 4, 6, and 7). No signal was detected, however, in RNA extracted from a wild-type mouse embryo (lane 10), from the Mov13 cell line (lane 8), or from a heterozygous embryo (lane 5). Collagen-specific bands detected by the cDNA probe (upper panel) in homozygous Mov13 fetuses (lanes 1, 3, 6, and 7) therefore represent transcripts derived from the human transgene. The intensity of the signal in the transgenic embryos carrying the human transgene was comparable to that of the wild-type embryo (lane 10), indicating that the human COL1A1 gene transcribed proα1(I) RNA as efficiently as the mouse gene did. The intensity of the human COL1A1 transcripts was slightly higher in Mov13 homozygotes than in heterozygotes (compare lanes 3, 6, and 7 with lanes 2 and 4). It is possible, therefore, that the presence of a functional mouse gene affects the level of stable transcripts from the human transgene.

To test whether the transgene was transcribed in an appropriate tissue-specific manner, we quantitated the steady-state mRNA of the human transgene and the mouse endogenous gene by an S1 nuclease protection assay. For this, a 1.4-kb *Xba*I-*Hin*dIII fragment extending from a position 113 bases 3′ of the human transcription start site into intron 1 was isolated from the human collagen construct pHCH2.3, and a 1.3-kb *Xba*I-*Xba*I fragment extending from a position 108 bases 3′ of the mouse transcriptional start site into intron 1 was isolated from the mouse collagen construct pI. The human transcript protects 109 bases of the human probe, whereas the endogenous mouse transcript protects 82 bases of the mouse probe (Fig. 4C). At the hybridization temperatures used, no signal arises from mouse-human cross hybridization owing to the sequence differences between the two genes (Fig. 4A). Human- and mouse-specific collagen transcripts in RNA extracted from different tissues of HucII animals were analyzed by the S1 protection assay. A signal at 82 base pairs, which is characteristic of mouse collagen-specific RNA, was strong in tissues such as heart, lung, and muscle, weaker in liver and kidney, and almost not detectable in brain and spleen. This is the expected tissue distribution of collagen RNA. Importantly, a band at 109 base pairs characteristic of the human collagen RNA was present in the same tissues as and at similar relative intensities to the endogenous mouse signal.

The results described above indicate that the human proα1(I) collagen gene is expressed not only in the same tissues as the endogenous mouse gene (Fig. 4), but also at comparable levels (Fig. 3). It is therefore unlikely that the perinatal death of homozygous Mov13 embryos carrying the HucII gene was due to insufficient or inappropriate expression of the human proα1(I) collagen gene.

Expression of the human proα1(I) collagen protein in wild-type and Mov13 mice. The production of stable collagen by the human transgene in Mov13 heterozygote cell cultures was assessed by SDS-polyacrylamide gel electrophoresis of [^{3}H]proline-labeled procollagen species (Fig. 5A). These gels

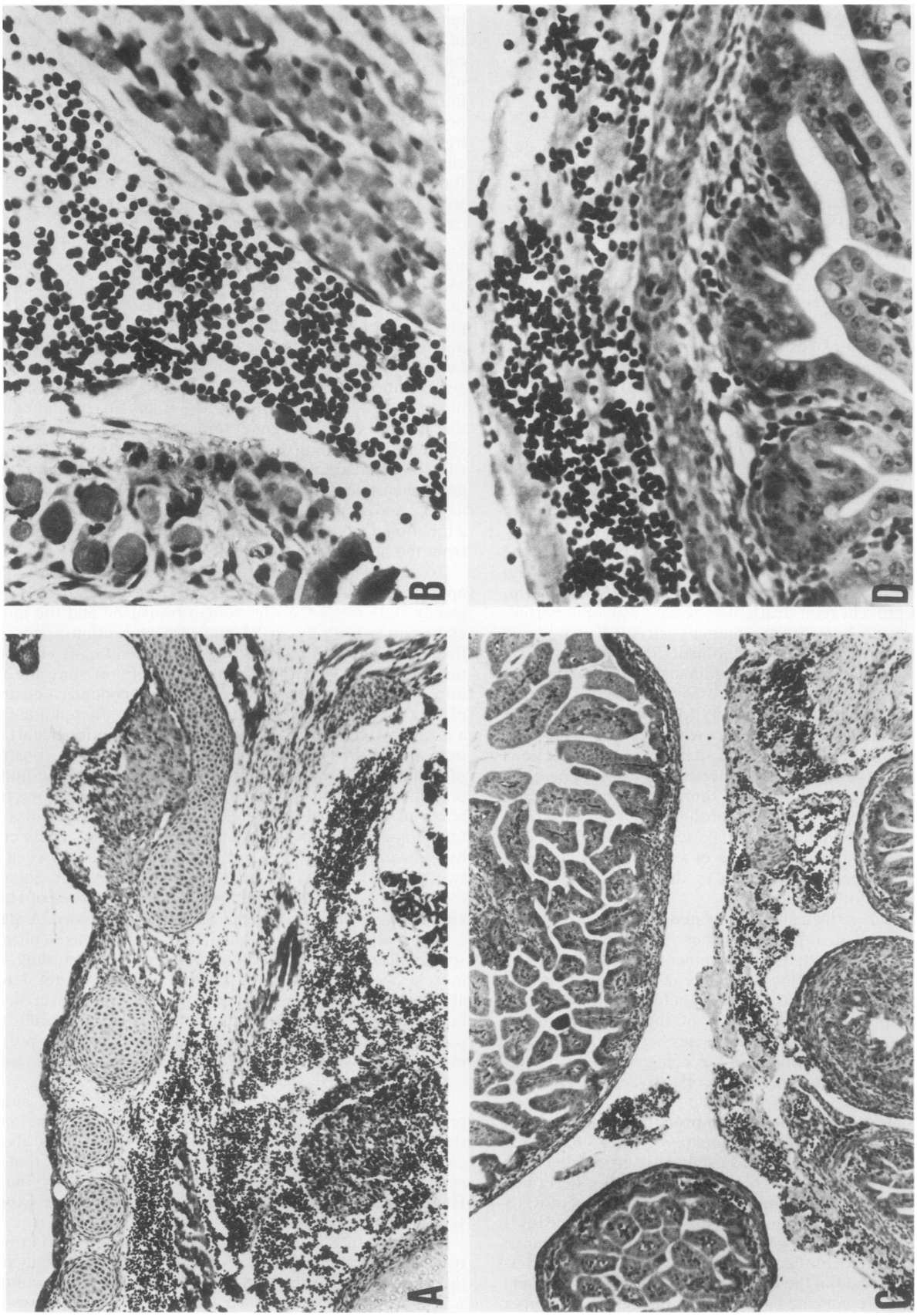

FIG. 2. Histology of representative areas of bleeding in HucII/Mov13 newborn mice. (A and B) Hemorrhage in neck region; (C and D) hemorrhage in abdominal cavity. Note fibrin and blood cells, indicating clotted blood. Magnification of panels A and C, ×82.5; magnification of panels B and D, ×330.

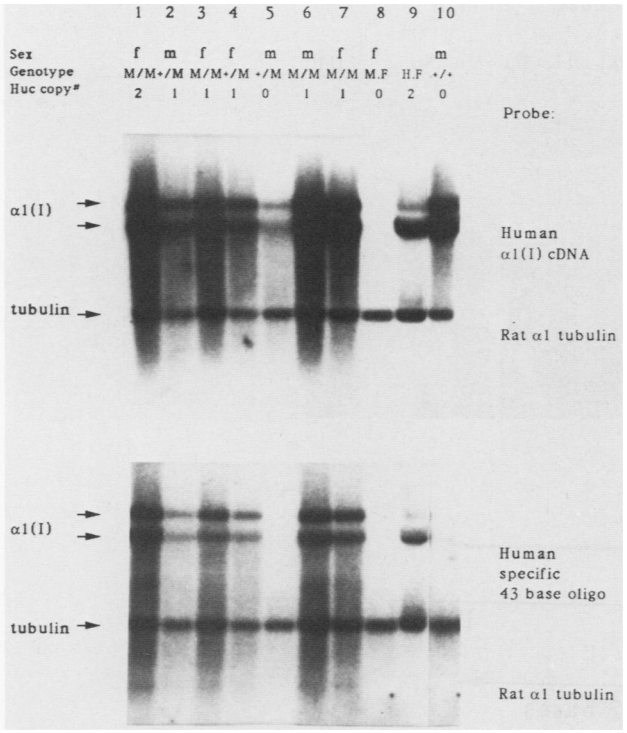

FIG. 3. Northern analysis of human proα1(I) collagen gene expression in Mov13 embryos carrying the HucII locus. The upper and lower panels are autoradiographs of duplicate filters. They were hybridized to a human α1(I) collagen cDNA clone (upper panel), which detects total proα1(I) collagen RNA, and to a human-specific probe (lower panel). To standardize the amount of RNA, we also hybridized filters with the rat α_1-tubulin probe. The genotype and number of copies of the human gene in the embryos are indicated above each lane. Abbreviations: H.F., human fibroblast cell line SV80; M.F., mouse fibroblast cell line derived from a homozygous Mov13 embryo.

were run under nonreducing conditions so that only the collagen α1(I) and α2(I) chains and the procollagen processing intermediates pNα1(I) and pNα2(I) were resolved. Under these conditions, cells from Mov13 homozygous mice containing the HucII transgene (lanes 4 and 5) produced pNα1(I) chains with an electrophoretic migration identical to authentic human pNα1(I) (lane 1). In contrast, mouse pNα1(I) chains (lanes 2 and 3) had a distinctive, slower migration. The reason for this migrational difference between human and mouse procollagens is not known, but most probably results from amino acid sequence differences causing altered SDS binding. However, this result clearly demonstrates, as expected, that human α1(I) procollagen is produced by cells from the Mov13 mice containing the HucII transgene. Six cell lines expressing HucII mRNA were analyzed, and all produced similar high levels of human procollagen protein. Detailed analysis of the cell culture collagen demonstrated that the collagen heterotrimers were stable to pepsin digestion and thus formed normal triple-helical molecules that were secreted normally by the cells (data not shown). The human α1(I) and mouse α2(I) chains of the heterotrimeric type I collagen molecules had an electrophoretic migration identical to that of control α1(I) and α2(I) molecules (Fig. 5A), indicating that these chains contained normal levels of posttranslational modifications of lysine (4). Normal levels of lysine hydroxylation and glycosylation suggest that the association of the human α1(I) and mouse α2(I) chains into a triple helix occurs efficiently, since interruptions to helix formation characteristically lead to increased lysine modification (4, 6). The human pNα1(I) and α1(I) in lane 5 appeared to have a slightly lower mobility than the corresponding bands in the human control cells (lane 1). This is probably due to overloading of the sample in lane 5.

The human-mouse interspecies heterotrimer in transgenic mouse tissues was also evaluated biochemically. Serial extraction of dermal samples with neutral salt buffer followed by pepsin showed that the collagen solubility of Mov13 mice expressing the transgene was indistinguishable from that of control animals. In all cases, approximately 14 to 18% of the collagen was extracted with neutral salt buffer, 70 to 80% was extracted by pepsin digestion, and 6 to 13% remained insoluble.

The nature of the pepsin-extracted collagen was further analyzed by gel electrophoresis (Fig. 5B). Animals of various genotypes were examined. The control (wt/wt) collagen (lane 1) was again indistinguishable electrophoretically from that of the Mov13 homozygotes, either hemizygous (lane 2) or homozygous (lane 3) for HucII, and of Mov/wt (lane 4) or wt/wt (lane 5) embryos carrying the human transgene. In all of these samples, the electrophoretic migration and quantity of the covalently cross-linked β components were comparable. The relative proportions of the β components and their ratio to α chains were also the same on comparing denaturant (4 M guanidine hydrochloride) and acetic acid extracts of the dermal collagens by SDS-polyacrylamide electrophoresis. Analyses of the contents of collagen, hydroxylysine, and mature cross-linking amino acids (pyridinolines) in calvareal and femoral bone samples also failed to demonstrate any differences between control animals and Mov13 mice expressing the HucII transgene.

Quantitation of the electrophoretic analyses of the serial dermal extracts is given in Table 3. These extraction procedures fully solubilized the type I and III collagens. The total amount of type I collagen in the tissue and the ratio of type I to type III collagen is similar in control mice and Mov13 mice expressing the human collagen transgene. Finally, ultrastructural examination of the tissues by electron microscopy demonstrated that the human-mouse hybrid type I collagen formed fibrils of normal dimensions and distribution (data not shown).

DISCUSSION

In this study, we investigated whether human proα1(I) collagen chains can associate with a mouse proα2(I) chain to form a functional type I collagen in the animal. To do so, we introduced a cosmid clone of the human COL1A1 gene into the germ line of Mov13 mice which are deficient in proα1(I) synthesis. For this, we first generated two transgenic mouse strains, HucI and HucII, which carried a single copy of the human gene in the germ line. Genetic evidence indicated that the transgene in the HucI strain was integrated within less than 0.22 cM of the endogenous COL1A1 gene on chromosome 11, precluding the use of this strain for breeding the transgene into Mov13 mice. The transgene in the HucII strain was integrated on the X chromosome and was crossed into the Mov13 mutant strain. Homozygous Mov13 embryos die of circulatory failure between days 13 and 14 of gestation (17). In contrast, homozygous embryos carrying one or two copies of the human COL1A1 gene developed normally to birth, indicating that the human transgene can give rise to type I collagen which is functional in the animal. However, all homozygous embryos died within 36 h after birth. This

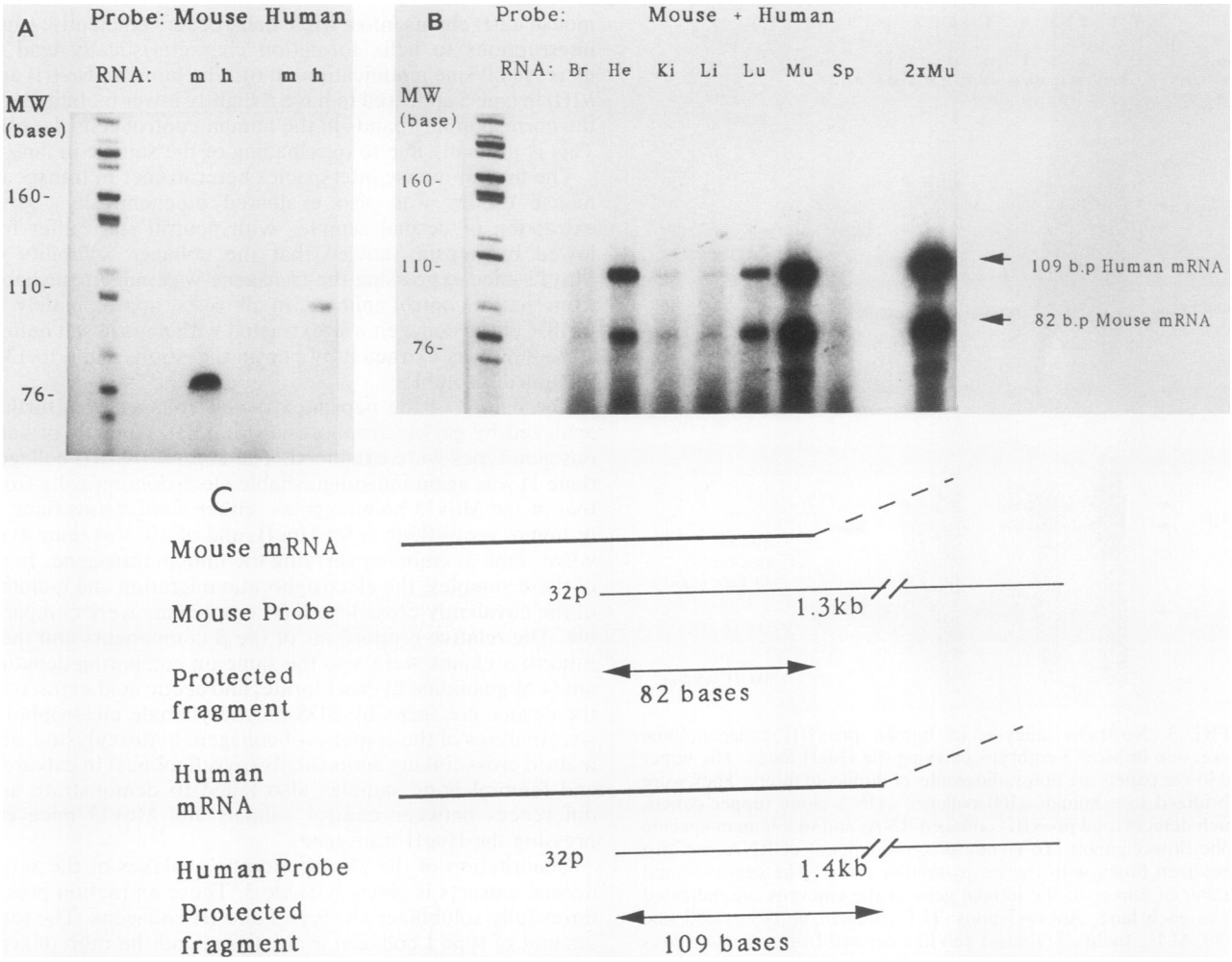

FIG. 4. S1 analysis of endogenous mouse and exogenous human proα1(I) collagen gene expression in 4-day-old HucII mice. (A) Control for the hybridization temperature. Mouse (m) and human (h) RNA (5 μg) was hybridized with the 3′-end-labeled mouse (at 58°C) and human (at 64°C) S1 probes, respectively. (B) Tissue-specific expression of mouse and human proα1(I) collagen gene. From left to right, the level of collagen gene expression in brain (Br), heart (He), kidney (Ki), liver (Li), lung (Lu), muscle (Mu), and spleen (Sp) tissue is shown. Each lane contained 5 μg of RNA, except the last lane, in which 10 μg of RNA from muscle was used to show that an excess amount of probes was used in the assay. The hybridization temperatures used here were same as in panel A. (C) Scheme of the S1 assay used to distinguish between mouse and human proα1(I) collagen transcripts. The top line represents exon 1 and part of intron 1 of the mouse proα1(I) collagen gene. The 1.3-kb mouse probe, extending from a position 108 base pairs (bp) 3′ of the transcriptional start site to intron 1 of the gene, is shown underneath the transcript. When this probe is hybridized with RNA at 58°C, an 82-base fragment is expected to be protected from S1 nuclease digestion by the mouse mRNA. The lower line shows exon 1 and part of intron 1 of human proα1(I) collagen gene. The 1.4-kb human probe, extending from a position 113 base pairs 3′ of the human transcriptional start site to intron 1, was hybridized with RNA at 64°C and is expected to yield a 109-base band protected by the human transcript.

could have been due to insufficient or ectopic expression of the transgene or to a subtle functional defect in the interspecies collagen molecule. Therefore, we analyzed the transcription pattern of the human COL1A1 gene and the collagen produced in the transgenic mice.

The human transgene, under the control of its own promoter including 1.6 kb of 5′-flanking sequences and 20 kb of 3′-flanking sequences, was expressed in HucII and HucI mice at levels comparable to the endogenous COL1A1 gene. An S1 nuclease protection assay also showed that the transgene was expressed in the appropriate tissue-specific manner. These results indicated that insufficient levels or inappropriate tissue distribution of the human proα1(I) RNA could not explain the perinatal death of the Mov13 homozygous animals. The level of the COL1A1 expression in previous experiments in which the same construct was transfected into SV40-transformed Mov13 tissue culture cells was much lower than in the transgenic animals (23). This may be have been due to negative control elements in the collagen promoter which are sensitive to cellular transformation (22).

Extensive biochemical characterization of the collagen produced in the transgenic animals revealed that the amounts of type I collagen present in the skin and bone of homozygous Mov13 newborns carrying the human transgene was comparable to those in normal wild-type mice. The stability of the triple helix and the cross-linking and morphology of the collagen fibrils were not distinguishable from those of normal mice. Therefore, by all criteria used, the interspecies heterotrimer was indistinguishable from the

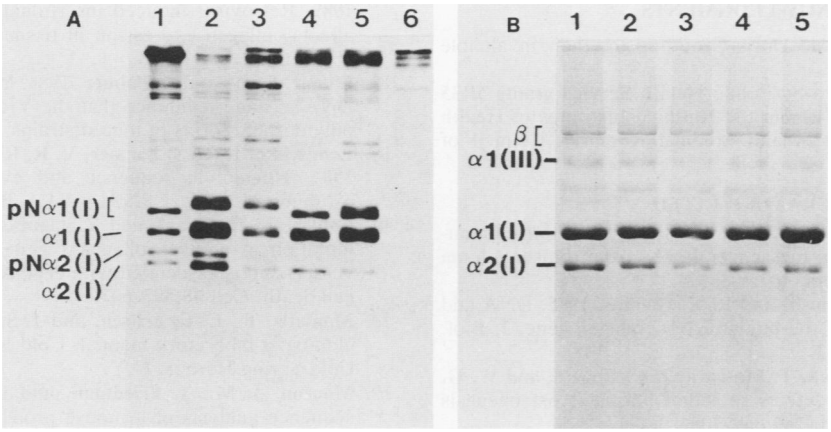

FIG. 5. SDS-polyacrylamide gel electrophoresis of collagen produced by Mov13 embryos carrying the HucII transgene. (A) Procollagens produced in cell culture were labeled with [^{3}H]proline and analyzed without reduction on 5% (wt/vol) polyacrylamide gels. Lanes: 1, human dermal fibroblasts (4); 2, SV40-transformed normal mouse fibroblasts (23); 3, Mov/Mov fibroblasts transfected with the normal mouse COL1A1 (23); 4 to 6, SV40-transformed cell lines derived from mouse embryos (lane 4, Mov/Mov, HucII/Y; lane 5, Mov/Mov, HucII/HucII; lane 6, Mov/Mov). (B) Samples representing pepsin extracts of equal dermis dry weight from day-19 embryos were electrophoresed on 5% gels after delayed reduction with 10% β-mercaptoethanol (29). Gels were stained with Coomassie blue. Lanes: 1, control mouse embryo dermis; 2, Mov/Mov, HucII/Y embryo; 3, Mov/Mov, HucII/HucII embryo; 4, Mov/wt, HucII/Y embryo; 5, wt/wt, HucII/HucII embryo. The electrophoretic migrations of type I collagen B components, α1(I) and α2(I) chains, pNα1(I) and pNα2(I) chains, and type III collagen α1(III) chains are indicated.

normal mouse type I collagen, revealing no structural abnormalities which could have explained the failure to rescue homozygous Mov13 mice beyond birth.

In contrast to our previous finding, in which the collagen produced by Mov13 cells transfected with the human COL1A1 gene (23), no hypermodification of the collagen molecules was seen in the transgenic mice. We believe that the hypermodification seen in the previous experiments was peculiar to the particular cell clone analyzed, since electrophoretic studies suggested that other collagens were also affected. An unexpected finding was that the migration of the mouse and the human pNα1(I) chain in SDS-gels differed, suggesting sequence difference in that region of the mouse and the human gene. This protein difference was seen in cells from transgenic fetuses and clearly demonstrated that the human proα1(I) collagen chain was indeed incorporated into a mouse-human interspecies collagen I heterotrimer.

The interspecies collagen I was functioning normally in vivo throughout embryonic development but did not prevent postnatal death of homozygous Mov13 mice, which was characterized by profuse bleeding. We suggest two possible explanations for the failure of the interspecies collagen I heterotrimer to promote the survival of homozygous Mov13 mice beyond birth. It is possible, first, that the slight sequence differences between the human and the mouse proα1(I) chain results in a subtle functional defect of the hybrid protein, which becomes apparent only after birth when blood pressure rises, exerting particular demands on the stability of the circulatory system. For example, the different migration of the human and the mouse pNproα1(I) may reveal a so far unrecognized function of the amino propeptide, which becomes manifest only when the human proα1(I) chain associates with the mouse proα2(I) chain. The HucII/Mov13 mouse system may therefore be useful for studying subtle structural parameters which are important for the normal function of the type I collagen.

TABLE 3. Quantitation of type I and III collagens in transgenic embryo dermis[a]

Genotype		Amt of type I collagen:	
Mov-13	HucII	(μg/mg [dry wt])[b]	% of total collagen[c]
wt/wt	wt/wt	96.5	68.3
wt/wt	HucII/HucII	127.8	75.4
Mov/wt	HucII/Y	101.5	64.8
Mov/Mov	HucII/Y	73.0	66.4
Mov/Mov	HucII/HucII	94.6	60.7

[a] Collagen types were quantified by electrophoresis and densitometry of collagen extracted by neutral salt buffer, pepsin digestion, and cyanogen bromide digestion of the insoluble residue. The collagen content was calculated by comparison with known collagen standards electrophoresed on adjacent gel tracks, and the amounts determined in each extract were combined to give the total tissue collagen content.

[b] Each value is the average of duplicate tissue analyses.

[c] Total collagen content is type I plus type III collagen.

Furthermore, we cannot exclude the possibility that a second gene distinct from the COL1A1 gene is affected by the retrovirus insertion as well and that the lack of function of this second gene causes the perinatal death in homozygous Mov13 embryos carrying the human COL1A1 transgene. To date, we have no evidence for another gene in the vicinity of the retrovirus insertion. We cannot exclude the possibility that a more distant gene is affected by the provirus. The available evidence, however, is consistent with the notion that a proviral genome may affect the function of only those gene-regulatory elements which are close to the insertion site rather than affecting the function of distant chromosomal regions. This is suggested by the observation that the Mov13 mutation affects the expression of the collagen α(1)I gene in a tissue-specific manner, repressing transcription in most mesenchymal cells but permitting expression in odontoblasts (14).

At present we are introducing the mouse COL1A1 gene into transgenic mice to distinguish between these models. Whatever the result of these studies might be, it is likely that the genetic manipulation of the Mov13 mutant strain will provide important insights into the function of type I collagen and/or other genes affected by the proviral insertion.

ACKNOWLEDGMENTS

We thank Ruth Curry and Doris Grotkopp for their invaluable assistance.

This work was supported by Public Health Service grants 5R35 CA44339 and P01 HL41484 from the National Institutes of Health and by the National Health and Medical Research Council of Australia.

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